Silibinin improves the cytotoxicity of methotrexate in chemo resistant human rhabdomyosarcoma cell lines

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ABSTRACT

الأهداف: دراسة إن كان للسيليبينين دور في السيطرة على المقاومة الكيميائية لميتوتريكسات الساركومة العضلية المخططة البشرية.

الطريقة: أجريت هذه الدراسة في قسم الأدوية والسموم، كلية الصيدلة، جامعة بغداد، بغداد، العراق خلال الفترة من اكتوبر 2012م حتى مارس 2013م. أجريت دراسة في المختبر لتحريض مقاومة MTX في خلية مثوتركسات المقاومة للساركومة العضلية. المخططة البشرية وتم علاج الخلايا بتراكيز مختلفة من MTX، أو السليبين لوحده، أو كلاهما. قمنا بتحديد حياة الخلية باستخدام مقايسة MTT.

النتائج: يساعد SDH في طريقة تركيز الخلايا المعتمدة على مقاومة الحساسية في خلايا MTX للوصول إلى أعلى تركيز سام لخلايا MTX، ويقلل من IC50 في MTX حوالي 17.8 طية. كما ارتبط انخفاض IC50 لخلايا MTX بشكل عكسي مع ارتفاع تركيز SDH (R=0.78، p=0.04).

خاتمة: يحسن SDH من حساسية ميتوتريكسات الساركومة العضلية المخططة البشرية للنشاط السمي لخلايا MTX في طريقة تركيز الخلايا المعتمدة.

Objectives: To investigate whether silibinin (SDH) could overcome chemoresistance of methotrexate (MTX)-resistant human rhabdomyosarcoma (hRD).

Methods: This study was conducted at the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq from October 2012 to March 2013. In this in vitro study, resistance to MTX was induced in hRD cell line, the cells were treated with different concentrations of MTX or SDH alone, and in combination. Cell viability was determined by tetrazolium assay.

Results: The SDH in a concentration-dependent pattern, enhanced the sensitivity of MTX-resistant cells to the maximum cytotoxic concentration of MTX, and decreased the IC_{50} (concentration resulting in 50% inhibition of cell growth) of MTX by 17.8 fold. The decrease in IC_{50} of MTX was negatively correlated with increasing SDH concentrations with $R^2 = 0.78$ and $p=0.04$.

Conclusion: The SDH improves the sensitivity of MTX-resistant hRD cell lines to the cytotoxic activity of MTX in concentration-dependent pattern.

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Drug resistance is one of the main causes of treatment failure and mortality in cancer patients. Drug resistance concomitant with an invasion has been found in many cancer cells isolated from patients with different types of tumors,[1] that cells have these properties may be due to an increase in metastatic potential following chemotherapeutic insults.[2] Thus, there is an urgent need for novel treatment strategies to overcome drug resistance and tumor metastasis. Methotrexate (MTX) is used, mostly in combination with other cytotoxic agents, for the treatment of many neoplasms including acute leukemia and certain solid tumors,[3] it is also used in non-neoplastic disorders, and as an anti-inflammatory

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and/or immunosuppressive drug.[4,5] After transport into the cytoplasmic milieu through the reduced folate carrier, MTX was polyglutamated through an energy consuming reaction catalyzed by folylpolyglutamate synthase (FPGS).[6] Resistance to the effect of MTX was reported after prolonged treatment with consequent attenuation of its efficacy. However, combination treatment of MTX with other drugs that could modulate the expression of genes involved in MTX resistance, and be an adequate strategy to prevent the development of resistance.[7] Many molecular mechanisms are suggested for MTX resistance, including decreased cellular uptake via reduced folate carriers (RFC).[8] The increase of MTX extrusion to the extracellular compartments was mainly attributed to the excessive expression many membrane-bound multi-drug resistance proteins, especially MRP1-4 group that contributed to a specific pattern of MTX resistance.[9] Treatment failure and remarkable toxicity of chemotherapeutic agents encouraged the search for many alternative approaches like the use of phytochemicals, which are thought to be effective with a wide margin of safety. Moreover, many current advances in drug development have revealed cancer preventive and curative efficacies of many phytochemicals.[10] Silibinin di-hemisuccinate (SDH), a flavonoid antioxidant from milk thistle (*Silybum marianum L.*), has been used extensively for many years for the treatment of liver disorders.[11] It recently received attention due to its chemopreventive and anticancer activity.[12] In vitro and in vivo studies have shown that SDH is a potent sensitizer for apoptosis induced by a range of anticancer drugs.[13,14] Additionally, several in vitro and in vivo combination studies of SDH and chemotherapeutic drugs were carried out to analyze the effects of such a combination on growth inhibition, cell cycle regulation, and apoptosis.[15-17] However, no report was found during the literature search for the involvement of SDH in sensitizing chemoresistant malignant cells to MTX. In this study, we evaluated the effect of SDH in increasing sensitivity of resistant human rhabdomyosarcoma (hRD) cells to MTX, and the dose response relationship for this effect.

Methods. This study was conducted at the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq from October 2012 to March 2013. Methotrexate (Lederle, Hampshire, UK) and SDH (Madaus, Koeln, Germany) stock solutions were obtained as pharmaceutical preparations at concentrations 2 mM (MTX) and 1 mM (SDH). These drugs were stored at 4°C. Immediately before use, they were diluted with RPMI 1640 prepared as for cell culture. The tetrazolium (MTT) (Sigma Chemical Co, Poole, Dorset, UK) was dissolved in phosphate buffered saline (PBS) to produce a stock solution of 5 mg/ml, which was stored at 4°C. The local scientific committee approved the research protocol. This study used human hRD line as previously described.[18] The hRD cell line was obtained from the Research Center of Biotechnology, Al-Nahrain University, Baghdad, Iraq. The hRD cell lines were maintained in RPMI 1640 (Sigma Chemical Co, Poole, Dorset, UK) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Scotland, UK), 2 mM L-glutamine (Sigma Chemical Co, Poole, Dorset, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were grown as attached monolayers, and were incubated at 37°C in a humidified atmosphere with 5% CO_2. Resistant hRD cells were obtained in the laboratory through the incubation with stepwise concentrations of MTX as previously described.[19] Cytotoxicity was assessed using the MTT assay as described by Mosmann.[20] Briefly, hRD cells ($2x10^5$ cells/mL) were plated in 100 µL in each well of 96-well round-bottomed microtiter plates. Methotrexate and SDH (each alone or in combinations), were added at the desired concentrations, and the plates were incubated at 37°C for 72 hours in a 5% CO_2 humidified atmosphere. At the end of the incubation period, 10 µL of a stock solution of 5 mg/mL MTT, were added to each well, and the plates were incubated for another 4 hours at 37°C. Absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (LabSystems Multiscan RC, Helsinki, Finland) at a wavelength of 540 nm with reference at 650 nm. Growth inhibition was measured by dividing the mean absorbance of treated wells per mean absorbance of control wells (drug-free wells), and is expressed as a percentage. The inhibitory concentrations of 50% of cells (IC_{50}) values were defined as the drug concentrations, at which cell growth was inhibited by 50% compared with drug-free controls. Values were expressed as mean ± standard deviation, the values were statistically evaluated using linear regression test, unpaired student's t-test, and one-way analysis of variance (ANOVA), supported by Bonferroni's post hoc analysis. Values with $p<0.05$ were considered significant. Analysis was performed using Graph Pad Prism software for Windows version 5 (Graph Pad Software Inc, San Diego, CA, USA).

Results. **Figure 1** shows the effect of different concentrations of MTX on the survival of MTX-sensitive hRD cell line, where maximum effect achieved

(14.2%) with 800 μM MTX. Meanwhile, in MTX-resistant hRD cells, the same concentration of MTX (800 μM) decreases cell survival only to 53.1%, and the IC_{50} for MTX was significantly increased ($p<0.05$) from 102.3 μM to 1258 μM (12.3 fold) (**Table 1**). In **Figure 2**, the maximum concentration of SDH (200 μM) decreases the MTX-sensitive cell survival to 68.7%, while in MTX-resistant cell line, this concentration of SDH decreased survival to 74%; the IC50 for SDH was significantly increased ($p<0.05$) by 5-fold, as shown in **Table 1**. **Figure 3** shows that incubation of MTX-resistant hRD cells with 800 μM MTX and increasing concentrations of SDH (0, 25, 50, 100 and 200 μM) improves the cytotoxic effect of the given concentration of MTX and produces dramatic decrease in cell survival, achieving only 2.4% survival with maximum concentration of SDH (200 μM). **Table 2** clearly shows that SDH decreases the IC_{50} for MTX in a concentration-dependent pattern, and the maximum SDH concentration significantly decreases ($p<0.05$) the IC_{50} for MTX to 17.8-fold compared to its value when used alone in MTX-resistant hRD cells. The decrease in IC_{50} of MTX was negatively correlated with increasing SDH concentrations, with $R^2 = 0.78$ and $p=0.04$ (**Figure 4**).

Discussion. Although many efforts are carried out to improve chemotherapeutic response in cancer cells, development of drug resistance remains a major challenge. In fact, only 50-60% of cancers are responsive to chemotherapy,[21] indicating that the outcome is still far from optimum. The use of alternative anti-cancer agents, particularly those obtained from natural sources, may be a good choice in this regard.[22] Silibinin is a natural chemical that shows promising anti-cancer activity in several studies, including a phase I clinical trials.[23,24] In vitro studies confirm that SDH at a range of 25-100 μmol/L inhibits cell viability, likely through

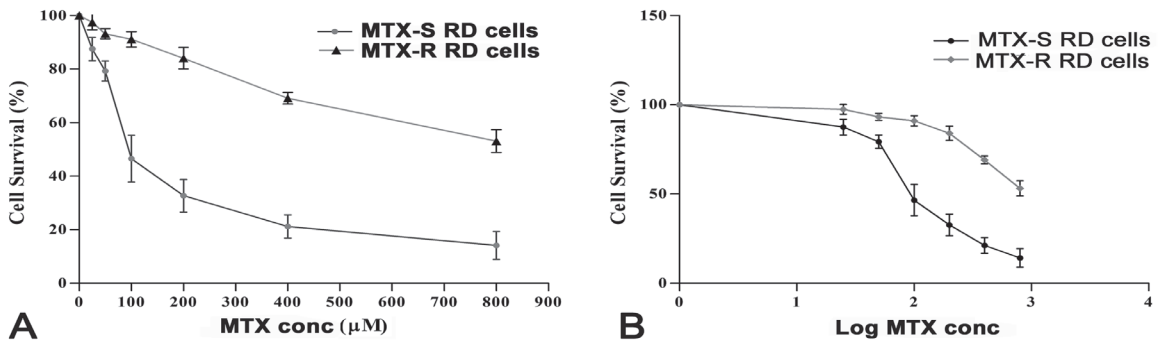

Figure 1 - Effects of different doses of methotrexate (MTX) on the survival of MTX-S (sensitive) and MTX-R (resistant) human rhabdomyosarcoma cell (RD) line expressed in ordinary (**A**), and log (**B**) concentration scale. SDH - silibinin

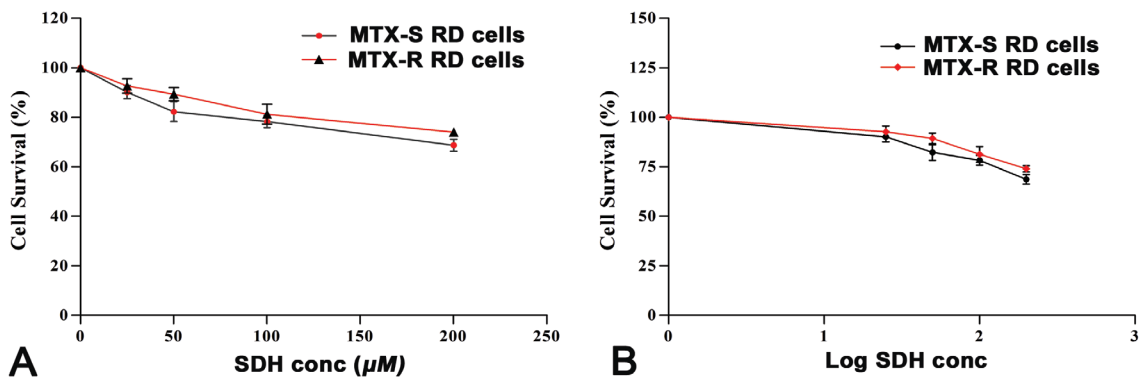

Figure 2 - Effects of different doses of silibinin on the survival of methotrexate (MTX)-sensitive (S) and MTX-resistant (R) human rhabdomyosarcoma (RD) cell line, expressed in ordinary (**A**), and log (**B**) concentration (conc) scale. SDH - silibinin

Table 1 - Sensitivity of MTX-sensitive and MTX-resistant hRD cell line to the effects of MTX and SDH (measured as IC_{50}).

Type of treatment	n	IC_{50} (μM)	
		MTX-sensitive hRD cells	MTX-resistant hRD cells
MTX	6	102.3 ± 13.4	1258 ± 168.1*
SDH	6	15848 ± 910.4	79432 ± 680.2*

IC50 - concentration resulting in 50% inhibition of cell growth, hRD - rhabdomyosarcoma; MTX - methotrexate, SDH - silibinin, *significantly different compared with MTX-sensitive hRD cells (p<0.05)

Table 2 - Effect of different concentrations of silibinin dihemisuccinate (SDH) on the sensitivity of MTX-resistant hRD cells to 800 μM MTX (measured as IC_{50}).

Silibinin (μM)	Methotrexate IC_{50} (μM)
0	1258 ± 168.1[a]
25	1122 ± 86.7[a]
50	562.3 ± 62.3[b]
100	158.5 ± 15.9[c]
200	70.8 ± 10.1[d]

IC_{50} - concentration resulting in 50% inhibition of cell growth, values with non-identical superscripts (a, b, c, d) are significantly different (p<0.05)

cell cycle arrest and induction of apoptosis.[25,26] While SDH has been evaluated in many types of tumors, very little is known regarding its effects in MTX-resistant hRD.

In the present study, we provide evidence that SDH improves the response to the cytotoxic effects in MTX-resistant hRD cells. There is a well-documented cross-resistance between MTX and SDH in MTX-resistant hRD cells. The IC_{50} of SDH in our model was 1258 ± 168.1 μM, and cytotoxicity was confirmed by MTT assay. These results are consistent with previous studies regarding the effect of the other polyphenol, the quercetin, in osteosarcoma cells.[27] In the present study, hRD cells were treated with different concentrations of MTX for 72 hand cell viability was measured by the MTT assay. As shown in **Figure 2** and **Table 1**, hRD cells were resistant to MTX in that the IC_{50} of MTX was 12.3-fold higher in these cells compared to their parent cell line. To investigate whether SDH was effective in improving sensitivity of resistant hRD cells to MTX, the cytotoxicity of combination of MTX with different concentrations of SDH in MTX-resistant hRD cells

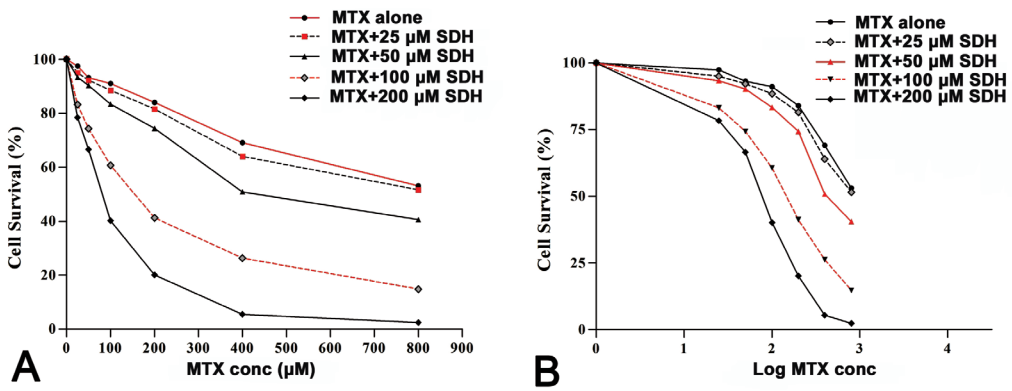

Figure 3 - Effects of different doses of silibinin on the survival of methotrexate (MTX)-resistant human rhabdomyosarcoma cells incubated with different concentrations of MTX, expressed in ordinary (**A**), and log (**B**) MTX concentration scale. SDH - silibinin

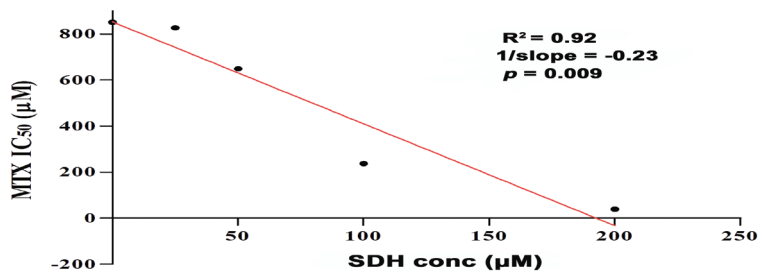

Figure 4 - Correlation between silibinin concentration and methotrexate (MTX) IC_{50} required to inhibit growth of human rhabdomyosarcoma cell line. IC_{50} - concentration resulting in 50% inhibition of cell growth, SDH - silibinin

was determined. The results showed that MTX/SDH combination produced a dramatic reversal of MTX resistance in hRD cells. As shown in **Table 2**, the IC_{50} for MTX decreased with increasing concentrations of SDH up to 200 μM. Silibinin reduced the IC_{50} of MTX, in a dose-dependent pattern from 1258 μM to 70.8 μM (decrease IC50 up to 17.8-fold). In addition to its well-known cytoprotective activity, SDH enhances the activity of chemotherapeutic agents.[28] In this respect, many researchers reported that SDH effectively increases the sensitivity of prostate cancer cells to the cytotoxicity induced by many chemotherapeutic agents, including doxorubicin, cisplatin, carboplatin, and mitoxantrone.[15-17] However, bioavailability of SDH was considered as a major problem that limits its therapeutic utility, it can be attributed to its large polyphenolic structure and low water solubility.[29] Accordingly, modified formulations have tried to solve this problem.[30] In many MTX-responsive cancers, the currently adopted treatment programs are based on a combination of MTX with other agents, including doxorubicin and cisplatin, where MTX is the most active one.[31] Despite the improvement of response, a considerable number of patients develop MTX resistance and die due to disease progression.[21] Accordingly, additional therapeutic agents need to be evaluated to improve the survival of MTX-resistant patients.

The use of sensitizers is one of the available strategies to reverse resistance to chemotherapy. Silibinin has been demonstrated to be an effective chemopreventive and chemotherapeutic agent,[32] without any toxic or adverse effects.[33,34] Therefore, it may have potential clinical application in combination chemotherapy. Although the present study did not reveal the exact mechanisms, through which SDH reverses chemoresistance, our results demonstrate that SDH produced a dramatic reversal of MTX resistance and enhanced MTX-induced cytotoxicity in hRD cells resistant to MTX. This finding in agreement with that reported by Zhou et al,[35] where SDH significantly restores the sensitivity of chemoresistant human ovarian carcinoma cells to paclitaxel. Many mechanisms are suggested for the antiproliferative activity of SDH, including the inhibition of the Cdk4 pathway, increase in p53 expression, down-regulation of surviving and cyclin D1, phosphorylation of ERK1/2 and nuclear phospho-p65, cleavage of caspases, and mitochondrial release of cytochrome c, and apoptosis-inducing factors.[36-39] Additionally, MTX is known to be a P-glycoprotein (P-gp) substrate, and emergence of resistance may be due to over expression of this membrane protein;[40] the inhibition of P-gp by SDH may lead to increased accumulation of MTX with a consequent increase in sensitivity of resistant cells to the cytotoxic activity of MTX.[41] Further studies are required to investigate the exact molecular mechanisms behind the effect of SDH in this regard.

In conclusion, SDH improves the sensitivity of MTX-resistant hRD cell lines to the cytotoxic activity of MTX in concentration-dependent pattern.

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