



Original Research Article

Sulphoraphane, by virtue of its antioxidant potential down-regulates HSP90 in leukemia cells

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ABSTRACT

Keywords

Leukemia;
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HSF1;
anti-oxidant
enzymes.

Persistent pro-oxidative state leading to oxidative stress is a key feature of cancer cells. Cancer cells generate more reactive oxygen species (ROS) than their normal counterparts. Heat shock proteins (HSPs), which are a highly conserved family of proteins that aid in proper folding, are induced in response to various forms of stresses including oxidative stress. Of all types of HSPs, HSP90 play a pivotal role in the pathogenesis of cancer. Targeting ROS and HSP90 could be a novel approach in cancer control. Inhibitors used are generally toxic and therefore adopting natural means in this regard is highly desirable. Isothiocyanates are a class of plant derived molecules showing anti-carcinogenic properties. Sulphoraphane (SFN), an isothiocyanate, by inducing anti-oxidant enzymes can quench ROS resulting in down-regulation of Heat shock factor (HSF1), HSP90 and its client proteins Bcr-Abl and c-Raf in leukemia cells. Similar trend was observed in lymphocytes subjected to oxidative stress.

Introduction

Cancer cells are under elevated oxidative stress, which is due to an imbalance between cellular anti-oxidant defence mechanism and ROS production in the cell (Acharya *et al.*, 2010). Cancer cells produce higher level of ROS than the normal cells and ROS is responsible for maintaining the cancer phenotype (Gibellini *et al.*, 2010). In tumor cells, ROS are enhanced due to oncogene

activation, lack of blood supply, decrease or inactivation of anti-oxidant enzymes, high metabolic activity (Wang and Yi, 2008; Hole *et al.*, 2011). Elevated ROS level has been documented in various types of cancer including leukemia (Hole *et al.*, 2011). ROS acts as a secondary messenger in cell signalling cascade and are important for several physiological processes (Acharya *et al.*, 2010). ROS are

regarded as oncogenic as they aid in tumor cell survival by promoting initiation, promotion, progression and metastasis of cancers (Wang and Yi, 2008; Hole *et al.*, 2011). Oxidative stress causes cellular damage to the proteins, lipids, membranes and DNA contributing to carcinogenesis (Khandrika *et al.*, 2009; Fearon and Faux, 2009; Wells *et al.*, 2009; Valko *et al.*, 2006). To combat the ROS induced oxidative damage, aerobic organisms have developed a variety of anti-oxidant defence mechanisms for maintaining their genetic stability. These include different anti-oxidant enzymes that act in cellular defence, such as catalase, super oxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione S transferase (GST) (Acharya *et al.*, 2010). Heat shock proteins (HSPs) are evolutionary conserved proteins that assist in proper folding and degradation of misfolded proteins (Sreedhar and Csermely, 2004; Soo *et al.*, 2008). HSPs are produced in response to different forms of stresses including oxidative stress (Jolly and Morimoto, 2000; Gorman *et al.*, 1999; Kalmar and Greensmith, 2009; Martindale and Halbrook, 2002). Cancer cells over-express HSPs in order to survive under the stressed environment (Ciocca and Calderwood, 2005, Khalil *et al.*, 2011). There are various types of HSPs, based on their molecular weights (Khalil *et al.*, 2011). Among all HSPs, HSP90 plays most important role in tumorigenesis (Bagatell and Whitesell, 2004). HSP90 interacts with various proteins of apoptotic cascade, thus preventing apoptosis (Lanneau *et al.*, 2007; Bagatell and Whitesell, 2004). It modulates various cellular signalling pathways (Powers and Workman, 2006), maintains stability and function of different kinases (Marcu *et al.*, 2002). Targeting HSP90 interferes with signalling proteins like Bcr-Abl, c-Raf etc

which impart survival to the cancer cells. HSP90 is regulated by a transcription factor, heat shock factor (HSF1) (Dai *et al.*, 2007; Solimini *et al.*, 2007). Therefore targeting HSP90 is an emerging area to treat cancer. Inhibitors of HSP90 are not free from toxicity; therefore identifying agents that will selectively kill malignant cells without affecting normal cells will widen the therapeutic approaches in cancer. Phytochemicals are a (Surh, 2003) good choice in this respect. Isothiocyanates present in cruciferous vegetables show various cancer fighting properties; sulphoraphane is one such naturally occurring isothiocyanate. Anticancer properties of sulphoraphane are attributable to its blocking and suppressing effects (Li *et al.*, 2010). Sulphoraphane induces phase II detoxification enzymes, inhibits phase I enzymes and acts as an effective modulator of cell death, cell cycle, angiogenesis, susceptibility to carcinogens, invasion and metastasis. It also possesses antioxidant activities (Xu *et al.*, 2012). Sulphoraphane inhibits various signalling molecules which are client proteins of HSP90.

Present study aims to elucidate the role of sulphoraphane, a natural isothiocyanate on expression of HSP90 and HSF1 in leukemia cells. It was further investigated whether quenching of ROS by natural means is associated with the down-regulation of HSP90 and HSF1.

Materials and Methods

Cell culture

Human chronic myelogenous leukemia cell line K-562, used in this study, were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (gentamycin,

penicillin and streptomycin). Cells were allowed to grow in a humidified 5% CO₂/95% air atmosphere at 37°C.

Isolation of lymphocytes

Heparinised blood from healthy individuals was layered on Histopaque 1700 and centrifuged at 1000 rpm for 20 min. Lymphocytes were collected from the buffy coat at the interface. Isolated lymphocytes were cultured in RPMI-1640 with 10% FBS and phytohaemagglutinin (PHA) 20 µg/ml.

Treatment protocol

K-562 cells were treated with different concentrations of sulphoraphane (0, 1, 5, 10, 20 µM) for 24 h. Lymphocytes were treated with various concentrations of sulphoraphane for 24 h, followed by insult with H₂O₂ (50 µM) for 1 h.

Assessment of cytotoxicity of sulphoraphane

MTT assay was performed to study whether sulphoraphane is cytotoxic to K-562 cells. This colorimetric assay measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl) - 2, 5 - diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenase enzyme. The tetrazolium compound MTT solution 50 µl (1.2 mg/ml in water) was added to the treated cells in each of the 96 wells and incubated for 4 h. MTT-formazan product was dissolved in DMSO, and the product was estimated by measuring absorbance at 570 nm in an ELISA plate reader. The amount of formazan produced is directly proportional to the number of living cells.

Western blotting

Western blot analysis of HSP90, HSF1,

Bcr-Abl, c-Raf was carried out using corresponding antibodies (Sarkar *et al.*, 2012; Sarkar *et al.*, 2013). Cell lysates containing equal amount of proteins were analysed on 12.5% SDS under reducing condition. Gel was then electroblotted on to a nitro cellulose membrane, which was subsequently incubated with primary and secondary antibodies and treated with BCIP/NBT to visualise the proteins. β-actin was used as a loading control.

Measurement of ROS

ROS level was measured by a fluorometric assay with 2', 7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is a small nonpolar, nonfluorescent, cell membrane permeable molecule, which gets diffused into cells. DCFH is oxidized by reactive oxygen species producing a highly fluorescent compound 2', 7'-dichlorofluorescein (DCF). The fluorescence intensity of DCF inside the cells is proportional to the amount of ROS produced. Briefly, 5x10⁵ cells were treated with different concentrations of sulphoraphane and harvested. Cells suspended in HEPES buffered saline (HBS, pH 7.4 containing NaCl 140 mM, KCl 5 mM, HEPES 10 mM, CaCl₂ 1 mM, MgCl₂ 1 mM, glucose 10 mM) were incubated with DCFH-DA (5µM) for 30 min in dark, at 37°C (Sinha *et al.*, 2010). Fluorescence intensity as measured in a spectrofluorimeter (excitation 504 nm; emission 529 nm) gives an estimation of ROS.

Measurement of antioxidant enzymes

For catalase measurement, cell supernatant, treated with ethanol (10 µl/ml) was kept in ice for 30 min. This was followed by addition of 1% Triton X-100 and kept on ice for 30 min. Cell

supernatant was then added to the assay mixture, contacting 0.5 M sodium phosphate buffer, pH 7.0 and 10 mM H₂O₂. The absorbance was noted at 240 nm. Catalase activity is defined as the amount of enzyme required to decompose 1 M of H₂O₂ per minute (Sinha *et al.*, 2007).

Superoxide dismutase (SOD) was measured on the basis of the ability of the enzyme to inhibit auto-oxidation of pyrogallol. The cytosolic supernatant was treated with 1% Triton X-100 and kept for 30 min at 4^o C. This was added to the assay mixture containing 0.05 M sodium phosphate buffer (pH 8.0), 0.1 mM EDTA and 0.27 mM pyrogallol. Absorbance was measured at 420 nm. 1 unit is defined as the amount of SOD required to produce ½ the minimal inhibition of pyrogallol auto-oxidation (Sinha *et al.*, 2007).

Statistical Calculation

Statistical calculation was performed using SPSS 10.0 (one way ANOVA followed by Dunett t-test).

Results and Discussion

Effect of sulphoraphane on lymphocytes isolated from normal healthy donors

MTT reduction assay reveals that sulphoraphane hardly shows any toxicity towards lymphocytes isolated from normal healthy donors [Fig 1(A)]. Relative absorbance at 570 nm corresponds to the number of live cells. The highest concentration of sulphoraphane used was 20 µM; for K-562 cells relative absorbance is 0.476 where as for lymphocytes it is 0.947, indicating that sulphoraphane shows differential toxicity in K-562 cells and lymphocytes.

Constitutive expression of HSF1 and HSP90

Western blot analysis clearly shows that the expression of HSP90 and the transcription factor HSF1 are constitutively high in K-562 cells compared to the normal lymphocytes [Fig 1(B)].

Effect of sulphoraphane on expression levels of HSF1 and HSP90

Sulphoraphane (1, 5, 10 and 20 µM) down-regulates the expressions of HSP90 dose dependently in K-562 cells as revealed by western blot analysis. HSF1, which is over-expressed in K-562 cells, is also negatively regulated by sulphoraphane in a concentration dependent manner [Fig 1(C)]. HSF1, which is a transcription factor regulating heat shock proteins, inhibits HSP90 by virtue of its down-regulation by sulphoraphane. 17-AAG is an inhibitor of HSP90. Western blot analysis shows that sulphoraphane (20 µM) can inhibit HSP90 as achieved by treatment with 3 µM 17-AAG [Fig 1(D)]. Results give a clear indication that sulphoraphane acts an inhibitor of HSP90.

Regulation of Bcr-Abl and c-Raf

Bcr-Abl, a tyrosine-kinase inhibitor is the first-line therapy for most patients with chronic myelogenous leukemia (CML). One mechanism by which Bcr-Abl signals in cells is by activation of onco-protein c-Raf (Nakamura *et al.*, 2011). Effect of sulphoraphane on these two onco-proteins has been looked into. Western blot results show that both the onco-proteins are negatively regulated by sulphoraphane and this down-regulation is dose dependent [Fig 1(E)]. The proto-oncogene c-Raf is

part of the ERK1/2 pathway as a MAP kinase kinase kinase (MAP3K) that functions downstream of the Ras subfamily and is a member of the serine/threonine-specific protein kinases (Avruch, 2007). HSP90 maintains the stability and function of these two vital oncoproteins. Inhibition of HSP90 blocks the chaperoning activity of the protein, resulting in the down-regulation of Bcr-Abl and c-Raf. Similar results are obtained with different concentrations of 17-AAG (0, 0.1, 0.5, 1.5 and 3 μ M) [Fig 1(F)], confirming that suppression of Bcr-Abl and c-Raf by sulphoraphane is due to inhibition of HSP90.

Modulation of H₂O₂ induced generation of ROS by sulphoraphane

Basal level of ROS in lymphocytes is low compared to K-562 cells [Fig 2 (A)]. It was intended to see whether generation of ROS in lymphocytes could affect the HSP90 and HSF1 level. Sulphoraphane effectively reduces the level of ROS in K-562 cells in a dose dependent way [Fig 2 (B)]. Hydrogen peroxide (H₂O₂) is simplest peroxide which acts as a strong oxidizer and due to the oxidizing capacity it is considered a highly reactive oxygen species. 50 μ M H₂O₂ induces ROS in lymphocytes, which can be reduced to the basal level with increasing concentration of sulphoraphane [Fig 2 (C)]. HSF1 and HSP90 level in lymphocytes are low compared to K-562 cells. Generation of ROS increases the expression of HSF1 and HSP90, which can be efficiently de-regulated by sulphoraphane [Fig 2 (D)]. Results clearly indicate that ROS is intricately related to the elevated expression of HSF1 and HSP90.

Activation of anti-oxidant enzymes by sulphoraphane

Sulphoraphane efficiently induces anti-oxidant enzymes like catalase [Fig 3 (A)] and superoxide dismutase (SOD) [Fig 3 (B)] in K-562 cells, thereby imparting protection against ROS. Sulphoraphane therefore could be considered as a potential anti cancer agent in leukemia cells.

HSP90 is an important heat shock protein which plays vital role in different cellular processes like cell proliferation, differentiation and apoptosis (Conte *et al.*, 2011). Therefore HSP90 as a target from therapeutic point of view has gained tremendous importance. Cancer cells continuously face many forms of stresses, which leads to generation of free radicals. Proper functioning of body depends on a balance between generation of free radicals and antioxidants (Lobo *et al.*, 2010). These cells can survive extreme environmental stimuli such as hypoxia, stresses due to chemotherapy and radiation. Exposure to free radicals results in significant damage to cellular proteins. Molecular chaperones like HSP90 protect such damaged proteins and are therefore highly expressed in malignant cells. Heat shock proteins (HSP) play an important role in maintaining protein stability and function (Wang *et al.*, 2004). Thus they help in cell survival under stressed conditions. They also aid in the process of tumorigenesis including leukemia. Aggressiveness of leukemia is partly due to higher expression of HSP90. Therefore HSP90 can be considered as an attractive target in carcinogenesis particularly CML (Zackova *et al.*, 2012).

Fig.1 Modulation of various parameters by sulphoraphane

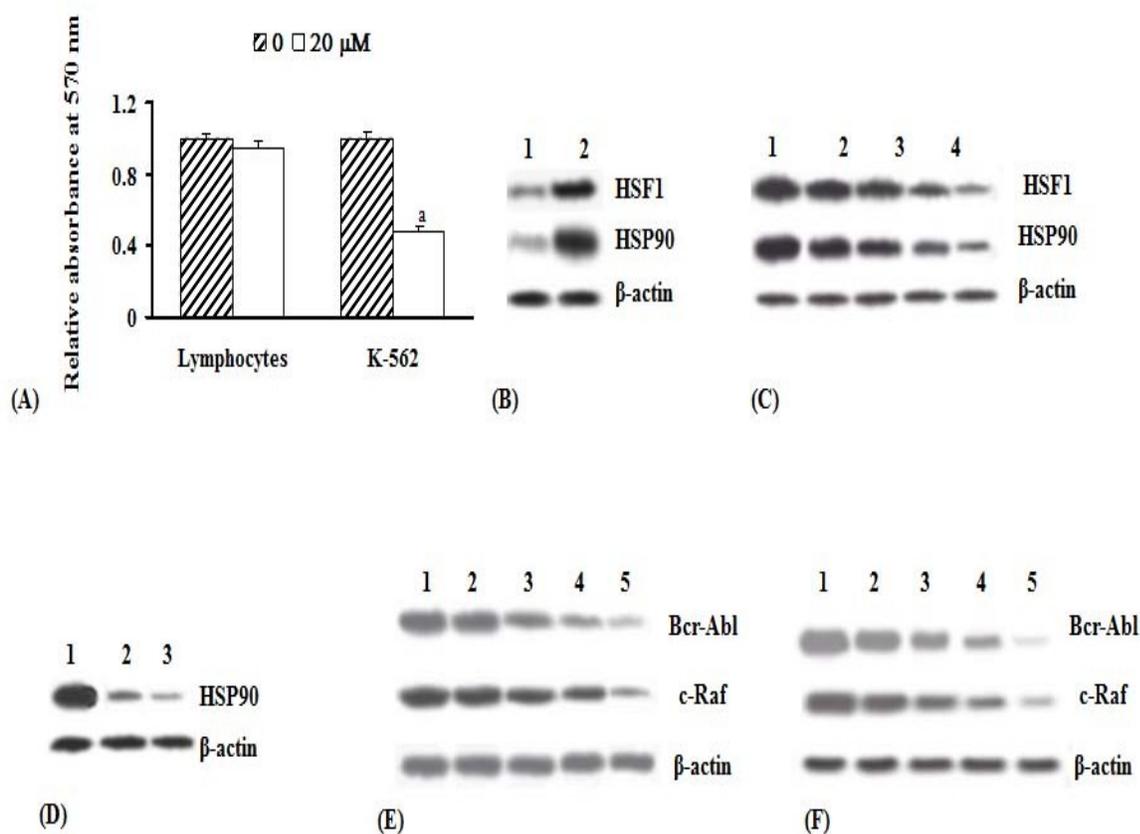


Fig.1

Fig 1(A) shows the differential toxicity of sulphoraphane in lymphocytes and K-562 as assessed by MTT assay. The experiment has been repeated thrice. Values represent mean \pm SE (n=3). Values are significant ^a(p<0.005), with respect to untreated cells. Fig 1(B) Constitutive expression of HSF1 and HSP90 as obtained by western blot analysis. Lanes 1 and 2 show the proteins bands in lymphocytes and K-562 cells respectively. Fig 1(C) shows the modulation of HSF1 and HSP90 in K-562 cells. Lanes 1 - 5 show the protein bands corresponding to sulphoraphane concentrations of 0, 1, 5, 10, 20 μ M. Fig 1(D) Constitutive expression of HSP90 (Lane 1), as modulated by sulphoraphane (20 μ M) and 17-AAG (3 μ M) has been shown in Lanes 2 and 3 respectively. Fig 1(E) Modulation of Bcr-Abl and c-Raf in K-562 cells by sulphoraphane. Cells are treated with sulphoraphane at concentrations 0 μ M (lane 1), 1 μ M (lane 2), 5 μ M (lane 3), 10 μ M (lane 4) and 20 μ M (lane 5) for 24 h. Western blot analysis using anti-Bcr-Abl and c-Raf antibodies has been performed. β -actin is used as control to ensure equal loading of proteins. Fig 1(F) Modulation of Bcr-Abl and c-Raf in K-562 cells by 17-AAG. Cells are treated with a specific HSP90 inhibitor 17-AAG for 24 h at concentrations 0 μ M (lane 1), 0.1 μ M (lane 2), 0.5 μ M (lane 3), 1.5 μ M (lane 4) and 3.0 μ M (lane 5). β -actin is used as a loading control.

Fig.2 Estimation of ROS and its modulation

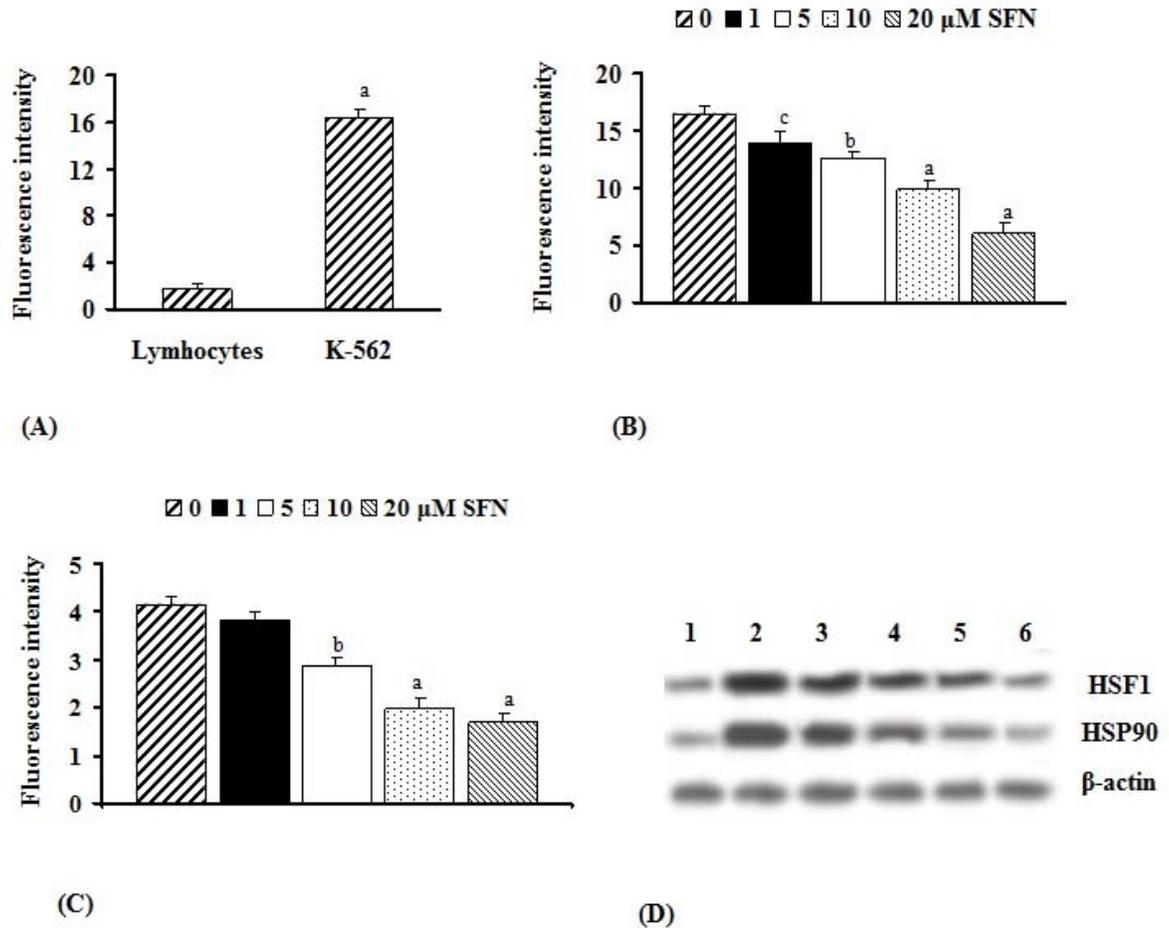


Fig. 2

Fig.2(A) represents the constitutive level of ROS in lymphocytes and K-562 cells. Experiments have been performed in triplicates. Values represent mean \pm SE (n=3). Values are significant ^a(p<0.001) with respect to the lymphocytes. Fig 2(B) ROS level as influenced by increasing concentrations of sulphoraphane has been depicted in bar diagram. Experiments have been performed in triplicates. Values represent mean \pm SE (n=3). They are significant ^a(p<0.001), ^b(p<0.005) and ^c(p<0.01) with respect to the untreated cells. Fig 2(C) shows the effect of sulphoraphane on lymphocytes. Experiments have been performed in triplicates. Values represent mean \pm SE (n=3). They are significant ^a(p<0.001), ^b(p<0.005) with respect to the untreated cells. Fig 2(D) shows how sulphoraphane protects lymphocytes challenged by H₂O₂. Fig 2(E) Induction of HSF1 and HSP90 by H₂O₂ in lymphocytes and their modulation. Lane 1 shows the constitutive level of HSF1 and HSP90 in lymphocytes. Lane 2, 3, 4, 5, 6 show the H₂O₂ induced expression of HSF1 and HSP90 as influenced by 0, 1, 5, 10, 20 μM sulphoraphane respectively.

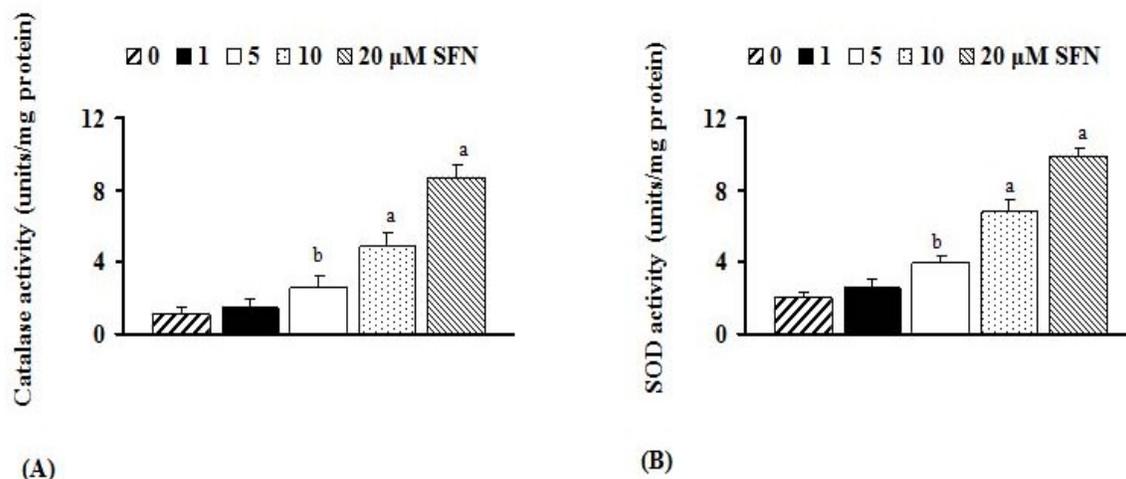
Fig.3 Induction of antioxidant defence enzymes**Fig. 3**

Fig 3(A) shows the catalase activity in K-562 cells with increasing doses of sulforaphane (0, 1, 5, 10, 20 μ M). The result obtained is the mean of three independent experiments. Values represent mean \pm SE (n=3). ^a(p<0.001), ^b(p<0.005) are significantly different compared with untreated cells. Fig 3(B) Bar diagram represents the SOD activity in K-562 cells with different doses of sulforaphane as described before. The experiment has been repeated thrice. Values represent mean \pm SE (n=3). Values are significant ^a(p<0.001), ^b(p<0.005) with respect to untreated cells.

Natural compounds are blessed with their medicinal values and are anti-carcinogenic. Role of these compounds in manipulating the expression of HSP90 has been highlighted in this study. Isothiocyanates are a group of compounds present in cruciferous vegetables (Roy *et al.*, 2012). Sulforaphane belonging to the isothiocyanate group of organosulfur compounds are commonly found in cauliflower, cabbage, broccoli, brussel sprouts etc. These compounds are unique as they are preferentially toxic to the cancer cells, sparing the normal counterparts. Therefore, using such compounds in therapy will be harmless.

Present study has been conducted in chronic myelogenous leukemia model K-

562, which over expresses HSF1 and HSP90. These biomarkers may be targeted in a non-toxic way using natural compounds so that healthy cells are hardly affected. Furthermore it became apparent that generation of ROS is a vital factor for up-regulation of HSP90. Quenching ROS by sulforaphane results in inhibition of HSF1 and subsequently HSP90. Two important client proteins of HSP90 namely Bcr-Abl and c-Raf has been down-regulated by sulforaphane. Similar results have been obtained when K-562 cells have been treated with 17-AAG, a specific inhibitor of HSP90. Results show that HSP90 plays a pivotal role in the signalling pathways controlling leukemogenesis.

Lymphocytes normally possess lower levels of ROS as compared to cancer cells (K-562). A stressed condition has been created in lymphocytes using H₂O₂, which simulates the development of carcinogenic process. Under this stressed condition the HSF1 and HSP90 levels have been found to be high compared to the unstressed lymphocytes. This induced ROS level can be well controlled by sulphoraphane. Quenching of ROS leads to inhibition of HSF1 and HSP90, two important prognostic markers in cancer. Sulphoraphane reduces the expression level of HSF1 and HSP90 in stressed lymphocytes as well as in K-562 cells, thereby proving that ROS is closely associated with higher expression of HSF1 and HSP90. Sulphoraphane has been found to increase the activity of anti-oxidant enzymes catalase and SOD; which in turn helps to reduce ROS levels in cancer cells. Out of control proliferation is a key feature of cancer cells and ROS (exogenous and endogenous) help in this regard (Gupta *et al.*, 2012). Agents that inhibit ROS can inhibit aberrant growth of cancer cells. This study shows that sulphoraphane, which is blessed with strong anti-oxidant properties, may be considered as an attractive compound in the battle against cancer although further studies are required to prove its potential in leukemia therapy.

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