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 μM). 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)**

Liver Fibrosis

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 (*Sylibum 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]

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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
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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% CO_2. 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 µM 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 µM 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 µl reaction mixture was prepared with 12.5 µl TaqMan Gene Expression Mastermix (Applied-Biosystems), 1 µl primers/probe mix, 5 µl sample DNA and 6.5 µl 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 (ΔCt) method. This method is used to determine the values of Δ cycle threshold (ΔCt) 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.

