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# Interruption of mitochondrial complex IV activity and cytochrome *c* expression activated $O_2^{\cdot-}$-mediated cell survival in silibinin-treated human melanoma A375-S2 cells via IGF-1R–PI3K–Akt and IGF-1R–PLC γ–PKC pathways ☆

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ABSTRACT

Silibinin was reported to have high cyto-toxicity in many malignant cell lines, however, it showed low cyto-toxicity in treatment of human melanoma A375-S2 cells and even protected these cells against certain stress insults. Reactive oxygen species was reported to have controversial effects on cancer chemotherapy. In this study we investigated the mechanism of reactive oxygen species generation and the role of reactive oxygen species in protecting cells against silibinin induced cyto-toxicity in A375-S2 cells. We found that silibinin induced the generation of large amount of superoxide anion ($O_2^{\cdot-}$) and small amount of hydrogen peroxide ($H_2O_2$) through down-regulating the activity of mitochondrial complex IV and the protein level of cytochrome *c*. We also discovered that $O_2^{\cdot-}$ generation activated insulin like growth factor-1 receptor (IGF-1R) and its down-stream phosphatidylinositol 3-kinases–Akt (PI3K–Akt) and phospholipase C γ–protein kinase C (PLC γ–PKC) signaling pathways, which were augmented by $H_2O_2$ scavenger catalase. Scavenging $O_2^{\cdot-}$ by superoxide dismutase (SOD) or inhibition of IGF-1R-PI3K–Akt and IGF-1R-PLC γ–PKC signaling pathways increased cell apoptosis. Therefore, $O_2^{\cdot-}$ mediated cell resistance to silibinin via activating IGF-1R-PI3K–Akt and IGF-1R–PLC γ–PKC pathways in silibinin treated A375-S2 cells.



## 1. Introduction

Reactive oxygen species is composed of a series of free radical molecules, including hydroxyl free radical (·OH) and $O_2^{\cdot-}$, as well as non-radical molecules, such as $H_2O_2$ and hypochlorous acid (HOCl) (Yu et al., 2008). $O_2^{\cdot-}$ and $H_2O_2$ are the primary sources of reactive oxygen species. Mitochondria from various aerobic organisms have been recognized as effective sources of $O_2^{\cdot-}$ and $H_2O_2$ (Murphy, 2009).

There are four complexes located at the mitochondrial inner- and outer-membrane to perform the electron transporting function. Complex I (NADH coenzyme Q reductase) accepts electrons from the Krebs cycle electron carrier nicotinamide adenine dinucleotide (NADH), and passes them to ubiquinone (UQ), which also receives electrons from complex II (succinate dehydrogenase). UQ passes electrons to complex III (cytochrome bc1 complex), which transfer them to cytochrome *c*. Then cytochrome *c* transfer the electrons to complex IV (cytochrome *c* oxidase), whereby two $H_2O$ molecules are produced by four electrons. Normally there are about 1–2% electron leaking from mitochondria, which reduced $O_2$ into $O_2^{\cdot-}$ (Murphy, 2009; Strathmann et al., 2010).The dysfunction of mitochondrial complexes activity or cytochrome *c* expression will trigger more reactive oxygen species generation (Lee and Xu, 2007).

Cancer cells produce larger amount of reactive oxygen species than normal cells since they have higher metabolic activities and mitochondrion is the most important organelle in organism metabolism (Bellance et al., 2009). The "two-faced" character of reactive oxygen species is substantiated by growing body of evidences. On one hand, reactive oxygen species can induce cellular senescence and apoptosis and therefore function as anti-tumorigenic species; on the other hand, moderate and sustained production of reactive oxygen species within cells can activate secondary survival signaling pathways such as ERK–MAPK pathway and NF-κB pathway, which induce and maintain cancer cell's oncogenic phenotype (Trachootham et al., 2008).

Silibinin (Fig. 1), a natural compound abstracted from milk thistle (*Silybum marianum*), is reported to have hepatoprotective properties

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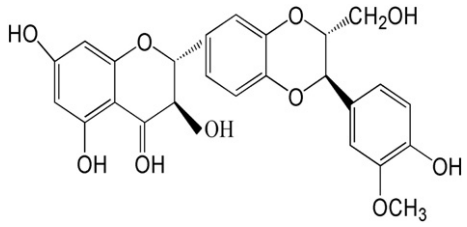


**Fig. 1.** Chemical structure of silibinin.

that protect liver cells against toxins both in vivo and vitro (Gazák et al., 2007). Silibinin also has anti-cancer effects against a series of cancer cell lines, including human prostate adenocarcinoma, breast carcinoma, cervical carcinoma, and lung carcinoma cells, and the mechanisms have been well studied (Kaur and Agarwal, 2007; Ramasamy and Agarwal, 2008). However, silibinin shows very low cyto-toxicity and even has cyto-protective effect against UV and mitomycin C-induced insults in human melanoma A375-S2 cells (Jiang et al., 2009; Li et al., 2004); the reason why A375-S2 cells have low sensitivity to silibinin is still not well understood. Recently, silibinin has been reported to induce protective reactive oxygen species generation in human breast cancer MCF-7 cells (Wang et al., 2010). However, owing to the high sensitivity to silibinin induced cell toxicity, MCF-7 cells are not a good model to investigate the protection mechanism. In this study, we focus on solving two problems: (1) to find out how, where and which types of reactive oxygen species are induced in A375-S2 cells and (2) to elucidate the cyto-protective mechanism mediated by reactive oxygen species in this cell line.

## 2. Materials and methods

### 2.1. Materials

Silibinin was obtained from China Institute of Biological Products (Beijing, China). The purity of silibinin was measured by HPLC and determined to be about 99%. Silibinin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The concentration of DMSO was kept below 0.1% in all the cell cultures, and did not exert any detectable effect on cell growth or death. 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), glutathione N-acetyl-L-cysteine (NAC), Glutathione (GSH), SOD, catalase, diphenyleneiodonium chloride (DPI), rotenone, stigmatellin, theonyltrifluoroacetone (TTFA), sodium azide ($NaN_3$), mitochondria isolation kit, cytochrome *c* oxidase assay kit, and acridine orange (AO), PKC inhibitor staurosporine, Akt1/2 kinase inhibitor 1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl) phenyl) methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate (A6730) were from Sigma Chemical (St. Louis, MO, USA). IGF-1R inhibitor tyrphostin (AG1024), PI3K inhibitor wortmannin, MPT pore inhibitor cyclosporin A (CSA), Raf-1 inhibitor 3-(3,5-Dibromo-4-hydroxy-benzylidene)-5-iodo-1,3-dihydro-indol-2-one (GW5074), ERK–MAPK inhibitor 2′-amino-3′-methoxyflavine (PD98059), p38 MAPK inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), rabbit polyclonal antibodies against cytochrome *c*, IGF-1R, p-IGF-1R, PI3K, p-PI3K, Akt, p-Akt, Raf-1, p-Raf-1, ERK, p-ERK, p38, p-p38, Bcl-2, p53, caspase 3, caspase 6, β-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Whole cell lysis buffer containing proteasome inhibitor was from Beyontime (Shanghai, China), and Electro chemiluminescence (ECL) was obtained from Thermo Scientific (Rockford, IL, USA). The tyrosine kinase activity assay kit was from Chemicon International (Temecula, CA, USA).

### 2.2. Cell culture

The human melanoma A375-S2 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C with 5% $CO_2$ in a humidified atmosphere.

### 2.3. Flow cytometric analysis of reactive oxygen species production

2′,7′-dichlorofluorescin diacetate (DCFH-DA) is a fluorescent probe of reactive oxygen species, which enters the cell and the acetate group on DCFH-DA was cleaved by cellular esterase, tripping the non-fluorescent polar DCFH inside. Subsequently the DCFH was oxidized by reactive oxygen species and yielded the fluorescent product DCF which could be examined by flow cytometry. Therefore, the intracellular DCF fluorescence intensity reflected the intracellular reactive oxygen species levels. $5\times10^5$ cells of each group were treated with 150 μM silibinin for 0, 5, 15, 30 min or 1, 3, 6, 12, 24 h, respectively, or treated with silibinin at 0, 25, 50, 75, 100, 150, 200 and 300 μM for 1 h, or treated with silibinin in the presence/absence of SOD (50, 100 U/ml) and catalase (400, 800 U/ml) for 1 h. The cells were stained with 10 μM DCFH-DA for 30 min at 37 °C, collected and re-suspended in 500 ul PBS. The samples were analyzed by a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the excitation wavelength at 480 nm and the emission wavelength at 525 nm (Wang et al., 2010).

### 2.4. Measurement of SOD and catalase activity

$1\times10^6$ cells per flask were pretreated with 150 μM silibinin for 0, 5, 15, 30 min or 1, 3, 6, 12, 24 h and collected. After that the cells were washed twice, resuspended in PBS and sonicated for 10 s on ice, then centrifuged at 1000×g for 15 min. For SOD activity determination, the supernatants were subjected to intracellular SOD activity assays. Total SOD activity was determined using the commercial assay kit (Jiancheng, Nanjing, China). The assay based on the reaction that SOD inhibits the oxidation of hydroxylamine by the xanthine–xanthine oxidase system (Wang et al., 2010). The mauve product (nitrite) produced by the oxidation of hydroxylamine has an absorbance peak at 550 nm. One unit of the SOD activity was defined as the amount of the sample that reduced the absorbance at 550 nm by 50%. Total SOD activity was determined using the commercial assay kit (Jiancheng, Nanjing, China). For determination of catalase activity determination, the supernatants were subjected to intracellular catalase activity assays. The catalysis of catalase on hydrogen peroxide can be terminated by adding ammonium molybdate. The remaining hydrogen peroxide reacted with ammonium molybdate to form yellow complex whose production was measured at 405 nm for the calculation of catalase activity. The amount of enzyme that decomposed 1 μM of hydrogen peroxide per minute per milligram protein was defined as one unit of catalase activity (Li et al., 2008). The enzyme activity in each sample was expressed as folds of control.

### 2.5. Fluorescent microscopy of reactive oxygen species production

$0.35\times10^5$ cells per well in six-well plate were treated with 150 μM silibinin in the presence/absence of SOD (50, 100 U/ml) and catalase (400, 800 U/ml) for 24 h. The cells were rinsed with ice cold PBS twice, and incubated with 10 μM DCFH-DA at 37 °C for 30 min. After incubation the cells were observed under fluorescent microscope (Olympus, Tokyo) with the excitation wavelength at 480 nm and the emission wavelength at 525 nm (Du et al., 2010).