

DMD#13987

## **Variable sulfation of dietary polyphenols by recombinant human sulfotransferase (SULT)**

### **1A1 genetic variants and SULT1E1**

Din Ung and Swati Nagar

Department of Pharmaceutical Sciences, Temple University School of Pharmacy,

3307 North Broad Street, Philadelphia PA 19140

DMD#13987

**Running title:** Sulfation of dietary polyphenols by SULT1A1 and SULT1E1

**Corresponding author:** Swati Nagar

Temple University School of Pharmacy

3307 North Broad Street

Philadelphia PA 19140

Telephone: (215) 707 9110

FAX: (215) 707 3678

Email: [swati.nagar@temple.edu](mailto:swati.nagar@temple.edu)

Number of text pages: 29

Number of tables: 1

Number of figures: 5

Number of references: 39

Number of words in

Abstract: 198

Introduction: 662

Discussion: 1262

List of non-standard abbreviations:

SULT: sulfotransferase

SNP: single nucleotide polymorphism

UGT: uridine diphosphoglucuronosyltransferase

PAPS: 3'-phosphoadenosine 5'-phosphosulfate

PCR: polymerase chain reaction

DMD#13987

MRP2: multi-drug resistance protein 2

BCRP: breast cancer resistance protein

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

DMD#13987

## Abstract

Human cytosolic sulfotransferases (SULTs) catalyze the sulfate conjugation of several important endo- and xenobiotics. Among the superfamily of SULT enzymes, SULT1A1 catalyzes the sulfation of small planar phenolic compounds, whereas SULT1E1 has a major role in estrogen conjugation. The human SULT1A1 gene has common single nucleotide polymorphisms (SNPs) which define three allozymes, SULT1A1\*1, \*2, and \*3. The enzyme kinetics of SULT1A1 allozymes and SULT1E1 were characterized for the polyphenolic substrates apigenin, chrysin, epicatechin, quercetin, and resveratrol. Purified recombinant SULT proteins were generated in a baculoviral-insect cell system, and incubated *in vitro* with each substrate to determine catalytic activity. The effect of polyphenol sulfation was examined in mammalian cell lines stably expressing SULT1E1. For all polyphenols investigated: ‘normal activity’ SULT1A1\*1 allozyme had significantly greater  $V_{\max}$  estimates than SULT1E1, and allele-specific differences in SULT1A1-mediated sulfation were observed. The polymorphic SULT1A1\*2 allozyme exhibited low activity toward apigenin, epicatechin, and resveratrol. SULT1A1\*1 and \*3 acted as ‘normal-activity’ allozymes for these substrates. Altered cellular proliferation was observed in MCF-7 cells stably expressing SULT1E1 upon treatment with chrysin, quercetin, or resveratrol, thus suggesting inactivation of these compounds by SULT1E1. These results suggest an important role for SULT isozymes and their pharmacogenetics in polyphenol disposition.

DMD#13987

Human cytosolic sulfotransferases (SULTs) catalyze the conjugation of several endogenous substrates such as hormones and neurotransmitters, and xenobiotics such as therapeutic drugs, dietary carcinogens, and dietary polyphenols (Coughtrie, 2002). Among the various SULT family members, human SULT1A1 catalyzes the sulfation of small phenolic compounds, and SULT1E1 is the major isozyme responsible for the sulfation of estrogens such as estrone and  $\beta$ -estradiol (Gamage et al., 2006). Common single nucleotide polymorphisms (SNPs) have been reported in the human SULT1A1 gene (Jones et al., 1995; Raftogianis et al., 1996). The common SULT1A1 allozymes include \*1, the \*2 variant defined by an Arg213His amino acid change due to a conversion from G to A at nucleotide 638, and the \*3 variant with an A to G conversion at nucleotide 667, defined by a Met223Val change. These SNPs lead to changes in thermal stability, enzymatic activity and altered degradation of the allozymes (Raftogianis et al., 1997; Raftogianis et al., 1999; Nagar et al., 2006). Recently, mutations in the SULT1E1 gene have also been reported; however, the mutation frequencies in the population studied were very low (Adjei et al., 2003). The possible association between SULT1A1 pharmacogenetics (SULT1A1 ‘normal activity’ \*1 and ‘low activity’ \*2 alleles) and cancer has been evaluated in several studies (Seth et al., 2000; Bamber et al., 2001; Zheng et al., 2001; Wang et al., 2002; Tang et al., 2003; Wu et al., 2003; Choi et al., 2005; Nowell et al., 2005; Shatalova et al., 2005). These studies provide epidemiological evidence for associations among diet, genetics, and cancer risk. Thus, there is a need for direct correlation studies among phase II enzyme pharmacogenetics, dietary carcinogen/chemopreventive intake, and altered *in vivo* phenotype.

The therapeutic potential of several anti-oxidant chemopreventive dietary polyphenols has generated renewed interest in these compounds (Baur and Sinclair, 2006; Wen and Walle, 2006).

DMD#13987

Flavonoids are a subclass of polyphenols, and different types of flavonoids are found in various food sources such as tea, grapes, green leafy vegetables and soy foods (Beecher, 2003; Manach et al., 2004). Dietary polyphenols have anti-oxidant properties and some inhibit angiogenesis and cancer cell proliferation *in vitro* (Brownson et al., 2002). Many polyphenols are conjugated in the human body to their sulfate and glucuronide conjugates, by the sulfotransferase (SULT) and uridine diphosphoglucuronosyltransferase (UGT) enzyme superfamilies, respectively (Pai et al., 2001; Manach et al., 2004). Sulfation of flavonoids and non-flavonoid polyphenols by human cytosolic SULT isozymes has been described in previous reports (Pai et al., 2001; Harris et al., 2004). It has been suggested that dietary polyphenols alter the bioavailability of endogenous estrogens by competing as substrates and/or inhibitors for human SULT1E1 and SULT1A1 isozymes, thus possibly contributing to variable breast cancer risk (Harris et al., 2004). Hepatic and extrahepatic conjugation of polyphenols such as resveratrol is thought to contribute to their low oral bioavailability (Walle et al., 2004). Resveratrol has recently received much attention for its chemopreventive and numerous other health benefits despite its low bioavailability (Baur et al., 2006; Baur and Sinclair, 2006).

Several studies have identified the potential of dietary polyphenols as chemopreventives, and the importance of their conjugation in decreased bioavailability. However, it remains to be determined how phase II metabolizing enzyme pharmacogenetics alters polyphenol disposition. We have previously characterized the *in vitro* enzyme kinetics of SULT1A1-mediated sulfation for the flavonoids chrysin, genistein, and quercetin (Nagar et al., 2006). In the previous study, we further characterized the SULT1A1\*1, \*2 or \*3 allozyme-dependent variation in sulfation of these flavonoids. The purpose of this study is to extend our understanding of polyphenol

DMD#13987

sulfation and the cellular effect of this process. We hypothesize that both SULT1A1 and SULT1E1 play important roles in the conjugation of numerous dietary polyphenols. We additionally hypothesize that SULT1A1 pharmacogenetics explains variable sulfation of these polyphenols. We therefore characterized in detail the enzyme kinetics of SULT1A1- (\*1, \*2, and \*3 allozymes) and SULT1E1-mediated sulfation of the following flavonoids: flavones (apigenin, chrysin) and flavonols (epicatechin, quercetin). Additionally, the sulfation of the non-flavonoid polyphenol resveratrol was evaluated.

DMD#13987

## **Materials and Methods**

### **Chemicals and reagents**

Apigenin, chrysin, quercetin dihydrate, (–)-epicatechin, and resveratrol were obtained from Sigma (St. Louis, MO). The co-substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS) had previously been purchased from Dr. H. Glatt (German Institute of Human Nutrition, Nuthetal, Germany). Radiolabeled <sup>35</sup>S-PAPS was obtained from PerkinElmer Life And Analytical Sciences, Inc. (Boston, MA). Analytical grade chemicals were used to prepare buffers and other reagents. Sf-9 cells and related media were obtained from Invitrogen (Carlsbad, CA). MCF-7 cells were obtained from ATCC (Manassas, VA) and its media was obtained from Mediatech, Inc. (Herndon, VA). Gibco RPMI Media 1640 for cell proliferation assays (with *L*-glutamine, without phenol red) was obtained from Invitrogen (Carlsbad, CA). All other cell culture reagents were purchased through Fisher Scientific International, Inc. (Hampton, NH).

### **Protein purification of recombinant SULT1A1 (\*1, \*2, and \*3) and SULT1E1**

Recombinant 6X-Histidine-tagged SULT1A1\*1, \*2, \*3, and SULT1E1 proteins were expressed in a baculoviral-insect cell system. SULT1A1\*1, \*2, \*3, and SULT1E1 constructs were cloned into the baculovirus expression vector pBLUEBac-His2A and cotransfected with 1 µg of BacVector-3000 viral DNA through liposome-mediated transfection into Sf-9 insect cells as previously described (Raftogianis et al., 1999; Nagar et al., 2006). Stocks of viral 6X-His-tagged SULT cDNA were used to infect Sf-9 cell cultures at 28°C for 48 hours and were harvested for protein purification. Bradford assays were performed on the purified proteins to determine their concentrations. Further analysis to verify the purity of the proteins included SDS-PAGE and Western blot with anti-SULT1A1 or anti-SULT1E1 antibodies. The purified proteins resulted in



DMD#13987

a single SULT1A1 or SULT1E1 band for each sample in Western blot analyses. Aliquots of purified protein were stored at  $-80^{\circ}\text{C}$  for future use.

### **Radiometric SULT assays**

The purified recombinant SULT proteins were characterized for catalytic activity toward the flavonoids and resveratrol with a radiometric assay (Anderson and Weinshilboum, 1980; Varin et al., 1987; Geese and Raftogianis, 2001). Initially, the conditions for linearity of protein amount and time were optimized. The reaction components were purified protein, substrate (apigenin, chrysin, quercetin dihydrate, (–)-epicatechin, or resveratrol) and co-substrate  $^{35}\text{S}$ -PAPS contained in 0.2-ml thin-wall PCR tubes with a final volume of 30  $\mu\text{l}$ . The reaction buffer was 10 mM potassium phosphate buffer pH 6.5. The duration of the incubations varied from 15–30 minutes at  $37^{\circ}\text{C}$ . The substrate concentrations ranged from 0.1  $\mu\text{M}$ –3000  $\mu\text{M}$ . The substrates were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 3.3% in all incubations. The range of total protein tested was 0.2  $\mu\text{g}$  to 2  $\mu\text{g}$ . The reactions were initiated by the addition of co-substrate 10  $\mu\text{M}$  PAPS ( $^{35}\text{S}$ -PAPS + cold PAPS in the ratio 1:9 v/v). Preliminary experiments were conducted with 10 – 30  $\mu\text{M}$  PAPS. Ten  $\mu\text{M}$  PAPS was found to be sufficiently high and was not depleted at the highest substrate concentrations studied. The reactions were quenched with the addition of 3  $\mu\text{l}$  2.5% aqueous glacial acetic acid, 6  $\mu\text{l}$  fresh 0.1 M tetrabutylammonium phosphate monobasic, and 150  $\mu\text{l}$  ethyl acetate. Vortexing and centrifugation at 1800 *g* for 3 min of the tubes resulted in separate aqueous and organic layers. To quantitate the reaction products, 100  $\mu\text{l}$  of the top organic layer was added to scintillation fluid for liquid scintillation counting. All incubations were carried out in triplicate, with appropriate negative controls (no substrate).

DMD#13987

### **Data analysis for enzyme kinetics**

Initially, a broad range of substrate concentrations was used to determine inhibitory substrate concentration. When inhibition was observed, the substrate inhibition model was utilized to try and obtain  $K_i$  estimates (Nagar et al., 2006). If the substrate inhibition equation failed to fit the observed data, the Michaelis-Menten equation was fit to a narrow range of substrate concentrations; parameter estimates were obtained by the following equation used to fit the data:

$$v = V_{\max} * [S] / (K_m + [S])$$

where  $v$  is the rate of the reaction,  $V_{\max}$  is the maximum velocity estimate,  $[S]$  is the substrate concentration, and  $K_m$  is the Michaelis-Menten constant. Prior to non-linear regression analysis, all data were transformed and Eadie-Hofstee curves plotted; the Michaelis-Menten model was fit only to data exhibiting linear Eadie-Hofstee plots. Non-linear regression was performed with GraphPad Prism® version 4.03 for Windows (GraphPad Software, Inc., San Diego, CA). A statistical comparison of the parameter estimates was performed using a two-sided  $t$ -test assuming normal distribution, where a  $p$  value less than 0.01 was deemed significant (Nagar et al., 2004).

### **Proliferative response of MCF-7 cells stably expressing SULT1E1 to polyphenols**

Generation of MCF-7 cells stably expressing SULT1A1\*1, \*2, or \*3 has been previously described (Nagar et al., 2006). MCF-7 cells stably expressing SULT1E1 were similarly generated. Multiple clones were isolated and expanded. Clones were characterized with Southern, Northern and Western blot analyses, and three SULT1E1 clones (#1, #10, and #22) were found to express comparable RNA as the cell lines expressing SULT1A1. Thus, these three

DMD#13987

clones were evaluated in subsequent studies. MCF-7 cells stably expressing SULT1E1 were treated with polyphenols (0–100  $\mu$ M) for 48 hours, and cell viability was determined with a standard Alamar Blue<sup>TM</sup> (Biosource International Inc., Camarillo, CA) assay. MCF-7 cells as negative controls, and MCF-7 cells stably expressing SULT1E1 (clones #1, #10, #22) and empty vector pCR3.1 were plated in 96-well plates. Cells were grown in RPMI 1640 (containing L-glutamine, but without phenol red) medium with 5% charcoal-dextran stripped fetal bovine serum overnight at 5% CO<sub>2</sub>, 37°C. The cell culture medium was replaced every 12 hours to remove endogenous estrogens. The next day, the cells were dosed with resveratrol, apigenin, chrysin, epicatechin, or quercetin (0–100  $\mu$ M) prepared in fresh media. After 48 hours, fluorescence was measured at an excitation wavelength of 530 nm and emission at 590 nm. Proliferation data were normalized such that cell growth with no treatment was set at 100%, for each cell line at each substrate concentration. All experiments were carried out in four replicates per assay condition. Proliferation among cell lines was compared statistically with ANOVA followed by Tukey's multiple comparison test (GraphPad Prism® version 4.03 for Windows).

DMD#13987

## Results

Sulfation kinetics of three polyphenols – apigenin, epicatechin, and resveratrol – via recombinant SULT1A1 and SULT1E1 were initially determined. Figure 1A depicts the sulfation of apigenin by SULT1E1, SULT1A1\*1, \*2, and \*3, with the respective Eadie-Hofstee plots depicted in figure 1B. Michaelis-Menten estimates are reported in table 1. The  $V_{\max}$  estimate for apigenin sulfation was significantly lower with SULT1E1 and SULT1A1\*2 compared with the \*1 protein. The  $K_m$  estimate with SULT1E1 was significantly higher than that with SULT1A1\*1. Inhibition was observed at high apigenin concentrations with all the proteins (fig 1C); inhibition could not be determined with SULT1A1\*2 due to low activity. A substrate inhibition model could not explain these inhibition data; therefore no  $K_i$  estimates were obtained.

Figure 2 depicts the kinetics of epicatechin sulfation. In figure 2A, epicatechin sulfation by SULT1E1 as well as by SULT1A1 allozymes did not exhibit inhibition in the concentration range studied. Eadie-Hofstee plots are shown in figure 2B. Comparison of the kinetic estimates (table 1) revealed significantly lower  $V_{\max}$  estimates and significantly higher  $K_m$  estimates for SULT1E1 and SULT1A1\*2 compared with the SULT1A1\*1 allozyme. Interestingly, the  $K_m$  estimate for the SULT1A1\*3 allozyme was significantly lower than that for SULT1A1\*1, leading to a  $V_{\max}/K_m$  estimate comparable with that of the ‘wild-type’ \*1 allozyme.

Resveratrol sulfation kinetics is depicted in figure 3, with corresponding kinetic estimates reported in table 1. As can be seen in figure 3A, resveratrol sulfation by either SULT1E1 or the SULT1A1 allozymes did not exhibit inhibition at the substrate concentrations evaluated. Figure 3B exhibits the Eadie-Hofstee plots for these data. The kinetic estimates for resveratrol sulfation

DMD#13987

via SULT1E1 were significantly different from the SULT1A1\*1 allozyme. This resulted in a lower  $V_{\max}/K_m$  estimate with SULT1E1 versus SULT1A1\*1 (table 1). Among the SULT1A1 polymorphic allozymes, both the variant \*2 and \*3 allozymes had significantly lower  $V_{\max}$  estimates than the \*1 allozyme. The  $K_m$  estimates were not statistically significantly different among the three SULT1A1 groups.

Next, the relative activities of recombinant SULT1E1 versus SULT1A1 toward the flavonoids chrysin and quercetin were evaluated. Figure 4A depicts sulfation kinetics of chrysin and quercetin by SULT1E1, while the corresponding Eadie-Hofstee plots are depicted in figure 4B. The parameter estimates are listed in table 1. We have previously reported the sulfation of these flavonoids by recombinant SULT1A1\*1, \*2, and \*3 allozymes (Nagar et al., 2006). For chrysin and quercetin, comparison of the published values for SULT1A1\*1-catalyzed sulfation with SULT1E1-catalyzed sulfation determined here revealed significant differences between the two proteins. For chrysin, SULT1A1\*1 had a significantly higher  $V_{\max}$  than the SULT1E1 protein, and a resultant  $V_{\max}/K_m$  ratio greater by an order of magnitude. Quercetin sulfation exhibited a significantly higher  $V_{\max}$  estimate via SULT1A1\*1, but a significantly lower  $K_m$  via SULT1E1. Thus the  $V_{\max}/K_m$  ratios were similar in this case (table 1). Inhibition was observed at high concentrations of both chrysin and quercetin (figure 4C). A substrate inhibition model could not explain these inhibition data; therefore no  $K_i$  estimates were obtained.

The effect of all five polyphenols on cell proliferation was evaluated next. MCF-7 cells exhibited similar proliferation profiles as cells expressing the empty vector pCR3.1 (data not shown). As shown in figure 5A, all five polyphenols inhibited cell proliferation at the highest concentration

DMD#13987

evaluated (100  $\mu\text{M}$ ). At 50  $\mu\text{M}$ , quercetin induced proliferation while epicatechin had no effect. Chrysin, apigenin, and resveratrol inhibited cell proliferation at the 50  $\mu\text{M}$  concentration. Figure 5B shows relative proliferation of negative control pCR3.1 empty vector-expressing MCF-7 cells versus cells stably expressing SULT1E1. Three SULT1E1 clones (#1, #10, and #22) were evaluated. There was no difference in proliferation among the cell lines upon treatment with 50  $\mu\text{M}$  of either apigenin or epicatechin. Quercetin (50  $\mu\text{M}$ ) induced proliferation in the negative control pCR3.1 expressing cells. This proliferation was inhibited by SULT1E1-expressing cells (clones #10 and #22) upon similar treatment. Chrysin and resveratrol both inhibited proliferation of pCR3.1-expressing cells, while all clones expressing SULT1E1 proliferated significantly more than the control cell line upon treatment.

DMD#13987

## Discussion

Dietary polyphenols have anti-oxidant properties and have been studied for their anticancer potential (Beecher, 2003; Baur and Sinclair, 2006). These compounds are sulfated by human SULT1A1 and SULT1E1, and are thought to interact with estrogens via this common metabolic pathway (Otake et al., 2000; Harris et al., 2004). SULT1A1 pharmacogenetics have been evaluated in detail (Raftogianis et al., 1996; Raftogianis et al., 1997; Raftogianis et al., 1999). Recently, genetic mutations in the human SULT1E1 gene, albeit at low frequencies, have been reported (Adjei et al., 2003). While epidemiologic studies have examined the association between SULT1A1 pharmacogenetics, dietary intake, and cancer risk, few studies have examined the genotype-phenotype correlation between polymorphic SULT1A1 and dietary polyphenol metabolism (Seth et al., 2000; Bamber et al., 2001; Williams et al., 2001; Zheng et al., 2001; Wang et al., 2002; Tang et al., 2003; Wu et al., 2003; Choi et al., 2005; Nowell et al., 2005; Shatalova et al., 2005). We have previously reported allele-specific differences in SULT1A1-catalyzed sulfation of chrysin and quercetin (Nagar et al., 2006). In the current study, we have characterized the sulfation of additional flavonoids by purified recombinant SULT1A1 and SULT1E1 proteins. Importantly, the effect of SULT1E1-catalyzed sulfation (and inactivation) of polyphenols on human breast cancer cells is additionally reported.

With the purified recombinant proteins expressed in a baculoviral / Sf-9 insect cell system, the 'normal activity' SULT1A1\*1 allozyme exhibited significantly greater  $V_{max}$  estimates for all the polyphenols studied when compared with the SULT1E1 protein (table 1). For epicatechin, resveratrol, and chrysin, the  $V_{max}/K_m$  estimates were at least an order of magnitude greater with SULT1A1\*1 compared to SULT1E1. Specific activities for epicatechin and quercetin have been

DMD#13987

reported with purified recombinant SULTs in transformed BL21 (DE3) cells (Pai et al., 2001). At 100  $\mu\text{M}$  epicatechin, our mean specific activity value for sulfation by SULT1E1 (300 pmol/min/mg protein) compares very well with that published by Pai *et al* (351 pmol/min/mg protein). Quercetin sulfation by recombinant SULT1E1 at 100  $\mu\text{M}$  substrate yielded a specific activity of 90 pmol/min/mg in our assays, compared with 610 pmol/min/mg reported by Pai *et al*. Differences in incubation conditions may account for this difference. To our knowledge, this study is the first report of apigenin sulfation kinetics catalyzed by recombinant SULT1E1. Chrysin sulfation has been reported in human liver samples as well as in human liver cell lines, and the  $V_{\text{max}}$  estimates we report are higher than those reported in human livers, possibly because we employed purified recombinant protein in our assays (Galijatovic et al., 1999; De Santi et al., 2000).

With regard to SULT1A1-catalyzed polyphenol sulfation, to our knowledge allele-specific sulfation of apigenin, epicatechin, and resveratrol by SULT1A1\*1, \*2, and \*3 allozymes has not been reported to date. Our results indicate a significantly lower  $V_{\text{max}}$  estimate for apigenin sulfation by SULT1A1\*2 compared to the \*1 allozyme. With epicatechin as the substrate, SULT1A1\*2 exhibited a lower  $V_{\text{max}}$  and a higher  $K_m$  than the \*1 variant, leading to a very low  $V_{\text{max}}/K_m$  ratio. SULT1A1\*3 on the other hand had a significantly lower  $K_m$  than \*1, and a comparable  $V_{\text{max}}/K_m$  estimate. Mean SULT1A1\*1 activity toward 100  $\mu\text{M}$  epicatechin (1173 pmol/min/mg) compared well with a previously reported value (1364 pmol/min/mg, Pai et al. 2001). Resveratrol sulfation by SULT1A1\*1 and \*3 allozymes showed comparable intrinsic clearance ( $V_{\text{max}}/K_m$ ) estimates despite significantly different  $V_{\text{max}}$  estimates. The SULT1A1\*2 allozyme had a significantly lower  $V_{\text{max}}$  for resveratrol sulfation compared with \*1. Thus, allele-



DMD#13987

specific differences in SULT1A1-mediated activity were observed for all the polyphenols studied. Data presented here are in agreement with literature reports where SULT1A1\*2 has been shown to be the 'low-activity' allele toward different classes of substrates (Raftogianis et al., 1999).

Conjugation of the polyphenol resveratrol at different positions, leading to various metabolites, has recently been described (Miksits et al., 2005). Miksits *et al* reported the formation of two sulfated metabolites via SULT1A1, with substrate inhibition and sigmoidal kinetics. That study generated recombinant SULT1A1 protein in a bacterial system, while we report the use of a baculoviral expression system. The variable protein source in the two studies partly explains the different results. It is also important to consider possible solvent effects: DMSO at a relatively high final concentration (3.3%) was employed in our incubations. The study by Miksits *et al* employed an HPLC assay for metabolite detection, while the radiometric assay employed here does not allow for differentiation between different sulfated metabolites. Despite this drawback, the estimates obtained here were useful in a comparative study among the SULT1E1, SULT1A1\*1, \*2, and \*3 allozymes. At the substrate concentration range evaluated, data transformation yielded linear Eadie-Hofstee plots (figures 1-4b), and the Michaelis-Menten equation fit the data well. Kinetic estimates obtained here, and those reported with recombinant SULT proteins in other studies, yielded comparable results (Pai et al., 2001). Additionally, as discussed below, cellular phenotypic data in cultured mammalian cells for substrates like quercetin and resveratrol correlate well with data from recombinant protein experiments. Current studies in our laboratory focus on developing HPLC-based assays to quantitate polyphenol conjugates for future work.

DMD#13987

While data obtained from purified recombinant protein provide useful enzyme kinetic parameters, it is critical to examine the effect of enzymatic inactivation of a substrate *in vivo*. Cultured cells provide an environment closer to the *in vivo* situation compared with purified protein. Additionally, in the case of potentially chemopreventive polyphenols, it is possible to evaluate the cellular effect of the substrate (in this case proliferative response) in cells in the presence or absence of the enzyme. To this end, we initially characterized the effect of varying concentrations of polyphenols on MCF-7 cells stably expressing pCR3.1 (fig 5A). Resveratrol has been reported to have an anti-proliferative effect on MCF-7 cells (Kim et al., 2004). Similar effects were observed in our assays in both MCF-7 cells and cells stably expressing pCR3.1. Next, the effect of polyphenol sulfation (and inactivation) was evaluated. At 50  $\mu\text{M}$  concentration, chrysin, apigenin, and resveratrol had an anti-proliferative effect on control cells, while quercetin induced proliferation. Cells stably expressing SULT1E1 reversed the effects of chrysin, quercetin, and resveratrol (fig 5B). Thus, sulfation of these polyphenols by SULT1E1 yielded a phenotypic response at the cellular level, and inactivation by SULT1E1-catalyzed sulfation reversed the proliferative effect. SULT1E1 is the major isozyme responsible for the sulfation of endogenous estrogens (Coughtrie, 2002). Therefore, it would be interesting to investigate the effect of estrogen + polyphenol cotreatment in cells stably expressing SULT1E1, to determine what effect this interaction elicits at the cellular level. It is possible that other mechanisms besides sulfation play a major role in the disposition and hence the effect of these polyphenols in MCF-7 cells. MCF-7 cells exhibit low glucuronidation capacity and UGT2B15 expression, and flavonoids are substrates for this human UGT isozyme (Harrington et al., 2006). Additionally, flavonoids such as chrysin (and its conjugated metabolites) are good substrates for

DMD#13987

the multi-drug resistance protein MRP2 transporter (Walle et al., 1999), and chrysin is known to inhibit the breast cancer resistance protein BCRP (ABCG2) in MCF-7 cells (Zhang et al., 2004).

In conclusion, this study evaluated the sulfation of five polyphenols with recombinant polymorphic SULT1A1 variants and SULT1E1. For most polyphenols examined, recombinant SULT1A1 exhibited greater activity than SULT1E1. Apigenin, epicatechin, and resveratrol exhibited SULT1A1 allele-specific variation in sulfation, with SULT1A1\*1 and \*3 acting as ‘normal-activity’ allozymes, and SULT1A1\*2 acting as a ‘low-activity’ allozyme. Altered cellular proliferation was observed in cells stably expressing SULT1E1 upon treatment with chrysin, quercetin, or resveratrol, possibly due to inactivation of these substrates by SULT1E1. Together, these results provide evidence for the role of SULT enzymes and their pharmacogenetics in polyphenol conjugation and cellular effects.

DMD#13987

**Acknowledgment:**

We would like to acknowledge Dr. Rebecca L. Blanchard for supplying constructs for recombinant protein expression. The technical input of Ms. Lisa Orfe toward the development of stable cell lines is acknowledged.

DMD#13987

## References:

- Adjei AA, Thomae BA, Prondzinski JL, Eckloff BW, Wieben ED and Weinshilboum RM (2003) Human estrogen sulfotransferase (SULT1E1) pharmacogenomics: gene resequencing and functional genomics. *Br J Pharmacol* **139**:1373-1382.
- Anderson R and Weinshilboum R (1980) Phenolsulfotransferase in human tissue: radiochemical enzymatic assay and biochemical properties. *Clinica Chemica Acta* **103**:79 - 90.
- Bamber D, Fryer A, Strange R, Elder J, Deakin M, Rajagopal R, Fawole A, Gilissen R, Campbell F and Coughtrie M (2001) Phenol sulphotransferase SULT1A1\*1 genotype is associated with reduced risk of colon cancer. *Pharmacogenetics* **11**:679 - 685.
- Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R and Sinclair DA (2006) Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**: 337 - 42.
- Baur JA and Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* **5**:493-506.
- Beecher G (2003) Overview of dietary flavonoids: nomenclature, occurrence and intake. *J Nutr* **133**:3248S-3254S.
- Brownson DM, Azios NG, Fuqua BK, Dharmawardhane SF and Mabry TJ (2002) Flavonoid effects relevant to cancer. *J Nutr* **132**:3482S-3489S.
- Choi JY, Lee KM, Park SK, Noh DY, Ahn SH, Chung HW, Han W, Kim JS, Shin SG, Jang JJ, Yoo KY, Hirvonen A and Kang D (2005) Genetic polymorphisms of SULT1A1 and

DMD#13987

- SULT1E1 and the risk and survival of breast cancer. *Cancer Epidemiol Biomarkers Prev* **14**:1090-1095.
- Coughtrie M (2002) Sulfation through the looking glass - recent advances in sulfotransferase research for the curious. *The Pharmacogenomics Journal* **2**:297-308.
- De Santi C, Pietrabissa A, Spisni R, Mosca F and Pacifici GM (2000) Sulphation of resveratrol, a natural compound present in wine, and its inhibition by natural flavonoids. *Xenobiotica* **30**:857-866.
- Galijatovic A, Otake Y, Walle UK and Walle T (1999) Extensive metabolism of the flavonoid chrysin by human Caco-2 and Hep G2 cells. *Xenobiotica* **29**:1241-1256.
- Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL and McManus ME (2006) Human sulfotransferases and their role in chemical metabolism. *Toxicol Sci* **90**:5-22.
- Geese W and Raftogianis R (2001) Biochemical characterization and tissue distribution of human SULT2B1. *Biochemical and Biophysical Research Communications* **288**:280 - 289.
- Harrington WR, Sengupta S and Katzenellenbogen BS (2006) Estrogen regulation of the glucuronidation enzyme UGT2B15 in estrogen receptor-positive breast cancer cells. *Endocrinology* **147**:3843-3850.
- Harris RM, Wood DM, Bottomley L, Blagg S, Owen K, Hughes PJ, Waring RH and Kirk CJ (2004) Phytoestrogens are potent inhibitors of estrogen sulfation: implications for breast cancer risk and treatment. *J Clin Endocrinol Metab* **89**:1779-1787.
- Jones AL, Hagen M, Coughtrie MW, Roberts RC and Glatt H (1995) Human platelet phenolsulfotransferases: cDNA cloning, stable expression in V79 cells and identification

DMD#13987

of a novel allelic variant of the phenol-sulfating form. *Biochem Biophys Res Commun* **208**:855-862.

Kim YA, Choi BT, Lee YT, Park DI, Rhee SH, Park KY and Choi YH (2004) Resveratrol inhibits cell proliferation and induces apoptosis of human breast carcinoma MCF-7 cells. *Oncol Rep* **11**:441-446.

Manach C, Scalbert A, Morand C, Remesy C and Jimenez L (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**:727-747.

Miksits M, Maier-Salamon A, Aust S, Thalhammer T, Reznicek G, Kunert O, Haslinger E, Szekeres T and Jaeger W (2005) Sulfation of resveratrol in human liver: evidence of a major role for the sulfotransferases SULT1A1 and SULT1E1. *Xenobiotica* **35**:1101-1119.

Nagar S, Remmel R, Hebbel R and Zimmerman C (2004) Metabolism of opioids is altered in liver microsomes of sickle cell transgenic mice. *Drug Metabolism and Disposition* **32**:98-104.

Nagar S, Walther S and Blanchard RL (2006) Sulfotransferase (SULT) 1A1 polymorphic variants \*1, \*2, and \*3 are associated with altered enzymatic activity, cellular phenotype, and protein degradation. *Mol Pharmacol* **69**:2084-2092.

Nowell SA, Ahn J, Rae JM, Scheys JO, Trovato A, Sweeney C, MacLeod SL, Kadlubar FF and Ambrosone CB (2005) Association of genetic variation in tamoxifen-metabolizing enzymes with overall survival and recurrence of disease in breast cancer patients. *Breast Cancer Res Treat* **91**:249-258.

DMD#13987

- Otake Y, Nolan AL, Walle UK and Walle T (2000) Quercetin and resveratrol potently reduce estrogen sulfotransferase activity in normal human mammary epithelial cells. *J Steroid Biochem Mol Biol* **73**:265-270.
- Pai TG, Suiko M, Sakakibara Y and Liu MC (2001) Sulfation of flavonoids and other phenolic dietary compounds by the human cytosolic sulfotransferases. *Biochem Biophys Res Commun* **285**:1175-1179.
- Raftogianis R, Wood T, Otterness D, Van Loon J and Weinshilboum R (1997) Phenol sulfotransferase pharmacogenetics in humans: association of common SULT1A1 alleles with TS PST phenotype. *Biochem Biophys Res Commun* **239**:298 - 304.
- Raftogianis RB, Her C and Weinshilboum RM (1996) Human phenol sulfotransferase pharmacogenetics: STP1 gene cloning and structural characterization. *Pharmacogenetics* **6**:473-487.
- Raftogianis RB, Wood TC and Weinshilboum RM (1999) Human phenol sulfotransferases SULT1A2 and SULT1A1: genetic polymorphisms, allozyme properties, and human liver genotype-phenotype correlations. *Biochem Pharmacol* **58**:605-616.
- Seth P, Lunetta K, Bell D, Gray H, Nasser S, Rhei E, Kaelin C, Iglehart D, Marks J, Garber J, Haber D and Polyak K (2000) Phenol sulfotransferases: hormonal regulation, polymorphism, and age of onset of breast cancer. *Cancer Research* **60**:6859-6863.
- Shatalova EG, Walther SE, Favorova OO, Rebbeck TR and Blanchard RL (2005) Genetic polymorphisms in human SULT1A1 and UGT1A1 genes associate with breast tumor characteristics: a case-series study. *Breast Cancer Res* **7**:R909-921.
- Tang D, Rundle A, Mooney L, Cho S, Schnabel F, Estabrook A, Kelly A, Levine R, Hibshoosh H and Perera F (2003) Sulfotransferase 1A1 (SULT1A1) polymorphism, PAH-DNA



DMD#13987

- adduct levels in breast tissue and breast cancer risk in a case-control study. *Breast Cancer Research and Treatment* **78**:217 - 222.
- Varin L, Barron D and Ibrahim RK (1987) Enzymatic assay for flavonoid sulfotransferase. *Anal Biochem* **161**:176-180.
- Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr. and Walle UK (2004) High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab Dispos* **32**:1377-1382.
- Walle UK, Galijatovic A and Walle T (1999) Transport of the flavonoid chrysin and its conjugated metabolites by the human intestinal cell line Caco-2. *Biochem Pharmacol* **58**:431-438.
- Wang Y, Spitz M, Tsou AM-H, Zhang K, Makan N and Wu X (2002) Sulfotransferase (SULT) 1A1 polymorphism as a predisposition factor for lung cancer: a case-control analysis. *Lung Cancer* **35**:137-142.
- Wen X and Walle T (2006) Methylation protects dietary flavonoids from rapid hepatic metabolism. *Xenobiotica* **36**:387-397.
- Williams J, Stone E, Fakis G, Johnson N, Cordell J, Meinel W, Glatt H, Sim E and Phillips D (2001) N-Acetyltransferases, sulfotransferases, and heterocyclic amine activation in the breast. *Pharmacogenetics* **11**:373 - 388.
- Wu M-T, Wang Y-T, Ho C-K, Wu D-C, Lee Y-C, Hsu H-K, Kao E-L and Lee J-M (2003) SULT1A1 polymorphism and esophageal cancer in males. *International Journal of Cancer* **103**:101-104.
- Zhang S, Yang X and Morris ME (2004) Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Mol Pharmacol* **65**:1208-1216.

DMD#13987

Zheng W, Xie D, Cerhan J, Sellers T, Wen W and Folsom A (2001) Sulfotransferase 1A1 polymorphism, endogenous estrogen exposure, well-done meat intake, and breast cancer risk. *Cancer Epidemiology, Biomarkers and Prevention* **10**:89 - 94.

DMD#13987

**Footnotes:**

This work was partially supported by grants to S.N. from the Cancer Research and Prevention Foundation, Alexandria VA, and Temple University Research Incentive Funds.

This work was presented as poster presentations at the 14<sup>th</sup> North American International Society for the Study of Xenobiotics (ISSX) meeting, Rio Grande, Puerto Rico and the 2006 annual meeting of the American Association of Pharmaceutical Scientists (AAPS), San Antonio TX.

DMD#13987

## Legends for figures

**Figure 1.** Kinetics of sulfation of dietary flavone apigenin by SULT1E1 and SULT1A1 allozymes. Purified recombinant SULT1A1\*1, \*3, SULT1E1 (0.5  $\mu$ g each) or SULT1A1\*2 (1  $\mu$ g) protein was incubated with substrate and 10  $\mu$ M  $^{35}$ S-PAPS for 15 min at 37°C, and the formation of radioactive sulfated product was quantitated with a radiometric assay as described under Methods. a) Michaelis-Menten curves for apigenin sulfation by SULT1E1, SULT1A1\*1, \*2, or \*3 at 0-50  $\mu$ M. Inset: Michaelis-Menten curve for the \*2 allozyme is depicted on an expanded y-axis scale at 0-100  $\mu$ M apigenin. Data expressed as mean  $\pm$  SD, n = 3 for all except n = 6 for \*2. Representative fitted lines are depicted; parameter estimates were obtained by fitting the kinetic models to actual data replicates. b) Eadie-Hofstee plots of Michaelis-Menten kinetics of apigenin sulfation by SULT1E1, SULT1A1\*1, \*2, or \*3. Data reported as mean, n = 3 for all except n = 6 for \*2. c) Apigenin sulfation by SULT1E1, SULT1A1\*1, or \*3, at 0-100  $\mu$ M substrate concentration, where inhibition is shown. Data reported as mean, n = 3 for all except n = 6 for \*3. A substrate inhibition equation (see Methods section) could not adequately describe these data; therefore  $K_i$  values were not obtained.

**Figure 2.** Kinetics of sulfation of dietary flavonol epicatechin by SULT1E1 and SULT1A1 allozymes. Purified recombinant SULT1A1\*1, \*3 (0.5  $\mu$ g each), SULT1A1\*2, or SULT1E1 (2  $\mu$ g each) protein was incubated with substrate and 10  $\mu$ M  $^{35}$ S-PAPS for 15 min at 37°C, and the formation of radioactive sulfated product was quantitated with a radiometric assay as described under Methods. a) Michaelis-Menten curves for epicatechin sulfation by SULT1E1, SULT1A1\*1, \*2, or \*3 at 0-3 mM. Insets: Michaelis-Menten curves for the \*2 allozyme and SULT1E1 is depicted on an expanded y-axis scale. Data expressed as mean  $\pm$  SD, n = 3.

DMD#13987

Representative fitted lines are depicted; parameter estimates were obtained by fitting the kinetic models to actual data replicates. b) Eadie-Hofstee plots of Michaelis-Menten kinetics of epicatechin sulfation by SULT1E1, SULT1A1\*1, \*2, or \*3. Data reported as mean, n = 3.

**Figure 3.** Kinetics of sulfation of dietary polyphenol resveratrol by SULT1E1 and SULT1A1 allozymes. Purified recombinant SULT1A1\*1, \*2, \*3 (0.5  $\mu\text{g}$  each), or SULT1E1 (0.2  $\mu\text{g}$ ) protein was incubated with substrate and 10  $\mu\text{M}$   $^{35}\text{S}$ -PAPS for 15 min (SULT1A1\*2, \*3, or SULT1E1 each) or 30 min (SULT1A1\*1) at 37°C, and the formation of radioactive sulfated product was quantitated with a radiometric assay as described under Methods. a) Michaelis-Menten curves for resveratrol sulfation by SULT1E1, SULT1A1\*1, \*2, or \*3 at 0-100  $\mu\text{M}$ . Insets: Michaelis-Menten curves for the \*2 allozyme and SULT1E1 is depicted on an expanded y-axis scale. Data expressed as mean  $\pm$  SD, n = 3. Representative fitted lines are depicted; parameter estimates were obtained by fitting the kinetic models to actual data replicates. b) Eadie-Hofstee plots of Michaelis-Menten kinetics of resveratrol sulfation by SULT1E1, SULT1A1\*1, \*2, or \*3. Data reported as mean, n = 3.

**Figure 4.** Kinetics of sulfation of dietary flavone chrysin and flavonol quercetin by SULT1E1 isozyme. Purified recombinant SULT1E1 (0.2  $\mu\text{g}$ ) protein was incubated with substrate and 10  $\mu\text{M}$   $^{35}\text{S}$ -PAPS for 20 min (chrysin) or for 30 min (quercetin) at 37°C, and the formation of radioactive sulfated product was quantitated with a radiometric assay as described under Methods. a) Michaelis-Menten curves for quercetin and chrysin sulfation by SULT1E1 at 0-10  $\mu\text{M}$ . Data expressed as mean  $\pm$  SD, n = 3. Representative fitted lines are depicted; parameter estimates were obtained by fitting the kinetic models to actual data replicates. b) Eadie-Hofstee

DMD#13987

plots of Michaelis-Menten kinetics of quercetin and chrysin sulfation by SULT1E1. Data reported as mean,  $n = 3$ . c) Sulfation of quercetin and chrysin by SULT1E1 at 0-100  $\mu\text{M}$  substrate concentration, where inhibition is shown. Data reported as mean,  $n = 6$ . A substrate inhibition equation (see Methods section) could not adequately describe these data; therefore  $K_i$  values were not obtained.

**Figure 5.** Cell proliferation upon polyphenol treatment of MCF-7 cells stably expressing human SULT1E1. a) Effect of varying concentrations of polyphenols (0-100  $\mu\text{M}$ ) on MCF-7 cells stably transfected with the empty vector pCR3.1. Response was measured with a standard cell proliferation assay as described under Methods. Data are expressed as mean,  $n = 4$ . Proliferation at 50  $\mu\text{M}$  polyphenol is highlighted, as this was the concentration used for subsequent assays. b) Effect of 50  $\mu\text{M}$  polyphenol on MCF-7 cells stably expressing human SULT1E1. Three clones, #1, #10, and #22 were evaluated. Data expressed as mean + SD,  $n = 4$ . ANOVA followed by Tukey's multiple comparison tests were used to determine statistically significant differences. Significant differences from pCR3.1 (control cell line) values are reported.  $*p < 0.05$ ; ns = not significant.

DMD#13987

**TABLE 1. Kinetic parameter estimates for the sulfation of polyphenols by SULT1E1 and SULT1A1 allozymes.**

Data are expressed as estimate  $\pm$  SE, n = 3. Estimate units are as follows:  $V_{\max}$ : nmol/min/mg;  $K_m$ :  $\mu$ M;  $V_{\max}/K_m$ : ml/min/mg.

SUBSTRATE	Estimate	Sulfotransferase			
		SULT1E1	SULT1A1*1	SULT1A1*2	SULT1A1*3
<b>Apigenin</b>	$V_{\max}$	$3.72 \pm 0.17^b$	$5.83 \pm 0.27$	$0.049 \pm 0.002^{a,b}$	$4.54 \pm 0.14$
	$K_m$	$5.3 \pm 0.65^b$	$1.72 \pm 0.36$	$2.64 \pm 0.59$	$2.57 \pm 0.41$
	$V_{\max}/K_m$	0.70	3.39	0.019	1.77
<b>Epicatechin</b>	$V_{\max}$	$0.36 \pm 0.024^b$	$3.79 \pm 0.16$	$0.025 \pm 0.0022^b$	$2.99 \pm 0.11$
	$K_m$	$961.8 \pm 175.7^b$	$241.6 \pm 53.86$	$419.3 \pm 151.8^b$	$65.08 \pm 11.15^b$
	$V_{\max}/K_m$	0.0004	0.016	5.96E-05	0.046
<b>Resveratrol</b>	$V_{\max}$	$1.22 \pm 0.05^b$	$6.81 \pm 0.22$	$0.33 \pm 0.02^b$	$4.4 \pm 0.19^b$
	$K_m$	$6.88 \pm 1.12^b$	$2.28 \pm 0.45$	$0.47 \pm 0.16$	$1.1 \pm 0.30$
	$V_{\max}/K_m$	0.18	3.0	0.70	4.0
<b>Chrysin</b>	$V_{\max}$	$1.6 \pm 0.10^b$	$8.4 \pm 0.14^c$		
	$K_m$	$4.5 \pm 0.65$	$2.5 \pm 0.3^c$		
	$V_{\max}/K_m$	0.36	$3.36^c$		
<b>Quercetin</b>	$V_{\max}$	$2.8 \pm 0.15^b$	$35.1 \pm 5.8^c$		
	$K_m$	$2.0 \pm 0.34^b$	$33.5 \pm 6.8^c$		
	$V_{\max}/K_m$	1.4	$1.05^c$		

<sup>a</sup> Data expressed as estimate  $\pm$  SE, n = 6.

<sup>b</sup> Estimate significantly different from \*1 as determined by a two-sided *t*-test, \*\**p* < 0.01.

<sup>c</sup> Data reported by Nagar et al. 2006.

Figure 1A

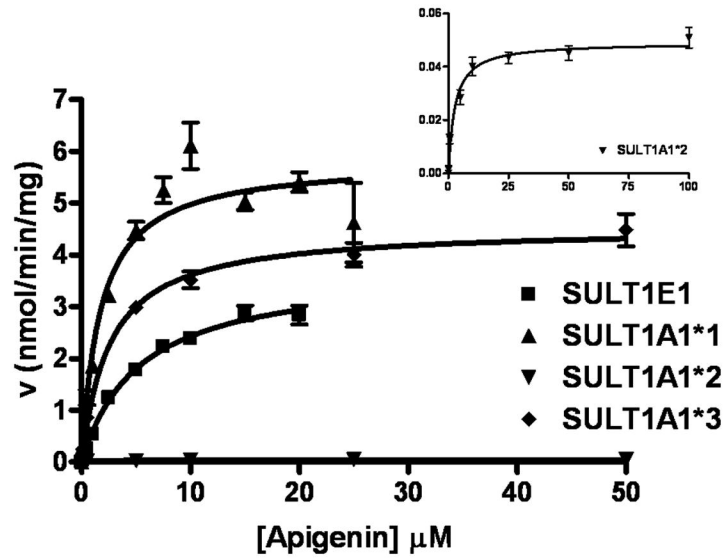


Figure 1B

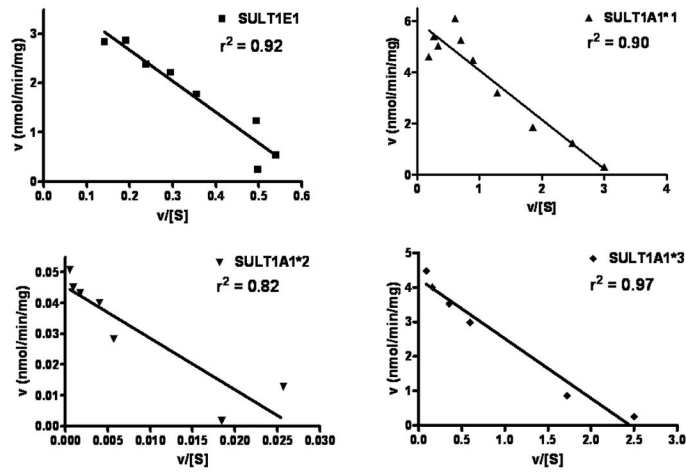


Figure 1C

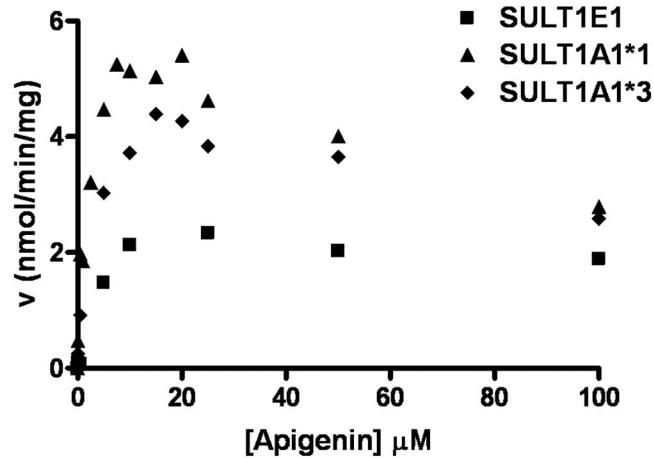




Figure 2A

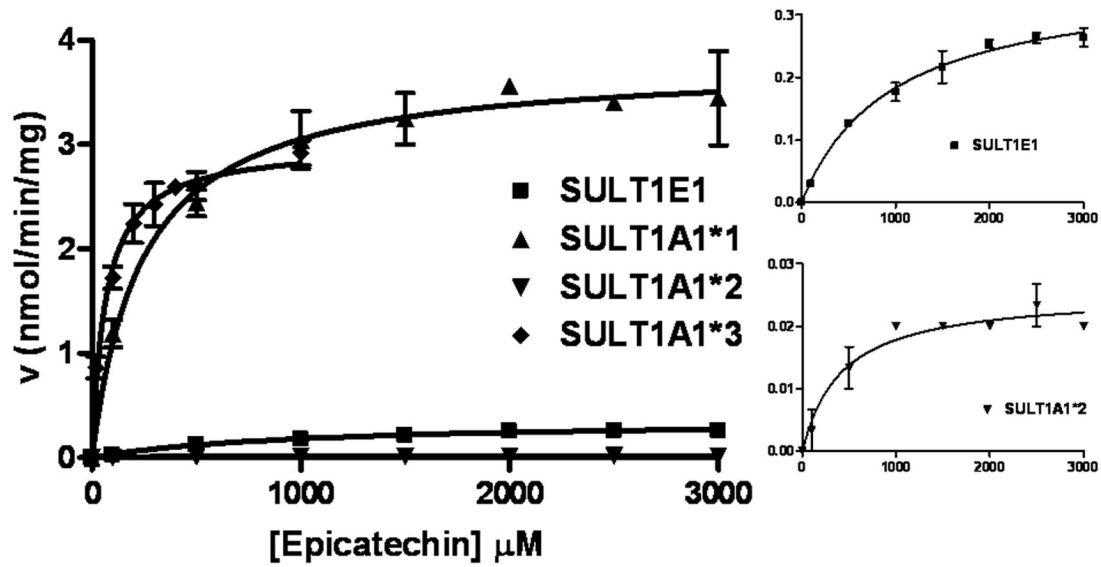


Figure 2B

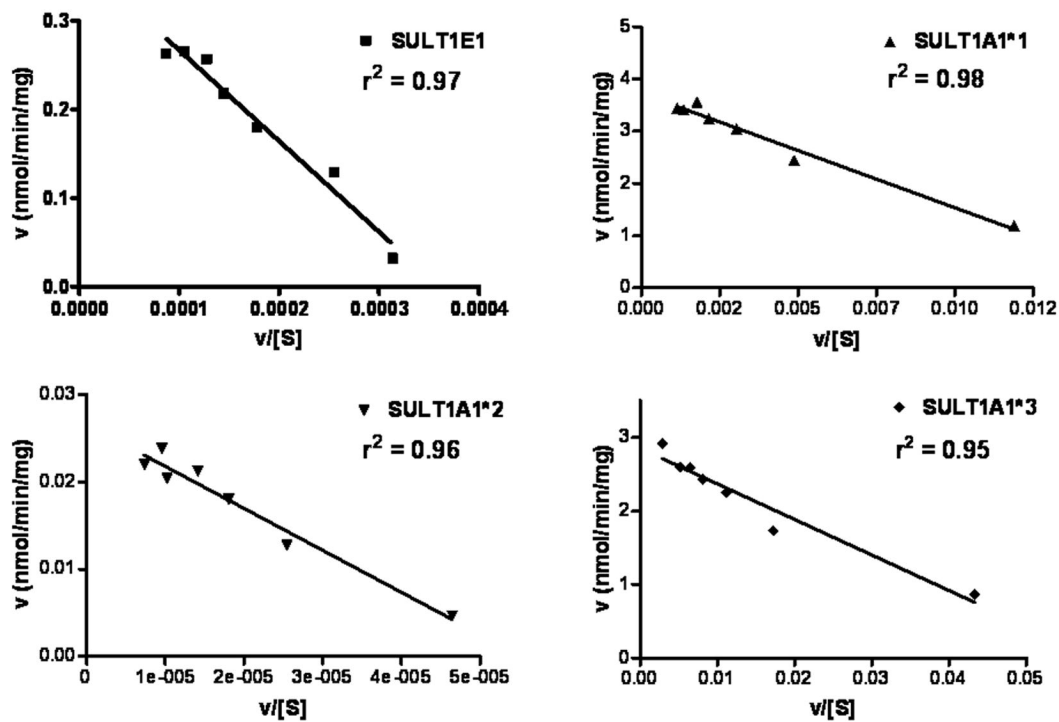


Figure 3A

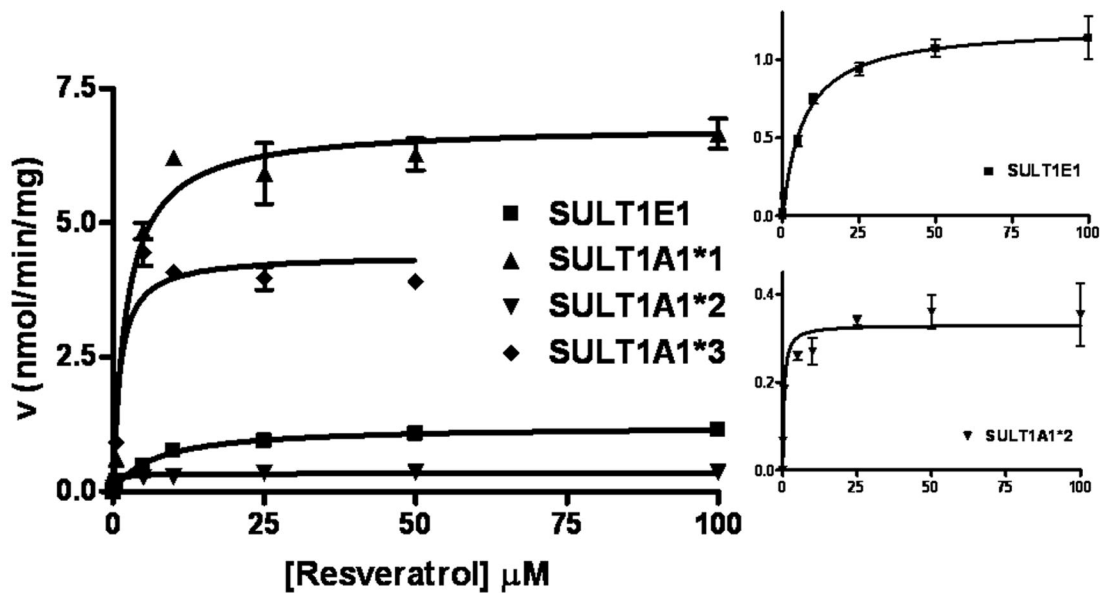


Figure 3B

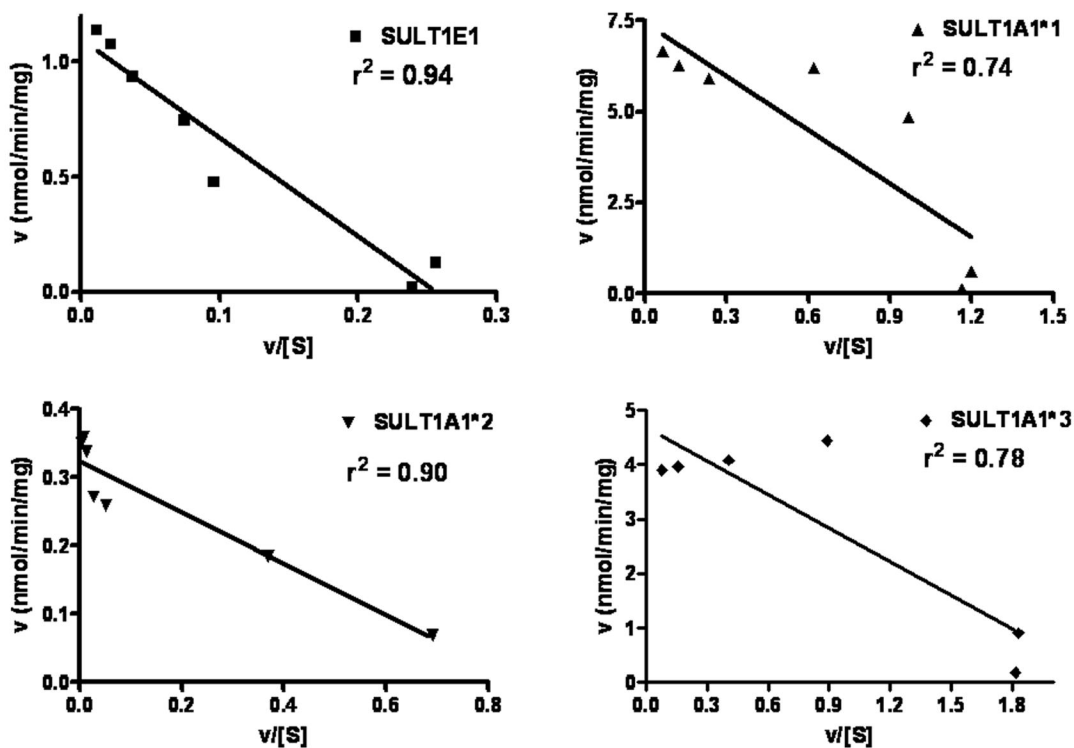


Figure 4A

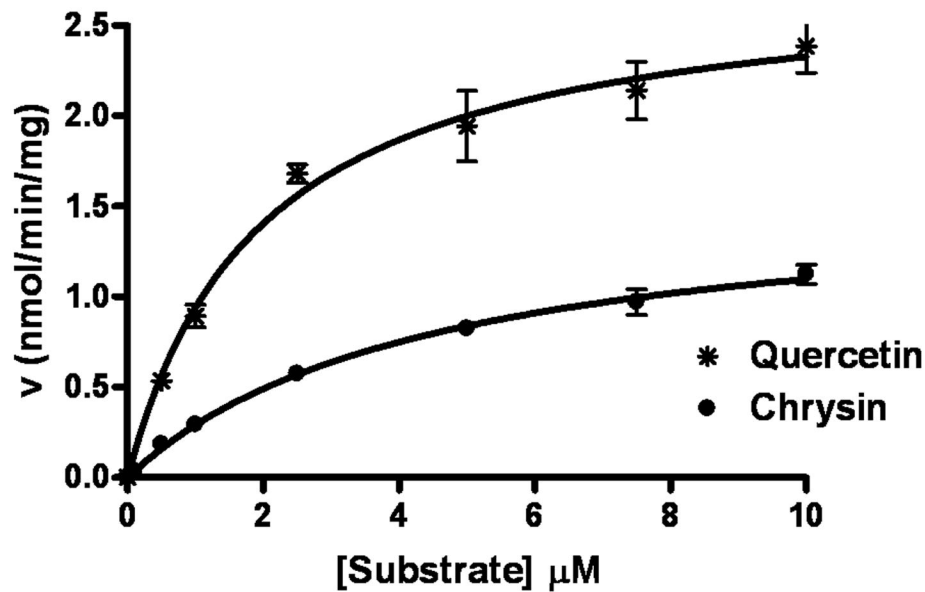


Figure 4B

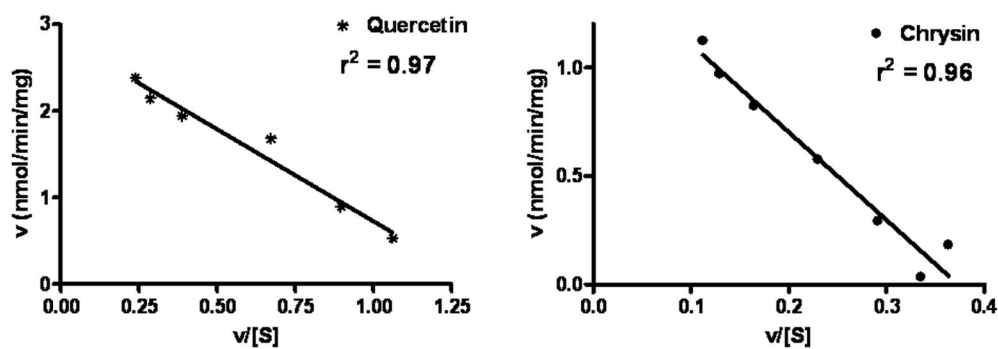


Figure 4C

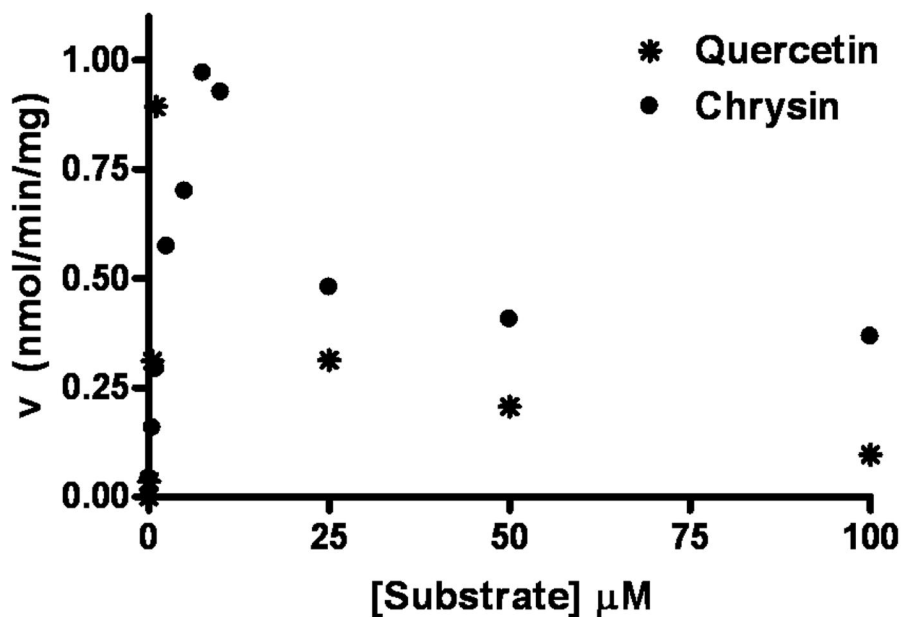


Figure 5A

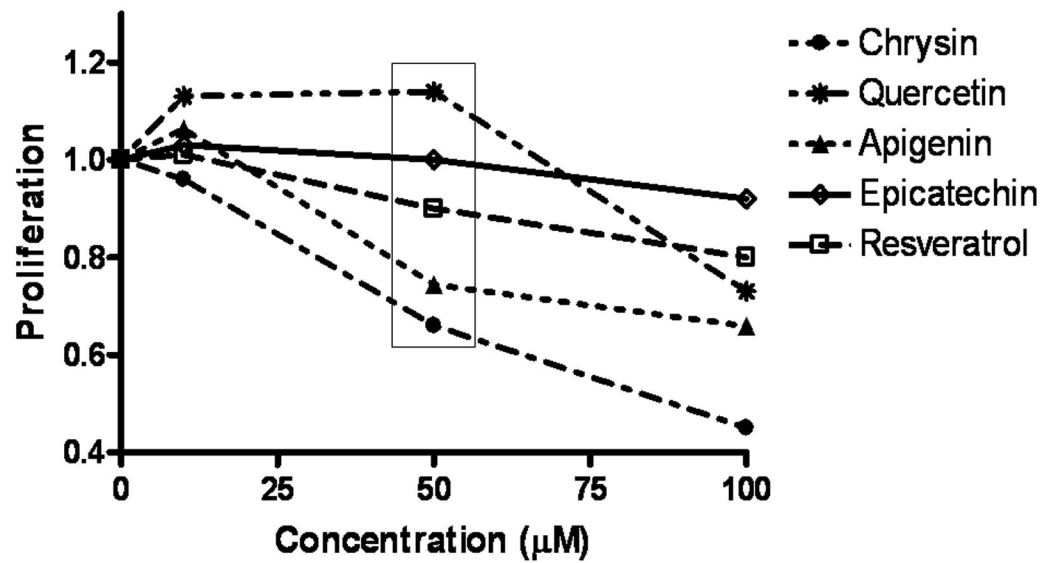


Figure 5B

