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ORIGINAL ARTICLE

Effects of silymarin and silymarin-doxorubicin applications on telomerase activity of human hepatocellular carcinoma cell line HepG2

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Summary

Purpose: *Hepatocellular carcinoma (HCC) is resistant to conventional chemotherapeutics such as doxorubicin. Milk thistle extract, or its active constituent silymarin has been used by cancer patients as an alternative and complementary agent. Telomerase activation is one of the initial events of HCC. In this study, we applied doxorubicin and silymarin for 72 hrs in order to test individual and combined effect of the agents on telomerase activity.*

Methods: *The effects of doxorubicin, silymarin, and their combination on the proliferation of HepG2 cell line were tested by MTT assay, and Checkerboard micro plate method was applied to define the nature of doxorubicin and silymarin interactions on the cells. Lipid peroxidations were assessed by thiobarbituric acid reactive substance (TBARS) level. Telomerase activity was determined according to the telomeric repeat amplification protocol (TRAP). Untreated cells were used as control group.*

Results: *Doxorubicin-silymarin combination had indifferent antiproliferative effects on HepG2 cells. Telomerase activity of the cells incubated with IC_{50} of doxorubicin and silymarin decreased to 72% ($p<0.05$). IC_{50} combinations of doxorubicin and silymarin caused 70% ($p<0.05$) reduction. All treatments except for the $½IC_{50}$ of silymarin caused significant increase in lipid peroxidation levels when compared to controls. TBARS levels did not significantly increase when doxorubicin and silymarin were applied in combination, which is in concordance with the indifferent drug interaction.*

Conclusion: *IC_{50} of both doxorubicin and silymarin alone and in combination inhibited telomerase activity. Mechanism of inhibition may be elucidated by further molecular studies.*

Key words: *checkerboard, doxorubicin, hepatocellular carcinoma, silymarin, telomerase activity*

Introduction

HCC is one of the most common cancers worldwide causing 250,000 deaths annually [1]. Liver transplantation and surgical resection considerably decrease mortality and increase survival, although these therapies are still effective in a restricted number of patients [2]. HCC is also highly resistant to conventional systemic chemotherapy. Only partial responses are obtained with doxorubicin among the chemotherapeutics used. Doxorubicin is a topoisomerase II inhibitor, DNA intercalator leading to DNA strand breaks and formation of reactive oxygen species (ROS) in cells [3-5].

Telomerase is a reverse transcriptase which synthesizes telomeric DNA by using its RNA as template. The enzyme is a ribonucleoprotein complex composed mainly of human telomerase reverse transcriptase (hTERT), telomerase associated protein (TEP1), and telomerase RNA (TERC). Reactivation of telomerase in cancer cells is par-

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ticularly important for cellular immortalization, making the telomerase a chemotherapy target. Telomerase is regulated both by special proteins such as p16 proteins and epigenetic mechanisms [6]. hTERT expression is the rate-limiting determinant of telomerase activation in HCC, and its activity increases according to the HCC grade of histological differentiation [7,8]. Increasing selectivity of chemotherapeutic agents to reduce their toxicity to normal cells is one of the most challenging issues when developing new chemotherapy strategies. Since most human cancers acquire the ability to activate telomerase and possess shorter telomeres as compared to most normal somatic tissues, inhibition of telomerase has become a potential target for selective cancer chemotherapy [9].

Silymarin, a flavonolignan, is extracted from the milk thistle (Silybum marianum) seeds. The plant is indigenous in Europe, USA and South America. Silymarin is composed of four flavonoid isomers: silibinin, isosilibinin, silydianin and silychristin. Silymarin and silibinin have been used as hepatoprotective agents against liver damage caused by hepatitis, cirrhosis, alcohol, and fungi for 2000 years. Silymarin has antioxidant and anticarcinogenic affects, and has been used as a complementary and alternative agent by cancer patients [10,11]. In the past 30 years, it is clinically used in Europe and Asia in the commercial form of Legalon™ and Silipide IdB1016 [12].

In this study we aimed to investigate in vitro interactions and combined effects of doxorubicin and silymarin on telomerase activity of HepG2 cells. In order to evaluate the effects on oxidative damage, lipid peroxidation levels were also tested.

Methods

Cell culture

In the present study, HepG2 human HCC cells, which are capable of metabolizing many nutrients, metabolites and xenobiotics were used. Cells were purchased from ATCC (American Type Culture Collection), and cultured according to the protocol provided by the ATCC as an attached type monolayer culture. Cells were maintained in Dulbecco's minimal essential medium (DMEM; Biochrom AG, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG), L-glutamine (Biochrom AG) and penicillin (10 000 U/ml and streptomycin (10 000 µg/ml) (Biological Industries, Israel) at 37°C in a humidified atmosphere of 5% CO_2 (Heraeus, Hanau, Germany).

Assay for cell proliferation

The effects of doxorubicin (AppliChem, Darmstadt, Germany), silymarin (Sigma-Aldrich, USA), and their combination on the proliferation of HepG2 cells were colorimetrically tested by biochemical reduction of MTT (3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich). Doxorubicin and silymarin were diluted from high to low concentrations (32-0.06 µM and 2400-4.69 ng/mL for doxorubicin and silymarin, respectively) and placed horizontally in 96-well microtiter plates. Cells were seeded to each well ($1x10^5$) with the exception of medium controls. The plates were incubated for 72 hrs, and then 20 µL of MTT solution (5 mg/mL) were added to each well. After incubation for 4 hrs, 100 µL sodium dodecyl sulfate (Sigma-Aldrich) solution (10% w/v) was added to each well. The plates were incubated overnight to allow the dissolution of formazan crystals. The inhibition of cell proliferation was determined by measuring the optical density of the chromogenic product at 540 nm with an ELISA reader (Biotek Instrument ELx800, USA). Inhibition of cell proliferation and inhibitory concentrations 50 (IC_{50}) values, which are drug concentrations at which 50% of cells are viable, were calculated from the logarithmic trend lines of the proliferation graphs.

A Checkerboard micro plate method was applied to study the effects of doxorubicin and silymarin interactions on cells as previously described [13]. The dilutions of doxorubicin (A) were made horizontally and the dilutions of silymarin (B) vertically in a microtiter plate in 100 µL. The cells were distributed to each well in 50 µL ($1x10^4$ cells). The cells were incubated for 72hrs at 37°C in a CO_2 incubator. The cell growth was determined by MTT staining as described above. Interaction was evaluated according to the following equations:

FICA= IC_{50A} in combination / IC_{50A} alone

FICB= IC_{50B} in combination / IC_{50B} alone

where FIC is the fractional inhibitory concentration.

The fractional inhibitory index (FIX) = $FIC_A + FIC_B$ demonstrates the effect of the combination of anticancer drug and resistance modifier. It is accepted that a FIX value 0.51-1.00 denotes additive effect and a FIX value less than 0.50 denotes a synergistic one. A FIX value 1.00-2.00 is considered as an indifferent effect, whereas a value greater than 2 indicates an antagonistic effect [14].

Treatments

Treatments for further studies were performed at IC_{50} (0.18 µM and 700 ng/mL for doxorubicin and silymarin, respectively) and ½IC_{50} (0.09 and 350 ng/mL for doxorubicin and silymarin, respectively) for 72 hrs.

Lipid peroxidation

Lipid oxidation was determined by the modified method of Ahn et al. [15]. Cells were seeded to 25 cm^2 culture flasks and treated with doxorubicin, silymarin and doxorubicin plus silymarin for 72 hrs. Trypsinized cells were resuspended in 1 mL PBS and lysed

with repetitive freezing and thawing at -20°C with vigorous vortexing in between freezing and thawing. Protein amount of lysates was determined according to Bradford method [16]. Twenty five microliters of 0.04M butylated hydroxytoluene (BHT; Sigma-Aldrich) in ethanol were added to prevent artificial increase of malondialdehyde during the experiment. After addition of 0.5 mL 30% (w/v) trichloro acetic acid (TCA; Sigma-Aldrich), the suspension was vortexed. Three milliliters of thiobarbituric acid (TBA; Sigma-Aldrich) / TCA (20mM TBA in 15% (w/v) TCA) solution was added and the samples were vortex mixed. Samples were incubated in boiling water for 1 h for color development. Samples were cooled to room temperature on ice and centrifuged at 4000 rpm for 5 min. Absorbance of the supernatants were measured at 532 nm (and 600 nm), and concentration of TBARS was determined by using extinction coefficient as 155 mM-1.cm-1. Lipid peroxidation was expressed as nmol TBARS per mg protein.

Assay for telomerase activity

Telomerase activity was determined according to the TRAP with slight modifications [17,18]. All the equipment was diethyl pyrocarbonate (DEPC) treated, and the solutions were prepared with DEPC treated distilled water. Briefly, $1x10^5$ cells were resuspended in 200 µL of ice-cold NP-40 lysis buffer, and incubated on ice for 30 min. The protein concentration of cell extracts was determined with Bradford assay [16]. One microliter of diluted extract (200 ng/µL) was added to 48 µL of reaction mixture containing 10X TRAP buffer (5 µL), 50X deoxynucleotide triphosphate mix (1 µL), TS primer (100ng/µL) (1 µL), 50X TRAP primer mix (1 µL), 5 units/µL Taq polymerase (0.4 µL), and 5% DMSO (2.5 µL). Each analysis included a positive control obtained from telomerase positive cell extract and a negative control containing NP-40 lysis buffer instead of extract. The tubes were incubated in thermocycler at 30°C for 30 min for the elongation of TS primer by telomerase, followed by 32 cycles of PCR amplification (95°C for 30 s, 52°C for 30 s and 72°C for 30 s).

Detection and analysis of TRAP products

PCR products (25 µL) were mixed with 6X loading dye (5 µL), and electrophoresed in 0.5X tris-borate-EDTA buffer on 10% nondenaturating polyacrylamide gel at 300 V for 2 hrs. Silver staining technique was used for visualization [18,19]. The gel was washed twice for 3-4 min with fixing solution containing 10% ethanol and 0.5% acetic acid. Then the gel was stained in 0.1% (w/v) $AgNO_3$ (Sigma) for 15 min followed by incubation in developing solution containing 1.5% (w/v) NaOH; Sigma) and 0.15% formaldehyde (Merck, Darmstadt, Germany). After appearance of the bands, the gel was photographed.

A sample was considered positive for TA when the characteristic ladder of TRAP products (TP) with 6 bp increments and a 36-bp internal control (IC) were observed, and negative when only IC was present. Densitometric analysis of the gel was carried out by ImageJ software (National Institutes of Health, Maryland, USA), and the relative telomerase activity (RTA) was calculated by the following formula:

$$RTA = \frac{(X_{TP}/X_{IC})}{(Tel_{TP}/Tel_{IC})}$$

where densitometric band intensities were designated by X and Tel for the samples and telomerase positive control, respectively. MCF-7 cell lysate was used as positive control [18].

Statistics

All data are expressed as mean ± standard error of the means (SEM). All statistical analyses were performed using SPSS Statistics 20.0 (SPSS Inc., Chicago, Ill, USA). In case of normal distribution and homogeneity of variances, treatments were statistically evaluated by one-way ANOVA analysis at 0.05 levels and *post hoc* Tukey analysis were carried out to find groups whose mean differences were significant.

Results

Antiproliferative effects of doxorubicin and silymarin on HepG2 cells

The effect of doxorubicin and silymarin on the proliferation of HepG2 cells for 72 hrs was examined by MTT assay. Both doxorubicin and silymarin significantly ($p<0.05$) inhibited growth of HepG2 cells in a concentration-dependent manner. IC_{50} for doxorubicin was 97.8 ng/mL (0.18 ± 0.03 µM), whereas it was 700 ± 40 ng/mL for silymarin (Table 1). Next, doxorubicin and silymarin were applied in combination for 72 hrs to test the combined antiproliferative effects on cells. The fractional inhibitory index of combined application of doxorubicin and silymarin was 1.76. Since the FIX value was between 1 and 2, doxorubicin-silymarin combination had indifferent antiproliferative effects on cells.

Effects of doxorubicin and silymarin on telomerase activity of HepG2 cells

Table 1. Results of the proliferation assay

	IC_{50} (ng/mL ± SEM)	*FIX ± SEM*	*Comment*
DOX	97.8 ± 10.9	-	
SLY	700.0 ± 40.0	-	
DOX+SLY	-	1.76 ± 0.28	Indifferent

Dox: doxorubicin, SLY: silymarin, SEM: standard error of the mean

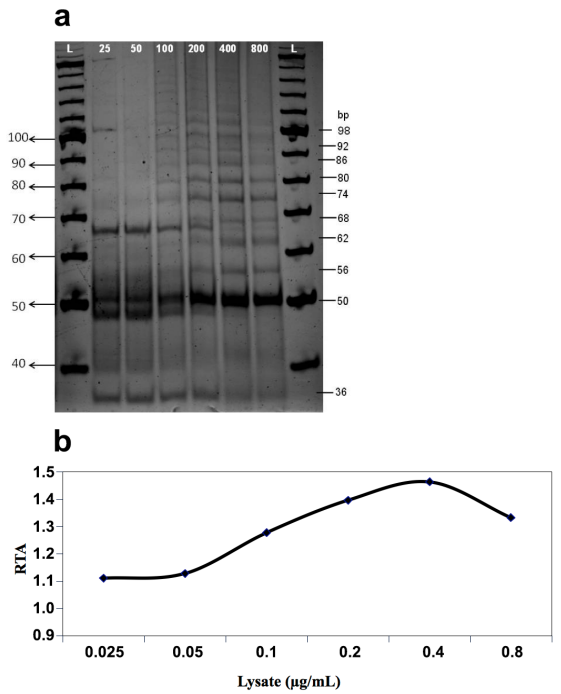

Figure 1. TRAP-silver staining assay. **(a)** TRAP products on 10% polyacrylamide gel. 36 bp products represent internal controls, and 50-98 bp products represent telomeric repeats with 6 bp intervals for all sample wells. L: bp ladders, 10, 25, 50, 100, 200, 400 and 800 represent the amount of protein in µg/mL. **(b)** Effects of cell lysate amount on relative telomerase activity (RTA) levels. RTA levels were linear with the lysate amount in the 25-400 ng range.

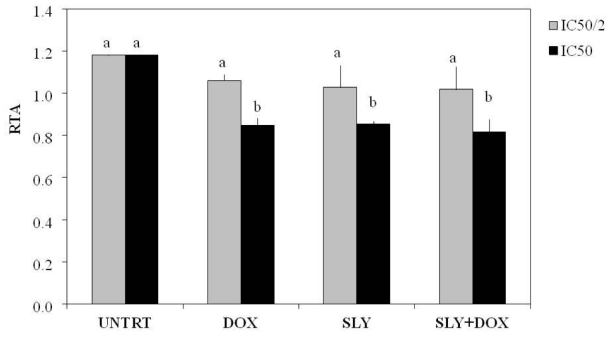

Figure 2. Relative telomerase activity (RTA) of doxorubicin and silymarin treated HepG2 cells. Different letters represent significant difference between groups with p<0.05.

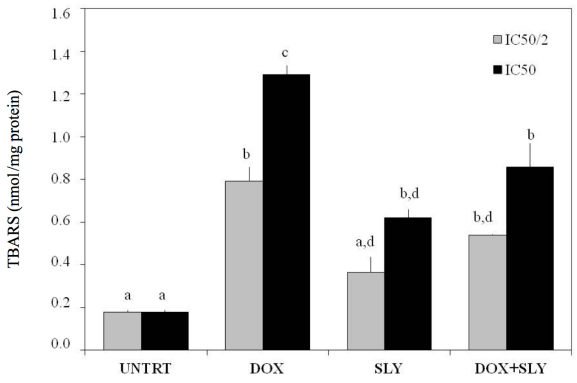

Figure 3. Lipid peroxidation (TBARS) levels of doxorubicin and silymarin treated HepG2 cells. Different letters represent significant difference between groups with p<0.05.

Both the characteristic TRAP ladder with 6 base pair increments starting from 50 bp and the 36 bp IC bands were visible after staining (Figure 1a). For calculation of RTA levels in HepG2 cells, MCF-7 cells were used as positive control. In order to perform quantitative analysis, RTA levels were calculated for different amounts of cell lysates. RTA levels were linear with the lysate amount (R^2=0.9) in 25-400 ng range (Figure 1b). When 800 ng of lysate were used, a decrease in RTA was observed, which may be correlated to the inhibition of PCR due to high protein concentration. Optimum cell lysate amount for quantitation was determined as 200 ng.

RTA levels decreased to 72% (p<0.05) in the cells incubated with IC_{50} (0.18 µM) doxorubicin application (Figure 2), whereas RTA level change was 90% in the cells incubated with half of the IC_{50}. IC_{50} of silymarin application reduced RTA to 72% (p<0.05). ½IC_{50} of silymarin application caused 87% reduction in RTA (statistically insignificant). 0.18 µM doxorubicin together with 700 ng/mL silymarin caused 70% (p<0.05) reduction in RTA, whereas 0.09 µM doxorubicin together with 350 ng/mL silymarin had no effect on RTA.

Lipid peroxidation levels in doxorubicin and silymarin treated HepG2 cells

According to TBARS levels (Figure 3), all treatments except the ½ IC_{50} of silymarin caused significant (p<0.05) increase in lipid peroxidation levels when compared to control (0.18±0.01 nmol/mg protein). TBARS in IC_{50} of doxorubicin treated cells was 1.29±0.04 nmol/mg protein, significantly (p<0.05) higher than the TBARS of half concentration treated cells (0.79±0.07 nmol/mg protein) and all other treatment groups.

Discussion

Doxorubicin inhibits cell proliferation, induces G2-M cell arrest or apoptosis [20,21]. Since only 20% of the HCC patients are sensitive to doxorubicin, new chemotherapy regimens including doxorubicin in combination with chemical bioactive compounds of plant origin have been investigated [3,4,22].

In this study, we initially tested the nature of doxorubicin and silymarin interaction in HepG2 cells. Doxorubicin and silymarin were applied to HepG2 cells in combination, and the interaction of the two agents was determined to be indifferent (Table 1). In previous studies on breast, colon, and prostate cancer cell lines, interaction of silymarin or its bioactive component silibinin with doxorubicin was reported to be synergistic [22-26].

In our study, the indifferent antiproliferative effect of silymarin and doxorubicin might be related to differences from previously used silymarin concentrations and the current cell line. The concentration range of silymarin used in combination assays was lower than those used in previous studies, however, it included the plasma concentrations (i.e. 300 ng/ml) achievable by orally taken dietary supplements [27]. In addition, cellular differences including involvement of different metabolic pathways and genetic factors may have impact on drug interactions.

Telomerase activation is one of the initial events of HCC. It is one of the rate limiting steps of hepatocarcinogenesis initiation and prognosis. In fact, more than 85% of the patients have high telomerase activity, and telomere lengths differ from neighboring cells [28]. Doxorubicin inhibits telomerase activation by triggering degradation of PinX1 protein which enables telomerase binding to telomeres [1]. However, there is limited data on antitelomerase efficacy of doxorubicin on cells of different origins. For example, doxorubicin was reported not to have antitelomerase activity on nasopharyngeal, testicular and squamous cell carcinoma cells, whereas it was found to inhibit telomerase activity of fibroblasts and T cells [29-32]. It is also noteworthy that the antitelomerase action of the agents is prominent only when the critical telomere length is attained [33]. In this study, we demonstrated that doxorubicin decreased telomerase activity of HCC in a concentration-dependent manner. It was previously reported that doxorubicin decreased the telomerase activity of hepatoma cells depending on the application concentration and duration [34]. Secondly, we have tested whether the dietary supplement silymarin had modulating effect on the telomerase activity either alone or in combination with doxorubicin. In fact, silymarin, either alone or in combination with other agents, was reported to decrease telomerase activity and hTERT expression of different cancer cell types [35-38]. According to our results (Figure 2), though silymarin caused a significant decrease in RTA of HepG2 cells, the RTA did not change when it was applied in combination with doxorubicin.

Silibinin, the bioactive component of silymarin, has prooxidant effects on cells, particularly in the presence of metal ions [4,39]. Prooxidant conditions with the effect of the metal ions increase the formation of superoxide anions ($O2^{-)}$ and hydroxyl radicals ($OH^{\cdot}$) from hydrogen peroxide (H_2O_2). Downstream oxidations like lipid peroxidation are the initial steps of the free radical induced cellular damage. Covalent modifications of the DNA, lipids, and proteins cause functional damages on vital pathways of the cells [4,40]. TBARS are the byproducts of the lipid peroxidation. According to TBARS levels (Figure 3), both IC_{50} and $½IC_{50}$ of doxorubicin application significantly increased lipid peroxidation. Silymarin effect, on the other hand, was concentration-dependent, i.e. only IC_{50} of silymarin application caused lipid peroxidation. Nonetheless, TBARS levels did not significantly increase when doxorubicin and silymarin were applied in combination, which is in concordance with the indifferent drug interaction.

In our previous study, the cytotoxic and genotoxic potential of silymarin was tested, and comparable results with doxorubicin were obtained [41]. We demonstrated that both silymarin and doxorubicin inhibited the growth of HepG2 cells in a concentration- and time-dependent manner (i.e. 24 vs 48 hrs). In this study, we applied both agents for 72 hrs in order to test the effect of the agents on telomerase activity, and revisited the effects on cytotoxicity with prolonged incubation time. In comparison to our previous results with 24 and 48 hrs incubation periods, IC_{50} of doxorubicin was lower whereas IC_{50} of silymarin was higher after 72 hrs indicating a time-dependent increase and decrease of the antiproliferative effect of doxorubicin and silymarin, respectively. Additionally, lipid peroxidation levels were concordant to our previous findings. In conclusion, in this study we determined that silymarin-doxorubicin interaction in HepG2 cells was indifferent after 72 hrs. IC_{50} of both doxorubicin and silymarin alone and their combination inhibited telomerase activity. The mechanism of inhibition may be elucidated by further molecular studies.

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