Fidarestat sensitized Caco-2 cells to Doxorubicin via increasing cell cycle-dependent cytotoxicity

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Research note

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Fidarestat sensitized Caco-2 cells to Doxorubicin via increasing cell cycle-dependent cytotoxicity

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Abstract:

Objectives: Anthracycline drugs such as Doxorubicin are considered one of the most active, wide-spectrum, and cost-effective drugs that have been used as a therapy for many cancers, but not for colorectal cancer. Recent reports indicate that inhibiting Aldose Reductase increases the sensitivity of human colorectal cancer cells to Doxorubicin. In the present study, we have investigated the effect of using an Aldose Reductase inhibitor (Fidarestat) as an adjuvant drug with Doxorubicin on colorectal cell line Caco-2, by studying cell viability, type of death, and cell cycle distribution in order to understand the pathways of this treatment.

Results: Our results indicated that Fidarestat significantly increased the sensitivity of Caco-2 cells to Doxorubicin in a concentration-independent manner. In addition, Fidarestat increased Doxorubicin-induced G2/M phase arrest as well as induced cell cycle arrest in the S phase, thus potentiated Doxorubicin cytotoxicity. Overall, our results demonstrated that a potent and clinically safe Aldose Reductase inhibitor, Fidarestat, sensitized Caco-2 cells to Doxorubicin via increasing Doxorubicin cell cycle-dependent cytotoxicity.

Keywords: Fidarestat, Doxorubicin, Caco-2, Anthracyclines, Aldose Reductase.

1. Introduction

Chemotherapy is a common therapeutic choice for Colorectal Cancer (CRC), particularly at the advanced stages and after surgical removal of the tumor [1]. However, chemotherapeutic drugs that have been advised for CRC such as 5- Fluorouracil and Oxaliplatin have serious side effects including fatigue, anemia, and the risk of changes in heart rhythm. Therefore, the researchers keep looking for alternatives to those drugs [2, 3]. One of the candidates is the Anthracyclines such as Doxorubicin, which has been used as a treatment for leukemia, lymphoma, and breast cancer but not for CRC because of the inherent resistance of this type of cancer cells to Anthracyclines, which at higher doses cause severe cardiotoxicity [4–6]. One of the approaches that have been proposed to cope with the resistance of CRC cells to Anthracyclines, is using adjuvant drug therapy along with chemotherapeutic drugs to prevent the growth of this cancer [7].

The expression of Aldose Reductase (AR) is reported in various forms of cancer [8]. It is reported also that the Inhibition of AR prevents CRC cells growth in cell culture and nude mice xenografts [9,10]. Furthermore, AR is also an obligatory mediator of the inflammatory changes caused by growth factor-induced cytotoxicity in CRC, which supports that AR inhibition could be a promising therapy for CRC [11]. Noticeably, One of the AR inhibitors, Fidarestat, has been found to increase the sensitivity of human CRC cells to Doxorubicin and limits Anthracyclines-induced cardiotoxicity in mice [12]. However, the usage of Fidarestat as an adjuvant drug in combination with Anthracyclines for enhancing antitumor efficacy still needs to be investigated in order to understand the pathways of this treatment. In this study, we have used Doxorubicin in the presence of Fidarestat as an adjuvant drug to treat CRC cell line Caco-2 and study cell viability, type of death, and cell growth patterns due to this treatment.

2. Materials and Methods

2.1. Materials

Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Trypsin-EDTA, L-Glutamine, Penicillin/Streptomycin solution and Dulbecco's Phosphate-buffered Saline (DPBS) were obtained from Sigma-Aldrich Ltd. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and Dimethylsulfoxide (DMSO) were obtained from Genaxxon Biosciences GmbH. Doxorubicin (DOX) was purchased from EBEWE Pharma GmbH Nfg. KG. Fidarestat (FID) was purchased from Cayman Chemicals.

2.2. Cell culture

Human Colorectal Carcinoma (Caco-2) cell line was generously gifted from the Human cell culture laboratories in Atomic Energy Commission of Syria. Caco-2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 20% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and 2mM L-Glutamine. Cells were maintained at 37 °C in a humidified atmosphere supplemented with 5% CO₂.

2.3. Cytotoxicity studies

Caco-2 cells were seeded at 20×10^3 cells/well in a 96-well plate and allowed to adhere overnight. Then cells were treated with either 1 μ M of doxorubicin alone for 48, 72 hours (hr) respectively, or with 1 μ M of doxorubicin in combination with Fidarestat. For wells to be treated with Doxorubicin in combination with Fidarestat, two protocols were followed: in the first protocol, cells were incubated for 24hr with various concentrations of Fidarestat (10, 30, 50 μ M). After that, cells were treated with 1 μ M of Doxorubicin for 48hr. In the second protocol, cells were treated with doxorubicin in combination with Fidarestat at the same point of time for 48, 72hr respectively

using the same concentration in the first protocol. At the end of the incubation period, cell viability was determined using the MTT assay as previously described [13]. The absorbance recorded at 540nm using Epoch™ Microplate Spectrophotometer. The results are representative of three independent experiments.

2.4. Cell death analysis

Caco-2 cells were seeded in a 6-well plate at a density of 1.5×10^5 cells/well and allowed to adhere overnight. After that, cells were incubated with 1μM Doxorubicin and 30μM Fidarestat alone or in combination for 48hr. For combined treatment, cells were incubated with 30μM Fidarestat for 24hr and then treated with 1μM Doxorubicin for 48hr. At the end of the incubation period, cells were harvested and stained according to BD Biosciences Apoptosis Detection Kit I (BD Biosciences, USA) manufacturer's instructions. Then Cells were analyzed directly on already set BD FACSCalibur flow cytometer (Becton–Dickinson, USA). Data were analyzed using Flowing Software 2.5.1.

2.5. Cell Cycle Analysis

Cell treatment was performed as described previously in cell death analysis. At the end of the incubation period, Cells were collected, washed with DPBS and stained for cell cycle analysis using BD Biosciences cell cycle test kit (BD Biosciences, USA). Cells were analyzed using BD FACSCalibur flow cytometry. Cell cycle distribution was analyzed using Flowing Software 2.5.1.

2.6. Statistical analysis

Graphpad prism version 8 software was used to perform the statistical analysis. All data are expressed as Mean± SEM. Comparisons of means were conducted using one-way ANOVA. P < 0.05 was considered statistically significant.

3. Results

3.1. Fidarestat enhanced the sensitivity of Caco-2 cells to Doxorubicin in a concentration-independent manner.

The combination of Doxorubicin (1 μ M) with Fidarestat (10 μ M) at the same point of time increased cell sensitivity to Doxorubicin. Approximately, a decrease of 15% in cell viability observed when Caco-2 cells were treated with Doxorubicin in combination with Fidarestat for 72hr compared to Doxorubicin alone (Fig.1A). In addition, about 5% decrease in cell viability was observed when cells were treated with the drugs combination for 48hr compared to Doxorubicin solo treatment (Fig.1B). However, the decrease in cell viability was greater when Fidarestat was applied 24hr before Doxorubicin (Approximately, a decrease of 12% in cell viability compared to Doxorubicin solo treatment) (Fig.1C). Moreover, using higher concentrations of Fidarestat (30, 50 μ M) did not increase cell response to Doxorubicin, which indicated that Doxorubicin sensitivity increment is Fidarestat concentration-independent.

Figure 1: MTT assay results showing the effect of Doxorubicin (1 μ M) alone or in combination with Fidarestat (10, 30, 50 μ M). (A) Caco-2 cells viability after 72hr of the same time of point treatment with the drug combination (B) Caco-2 cells viability after 48hr of the same time of point treatment with drug combination. (C) Cell viability after 24hr treatment with Fidarestat then Doxorubicin for 48hr. Values are Mean $\pm$ SEM (n = 6), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: p > 0.05.

3.2. Fidarestat pre-treatment increased Doxorubicin-induced cell death

Fidarestat induced cell apoptosis in small percentage of cells (Figure.2. B). whereas Doxorubicin induced necrotic cell death (Figure.2.C). Moreover, the number of necrotic cells was significantly increased when Fidarestat was applied 24hr before Doxorubicin (about 33.92% compared to 26.88% in Doxorubicin solo treatment) (Figure.2.D).

Figure2: Cell death in Caco-2 cells treated with Doxorubicin alone or in combination with Fidarestat for 48hr: Annexin V/PI staining analyzed by flow cytometry. (A) Control. (B) 30 μ M Fidarestat. (C) 1 μ M Doxorubicin. (D) 1 μ M Doxorubicin in combination with 30 μ M Fidarestat.

3.3. Fidarestat pre-treatment induced S phase arrest and increased Doxorubicin-induced cell cycle arrest in the G2/M phase

Cell cycle analysis results showed that Fidarestat treatment increased cell number in the S phase compared to control cells (from $24.32 \pm 0.74\%$ to $29.98 \pm 0.59\%$) (Figure.3A–B). Doxorubicin treatment alone significantly increased cell population in the G2/M phase compared to control cells (from $13.38 \pm 0.87\%$ to $21.83 \pm 0.12\%$) with a slight increase in sub-G1 population (from $7.04 \pm 0.64\%$ to $10.18 \pm 0.32\%$) (Figure.3C). Interestingly, Doxorubicin combination with Fidarestat induced significant cell cycle arrest at the S phase ($32.28 \pm 0.27\%$ compared to $24.32 \pm 0.74\%$ in control cells), which didn't observe in Doxorubicin solo treatment (Figure.3D). Furthermore, it increased cell population in the G2/M phase compared to Doxorubicin solo treatment (from $21.83 \pm 0.12\%$ to $26.06 \pm 0.8\%$) (Figure.3D).

Figure.3: Cell cycle in Caco-2 cells treated with Doxorubicin alone or in combination with Fidarestat for 48hr; PI staining analysed using flow cytometry. (A) Control. (B) 30 μ M Fidarestat. (C) 1 μ M Doxorubicin. (D) 1 μ M Doxorubicin in combination with 30 μ M Fidarestat. (E) Differences in cell cycle phase percentage relative to total phases. Each data point represents the Mean ($\pm$ SEM) of two independent experiments.

4. Discussion

In our present study, we have demonstrated that Fidarestat sensitized Caco-2 cells to Doxorubicin treatment, which is consistent with Sonowal et al (2017) results, who demonstrate that Fidarestat increases Doxorubicin-sensitivity in HT29 and SW480 cells [12]. However, we further found that this increment is Fidarestat concentration- independent. We believe that the chemo-sensitizing effect of Fidarestat is a result of the inhibition of drug transporters [14]. It has been reported that Fidarestat pre-treatment prevents Doxorubicin-induced upregulation of drug transporters and limits the efflux of Doxorubicin, which in turn enhances its intracellular accumulation [12]. This could explain the observed increase in Doxorubicin-sensitivity in our study. In addition, Fidarestat chemo-sensitizing effect could be a result of preventing Doxorubicinol formation. Since AR catalyzes the conversion of Doxorubicin to the less toxic Doxorubicinol [15,16], it is possible that the inhibition of AR could have prevented Doxorubicin conversion to Doxorubicinol, resulted in a higher level of Doxorubicin and Consequently enhances its cytotoxic effect.

Another important finding was that Fidarestat induced cell apoptosis in a small percentage of cells, which is consistent with a previous study on cholangiocarcinoma that used Epalrestat to inhibit AR [17]. It is likely that Fidarestat treatment induced cell apoptosis because it is a specific inhibitor of AR isoform AKR1B10, which is involved in cancer cell proliferation. However, further research should be undertaken to investigate the apoptotic effect of Fidarestat. Additionally, our study found that treatment of cells with Doxorubicin in combination with Fidarestat led to a significant increase in necrotic cells. The observed increase in cell death might have been a result of the increased accumulation of Doxorubicin that led to more cytotoxicity. However, another explanation might be obtained from the cell cycle analysis. As mentioned in the literature, Doxorubicin causes cell cycle arrest in various types of cancer through intercalation into cellular

DNA in the S phase, or the G2/M phase, it also poisons Topoisomerase II resulting in S and G2/M phase arrests [18–20]. In our current study, Doxorubicin solo treatment resulted in G2/M phase arrest but didn't result in S phase arrest. However, Fidarestat pre-treatment resulted in Doxorubicin-induced S phase arrest. This result may be explained by the fact that tumour cells could be sensitized to Doxorubicin by agents that induce S-phase accumulation, which generates higher sensitive-cell population to S-phase specific agents such as Doxorubicin [21,22]. Thus, the observed S phase arrest resulted from Fidarestat pre-treatment could be attributed to Fidarestat-induced cell cycle arrest in the S-phase, which led to excessive sensitivity to Doxorubicin. Besides, Ling et al (1996) reported that synchronized P388 cells in the S phase were more sensitive to Doxorubicin than cells in the G1-phase [23], which supports our vision that Fidarestat-induced S phase arrest could be partly the reason for potentiating Doxorubicin cytotoxicity. Moreover, Fidarestat pre-treatment resulted in a greater cell accumulation in the G2/M phase in Doxorubicin treated cells. Several reports have shown that Increasing G2/M arrest is associated with enhanced cell death [24]. Hence, Fidarestat pre-treatment increased cell death by increasing cell arrest in the G2/M phase. Altogether, these results indicate that Fidarestat sensitized Caco-2 cells to Doxorubicin through potentiating Doxorubicin cell cycle-dependent cytotoxicity, which gives a clearer interpretation of the chemo-sensitizing effect of Fidarestat.

In conclusion, our study showed that Fidarestat improved Doxorubicin-induced cytotoxicity in CRC cells through inducing cell cycle arrest and enhancing cell death. This demonstrates new explanation to the sensitizing effect of Fidarestat to Doxorubicin and providing a strong evidence that a potent and clinically safe AR inhibitor, Fidarestat, could be a candidate as an adjuvant drug to increase the cytotoxicity of Doxorubicin in CRC cells.

201 **5. Limitations**

202 This study had some limitations. First, we have used only one cell line, further studies on another
203 cell lines should be conducted to confirm our results. Second, no replication for cell death analysis
204 because of the limited quantity of Fidarstat, which also limited our research work and our
205 investigation on cell death and cell cycle; Hence future studies are necessary in order to elucidate
206 the molecular mechanisms underlying the effect of Fidarestat on cell death and cell cycle.

207 **Abbreviations:**

208 CRC: Colorectal cancer

209 AR: Aldose Reductase

210 DMEM: Dulbecco's Modified Eagle's medium

211 FBS: Fetal Bovine Serum

212 DPBS: Dulbecco's Phosphate-buffered Saline

213 MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

214 DMSO: Dimethylsulfoxide

215 GFP: Green Fluorescent protein

216 DOX: Doxorubicin

217 FID: Fidarestat

218 AKR1B10: Aldo-keto reductase family 1 member B10.

219 **Declarations'**

220 **Ethics approval and consent to participate**

221 Not applicable.

222 **Consent for publication**

223 Not applicable.

224 **Availability of data and materials**

225 All data generated or analyzed during this study are included in this published article.

226 **Competing interests**

227 The authors declare that they have no competing interests.

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230 **Authors' contributions**

231 MH and FM designed the study. MH carried out the laboratory work. MH and Abdulmunim

232 Aljapawe analyzed the data. MH drafted the paper. FM revised the paper. All authors read and

233 approved the final paper

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Figures

Figure 1

MTT assay results showing the effect of Doxorubicin (1 μ M) alone or in combination with Fidarestat (10, 30, 50 μ M). (A) Caco-2 cells viability after 72hr of the same time of point treatment with the drug combination (B) Caco-2 cells viability after 48hr of the same time of point treatment with drug combination. (C) Cell viability after 24hr treatment with Fidarestat then Doxorubicin for 48hr. Values are Mean $\pm$ SEM (n = 6), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: p > 0.05.

Figure 2

Cell death in Caco-2 cells treated with Doxorubicin alone or in combination with Fidarestat for 48hr: Annexin V/PI staining analyzed by flow cytometry. (A) Control. (B) 30 μ M Fidarestat. (C) 1 μ M Doxorubicin. (D) 1 μ M Doxorubicin in combination with 30 μ M Fidarestat.

Figure 3

Cell cycle in Caco-2 cells treated with Doxorubicin alone or in combination with Fidarestat for 48hr; PI staining analysed using flow cytometry. (A) Control. (B) 30μM Fidarestat. (C) 1μM Doxorubicin. (D) 1μM Doxorubicin in combination with 30μM Fidarestat. (E) Differences in cell cycle phase percentage relative to total phases. Each data point represents the Mean ($\pm$ SEM) of two independent experiments.