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RESEARCH ARTICLE

Chemotherapeutic activity of Silymarin combined with doxorubicin liposomes in 4T1 breast cancer cells

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ABSTRACT

Cancer is the second leading cause of death worldwide. Despite great efforts over many years, today cancer treatment is not very effective. The main reasons for cancer chemotherapy failure are high cytotoxicity, low response rates in solid tumors, and development of resistance. Different experimental studies have shown that drug combination using low toxicity natural compounds such as polyphenols can reduce the required dose of cytotoxic drugs for cancer treatment. The polyphenolic compound, Silymarin (SLM), is an active extract from the seeds of the plant milk thistle (Silybum Marianum). It is well known for its hepatoprotective, antioxidant and chemoprotective effects. In the present study, we investigated whether the combination of Silymarin with Caelyx® (commercial doxorubicin liposome (DXL)) could enhance the cytotoxicity on 4T1 breast cancer cells *in vitro*. For this, 4T1 breast cancer cells were exposed to Silymarin, DXL and their combination at different molar ratios, to elucidate if the two drugs could dictate synergistic effect *in vitro*. Results indicated that SLM-DXL combination at 100 and 300 molar ratios, exert synergistic growth-inhibitory effects. These synergistic effects were observed only at lower SLM-DXL concentrations. In conclusion, it is conceivable that in SLM-DXL combination chemotherapy, drug ratios play a key role which determine the final response following treatment. Thus, using liposomes as targeted drug delivery systems, it would be possible to achieve appropriate combination of the two drugs at correct doses and correct administration intervals clinically.

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INTRODUCTION

Cancer is the second cause of death worldwide. It is expected that its incidence is growing worldwide due to the aging of the population (1). Despite intensive research aimed at discovering anticancer agents and different improvements made in chemotherapeutic regimens, cancer treatment today is inadequate: it is not very effective and accompanied by several side effects (2). Indeed, most chemotherapeutic agents effectively target fast dividing cells and there is no distinction in their functionality between cancerous cells and the fast growing normal cells. Therefore, normal cells are

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also damaged and a wide variety of side effects, such as vomiting, nausea, cardiotoxicity, hepatotoxicity, nephrotoxicity, ototoxicity, immunosuppression, myelosuppression, hemorrhage, anemia, malnutrition, and non-specific neurocognitive problems are observed (3).

One prototypic example of cytotoxic drugs is doxorubicin (DXL). It is an antitumor anthracycline antibiotic commonly used to treat a variety of cancers. Despite the introduction of doxorubicin against malignant tumors, its use in clinical chemotherapy is limited due to progressive and clinically significant cardiotoxic effects (4, 5). The above mentioned toxic manifestations clearly suggest that there is an urgent need for nontoxic and clinically effective treatments of cancer that eliminate problems associated with conventional chemotherapies (2).

One of the main reasons of failure in cancer treatment is high cytotoxicity, development of resistance, and low response rates of chemotherapy in solid tumors (6). It is clear that the dosage of a cytotoxic drug is a critical factor in both its effectiveness and toxicity. In the low doses, it will not be effective against tumor, but with increasing the dose, the toxicity signs are prominent (3).

In recent years, with the aim of developing more efficacious strategies while reducing systemic toxicity, efforts have been directed toward combination therapy. Naturally occurring agents with different mechanisms of action and non-overlapping toxicities, are suggested as a promising candidates in synergistic combination therapy of cancer (7-9).

Several experimental studies suggested using combination of low toxic natural compounds such as polyphenols, to reduce the required dose of cytotoxic agents in the treatment of cancer (10-14).

Silymarin (SLM) is an active extract from the seeds of the plant milk thistle (*Silybum Marianum).* The plant milk thistle has been used by mankind for a long time as a food in some parts of the world (15-17). SLM extract and its main component, silybin, are well known for their antioxidant, hepatoprotective and chemoprotective effects. Recently, increasing evidences highlighted significant anti-neoplastic activity of these agents in a variety of *in vitro* and *in vivo* cancer models, including skin, breast, lung, colon, bladder, and prostate (4, 5, 17, 18).

On the other hand, the modulatory effect of SLM on some chemotherapeutic drugs was demonstrated by its ability to strengthen the cytotoxic effect of doxorubicin and cisplatin against breast and prostate cancer cells (13, 19-22). However, so far no study has been carried out to determine the effects of different drug ratios on the chemotherapeutic efficacy of the combination of the two drugs.

In the present study, we investigated cytotoxic effects of SLM-DXL combination at different molar ratios, in liposomes as a nanocarrier, on 4T1 breast cancer cells and focused our efforts on elucidating which ratios of the two drugs in combination, could show antitumor activity *in vitro.*

MATERIALS AND METHODS

Materials

SLM was obtained from sigma (USA). Commercially available doxorubicin liposomes, Caelyx®, was purchased from Behestan Darou Company (Tehran, Iran). Roswell Park Memorial Institute 1640 medium (RPMI 1640) was purchased from GIBCO (USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was from Promega (Madison, WI). 4T1 cell line was obtained from Pasteur Institute (Tehran, Iran).

Trypan blue, isopropanol, DMSO and chloroform were purchased from Merck (Darmstadt, Germany). All other solvents and reagents were used as chemical grade.

Combination study

SLM was dissolved in DMSO at the final concentration of 40 mg/ml. For MTT, the concentration ranged from 5-500 nmol/ml. For Caelyx®, the concentration ranged from 0.1-2 nmol/ml. For combination study, SLM/DXL were used at 100, 300, 600 and 1200 molar ratios, respectively.

Cell viability assay

MTT assay was used to determine cell viability. For this, 96-well plates were seeded with 2500 4T1 cells/well. Each plate included untreated cells and medium without cells, respectively. After an overnight incubation at 37 °C, 5% CO_2, the medium was carefully aspirated off, avoiding removal of the cells and replaced with fresh medium (200 µl) containing up to 100 µl of serial dilution of drug formulations. Then, plates were incubated at 37 °C, 5% CO_2 for 48 hours. Four hours before ending incubation, the medium was carefully aspirated off and replaced with 100 µl FCS free cell cultured medium containing 10 µl of MTT solution. In living

cells, mitochondrial dehydrogenases can convert soluble MTT yellow dye to an insoluble purple formazan precipitate by cleavage of the tetrazolium ring. This conversion has been used to develop an assay system for measurement of cell viability. The produced insoluble formazan was dissolved by adding 200 µl DMSO (Merck, Germany) and its optical density (OD) was read with a multi-well scanning spectrophotometer at a wavelength of 570 nm. 4T1 cell cultured wells containing 200 µl RPMI cell culture medium used as positive control in each plate (23).

The percentage of cytotoxicity was calculated according to following formulas:

$$\%\text{Cytotoxicity} = 100 * \left(1 - \frac{\text{mean absorbance of drug treated cells} - \text{mean absorbance of blank}}{\text{mean absorbance of positive control cells} - \text{mean absorbance of blank}}\right)$$

$$\%\text{Viability} = 100 - \%\text{Cytotoxicity}$$

The half maximal inhibitory concentration (IC_50) values of DXL and SLM and combination indexs (CI) of mixture thereof in 4T1 cells were calculated by the CalcuSyn software Version 2.1 (Biosoft, Cambridge, UK). The drugs interaction was claimed synergistic if CI<1, and antagonistic if CI>1.

RESULTS AND DISCUSSION

Cytotoxicity of DXL and SLM were measured using MTT assay. The IC_{50} values for SLM and DXL in 4T1 cells are shown in Table1. Table 2 shows the IC_{50} values for combined SLM and DXL in 4T1 cells at different of SLM to DXL molar ratios.

Results of the *in vitro* study suggest that apart from individual agents comprising the combination, the ratios and dosages of each agent are also critical for obtaining synergistic or antagonistic effects.

Table 1. Half maximal inhibitory concentration values (IC_{50}s) of SLM and DXL against 4T1

Cell line	IC_{50}(nM/ml)	
	SLM	DXL
4T1	77.36 ± 14.21	0.39± 0.15

Table 2. Half maximal inhibitory concentration values (IC_{50}s) of combined SLM and DXL in 4T1 cells

	IC_{50} (nM/ml)	
SLM:DXL Molar ratio	SLM	DXL
100	2.22 ± 0.20 [a]	0.02 ± 0.005 [b]
300	7.58 ± 1.16 [c]	0.02 ± 0.004
600	140.16 ± 24.30	0.22 ± 0.04
1200	137.80 ± 5.37	0.11 ± 0.01

[a] p<0.006, [b] p<0.02 , [c] p<0.007

The combination therapy has known as an important strategy for better long-term prognosis with reduced side effects in the treatment of cancer (24). Different studies have shown that various combinations of drugs and polyphenols or other natural products can work through the multiple mechanisms such as influencing on different biosynthetic pathways, blocking the functioning and maintenance of some essential macromolecules (25). Polyphenols are among phytochemicals that bear excellent anti-oxidant and anti-inflammatory features, as well as modulatory effects on the cell signaling pathways. The use of these compounds however, as anti-cancer agents alone may not be as effective. Promising *in vitro* and *in vivo* experiments highlights the efficacy of using polyphenols in combination with chemotherapeutics over the conventional anti-neoplastic drugs and their possible application in the clinical settings (26). For example, liposomes bearing combination of doxorubicin and curcumin showed the highest cytotoxicity against A549 cells compared to doxorubicin liposomes (27).

Considering the synergistic and antagonistic action of SLM on DXL and other cytotoxic drugs, some studies suggest that this effect is partially due to the influence of SLM on the active transport of drugs through the cell membrane transporters (20, 28, 29). Some cell membrane transporters such as P-gp (P-Glycoprotein), BCRP (breast cancer resistance protein) and MRPs (multidrug resistance protein) are drug efflux transporters. They are able to efflux various anticancer drugs out of the cancer cells. Therefore, overexpression of such transporter proteins can lead to a significant decrease in the intracellular drug concentrations, resulting in MDR (Multidrug-resistant) to a broad spectrum of cytotoxic drugs (29). It is reported that SLM can inhibit the action of P-gp, BCRP and MRP-1 through a competitive inhibition of substrate transport mechanism. It is the most common mechanism appears to be involved, but other mechanisms have also been reported (28, 30). According to our results, it seems that only the lower concentrations of SLM could inhibit doxorubicin transportation through the membrane transporters.

Though in several experimental *in vitro* studies, silybin enhanced cytotoxicity of various chemotherapieutics including doxorubicin,

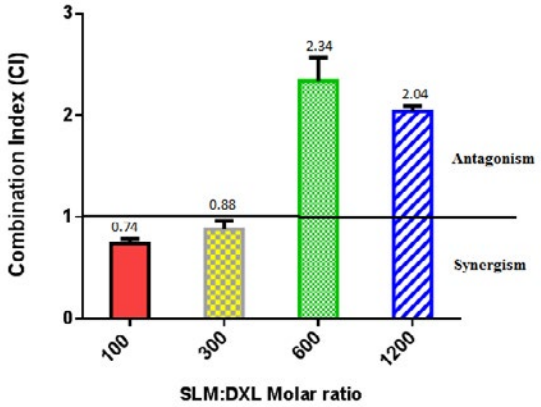

Fig. 1. Combination indexes (CIs) of DXL and SLM mixture at different SLM to DXL molar ratio in 4T1 cells. Plates were seeded with 2500 4T1 cells/well. After an overnight incubation, the medium was replaced with medium containing serial dilution of drug combination at SLM:DXL 100, 300, 600 and 1200 molar ration. After 48 hours' incubation, the medium was replaced with medium containing MTT. The optical density (OD) was read with a multi-well scanning spectrophotometer at a wavelength of 570 nm.

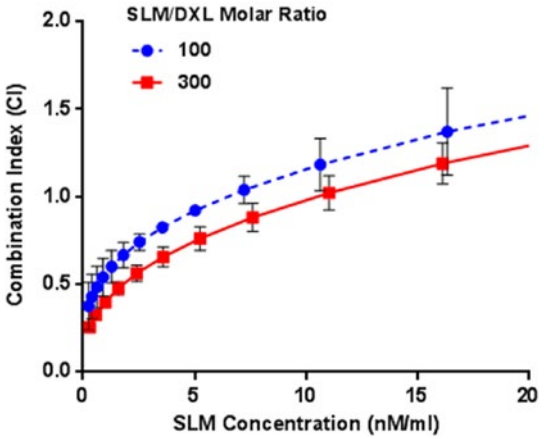

Fig. 2. Combination indexes (CIs) of DXL and SLM mixture at the different SLM concentrations in 4T1 cells. Plates were seeded with 2500 4T1 cells/well. After an overnight incubation, the medium was replaced with medium containing serial dilution of drug combinations at increasing SLM concentrations (0-20 nM/ml). After 48 hours' incubation, the medium was replaced with medium containing MTT. The optical density (OD) was read with a multi-well scanning spectrophotometer at a wavelength of 570 nm.

cisplatin and carboplatin in different cell lines (13, 20, 31), this information for prediction of clinical activity has been questionable.

While in an *in vitro* system, the drug concentrations and their ratios can be tightly controlled, following administration of combined conventional anticancer agents in an animal model, each individual agent will be distributed, metabolized and eliminated independently of the other. Therefore in the most cases, it can be very complicated to control the ratio of the combined drugs reaching the tumor site (32).

As shown in (Tables 1) and (Table 2), there are significant differences in the cytotoxicity of SLM and DXL compared with their combination at 100 ($p<0.006$ and $p<0.02$) and 300 ($p<0.007$ and $p<0.02$) SLM- DXL molar ratios, respectively on 4T1 cells.

Combination indexes (CIs) of DXL and SLM mixture at different molar ratios of SLM to DXL in 4T1 cells are shown in (Fig. 1). Results indicate that synergistic effects are present only at the lower concentrations of SLM-DXL (100 and 300 molar ratios), and at higher ratios antagonistic effects were observed. (Fig. 2) and (Fig. 3) revealed that at these molar ratios, synergistic effects can only be observed at the lower concentrations of SLM and DXL.

The results of the present study indicate that SLM can synergize the cytotoxic effects of DXL

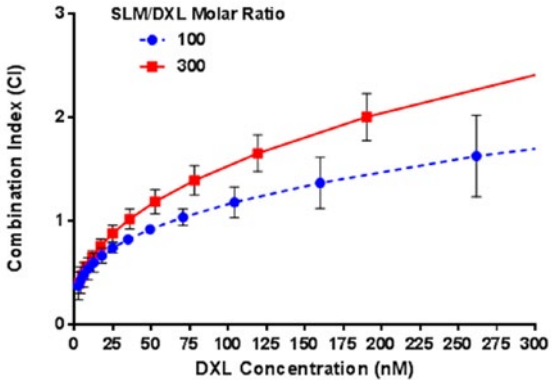

Fig. 3. Combination indexes (CIs) of DXL and SLM mixture at the different DXL concentrations in 4T1 cells. Plates were seeded with 2500 4T1 cells/well. After an overnight incubation, the medium was replaced with medium containing serial dilution of drug combinations at increasing concentrations of DXL (0-300 Nm). After 48 hours' incubation, the medium was replaced with medium containing MTT. The optical density (OD) was read with a multi-well scanning spectrophotometer at a wavelength of 570 nm.

only at lower molar ratios of SLM-DXL (100, 300), and at higher ratios, antagonistic effects were observed. It has also been shown that at these molar ratios, synergistic effects can be observed only at lower SLM (< 10 nM/ml) and DXL (<40 nM) concentrations, respectively.

CONCLUSION

Some targeted drug delivery systems, such as the liposomes can provide opportunities to maintain drug ratios following injection. They can minimize first-pass metabolism/distribution of entrapped drugs and thus induce their accumulation at the tumor site. Together, such targeted drug delivery systems with capability of simultaneous co-encapsulation of two or even more drugs can control pharmacokinetic of drug combinations that may not be achieved with conventional formulations.

In conclusion, the success of combination chemotherapy could be provided by using targeted drug delivery systems possessing correct combination of drugs at the correct doses and correct administration intervals. The current study focused on the combination of SLM with commercial doxorubicin liposomes *in vitro*. However, based on the current promising results, it can be further developed to liposomes co-encapsulating these two agents. In this manner, we will be able to test the ratio-dependent antitumor activity of combined drugs *in vivo*. However, further in-depth mechanistic studies, *in vivo* animal experiments are needed to test the value of SLM and its components in combination therapy of cancers.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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