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ORIGINAL ARTICLE

# Sulforaphane exerts anticancer effects on human liver cancer cells via induction of apoptosis and inhibition of migration and invasion by targeting MAPK7 signalling pathway

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## Summary

***Purpose:*** *This study was undertaken to investigate the anticancer effects of Sulforaphane against liver cancer and to elucidate the underlying molecular mechanisms.*

***Methods:*** *WST-1 assay was used to monitor the proliferation rate. DAPI and annexin V/propidium iodide (PI) staining was used for apoptosis. Flow cytometry was used for cell cycle analysis. Wound healing and transwell assays were used to monitor cell migration and invasion. The protein expression was determined by western blot analysis.*

***Results:*** *Sulforaphane decreased the viability of the liver cancer HepG2 cells and exhibited an $IC_{50}$ of 9 μM. This molecule exerted very low toxic effects on the normal AML12 hepatocytes and exhibited an $IC_{50}$ of 100 μM. Flow cytometry analysis showed that Sulforaphane triggered G2/M arrest of the liver HepG2 cancer cells. DAPI staining revealed that Sulforaphane triggered apoptotic death of HepG2 cells which was accompanied with activation of caspases 3 and 9, up-regulation of Bax and downregulation of Bcl-2. Transwell assay showed that Sulforaphane inhibited the migration and invasion of the HepG2 liver cancer cells in a dose-dependent manner. The effects of Sulforaphane were also investigated on the MAPK7 signalling pathway and it was found that the molecule could block this pathway in HepG2 cells.*

***Conclusion:*** *Sulforaphane may prove essential in the development of systemic therapy for liver cancer.*

***Key words:*** *Liver cancer, apoptosis, migration, invasion, proliferation*

## Introduction

Plants are amazing natural chemical factories with the capability to synthesize a wide array of chemical scaffolds with enormous pharmacological potential [1]. Studies have shown that the consumption of cruciferous plant parts is associated with decreased risk of cancer development [2]. This is mainly believed to be due to the presence of several isothiocyanates [3]. Vegetables such as broccoli and cabbage are considered to be rich sources of isothiocyanates such as sulforaphane [4]. Several *in vitro* and *in vivo* studies have shown that sulforaphane exhibits strong anticancer effects [5] and has been shown to arrest the growth of epithelial carcinomas and breast cancer [6,7]. Multiple mechanisms have been shown to be responsible for the anticancer effects of sulforaphane which include apoptosis, autophagy and cell cycle arrest to name a few [8-10]. For example, in colon cancer cells, sulforaphane induces apoptosis and cell cycle arrest and in prostate cancer cells sulforaphane triggers autophagy [9,10]. Nonetheless, the anticancer effects of this molecule have not been thoroughly explored against the human liver cancer. Therefore, this study was undertaken to investigate the anticancer effects of sulforaphane against the human liver cancer cells. Liver cancer is the 5$^{th}$ and 8$^{th}$

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prevalent type of cancer in males and females, respectively. The risk of liver cancer has been reported to increase with age [11]. Around 0.56 million new cases of liver cancer are reported annually world over. Additionally, the frequency of liver cancer is comparatively higher in developing countries [12]. The adverse effects of currently available inefficient chemotherapy remarkably obstructs the treatment of liver cancer [13]. This has pressed the pursuit for new drugs from plants and herein we found that sulforaphane inhibits the growth of the human liver cancer cells by triggering cell cycle arrest and autophagy. Taken together, this study revealed the potential of sulforaphane as lead molecule for the development of liver cancer systemic therapy.

## Methods

*Cell proliferation assay*

The proliferation rate of the human HepG2 liver cancer cells and normal AML12 hepatocytes was monitored by WST-1 assay. In brief, liver cancer cells were cultured in 96-well plates at a density of $2\times10^5$ cells/well and treated with 0 to 200 µM concentrations of sulforaphane for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for another 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation.

*DAPI and Annexin V/PI*

The HepG2 liver cancer cells were cultured in 24-well plates for 24 h at 37°C. The cells were then collected by centrifugation and washed with phosphate buffered saline (PBS). After this, the cells were stained 1.2 mM DAPI for 5 min. The HepG2 cells were then washed with PBS and then observed both by fluorescence and phase contrast microscopy. For annexin V/PI assay, HepG2 cells ($5\times10^5$ cells/well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC/PI. The percentage of apoptotic HepG2 cells was determined by flow cytometry.

*Wound healing assay*

The HepG2 cells were cultured till 80% confluence. This was followed by removal of Dulbecco's modified Eagle's medium (DMEM) and subsequent washing with PBS. Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again under an inverted microscope

*Cell invasion assay*

The effects of sulforaphane on the invasion of the HepG2 cells was determined by transwell chamber assay with Matrigel. The HepG2 cells were transfected with miR-187 mimics and around 200 ml cell cultures were placed onto the upper chamber and only DMEM was placed in the bottom chamber. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200x magnification.

*Western blot analysis*

The HepG2 cells were then lysed in RIPA lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were separated by 10% SDS-PAGE, followed by transferring to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C for overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

*Statistics*

The experiments were performed in triplicate. The values presented are mean of three experiments ± SD. *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant. Student's *t*-test using GraphPad prism 7 software was employed for statistical analysis.

## Results

*Sulforaphane exerts antiproliferative effects on the human liver cancer cells*

The assessment of the antiproliferative effects of sulforaphane (Figure 1A) on the liver cancer cells and normal hepatocytes was carried out by WST-1 cell proliferation assay at concentrations

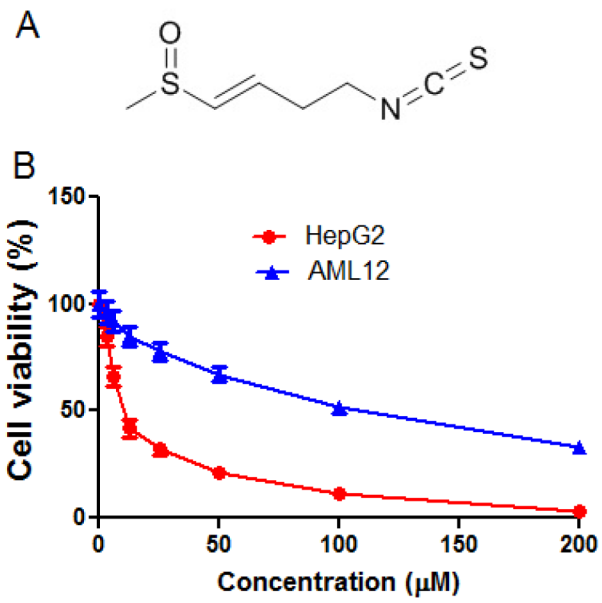


**Figure 1. A:** Chemical structure of sulforaphane. **B:** WST-1 assay showing the effects of sulforaphane on the proliferation of HepG2 and AML12 cells. The experiments were performed in triplicate and expressed as mean ± SD (p<0.05).

ranging from 0 to 200 μM. The results showed that sulforaphane suppressed the growth of the liver cancer HepG2 cells in a dose-dependent manner and an $IC_{50}$ of 9 μM was observed for sulforaphane against the HepG2 cells (Figure 1B). However, the growth of the normal human hepatocytes was minimally effected by sulforaphane as evidenced from the $IC_{50}$ of 90 μM.

*G2/M arrest of liver cancer cells induced by sulforaphane*

The HepG2 liver cancer cells were treated with various concentrations of sulforaphane and the distribution of HepG2 cells at each phase of the cell cycle was determined by flow cytometry. The results showed that the G2/M phase cells increased remarkably upon sulforaphane treatment. The percentage of G2/M phase cells were 19, 35.5, 51.7 and 68 % at 0, 4.5, 9 and 18 μM concentrations of Sulforaphane respectively, suggestive of G2/M arrest of the HepG2 cells (Figure 2). Western blot analysis was also performed to examine the effects of Sulforaphane on the expression of cyclin B1. The results showed that sulforaphane inhibited the expression of cyclin B1 in a concentration-dependent manner (Figure 3).

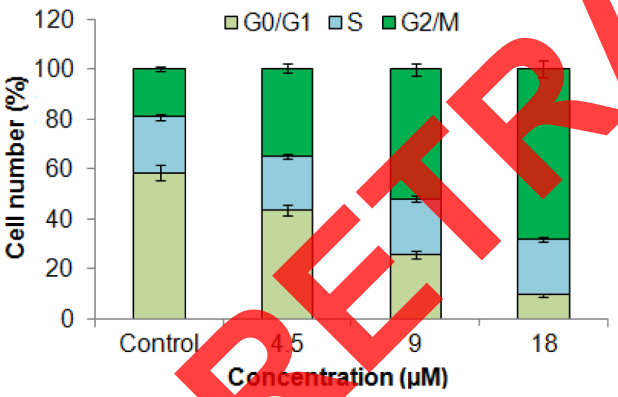


**Figure 2.** Effect of sulforaphane on the distribution of the HepG2 cells as determined by flow cytometry. The Figure shows that the percentage of the G2/M phase cells increased concentration-dependently ($p<0.05$). The experiments were performed in triplicate and expressed as mean ± SD.

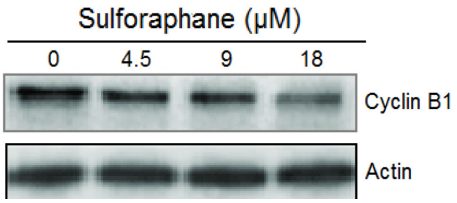


**Figure 3.** Effect of sulforaphane on the expression of the cyclin B1 as depicted by western blot analysis. The Figure shows that upon sulforaphane treatment of HepG2 cells the cyclin B1 expression decreased concentration-dependently. The experiments were performed in triplicate.

*Apoptotic cell death of HepG2 cells by sulforaphane*

Sulforaphane-induced apoptosis in the HepG2 cells was ascertained by DAPI staining of the sulforaphane-treated HepG2 liver cancer cells. The results of DAPI staining (Figure 4) showed that the molecule caused nuclear fragmentation of the HepG2 cells in dose-dependent manner, suggestive of apoptosis. The Annexin V/PI staining showed

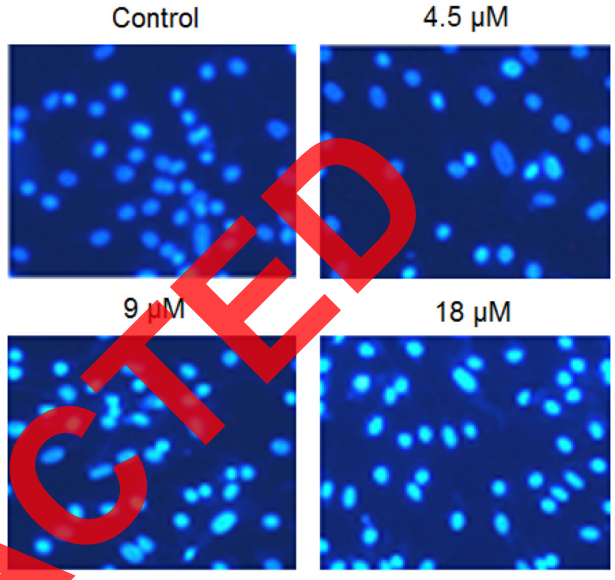


**Figure 4.** DAPI staining showing that sulforaphane causes nuclear fragmentation of the HepG2 cells. The experiments were performed in triplicate.

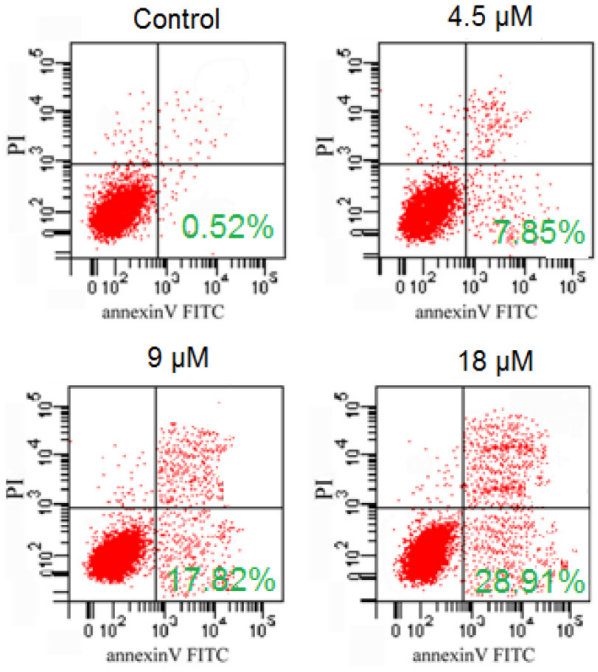


**Figure 5.** Annexin V/PI staining showing that the apoptotic HepG2 cell percentage increases with increase in the concentration of sulforaphane. The experiments were performed in triplicate.

that the percentage of apoptotic HepG2 cells increased with increase in the concentration of sulforaphane (Figure 5). The percentage of apoptotic HepG2 cells was 0.52, 7.85, 17.82 and 28.91% at the sulforaphane concentrations of 0, 4.5, 9 and 18 μM. Western blot analysis was carried out to examine the effects of sulforaphane on the expression of apoptosis biomarker proteins which showed that sulforaphane increased the expression of Bax and decreased the expression of Bcl-2. Additionally, the western blotting revealed that sulforaphane enhanced the cleavage of the capase-3 and -9 in a dose-dependent manner (Figure 6).

*Inhibition of cell migration and invasion of HepG2 cells by sulforaphane*

The effects of sulforaphane were investigated on the migration of the HepG2 cells at $IC_{50}$ by wound healing assay. The results showed that sulforaphane inhibited significantly the migration of the HepG2 cells at $IC_{50}$ as indicated by wound width (Figure 7). Transwell assay was used to assess the effects of sulforaphane on the invasion of the HepG2 cells which revealed that the invasion the HepG2 cells was decreased considerably upon treatment with different doses of sulforaphane (Figure 8).

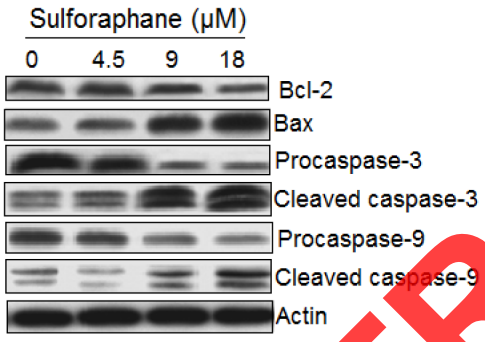


**Figure 6.** Effect of sulforaphane on the expression of the apoptosis-related proteins as depicted by western blot analysis. The Figure shows that the expression of Bax, cleaved caspase-3 and -9 increased while that of Bcl-2 decreased dose-dependently in HepG2 cells. The experiments were performed in triplicate.

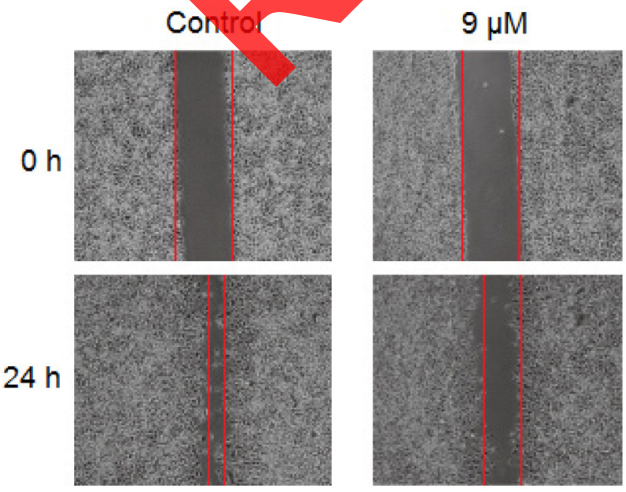


**Figure 7.** Wound healing assay showing that sulforaphane inhibits the migration of the HepG2 cells. The experiments were performed in triplicate.

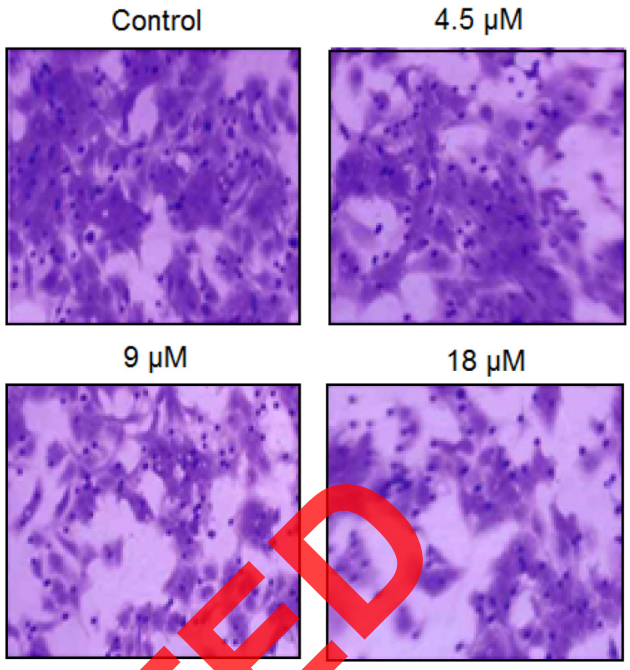


**Figure 8.** Transwell assay showing that sulforaphane inhibits the invasion of the HepG2 cells. The experiments were performed in triplicate.

*Inhibition of MAPK7 expression in the HepG2 cells by sulforaphane*

The effects of sulforaphane were also investigated on the expression of the MAPK7 in the HepG2 cells. The results showed that the expression of MAPK7 was significantly and concentration-dependently decreased upon treatment with Sulforaphane (Figure 8).

## Discussion

Accounting for more than 9% of all the cancer-related mortality, liver cancer was the 5th most prevalent cancer in 2012 [14]. Because of its aggressive nature and very poor survival rates, liver cancer remains one of the main health issues across the globe [15]. To combat the growing incidence and improve the survival rate of the liver cancer patients, it is important to develop novel, effective and efficacious drugs. Isothiocyanates of plant origin have gained importance as anticancer agents over last few decades [16]. This study evalu-

ated the anticancer effects of an important isothiocyanate, sulforaphane. The results have shown that sulforaphane suppressed the growth of liver cancer cells in a selective manner with minimally toxic effects on the normal cells. Previous studies have also shown that sulforaphane exhibits the potential to halt the growth of cancer cells. For example, it has been reported to suppress the growth of Barrett adenocarcinoma and colon adenocarcinoma cells [17,18]. The flow cytometric analysis of the sulforaphane-treated HepG2 cells showed that it induces G2/M cell cycle arrest which was also accompanied by depletion of cyclin B1 expression. The cell cycle arrest inducing property of sulforaphane has also been reported previously [19-21]. It has been shown to cause growth inhibition of leukemia cells by inducing cell cycle arrest [19]. Similarly, it has also been reported to induce cell cycle arrest in pancreatic and ovarian cancer cells, further validating the results of the present investigation [20,21]. Sulforaphane has also been shown to cause apoptosis of cancer cells, for example, it has been reported to cause apoptosis in breast, prostate and lung cancer cells [22-24]. Therefore, we carried out DAPI staining of the sulforaphane-treated liver cancer cells to assess if it also induces apoptosis in the liver HepG2 cancer cells. The results showed that this molecule induces apoptosis in the liver cancer cells which was also accompanied by the activation of caspases and enhancement of the Bax/Bcl-2 ratio. The effects of sulforaphane were also examined on the migration and invasion of the HepG2 cells which is in concordance with a previous study wherein it was reported to inhibit the migration and invasion of oral cancer and glioblastoma cells [25,26]. The effects of sulforaphane were finally examined on the MAPK7 by western blot analysis and the results showed that sulforaphane caused a remarkable and concentration-dependent decrease in the expression of MAPK7, suggestive of the anticancer potential of this molecule.

## Conclusion

Taken together, the results of the present study indicate that sulforaphane is a potent anticancer agent and exerts anticancer effects on human liver cancer cells via induction of cell cycle arrest and apoptosis. It also inhibits the migration and invasion of the liver cancer cells and may prove useful in the development of systemic therapy for liver cancer.

## Conflict of interests

The authors declare no conflict of interests.



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