Silibinin Inhibits Proliferation and Migration of Human Hepatic Stellate LX-2 Cells

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Background: Proliferation of hepatic stellate cells (HSCs) play pivotal role in the progression of hepatic fibrosis consequent to chronic liver injury. Silibinin (SBN), a flavonoid compound, has shown to possess cell cycle arresting potential against many actively proliferating cancers cell lines. The objective of this study was to evaluate the anti-proliferative and cell cycle arresting properties of SBN in rapidly proliferating human hepatic stellate LX-2 cell line. **Methods:** LX-2 cells were fed with culture medium supplemented with different concentrations of SBN (10, 50 and 100 μ M). After 24 and 96 h of treatment, total cell number was determined by counting. Cytotoxicity was evaluated by trypan blue dye exclusion test. The expression profile of cMyc and peroxisome proliferator-activated receptor- γ (PPAR- γ) protein expressions was evaluated by Western blotting. Oxidative stress marker genes profile was quantified using qPCR. The migratory response of HSCs was observed by scrape wound healing assay. **Results:** SBN treatments significantly inhibit the LX-2 cell proliferation (without affecting its viability) in dose dependent manner. This treatment also retards the migration of LX-2 cells toward injured area. In Western blotting studies SBN treatment up regulated the protein expressions of PPAR- γ and inhibited cMyc. **Conclusion:** The present study shows that SBN retards the proliferation, activation and migration of LX-2 cells without inducing cytotoxicity and oxidative stress. The profound effects could be due to cell cycle arresting potential of SBN. (J CLIN EXP HEPATOL 2016;6:167-174)

Hepatic fibrosis is the pathological consequence of chronic liver diseases, which can ultimately lead to cirrhosis and hepatocellular carcinoma. A wide spectrum of chronic liver injuries, including viral hepatitis, cholestasis, chronic ethanol consumption, non-alcoholic steatohepatitis, and non-alcoholic fatty liver disease, can cause chronic hepatic inflammation and wound healing process in the liver, consequent to fibrosis.¹ It has been well demonstrated that following liver injury,

the hepatic stellate cells (HSCs) undergo “activation”, which is the stage of transition of quiescent cells into proliferative, fibrogenic, and contractile myofibroblasts.² HSCs activation causes progressive accumulation of extracellular matrix (ECM) in perisinusoidal space, eventually leading to liver fibrosis, and finally to cirrhosis. The increase in ECM synthesis is sustained by increased secretion of ECM components per cell as well as by an increase in the number of activated HSCs. *In vitro* and *in vivo* studies have shown that proliferation and migration of HSCs toward the areas of tissue remodeling may be an additional factor contributing to wound healing and fibrosis.^{3,4} Hence, prevention of HSC proliferation together with its migration toward the microenvironment of injury in the liver regarded as one of the key strategies to reduce the progression of hepatic fibrosis.

Silibinin (SBN) is the most active component of silymarin present in milk thistle (*Silybum marianum*). Its mechanism of action is complex and highly beneficial in protecting hepatocytes. Overwhelming evidence confirms the protective effect of SBN against various drug and chemical induced hepatotoxicity and oxidative stress *in vivo*.⁵⁻⁸ Conversely, *in vitro* SBN is reported to induce reactive oxygen species (ROS) mediated oxidative stress induced cell death in various cancer cell lines.⁹⁻¹¹

Keywords: cytotoxicity, hepatic stellate cells, wound healing, oxidative stress

Received: 24.09.2015; **Accepted:** 6.01.2016; **Available online:** 13 January 2016

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Abbreviations: AKR1C1: aldo-keto reductase family 1, member C1; ARE: antioxidant responsive element; CDKI: cyclin dependent kinase inhibitor; CYP450: cytochrome P450; DMSO: dimethylsulphoxide; DMEM: Dulbecco's modified Eagle's medium; ECM: extracellular matrix; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HMOX1: heme oxygenase (decycling) 1; HSCs: hepatic stellate cells; NQO1: NAD(P)H dehydrogenase, quinone 1; Nrf-2: nuclear respiratory factor; PPIA: peptidylprolyl isomerase A; PPAR- γ : peroxisome proliferator-activated receptor- γ ; qPCR: quantitative polymerase chain reaction; ROS: reactive oxygen species; SBN: silibinin; TXNRD1: thioredoxin reductase 1 <http://dx.doi.org/10.1016/j.jceh.2016.01.002>

Peroxisome proliferator-activated receptor- γ (PPAR- γ) is a member of steroid/thyroid hormone nuclear receptor super family, and it is reported to be reduced dramatically in activated and proliferated HSCs both *in vitro* and *in vivo*.¹² Treatment of culture activated HSCs with PPAR- γ ligands reversed its activation by inhibiting collagen production and blocking PPAR- γ mediated cellular proliferation.¹³ These results clearly indicate the role of PPAR- γ in regulation of HSCs activation in liver fibrogenesis. cMyc is an oncoprotein, which prevents cell cycle progression by controlling the expression of p21 and p27 cyclin dependent kinase inhibitor (CDKI) proteins which are growth inhibitor signals. And thus, it plays a vital role in initiation of cell cycle progression.¹⁴ On the contrary, downregulation of cMyc causes cell cycle arrest by the activation of above two CDKI proteins. Activation of cMyc in quiescent cells is sufficient to induce cell cycle progression even in the absence of growth factors.¹⁵

Over the past three decades, mechanisms of fibrosis have focused on HSCs, which become fibrogenic myofibroblasts during injury through 'activation', and are at the nexus of efforts to define novel drug targets.² Clearance of activated and proliferated HSCs from injured liver could be one of the clear therapeutic strategies to reduce the progression of hepatic fibrosis. Moreover, studies regarding the oxidative stress inducing potential of SBN on non-cancer pathologies, especially against HSCs are not available. Hence, in this study, we investigated the anti-proliferative, anti-migratory, and oxidative stress inducing potentials of SBN in human hepatic stellate LX-2 cell line.

MATERIALS AND METHODS

Chemicals

SBN, dimethylsulphoxide (DMSO), and trypan blue solution (0.4%) were purchased from M/s. Sigma-Aldrich Chemicals, Brussels, Belgium. All other chemicals used in various biochemical and molecular assay procedures in this study were purchased locally in Belgium.

Cell Line

LX-2 cells (human HSC line) used for this study were obtained from Dr. S.L. Friedman, Mount Sinai School of Medicine, New York. These cells are derived from normal human HSC that are spontaneously immortalized.¹⁶ The LX-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; with high glucose, L-glutamine and without sodium pyruvate) containing 1% of fetal bovine serum (FBS, Gibco, Belgium) and penicillin-streptomycin (Life Technologies, Belgium). Cells in passages 5–10 were used in this study. Cultures were maintained at 37° C in a fully humidified atmosphere containing 5% CO₂. Upon reaching confluency, the cells were trypsinized, passaged, and maintained for subsequent studies. Cultured cells

were imaged and photographed on Leica DMIL inverted microscope.

Treatment

SBN was dissolved in 0.1% DMSO (v/v) to prepare 10 mM stock solution. This stock SBN solution was diluted suitably with medium containing 1% FBS to obtain a working concentration of 10, 50 and 100 μ M of SBN. In all the dilutions, the concentration of DMSO never exceeded 0.1%. SBN treatment was given to the LX-2 cells as for 24 and 96 h. From the practical point of view, it is significant to stress that the effects of SBN reported in the present study were obtained in the dose range 10, 50 and 100 μ M and compatible with the serum concentrations of the drug observed after oral administration in human.¹⁷

Cell Counting and Cytotoxicity Assay

After 96 h of treatment, cells were collected by 0.05% trypsin (Life Technologies, Belgium) application. Total cell number was determined by counting each sample in triplicate using a KOVA Glasstic® Slide 10 under Leica DMIL inverted microscope. Viability was also evaluated by the trypan blue dye exclusion assay.

Quantitative Real Time RT-PCR

Total RNA was extracted with tripure reagent (Roche) according to the manufacturer's instructions. Briefly, cells were lysed with the reagent, chloroform was added, and cellular RNA was precipitated by isopropyl alcohol. After washing with 70% ethanol, the RNA pellet was dissolved in nuclease-free water and then quantified. Total RNA was reverse transcribed to complementary DNA (cDNA) using high capacity cDNA reverse transcription kit, AB Applied Biosystem. For qPCR, each sample, a 25 μ l reaction mixture was prepared with 12.5 μ l TaqMan Gene Expression Mastermix (Applied-Biosystems), 1 μ l primers/probe mix, 5 μ l sample DNA and 6.5 μ l DEPC-treated water (Invitrogen). All samples were analyzed in triplicate with a TaqMan standard 40-cycle amplification program with both annealing and elongation performed at 60 °C (10 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 90 s at 60 °C). DEPC water used as negative control. As an endogenous control GAPDH or PPIA was used (Applied Biosystems). Data were analyzed with a comparative threshold cycle (Δ Ct) method. This method is used to determine the values of Δ cycle threshold (Δ Ct) by normalizing the average Ct value of each treatment with the value of each opposite endogenous control (GAPDH or PPIA). Then, calculation of $2^{-\Delta\Delta Ct}$ of each treatment was performed as described by Livak and Schmittgen.¹⁸ Table 1 depicted the primers were used as a reference gene for normalization. Step One Software (version 2.2; AB Applied Biosystems) was used to analyze results.

Table 1 List of Primers/Antibodies Used for the qPCR/Western Blotting.

Primer/probe used				NCBI reference	
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)				NM_002046.3	
Peptidylprolyl isomerase A (PPIA)				NM_021130.3	
NAD(P)H dehydrogenase, quinone 1 (NQO1)				NM_000903.2	
Thioredoxin reductase 1 (TXNRD1)				Hs00182418_m1	
Aldo-keto reductase family 1, member C1 (AKR1C1)				NM_001353.5	
Heme oxygenase (decycling) 1 (HMOX1)				NM_002133.2	
Antibodies					
Antibody	Mol. weight (kDa)	Origin	Dilution	Type	Reference no.
cMyc	60	Rabbit	1:1000	Polyclonal	Cell signaling (# 9402)
PPAR-γ	54	Rabbit	1 μg/ml	Polyclonal	Abcam (Ab27649)
β-Actin	42	Mouse	1:20,000	Monoclonal	Abcam (AB6276)

Western Blot Analysis

LX-2 cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% deoxycholate sodium and 0.1% protease) and phosphatase inhibitor cocktails (Sigma-Aldrich, Belgium). After extraction, protein concentration was estimated by the Bradford method (Biorad Laboratories) with bovine serum albumin as standard. Total protein extracts were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond^{TM}-ECL, Amersham, CA). The membranes were blocked with 5% bovine serum albumin, probed overnight at 4 °C with primary antibodies and 2 h at room temperature with corresponding secondary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence with protein A-horseradish peroxidase and the SuperSignal chemiluminescent system (Pierce, Rockford, IL). Details of primary and corresponding secondary antibodies used for this study are given in Table 1.

Scrape-Wound Healing Assay

LX-2 cells were plated at 20,000 cells/cm² in collagen coated (288 μ g/plate) 6 well plates and grown for 48 h. Then the medium was aspirated, and the cell-coated surface was scraped with a 200 μ l pipette tip in a single stripe. The scrape-wounded surface was washed twice with Dulbecco's phosphate-buffered saline and then the wounds in the cultures were treated with DMSO and different SBN concentrations and allowed to heal for 24 h at 37 °C inside the incubator. Migration of cells into wounded areas was evaluated with an inverted microscope and photographed. The average extent of wound closure was evaluated by multiple measurements of the width of the wound space, for each of these cases.

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA), and Newman Keuls' multiple comparison tests

were performed to assess the significance of differences in the means of various treatment groups, using Graph pad prism software. The values were tabulated and presented as mean $\pm$ S.D. The *p* value < 0.05 was considered as statistically significant.

RESULTS

Effect of SBN on Cytotoxicity and Viability of LX-2 Cells

The viable LX-2 cells were observed as cultured activated and acquired myofibroblast like phenotypes, which were the characteristic features of the activated HSCs in fibrotic liver diseases (Figure 1). The present study shows that DMSO and SBN treatments did not produce any cytotoxic effects, and the cell viability was more than 90% as compared to control during their exposure for 96 h in serum supplemented medium (Figure 2A).

Effect of SBN on Proliferation of LX-2 Cells

Treatment of LX-2 cells at different concentrations of SBN for 96 h in serum supplemented medium produced a dose-dependent fall in the proliferative activity of LX-2 cells as compared to control and DMSO treatments (*P* < 0.01; *P* < 0.001; *P* < 0.001). The anti-proliferative activity of SBN on LX-2 cells was at the maximum in the highest concentration (100 μ M) (Figure 2B).

mRNA Expression of Nuclear Respiratory Factor (Nrf) Targeted Oxidative Stress Markers Genes in LX-2 Cells

The expression profile of Nrf targeted above marker genes such as NQO1, HMOX-1, TXNRD1 and AKR1C1 was evaluated in LX-2 cells exposed to SBN for 96 h. SBN treatments (10, 50 and 100 μ M) did not produce any significant change in the relative mRNA expressions of all the above marker genes in LX-2 cells as indicated by

Figure 1 Morphology of LX-2 cells treated with silibinin for 96 h. (A) Control, (B) DMSO, (C–E) SBN 10, 50 and 100 μ M treatments respectively (200 $\times$).

Figure 2 Effect of SBN treatment on LX-2 cells (A) cytotoxicity, (B) cell proliferation, (C) oxidative stress. Values are presented as mean $\pm$ S.D. of 3 nos. of observations for 96 h of different concentrations of SBN exposure respectively. Multiple comparisons between treatment groups were performed by using Newman–Keul’s test. *Control compared to DMSO and SBN treatment groups. #DMSO group compared to all the SBN treated groups. ** $P < 0.01$; *** $P < 0.001$. ## $P < 0.01$; ### $P < 0.001$.

qPCR analysis, and they were comparable to control (Figure 2C). These evaluations clearly demonstrate that SBN treatment does not induce oxidative stress in LX-2 cells.

Expression of cMyc and PPAR- γ Proteins by Western Blotting

cMyc protein expression shows a dose-dependent decrease in its expression profile in SBN treated LX-2 cells as compared to DMSO treatment. The PPAR- γ protein expression was increased upon SBN exposure in LX-2 cells. The expression was prominent at 10 μ M of SBN exposure as compared to DMSO treatment (Figure 3). cMyc and PPAR- γ involved in cell cycle arrest and inhibition of HSCs activation respectively. These results indicate that SBN treatments cause cell cycle arrest.

Effect of SBN on Wound Healing and Migratory Properties of LX-2 Cells

The wound healing and migration properties of LX-2 cells were studied in collagen pre-coated culture plates. A scrape wound created on the monolayers of LX-2 cells was exposed to SBN treatments (10, 50 and 100 μ M) and were observed 24 h after treatments. While there was very meager migration of cells in controls, which were maintained

Figure 3 Effect of SBN (24 h) on the protein expression of cMyc and PPAR- γ on LX-2 cells. β -Actin used as an internal control for optimization (representative images of at least three experiments in triplicate).

Figure 4 Inhibitory effects of SBN on HSC motility in wound healing model. A scrape wound was generated in the cell layer to remove a linear area of cells. 10% FBS used as a positive control (representative images of at least three experiments in triplicate).

in serum free and 1% serum supplemented condition. None of the SBN treatments produced migration and wound closure in 1% serum supplemented culture medium (Figure 4). In positive control, there was complete migration and closure of wound in LX-2 cells cultured in 10% serum supplemented medium indicating that hastens rapid closure of wound.

DISCUSSION

Hepatic fibrosis combined with cirrhosis is considered a precancerous state that provides the proper microenvironment for tumor development.^{19,20} Proliferation along with migration of HSCs to the site of liver injury is one of the earlier events during the pathological progression of hepatic fibrosis.^{4,21} Suppression of proliferation by administration of some therapeutic agents has been shown to mitigate the proliferation of HSCs and thereby to delay the process of fibrosis.^{22,23} This can be achieved either by inhibition of HSCs proliferation and/or induction of cell death to get rid of activated HSCs from injured liver. The latter strategy is commonly employed for the induction of oxidative stress of the actively proliferating cancerous cells^{9–11} *in vitro* studies.

In recent studies, several plant-derived compounds have been employed in LX-2 cells for the study of hepatic fibrosis and promising antifibrotic effects were realized.^{24,25} Studies using LX-2 cells have also opened several

avenues in the field of hepatic fibrosis. For instance, role of silent information regulator 1 (SIRT1) was disclosed and the role of SIRT1 in the reversion of activated LX-2 cells was confirmed.²⁶ In a previous study it was reported that antiproliferative effect of SBN in human primary HSCs with low concentrations and short exposure period.²⁷ However, evaluation of antiproliferative, migratory and oxidative stress inducing potential of SBN in LX-2 cells is scanty, and this study is probably the first of its kind.

In order to ascertain, whether SBN treatments at various concentrations themselves are cytotoxic to LX-2 cells, an attempt was made to evaluate its toxicity by investigation of the cell viability assay by trypan blue test in serum supplemented culture conditions. SBN exposure at different concentrations did not affect the LX-2 cell viability. Ironically, SBN exposure has been reported to induce cytotoxicity in various cell lines.^{28,29} In contrast to these reports, we have observed that SBN exposure is not cytotoxic in LX-2 cells. It is likely that this discrepancy in cytotoxic response to SBN exposure could be due to different mechanisms of action of SBN on various cell types as being proposed by Zhang et al.³⁰ SBN treatment shows a dose-dependent fall in active proliferation of immortalized LX-2 cells. These observations clearly indicate that SBN treatments retard active proliferation of LX-2 cells without affecting its cell viability.

Several studies have proposed that SBN might exhibit anti-proliferative effect by enhancing cell cycle arrest,

apoptosis, cellular senescence and induction of oxidative stress in actively proliferating cancer cell lines.^{9,30,31} In fact, it was shown that SBN inhibits growth factors induced human primary HSCs proliferation in dose dependant manner.²⁷ In contrast, our previous studies show that 96 h of SBN treatment does not induce apoptosis and cellular senescence in LX-2 cells.⁶ Since the observation that SBN treatments does not affect the cell viability but still retards active proliferation of LX-2 cells, it could be reasonable to assume that SBN-induced fall in proliferation in the present study could be due to enhanced suppression of their growth as the consequence of cell cycle arrest.

cMyc is a transcriptional factor, which plays a vital role in the regulation of p21 and p27 proteins. Amati et al.¹⁵ have shown that increase in the level of cMyc directly inhibits the activation of p21 and p27, which eventually leads to cell cycle progression, especially in the late G1 phase. It has been concretely proved that activation of p21 and p27 prevents the binding of CDK2 with cyclin E causes cell cycle arrest in several *in vitro* cell lines.^{32,33} In this study, SBN caused a dose-dependent decrease in cMyc protein expression. It is likely that the downregulation of cMyc protein expression accompanied by up regulation of p21 and p27 would have caused the cell cycle arrest through the inhibition of cyclin E binding with CDK2. SBN has been shown to downregulate the expression of cMyc and thereby arrest cell cycle progression in several *in vitro* cell lines,^{34,35} and our current results are in agreement with these reports.

Furthermore, studies have shown that PPAR- γ activity is expressed highly in quiescent HSCs of normal liver and its activity are reduced in activated rat and human HSCs, which undergo proliferation.^{12,13} Additionally, it has been shown that the treatment of culture activated HSCs with PPAR- γ ligands reversed the collagen production and activation of HSCs.¹³ These results show that increase in PPAR- γ protein expression is high in non-proliferating quiescent cells, and it is a valid parameter in differentiating the quiescent from activated cells. Interestingly, LX-2 cells treated with SBN shows normal expression of PPAR- γ at higher concentrations and it increased the expression in lower concentrations as compared to DMSO treatment indicating the fact that LX-2 cells have not undergone the proliferative state, and they remain quiescent. Our current reports are in agreement with the above reports.

SBN is said to be biotransformed by phase-I reactions involving CYP450 and eventually detoxified by phase-II detoxifying enzymes. Thus, it is said to exert effects on both phase-I and II metabolic reactions.³⁶ Nrf-2 critical transcription factors are the group of phase-II detoxifying enzymes, which are responsible for phase-II biotransformation. The NQO1, HMOX1, and TXNRD1 are the major downstream genes of Nrf-2 transcription factors. The

NQO1 acts as superoxide scavenger, and it prevents the generation of ROS such as superoxide and hydrogen peroxide.^{37,38} HMOX1 is an essential enzyme in heme catabolism and is induced by oxidative stress. Both *in vitro* and *in vivo* studies have reported that HMOX1 is susceptible to oxidative stress.³⁹ TXNRD1 gene codes for the cytosolic enzyme, and it regulates the levels of intracellular ROS, which are required for normal cell proliferation but are toxic to the cells at excessive levels. The enzyme thioredoxin carries numerous essential roles, such as protein disulfide reduction, protein repair and its folding, regulation of apoptosis, cellular proliferation and protection against oxidative stress.⁴⁰ The gene AKR1C1 plays a major role against induction of electrophilic ROS *via* the involvement of cellular defensive protein *i.e.*, antioxidant responsive element (ARE). We observed that SBN treatment at all the concentrations investigated did not produce any significant change in the mRNA expressions of all the above said Nrf-2 critical transcription factors. These results prove beyond doubt that SBN does not cause oxidative stress in rapidly proliferating LX-2 cells. Reports regarding the mRNA expressions profile of the above said Nrf-2 critical transcription factors of oxidative stress during SBN treatment in LX-2 cells are not available in previous literature and ours is probably the first of its kind.

Migration is a normal critical stride in the recruitment of activated HSCs in areas of injury during the process of wound healing and hepatic fibrogenesis.⁴¹ Several *in vitro* and *in vivo* studies have shown concrete evidences of HSCs migration toward the areas of tissue remodeling.^{3,4} In this study, LX-2 cells exposed to SBN at increasing concentrations for 24 h in serum supplemented medium show a decrease in the migration of the LX-2 toward the scrap wound induced in the culture. This delaying process of HSCs migration toward wound area and the anti-proliferative responses of proliferating cells by SBN treatments could be utilized beneficially toward delaying hepatic fibrosis. Trappoliere et al.²⁷ reported that 6 h SBN exposure reduced PDGF induced migration of human primary HSCs in serum free medium, and our present observation has shown similar correlation in delayed migration of LX-2 cells toward scrape wound healing response.

CONCLUSION

In conclusion, the present study shows that SBN retards the proliferation, activation, and migration of LX-2 cells without inducing cytotoxicity and oxidative stress. The profound effects could be due to cell cycle arresting potential of SBN. Hence, detailed further studies are warranted on these lines.

CONFLICTS OF INTEREST

All authors have none to declare.

ACKNOWLEDGMENTS

ED gratefully acknowledges the support of a 20 months scholarship obtained thanks to the MAHEVA ERASMUS MUNDUS Action 2 European project (Agreement No. 2010-2372/001-001-EMA2, coordinator Montpellier2). MN is a researcher of the Institute of Experimental and Clinical Research (IREC).

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