# Dialyl-sulfide with chalcone prevent breast cancer promoting SULT1E1 malregulations and oxidant-stress dependant HIF1a-MMPs induction.

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**Abstract**

Background: In some breast cancers, decreased estrogen-sulfotransferase (SULT1E1) and its inactivation caused by oxidative-stress lead to elevated E2 levels as well as hypoxia-inducible tissue-damaging factors. Methods: Here, matrix-metalloproteases (MMP2/9) activity and SULT1E1-HIF1α protein/gene expression (Western-blot/RTPCR) were assessed in human breast-cancers versus their adjacent-tissues. Oxidant-stress neutralizer, chalcone (α,β unsaturated ketone) and SULT1E1-inducer dialyl-sulfide (source garlic; Allium sativum) were tested to prevent cancer causing factors in rat, in-vitro and in-vivo model. The antioxidant-enzymres SOD1, catalase, GPx and LDH, and matrix-degenerating MMP2/9 activities were assessed (gel-zymogram). Histoarchitecture (HE-staining) and tissue SULT1E1-localization (immuno-histochemistry) were screened. Extensive statistical-analysis were performed. Results: Human cancer-tissue expresses higher SULT1E1, paralleling HIF1α protein/mRNA owing to lower LDH activity. In addition, increase of MMP2/9 activities commenced tissue damage. However, chalcone and DAS significantly induced SULT1E1 gene/protein, and suppressed HIF1α expression, and MMP2/9 activities in rat tissues. Correlation of individual parameter statistics and group statistics of t-test suggest significant correlation of oxidative-stress (MDA) with SULT1E1 (p=0.006), HIF1α (p=0.006) protein-expression. The NPSH showed a negative correlation (p=0.001) with HIF1α, These two proteins and SULT1E1 mRNA expressions in human breast tumor were significantly higher ($p<0.05$) compared to the adjacent tissues. Pearson correlation data suggest, SULT1E1 is correlated with NPSH in different exposure groups. Conclusions: Breast cancers associate with SULT1E1, HIF1α and MMPs deregulations. Higher SULT1E1-protein in advanced cancer, remain inactive in oxidant oxidative environment and may be re-activated in chalcone induced reducing-state. Moreover, DAS induced SULT1E1 mRNA expression augments its protein increment. Synergistic drug-effects commenced HIF1α and MMPs suppression. Further studies are necessary.

## INTRODUCTION

Breast cancer is the most common female cancer globally, representing nearly a quarter (25%) of all cancers. Organic carcinogens covalently bind DNA formingstable and depurinating adducts. The compound 4-hydroxyestradiol and estradiol-3, 4-quinone (E1(E2)-3,4-Q)) produces significantly higher levels of depurinating adduct and smaller stable adduct (1). These estrogen-DNA adducts are quickly lost by the cleavage of the glycosyl bond from DNA and create apurinic sites that can cause cancer (2,3). Mounting evidences demonstrate that a few E2 metabolites (i.e. CE-3, 4-Q) interact with DNA and form 4-OHE1(E2)-1-N3Ade and 4-OHE1(E2)-1-N7Gua that causes DNA mutation and potential cellular transformation (4,5).

The use of human breast epithelial cell lines such as MCF-10F, an immortalized, non-transformed estrogen receptor (ER)-α-negative cell line has identified the initiation of cancer by estrogen-DNA adducts. Results indicate that beside the alteration of E2-ER signaling, genotoxic effects via estrogen metabolite are partly responsible for the malignant transition . These findings support the hypothesis that the availability of the active estrogen is a critical determinant in the treatment of some breast cancer.

A large percentage of breast cancers are sensitive to estrogen and have a good response to endocrine therapy based on selective ER modulators (SERM) i.e. tamoxifen (7) and fulvestrant (8). Reduction in estrogen synthesis by aromatase-inhibitors of is also a vital strategy (9). Beyond these strategies, estrogen metabolizing enzymes are given less priority for research. SULT1E1, a phase II metabolizing enzyme that is located in the cytoplasm and responsible for sulfonation of estrogen (10). SULT1E1 polymorphisms are shown to be a risk factor for breast and endometrial cancers, (11) suggesting that its modulation might be an attractive strategy in the prevention, management and or treatment of breast cancer. In contrary, the steroid sulfatases (STs) converts water soluble sulfated-estrogen to insoluble estrogen in the target tissues. Roughly, it sketches that activation of SULT1E1 and inhibition of STS might work in favor of breast cancer treatment.

The induction of SULT1E1 along with anti-breast cancer action of TM208 and tamoxifen (ER antagonist) may bring better treatment outcomes in hormone ER+ breast cancers. Estrogen is differentially metabolized in healthy ovarian surface epithelium (OSE) and in epithelial ovarian cancer (EOC) cells. Total mRNA assay by qRT-PCR revealed significantly higher SULT1E1 mRNA expression in OSE than in EOC. Inflammatory cytokines further augmented the local production of E2 by stimulating STS and suppressing SULT1E1 (12). It was concluded that the local estrogen metabolism may be a target for EOC treatment. SULT1E1 is transcriptionaly regulated under oxidative stress, and is also a target of NRF2. Oxidative stress responses augment Nrf-2 and HIF-1a in breast cancer cell. Hypoxia Inducing Factor (HIF) target genes in every step of the metastatic process. Digoxin which blocks HIF action eventually decreases primary tumor growth, vascularization and invasive metastasis in animal models of breast cancer (13,14). Degeneration of extracellular matrix is implicated as the metastatic growth factor and MMPs are highly expressed in advanced breast cancer. A high expression of MMP-2 and MMP-9 was found in >75% of breast cancer patients (15). So, it is suggested that the restriction in HIF1α and MMP activities may have therapeutic prospects against breast cancer.

In our earlier study, we explored that the pre-tumorigenic condition induced by ethylnitrosourea (ENU) and E2 showed impairment of SULT1E1 expression and E2 regulations via oxidant-stress signaling (16). Thus, induction and activation of SULT1E1 might become cancer prevention and treatment startegy. An earlier study demonstrated that dialylsulfide (DAS) treatment caused the nuclear accumulation of constitutive androstane receptor (CAR) resulting in a significant increase of SULT1E1 mRNA and protein in the liver of female mice. DAS effectively reduces lipid peroxidation, recovering cell viability, attenuating DNA strand breaks in cultured breast cancer MCF-10A cells (17). Despite of significant SULT1E1 induction by DAS, the endogenous E2 level was unaltered and no increase in estrone sulfate level was noticed. Contrarily, the clearance of exogenously administrated E2 was accelerated in DAS treated mice (18). This suggests that under variable oxidative stress condition, SULT1E1 transits between gain and loss of enzyme activity Our previous report suggests that oxidative stress induced SULT1E1 modifications may be similar in human breast cancer tissue and experimental animal model (16). Further study demonstrates that transcriptional regulation of stress, promotes Nrf-2 and proinflammatory marker NFkβ through E2 mediated signaling. These signallings are also associated with breast cancer initiation and severity. So, a reducing environment is required to keep SULT1E1 active which may keep E2 levels under control, SULT1A1 also sulfoconjuagtes E2 but at higher concentration and definitely contributes to control the E2 pool. Chalcone posses oxidative stress relieving property and and act as antioxidant in different diseases (19). Hence, we opted for Chalcone in this study to induce cellular reducing environment to keep SULT1E1 active together with Dialylsulfide that induces mRNA/protein expression of SULT1E1. Our present study may have some therapeutic implications in breast cancers.

## MATERIALS AND METHODS

### Ethical clearance and fulfillments of other regulatory affairs

This is to state that the present study was carried out in accordance with the National Institutes of Health, USA guidelines and the institutional ethical concerns, relevant guidelines and regulations were maintained throughout the investigation. This study confirms that all experimental protocols were approved by the institutional (Oriental Institute of Science and Technology) Ethics Committee (oist/EC/hu/bt/16/). This

is also to state that informed consent was obtained from all participants who were at their post-menopausal age.

Female Wistar rats were purchased from a small-animal firm house (Govt. registered) that follows all ethical norms and maintain requisite regulatory affairs. The firm house is a Government accredited (CPCSEA-Committee for the Purpose of Control and Supervision of Experiments on Animals: Reg. no 1A2A/PO/BT/S/15/CPCSEA. organization under the Dept of Animal Husbandry and Dairy, Ministry of Agriculture and Farmer's Welfare, Govt. of India. Proper permissions for all animal experiments, were obtained from the Institutional (Oriental Institute of Science and Technology) Review Board.

**Inclusion and exclusion criteria**

Inclusion criteria- Patients only with breast carcinoma were included. Tumors were collected only from patients undergoing mastectomy. Those patients were also included who had a large gap of time between chemotherapy and mastectomy.

Exclusion criteria- Women suffering from endometriosis, pelvic inflammatory disease, tuberculosis, or any kind of liver and kidney disease, ovarian cancers, Poly cystic ovarian syndrome, colon carcinoma, lung carcinoma, pregnancy, menstruation or any other infectious disease like HIV, HPV, HCV and Hepatitis B were excluded.

**Details of the participants**

Detailed information about randomly selected patients (maintaining both-side anonnimity and double blind random screen) who donated their tumor samples has been provided in S-Table 1. The S-Table 1 informs about the patient's age (44.69 ± 7.55 years; mean ± SD) and body weight (56.08± 6.63 Kg; mean ± SD), livelihood status, nutritional status, grade of the disease, tumor size and the details regarding the inclusion of the lymph nodes and metastasis.

**Breast tumor sample collection**

The study was conducted in Oriental Institute of Science and technology and a total of 23 breast tumor samples were obtained from the local District Medical College and Hospital with proper ethical clearance. Breast tumors are diagnosed clinically; breast cancers were classified on the basis of TNM (The extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M)) staging and grades. In some cases down staging of cancer with chemotherapy was done prior to surgery and samples were collected. In this regard, this is to mention that tumor sample and corresponding surrounding tissues were collected separately soon after surgery and stored at -20°C. A small part of the tissue was also stored in formalin for histology and immunohistochemistry. We sincerely thank Dr. Guangping Chen of Department of Physiological Sciences of Oklahoma State University for providing the primary antibody against rSULT1E1 and hSULT1E1. Anti- Nrf-2 and NFkβ antibodies were used in the current study.

**Treatment of animal model (rats) with Diallyl sulfide and Chalcone.**

The animal experiments were designed to study short term, and long term effect of the drugs. The results may provide us with valuable information of duration required by these drugs to affect the desired expression and activation of SULT1E1. Three different sets of experiment were used for evaluating the effect of diallylsulfide and chalcone. Two of the experiment were in-vivo and one in vitro experiment.

**In- vitro (3 hour, 5 hour and overnight treatment)**

*Preparation of single cell suspension of rat hepatocyte*

The animal experiments were performed in the Oriental Institute of Science and Technology, Cell and Mol. Therap. Lab. animal facility and surgery chamber. The fresh and laboratory acclimatized animals were anesthetized and then euthanized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Fifty grams of rat liver tissue processed to have single cell suspension. A nylon mesh was used to scrape the tissue in DBSS Buffer. The scarpped product was centrifuged at $120\times g$ . The cellular pellet was washed

with constituted L-15 media. A final suspension of the cells was made in 10 ml of constituted L-15 media containing 1500 mg/l D-glucose, 1% penicillin and streptomycin, 20% FBS, 1% Glutamine. 650 µl of media containing cell was added to each petri plate comprising of 9350 µl of constituted L-15 media containing the required drug.

Three in vitro groups based on duration of exposure were considered for this study. . Grp 1-3hr, Grp 2-5 hr and Grp 3-12 hrs exposure. Each petri dish was provided with 650 µl of cell suspension (from stock) and each group comprised of 4 plates containing a total of 10 ml solution such as Plate-1 (control): 650µl of cell suspension+ 9250 µl buffer, Plate-2: DAS-100 µM+ buffer (making up to 10 ml), Plate-3: Chal-100µM+buffer (making up to 10 ml), and Plate -4: DAS+Chal 100µM each + buffer (making up to 10 ml).

**In –vivo (24, 48, 72 hours treatment)**

Animals were divided in 3 groups, Group 1-control, Group 2-Diallysulfide (DAS), 80 mg DAS/100gm b.w., Group 3- Chalcone, 4 mg/100gm b.w., Group 4- Combination of DAS (80 mg) and Chalcone (4mg). The animals were treated with the drug or drug combination by gavages. DAS and chalcone were solubilized in 0.1% ethanol (Ethanol+water) to get the required concentration. The current dose of DAS and chalcone was screened after consultation with some previously published reports (17-19). In addition, some dose response studies were performed in our lab to validate the selected dose. The present dose demonstrated significant responses on some genes expression like SULT1E1 and this dose did not initiate any toxicity which was evaluated from animal body weight, organ weight/body weight ratios, liver and kidney function test and haematology or other toxicity studies. Control group was given same amount of 0.1 % ethanol solution as. Different groups had 3-5 animals per group and animals were serially sacrificed at 24, 48, 72 hours after drug administration, a second dose was given to a single animal at 72 hour and was sacrificed on the $5^{th}$ day of the experiment i.e. at 120 hours or after 48 hours of the second dose.

**In –vivo (7 day treatment)**

Four group having four animals each was taken for this experiment. Group 1 was control, Group 2 was treated per day with 20 mg of DAS, Group 3 was treated with 8 mg of chalcone and Group 4 was treated with a combination of DAS (20mg) + Chalcone (8mg). These animals were treated for 7 days and sacrificed on the $8^{th}$ day. All animals were kept under UV radiation for 2 hours a day, until sacrificed. . UV radiation was given for the purpose of immune compromisation.

**Cytosol preparation of rat liver tissues**

Immediately after animal sacrifice the liver tissue were cleaned in phosphate buffer and homogenized (30 % w/v) in ice-cold phosphate buffer (0.1 mol /L, pH 7.4) then centrifuged at 10,000 rpm at $4^{o}$C for 30min. The supernatant (cytosol) was collected and stored at -20.C in different aliquots for further assays.

**Cytosol preparation of human breast tissue sample**

Breast tumor and the corresponding surrounding tissues were homogenized (30% w/v) in ice-cold phosphate buffer (0.1 mol /L, pH 7.4); andcentrifuged at 10,000 rpm at $4^{o}$C for 30min. The supernatant (cytosol) was collected and stored at -20.C in different aliquots for further assays.

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Western blot was conducted as in Maiti et al. 2007 with a slight modification. A 12 % denaturing gel was loaded with 25 µg of protein (obtained from the rat liver tissue of animals treated with a single dose of DAS, Chalcone and DAS + Chal for 24, 48and 72 hours) and electrophoresis carried out at 100v for 3 hours, transfer to nitrocellulose membrane was done at 100v for 2 hours. The membrane was washed and incubated with primary antibodies (anti rHIF1α and anti rSULT1E1) and secondary antibodies as mentioned in the referenced protocol (20). The membrane were treated with Diaminobenzidine (DAB) until the brown colored bands were developed.

**MMP 2/9 zymographic analysis in breast cancer and surrounding tissues.**

The cytosols fromtumorand its corresponding surrounding tissue were assayed for MMP activity. One percent type B-gelatin solution prepared in water, 8 % resolving gel is prepared containing 1% B-gelatin solution. Samples were prepared in MMP dye and loaded in gel to run at 110V for 60 minute. After completion of electrophoresis the gel wasactivated by washing buffer (as mentioned in the referenced protocol) and then incubated in zymographic gel development buffer pH 7.4 at 37$^{o}$C for 42 hours. Finally the gel was stained in amide black or Coommasie blue solution for 1 hour at room temperature in a shaker. The gels were then destained with solution I for 15 to 30 minutes, followed by destaining solution II for 3 to 5 hours until clear MMP bands appeared. After washing the destaining solution the gel was incubated in a gel preservative solution for 15 minutes and finally scanned with a digital scanner (21)

**Determination of SULT1A1 activity in human tissues by PNPS assay method.**

The β-Naphthol sulfation activity was determined from the tumor tissue and its surrounding. This assay determines phenol sulfation activities of different isoforms of phenol sulphating SULTs. A reaction mixture containing 50mM Tris buffer, pH 6.2, 5mM PNPS, 20 M PAPS, and 0.1mM -naphthol was used to measure the sulfation activity. Tumor and surrounding tissue cytosols (50 µg protein) were used as the enzyme source in a total reaction volume of 250 µl. After 30 min incubation at 37$^{o}$C in a shaking water bath, the reaction was stopped by adding 250µl of 0.25M Tris, pH 8.7. The reaction mixtures were read at 401 nm in a spectrophotometer. Specific activity (SA) was expressed as nmole/minute/mg of protein (22).

**RT-PCR of SULT1E1 from rat hepatocytes**

The RNA was isolated from hepatocyte of rats treated with DAS, Chalcone or their combination for 24, 48 and 72 hours. 1 µg of whole RNA utilized to perform reverse transcription PCR using Qiagen one step RT-PCR kit and SULT1E1 primer following the instructions provided in the kit. Total RNA concentration was measured by a NanoDrop Microvolume Spectrophotometers at 260 nm (A260 reading = 40 µg/ ml RNA) and its purity was tested from the ratio of A260/A280. The master mixprepared with stipulated proportions of ingredients including enzymes, dNTPs, template RNA and gene specific primer (both for human and rat SULT1E1) pairs (F 5'-CTTCCAGTATCATTTTGGGAAAAG-3' and R 5'-TGGATTGTTCTTCATCTC-3', all the primer specificity was satisfactory, that was tested by universal nucleotide alignment). To compare with control, 500 bp cDNA of rat β-actin and 200 bp cDNA of human β-actin were synthesized and loaded in the gel. The primer pair F 5'-GATGTACGTAGCCATCCA-3'/R 5'-GTGCCAACCAGACAGCA-3' for the synthesis of rat β-actin and F 5'-GGCGGCAACACCATGTACCCT-3'/R 5'-AGGGGA GGGACTCGTCATACT-3' for human β-actin were designed using the GeneFisher primer designing software and published earlier (20, 22). PCR was carried out in Eppendorf® Mastercycler® and the reaction program included reverse transcription at 50$^{o}$C for 30 min, PCR activation step 95$^{o}$C for 15 min, denaturation 94$^{o}$C for 1 min, annealing 64$^{o}$C for 1 min and extension at 72$^{o}$C for 1 min (30 cycles). Final extension was allowed for 10 min at 72$^{o}$C. PCR products were run an agarose gel electrophoresis image was captured by a BioRad gel doc system.

**Activities assay of SOD, catalase, GPx and LDH activities by gel zymogram**

*Super oxide dismutase.* Three different SOD activities were assayed, first in breast tumor and corresponding surrounding tissue, second in the serum of animal model treated with DAS, Chalcone and DAS+Chalcone for 7 days, 24, 48 and 72 hours and thirdly in the in vitro treated heapatocytes (3, 5 and 12 hour). A tablet of nitro blue tetrazolium (NBT) dissolved in 30 ml of water and the non-denaturing (10%) acrylamide gel kept soaked in it for 30 minfollowed by 40 ml SOD solution containing 0.028 M tetramethylethylenediamine (TEMED), 2.8 x 10-5 M riboflavin, and 0.036 M potassium phosphate at pH 7.8) for 15 minin shaking condition. Finally the gel was placed on clean acetate sheet and illuminated for 5 to 15 min and thenunder a UV illuminator (23)

*Catalase.* A non-denaturing gel (8%) was loaded with a 25µg of cytosol (from in-vitro treated rat hepatocytes for 3, 5 and 12 hours with DAS, Chalcone and DAS+Chalcone) and electrophoresed for 3 h at 40 mA at 4 °C. The gel was washed 3 × 10 min in distilled water and incubated in 0.003% $H_2O_2$ for 10 minutes, followed

by staining with 2% ferric chloride and 2% potassium ferricyanide. In this critical step two reagents were not mixed prior to staining rather poured together directly on top of the gel. The gel became greenish blue except in the position containing the catalase. After rinsingwith distilled water the gel was scanned when the maximum contrast between the band and the background was obtained (23).

*Glutathione peroxidase.* A non-denaturing gel (8%) was loaded with a 150 µg of protein obtained from rat hepatocytes treated for different times with DAS and/or Chalcone. Electrophoresis carried out for 3 h at 40 mA at 4°C. The gel was washed 3 × 10 min in distilled water containing 1 mM GSH and incubatedin 0.008% cumene hydroperoxide for 10 minutes. Then 1% ferric chloride (w/v) containing 1% GSH and 1% potassium ferricyanide (w/v) containing 1% GSH were prepared separately and poured together directly on the gel. When achromatic bands begin to form (5 – 15 min), the stain was poured off and the gel was washed extensively with distilled water. The image of the bands demonstrated GPx activity was evaluated. The cumene hydroperoxides instead of $H_2O_2$ performed as a good substrate and the GPx (contains a selenium in the active site) can utilize this hydroperoxide to determine total peroxidase activity according to the established protocol as described in the referenced article (23).

*Lactate dehydrogenase (LDH).* LDH zymogram in tumour and its corresponding surrounding tissue obtained from breast cancer pateints,was performed by using a standard protocol (24) with slight modification. LDH zymograms were obtained by separating 10 mg of protein on a native PAGE gel of 8 % for 2 h at 4 W, then soaked in water followed by washing twice with water. The LDH activity was detected by soaking the gel in a staining solution containing 0.1 M Tris-HCl (pH 8.0), 750 mM NAD1, 200 mM lithium lactate, 0.1 mg of NBT - nitroblue tetrazolium per ml, and 0.02 mg of phenazine methylsulfate per ml. Enzyme activity bands were developed from the conversion of NAD1 to NADH, which were clearly visible. The gel was scanned under the gel doc system.

**Determination of oxidative status and cytotoxicity parameters in rat hepatocytes**

**Estimation of Malondialdehyde (MDA) Levels**

The MDA was estimated both from liver tissue and serum samples. Tissue was homogenized (10 % w/v) in the ice-cold phosphate buffer (0.1 mol /L, pH 7.4) and the homogenate was centrifuged at 10,000 rpm at 4$^{o}$C for 10 min. The MDA assay was conducted using the supernatant following the protocol (25). To chelate iron and reduce its interference in peroxidation reaction of unsaturated fatty acid, 1 mM EDTA was used in the reaction mixture. To reduce the interference caused by a yellow-orange colour produced by some carbohydrates, the reaction mixture was heated at 80$^{o}$C instead of 100$^{o}$C. Finally, the MDA was measured and calculated utilizing the molar extinction coefficient of MDA (1.56 x 105 cm2/ mmol).

**Estimation of Non Protein Soluble Thiol (NPSH)**

The NPSH in serum and liver tissue homogenates (prepared in 0.1 M phosphate buffer, pH 7.4) were determined by the standard DTNB (5, 5'- dithiobis-2-nitrobenzoic acid) method with a slight modification (26). In brief, the protein was precipitated by trichloroacetic acid and clear cytosol was added to 0.1 M sodium phosphate buffer containing 5 µM DTNB. The level of NPSH was determined against a GSH standard curve.

**Evaluation of general toxicity**

Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), NPSH and MDA were measured from the rat serum treated with DAS, Chal, and DAS + Chalcone for 24, 48 and 72 hours. SGPT and SGOT were measured by standard protocol with the assay kits (Ranbaxy, India or other reputed company). Total protein from serum was measured following the standard protocol (27).

**Histoarchitechture studies of cancer tissues**

Breast tumor tissue and its corresponding surrounding tissues were embedded in paraffin, serially sectioned at 5 µM by an automated cryostat slicing machine (Leica Biosystems), stained with eosin and hematoxylin (Harris), and observed under a microscope (Nikon, Eclipse LV100, magnification 20X) to study the tissue histoarchitecture.

### Immunohistochemistry for SULT1E1

Tumor and its corresponding surrounding tissues were embedded in paraffin, serially sectioned at 5μM by an automated cryostat slicing machine (Leica Bio systems). Sections were deparaffinized by baking at 60 c followed by xylene treatment, downgraded alcohol and water. Slides were washed with PBST containing 1% casein for 10 minutes, tissue sections were incubated with 5% casein for 30 minutes for preventing non-specific binding followed by overnight incubation in primary antibody SULT1E1 and HIF1αin 1% casein PBST, washed with 1% PBST and incubated in 1% casein PBST containing secondary antibody for 1 hour, washed with 1% PBST followed by water and stained with chromogenic substrate DAB for 3 minutes and then washed with water. Slides were fixed with mounting medium and observed under a microscope (Nikon, Eclipse LV100, magnification 20X) to study the SULT1E1 expression and localization.

### Statistical analysis

For the statistical evaluations of various factors, the SPSS for Windows statistical software suite (SPSS Inc., Chicago, IL, USA, 2016) was employed. Data from all parameters were collected from multiple independent set as well as replicated from a single set of experiments. The Kolmogorov-Smirnov test was used to examine the data distribution pattern. Students t' test analysis and multiple comparison ANOVA tests were used to evaluate the baseline continuous variables and the results of the analyses. The level of significance were denoted within the 95% confidence limit and represented as $p<0.001$ to $p<0.05$.

## RESULTS

### Statistical analysis of relation between Cancer manifestation and this disease causing factors

The protein density and mRNA density (in surrounding and tumor) from western blot and RT-PCR data suggests that both PD and MD of 1E1 and HIFα are significantly increased in the tumor groups (Table 1 and Fig 1). Present correlation study suggests that tissue MDA level is positively associated with SULT1E1 tissue expression. The 1E1 is an adaptive enzyme that catalyze the inactivation of E2 by forming E2S. More oxidative stress as evident in high MDA level has some initial role in active E2 nullification. MDA is negatively associated with HIFα expression. At the initial phase of MDA related stress response has more adaptive responses of lower HIFα. But in breast cancer tissue it is noticed that, at higher disease state with prolonged oxidative stress exposure and high MDA level HIFα is increased significantly and Nrf2 is shown to positively associated with HIFα induction. NPSH and uric acids are regarded as the endogenous soluble antioxidants those may have some protective role against the oxidant-stress-induced 1E1 regulations and Nrf2, HIFα induction. Present results and correlation data suggest that NPSH and uric acids are negatively correlated with HIFα expression. Lower antioxidant level augments the HIFα level (Table-2). The data from this table are represented from DAS and chalcone related studies shown in the figure from in fig 4, 5 and 6. Table 3 suggests that in control, DAS and Chalcone groups, NPSH and 1E1 are found to be associated. When in control and in DAS these are positively associated in Chal- group NPSH and 1E1 are shown to be negatively associated. No significant association was noticed in DAS and Chalcone groups.

Table 4 suggests that, in case of time-based exposure NPSH and 1E1 and 1E1 are found to be positively associated and 3 and 24 hrs of exposure. No significant relation was noticed in 5 hrs exposure. New sample size and internal variability may be the cause for this.

### SULT1E1 protein expression in Tumor and surrounding tissues

Expression of SULT1E1 protein in tumour was comparatively higher than corresponding surrounding tissues. A few surrounding tissue have shown an increased SULT1E1 expression, whose corresponding tumor shows more intense SULT1E1 expression. Our findings abide by earlier study that suggests that SULT1E1 is expressed more in the tumor tissues as compared to their surroundings, and our studydid the first attempt to explain that, though SULT1E1 is overexpressed in tumor it remains inactive (28). Considering both the studieswe utilized DAS and Chalcone separately as SULT1E1 inducer and reducing agent and also in combination to mainatian an elevated active SULT1E1 state.

**HIΦ1α προτειν εξπρεσσιον ιν ρατ λιερ τισσυε τρεατεδ ωιτη ΔΑΣ ανδ ςηαλςονε**

The expression of HIF1α in tumor tissue was remarkably higher when compared to their corresponding surrounding tissues which was noticed in SULT1E1 expression. HIF-1 plays important roles in breast cancer metastasis by mediating hypoxia-induced expression of mRNA-encoding genes. HIF-1 also regulates the expression of non-coding RNAs, which are critical regulators of migration, invasion, and metastasis (14).

**Superoxide dismutase (SOD1) and Lactate dehydrogenase (LDH) activities in breast cancer tissue**

The antioxidant enzyme superoxide dismutase (SOD) activity was found to be higher in the tumor as compared to its surrounding (Fig-1a, SOD1). In essence, lactate dehydrogenase (LDH) is an enzyme necessary for the conversion of sugar into cellular energy. An elevated total serum SOD level is a predictor of tissue damage and inflammation (29). Another study has shown loss of LDH-B expression as an early and frequent event in human breast cancer (30). In our current study, we found the loss of LDH 3, 4 and 5 in breast cancer as compared to the surrounding whereas LDH 2 seems to be equally expressed both in the surrounding and the tumor. LDH has 2 subunits, subunit A and subunit B in different proportion in each type of LDH. In this study, we found that the expression of Subunit A is reduced or almost lacking in breast tumor. Therefore, the less is the expression of LDH 3, 4 and 5 in tumor tissue (Fig-1a).

**Matrix metalloproteases (MMP 2 and 9) activities**

Breast tissue expressed both MMP2 and MMP9. Compared to the surrounding tissue, the tumour tissue had a higher expression of MMP9. MMP9 was not similarly expressed in all the tumors, some tumors had less, whereas some had more MMP9. MMP2 expression was less in the tumor in comparison to the corresponding surroundings. MMPs are vital in cancers. MMP-9 can cleave many extracellular matrix (ECM) proteins to regulate ECM remodeling. Several plasma surface proteins can also be cleaved by it, releasing them from the cell surface. MMP-9 has been widely found to relate to the pathology of cancers, including but not limited to invasion, metastasis and angiogenesis (31).

**Histopathology of human breast cancer sample**

In the tissue surrounding the tumors, few normal ducts lobules in a fibrous stromal along with adipose tissue are identified (Fig-2, Surrounding).The tumor tissue shows ductal carcinoma in-situ and micro invasive cells. Hematoxylene-eosin stained breast cancer tissue shows several mitosis and pleomorphic nuclei. Irregular and undifferentiated ducts are present in the tumor tissue (Fig-2).

**Immunohistochemistry of SULT1E1 of human breast cancer sample**

Immunohistochemistry results show that the tumor and surrounding both are positive for SUT1E1. The strength of SULT1E1 staining in tumor is stronger than corresponding surrounding tissue. The distribution and localization of SULT1E1 was noticed in stromal tissue, adipose lining and nucleus in both tumor and corresponding surrounding tissues. Tumor tissue was darkly stained for SULT1E1 compared to the surrounding suggesting increased expression (Fig-2).

**SULT1A1 activity of human breast cancer sample**

Sulfotransferase also utilizes PAPS as sulfonate donor to catalyze the sulfate conjugation of a wide variety of acceptor molecules bearing a hydroxyl or an amine group including estrogen. To find whether E2 is controlled by other E2 regulating enzymes such as SULT1A1, we did SULT1A1 activity assay. Interestingly we found no difference in the SULT1A1 activity between tumor and their surrounding tissue (Fig-1b). Thus, there is no SULT1A1 interference in the final outcome of E2.

**SULT1E1 protein expression (Animal experiment)**

**In vivo experiment**

Following a single dosage of DAS on day 1, the hepatic tissue animal group treated with DAS demonstrated a progressive increase in the SULT1E1 expression with increasing duration. SULT1E1 expression was comparable to DAS 48 hours after DAS 2$^{nd}$ dose group. On the other hand, in 24 and 48 hours groups of chalcone treated animals expressed highest SULT1E1 but the expression gradually decreased with increasing duration. The combination group (DAS + Chal) showed an expression level less than the DAS or Chalcone alone group but greater than the control group (Fig-4a).

**In vitro experiment**

The DAS group expressed SULT1E1 more than the control group during the in-vitro therapy, and this expression gradually increased over time. The chalcone group had less SUT1E1 expression than the control and gradually decreased with increasing time. Whereas the combination group had highest expression at the first 3 hours of incubation, decreased at 5 hours and remain undetected at 12 hours of incubation (Figure-4b). However, 3hr DAS+Chal treated tissue showed the best result with highest level of SULT1E1 and then gradually decreased in 5 hr and least is found in 12 hr. Thus, DAS has direct effect on the expression of SULT1E1 and Chalcone has some moderate effect.

**Massenger RNA (mRNA) expression studies of SULT1E1**

When administered for a period of 7 days, it was discovered that DAS and Chalcone, both alone and collectively, were able to increase mRNA in comparison to the animals in the control group via inducing transcription of the SULT1E1 gene. SULT1E1 mRNA was highly expressed in the DAS treated cells. Chalcone also expresses SULT1E1 mRNA but less than DAS. Whereas DAS+Chal treated showed highest SULT1E1 in comparison to DAS alone and Chalcone alone group but a single animal had low SULT1E1 mRNA. These expressions were all higher than the control group (Fig-4c).

**Catalase**

**In - vitro Treatment for 3, 5 and 12 hours**

The enzymatic activity of catalase was low in DAS, Chalcone and DAS + Chalcone combination group in comparison to the untreated tissue. Catalase Activity gradually decreased in the in the DAS treated tissues with time, and the same was true for the Chalcone treated tissue. Among all the treated groups the highest activity was found in the DAS+Chalcone treated tissue for 3 and 5 hours, however no activity was found in the DAS + Chalcone- 24 hr treated tissue (Fig-3b).

**SOD (24 to 72 hours)**

The SOD activity remained unchanged in the DAS alone, chalcone alone group with a slight decrease of activity in DAS group. The combination group showed a decrease in the activity in the DAS+Chalcone (48 hr) and then markedly increased in the 72 hour group and the group treated with the 2nd dose. DAS alone and Chalcone alone group showed comparatively less SOD activity than the control or untreated group. The combination group of DAS+ chalcone showed a significant increase in SOD activity on treatment with the 2$^{nd}$ dose after 72 hours of the 1$^{st}$ dose. Among the 3 control animals, SOD activity was high in 2 animals and less in the 3$^{rd}$. The DAS treated 24, 48 and 72 hour animal showed Less SOD than first 2 controls. Animal treated with 2$^{nd}$ dose of DAS had higher SOD activity. The chalcone treated group had low SOD activity than control and DAS group, but the 2$^{nd}$ dose had high SOD activity. Interestingly DAS+Chal had the highest SOD activity, which gradually increased with duration till 72 hrs (Fig-3c).

**SOD activity (7 days or 3 to 12 hours treatment)**

The SOD activity was slightly less in the DAS group as compared to the control. The chalcone group also displayed lower SOD activity in half of the animal as compared to the control and DAS group. The other half shows SOD activity similar to the control group. The entire group showed comparatively lower SOD activity compared to the control (Fig-3a).

The control group showed SOD activity, and DAS at 3 hour showed SOD activity but 5 and 12 hours showed negligible SOD. DAS+Chalcone at 5 and 12 hour showed some activity but less than the control (Fig-3b).

**Glutathione peroxidase (GPx) activity study by gel zymogram**

The GPx activity was highest in the control group (Untreated) as compared to other group of animal such as DAS alone, Chalcone alone and DAS + Chalcone group. The GPx activity was significantly lower in the DAS group than it was in the chalcone, which was likewise significantly lower. The DAS + Chalcone group a gradual increase in the GPX activity with duration and the highest activity among them all was found in the DAS + Chalcone 2$^{nd}$ dose group (Fig-3c).

The control group showed some GPx activity and there was no activity in the DAS treated animal group. The Chalcone group showed minute activity. The Das + Chal group showed high Gpx activity. The GPx activity was found to be lower in the DAS, Chalcone group as compared to the control group of animal. The GPx activity was stimulated in the DAS+Chalcone group of animal's hepatic tissue (Fig-3a).

**Εξπρεσσιον οφ HIΦ1α προτειν**

HIF1 expression was high in the DAS-24 hr group and then appeared to gradually decline with time as well as with the second dosage. Yet, in the Chalcone-treated group, HIF1 is observed to gradually express more with time and the second dose. In the DAS+Chalcone group of animal hepatic tissue, the expression of HIF1 was essentially the same. This justifies why DAS+chal have little impact on the HIF1 group (Fig-4a, b). The band density of HIF1 expression is depicted in the bar diagram, which also clearly shows the pattern of HIF1 expression. (Fig-4c).

**Oxidative stress related parameters; Malondialdehyde (MDA) and Non protein soluble thiol (NPSH)**

The level of non protein thiol in vitro experiment increased after 3, 5 and 24 hrs of incubation with DAS. Das+ Chalcone incubation also causes NPSH induction as compared to the control cells (Fig-5a). In the in vivo experiment, NPSH was negligibly increased in the DAS alone and Chalcone alone treatedgroup, whereas a slight increase was noticed in DAS+Chal, 48 hr, 72hr and 2$^{nd}$ dose treatment (Fig-5b). Serum NPSH was found to be reduced in the Chalcone alone group, and the other entire group had NPSH similar to the control, showing no changes in the redox status in terms of NPSH.

Elevated MDA was seen in DAS-treated mice, demonstrating high MDA production with the second dose of DAS, suggesting that DAS does appear to trigger lipid peroxidation. During 48 and 72 hours, Chalcone 2nd dosage and DAS + Chalcone also had a higher MDA, in contrast to the other groups, which displayed MDA similar to the control group (Fig-5c). Serum MDA levels remain stable (Fig-5g). This means that neither DAS nor Chalcone cause systemic lipid peroxidation.

**General toxicity**

Except for the DAS alone group, neither the Chalcone alone group nor the DAS+Chalcone group had raised SGOT or SGPT levels. Hence, these medications lacked such liver damage. So, these drugs can be used as therapeutic agents (Fig-5d).

**DISCUSSIONS**

Our present result of DAS induction of SULT1E1 has been supported by a previous finding of 1E1 induction by this drug in mouse liver. Recently, mouse SULT1E1 was shown to be induced by many chemicals/composition including garlic extract (*Allium sativum* **,** rich with DAS) that activate CAR (18).This suggests that use of crude garlic extract may have some beneficial effect in E2 dependant cancer.

In this study, we sought to combine the idea of SULT1E1 induction by DAS with the creation of a reducing environment by chalcone. An enhanced level of SULT1E1 has been found in tumour tissues in earlier research as part of an adaptation mechanism to control active E2. However, the 1E1 activity may be insufficient to counter the significant surge of E2 in E2 dependent breast cancer. Limitations in 1E1 activity might be due

to its inactivation in oxidative stress in breast cancer (28), where Cys83 of SULT1E1 may remain in oxidized state and hinders E2 binding and sulfo-conjugation by this enzyme (28,20). Eventually, it allows elevated active estrogen leading to a carcinogenic effect. This study hypothesized that the induction of SULT1E1 by DAS and its activation by chalcone may significantly control E2 level and the disease pathogenesis resulting in an increase in patients' survival.

Rat treated with DAS and Chalcone demonstrated elevated SULT1E1 expression at different treatment schedule and DAS alone also showed a consistent higher and chalcone moderate SULT1E1 expression (Fig-2a and 2b). This abides by an earlier study that SULT1E1 is an oxidative stress responsive gene that gender specifically affects liveri/reperfusion injury (32, 28). Chalcone has been shown to create a reducing environment that helps to keep SULT1E1 active in the present study.

The highest expression was noticed in DAS + Chalcone, 3hr in-vitro experiment. Both in vivo and in vitro experiments provide us a clear outlook that this drug combination may be an effective therapeutic strategy against E2 dependant breast cancer. Oxidative stress is known to induce HIF1α via Nrf2 which has been shown to be induced in human ovarian cancer may have possible role in breast cancer also. Role of Nrf2 and NfkB has been demonstrated in human breast cancer tissue and in experimental rodent model (28, 33). Nrf2 binds at 32 kb upstream of HIF1α to an antioxidant response element and causes its transcription. Nrf2 activation and HIF1α upregulation are found to be associated with each other in certain selected type of cancers. A link between Nrf2 and HIF1α led us to investigate HIF1α, since Nrf2 is completely associated with oxidative stress management. Oxidative stress is linked with SULT1E1 induction and activation via Nrf2 (34).

Potential experimental models of rat hepatocytes exposed to current drugs were used to test their metabolism and their ability to regulate hepatic gene expressions (35,36). In the current study, chalcone group induces catalase in the rat hepatocytes in 24 to 72 hours exposure. Beside its reducing effects, chalcone showed strong antioxidant effect and activate the catalase and both drugs strongly activate the SOD activity also. Catalase was moderately induced in both drugs treated group (Fig-3). Superoxide dismutase and catalase mimetic-drugs MnTmPyP and 134 have distinct impacts on breast cancer cell proliferation via TNFa-induced NF-B regulations confirm our findings that proposed drugs may have positive effects (37). This result may be linked to our previous study on human breast tumor sample (28). Anti-invasive and anti-angiogenic potential of chalcone derivatives acted as an HIF-1 inhibitor (38) which is also noticed in our study.

DAS and chalcone mediated induction of anti-stress responses may be via activated Nrf2, thereby, increasing of antioxidant enzyme i.e. SOD, catalase and GPx supporting to oxidative-stress induction and its possible adaptations have been noticed in the current study. The novel combination of DAS and chalcone might be invaluable, because oxidative stress not only inactivates SULT1E1 but also augments Nrf-2 and HIF-1a in breast cancer. The NPSH levels elevated in DAS or Chalcone alone and in combination group (Fig-5a, 5b). This ascertains the induction of the reducing environment and minimization of oxidative stress which might be strongly supportive in E2 related cancers. However, there is some amount of lipid peroxidation products (MDA) are noticed in the liver tissue of the reats of $2^{nd}$ dose DAS and DAS+Chalgroups (48 and 72 hours). An extra amount of antioxidant (vitamin C and E) as supportive to this combination would be able to protrct the tissue (Fig-5c). Report suggests that some chemotherapeutic applications may cause some level of systemic stress. Several phytomaterials have demonstrated therapeutic efficacy against certain ailments. As for example, cardamonin inhibited the Nrf2-dependent ROS scavenging system in addition to suppressing HIF-1a, which led to an increase in intracellular ROS levels. As a result of decreased glucose absorption and the production of lactic acid, it significantly aided ROS-induced apoptosis in breast cancer cells (39).

Cancer subclones are encouraged by intramural hypoxia in metastasis. HIF-1 expression levels at various clinical phases of the illness predict the outcome for several malignancies, including breast cancer. Tumor tissue in the current study has higher HIF1 protein expression than does surrounding tissue (Fig-1f). In the DAS + Chalcone combination group, we observed that both DAS and Chalcone had a substantial inhibitory effect on the HIF1 (Fig- 4a & 4b). This finding confirms our hypothesis that the use of these

medications in combination provides a high level of clinical benefit. An earlier work demonstrated that histone-deacetylase (HDAC) inhibitors can target the HIF-1 protein owing to von Hippel-Lindau (VHL) protein-dependent degradation, suggesting a potential connection between oxidative stress-controlled HIF-1a and cell cycle regulation. Our current study was supported by a previous study which has shown that diallyl trisulfide (DAS) dose-dependently inhibited HIF-1α in hypoxic MDA-MB-231 cells thereby inhibiting hypoxia-induced pre metastatic changes and angiogenesis in these cells. The angiogenic responses in high rate of tumerigenic growth may be terminated by the combined effects of DAS and Chalconein addition to their SULT1E1induction effects.

The MMP9 was mainly expressed in tumor tissue and less expressed in surrounding tissue; some of those have higher and variable expressions due to inter-individual variability. The MMP2 was expressed less in the tumor tissues. The MMPs can cleave several plasma surface proteins causing their release from the cell surface. The MMP has been widely found to relate to the pathology of cancers including but not limited to invasion, metastasis and angiogenesis (31). Under hypoxic stress, HIF-1a rapidly accumulates and activates hundreds of genes including MMPs in breast cancer patients (40). Our preliminary findings on the potential control of breast cancer and metastatic status by using DAS and chalcone combination may be significant from a therapeutic standpoint. Chalcone also triggered the mitochondrial apoptotic signaling by increasing the amount of Bax and Bak and reducing the level of Bcl-2 and Bcl-X (L), and subsequently activated caspase-9 in MCF-7 and MDA-MB-231 cells (41). Chalcone may therefore operate as an apoptotic signal inducer for malignant cell death by activating caspase through the mitochondrial Bcl-2/BAX pathway.

Lactate dehydrogenase is responsible for conversion of sugar into energy in cells. Alterations in LDH may be related to anaerobic oxidation and metabolic status that eventually occur in hypoxic tissues that have been damaged by cancer. Earlier studies show loss of LDH-B expression as an early event that is frequently occurs in breast cancer (30). We found loss of LDH 3, 4 and 5 in breast cancer in comparison to the surrounding and LDH 2 seems to be equally expressed both in the surrounding and the tumor. In the current study expression of Subunit A is reduced or almost lacking in breast tumor. Our findings interpret that, less is the expression of LDH 3, 4 and 5 in tumor tissue (Fig-1a). Alterations with inter-individual variability in LDH activity has been linked to the catalase regulations in the breast cancer patients since oxidative stress have dual effects on antioxidant state and hypoxia mediated energy regulations. Once again this is also related to the HIF1a responses noticed in the current study (42). Report showed a higher LDH-A expression in the tumor that has been associated to metastatic breast cancer. Supportive to our present LDH result I link may be pointed between hypoxia induced LDH and HIF1a expressions as different stages of breast cancer predictor (43).

According to the current study there is no alteration in the SULT1A1 activity in both breast cancer tissue and its surrounding tissues (Fig-1b) suggesting that 1A1 mediated non-specific E2-sulafation is not taking place which supports more important role of 1E1. Altered regulations of 1A1 and/or its polymorphism have been linked to several types of cancer. However, under a chemotherapeutic setting the complex role of 1A1 cannot be easily predicted. As for example, possible interaction of anti-cancer anti-estrogenic drug tamoxifen with the SULT1A1 may be considered here. The anti-cancer therapeutic effect of tamoxifen and melatonin may be somewhat influenced by 1A1's biotransformation of these medicines. (44).

SULT1E1 mRNA was also induced in DAS, Chalcone and DAS + Chalcone groups (Fig-2c). Figures 2a and 2b from the RT-PCR and western blot experiments indicate that DAS and Chalcone or DAS alone acts at both the transcriptional and translational levels (Figure 7). Therefore DAS may become a multifunctional drug that inhibits HIF-1α and induces SULT1E1at the transcriptional and translational levels. Previous studies from our lab have shown that SULT1E1 expression either increases in the tumor with increased oxidative stress at late stages of the disease or reduces at the initial stages of the disease to let E2 be active and gradually increases with the disease pathogenicity (28), but remains inactive due to elevated oxidative stress, as shown in our animal studies (ENU paper) and another earlier studies (20). The current study shows no alterations in the SULT1A1 activity in the breast tumor and surrounding tissue explaining that the breast cancer may have little or no role of SULT1A1 mediated nonspecific metabolism of estrogen. Direct use of

breast cancer tissue or animal model might be better for the therapeutic study. In the present investigation, we focused less on the patho-physiological state but more pointed on the inter-relation of some disease related genes and proteins expression. It is known that the liver is the port of entry and metabolism of any exogenous and endogenous drugs including estrogen which are significantly catabolized by the phase I and phase II enzymes. Liver produces and circulates (to target organs like breast, endometrium) significant amounts of estrogens and liver generates different estrogen-metabolizing enzymes like SULT1E1, STS and others. The mRNA and protein tested in the current investigation are highly expressed in liver at basal level and significantly respond by a modulator. It is shown that dexamethasone (DEX) treatment increased hepatic and MCF-7/VEGF tumor expression of *Sult1e1/SULT1E1* (43 45). Glucocorticoid receptor expression induced by DEX can also augment SULT1E1 expression in mouse liver and MCF-7 resulting in more E2 sulfation/inactivation and tumor growth inhibition (46,16). Liver is the competent source for study a large number of genes/proteins expression. Other important point is that liver is an important target of breast cancer metastasis. Report reveals that breast cancer liver metastasis (BCLM) is linked with poor prognoses of this disease. Tumor intrinsic subtype demonstrates preferential metastasis to liver with several types of breast cancer (47). In this cancer, it is an adaptive strategy by increasing SULT1E1 gene and protein expression, tissues makes an initial attempt to get rid of extra amount of E2. But due to more oxidative environment, SULT1E1 cannot perform properly. In the mean time, high rate of cell division especially in the tumor region with comparison to its surrounding, oxidative stress further increases, hypoxic environment worsen the situation by increasing HIF1a, Nrf2, NfkB and eventually tissue-degenerating MMPs expressions, these have been shown in our current study and in several earlier studies. So, to counteract the fast rate kinetics of tumor growth and adversely functioning of these genes/proteins, application of some therapeutic measure might help. In the current study DAS and chalcone combination has been shown to significantly increase SULT1E1 expression/activation, and decrease oxidative stress, HIF1a, MMPs expressions.

## CONCLUSIONS

The current study explores the events of the redox dependent adverse regulations of SULT1E1 and possible foul-play of estradiol in breast cancer tissues of postmenopausal women. This has been linked to the induction of HIF1α and MMP2/9 activation. In an attempt towards the therapeutic approach, we have demonstrated that dially sulfide is a good inducer of SULT1E1 gene and protein which significantly decreased the HIF1α and MMPs. In addition chalcone played an ideal rectifier role of cellular redox environment. Together, the two drugs may be applied as effective therapeutic materials against some forms of breast cancer. To make definitive statements, proper pharmacological testing must be performed.

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**Figure legends**

**Figure 1.** 1a. Expression of SOD1, LDH 2, 3&4, and MMP2, MMP9 in human breast tumor (n=3) and its corresponding surrounding tissues (n=3): Lane distribution-1, 2, 3-surounding & 4, 5, 6 –Tumor.

1b. Activity assay of SULT1A1 in human tumor tissue (n=3) and its corresponding surrounding areas (n=3).

1c. SULT1E1 (protein) expression in tumor (n=6) tissue and their corresponding surrounding (n=6) tissue are calculated and densitometric data are presented. (blot image not shown). Level of significance in Student's t –test is, b= $p<0.01$.

1d. HIF1α (protein) expression in tumor tissue (n=6) and their corresponding surrounding (n=4) tissue with densitometric analysis. Molecular weight marker β-galactosidase (120kD) was used in this study. Human β-actin was used to verify the control protein. Level of significance in Student's t –test is, b= $p<0.01$.

1e. SULT1E1 (gene) expression RTPCR data in tumor (n=5) tissue and their corresponding surrounding (n=3) tissue are calculated and densitometric data are presented. Level of significance in Student's t –test is, b= $p<0.01$.

**Figure 2.** Immunohistochemistry- SULT1E1 expression in tumor tissue and adjacent surrounding tissue. HE staining- Histoarchitecture of tumor and adjacent surrounding tissue.

**Figure 3.** 3a. Gel zymographic data of SOD activity in serum and liver tissue and GPx activities in liver tissues of rats treated with DAS, chalcone, or DAS+chalcone.

3b. Gel zymographic data of SOD and catalase activity in the liver tissues of rat treated for 3, 5 or 12 hours with DAS, chalcone or DAS+chalcone.

3c. Gel zymographic data of SOD and GPx activity in liver tissue of rats treated for 24, 48 and 72 hours with DAS, chalcone or DAS+chalcone.

**Figure 4.** 4a. Expression of rSULT1E1 protein and its densitometric analysis from rat treated with DAS, chalcone (Chal) or DAS+chalcone for 24, 48 and 72 hours and in one group DAS+chalcone with a second dose of DAS. Expression of rβ-actin was studied from all these experimental groups. Lane distribution- details are mentioned at the bottom of the lanes.

4b. Expression of rSULT1E1 protein and its densitometric analysis in rat liver cells (primary culture) treated with DAS, chalcone or DAS+chalcone for 3, 5 and 12 hours. Lane distribution- details are mentioned bottom of the lanes. Two molecular weight marker M1 and M2 were also run to verify SULT1E1 protein.

4c. Expression of rSULT1E1 mRNA from the liver of rat treated with DAS, chalcone or Das+chalcone and their densitometry data are presented. Expression of rβ-actin mRNA was also screened as a loading control.

**Figure 5.** Expression of rHIF1α protein and its densitometric analysis in rat model treated with DAS, chalcone (Chal) and DAS+chalcone for 24, 48 or 72 hours and a group with chalcone second dose. Lane distribution of -5a, As mentioned at the bottom of the blot image.

5b. Densitometric analysis and relative band densities of fig. 5a are presented as bar in the diagram.

5c. Gel zymographic data of MMP2 and MMP9 from the liver tissues of rat treated with DAS, chalcone or DAS + Chalcone for 7 days treatment. 5e. Gel zymographic data of MMP2 and MMP9 from the liver tissues of rat treated with DAS, chalcone or DAS + chalcone for 24, 48 or 72 hours.

**Figure 6.** Different parameters were measured from rat liver tissue after in-vitro treatment with DAS, chalcone or DAS+chalcone and data are presented as bar diagram. The representation of bars are mentioned in the figures-Individual data from in vitro experiments are presented in fig 6a-6c. Different groups had 3-5 animals per group as depicted in the picture and animals were serially sacrificed at stipulated time interval. In vivo experimental data from 7 days treatment groups are presented in fig 6d-6g. Bars represent the means ± SE from 4 animals in each group. Levels of significances of difference between two groups are verified Student's t test.

**Figure 7** Schematic representation of breast cancer association with redox-regulated SULT1E1 dysfunction and HIF1a/MMPs up-regulations. The prominent therapeutic role of Dialylsulfide (DAS) and chalcone via reversal of these protein dysfunctions has been summarized.

Table 1. The protein density and mRNA density (in surrounding and tumor) from western blot and RT-PCR data

| **Group Statistics** | **Group Statistics** | **Group Statistics** | **Group Statistics** | **Group Statistics** | **Group Statisti** |
|---|---|---|---|---|---|
| Type | Type | N | Mean | SD | SE |
| SULT_PD | Surr | 6 | 1.6667 | 0.81650 | 0.33333 |
| | Tumor | 6 | 2.9333 | 0.55737 | 0.22755 |
| HIFaPD | Surr | 4 | 1.8750 | 0.85391 | 0.42696 |
| | Tumor | 6 | 3.6667 | 1.03280 | 0.42164 |
| SULT1E1_mD | Surr | 3 | 1.2000 | 0.20000 | 0.11547 |
| | Tumor | 4 | 3.2250 | 1.18708 | 0.59354 |

Table 2. Statistical analysis of Pearson's correlations amongst different oxidative-stress parameters and SULT1E1 expressions in three different diseases conditions in the tumor/surrounding tissues

**Correlations**

| | | MDA |
|---|---|---|
| MDA | r | 1 |
| | P | |
| | N | 34 |

| | | |
|---|---|---|
| NPSH | r | 0.090 |
| | P | 0.611 |
| | N | 34 |
| URIC_ACID | r | 0.026 |
| | P | 0.888 |
| | N | 32 |
| HIFa_Expr | r | -.509** |
| | P | 0.006 |
| | N | 28 |
| SULT1E1_Expr | r | .486** |
| | P | 0.006 |
| | N | 30 |
| . Correlation is significant at the 0.01 level (2-tailed). | **. Correlation is significant at the 0.01 level (2-tailed). | **. Corre |

Table 3. The correlation data suggest that in Control, DAS and Chalcone groups, NPSH and SULT1E1 are found to be associated.

**Correlations**

| Type | Type | Type |
|---|---|---|
| CONTROL | NPSH | Pearson |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson |
| | | Sig. (2-ta |
| | | N |
| DAS | NPSH | Pearson |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson |
| | | Sig. (2-ta |
| | | N |
| CHAL | NPSH | Pearson |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson |
| | | Sig. (2-ta |
| | | N |
| CHAL,DAS | NPSH | Pearson |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson |
| | | Sig. (2-ta |
| | | N |
| . Correlation is significant at the 0.01 level (2-tailed). | **. Correlation is significant at the 0.01 level (2-tailed). | **. Corre |
| . Correlation is significant at the 0.05 level (2-tailed). | *. Correlation is significant at the 0.05 level (2-tailed). | *. Correl |

Table 4. Time dependent correlation data of different groups of DAS, Chalcone exposed rat

**Correlations**

| Hour | Hour | Hour |
|---|---|---|
| 3.00 | NPSH | Pearson C |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson C |
| | | Sig. (2-ta |
| | | N |
| 5.00 | NPSH | Pearson C |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson C |
| | | Sig. (2-ta |
| | | N |
| 24.00 | NPSH | Pearson C |
| | | Sig. (2-ta |
| | | N |
| | SULT1E1 | Pearson C |
| | | Sig. (2-ta |
| | | N |
| . Correlation is significant at the 0.01 level (2-tailed). | **. Correlation is significant at the 0.01 level (2-tailed). | **. Corre |
| . Correlation is significant at the 0.05 level (2-tailed). | *. Correlation is significant at the 0.05 level (2-tailed). | *. Correl |

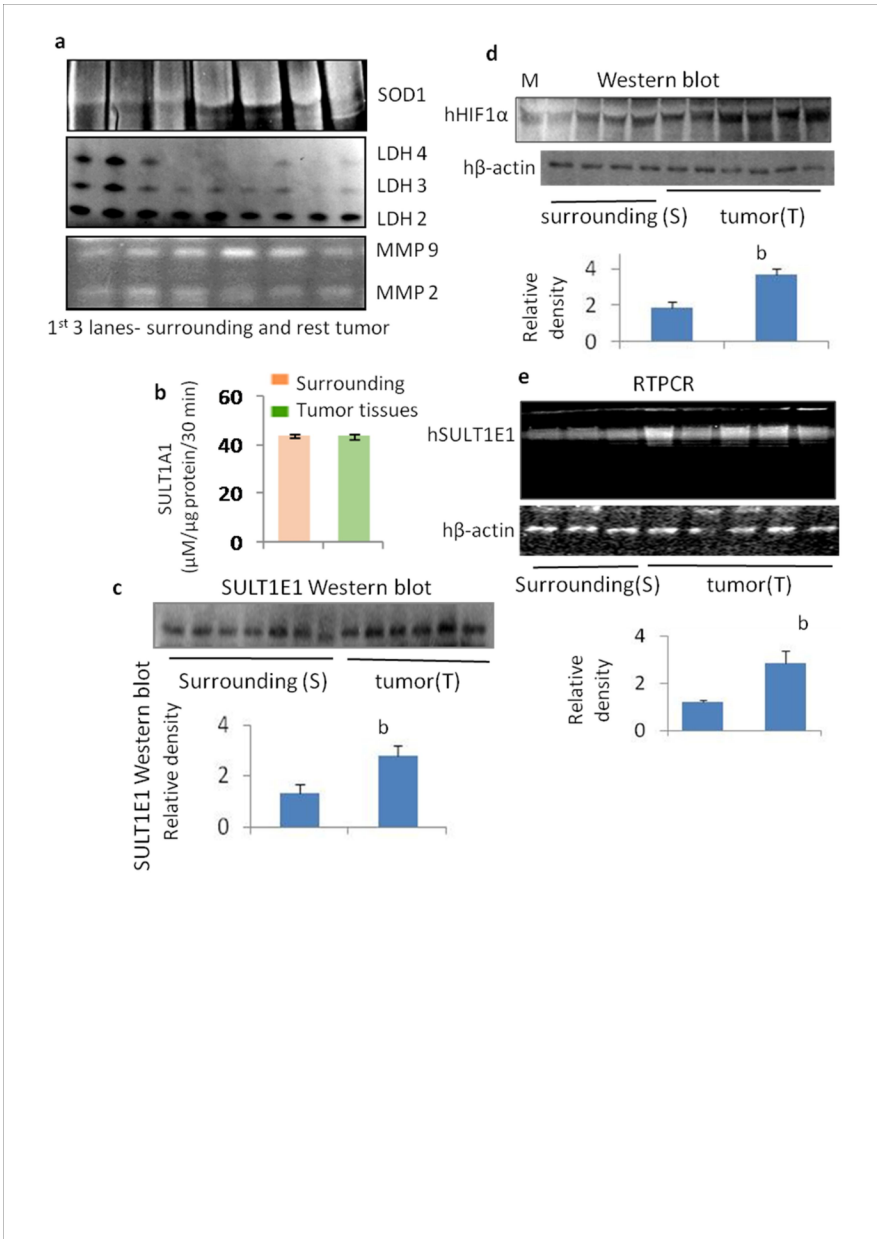


Figure 1

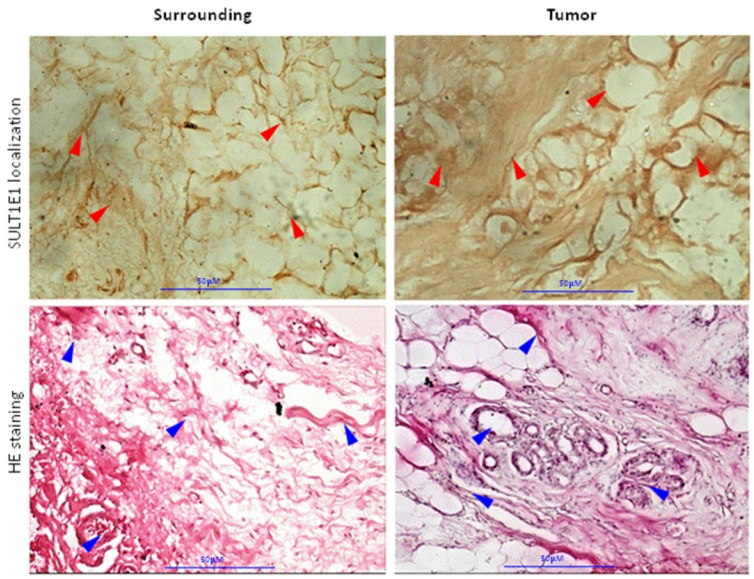


Figure 2

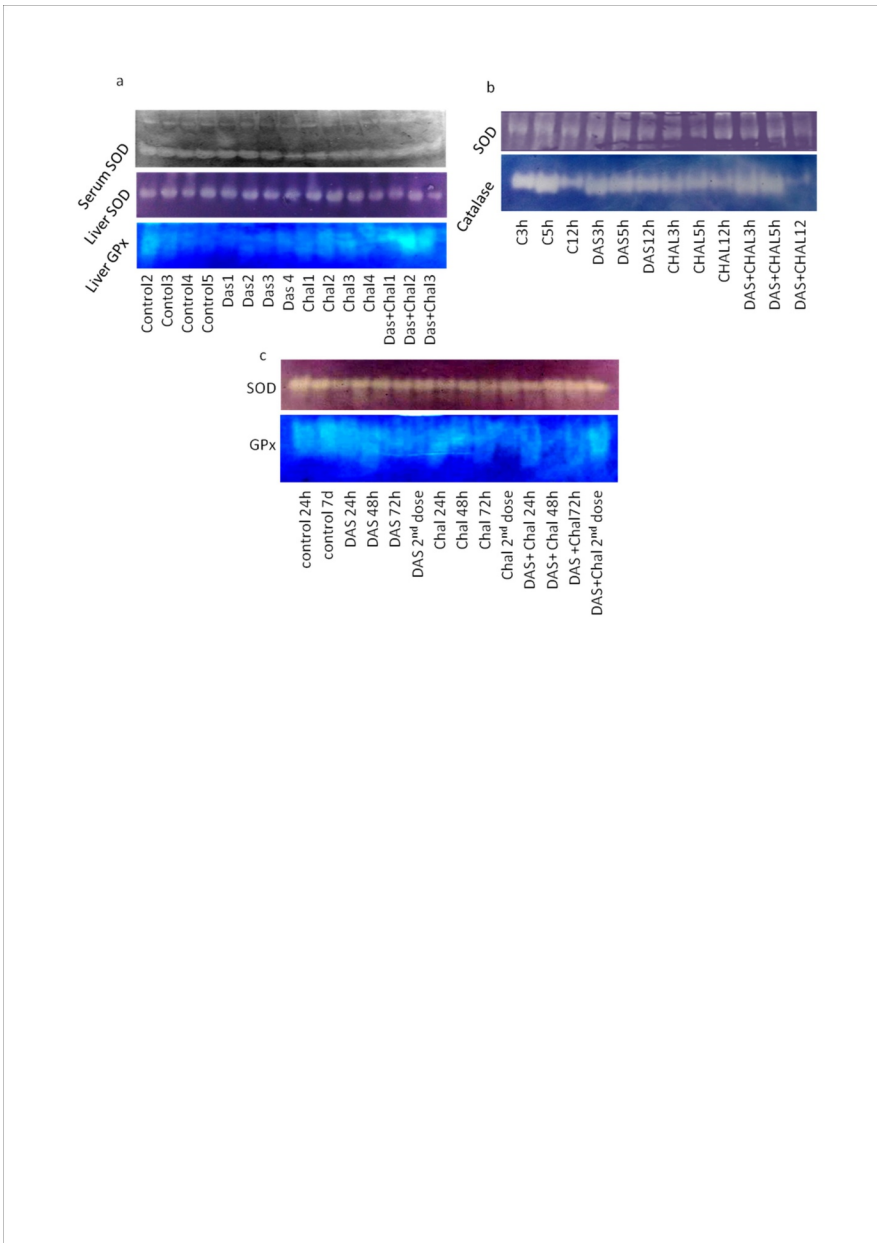


Figure 3

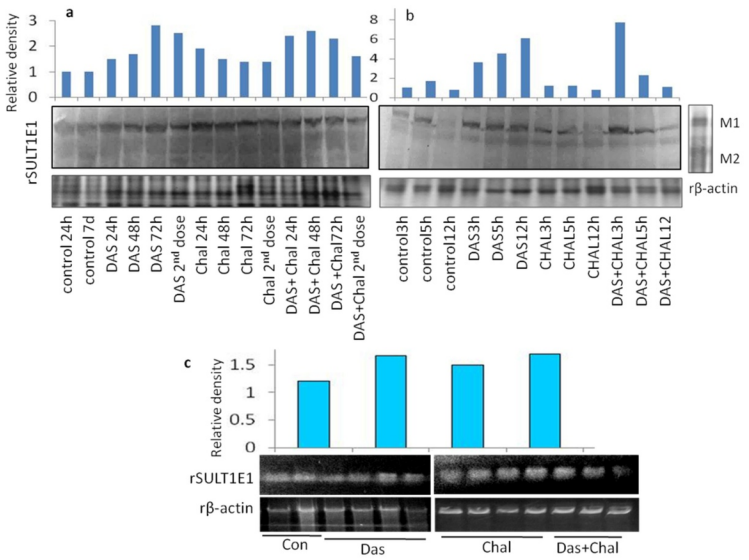


Figure 4

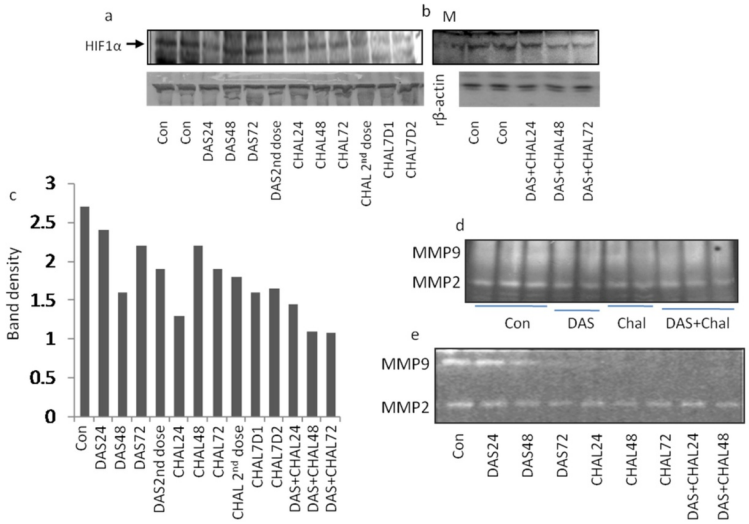


Figure 5

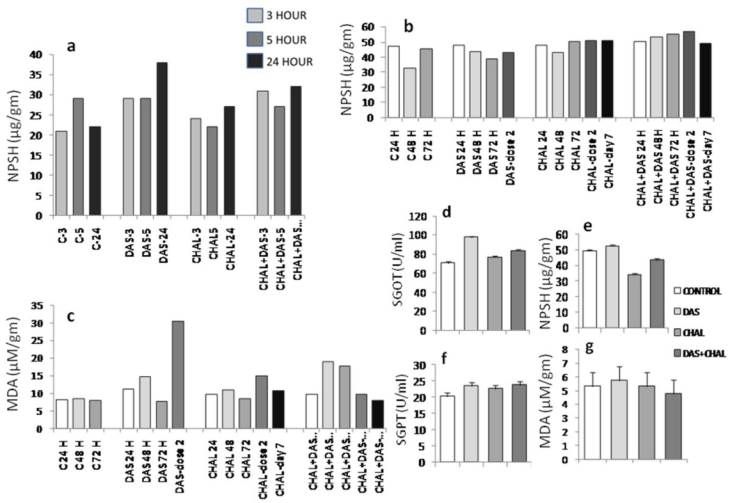


Figure 6

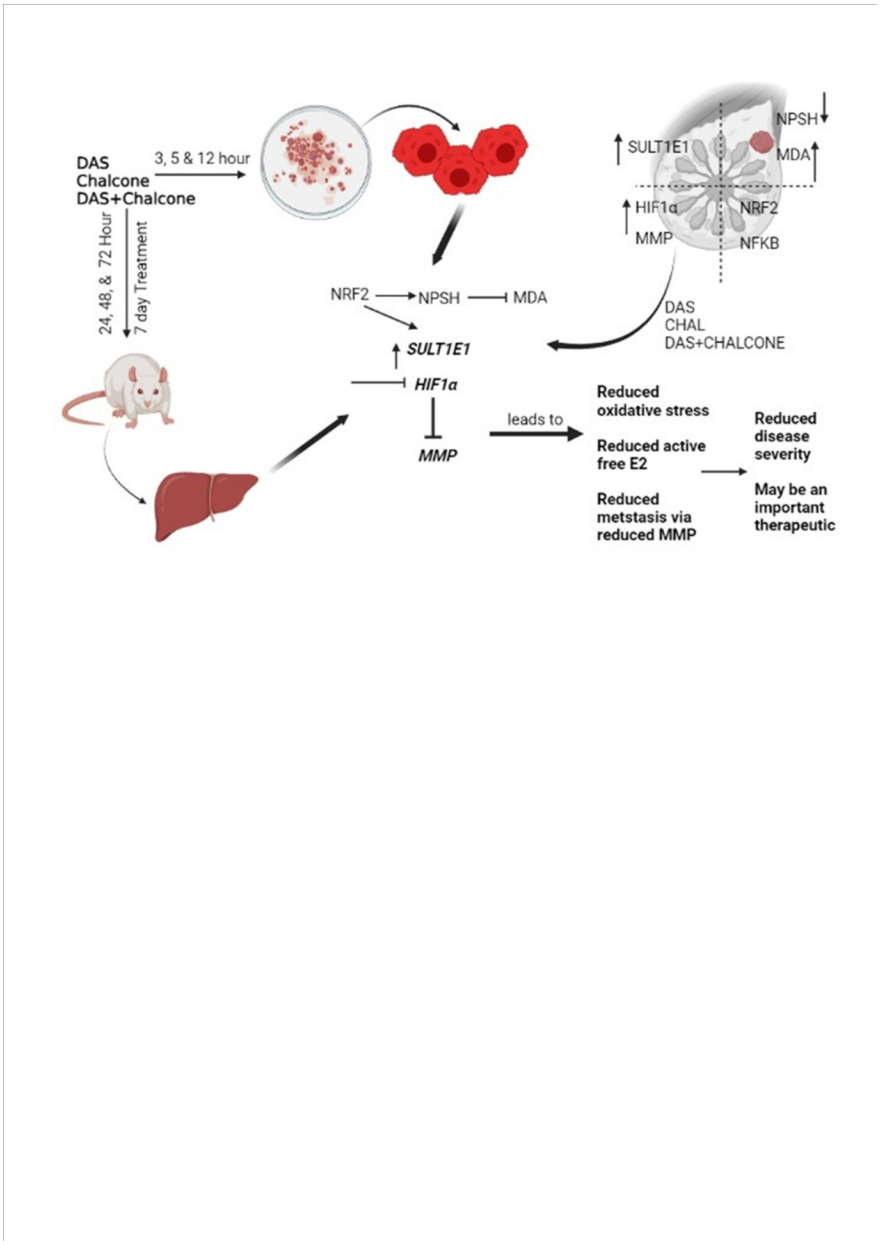


Figure 7