Differential Inhibition of Human Platelet Aggregation by Selected *Allium* Thiosulfinates

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Thiosulfinates (TSs) have been implicated as a principle source of the antiplatelet property of raw onion and garlic juice. The in vitro responses of human platelets to dosages of four TSs were measured using whole blood aggregometry and compared by regression analysis. Of the compounds evaluated, methyl methane-TS (MMTS), propyl propane-TS (PPTS), and 2-propenyl 2-propene-TS (allicin) are present in freshly cut *Allium* vegetables, whereas ethyl ethane-TS (EETS) has not been detected. All TSs were synthesized using a model reaction system. PPTS and allicin had the strongest antiplatelet activity at 0.4 mM, inhibiting aggregation by 90 and 89%, respectively. At the same concentration, EETS and MMTS were significantly weaker, inhibiting 74 and 26%, respectively. Combinations of TSs were not additive in their inhibition of aggregation, indicating that the antiplatelet potential of *Allium* extracts cannot be easily predicted by quantifying organosulfur components. EETS, PPTS, and allicin were significantly more potent platelet inhibitors than aspirin at nearly equivalent concentrations.

Keywords: *Aggregometry; Allium; platelets; thiosulfinates; antiplatelet*

INTRODUCTION

Platelet aggregation plays a central role in thrombosis (clot formation). The presence of a thrombus in an artery providing blood to the heart is the most common cause of acute coronary syndromes such as myocardial infarction and angina (Davies and Thomas, 1984; Fuster et al., 1992). Inhibitors of aggregation can provide protection against these symptoms that affect millions of people worldwide. Acetylsalicylic acid (aspirin) is one such inhibitor. The chances of a second heart attack can be reduced by as much as 40% by taking aspirin daily (Patrono, 1994). Increasing the level of natural platelet inhibitors in the diet may also reduce the risk of developing cardiovascular disorders mediated by platelet aggregation.

Previous research in our laboratory and others has shown that juice from both onion (*Allium cepa*) and garlic (*Allium sativum*) inhibits platelet aggregation in human blood in vitro (Goldman et al., 1995; Srivastava, 1984; Bordia, 1978). The flavors and aromas (Lancaster and Boland, 1990), as well as the antiplatelet activity (Goldman et al., 1996) of onions and other alliums are attributed to the organosulfur products of the transformation of *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs) by the enzyme alliinase and subsequent reactions. The ACSOs detected in intact onion are *S*-1-propenyl-L-cysteine sulfoxide (1-PeCSO), *S*-methyl-L-cysteine sulfoxide (MCSO) (Thomas and Parkin, 1994; Yoo and Pike, 1998), and in some studies, *S*-propyl-L-cysteine sulfoxide (PCSO) (Edwards et al., 1994). *S*-2-Propenyl-L-cysteine sulfoxide (2-PeCSO) is the predominant ACSO in garlic, with smaller amounts of MCSO and 1-PeCSO also present. *S*-Ethyl-L-cysteine sulfoxide (ECSO) has been detected in trace amounts in various *Allium* species, including garlic (Kubec et al., 2000). In intact *Allium* tissues, ACSOs are located in the cytosol, where they are protected from lysis by alliinase stored in vacuoles (Lancaster and Collin, 1981). Enzyme and substrates react rapidly upon disruption of the tissue.

ACSOs are cleaved by alliinase to sulfenic acids, ammonia, and pyruvate (Figure 1). Sulfenic acids are highly reactive and transient compounds. The majority of 1-propenylsulfenic acid rearranges to form propanethial sulfoxide, the lachrymatory factor, a compound that induces tearing in humans (Block, 1992). In fresh tissue, sulfenic acids with the same or different alk(en)yl substituents condense to form thiosulfinates (TSs). The TS profile of onion extract includes compounds with all nine possible substituent combinations with the exception of 1-propenyl 1-propene-TS (Block et al., 1992). The compound 1-propenyl 1-propene-TS has not been detected in *Allium* tissues. It may be oxidized to form bissulfine or cyclized to form zwiebelanes or react with other sulfenic acids to form cepaenes (Block, 1992). A similar series of eight TSs containing combinations of methyl, 1-propenyl, and 2-propenyl substituents is found in garlic homogenates (Lawson et al., 1991a). Although ECSO has been detected, the presence of ethyl-containing TSs has not been reported. Reduced temperatures and acid media favor the stability of TSs (Parkin and Shen, 2000). In nonpolar media such as oil macerations, TSs are likely transformed to vinyl dithiins and ajoenes (Lawson et al., 1991b). Steam distillation of TSs yields dialk(en)yl sulfides, disulfides, and trisulfides.

Many of the organosulfur compounds described above have demonstrated in vitro antiplatelet activity. Lawson et al. (1992) demonstrated that some TSs, dialk(en)yl sulfides, disulfides, and trisulfides, vinyl dithiins, and ajoenes, that are found in garlic preparations have in vitro antiplatelet activity in whole blood. In their study,

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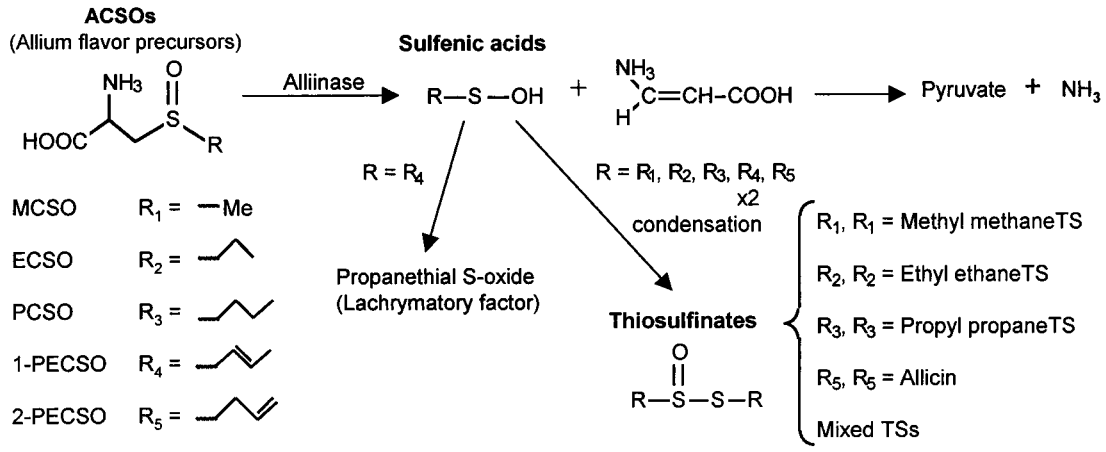

Figure 1. *Allium S*-alk(en)yl-L-cysteine sulfoxides and thiosulfinate formation.

TSs were found to provide nearly all of the antiaggregatory activity of garlic clove homogenate in whole blood. Many of the other organosulfur compounds detected were shown to inhibit aggregation but were not present in sufficient quantities to contribute significantly to the overall antiplatelet effect. Cepaenes, compounds found in larger quantities in onion than in garlic, have also been shown to inhibit in vitro aggregation in platelet-rich plasma (Morimitsu et al., 1992).

In vitro platelet aggregation can be assayed with either a light transmission or an electrical impedance aggregometer. Results from both instruments may exhibit large variances both among and within platelet donors (Goldman et al., unpublished data). Consequently, it is desirable to employ an experimental design with several donors and multiple subsamples. In such a scheme, every treatment is measured multiple times with each donor's platelets. Isolation and purification of individual organosulfur compounds from onion and garlic tissue in large enough quantities for testing in this type of design are difficult. A model reaction system for the synthesis of milligram amounts of bis-TSs [compounds with two identical alk(en)yl substituents flanking the TS functional group] has been developed in our laboratory (Shen et al., 1998). Enough pure TS can be prepared in a single reaction to be tested for inhibition of aggregation at several concentrations using the experimental format described.

Previous investigations have not determined the structure−function relationships of the alk(en)yl substituents in TS antiplatelet activity. Earlier studies have either tested fixed amounts of some TSs (Morimitsu et al., 1992) or measured their IC_{50} values (the concentration necessary to inhibit platelet aggregation by 50%) (Lawson et al., 1992). The principal objective of this study was to compare the dosage response profiles of four bis-TS inhibitors of in vitro platelet aggregation. A second experiment was performed to determine the net effect of combining different bis-TSs on platelet inhibition. The antiplatelet activities of TSs and aspirin were compared in a third experiment.

EXPERIMENTAL PROCEDURES

Preparation of Thiosulfinates. Thiosulfinates were prepared as described (Shen and Parkin, 2000). *S*-Propyl-L-cysteine, *S*-2-propenyl-L-cysteine, and ACSOs were prepared as described (Synge and Wood, 1956; Freeman and Whenham, 1975; Lancaster and Kelly, 1983). MCSO, ECSO, PCSO, and 2-PeCSO were synthesized by oxidizing *S*-alk(en)yl-L-cysteines with hydrogen peroxide. Four bis-TSs were prepared in reactions containing individual ACSO precursors and alliinase [isolated as described by Thomas and Parkin (1994)]. Of these, MMTS is present in freshly cut onion and garlic. PPTS arises in onion, whereas allicin is produced in garlic. EETS has not been detected in *Allium* tissues. The preparations were extracted with chloroform. The chloroform was allowed to evaporate immediately afterward, and TSs were reconstituted in water adjusted to pH 4−5 with dilute HCl. TS preparations were kept at 4 °C and used within 24 h of synthesis. TS purity was >99% when evaluated by ^{1}H NMR. Concentrations were determined by HPLC as described (Shen et al., 1998).

Whole Blood Aggregometry. Blood was drawn from three to five healthy donors by venipuncture (University of Wisconsin Health Sciences Human Subject Committee protocol 199-153) through a 21 gauge needle into a syringe containing the anticoagulant sodium citrate (1 volume of 3.8% sodium citrate to 9 volumes of blood) and mixed gently. An equal volume of Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, and 150 mM NaCl) was added to the blood and mixed by inversion. The blood was stored at 20−22 °C during the experiment and used within 4 h of venipuncture.

In vitro platelet aggregation was measured with a whole blood electrical impedance aggregometer (Chrono-log Corp.). For all experiments, 1 mL of blood/TBS was transferred to cuvettes containing a stirbar. After incubation at 37 °C for 3 min, 150 μL of TS (final concentrations in whole blood are reported), aspirin, or TBS was added. Cuvettes were incubated at 37 °C for a further 4 min. The aggregometer electrodes were then inserted into the blood mixture. Platelet aggregation was induced by adding 5 μg/mL collagen (Chrono-log Corp.). Change in electrical impedance between the electrodes was then recorded over 7 min with stirring. The change in impedance is proportional to the amount of platelet aggregation. The change in impedance at 6 min was used for all calculations. All treatments were evaluated with each donor's blood four times.

Experimental Design. The inhibition of platelet aggregation by various dosages of MMTS, EETS, PPTS, and allicin was determined. Concentrations ranging from no effect to complete inhibition of aggregation were chosen for each compound on the basis of the results of pilot studies. TSs were diluted in TBS immediately prior to testing. Data were analyzed as the percentage inhibition based on a non-TS control to which an equivalent volume of TBS diluent had been added. A multiple regression model of the concentrations of the four compounds versus percentage inhibition of aggregation was constructed. Included in the model were linear, quadratic, and cubic transformations of the concentrations. Indicator columns were added to compare the intercepts and slopes for compounds, donors, and compound*donor interactions. This regression construct enables additional sums of squares analysis for comparing the regression lines of com-

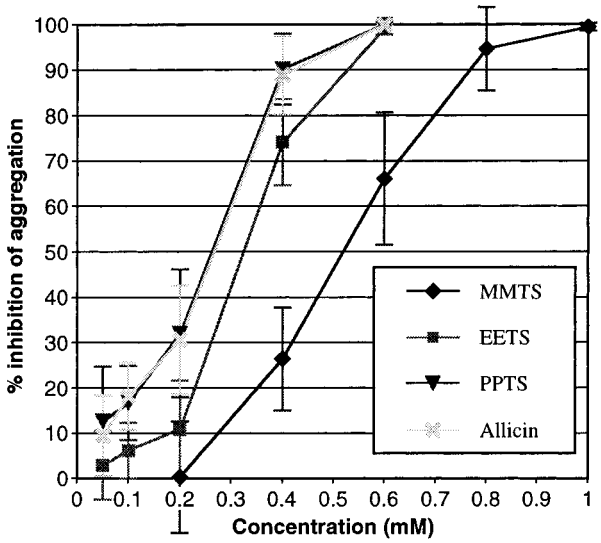

Figure 2. Dosage inhibition of in vitro platelet aggregation by thiosulfinates.

pounds, donors, and compound*donor interaction. Donor and interaction regression terms were pooled when the additional sums of squares test results were insignificant ($p > 0.10$). TS dosage response patterns were compared by determining whether pooling compound terms changed the regression model significantly. The terms were considered significantly different when additional sums of squares p values were <0.05. The IC_{50} was estimated for each TS using a simple regression of concentration versus percent inhibition of aggregation.

To determine whether mixing TSs has an additive effect on platelet aggregation, MMTS, PPTS, and allicin were tested in combinations. MMTS was used at a dosage of 0.4 mM, whereas final concentrations of PPTS and allicin were 0.2 mM. All compounds were tested singly and in all possible two-way combinations. Change in impedance values were analyzed as a multiple two-way interaction using the SAS System for Mixed Models. Donors were considered a random effect and TS combinations a fixed effect.

The platelet inhibitory effect of acetylsalicylic acid (aspirin) was measured at 0.36 mM, the approximate concentration in the blood of a 70 kg human following a dosage of two 325 mg tablets assuming complete absorption into the circulation. Aspirin was dissolved in 95% ethyl alcohol prior to dilution and then diluted in TBS so that the final level of ethyl alcohol in whole blood was 0.0475%. Platelet aggregation was compared to nonaspirin controls containing an equal amount of ethyl alcohol. In the same experiment the antiplatelet activities of MMTS, EETS, PPTS, and allicin were measured at 0.4 mM. The least squares mean for each compound treatment was estimated.

RESULTS AND DISCUSSION

Platelet Aggregation Dosage Response to Thiosulfinates. All four bis-TSs significantly inhibited platelet aggregation but to various degrees (Figure 2). Allicin and PPTS were the most effective inhibitors, with little difference between them. EETS was somewhat less potent, and MMTS was the weakest. At 0.4 mM, MMTS inhibited 26 ± 11% of platelet aggregation, whereas EETS, PPTS, and allicin inhibited 74 ± 10, 90 ± 8, and 89 ± 9%, respectively. Additional sums of squares tests from the regression analysis were insignificant ($p > 0.25$) for compound intercept terms as well as second- and third-order slope terms for donor. Consequently, it was possible to pool these terms in the final regression analysis model for compound dosage response comparisons. Intercept terms for donors were found to be highly significant ($p < 0.001$) and were therefore retained in the model. All PPTS and allicin regression terms were found to be equal (Table 1). All slope terms for MMTS were significantly different from those of the other TSs. EETS differed from PPTS and allicin for first- and second-order terms only.

Table 1. Differential Response of Platelets to Dosages of Four Bis Thiosulfinates[a]

A.

	MMTS	EETS	PPTS	Allicin	
MMTS		*	*	*	H_0: Third order terms equal
EETS	*		NS	NS	
PPTS	*	*		NS	
Allicin	*	*	NS		

H_0: Both second and third order terms equal

B.

	MMTS	EETS	PPTS	Allicin	
MMTS		*	*	*	H_0: First, second and third order terms equal
EETS	NS		*	*	
PPTS	NS	NS		NS	
Allicin	NS	NS	NS		

H_0: Intercepts equal

[a] Additional sums of squares analysis was used to compare terms of the different thiosulfinate regressions of dosage versus inhibition of platelet aggregation. Results from comparisons of third-order regression terms and both second- and third-order regression terms for different pairs of thiosulfinates are above and below the diagonal of ***A***, respectively. ***B*** contains results from comparisons of first-, second-, and third-order terms together and intercept terms above and below the diagonal, respectively. All insignificant result (NS) had p values >0.25. All comparisons resulting in significance (*) had p values <0.001.

Table 2. Predicted Bis Thiosulfinate Platelet Inhibitory IC_{50}[a] Value

compound	predicted IC_{50} (mM)	SD[b]
MMTS	0.55	0.02
EETS	0.32	0.02
PPTS	0.27	0.02
allicin	0.27	0.02

[a] Concentration required to inhibit 50% of platelet aggregation. [b] Standard deviation.

The differential antiplatelet activity of bis-TSs indicated that the alk(en)yl substituents contribute significantly to the inhibitory function of these compounds. The presence of a double bond between carbons 2 and 3 in bis-TSs with substituents consisting of three carbon atoms did not affect activity, as there were no substantial differences between the dosage regressions of PPTS and allicin. Predicted IC_{50} values (the concentration required to inhibit platelet aggregation by 50%) for the TSs are reported in Table 2. Other researchers (Morimitsu et al., 1992; Lawson et al., 1992) have used a logarithmic transformation of concentration in their calculation of IC_{50}. In this study, transformations were not used because they did not improve the predictive value of the regressions and resulted in very similar values. There were substantial differences between the IC_{50} values from our study and those from others. We found the IC_{50} of allicin to be 0.2728 mM, whereas

Table 3. Platelet Inhibitory Activity of Thiosulfinate Combinations[a]

treatment	% aggregation inhibited
MMTS (0.4 mM)	28
PPTS (0.2 mM)	35
allicin (0.2 mM)	27
MMTS (0.4 mM) + PPTS (0.2 mM)	95
MMTS (0.4 mM) + allicin (0.2 mM)	94
PPTS (0.2 mM) + allicin (0.2 mM)	85

[a] Standard error of differences of LS means = 7.2%.

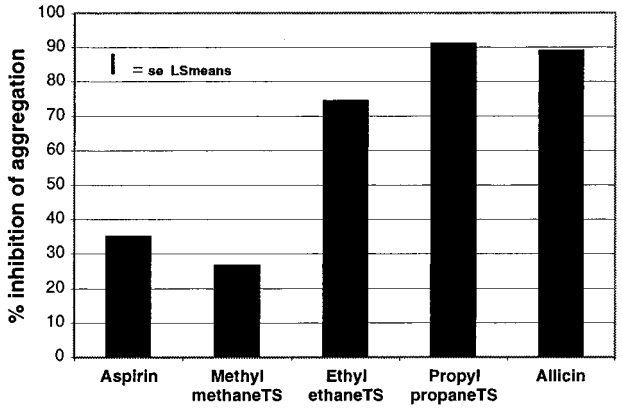

Figure 3. Inhibition of platelet aggregation by aspirin (0.36 mM) and thiosulfinates (0.4 mM).

Lawson et al. (1992) reported a value of 0.09 mM in whole blood. Others have reported values ranging from <0.01 (Mohammed and Woodward, 1986) to >0.4 mM (Block et al., 1986) in platelet-rich plasma aggregometry. In these previous studies, different agonists and agonist concentrations were used, the method of donor sampling is not described, and in some cases it is unclear whether multiple donors were used. This may account for some of the discrepancies in IC_{50} predictions. In this study there was a large variation in the responses of platelets from different donors to the compounds, as indicated by the highly significant donor intercept and first-order slope terms. This demonstrates the importance of including multiple donors in aggregometry studies.

Inhibitory Response of Thiosulfinate Combinations. Combining TSs had greater than an additive effect on inhibition of platelet aggregation (Table 3). The interaction terms for all three two-way combinations were significant ($p < 0.05$). The apparent synergism of TSs may simply be due to the nonlinear nature of the platelet response to dosage. Quadratic and cubic terms were both highly significant in the TS dosage experiment. Alternatively, mixed TS compounds with greater inhibitory effects than the bis forms may be present. Binary mixtures of homologous TSs can undergo exchange reactions to yield mixed, heterologous TSs (Parkin and Shen, 2000). However, this is unlikely to occur in the brief time of incubation (10 min total) and at the pH of blood. The lack of an additive effect of TS combinations suggests that the antiplatelet potential of *Allium* extracts cannot be evaluated by alk(en)yl composition analysis or by quantifying the organosulfur compounds present. A better understanding of the mechanistic basis of the platelet inhibitory effects of TSs and the interactions between TSs is needed.

Comparative Antiplatelet Activities of Thiosulfinates and Aspirin. Platelet aggregation in the presence of the solvent used for aspirin (0.0475% ethyl alcohol in TBS) was not significantly different ($p = 0.3$) from aggregation in the standard control with TBS. EETS, PPTS, and allicin at 0.4 mM inhibited platelet aggregation significantly more than 0.36 mM aspirin (Figure 3; differences in LS means, $p < 0.001$). Percentage inhibition by aspirin was greater than by MMTS ($p = 0.08$). The significantly greater antiplatelet potency of PPTS and allicin compared to aspirin implies that including *Allium* preparations in a form that contains TSs in the diet may be of therapeutic value to those with platelet-mediated cardiovascular disorders. Although some TSs were more potent inhibitors of in vitro platelet aggregation than aspirin, this may not be true in vivo. The efficiency of gastrointestinal absorption of TSs and their fate in the human body are unknown. The in vivo antiplatelet activity and antithrombotic potential of TSs remain undocumented and are the focus of continuing studies.

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