

## The metabolic bioactivation of caffeic acid phenethyl ester (CAPE) mediated by tyrosinase selectively inhibits glutathione S-transferase

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### ARTICLE INFO

#### Article history:

Received 28 October 2010

Received in revised form 24 March 2011

Accepted 25 March 2011

Available online 31 March 2011

#### Keywords:

Melanoma

Quinone

Glutathione

GST

Caffeic acid phenethyl ester

MRP

### ABSTRACT

Glutathione S-transferase (GST) and multidrug resistance-associated proteins (MRPs) play major roles in drug resistance in melanoma. In this study, we investigated caffeic acid phenethyl ester (CAPE) as a selective GST inhibitor in the presence of tyrosinase, which is abundant in melanoma cells. Tyrosinase bioactivates CAPE to an *o*-quinone, which reacts with glutathione to form CAPE-SG conjugate. Our findings indicate that 90% CAPE was metabolized by tyrosinase after a 60-min incubation. LC–MS/MS analyses identified a CAPE-SG conjugate as a major metabolite. In the presence of tyrosinase, CAPE (10–25  $\mu$ M) showed 70–84% GST inhibition; whereas in the absence of tyrosinase, CAPE did not inhibit GST. CAPE-SG conjugate and CAPE-quinone (25  $\mu$ M) demonstrated  $\geq$ 85% GST inhibition via reversible and irreversible mechanisms, respectively. Comparing with CDNB and GSH, the non-substrate CAPE acted as a weak, reversible GST inhibitor at concentrations  $>50$   $\mu$ M. Furthermore, MK-571, a selective MRP inhibitor, and probenecid, a non-selective MRP inhibitor, decrease the IC<sub>50</sub> of CAPE (15  $\mu$ M) by 13% and 21%, apoptotic cell death by 3% and 13%, and mitochondrial membrane potential in human SK-MEL-28 melanoma cells by 10% and 56%, respectively. Moreover, computational docking analyses suggest that CAPE binds to the GST catalytic active site. Caffeic acid, a hydrolyzed product of CAPE, showed a similar GST inhibition in the presence of tyrosinase. Although, as controls, 4-hydroxyanisole and L-tyrosine were metabolized by tyrosinase to form quinones and glutathione conjugates, they exhibited no GST inhibition in the absence and presence of tyrosinase. In conclusion, both CAPE and caffeic acid selectively inhibited GST in the presence of tyrosinase. Our results suggest that intracellularly formed quinones and glutathione conjugates of caffeic acid and CAPE may play major roles in the selective inhibition of GST in SK-MEL-28 melanoma cells. Moreover, the inhibition of MRP enhances CAPE-induced toxicity in the SK-MEL-28 melanoma cells.

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### 1. Introduction

Melanoma is the most lethal cancer of skin, and the number of melanoma cases has doubled in the past 20 years [1]. Surgical resection can be used to cure malignant melanoma in early stages,

but once it has metastasized to other organs, chemotherapy is the only option available with limited success partly due to drug resistance. Among all the reasons that are significantly responsible for melanoma cancer drug resistance, the over-expression of glutathione S-transferase (GSTs) and multidrug resistance-associated proteins (MRPs) may have critical roles [2–4].

GST (glutathione S-transferase) is an important enzyme in detoxification of a broad range of compounds. An important role of GST is to biotransform xenobiotics and other endogenous toxic compounds. It initiates the conjugation of hydrophobic electrophilic toxic substances including drugs, carcinogens, herbicides, and insecticides with the tripeptide glutathione (GSH) [5]. Over expression of GST may increase detoxification and circumvent the cytotoxic action of anticancer agents leading to multi-drug resistance (MDR) [3]. For instance, the current alkylating agents for cancer therapy are substrates for GST in tumor which leads to the development of multi-drug resistance (MDR) [3]. GSTs also

*Abbreviations:* CAPE, caffeic acid phenethyl ester; CA, caffeic acid; 4-HA, 4-hydroxyanisole; MEM, Modified Eagle Medium Alpha; FBS, fetal bovine serum; PBS, phosphate buffered saline; MEM, Minimum Essential Medium; MTT, (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide); DETAPAC, diethylenetriaminepentaacetic acid; GST, glutathione S-transferase; MRP, multidrug resistance-associated protein; TMRM, tetramethyl rhodamine methyl ester; GSH, glutathione; MDR, multi-drug resistance; EA, ethacrynic acid; CDNB, 1-chloro-2,4-dinitrobenzene; EA-SG, ethacrynic acid glutathione conjugate; CA-SG, caffeic acid glutathione conjugate; CAPE-SG, caffeic acid phenethyl ester glutathione conjugate.

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play a role in the detoxification of superoxides, peroxides, and hydroxyl radicals [6]. GST utilizes GSH to scavenge the toxic reactive xenobiotics, which are responsible for the production of oxidative stress and cell toxicity; this is one of the important parts of the defense mechanism against carcinogenic and toxic effects of toxic compounds [7]. In a study of GST expression, it was shown that GST is highly expressed in melanoma cells when compared to the normal cells [2].

Furthermore, the co-expression of MRPs with GSTs may play a major role in protection of cancer cells from anticancer agents [8,9]. It was previously reported that MRP proteins are responsible for the active transport across biological membrane [10]. MRPs were also shown to confer the resistance to several vinca alkaloids, anthracyclines and epipodophyllotoxins [11]. Moreover, it was reported that the detoxification of several anti-neoplastic agents was due to mixed action of both GSTs and MRPs [8,9,12]. It was also shown that human melanoma cells express high levels of both GSTs and MRPs [2,13].

To enhance selective drug delivery to melanoma, we have recently used tyrosinase, as a primary molecular target for bioactivation of caffeic acid phenethyl ester (CAPE) [1,14]. This is because tyrosinase is over-expressed and up-regulated in melanoma [15]. CAPE is an ester analog of caffeic acid and is an active component of propolis [16]. CAPE also exhibits antibacterial, anti-inflammatory, anti-viral, and anti-cancer properties. Recently, we investigated CAPE selective toxicity towards melanoma cells [1,14]. Bioactivation of CAPE by melanoma tyrosinase leads to the formation of quinone which causes selective toxicity towards melanoma cells compared to non-melanoma cell lines [1,14].

In the current study, our aims were to investigate CAPE and its quinone and glutathione conjugate metabolites, which are formed as a result of the CAPE's bioactivation by tyrosinase, as selective inhibitors of GST in melanoma cells, as a secondary molecular target, compared to non-melanoma cells, which do not express tyrosinase. The natures of GST inhibition, including reversibility, irreversibility, competitive and non-competitive inhibitions were investigated. We also tested the effect of MK-571, a selective MRP inhibitor [17] and probenecid, a non-selective inhibitor of MRP protein [18], in combination therapy with CAPE in human SK-MEL-28 melanoma cells.

## 2. Materials and methods

### 2.1. Materials

Glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), and all other materials, solvents and reagents used in this work were analytical grade with the highest degree of purity and were purchased either from Sigma–Aldrich, St. Louis, MO or Fisher-Scientific, Pittsburgh, PA. Glutathione S-transferase (GST) was purchased from Sigma–Aldrich (Cat. No. G8642). The isozyme is identified as hGSTP1-1 with 26 U/mg solid (55 U/mg protein). One unit will conjugate 1.0  $\mu$ mol of CDNB with reduced glutathione per min at pH 6.5 at 25 °C and the source of this enzyme is human placenta [19,20]. Mushroom tyrosinase was used throughout this study, as the purified human tyrosinase is unavailable commercially. Mushroom tyrosinase was purchased from Sigma–Aldrich (Cat. No. T3824), with 4276 U/mg solid. One unit of tyrosinase leads to  $\Delta A_{280\text{ nm}}$  of 0.001 per in at pH 6.5 at 25 °C in 3 mL reaction mix containing L-tyrosine, with an isoelectric point of 4.7–5.0. Because the compounds were dissolved in DMSO, the final concentration of DMSO was 1% v/v in cell culture media of the cells treated with drugs. Therefore, the media for control cells contained 1% v/v DMSO in the experiment. Phosphate buffered saline (PBS) was used as a vehicle to dissolve glutathione.

### 2.2. Cell lines and culture conditions

Modified Eagle Medium Alpha (MEM) (1 $\times$ ) (Cat. No. 32571-036), Leibovitz's L-15 Medium (Cat. No. 30-2008), fetal bovine serum (FBS) (Cat. No. 10082-139) were purchased from American Type Culture Collection (ATCC<sup>®</sup>), Manassas, VA. Versene (1 $\times$ , 0.2 g EDTA 4Na/L in phosphate buffered saline) (1:5000 Cat. No. 15040-066) were purchased from Invitrogen, Grand Island, NY. RPMI medium 1640 (1 $\times$ ) (Cat. No. 11875-119) was obtained from Invitrogen Corporation, Grand Island, NY. Human SK-MEL-28 cell line was obtained from ATCC<sup>®</sup>, Manassas, VA.

### 2.3. Identification of CAPE-SG conjugate metabolite using electrospray tandem mass spectrometry

LC/MS–MS method [21] was used to identify a CAPE glutathione (CAPE-SG) conjugate. Tyrosinase (20 U/mL) was added to a mixture of CAPE (62.5  $\mu$ M) and glutathione (200  $\mu$ M) in a final volume of 2 mL phosphate buffer (0.1 M, pH 6.5). The mixture was incubated for 5 min at 37 °C. The CAPE-SG conjugate was extracted using solid phase strata-X 33  $\mu$ m polymeric reversed phase extractor (Cat. No. 8B-S100-TAK, Strata, Torrance, CA, USA). Activation of the solid phase reversed phase extractor was performed by using 1 mL of water, 1 mL of methanol and 1 mL of water in sequence. One milliliter of the reaction solution was run through solid phase reversed phase extractor under a stream of nitrogen gas. Then, the solid phase extractor was washed with 100  $\mu$ L water. The product was collected using 500  $\mu$ L of methanol. The solvent was subsequently evaporated under a stream of nitrogen gas at 37 °C on a hot plate (N-Evap, Organomation Associates Inc., Berlin, USA). Five hundred microliter of acetonitrile was added to dissolve the sample. A similar procedure was performed for control solution containing CAPE and GSH in the absence of tyrosinase.

The above sample was analyzed by electrospray tandem mass spectrometry in positive mode with a Varian 1200L triple quadrupole mass spectrometer (Varian Inc., Palo Alto, CA, USA). Direct infusion of samples into the mobile phase stream was used to detect protonated parent ions and to determine precursor-product ion transitions. An isocratic flow of 50% water with 0.1% formic acid, and 50% acetonitrile at 0.1 mL/min was used for chromatographic separation on a Synergi 4u Max-RP column (75  $\times$  2.1 mm) (Varian Inc., Lake Forest, CA, USA) protected by a Synergi 4u Max-RP guard column (Varian Inc., Lake Forest, CA, USA). The electrospray housing temperature was set at 40 °C, and the nitrogen drying gas temperature was held at 250 °C. Argon was used as the collision gas at 1.4 mTorr pressure, with collision energies between 8 and 22.5 V, depending on the precursor-product ion transition. The capillary voltage was 56 V and the detector multiplier voltage was 1500 V. Varian MS Workstation version 6.9 software was used for data acquisition and processing. Using selective/multiple reaction monitoring technique, each sample reaction was monitored for detection of parent ion  $m/z = 590$  for CAPE-SG and parent ion  $m/z = 285$  for CAPE, and their respective predicted daughter ions on the LC/MS/MS detector. Separate samples of CAPE and GSH were used as controls to predict possible daughter ions for CAPE-SG in selective/multiple reaction monitoring mode.

### 2.4. GST substrate assay

GST (0.1 U/mL) was added to a mixture of CAPE (50, 100 and 250  $\mu$ M) and GSH (500  $\mu$ M) in a final volume of 2 mL phosphate buffer (0.1 M, pH 6.5). The mixture was pre-incubated for 15, 30 and 60 min at 37 °C. A 250  $\mu$ L aliquot was added to trichloroacetic acid (25  $\mu$ L; 30% w/v), vortexed and left at room temperature for 5 min. A 100  $\mu$ L aliquot of the supernatant was added to a mixture of Ellman's reagent (DTNB) (62.5  $\mu$ L; 2 mg/mL) and Tris/HCl buffer

(875  $\mu\text{L}$ ; 0.1 M, pH 8.9), and then vortexed. The absorbance of the solution was monitored at 412 nm [22]. Glutathione consumption was used as a marker to evaluate if CAPE were a substrate for GST. Similar experiments were performed on 4-HA, CA, and tyrosine to evaluate them as GST substrates. CDNB was used as a positive control. Ellman's reagent (DTNB) method was used to measure the extent of GSH consumption as previously described [1,14,23].

### 2.5. The inhibition of human placenta GST by CAPE

UV-Vis spectroscopy method was used to elucidate the inhibition of GST by CAPE. Human placenta GST inhibition was assayed by the method of Tuna et al. [24]. For a typical assay, the reaction mixture of 2.5 mL contained potassium phosphate buffer (100 mM, pH 6.5), glutathione (1 mM) and CDNB (200  $\mu\text{M}$ ), with 0.05 U/mL of human placenta GST [24]. Various concentrations of CAPE (10, 25 and 50  $\mu\text{M}$ ) were added to the above reaction mixture to investigate the inhibition of GST. The cuvette containing the reaction mixture had a water-jacket supported by a pump circulating water at a constant temperature of 25 °C. The time-dependent changes in  $A_{340\text{ nm}}$  were recorded for 6 min using a GBC UV-Vis spectral spectrophotometer (GBC Scientific, Victoria, Australia). The absorbance difference between 6 and 1 min was used to calculate the extent of GST inhibition. Absorbance was less than 1.0 at 6 min. The GST activity was calculated using the following formula: GST activity (mM/min) =  $[(\Delta A_{340\text{ nm}}/\text{min of sample}) - (\Delta A_{340\text{ nm}}/\text{min of blank})]$  divided by  $9.6\text{ mM}^{-1}\text{ cm}^{-1}$  (extinction coefficient of DNP-SG at  $A_{340\text{ nm}}$ ) [25]. Where  $\Delta A$  was the absorbance difference between 6 and 1 min at 340 nm. However the unit was converted to  $\mu\text{M}/\text{min}$  (nmol/mL/min) to graph Lineweaver-Burk plot. All experiments were determined in triplicate in buffers equilibrated at constant room temperature. Similar procedures were performed for 4-hydroxyanisole (4-HA), caffeic acid (CA), and tyrosine.

### 2.6. GST inhibition by CAPE-quinone (CAPE in the presence of tyrosinase)

In order to investigate the inhibition of GST by CAPE-quinone, tyrosinase (20 U/mL) was added to a mixture of CAPE (25  $\mu\text{M}$ ) and GST (0.1 U/mL) in a final volume of 2.5 mL phosphate buffer (0.1 M, pH 6.5). The mixture was incubated for 30 min at 37 °C to permit CAPE metabolism to CAPE-quinone by tyrosinase. To investigate the reversible or irreversible nature of GST inhibition by CAPE-quinone, mixture was filtered through Millipore centrifugal filter units with 10 K molecular weight cutoff to isolate GST from the reaction mixture (UFC801024, Amicon Ultra, Carrigtwohill, Ireland). Then, glutathione (1 mM) and CDNB (200  $\mu\text{M}$ ) [24] were added to the solution recovered from the Millipore centrifugal filter unit and made the final volume to 2.5 mL with phosphate buffer (100 mM, pH 6.5). The cuvette containing the reaction mixture had a water-jacket supported by a pump circulating water at a constant temperature of 25 °C. This mixture was used to investigate the irreversible inhibition of GST by CAPE-quinone using UV-Vis spectroscopy method as mentioned in Section 2.5. Control solutions containing GST or GST/tyrosinase were also used to assess the recovery for GST enzyme activity in the absence of tested compounds. The percentage inhibition of the enzyme activity by various inhibitors was calculated by comparing the results with GST activities in the controls. A similar approach was used to investigate the nature of GST inhibition by caffeic acid (CA).

### 2.7. GST inhibition by CAPE-SG conjugate (CAPE in the presence of tyrosinase and glutathione)

CDNB method was used to measure GST activity [24]. This experiment was performed to investigate the reversible and

irreversible nature of GST inhibition by CAPE-SG conjugate. Tyrosinase (20 U/mL) was added to a mixture of CAPE (25  $\mu\text{M}$ ), glutathione (100  $\mu\text{M}$ ) and GST (0.1 U/mL) in a final volume of 2.5 mL phosphate buffer (0.1 M, pH 6.5). The mixture was incubated for 30 min at 37 °C to allow the formation of CAPE-SG conjugate in the presence of tyrosinase and glutathione. The rest of experiment was similar to what described in the previous section. A similar approach was used to investigate the nature of GST inhibition by caffeic acid glutathione conjugate.

### 2.8. Competitive and non-competitive GST inhibition by CAPE, CAPE-quinone and CAPE-SG conjugate

CDNB method [24] was used to investigate the nature of GST inhibition and the inhibition constant ( $K_i$ ) [26] of CAPE, CAPE-quinone, and CAPE-SG conjugate by Lineweaver-Burk plots. Briefly, tyrosinase (20 U/mL) was added to a mixture of CAPE (10  $\mu\text{M}$ ), and GSH (1 mM) in phosphate buffer (0.1 M, pH 6.5). The mixture was incubated for 30 min at 37 °C to allow the formation of CAPE-SG conjugate. Then, GST (0.02 U/mL), CDNB (0.2–1 mM) were added to the solution mixture. The cuvette containing the reaction mixture had a water-jacket supported by a pump circulating water at a constant temperature of 25 °C. The absorbance at 340 nm was monitored for 6 min using UV-Vis spectroscopy method at 340 nm. The change in absorbance between 6 and 1 min was used as the indication of the reaction rate. The GST activity was calculated as described in Section 2.5. All the experiments were performed on three different days using freshly prepared GST, CDNB, CAPE, GSH and tyrosinase stock solutions on a daily basis. The averages of data points were used to graph the Lineweaver-Burk plot to determine the nature of competitive and non-competitive inhibition. The  $K_i$  values were determined according to a method published previously [26]. The control reaction contained GST (0.02 U/mL), GSH (1 mM) and CDNB (0.2–1 mM). The concentration of CDNB was 1 mM, when the nature of GST inhibition was investigated with respect to GSH (0.2–1 mM).

Similar experiments were performed to investigate the nature of GST inhibition with respect to GSH and CDNB by CAPE-quinone (10  $\mu\text{M}$ ), CAPE (100  $\mu\text{M}$ ) and EA (20  $\mu\text{M}$ ) with concentrations of GSH and CDNB ranging from 0.2 to 1 mM as discussed above. The inhibitory constants  $K_i$  of competitive and non-competitive inhibitions were calculated from following formulas [26].

$$\text{Competitive Inhibition : } K_i = K_m \times [I]/(K_m^* - K_m)$$

$$\text{Non-competitive Inhibition : } K_i = V_{\text{max}}^* \times [I]/(V_{\text{max}} - V_{\text{max}}^*)$$

Mixed Inhibition :

$$(a) K_i = V_{\text{max}}^* \times K_m \times [I]/((V_{\text{max}} \times K_m^*) - (V_{\text{max}}^* \times K_m))$$

$$(b) K_i' = V_{\text{max}}^* \times [I]/(V_{\text{max}} - V_{\text{max}}^*)$$

where  $K_m$  and  $K_m^*$  values are Michaelis-Menton constants,  $V_{\text{max}}$  and  $V_{\text{max}}^*$  values are maximum reaction rates, and  $K_i$  is the inhibition constant.  $K_m$  and  $V_{\text{max}}$  values were obtained in the absence of the inhibitor.  $K_m^*$  and  $V_{\text{max}}^*$  were obtained in the presence of inhibitors (CAPE, CAPE-SG, CAPE-quinone, or EA).  $[I]$  is the concentration of the inhibitor.

### 2.9. Cell culture

The cell culture experiments were performed as described previously [1]. Human SK-MEL-28 melanoma cells were cultured in MEM Alpha media supplemented by 10% FBS. After reaching a confluency of 90%, the cells obtained were used for cytotoxicity studies.

### 2.10. The inhibition of human SK-MEL-28 melanoma GST by CAPE

CDNB method was used to investigate the GST inhibition in melanoma cells [24,27]. A suspension of 200,000 cells in 500  $\mu$ L MEM Alpha media supplemented with 10% FBS was used for this study. The cell suspension was sonicated for 3–5 s and then centrifuged for 15 min at 13,000 rpm. The supernatant was taken into a tube to which glutathione (1 mM), CAPE (15, 25, 50, 100  $\mu$ M), CDNB (1 mM) and tyrosinase (10 U/mL) were added in a final volume of 2.5 mL phosphate buffer (100 mM, pH 6.5). The reaction mixture was used to investigate the inhibition of GST in human SK-MEL-28 melanoma cells by CAPE using UV–Vis spectroscopy method as described in Section 2.5. A similar procedure was used to study GST inhibition in the presence of 4-hydroxyanisole (4-HA), caffeic acid (CA), and tyrosine.

### 2.11. MTT cell cytotoxicity assay in SK-MEL-28 melanoma cells

The cytotoxicity assay was evaluated using yellow tetrazolium dye (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) (MTT) [28,29]. SK-MEL-28 cells were obtained from 90% to 95% confluent cell cultures, seeded at 40,000 cells/well in 24 well plate, and grown in 500  $\mu$ L fresh MEM Alpha media (supplemented by 10% FBS). After 24 h of seeding, cells were treated with CAPE, CA, 4-HA and tyrosine at 15, 30, 50 and 100  $\mu$ M for 48 h before measuring the cell viability using MTT assay [28]. An equal volume of DMSO was added to control cells so that final concentration of DMSO was 1%. An analysis of variance (ANOVA) was carried out to compare the percentage of surviving cells for each compound at various concentrations followed by Bonferoni's post *t*-test.

### 2.12. Effect of modulators on CAPE toxicity

The biochemical mechanism of CAPE toxicity in SK-MEL-28 cells was performed using various modulators [14] using MTT assay as described in the previous section. After 24 h of cell seeding, ethacrynic acid (EA) (2  $\mu$ M) a GST inhibitor [30,31], and MK-571 (10  $\mu$ M), a selective MRP inhibitor [17] and probenecid (500  $\mu$ M), a non-selective MRP inhibitor [18] were added to cell culture media. After 1 h cells were treated with CAPE (15 and 30  $\mu$ M) for 48 h. MTT assay was used to assess the effect of modulators on CAPE toxicity as described in Section 2.11.

### 2.13. Apoptosis assay

Apoptotic cells were identified by FITC-conjugated Annexin V and propidium iodide using Annexin V-FITC apoptosis assay kit (Cat. No. PF032, Calbiochem, La Jolla, CA, USA) according to a method published by us previously [14]. Briefly, SK-MEL-28 cells were seeded at 160,000 cells/well in six-well plates in MEM media and allowed to attach overnight. After seeding, MK-571 (10  $\mu$ M), a selective MRP inhibitor [17] and probenecid (500  $\mu$ M), a non-selective MRP inhibitor [18] were added to the cell culture media. After 1 h, CAPE (15 and 30  $\mu$ M) was added to the wells. 48 h after incubation, cells were detached using versene solution (300–350  $\mu$ L) (Cat. No. 15040, GIBCO, Carlsbad, CA). Collected cells were incubated with 5  $\mu$ L of Annexin FITC and 5  $\mu$ L of propidium iodide in 100  $\mu$ L of binding buffer for 15 min as described in Calbiochem Annexin V-FITC apoptosis assay kit (Cat. No. PF032, Calbiochem, La Jolla, CA, USA) [32]. The experiments were performed in triplicates.

### 2.14. Mitochondrial membrane potential assay

To investigate the role of MRP in the biochemical mechanism of CAPE toxicity in SK-MEL-28 cells, mitochondrial membrane

potential was determined in the presence and absence of MK-571, a selective MRP inhibitor [17] and probenecid, a non-selective MRP inhibitor [18], using tetramethyl rhodamine methyl ester (TMRM) fluorescent dye [33]. Using a method published by us previously [14]. Briefly, SK-MEL-28 melanoma cells were seeded at 160,000 cells/well in six well plates in MEM media and allowed to attach overnight. After seeding, MK-571 (10  $\mu$ M) and probenecid (500  $\mu$ M) were added to the respective wells 1 h prior to the addition of CAPE (15 and 30  $\mu$ M). After 48 h incubation, 20  $\mu$ L (50 nM) tetramethyl rhodamine methyl ester (TMRM) (Cat. No. T668, Invitrogen A/S, Taastrup, Denmark) was added [33]. The stained cells were analyzed using flow cytometer (Accuri C6 flow cytometer, Ann Arbor, MI). The 10,000 events were acquired. Arithmetic mean values of fluorescence signal in arbitrary units were determined for each sample in triplicate.

### 2.15. Docking calculations

AutoDock tools 1.5.2 and AutoDock 4.0 [34,35] were used to predict possible interactions between GST and CAPE or EA. During the calculations, the protein and ligands were treated as rigid groups. The predicted bound configuration with the lowest free energy was chosen for each ligand. Crystal structures of both wild-type GST (PDB ID 11GS) [36,37] and the C47S/Y108V GST double mutant (PDB ID 3KM6) [36] were applied for this study.

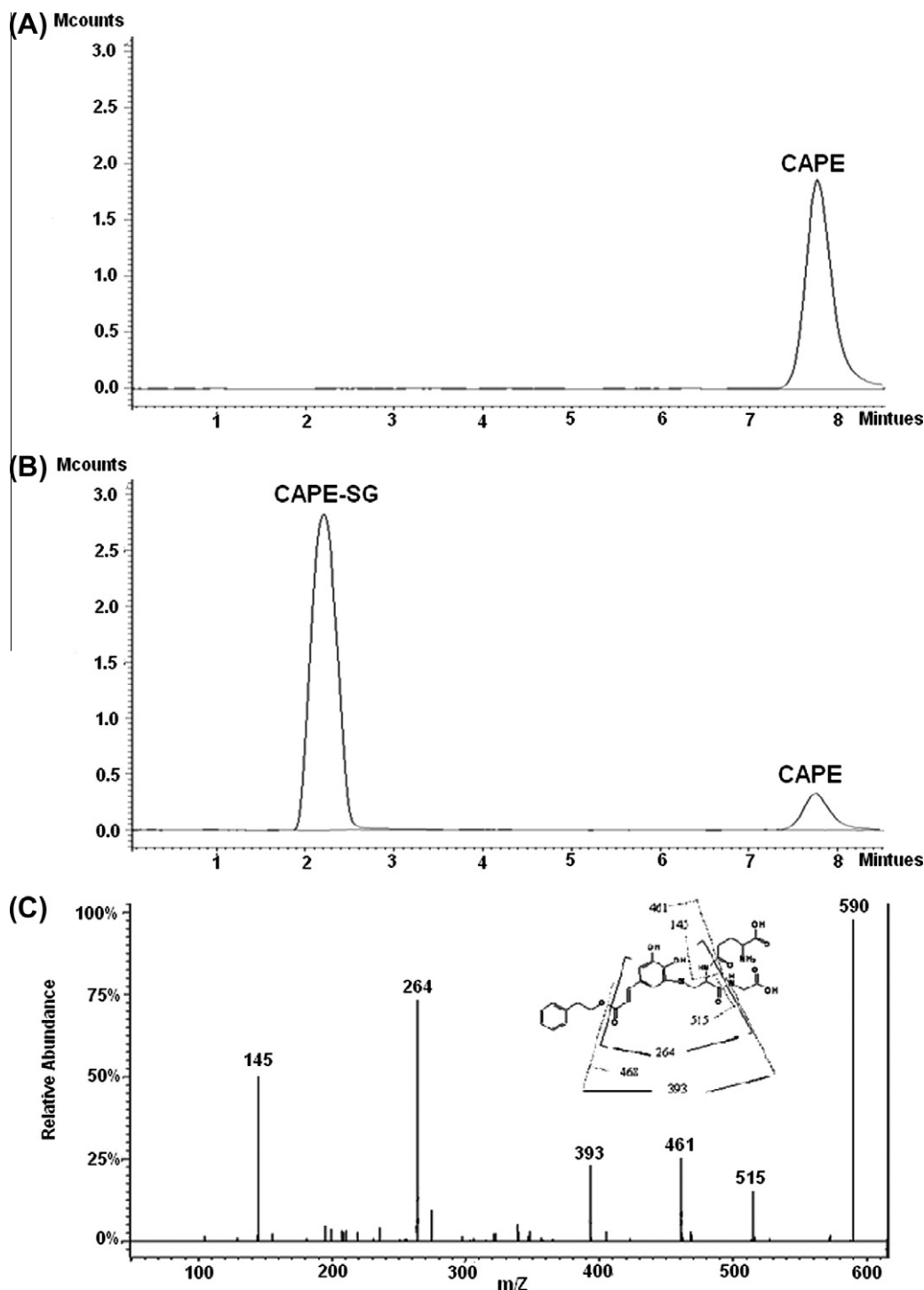
### 2.16. Statistical analysis

Results from three replicate experiments have been reported as mean  $\pm$  SD. An analysis of variance (ANOVA) was carried out to compare the percentage of surviving cells and percentage of GST inhibition for each compound at various concentrations followed by Bonferoni's post *t*-test.

## 3. Results

### 3.1. Glutathione conjugate of CAPE metabolite by LC–MS/MS

LC–MS/MS was used to identify the oxidized product of CAPE. The retention times on HPLC for the CAPE-SG conjugate and CAPE were 2.2 and 7.8 min, respectively (Fig. 1A and B). This was expected as CAPE-SG conjugate should be more hydrophilic than CAPE; and hence to elute faster. Using selective/multiple reaction monitoring, the parent ions were monitored for CAPE-SG and CAPE, simultaneously. Fig. 1A and B illustrate two overlaid detection windows for  $m/z = 590$  (CAPE-SG) peak and  $m/z = 285$  (CAPE) peak on the LC/MS/MS detector. There was no peak observed at 2.2 min when CAPE and glutathione incubated in the absence of tyrosinase implying that the peak at 2.2 min was formed only after CAPE bioactivation by tyrosinase and a subsequent reaction with glutathione (Fig. 1A and B). The 2.2 min peak was not formed when CAPE was incubated with tyrosinase (data not shown). The CAPE incubation with GSH also showed one peak at 7.8 min indicating that CAPE does not react with GSH in the absence of tyrosinase. These results indicated that incubation of CAPE, tyrosinase, and glutathione formed a major product that was eluted at 2.2 min. To characterize the product, LC–MS/MS analysis of parent ion was carried out. Further analysis of the peak at 2.2 min using tandem mass spectrometry in positive ion mode indicated a mono CAPE-SG conjugate at  $m/z$  [MH]<sup>+</sup> 590. Individual samples of CAPE and GSH were used as controls to predict possible daughter ions for CAPE-SG conjugate in selective/multiple reaction monitoring using LC–MS/MS analyses. Subsequent LC–MS/MS analyses of the parent signal [MH]<sup>+</sup> = 590  $m/z$  exhibited parent



**Fig. 1.** LC-MS/MS of CAPE-SG conjugate. Using selective/multiple reaction monitoring, both figures A and B represent two overlaid detection windows for  $m/z = 590$  (CAPE-SG) peak and  $m/z = 285$  (CAPE) peak on the LC/MS/MS detector. (A) After 5 min incubation of CAPE ( $62.5 \mu\text{M}$ ) with glutathione ( $200 \mu\text{M}$ ), the HPLC chromatogram revealed a peak at 7.8 min indicating that CAPE-SG conjugate was not formed in the absence of tyrosinase. (B) The glutathione conjugate of CAPE was determined by HPLC after 5 min incubation of reaction mixture containing CAPE ( $62.5 \mu\text{M}$ ), glutathione ( $200 \mu\text{M}$ ), and tyrosinase ( $20 \text{ U/mL}$ ). The peaks at 2.2 min and 7.8 min are CAPE-SG conjugate and CAPE, respectively. Note, the significant decrease in the intensity of CAPE peak at 7.8 min and the appearance of a new peak at 2.2 min. (C) The MS/MS analysis of the peak at 2.2 min showed CAPE-SG conjugate as the major metabolite ( $m/z 590 [\text{M}+\text{H}]^+$ ). The major daughter ions identified were 145, 264, 393, 461, 468, 515, and 590  $m/z$ . The MS/MS analysis of the CAPE and glutathione reaction mixture detected only CAPE ( $m/z 285 [\text{M}+\text{H}]^+$ ), indicating that CAPE-SG conjugate was not formed in the absence of tyrosinase.

CAPE-SG conjugate ion at  $m/z 590 [\text{M}+\text{H}]^+$  and daughter ions at 515  $[\text{M}-\text{glycine}]^+$ , 468  $[\text{M}-\text{phenethyloxy}]^+$ , 461  $[\text{M}+\text{H}-\text{glut}+\text{H}]^+$ , 393  $[\text{M}-\text{phenethyloxy}-\text{glycine}+\text{H}]^+$ , 264  $[\text{M}-\text{phenethyloxy}-\text{glycine}-\text{glu}]^+$ , and 145  $[\text{glut}+\text{NH}]^+$  (Fig. 1C).

### 3.2. GST mediated glutathione consumption assay

GSH consumption was used as a biomarker to evaluate CAPE, CA, 4-HA, and tyrosine as substrate for GST. The study found that

none of these tested compounds, including CAPE, 4-HA, tyrosine, and CA, was a substrate for GST. CDNB was reported previously to be a substrate of GST [38] and was used as a positive control. On a molar basis, 0.6 mol glutathione was consumed per mole of CDNB, when CDNB was metabolized by GST at 60 min incubation.

### 3.3. The inhibition of human placenta GST by CAPE-quinone, CAPE-SG conjugate and CAPE

CAPE alone did not inhibit GST activity at concentrations <25  $\mu\text{M}$ ; however, it marginally inhibited GST activity by 13% at a higher concentration of 50  $\mu\text{M}$  (Fig. 2A). Caffeic acid (Fig. 2B), a hydrolyzed product of CAPE, 4-HA, a substrate for tyrosinase [39] and tyrosine, a natural substrate of tyrosinase [40] did not show any inhibition of GST at concentrations of 10–50  $\mu\text{M}$ . In contrast, CAPE-quinone, formed by bioactivation of CAPE in the presence of tyrosinase was a potent GST inhibitor, which decreased the human placenta GST activity by 70% and 93% at concentrations 10 and 50  $\mu\text{M}$ , respectively (Fig. 2A). Similarly, it was found that caffeic acid-quinone at concentrations of 10–50  $\mu\text{M}$  inhibited GST activity by 23–67% (Fig. 2B), whereas 4-HA-quinone (50  $\mu\text{M}$ ) and tyrosine-quinone (50  $\mu\text{M}$ ) showed no significant GST inhibition (data not shown).

Interestingly, it was found CAPE-SG conjugate 10–50  $\mu\text{M}$ , formed as a result of CAPE bioactivation by tyrosinase in the presence of glutathione, inhibited GST activity by 68–96% (Fig. 2A). Similarly, caffeic acid glutathione (CA-SG) conjugate also inhibited GST activity by 19–61% (Fig. 2B). Ploemen et al. also reported similar findings on CA-SG conjugate [41]. In contrast, neither 4-HA-SG conjugate nor tyrosine-SG conjugate inhibited GST activity (data not shown). The order of the GST activity inhibition for CAPE in descending order was CAPE-quinone  $\geq$  CAPE-SG conjugate  $\gggg$  CAPE. The order of GST activity inhibition for caffeic acid, a hydrolyzed product of CAPE, in descending order was CA-Quinone  $>$  CA-SG conjugate  $\gggg$  CA (Fig. 2).

### 3.4. Irreversible and reversible nature of GST inhibition by CAPE-quinone, CAPE-SG conjugate and CAPE

The 10 K Millipore filter was used to separate GST from the reaction mixture. Although CAPE-SG conjugate (25  $\mu\text{M}$ ) showed significant GST inhibition (Fig. 3A), the activity of GST was recovered after filtering the reaction mixture through 10 K Millipore filter (Fig. 3B), indicating that CAPE-SG conjugate inhibited GST in a

non-covalent binding fashion that were reversible. As shown, CAPE-quinone inhibits GST significantly (Fig. 3A). In contrast, when the reaction mixtures were filtered through 10 K Millipore filter, the recovered GST from the filter did not show enzymatic activity (Fig. 3B), suggesting that CAPE-quinone inhibited GST through irreversible covalent binding. Tyrosinase alone did not have an inhibition effect on GST (data not shown). A similar result was observed for CA-SG and CA-quinones indicating that CA-SG inhibited GST reversibly whereas CA-quinone inhibited GST irreversibly (Fig. 3).

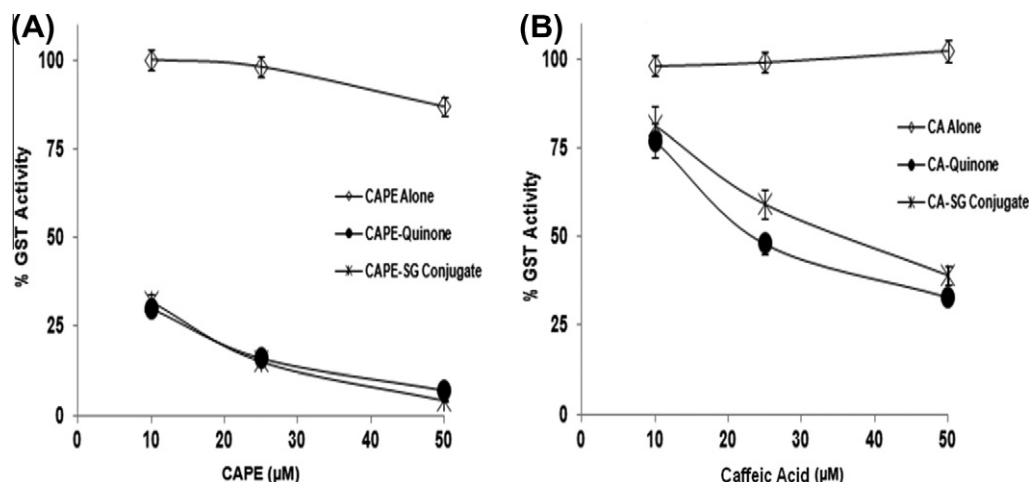
### 3.5. Competitive and non-competitive GST inhibition by CAPE, CAPE-quinone, CAPE-SG conjugate and EA

In order to evaluate the nature of GST inhibition by CAPE, CAPE-quinone, CAPE-SG conjugate and EA, the activity of GST enzyme was measured with respect to CDNB (0.2–1 mM) and GSH (0.2–1 mM). As shown in (Fig. 4A and B), the reciprocal plots of  $1/[\text{rate}]$  versus  $1/[\text{CDNB}]$  have the inhibitor and no inhibitor lines intercepting above the  $1/[\text{CDNB}]$  axis, suggesting that CAPE and CAPE-quinone are mixed inhibitors of GST with respect to CDNB with an apparent  $K_i$  and  $K'_i$  of 233 and 451  $\mu\text{M}$  for CAPE, and 2.7 and 8.8  $\mu\text{M}$  for CAPE-quinone (Fig. 4A and B and Table 1). However, CAPE-SG conjugate and EA inhibited GST via a competitive mechanism as the “no inhibitor” and “inhibitor” lines intercepted on the  $[1/\text{rate}]$  axis with an apparent  $K_i$  of 3.1 and 3.0  $\mu\text{M}$ , respectively (Fig. 4C and D and Table 1).

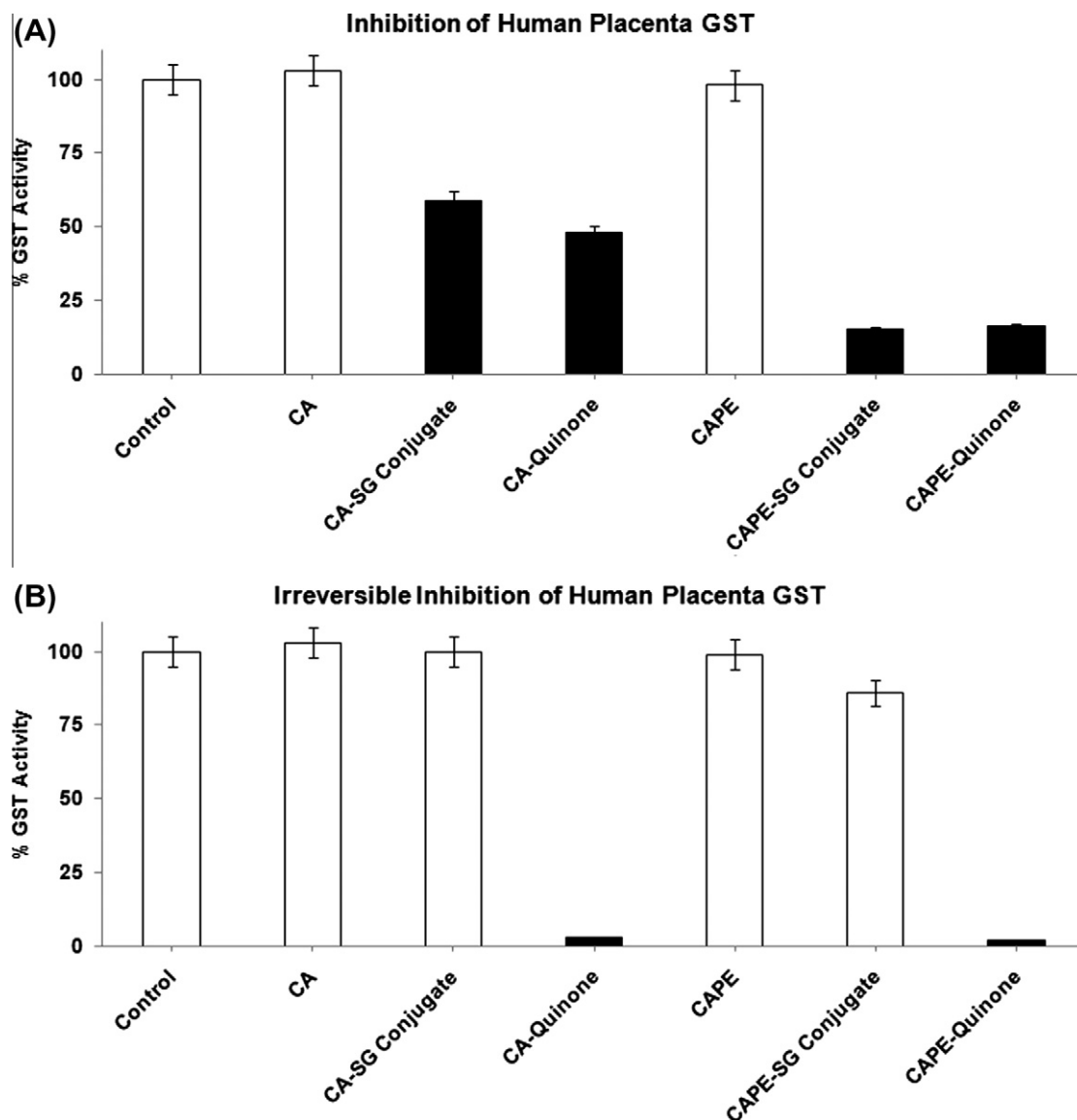
With respect to GSH (0.2–1 mM, Fig. 4E and G), CAPE and CAPE-SG conjugate showed mixed inhibitions of GST with an apparent  $K_i$  and  $K'_i$  of 942 and 2990  $\mu\text{M}$  for CAPE, respectively, and 2.6 and 14  $\mu\text{M}$  for CAPE-SG conjugate, respectively. However, CAPE-quinone acted as a competitive inhibitor of GST with an apparent  $K_i$  of 0.7  $\mu\text{M}$  (Fig. 4F and Table 1). On the other hand, EA acted as a non-competitive inhibitor of GST with an apparent  $K_i$  of 12  $\mu\text{M}$  (Fig. 4H and Table 1).

### 3.6. Inhibition of human SK-MEL-28 melanoma GST by CAPE

In the presence of tyrosinase, CAPE (15–100  $\mu\text{M}$ ) showed considerable and selective inhibition of GST in human SK-MEL-28 melanoma cell homogenate resulting in 61–86% inhibition (Fig. 5). Similarly, caffeic acid (15–100  $\mu\text{M}$ ) showed 21–53% GST inhibition whereas 4-HA (15–100  $\mu\text{M}$ ) showed only 4–18% GST inhibitions. Tyrosine (15–100  $\mu\text{M}$ ) did not show any significant GST inhibition (Fig. 5). These findings suggest that CAPE and its hydrolyzed



**Fig. 2.** The inhibition of GST. The inhibitory effects of CAPE and caffeic acid (a hydrolyzed product of CAPE) on human placenta GST with respect to CDNB. (A) CAPE-SG conjugate and CAPE-quinone at concentration of 10–50  $\mu\text{M}$  demonstrated 68–96% and 70–93% GST inhibition, respectively. (B) CA-SG conjugate and caffeic acid-quinone at concentrations of 10–50  $\mu\text{M}$  demonstrated 19–61% and 23–67% GST inhibition, respectively. At higher concentrations, CAPE alone showed negligible GST inhibition. 4-HA-SG conjugate, 4-HA-quinone, 4-HA, tyrosine-SG conjugate, tyrosine-quinone and tyrosine at concentrations of 10–50  $\mu\text{M}$  did not show GST inhibition (data not shown).



**Fig. 3.** Reversible and irreversible nature of GST inhibition. (A) CAPE-SG conjugate, CAPE-quinone, CA-SG conjugate (caffeic acid glutathione conjugate), and CA-quinone showed significant GST inhibition. (B) Irreversible inhibition of human placenta GST. CAPE-quinone and CA-quinone showed significant irreversible inhibition of GST, whereas the GST activity was recovered after removing CAPE-SG and CA-SG conjugates by filtering the reaction mixture through Millipore centrifuge filter unit, indicating that both glutathione conjugates of CAPE and CA were reversible GST inhibitors.

product caffeic acid were significantly more potent in inhibiting GST in human SK-MEL-28 melanoma cell homogenate than 4-HA and tyrosine.

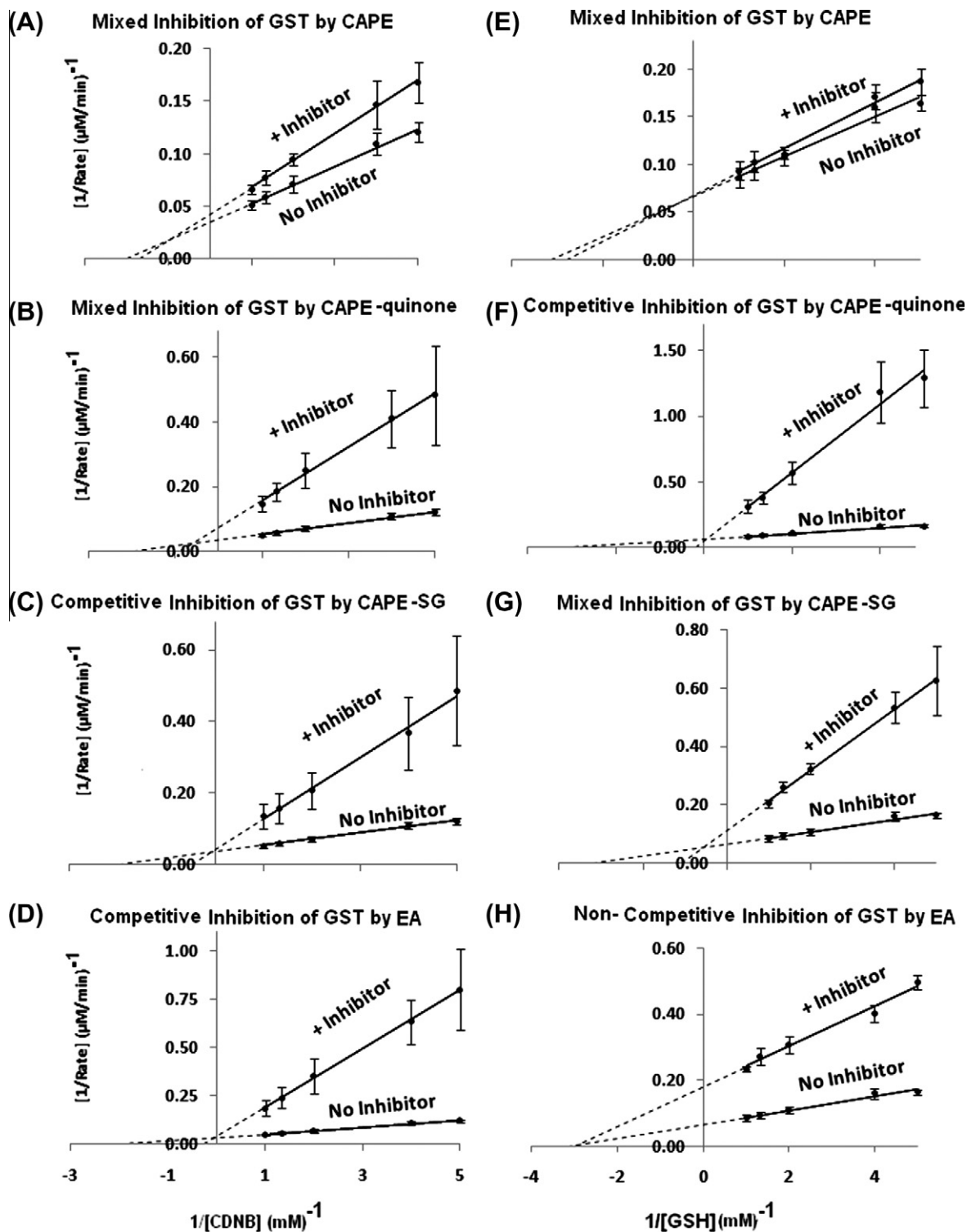
### 3.7. MTT cell cytotoxicity assay in SK-MEL-28 melanoma cells

MTT assay was performed to evaluate the anti-proliferative effects of CAPE, CA, 4-HA and tyrosine. CAPE showed significant cytotoxic effects towards human melanoma SK-MEL-28 cells in comparison to CA, 4-HA and tyrosine. After 48 h incubation, CAPE (15  $\mu\text{M}$ ) showed 48% cell toxicity towards SK-MEL-28 melanoma cells whereas CA, 4-HA and tyrosine at 30  $\mu\text{M}$  demonstrated 2–27% cell toxicity (data not shown). The  $\text{IC}_{50}$  (48 h) values of CAPE and 4-HA were 15 and 60  $\mu\text{M}$  in SK-MEL-28 melanoma cells, respectively, whereas the  $\text{IC}_{50}$  (48 h) values of CA and tyrosine were 2.3 mM and >5 mM, respectively. These findings suggested that CAPE was more effective as a cytotoxic agent towards SK-MEL-28 melanoma cells than other tested compounds.

### 3.8. The biochemical mechanism of CAPE toxicity in SK-MEL-28 melanoma cells

To investigate the biochemical mechanism of CAPE toxicity in SK-MEL-28 melanoma cells, the cell viability was determined in the presence and absence of modulators using MTT assay, Annexin V-PI apoptosis assay, and mitochondrial membrane potential measurement.

Using MTT assay, MK-571 (10  $\mu\text{M}$ ), a selective MRP inhibitor [17] and probenecid (500  $\mu\text{M}$ ), a non-selective MRP inhibitor [18], decreased the  $\text{IC}_{50}$  value of CAPE (15  $\mu\text{M}$ ) by 13% and 21%, respectively, and of CAPE (30  $\mu\text{M}$ ) by 12% and 9%, respectively (Table 2). These findings suggest that CAPE or its cytotoxic metabolites are substrate for MRP and hence can be detoxified by this efflux pump. Therefore, the inhibition of MRP may enhance CAPE induced toxicity in SK-MEL-28 cells. On the other hand, ethacrynic acid (EA) (2  $\mu\text{M}$ ), a GST inhibitor [30,31] did not affect the  $\text{IC}_{50}$  value of CAPE towards melanoma cells (Table 2). This could be because both CAPE-quinone and CAPE glutathione conjugate are potent GST inhibitors (Table 1),



**Fig. 4.** Lineweaver–Burk plots: competitive and non-competitive nature of GST inhibition. The experiments were performed on three different days using freshly prepared GST, CDNB, CAPE, GSH and tyrosinase stock solutions on a daily basis. The average of data points was used to graph the Lineweaver–Burk plot to determine the nature of competitive and non-competitive inhibition of GST. (A) Mixed GST inhibition by CAPE with respect to CDNB. (B) Mixed GST inhibition by CAPE-quinone with respect to CDNB. (C) Competitive GST inhibition by CAPE-SG conjugate with respect to CDNB. (D) Competitive GST inhibition by ethacrynic acid (EA) with respect to CDNB. (E) Mixed GST inhibition by CAPE with respect to GSH. (F) Competitive GST inhibition by CAPE-quinone with respect to GSH. (G) Mixed GST inhibition by CAPE-SG conjugate with respect to GSH. (H) Non-competitive GST inhibition by ethacrynic acid (EA) with respect to GSH. The unit of GST activity is  $\mu\text{M}/\text{min}$  (nmol/mL/min).

which may explain why ethacrynic acid did not enhance CAPE toxicity in melanoma cells.

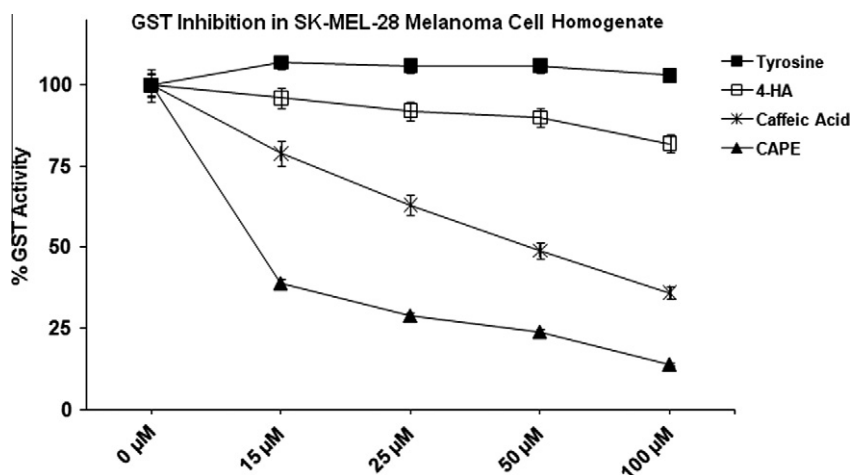
Using Annexin V apoptosis assay kit, MK-571 (10  $\mu\text{M}$ ) and probenecid (500  $\mu\text{M}$ ), MRP inhibitors, increased the CAPE (15  $\mu\text{M}$ ) induced apoptotic cell death by 3% and 13%, respec-

tively. MK-571 and probenecid also increased the CAPE (30  $\mu\text{M}$ ) induced apoptotic cell death by 7% and 16%, respectively (Fig. 6). Probenecid (500  $\mu\text{M}$ ), a non-selective MRP inhibitor, approximately doubled the CAPE-induced apoptotic cell death at 15 or 30  $\mu\text{M}$  (Fig. 6).

**Table 1**

Summary results for the nature of GST inhibition and  $K_i$  values. Mechanisms of GST inhibition by CAPE, CAPE-quinone, CAPE-SG conjugate and EA with their respective  $K_i$  values with respect to CDNB and GSH are summarized. EA was used as a positive control, as it is a well known GST inhibitor [38]. The  $K_i$  values for competitive and non-competitive inhibitions were estimated as discussed in Sections 2.5 and 2.8 [26].

	Mechanism of GST inhibition					
	With respect to CDNB (0.2–1 mM)			With respect to GSH (0.2–1 mM)		
	Inhibition	$K_i$ ( $\mu\text{M}$ )	$K'_i$ ( $\mu\text{M}$ )	Inhibition	$K_i$ ( $\mu\text{M}$ )	$K'_i$ ( $\mu\text{M}$ )
CAPE	Mixed	233	451	Mixed	942	2990
CAPE-quinone	Mixed	2.7	8.8	Competitive	0.7	–
CAPE-SG conjugate	Competitive	3.1	–	Mixed	2.6	14
Ethacrynic acid	Competitive	3.0	–	Non-competitive	12	–



**Fig. 5.** The inhibition of GST in human SK-MEL-28 melanoma cell homogenate. CAPE (15–100  $\mu\text{M}$ ) led to a dose dependent GST inhibition ranging from 61 to 86% in human melanoma SK-MEL-28 cell homogenate. CA (15–100  $\mu\text{M}$ ) showed 21–53% GST inhibition whereas 4-HA (15–100  $\mu\text{M}$ ) and tyrosine (15–100  $\mu\text{M}$ ) did not show significant inhibition in comparison to CA (caffeic acid) and CAPE.

**Table 2**

Toxicity of CAPE in the presence of MK-571, probenecid and EA. Data represent the  $\text{IC}_{50}$  values from the MTT assay. MK-571, a selective MRP inhibitor [17], and probenecid, a non-selective MRP inhibitor [18], decreased the  $\text{IC}_{50}$  value of CAPE (15  $\mu\text{M}$ ) by 13% and 21%, respectively, and of CAPE (30  $\mu\text{M}$ ) by 12% and 9%, respectively. Ethacrynic acid (EA), a GST inhibitor [30,31], did not show any significant decrease in the  $\text{IC}_{50}$  of CAPE in SK-MEL-28 cell line. Cells were pretreated with MK-571, probenecid and EA for 1 h prior to the addition of CAPE.

	No modulator	MK-571 (10 $\mu\text{M}$ )	Probenecid (500 $\mu\text{M}$ )	Ethacrynic acid (2 $\mu\text{M}$ )
Cells	100 $\pm$ 4	98 $\pm$ 3	97 $\pm$ 5	97 $\pm$ 4
Cells (1% DMSO)	98 $\pm$ 5	97 $\pm$ 1	99 $\pm$ 6	96 $\pm$ 3
CAPE (15 $\mu\text{M}$ )	52 $\pm$ 2	39 $\pm$ 3*	31 $\pm$ 2*	51 $\pm$ 2
CAPE (30 $\mu\text{M}$ )	38 $\pm$ 2	26 $\pm$ 1*	29 $\pm$ 1*	39 $\pm$ 4

\* Significance difference ( $P < 0.05$ ).

The combination of MK-571 and probenecid with CAPE further decreased mitochondrial membrane potential in SK-MEL-28 cells for CAPE (15  $\mu\text{M}$ ) by 10% and 56%, respectively and for CAPE (30  $\mu\text{M}$ ) by 29% and 68%, respectively (Fig. 7). Probenecid (500  $\mu\text{M}$ ), a non-selective MRP inhibitor, approximately increased the effect of CAPE (15–30  $\mu\text{M}$ ) on mitochondrial membrane potential for 8–11-fold. On the other hand, MK-571 (10  $\mu\text{M}$ ), a selective MRP inhibitor, approximately increased the effect of CAPE (15–30  $\mu\text{M}$ ) on mitochondrial membrane potential for 3–4-fold. The modulators had no significant toxicity effect when incubated alone with SK-MEL-28 cells (Table 2 and Figs. 6 and 7).

### 3.9. Molecular docking

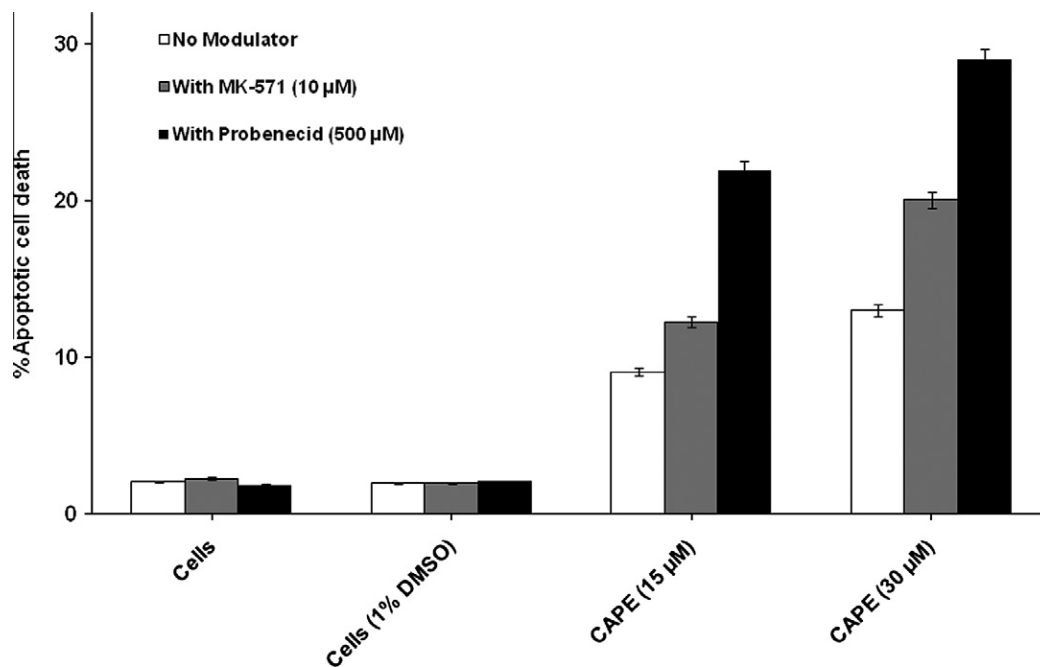
Computer prediction of the interactions between wild-type GST and CAPE or EA showed that the bound CAPE overlaps with both glutathione-binding site (G site) and substrates-binding (H site) of the enzyme (Fig. 8E); while bound EA only overlaps with the

H site (Fig. 8C). Similar results were obtained from the wild type GST and C47S/Y108V GST mutant (Fig. 8D and F). The molecular modeling also revealed that the binding sites of CAPE and EA could potentially overlap, which may explain why EA, a GST inhibitor, did not enhance CAPE toxicity in melanoma cells.

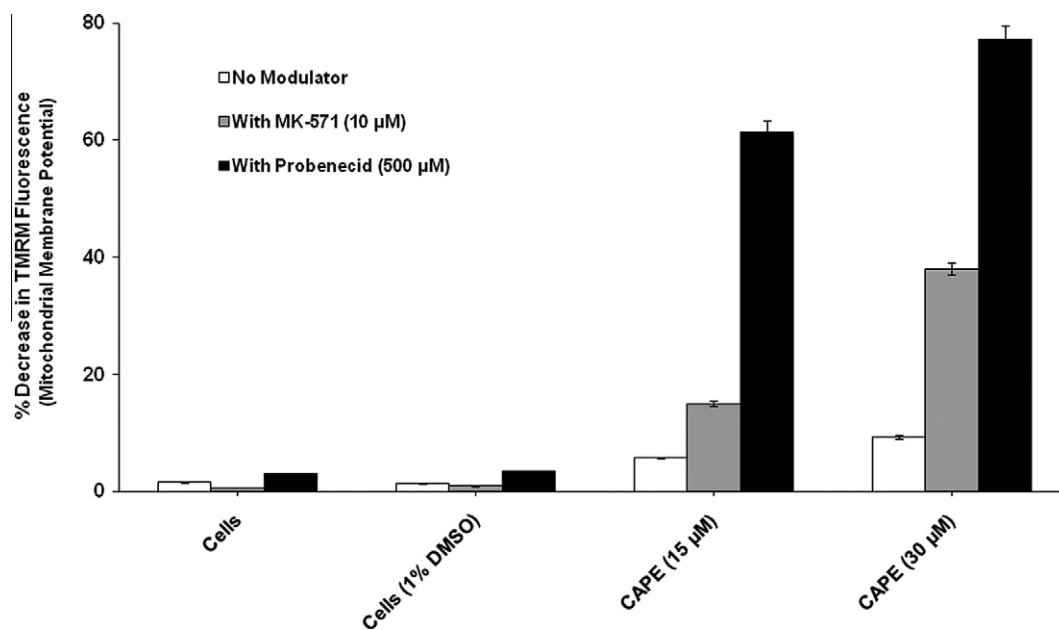
## 4. Discussion

CAPE is a caffeic acid phenethyl ester derivative which is a naturally occurring hydroxycinnamic acid found in propolis [16]. It is also known to be highly stable in human plasma [42]. In our earlier work, we have used tyrosinase as a primary molecular target to bioactivate CAPE to cytotoxic agents [1,14]. We also showed that CAPE was metabolized by tyrosinase to CAPE-quinone, depleted GSH, and selectively toxic towards melanoma cells *in vitro* and *in vivo* [1,14].

In the current work, we have used GST as a second molecular target in melanoma as GST plays an important role in multidrug



**Fig. 6.** Apoptosis assay. One hour of pretreatment of cells with MK-571, a selective MRP inhibitor, and probenecid, a non-selective MRP inhibitor, significantly increased CAPE induced apoptotic cell death. The effect of probenecid was significantly higher than MK-571.

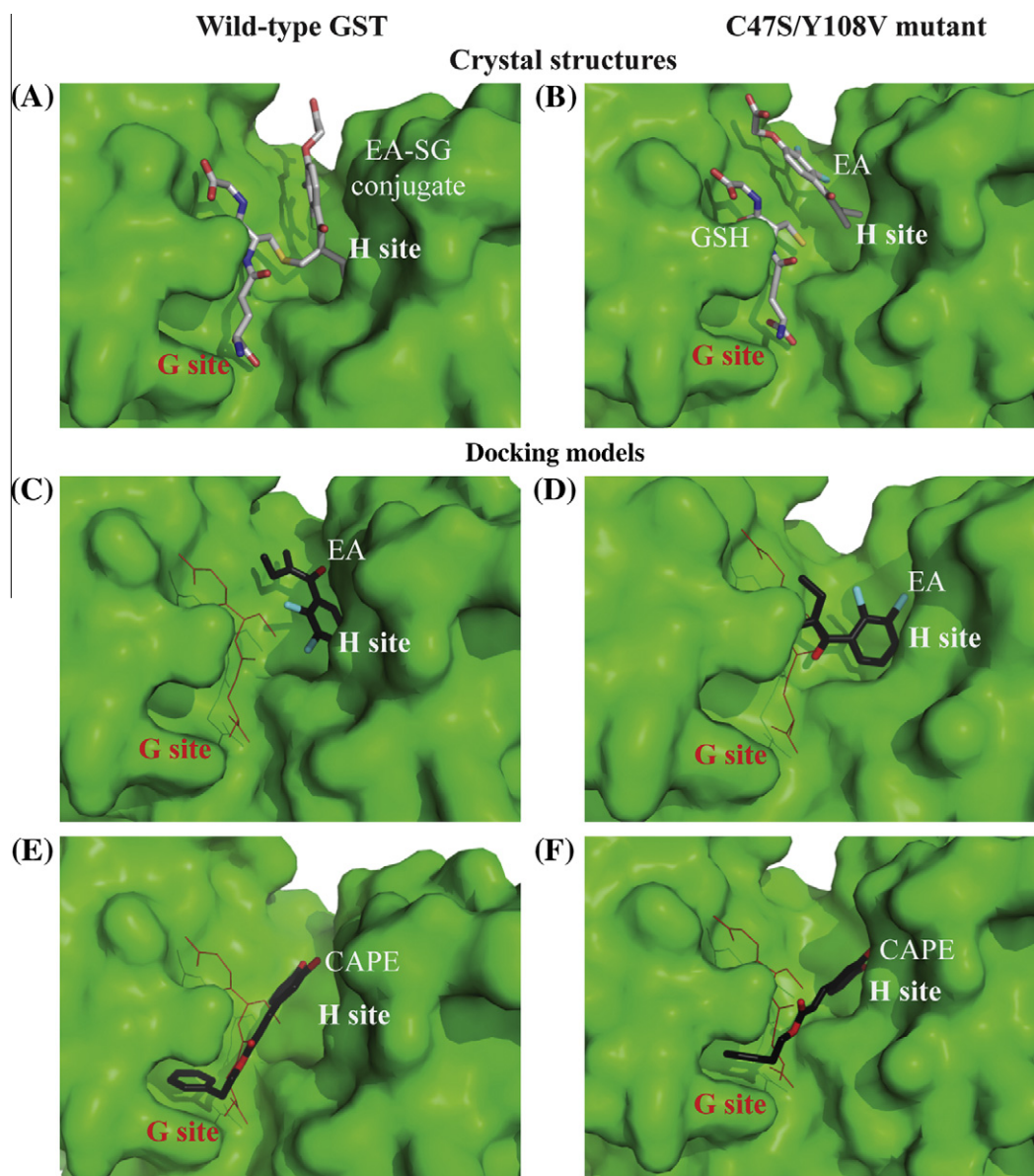


**Fig. 7.** Mitochondrial membrane potential. One hour of cell pretreatment with MK-571, a selective MRP inhibitor, and probenecid, a non-selective MRP inhibitor, significantly decreased mitochondrial membrane potential. The effect of CAPE and probenecid combination was more significant than CAPE in combination with MK-571.

resistance [9,43] due to the over expression in melanoma [2,13]. GST is one of the essential targets in the current development of cancer therapy because of resistance to various anticancer agents [13]. GST also has a vital role in glutathionylation of cellular proteins in cancer, which considered as an important protective mechanism against oxidative stress [44]. GSTP1 enzyme cooperates with multidrug resistance-associated protein (MRP) to protect the melanoma tumors from the anti-melanoma agents. Synergistic effects of both GSTP1 and MRP lead to melanoma drug resistance [9,45]. The important element in the failure of chemotherapy of melanoma is drug resistance [46]. Melanoma is one of the most

aggressive form of skin cancers and resistant to all current modalities of cancer therapy [46]. Among all resistance mechanisms involved in the melanoma therapy, the over expression of GST and MRP may play critical roles [2,13,47].

A study by Laio reported that CAPE has anti-metastatic activity in mouse colon carcinoma CT26 cells [48]. Recently, Chung group showed that CAPE also had inhibitory effects on human hepatocellular carcinoma HepG2 cell line [49]. Grunderger group showed that CAPE exhibited a significantly greater sensitivity to human tumor cell lines than normal cell lines [50]. Our previously reported work also showed that CAPE was an anti-melanoma agent [1,14].



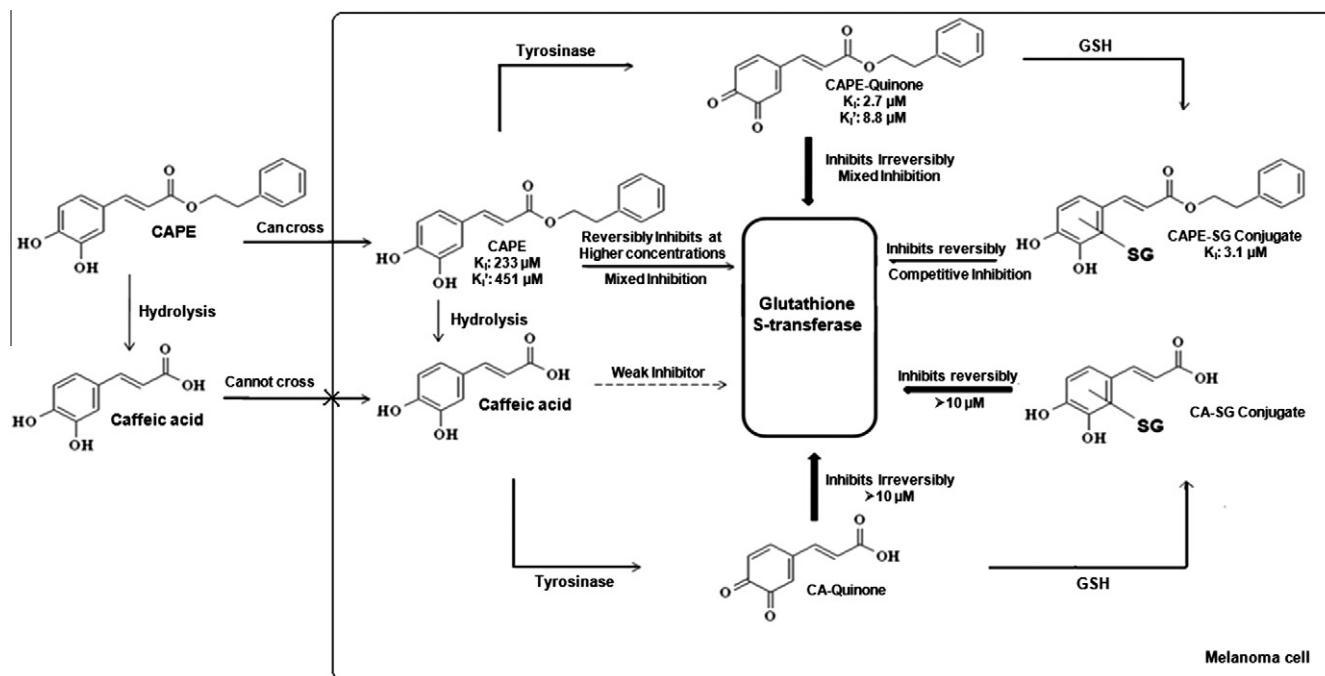
**Fig. 8.** Computational docking of CAPE and EA in the active sites of GST. The computational prediction of the interactions between GST and CAPE or EA was performed as described in Section 2. Sticks represent the ligands. Green surfaces represent the wild-type GST or C47S/Y108V double mutants GST. The GSH-binding site (G site) and the substrate-binding site (H site) are indicated. (A and B) X-ray crystal structures show EA-SG conjugate bound to the wild-type GST (PDB ID 11GS), and EA/GSH to C47S/Y108V mutant (PDB ID 3KM6) [36,37]. (C–F) The predicted bound conformation of EA (C and D) and CAPE (E and F), shown in black sticks, in the active sites of both wild type and C47S/Y108V GST mutant, respectively. The GSH, which was not included in the docking calculations, is present as red line to indicate the G site.

However, none of these studies demonstrated that CAPE selectively inhibits GST in melanoma cells. Moreover, these previous studies did not investigate the role of tyrosinase in the inhibition of GST by CAPE.

The current study is mainly focused on investigating CAPE as a selective GST inhibitor in the presence of tyrosinase. Such a selective inhibitor will be useful in the treatment of melanoma. Our findings indicate that CAPE-SG conjugate ( $\geq 10 \mu\text{M}$ ) and CAPE-quinone ( $\geq 10 \mu\text{M}$ ) significantly inhibit GST whereas CAPE ( $< 50 \mu\text{M}$ ) did not inhibit GST. We have found that CAPE and caffeic acid were weak and reversible inhibitors of GST, whereas CAPE-SG conjugate, CA-SG conjugate [21], CAPE-quinone, and CA-quinone all were potent inhibitors of GST. It was also found that CAPE-SG conjugate was a reversible competitive and mixed GST inhibitor with respect to CDNB and GSH, respectively (Table 1). On the other hand, CAPE-quinone was an irreversible mixed and competitive inhibitor of GST with respect to CDNB and GSH, respectively. For

the first time, we also identified a mono glutathione conjugate of CAPE when CAPE was incubated with tyrosinase and glutathione. Previously, we also reported a mono glutathione conjugate of 4-HA [51] and a mono glutathione conjugate of caffeic acid [52]. Tyrosinase was also investigated as an endogenous substrate of tyrosinase [40]. Our results indicate that all four compounds CAPE, caffeic acid (a hydrolyzed product of CAPE), 4-HA, and tyrosine were substrates for tyrosinase but not for GST. CAPE-SG conjugate and CAPE-quinone inhibited GST significantly, clearly indicating an additional role for tyrosinase in selective inhibition of GST in melanoma cells by intracellularly bioactivating CAPE (Scheme 1). However, among all tested compounds only CAPE demonstrated significant cytotoxic effects towards SK-MEL-28 cells at low concentrations, which could be due to its high lipid solubility (LogP 3.38) and cell membrane permeability.

Literature search on previous works has also shown that glutathione conjugates are involved in inhibition of GST [53,54].



**Scheme 1.** Summary of selective GST inhibition by CAPE in the presence of tyrosinase with respect to CDNB. CAPE can cross biological cell membrane due to its high lipid solubility whereas caffeic acid, an organic anion, cannot cross cell membrane due to its ionization at physiological pH of 7.4 (more than 99.9% is ionized at pH 7.4). CAPE acts as an anti-melanoma agent through tyrosinase prodrug bioactivation to cytotoxic *o*-quinone, which further is conjugated with glutathione to form CAPE-SG conjugate. CAPE-quinone inhibits GST via an irreversible mixed mechanism with respect to CDNB whereas CAPE-SG conjugate inhibits GST via a reversible competitive mechanism with respect to CDNB. CAPE alone at high concentrations (>50 μM) marginally inhibits GST reversibly by a mixed mechanism of inhibition with respect to CDNB. In addition, CAPE can potentially be hydrolyzed to caffeic acid intracellularly, which can be metabolized by tyrosinase to form CA-quinone and CA-SG conjugate to inhibit GST irreversibly and reversibly, respectively.

One recent study revealed that GSH conjugate of doxorubicin inhibited GST activity [53]. Doxorubicin showed an increased cytotoxicity not only towards MDR cells but also doxorubicin sensitive cells suggesting that glutathione conjugates of drugs such as doxorubicin may play a vital role in the GST expression and hence drug induced cytotoxicity. Lyttle group also reported that a number of compounds which are coupled to the thiol group of glutathione showed significant inhibition of GST activity [54]. Tyrosinase catalyzes the metabolism of quercetin to quinone and glutathione adducts [55]. Thilakavathy group reported that the quinone metabolite is more potent in inhibiting GST than quercetin itself [55–57]. Previously, Ploemen et al. [41] also reported that caffeic acid glutathione conjugate as a potent inhibitor of GST. Similarly, our data suggest that the bioactivation of CAPE by tyrosinase leads to the formation of CAPE-quinone, which is a more potent inhibitor of GST than CAPE alone.

In addition, % glutathione consumption mediated by human placenta GST in the absence of tyrosinase was used to investigate if CAPE, CA, 4-HA, and tyrosine were substrates for GST. At 60 min incubation, none of the above compounds found to be a substrate for GST.

Ethacrynic acid (EA) is known as a GST inhibitor [58] because of its  $\alpha,\beta$ -unsaturated carbonyl functional group [30,31]. EA can inhibit cell proliferation at higher concentrations, and is also able to enhance the cytotoxicity of many anticancer agents [59]. Because our data suggested that CAPE acted as a selective GST inhibitor in melanoma cells due to its bioactivation with tyrosinase, the co-incubation of EA with CAPE did not enhance CAPE induced toxicity in SK-MEL-28 melanoma cells, which express tyrosinase (Table 2) [28].

Computational docking of EA into the dimer interface of GST was previously reported and tested experimentally [36]. Our molecular docking studies also showed that CAPE and EA bind to the active site of GST (Fig. 8), suggesting that EA and CAPE may

act as GST inhibitors. The binding modes of both CAPE-quinone and CAPE-SG conjugates may differ from that of the un-conjugated CAPE molecule. These molecular modeling suggest that CAPE and CAPE-SG conjugate may share the same binding sites with EA and EA-SG conjugate on GST.

The GST inhibition results suggested that the nature of GST inhibition by CAPE and CAPE-quinone are mixed whereas the nature of GST inhibition by CAPE-SG and EA are competitive with respect to CDNB (0.2–1 mM) (Fig. 4A–D and Table 1). With respect to GSH, the nature of GST inhibition by CAPE and CAPE-SG is different from ethacrynic acid (EA) and CAPE-quinone. GST inhibition studies at 0.2–1 mM concentration of GSH (Fig. 4E–H) suggested that CAPE and CAPE-SG are mixed inhibitors of GST with respect to GSH whereas CAPE-quinone and EA are competitive and non-competitive inhibitors of GST with respect to GSH, respectively. Awasthi et al. has suggested that EA is a non-competitive inhibitor of GST with K<sub>i</sub> of 11.5 μM whereas EA-SG conjugate is a competitive inhibitor of GST with K<sub>i</sub> of 1.5 μM with respect to both CDNB and GSH [38].

The over-expression of GST is not always conferring a significant protection from the anticancer agents. GST must be co-expressed with MRPs to protect cells from anticancer agents [8,9,60]. It was previously reported that the detoxification of anticancer agents is a combined effect of both GSTs and MRPs [8,12]. This study also investigated the role of MRP in CAPE induced toxicity in human SK-MEL-28 melanoma cells. Our results showed that CAPE induced toxicity in SK-MEL-28 cells are enhanced by BSO, a glutathione biosynthesis inhibitor [61] (data not shown), and MK-571, a selective MRP inhibitor [17] and probenecid, a non-selective MRP inhibitor [18]. An increase in toxicity of CAPE in the presence of BSO in SK-MEL-28 sounds reasonable because glutathione depletion plays a vital role in CAPE toxicity towards melanoma cells as reported previously [1,14]. Most anticancer agents become resistant due to high levels of GST and high levels

of glutathione in tumor cells, which makes anti-neoplastic agents inactive. Melanoma is one of the most chemo-resistant cancers, which expresses high levels of GST [2,13] and high levels of MRP [13]. MK-571, a selective MRP inhibitor [17], and probenecid, a non-selective MRP inhibitor [18], increased CAPE induced cell toxicity, apoptosis, and significantly decreased mitochondrial membrane potential in SK-MEL-28 melanoma cells when co-incubated with CAPE. However, the effect of probenecid, a non-selective MRP inhibitor, was more significant than MK-571, a selective MRP inhibitor, suggesting other transporters may also play a role in CAPE induced toxicity. Previous literature also suggested that GST and MRP can act in synergy to protect cancer cells from toxicity of anticancer agents [62].

In summary, for the first time, we showed that CAPE acts as a selective inhibitor of GST in the presence of tyrosinase. Our investigation describes tyrosinase-catalyzed activation of CAPE to a quinone intermediate that reacts spontaneously with GSH to form a CAPE-SG conjugate that competitively and reversibly inhibits GST with respect to CDNB, while CAPE alone is not inhibitory. The CAPE quinone also irreversibly inhibits GST in the absence of GSH, likely by inactivation via alkylation of a key cysteine residue on GST. The irreversible inhibition by CAPE of GST was shown in cell lysates, but not in intact cells, which contain GSH in mM levels [63]. Similar results were found with caffeic acid, albeit with less potent dose-response, and in agreement with results with caffeic acid reported by Ploemen et al. [41]. GST and MRP play vital roles in the protection of cancer cells against cancer therapy. The toxicity of CAPE was enhanced by MK-571, a selective MRP inhibitor, and probenecid, a non-specific MRP inhibitor, suggesting MRP as a CAPE resistance factor, presumably via efflux of the CAPE-SG conjugate.

Because tyrosinase is expressed in melanoma, this may allow selective inhibition of GST as a secondary target in melanoma cells compared to non-melanoma cells that do not express tyrosinase, in addition to the direct toxicity of CAPE due to the selective activation and cytotoxicity of the CAPE quinone. Therefore, bioactivated CAPE and its GSH conjugates selectively inhibit GST in the presence of tyrosinase. It is expected that these metabolites selectively inhibit GST in melanoma cells as they contain tyrosinase. Here, the selective inhibition refers to selective inhibition of GST in melanoma cells versus non-melanoma tissue or cells. In addition to selective GST inhibition in melanoma cells, CAPE and its derivatives may also mediate a variety of biologically and toxicologically relevant processes and reactions, which were not investigated in this study.

Our study suggests that tyrosinase plays a major role in the bioactivation of CAPE, which leads to significant and selective inhibition of GST in human SK-MEL-28 melanoma cells. It also suggests a role for MRP in the biochemical mechanism of CAPE induced toxicity in melanoma cell line.

### Financial support

The work was supported by NCI/NIH 1R15CA122044-01A (to M.M.); National Institutes of Health Grants R21HL087895 and R01GM095538 (to L.G.) and Texas Norman Hackerman Advanced Research Program 010674-0034-2009 (to L.G.).

### Conflict of interest statement

The authors state no conflict of interest.

### Acknowledgements

The work was supported by NCI/NIH, 1R15CA122044-01A1 (to M.M.). The work was also partially supported by National Institutes of Health Grants R21HL087895 and R01GM095538 (to L.G.) and

Texas Norman Hackerman Advanced Research Program 010674-0034-2009 (to L.G.). L.G. and M.S.Y. performed the computational docking and prepared the related figure and text. H.T. helped with LC-MS/MS experiments.

### References

- [1] S.K. Kudugunti, N.M. Vad, E. Ekogbo, M.Y. Moridani, Efficacy of caffeic acid phenethyl ester (CAPE) in skin B16-F0 melanoma tumor bearing C57BL/6 mice, *Invest. New Drugs* 29 (1) (2009) 52–62.
- [2] D. Schandorf, A. Makki, C. Stahr, A. van Dyck, R. Wanner, G.L. Scheffer, M.J. Flens, R. Scheper, B.M. Henz, Membrane transport proteins associated with drug resistance expressed in human melanoma, *Am. J. Pathol.* 147 (6) (1995) 1545–1552.
- [3] B.D. Banerjee, V. Seth, A. Bhattacharya, S.T. Pasha, A.K. Chakraborty, Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers, *Toxicol. Lett.* 107 (1–3) (1999) 33–47.
- [4] A. Sau, F. Pellizzari Tregno, F. Valentino, G. Federici, A.M. Caccuri, Glutathione transferases and development of new principles to overcome drug resistance, *Arch. Biochem. Biophys.* 500 (2) (2010) 116–122.
- [5] S. Mena, A. Ortega, J.M. Estrela, Oxidative stress in environmental-induced carcinogenesis, *Mutat. Res.* 674 (1–2) (2009) 36–44.
- [6] K. Johansson, K. Ahlen, R. Rinaldi, K. Sahlander, A. Siritantikorn, R. Morgenstern, Microsomal glutathione transferase 1 in anticancer drug resistance, *Carcinogenesis* 28 (2) (2007) 465–470.
- [7] R. Pastila, D. Leszczynski, Ultraviolet-A radiation induces changes in cyclin G gene expression in mouse melanoma B16-F1 cells, *Cancer Cell Int.* 7 (2007) 7.
- [8] C.S. Morrow, P.K. Smitherman, S.K. Diah, E. Schneider, A.J. Townsend, Coordinated action of glutathione S-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1- and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells, *J. Biol. Chem.* 273 (32) (1998) 20114–20120.
- [9] P. Depeille, P. Cuq, I. Passagne, A. Evrard, L. Vian, Combined effects of GSTP1 and MRP1 in melanoma drug resistance, *Br. J. Cancer* 93 (2) (2005) 216–223.
- [10] S.P. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line, *Science* 258 (5088) (1992) 1650–1654.
- [11] P. Borst, R. Evers, M. Kool, J. Wijnholds, The multidrug resistance protein family, *Biochim. Biophys. Acta* 1461 (2) (1999) 347–357.
- [12] M. O'Brien, G.D. Kruh, K.D. Tew, The influence of coordinate overexpression of glutathione phase II detoxification gene products on drug resistance, *J. Pharmacol. Exp. Ther.* 294 (2) (2000) 480–487.
- [13] A. Moral, J. Palou, A. Lafuente, R. Molina, J. Piulachs, T. Castel, M. Trias, Immunohistochemical study of alpha, mu and pi class glutathione S transferase expression in malignant melanoma. MMM Group. Multidisciplinary Malignant Melanoma Group, *Br. J. Dermatol.* 136 (3) (1997) 345–350.
- [14] S.K. Kudugunti, N.M. Vad, A.J. Whiteside, B.U. Naik, M.A. Yusuf, K.S. Srivenugopal, M.Y. Moridani, Biochemical mechanism of caffeic acid phenylethyl ester (CAPE) selective toxicity towards melanoma cell lines, *Chem. Biol. Interact.* 188 (1) (2010) 1–14.
- [15] Y.M. Chen, W. Chavin, Tyrosinase activity in a highly pigmented human melanoma and in Negro skin, *Proc. Soc. Exp. Biol. Med.* 145 (2) (1974) 695–698.
- [16] L. Guarini, Z.Z. Su, S. Zucker, J. Lin, D. Grunberger, P.B. Fisher, Growth inhibition and modulation of antigenic phenotype in human melanoma and glioblastoma multiforme cells by caffeic acid phenethyl ester (CAPE), *Cell Mol. Biol.* 38 (5) (1992) 513–527.
- [17] J. Sun, S. Usune, Y. Zhao, K. Migita, T. Katsuragi, Multidrug resistance protein transporter and Ins(1,4,5)P<sub>3</sub>-sensitive Ca<sup>2+</sup>-signaling involved in adenosine triphosphate export via Gq protein-coupled NK2-receptor stimulation with neurokinin A, *J. Pharmacol. Sci.* 114 (1) (2010) 92–98.
- [18] E. Vamos, K. Voros, D. Zadori, L. Vecsei, P. Klivenyi, Neuroprotective effects of probenecid in a transgenic animal model of Huntington's disease, *J. Neural. Transm.* 116 (9) (2009) 1079–1086.
- [19] K. Asaoka, Affinity purification and characterization of glutathione S-transferases from bovine liver, *J. Biochem.* 95 (3) (1984) 685–696.
- [20] C.Y. Lee, Multiple forms of mouse glutathione S-transferases, *Biochem. Soc. Trans.* 12 (1) (1984) 30–33.
- [21] M.Y. Moridani, H. Scobie, P.J. O'Brien, Metabolism of caffeic acid by isolated rat hepatocytes and subcellular fractions, *Toxicol. Lett.* 133 (2–3) (2002) 141–151.
- [22] D. Gergel, A.I. Cederbaum, Interaction of nitric oxide with 2-thio-5-nitrobenzoic acid: implications for the determination of free sulfhydryl groups by Ellman's reagent, *Arch. Biochem. Biophys.* 347 (2) (1997) 282–288.
- [23] M.Y. Moridani, M. Moore, R.A. Bartsch, Y. Yang, S. Heibati-Sadati, Structural toxicity relationship of 4-alkoxyphenols' cytotoxicity towards murine B16-F0 melanoma cell line, *J. Pharm. Pharm. Sci.* 8 (2) (2005) 348–360.
- [24] G. Tuna, G.K. Erkmen, O. Dalmizrak, A. Dogan, I.H. Ogus, N. Ozer, Inhibition characteristics of hypericin on rat small intestine glutathione-S-transferases, *Chem. Biol. Interact.* 188 (1) (2010) 59–65.
- [25] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, *J. Biol. Chem.* 249 (22) (1974) 7130–7139.

- [26] S. Oetari, M. Sudibyo, J.N. Commandeur, R. Samhoedi, N.P. Vermeulen, Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver, *Biochem. Pharmacol.* 51 (1) (1996) 39–45.
- [27] Y.R. Pokharel, E.H. Han, J.Y. Kim, S.J. Oh, S.K. Kim, E.R. Woo, H.G. Jeong, K.W. Kang, Potent protective effect of isoimperatorin against aflatoxin B1-inducible cytotoxicity in H4IIE cells: bifunctional effects on glutathione S-transferase and CYP1A, *Carcinogenesis* 27 (12) (2006) 2483–2490.
- [28] N.M. Vad, G. Yount, D. Moore, J. Weidanz, M.Y. Moridani, Biochemical mechanism of acetaminophen (APAP) induced toxicity in melanoma cell lines, *J. Pharm. Sci.* 98 (4) (2009) 1409–1425.
- [29] N.M. Vad, S.K. Kudugunti, D. Graber, N. Bailey, K. Srivenugopal, M.Y. Moridani, Efficacy of acetaminophen in skin B16-F0 melanoma tumor-bearing C57BL/6 mice, *Int. J. Oncol.* 35 (1) (2009) 193–204.
- [30] K.D. Tew, S. Dutta, M. Schultz, Inhibitors of glutathione S-transferases as therapeutic agents, *Adv. Drug Deliv. Rev.* 26 (2–3) (1997) 91–104.
- [31] M.L. Iersel, J.P. Ploemen, I. Struik, C. van Amersfoort, A.E. Keyzer, J.G. Schefferlie, P.J. van Bladeren, Inhibition of glutathione S-transferase activity in human melanoma cells by alpha,beta-unsaturated carbonyl derivatives. Effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and trans-2-hexenal, *Chem. Biol. Interact.* 102 (2) (1996) 117–132.
- [32] X.J. Shang, G. Yao, J.P. Ge, Y. Sun, W.H. Teng, Y.F. Huang, Procyandin induces apoptosis and necrosis of prostate cancer cell line PC-3 in a mitochondrion-dependent manner, *J. Androl.* 30 (2) (2009) 122–126.
- [33] A. Wong, L. Cavellier, H.E. Collins-Schramm, M.F. Seldin, M. McGrogan, M.L. Savontaus, G.A. Cortopassi, Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells, *Hum. Mol. Genet.* 11 (4) (2002) 431–438.
- [34] G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function, *J. Comput. Chem.* 19 (1998) 1639–1662.
- [35] M.F.S. Sanner, The Scripps Python: a programming language for software integration and development, *J. Mol. Graphics Mod.* 17 (1999) 57–61.
- [36] I. Quesada-Soriano, L.J. Parker, A. Primavera, J. Wielens, J.K. Holien, J.M. Casas-Solvas, A. Vargas-Berenguel, A.M. Aguilera, M. Nuccetelli, A.P. Mazzetti, M.L. Bello, M.W. Parker, L. Garcia-Fuentes, Diuretic drug binding to human glutathione transferase P1-1: potential role of Cys-101 revealed in the double mutant C47S/Y108V, *J. Mol. Recognit.* (2010).
- [37] A.J. Oakley, M. Lo Bello, A.P. Mazzetti, G. Federici, M.W. Parker, The glutathione conjugate of ethacrynic acid can bind to human pi class glutathione transferase P1-1 in two different modes, *FEBS Lett.* 419 (1) (1997) 32–36.
- [38] S. Awasthi, S.K. Srivastava, F. Ahmad, H. Ahmad, G.A. Ansari, Interactions of glutathione S-transferase-pi with ethacrynic acid and its glutathione conjugate, *Biochim. Biophys. Acta* 1164 (2) (1993) 173–178.
- [39] M.Y. Moridani, Biochemical basis of 4-hydroxyanisole induced cell toxicity towards B16-F0 melanoma cells, *Cancer Lett.* 243 (2) (2006) 235–245.
- [40] J. Choi, S.J. Bae, Y.M. Ha, J.K. No, E.K. Lee, J.S. Lee, S. Song, H. Lee, H. Suh, B.P. Yu, H.Y. Chung, A newly synthesized, potent tyrosinase inhibitor: 5-(6-hydroxy-2-naphthyl)-1,2,3-benzenetriol, *Bioorg. Med. Chem. Lett.* 20 (16) (2010) 4882–4884.
- [41] J.H. Ploemen, B. van Ommen, A. de Haan, J.G. Schefferlie, P.J. van Bladeren, In vitro and in vivo reversible and irreversible inhibition of rat glutathione S-transferase isoenzymes by caffeic acid and its 2-S-glutathionyl conjugate, *Food Chem. Toxicol.* 31 (7) (1993) 475–482.
- [42] N. Celli, L.K. Dragani, S. Murzilli, T. Pagliani, A. Poggi, In vitro and in vivo stability of caffeic acid phenethyl ester, a bioactive compound of propolis, *J. Agric. Food Chem.* 55 (9) (2007) 3398–3407.
- [43] S.C. McNeely, A.C. Belshoff, B.F. Taylor, T.W. Fan, M.J. McCabe Jr., A.R. Pinhas, J.C. States, Sensitivity to sodium arsenite in human melanoma cells depends upon susceptibility to arsenite-induced mitotic arrest, *Toxicol. Appl. Pharmacol.* 229 (2) (2008) 252–261.
- [44] D.M. Townsend, Y. Manevich, L. He, S. Hutchens, C.J. Pazoles, K.D. Tew, Novel role for glutathione S-transferase pi. Regulator of protein S-glutathionylation following oxidative and nitrosative stress, *J. Biol. Chem.* 284 (1) (2009) 436–445.
- [45] Y.C. Awasthi, R. Sharma, S. Yadav, S. Dwivedi, A. Sharma, S. Awasthi, The non-ABC drug transporter RLIP76 (RALBP-1) plays a major role in the mechanisms of drug resistance, *Curr. Drug Metab.* 8 (4) (2007) 315–323.
- [46] H. Helmbach, E. Rossmann, M.A. Kern, D. Schadendorf, Drug-resistance in human melanoma, *Int. J. Cancer* 93 (5) (2001) 617–622.
- [47] L. Serrone, P. Hersey, The chemoresistance of human malignant melanoma: an update, *Melanoma Res.* 9 (1) (1999) 51–58.
- [48] H.F. Liao, Y.Y. Chen, J.J. Liu, M.L. Hsu, H.J. Shieh, H.J. Liao, C.J. Shieh, M.S. Shiao, Y.J. Chen, Inhibitory effect of caffeic acid phenethyl ester on angiogenesis, tumor invasion, and metastasis, *J. Agric. Food Chem.* 51 (27) (2003) 7907–7912.
- [49] T.W. Chung, S.K. Moon, Y.C. Chang, J.H. Ko, Y.C. Lee, G. Cho, S.H. Kim, J.G. Kim, C.H. Kim, Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism, *FASEB J.* 18 (14) (2004) 1670–1681.
- [50] D. Grunberger, R. Banerjee, K. Eisinger, E.M. Oltz, L. Efron, M. Caldwell, V. Estevez, K. Nakanishi, Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis, *Experientia* 44 (3) (1988) 230–232.
- [51] M.Y. Moridani, H. Scobie, A. Jamshidzadeh, P. Salehi, P.J. O'Brien, Caffeic acid, chlorogenic acid, and dihydrocaffeic acid metabolism: glutathione conjugate formation. Metabolic activation of 4-hydroxyanisole by isolated rat hepatocytes, *Drug Metab. Dispos.* 29 (11) (2001) 1432–1439.
- [52] J. Choi, S.J. Bae, M. Ha, J.K. No, E.K. Lee, J.S. Lee, S. Song, H. Lee, H. Suh, B.P. Yu, H.Y. Chung, A newly synthesized, potent tyrosinase inhibitor: 5-(6-hydroxy-2-naphthyl)-1,2,3-benzenetriol, *Bioorg. Med. Chem. Lett.* 20 (16) (2010) 4882–4884.
- [53] T. Asakura, A. Sasagawa, H. Takeuchi, S. Shibata, H. Marushima, S. Mamori, K. Ohkawa, Conformational change in the active center region of GST P1-1, due to binding of a synthetic conjugate of DXR with GSH, enhanced JNK-mediated apoptosis, *Apoptosis* 12 (7) (2007) 1269–1280.
- [54] M.H. Lyttle, M.D. Hocker, H.C. Hui, C.G. Caldwell, D.T. Aaron, A. Engqvist-Goldstein, J.E. Flatgaard, K.E. Bauer, Isozyme-specific glutathione-S-transferase inhibitors: design and synthesis, *J. Med. Chem.* 37 (1) (1994) 189–194.
- [55] T. Thangasamy, S. Sittadjody, S. Lanza-Jacoby, P.R. Wachsberger, K.H. Limesand, R. Burd, Quercetin selectively inhibits bioreduction and enhances apoptosis in melanoma cells that overexpress tyrosinase, *Nutr. Cancer* 59 (2) (2007) 258–268.
- [56] J.J. van Zanden, O. Ben Hamman, M.L. van Iersel, S. Boeren, N.H. Cnubben, M. Lo Bello, J. Vervoort, P.J. van Bladeren, I.M. Rietjens, Inhibition of human glutathione S-transferase P1-1 by the flavonoid quercetin, *Chem. Biol. Interact.* 145 (2) (2003) 139–148.
- [57] M. Kurata, M. Suzuki, K. Takeda, Effects of phenol compounds, glutathione analogues and a diuretic drug on glutathione S-transferase, glutathione reductase and glutathione peroxidase from canine erythrocytes, *Comp. Biochem. Physiol. B* 103 (4) (1992) 863–867.
- [58] F.J. Sanchez-Gomez, B. Diez-Dacal, M.A. Pajares, O. Llorca, D. Perez-Sala, Cyclopentenone prostaglandins with dienone structure promote cross-linking of the chemoresistance-inducing enzyme glutathione S-transferase P1-1, *Mol. Pharmacol.* 78 (4) (2010) 723–733.
- [59] S. Aizawa, K. Ookawa, T. Kudo, J. Asano, M. Hayakari, S. Tsuchida, Characterization of cell death induced by ethacrynic acid in a human colon cancer cell line DLD-1 and suppression by N-acetyl-L-cysteine, *Cancer Sci.* 94 (10) (2003) 886–893.
- [60] A. Harbottle, A.K. Daly, K. Atherton, F.C. Campbell, Role of glutathione S-transferase P1, P-glycoprotein and multidrug resistance-associated protein 1 in acquired doxorubicin resistance, *Int. J. Cancer* 92 (6) (2001) 777–783.
- [61] P. Horak, E. Sild, U. Soomets, T. Sepp, K. Kilk, Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches, *J. Exp. Biol.* 213 (Pt 13) (2010) 2225–2233.
- [62] P. Depeille, P. Cuq, S. Mary, I. Passagne, A. Evrard, D. Cupissol, L. Vian, Glutathione S-transferase M1 and multidrug resistance protein 1 act in synergy to protect melanoma cells from vincristine effects, *Mol. Pharmacol.* 65 (4) (2004) 897–905.
- [63] A. Ortega, J. Carretero, E. Obrador, J.M. Estrela, Tumoricidal activity of endothelium-derived NO and the survival of metastatic cells with high GSH and Bcl-2 levels, *Nitric Oxide* 19 (2) (2008) 107–114.