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Perspectivas teóricas na interação entre varfarina e compostos de alho com um estudo in silico de cyp3a4

Theoretical perspectives on the interaction between warfarin and garlic compounds with an in silico study of cyp3a4

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RESUMO

Alho (Allium sativum L.) é um dos suplementos alimentares mais consumidos no mundo e tem múltiplas propriedades biológicas. Entre todas as moléculas obtidas do alho, S-alil-L-cisteína (SAC), S-metil-L-cisteína (SMC) e S-alilmercaptocisteína (SAMC) são destacadas. Existem estudos in vitro e in vivo que correlacionam as interações destas moléculas com drogas medicinais como varfarina pela competição no sítio de ligação das isoenzimas do citocromo P450 (CYP). Varfarina é um anticoagulante oral pertencente à classe dos antagonistas da vitamina K e metabolizada por CYP3A4, 2C9 e 2C19. Este artigo consiste em uma revisão mostrando estudos com extratos e/ou compostos isolados do alho, vias metabólicas e consequências biológicas considerando interações farmacológicas. Os resultados revelaram que os extratos de alho expressam uma atividade inibitória em CYP3A4, 2C9 e 2C19. A inibição da CYP3A4 foi maior que 50% para SAC e SAMC. O experimento in silico foi realizado para SAC, SMC e SAMC na isoenzima CYP3A4 em que SAMC mostrou menor energia de interação (-85,9 Kcal mol^{-1}). (R)-varfarina foi estudada na mesma cavidade molecular deste sítio ativo e mostrou menor valor de energia de interação (-101,1 Kcal mol^{-1}) em comparação com três compostos, o que pode sugerir que varfarina mostrou melhor afinidade com CYP3A4. Consequentemente, SAMC interage melhor com CYP3A4, seguida de SAC e SMC (-80,4 e -70,2 Kcal mol^{-1}, respectivamente). Estes resultados indicam que mercaptocisteína mostra melhor encaixe com o sítio ativo da CYP3A4 humana. Então, estas interações podem potencializar o risco de sangramento em pacientes durante terapia com varfarina, pois alguns dos compostos de alho inibem a CYP responsável pela biotransformação de (R)-varfarina. Estes achados sugerem que o consume de alho deveria ser monitorado em pacientes que recebem terapia anticoagulante com varfarina e os profissionais da saúde devem estar conscientes deste potencial de interação.

Descritores: S-alil-L-cisteína, S-metil-L-cisteína, S-alilmercaptocisteína, Allium sativum L., varfarina, CYP3A4.

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ABSTRACT

Garlic (*Allium sativum* L.) is one of the most consumed food supplements in the world and has multiple biological properties. Among all molecules obtained from garlic, S-allyl-L-cysteine (SAC), S-methyl-L-cysteine (SMC) and S-allylmercaptocysteine (SAMC) are highlighted. There are *in vitro* and *in vivo* studies which correlate the interactions of these molecules with medicinal drugs such as warfarin by competition site in the cytochrome P450 isoenzymes (CYP). Warfarin is an oral anticoagulant belong to vitamin K antagonist class and metabolized by CYP 3A4, 2C9 and 2C19. This article consists in a review showing studies with extracts and/or isolated garlic compounds, metabolic pathways and biological consequences considering drug interactions. The results revealed that garlic extracts express an inhibitory activity in CYP 3A4, 2C9 and 2C19. Inhibition of CYP3A4 were greater than 50% for SAC and SAMC. *In silico* experiment was performed for SAC, SMC and SAMC in the CYP3A4 isoenzyme which SAMC showed lower energy of interaction (-85.9 Kcal mole^{-1}). (*R*)-warfarin was docked in same molecular cavity from this active site and showed lower value of energy interaction (-101.1 Kcal mole^{-1}) in comparison with three compounds, which may suggest the warfarin showed better affinity with CYP3A4. Consequently, SAMC interacts better with CYP3A4, followed by SAC and SMC (-80.4 and -70.2 Kcal mole^{-1}, respectively). These results indicate the mercaptocysteine shows better fit with the active site of human CYP3A4. So, these interactions may potentiate the risk of bleeding in patients during warfarin therapy, because some of the garlic compounds inhibit the CYP enzymes responsible for the biotransformation of (*R*)-warfarin. These findings suggest the consume of garlic should be monitored in patients receiving warfarin therapy and health professionals must be aware of this interaction.

Descriptors: S-allyl-L-cysteine, S-methyl-L-cysteine, S-allylmercaptocysteine, *Allium sativum* L., warfarin, CYP3A4.

INTRODUCTION

Warfarin has been used in anticoagulant therapy in prevention or treatment of chronic conditions like, cardioembolic ischemic stroke, deep vein thrombosis and pulmonary embolism[1]. The necessity for a chronic use reveals a tendency to interactions causing adverse effects, such as, internal bleeding or formation of a clot[2]. Warfarin has narrow therapeutic index and it must be dosed regularly using a blood test called prothrombin time (PT) with an international normalized ratio (INR)[3]. However, several compounds from food, plants and other drugs are able to promote alterations in PT and/or INR levels through direct interaction with warfarin or biological pathway that indirectly affects warfarin actions[4,5]. Considering all compounds able to interact with warfarin, *Allium sativum* L., known as garlic, has been studied over the years[6,7,8].

Allium sativum L. has been used as a food supplement around the world and possess known biological benefits since adjuvant in cancer therapy, blood lipid lowering agent, hypotensive effect and others effects[8,9]. A variety of sulphur and non-sulphur molecules that has been associated with these benefits such as allicin, ajoene, S-allyl-L-cysteine and allyl sulfide[10].

In vitro and *in vivo* studies were developed to understand the behavior of garlic compounds in specific cells and sites of action, like platelet aggregation, liver cytochrome enzymes (CYP) and coagulation

factors[11,12,13]. These studies were conducted with isolated compounds and aqueous/alcoholic extracts, among the extracts, one specific has proven to be beneficial to cardiovascular diseases and it is called an aged garlic extract (AGE)[9].

In vitro and *in vivo* assay are generally used to understand the molecular interactions mechanisms. However, cost, animal use, time, material/ supply achievement and other factors must be considered in these experiments[14]. In this sense, computational tools are interesting approaches based on theoretical methods of calculation in drug design to understand molecular structure and its physicochemical properties with biological interest[15]. Theoretically, *in silico* tests are able to provide the characteristic of the molecules on specific situations. So, it is possible to explain the interaction between warfarin and garlic compounds, since provided that it indicates the likely sites of action[6,7]. In this sense, it is necessary to provide scientific data from the molecules of interest to promote *in silico* assay.

The objective of this review is to discuss the relation of main components of *Allium sativum* and warfarin based in scientific literature and *in silico* evaluation of S-allyl-L-cysteine (SAC), S-methyl-L-cysteine (SMC) and S-allylmercaptocysteine (SAMC) in CYP3A4 isoenzyme to understand this interaction.

1. Material and methods

2.1. Review parameters

This article was developed by searching in PubMed, Scopus, Google Scholar and Scielo databases considering warfarin behavior in the organism and the composition, biological activities, *in vitro* and *in vivo* studies with garlic extracts and isolated compounds and its relation with warfarin action. The review was performed using the following keywords "*Allium sativum"*, "garlic", "warfarin", "CYP enzymes", "platelet activity", "phytochemistry", "platelet aggregation".

2.2. Molecular modeling studies

Computational studies were performed with the main water-soluble compounds of garlic, SAC, SMC and SAMC, aiming to obtain information which assists the understanding of the biological effects. The DFT B3LYP/6-311G* basis in gas phase methodology, evaluable in Spartan'08 for Windows software (Wavefunction Inc., Irvine, USA) was applied in the geometry optimization and conformational analysis. The geometry of compounds was optimized followed by submitting to systematic conformational analysis with torsion angle increment set of 30^{o} in the range $0\text{-}360^{o}$. The lowest energy conformer for chemical structure was saving in mol2 file before to use in docking studies.

The structure of human CYP3A4 encoded by PDB ID: 4D75[16] was downloaded from Protein Data Bank (PDB)[17], before to perform the docking studies, and this 3D structure was prepared by remove the water molecules and adding polar hydrogens using Autodock Tools 1.5.6[18]. Docking studies were performed using iGemdock software[19] in which the Individual binding poses to three compounds was assessed and submitted to dock in the active site of the CYP3A4.

Docking calculations were performed at drug screening Docking Accuracy Setting with GA parameters set for population size, generation and number of solutions as 200, 70 and 3, respectively, and Gemdock score function of hydrophobic and electrostatic (1:1 preference). iGemdock software was used to indicate the pharmacological interactions between CYP3A4 protein and the compounds studied.

BIBLIOGRAPHIC REVIEW

1.1. Cytochrome P450

Cytochrome P450 is a broad and diverse family of proteins responsible for Phase I metabolism where a drug or unknown substance is modified by reactions of oxidation, reduction and hydrolysis turning into a more water-soluble compound for easier excretion (clearance)[20]. These enzymes are responsible for the biotransformation of many important endogenous compounds and play a vital role in the detoxification of numerous xenobiotics. However, these enzymes are able to convert inert compounds to toxic electrophilic derivatives[21,22].

Cytochrome P450 showed different isoenzymes family known as CYPs, indicating the family, subfamily and gene (e.g. CYP2E1) which is affected for the xenobiotic and it started to be studied from the 50's. The initial experiments revealed that some substances cause an induction or inhibition of these enzymes, but the molecular structure was not elucidated[23]. CYPs were purifying from liver microsomes using chromatographic technics and recombinant DNA technology. These approaches were successful to classify the human P450s based on major substrate class, such as, sterols, xenobiotics, fatty acids, eicosanoids and vitamins, also were discovered some enzymes with unknown activity[21]:

- Sterols: 1B1, 7A1, 7B1, 8B1, 11A1, 11B1, 11B2, 17A1, 19A1, 21A2, 27A1, 39A1, 46A1, 51A1
- Xenobiotics: 1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5, 3A7
- Fatty Acids: 2J2, 4A11, 4B1, 4F12
- Eicosanoids: 4F2, 4F3, 4F8, 5A1, 8A1
- Vitamins: 2R1, 24R1, 26A1, 26B1, 26C1, 27B1
- Unknown: 2A7, 2S1, 2U1, 2W1, 3A43, 4A22, 4F11, 4F22, 4V2, 4X1, 4Z1, 20A1, 27C1

This knowledge allowed *in vitro* studies in order to evaluate the effect of drug in cell culture. Therefore, studies for unknown substances can be performed using a recognized drug based in its behavior in specific

CYPs and are identified as CYP probes as described for dextromethorphan (CYP2D6 probe) which this molecule is entirely metabolized by this isoenzyme[24,25].

There are 57 CYPs identified in the cytochrome P450 human and these genes are expressed differently when the human organism are exposed to determinate substance. However, only 6 isoforms are mainly interested in drug metabolism, among them CYP3A4 and CYP2C9 are 43% of the total[26]. If CYP1A2 is included, the three isoforms together are responsible for phase I metabolism and is involved in the clearance of approximately 70% of approved drugs[27].

CYP2C9 is responsible for the metabolic clearance of small lipophilic and acidic compounds such as the selective serotonin reuptake inhibitor fluoxetine[28], the (*S*)-warfarin[29], the lipid lowering drug fluvastatine and many non-steroidal anti-inflammatory drugs (e.g. flurbiprofen, diclofenac, ibuprofen, and naproxen)[30-34].

CYP3A4 acts on most lipophilic substrates and is known to metabolize more than 50% of marketed drugs, while CYP2D6 exhibits a preference for positively charged molecules, usually nitrogenous bases[35]. CYP1A2 and CYP2E1 acts on polyaromatic hydrocarbons (PAH) and smaller molecules, respectively. CYP families 1, 2 and 3 are the main contributors to the metabolism of more than 90% of clinical drugs while other CYP families (CYP4, 7, 11, 17, 19, and 21) are involved in the metabolism of endogenous steroids, bile acids and eicosanoids[21,36,37].

Therefore, CYP3A4 is the most abundant CYP in human liver and small intestine and it is also expressed in lung, stomach, colon, brain and adrenal and it is not expressed in kidney, prostate, testis or thymus. This isoenzyme is responsible for catalyzing the reactions of desaturation, oxidative carboxylic acid ester cleavage and oxidation of a nitrile to an amide and conversion of cholesterol to 4β-hydroxycholesterol[38,39,40]. Considering xenobiotics such as drugs, CYP3A4 is main responsible for the Phase I reaction of many important drugs, like simvastatin, finasteride, cyclosporin, indinavir, sildenafil and warfarin[41-47].

3.2. Hemostasis

The process of homeostasis is a balance between coagulation and fibrinolysis. Normally, inactivated platelets circulate in a discoid shape and possess numerous surface glycoprotein receptors for adhesive ligands, like fibrinogen, collagen or von Willebrand factor (Factor VIII). Therefore, there are membrane receptors to signal transduction and biochemical activation, like adenosine diphosphate receptors (ADP) ($P2Y_1$ and $P2Y_{12}$), thrombin receptors (PAR-1 and PAR-4) and adrenergic receptors[48].

When there is a lesion in a blood vessel, platelets reach and fixate, occurring a signaling process, mainly by the release of Factor VIII, collagen (type I and III) and fibronectin. Factor VIII is rapidly immobilized by collagen, present in the endothelium, and binds to platelets via a surface receptor glycoprotein Ibα (GPIbα), this complex is able to recruit more platelets in a high shear blood flow[49]. Posteriorly, there is an activation of protein kinase C and increased cytosolic calcium, then calcium activates phospholipase A_2 (PLA_2) to liberate arachidonic acid (ARA), which is a substrate, for cyclo-oxygenase (COX-1 in platelets) generating PGG_2 and PGH_2 as initial

products. After, thromboxane synthase acts leading to thromboxane A_2 formation (TXA_2). Also, ARA promotes the release of α- and dense granules, like ADP, factor V, protein S, guanosine triphosphate (GTP), magnesium, calcium and serotonin[50,51]. TXA_2 binds to TP (G-protein coupled) receptor causing a cascade of reactions leading to increase of cytosolic calcium levels.

The release of ADP, TXA_2 and serotonin leads to the recruit of more platelets resulting in formation of factors Va and Xa, phospholipids and calcium complex that activates prothrombin to thrombin (Factor IIa) and this molecule converts soluble fibrinogen (Factor I) to insoluble fibrin. Red blood cells, white blood cells and platelets are united by fibrin which cross-links to factor XIIIa (activated by thrombin) providing structural stability of the clot[52].

After healing the injured vessel, plasmin is able to lysis the thrombus by tissue plasminogen activator (tPA) or urokinase (uPA) generating fibrin degradation products, then ending the coagulation process[53]. There are drugs that interfere on three sites of coagulation: inhibitors of platelet aggregation (e.g. acetylsalicylic acid), inhibitors of thrombin (e.g. heparins, fondaparinux) and activated factor X (e.g. rivaroxaban, edoxaban)[47,54]. Therefore, the interference on vitamin K metabolism is caused by oral anticoagulants drug like warfarin[55].

3.3. Characterization of Warfarin

Warfarin, from coumarinic group, is used in anticoagulant therapy in many countries, with well-established protocols designed for treatment and prevention of various pathologies such as deep vein thrombosis and pulmonary thromboembolism and is available as a racemic mixture under the name of Coumadin®[1].

Warfarin shows almost complete bioavailability and about one hour are detectable in plasma. Some foods, like green vegetables, may interfere with its absorption, because they contain vitamin K in different concentrations. When it reaches the blood circulation, quickly this drug complex almost completely with plasmatic proteins, mainly albumin, and it does not cross the hematoencephalic barrier. In the liver, warfarin inhibits the vitamin K reductase which is responsible for converting the oxidized vitamin K to the reduced form slowing the natural coagulation process, after that it is metabolized by CYP450 isoenzymes: 2C9, 2C19, and 3A4, generating hydroxylated compounds[56,57] (Figure 1).

Warfarin is a racemic drug with equal R:S proportion (1:1) which are metabolized mainly by Phase I reaction. *(R)*-warfarin (CAS: 5543-58-8) is reduced to alcohols products by CYP3A4 and CYP1A2 that are excreted in urine while *(S)*-warfarin (CAS: 5543-57-7) is oxidized to 7-OH-warfarin by CYP2C9, which is eliminated in the bile[58] (Figure 1). Also, over 90% of metabolites are inactive and traces of unmetabolized warfarin are detected in urine[59,60]. The enantiomers of warfarin reduce nearly 30-50% of each vitamin K dependent coagulation factor (II, VII, IX and X) taking 2 to 3 days of treatment to reach the maximum anticoagulant effect[47].

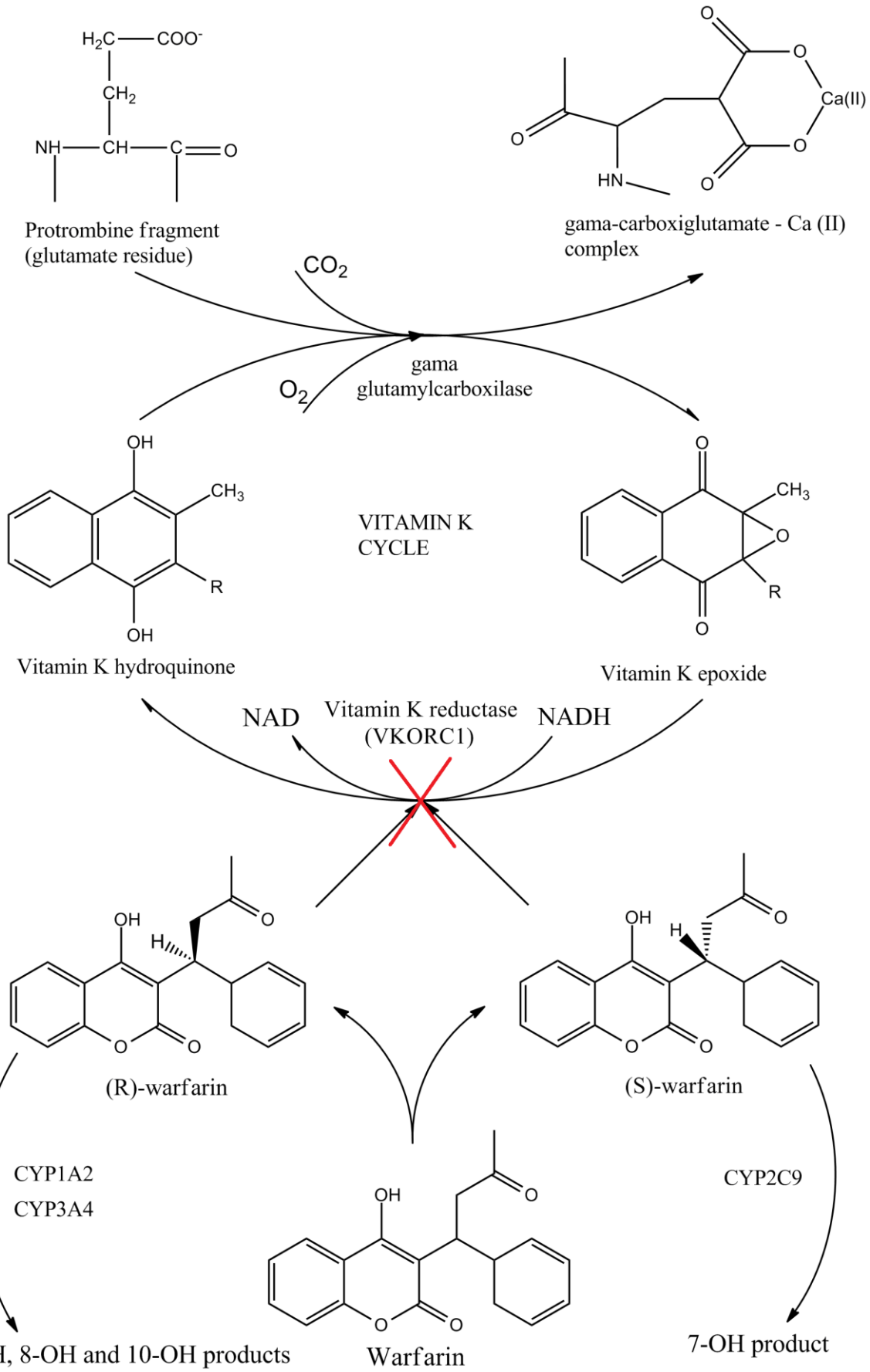

Figure 1 – Biological mechanism of warfarin[47]

Warfarin possesses a low therapeutic index and there is a need for monitoring by the PT levels along with INR standardization. INR is a calculus that correlates the PT of a patient by a known prothrombin time provided by a laboratory. In this calculus, there is an exponent denominated International Sensitivity Index (ISI), a number that represents the quality of the reagents used[3]. According to the equation 1:

$$INR = \left(\frac{PT_{patient}}{PT_{control}}\right)^{ISI}$$

Equation 1 – Determination of INR

Many cardiovascular pathologies require warfarin therapy, however there is not a fixed INR value for all heart diseases. So, specialized clinics and hospitals developed protocols that establish a range of INR values that correlates with the clinical situation, which is showed in the Table 1.

Table 1. Clinical situation and therapeutic range (INR) according to Clinical Hospital of Porto Alegre[61]

Clinical situations	Therapeutic range (INR)
Treatment of DVP (First episode)	2.0 – 3.0
Treatment of DVP (Second episode)	2.0 – 3.0
Chronic atrial fibrillation (persistent or intermittent) or Atrial flutter, in the presence of at least one of the factors: Previous ischemic EVA or TIA CHADS2 or CHADS-VASC scores ≥ 2 Rheumatic valvulopathy	2.0 – 3.0
Biological valve prosthesis	2.0 – 3.0
Rheumatic mitral valve disease with sinus rhythm in the presence of at least one of the factors: Regurgitation: thrombus in left atrium or previous systemic embolism Stenosis: left atrium diameter > 55 mm or previous systemic embolism	2.0 – 3.0
Myocardial infarction with mural thrombus or with severe anterior wall akinesia	2.0 – 3.0
Metallic valve prosthesis in aortic position	2.0 – 3.0
Metallic valve prosthesis in mitral position	2.5 – 3.5

DVP, deep vein thrombosis; EVA, encephalic vascular accident; TIA, transient ischemic attack.

There are some studies considering the interactions of warfarin with herbal medicine and/or food supplement such as garlic (*Allium sativum* L.). Indeed, consume of garlic in patients treated with warfarin or *in vitro*/ *in vivo* assays indicate a potentiation of warfarin effects. However, there are not providing extensive conclusion[62,63].

1.2. Phytocomposition of garlic

Garlic (*Allium sativum* L.) is one of the most consumed food supplement in the world and has multiple biological properties. Garlic consume has been associated with positive results as anticancer and antiparasitic agent as well as hypotensive effect and other biological effects[64-66]. Some garlic compounds are recognized agents which generate positive effects to immunology and hematologic systems in human body[67].

Its main constituents are sulphur-containing (i. e. allicin, aliin, ajoenes, vinyldithiins) and non-sulphur-containing compounds (i. e. allixin and saponins)[68,69]. In the literature, the biological effects are related to the sulphur-containing ones. Therefore, garlic also contains flavonoids/isoflavonoids (nobiletin, quercetin, rutin, and tangeretin), terpenes (e.g. citral, geraniol, linalool, and α- and β-phellandrene), prostaglandins and polysaccharides[70.]

Rahman (2007) also describes that garlic contains proteins, fibres and free amino acids, along with phosphorous, potassium, sulphur, zinc, moderate levels of selenium and vitamins A and C, and low levels of calcium, magnesium, sodium, iron, manganese and B-complex vitamins. Approximately 97% are water-soluble compounds with 0.15-0.7% being oil-soluble compounds[71].

The compound that is found in major proportion is allicin, along with the other sulphur-containing constituents, performs the most biological effects, and, also, is responsible for the characteristic garlic odor[72]. Allicin is not found *in natura* in garlic until its processing such as crushed, cut, chewed, dehydrated, pulverized, or exposed to water which activates the enzyme alliinase that metabolizes alliin to allicin. Then quickly decomposes to other compounds, such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and ajoene[73]. These compounds, as well as residual of intact allicin, react with bacteria from gastrointestinal tract, adding cysteine amino acid forming SAMC[74]. This mechanism is shown in the Figure 2.

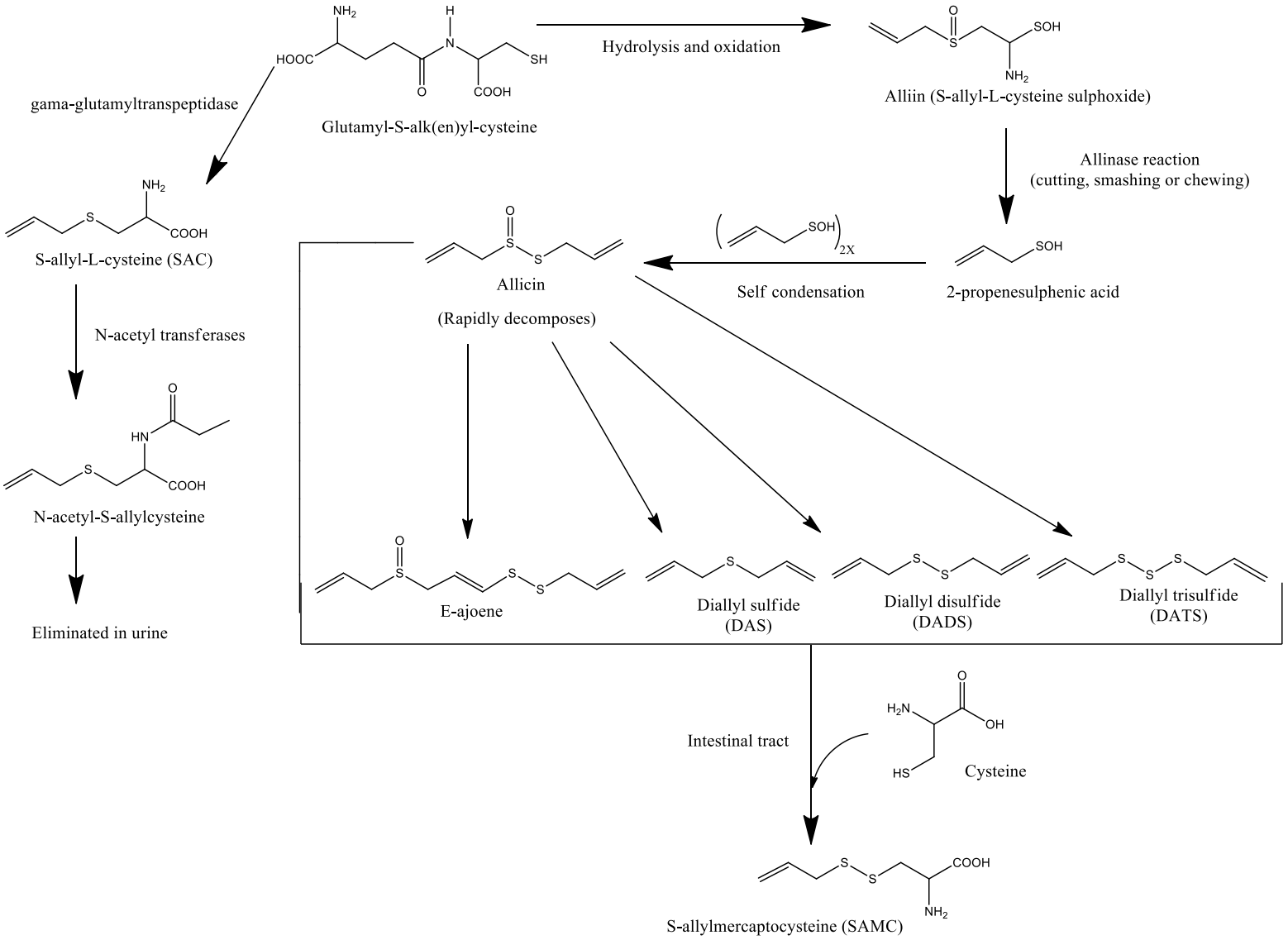

Figure 2 – Formation of organo-sulphur compounds and their respective modifications[67,75]

Another class of compounds, i. e. ɣ-glutamyl-L-cysteine peptides, are present too and once garlic is incubated with a water/ethanol mixture, in time, these peptides are converted to SAC, SMC and SAMC (Figure 3), this is called an aged garlic extract (AGE)[9,76].

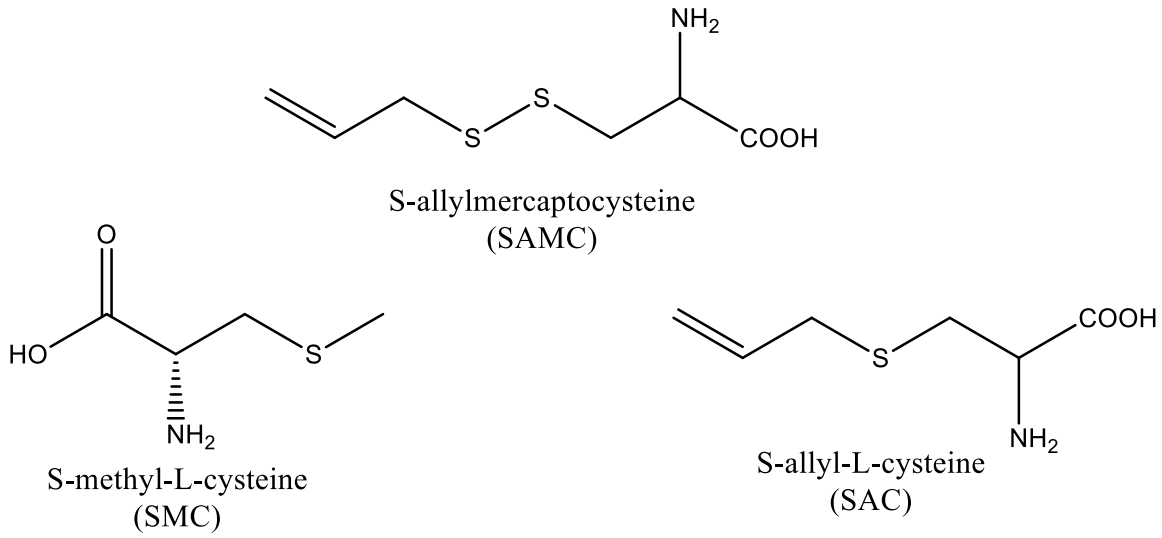

Figure 3 – Molecular structures of water-soluble compounds of garlic (PubChem, 2018)[77,78,79]

Table 2 – Physical and chemical properties of three selected compounds of garlic[80].

Compounds	Log P	Boiling point (°C)	Melting point (°C)	pKa	Solubility (mg/mL)
SAC	-1.78	346.79	145.23	2.53 / 9.14	10.30
SMC	-2.40	304.35	124.45	2.44 / 9.15	57.20
SAMC	-2.43	415.57	179.63	2.04 / 8.90	7.77

SAC, S-allyl-L-cysteine; SMC, S-methyl-L-cysteine; SAMC, S-allylmercaptocysteine.

There are studies *in vitro* and *in vivo* which correlate the interactions of these garlic compounds with metabolic pathways in human and animal organisms, such as, the cytochrome P450 enzymes (CYP450). In warfarin, these interactions may potentiate the risk of bleeding, because some of the garlic compounds inhibit the CYP enzymes responsible for the biotransformation of warfarin enantiomers[81].

2. RESULTS AND DISCUSSION

2.1. Evidence of interaction of garlic extracts and isolated compounds with platelet activity and CYP enzymes

The initial evidence of change in blood parameters linked to consumption of garlic came in the form of case reports, initially the signs observed were prolonged bleeding episodes and decreased platelet aggregation, however it was considered to be of minimal clinical significance until the early 2000s[82]. Over the years, other studies evaluated the mechanism of CYP enzymes for a better understanding of herb-drug interactions and its implications in anticoagulant therapy with warfarin[83-85].

A study of Borrelli, Capasso and Izzo (2007) shows that garlic has complex cardiovascular effects in antiplatelet, antithrombotic and fibrinolytic activity[86]. Three cases were reported:

- A spontaneous spinal epidural hematoma causing paraplegia secondary to a qualitative platelet disorder from excessive garlic ingestion;
- An increase of the PT was observed in a healthy 32 years old woman six day before a cosmetic surgery. The subject admitted to heavy garlic dietary intake preoperatively and was taken completely off garlic.
- A case of bilateral retrobulbar hemorrhage with elevated intraocular pressure during strabismus surgery has been reported in a 54 years old woman. The patients stated that he had been taking odorless garlic tablet ingestion prior to surgery. On the day prior to surgery, she had consumed five tablets (approximately 5 g of an equivalent fresh bulb). So, excessive garlic intake can be associated with coagulation alterations.

Garlic compounds in tablets and capsules shows inhibitory activity on the CYP3A4, CYP2C9 and CYP2C19 enzymes[87]. Also, platelet aggregation was studied using alcoholic and aqueous extracts of garlic when induced by arachidonic acid, collagen, ADP, adrenaline and A23187* (*calcimycin: a mobile ion-carrier that forms stable complexes with divalent cations and it is produced through fermentation of *Streptomyces chartreusensis*). The results showed greater inhibitory activity of alcoholic extract of garlic compared to the aqueous form[88]. Torres-Urrutia and colleagues (2011) observed the antiplatelet activity of a methanolic extract of garlic which platelets activated by ADP had a 25.2% of inhibition while no effect was observed with arachidonic acid[89].

A study of Markowitz and colleagues (2003) with administration of tablets containing garlic extract in 600 mg twice daily for 14 days with 14 healthy volunteers for evaluation of CYP3A4 and CYP2D6 behavior revealed no significant differences in their pharmacokinetics. Also, it was evaluated the allicin concentration in the tablets after aqueous dissolution assay using 3 tablets and 15 mL aqueous (0,5% phosphoric acid with pH 1.5 to inhibit alliinase activity during 30 min). Each tablet was described to possess 600 μg of allicin, however a medium value of 300 ± 60 μg per tablet was found, confirming the instability this molecule. Therefore, no change was observed for SAC levels[90]. In another study, Amagase (2006) verified that when allicin itself was kept at 20°C for 20 h, it decomposed to DADS (66%), DAS (14%), DATS (9%), and sulfur dioxide[91]. Similar behavior is also described in intestinal bacteria with alliinase activity[92].

Considering platelet function, this parameter was measured based in consume of capsule containing 9.9 g of garlic in two different times: before and after 4 hours from consumption. Adrenaline-induced platelet aggregation was found to be significantly reduced ($p < 0.05$). There was no change in platelet aggregation in response to ADP or collagen[93]. In vitro studies found that fresh garlic, odorless garlic tablets, garlic oil capsules, freeze dried garlic, and AGE exhibited inhibitory effects on CYP2C9*1, 2C19, 3A4, 3A5, and 3A7.

Furthermore, it was performed an experiment with garlic extracts in different concentrations and immortalized human cells lineage (Fa2N-4) which also expressed inhibitory effect on CYP2C9, but not on CYP3A4 in the conditions studied[62]. In a study developed by Leite and colleagues (2016), E-ajoene was isolated together other three compounds, expressed a non-competitive inhibitory activity in the COX interfering with the formation of prostaglandin H_2, a precursor of the thromboxane A_2[94]. Another compound, allicin (S-allyl cysteine sulphoxide), is responsible for the inhibitory platelet aggregation without affecting the COX, lipoxygenase, thromboxane synthetase, vascular prostacyclin and cyclic AMP levels[95].

Wu and colleagues (2002) performed a test in rats who received garlic oil with DAS, DADS and DATS three times a week for six weeks and confirmed by immunoblot that there was an induction of four liver enzymes (CYP1A1, CYP2B1, CYP 2E1 and CYP 3A1). Even, the correlation between the enzyme activity modulation and the number of sulphur atoms was observed, then DAS showed greater induction compared to the others[35].

Considering health effects from AGE compounds, Allison and colleagues (2012) observed that AGE inhibits platelet activation by increasing intracellular cAMP and reducing the interaction of GPIIb/IIa receptor with fibrinogen[96]. This experiment was conducted with a pool of platelets collected in healthy volunteers who had not taken any medication that could interfere with platelet function for two weeks prior to blood donation. Platelets were treated with aggregation agonists and AGE at concentrations ranged 1.56% to 25% (v/v). In addition, fluorescence and immunoassay technics were also used for complementary results. The effect of AGE on ADP-activated platelets showed that concentrations between 3.12 and 12.5% significantly reduced the number of platelets bound to immobilized fibrinogen by approximately 40% when compared to the control ($p<0.05$)[96].

Steiner and Li (2001) evaluated the effect of AGE in platelets too by a double-blind, crossover, placebo-controlled experiment with n = 34 (men and women) using SAC as a marker level for 44 weeks. The first 6 weeks there was no supplementation, for the next 6 weeks, the participants received 3 capsules per day of AGE (800 mg), increasing to 6 capsules per day on the next 6 weeks and to 9 capsules per day on the next 6 weeks, there was a 2 weeks washout period and then the treatment with the placebo started with the same conditions of the AGE administration. Blood was sampled every 2 weeks and processed for platelet aggregation and adhesion studies, as well as SAC measurement levels[97].

Their findings showed that platelet adhesion, evaluated with collagen, von Willebrand factor and fibrinogen-coated surfaces, for low-shear rates (30 platelets per second), there was a small, but significant reduction of platelet adhesion when 4.8 to 7.2 g AGE per day were consumed. The adhesion to fibrinogen was inhibited by AGE at all concentrations tested, for the last dosage (7.2 g per day) it was reduced by 33% compared

to baseline or placebo group. For the von Willebrand factor, the reduce in platelet adherence was only significant at the higher dosage (7.2 g per day). For the correlation of SAC levels in AGE supplementation, the SAC level achieved higher serum concentration at 4.8 g AGE per day (~140 ppb) when compared to baseline (~60 ppb), also is important to reveal that the placebo group also showed and increase on SAC levels with the rise of AGE consumption[97].

Moreover, an experiment using eight water soluble compounds (alliin, cycloalliin, methylin, SMC, SAC, N-acetyl-S-allyl-L-cysteine, SAMC, γ-glutamyl-S-allyl-L-cysteine) evaluated the CYP enzyme behavior in the 1A2, 2B6, 2C9, 2C19, 2D6 and 3A isoforms on human liver microsomes, and revealed that only SMC and SAC caused an inhibition greater than 50% on CYP3A4. Other compounds presented a lower than 50% ratio[98].

Different mechanisms may be evolved in coagulation, liver enzymes inhibition or induction. Once this is related to warfarin therapy, considering its pharmacologic behavior, is very concerning because garlic compounds may potentialize the anticoagulation effect leading to internal hemorrhage[71-73,78,87]. Also there is another topic to consider that is the way garlic is prepared and consumed.

Firstly, crushing, dehydrating, cutting or chewing the cloves releases aliinase, a vacuolar enzyme, reacts with alliin generating sulphenic acid intermediates and then this compounds form thiosulphinates, mainly allicin. There is a rapidly decomposition which other compounds are generated and another pathway promotes the reaction between γ-glutamylcysteine and γ-glutamyltranspeptidase that generates SAC (Figure 2)[67,74].

Cavagnaro and colleagues (2007) studied the effect of cooking on garlic antiplatelet activity and thiosulfinates content, compounds generated from allicin. This study evaluated three cooking methods (convection oven, boiling and microwave oven) on two preparations of garlic tissues: uncrushed (whole cloves) and crushed garlic. The results for convection oven were that uncrushed and crushed garlic until 3 min of heating showed no difference in inhibition of platelet aggregation activity compared to raw sample. In 6 min the crushed oven-heated sample had a fall from ~70% to ~20% of inhibitory activity and uncrushed oven-heated sample fall from over 80% to 0%. For boiling treatment, the results were similar to convection oven as to microwave oven. All methods rapidly degraded allicin content, but addition of raw garlic to microwaved-uncrushed garlic restored a full complement of antiplatelet activity that was completely lost without the raw garlic addition, suggesting that special attention to garlic preparation for consumption can improve its nutritional value[99].

All these studies indicate there is an interaction of garlic extracts or isolated compounds with platelet activity and CYP modulation. The main effect of garlic, especially SAC, SMC and SAMC, is the inhibition of CYP3A4, the same isoenzyme that metabolizes the (*R*)-warfarin. Although it was not determined which dose/frequency of these garlic compounds affects the biological pathways related to coagulation, it was showed that the interaction between garlic compounds and warfarin therapy may be harmful to the patient. We suggest that this behavior could be observed in clinical analysis laboratories by alterations of INR values depending on patient clinical conditions, similar to descripted on the literature[100]. In this sense *in silico* studies can be a

complement assay, indicating possible common binding sites and consequently predict adverse pharmacological effects.

4.1. In Silico Studies: A case of CYP3A4 and S-allyl-cysteine derivatives interactions

Molecular docking studies were performed on the three bioactive compounds using the conformer of minimal energy of both derivatives, generated by systematic search in Spartan software, which were used as individual poses. According to Bibi (2008) the CYP3A4 is the most abundantly *drug metabolizing* enzyme in humans which is responsible for the breakdown of over 120 different drugs, and therefore the inhibition may result in toxic effects[101]. Docking procedure that selectively bind to the active site of each poses of compounds were assessed and their interactions in the active site of the enzyme were analyzed.

A molecular docking model of SAC, SMC, and SAMC ligands and interactions with CYP3A4 (using PDB model code: 4D75) are showed below and may be used to understanding the affinity to active site of protein. All molecular docking data are showed in Table 3 and 4. The order of interaction is SAMC, which showed lower energy of interaction (-85.9 Kcal mole^{-1}) in comparison with other two, followed by SAC and SMC (-80.4 and -70.2 Kcal mole^{-1}, respectively). These results indicate the mercaptocysteine shows better fit with the active site of human CYP3A4.

Chemical structure of (*R*)-warfarin was docked in same molecular cavity from this active site and this showed higher value of energy interaction (-101.1 Kcal mole^{-1}) in comparison with three compounds, which may suggest the warfarin showed better affinity with CYP3A4. The main interactions generated by this model are observed are showed in Table 4, which the main pose of three compounds are located in molecular region near to heme from CYP3A4 (Figure 4). In this case, the hydrogen bonding interactions happen with heme, and with ILE 369 in case of SAMC. Electrostatic interactions were observed to all allyl and methyl derivatives but not with (*R*)-warfarin.

Other interaction such as van der Waals are observed with ARG 105, SER 119, ARG 212, ALA 305, THR 309, and with heme of central core of CYP3A4. ARG 105 is an important amino acid residue involved in interaction of CYP3A4 with the drugs daunorubicin and cytarabine and ARG 212 is also identified as an important in the binding of substrates to this enzyme[102,103]. Kaur and colleagues (2016) reported the ALA 305 and THR 309 as additional amino acid residues involved in this CYP inhibition[16].

In case of study performed in this work, the prediction of interaction with the same residues described in literature may suggest a molecular mechanism of action of allyl and methyl derivatives. These data suggest also the S-allyl and methyl cysteine derivatives which are the components of garlic water extract may be one of main compounds causing inhibition of CYP3A4. Our *in silico* findings showed that S-allyl and methyl cysteine derivatives may cause CYP3A4 inhibition. So, it is possible these molecules interfere in warfarin metabolization, specifically (*R*)-warfarin, considering CYP3A4 is an isoenzyme responsible for warfarin biotransformation.

Consequently, period of warfarin activity may be increase considering this interaction. According to other studies, bleeding risk is increased when consume of garlic is associated with warfarin therapy[86,91].

Table 3. Docking results and the main interactions of van der Waals, H-bonding and electrostatic to cysteine derivatives from garlic in human CYP3A4 (4D75), using IGemdock software.

Compounds	**Affinity Energy (Kcal mole^{-1})**	**VDW**	**H-bonding**	**Electrostatic**
SAMC	-78.19	-53.68	-18.98	-5.33
SAC	-73.20	-48.27	-18.85	-6.08
SMC	-70.21	-43.88	-20.53	-5.81
(R)-warfarin	-101.1	-75.9	-25.2	0.0

SAC, S-allyl-L-cysteine; SMC, S-methyl-L-cysteine; SAMC, S-allylmercaptocysteine; VDW, van der Waals.

Table 4. Central pharmacological interactions and residues involved in the binding site between cysteine derivatives from garlic in human CYP3A4 using IGemdock 2.1 and visualization using Chimera (v. 1.10.1).

Compounds	Main Molecular Interactions							
	H-bonding		van der Waals					
	ILE 369	Heme	ARG 105	SER 119	ARG 212	ALA 305	THR 309	Heme
SAMC	-3.3	-32.7	0.0	-0.5	-1.0	-7.7	-4.2	-29.8
SAC	0.0	-47.5	-0.3	-0.2	0.0	-4.8	-2.6	-16.0
SMC	0.0	-42.5	-0.5	-0.2	-0.1	-4.2	-1.4	-7.2
(R)-warfarin	0.0	-25.2	-4.4	-4.7	-4.5	-2.9	-1.5	-10.6

ALA, alanine; ARG, arginine; ILE, isoleucine; SER, serine; THR, threonine.

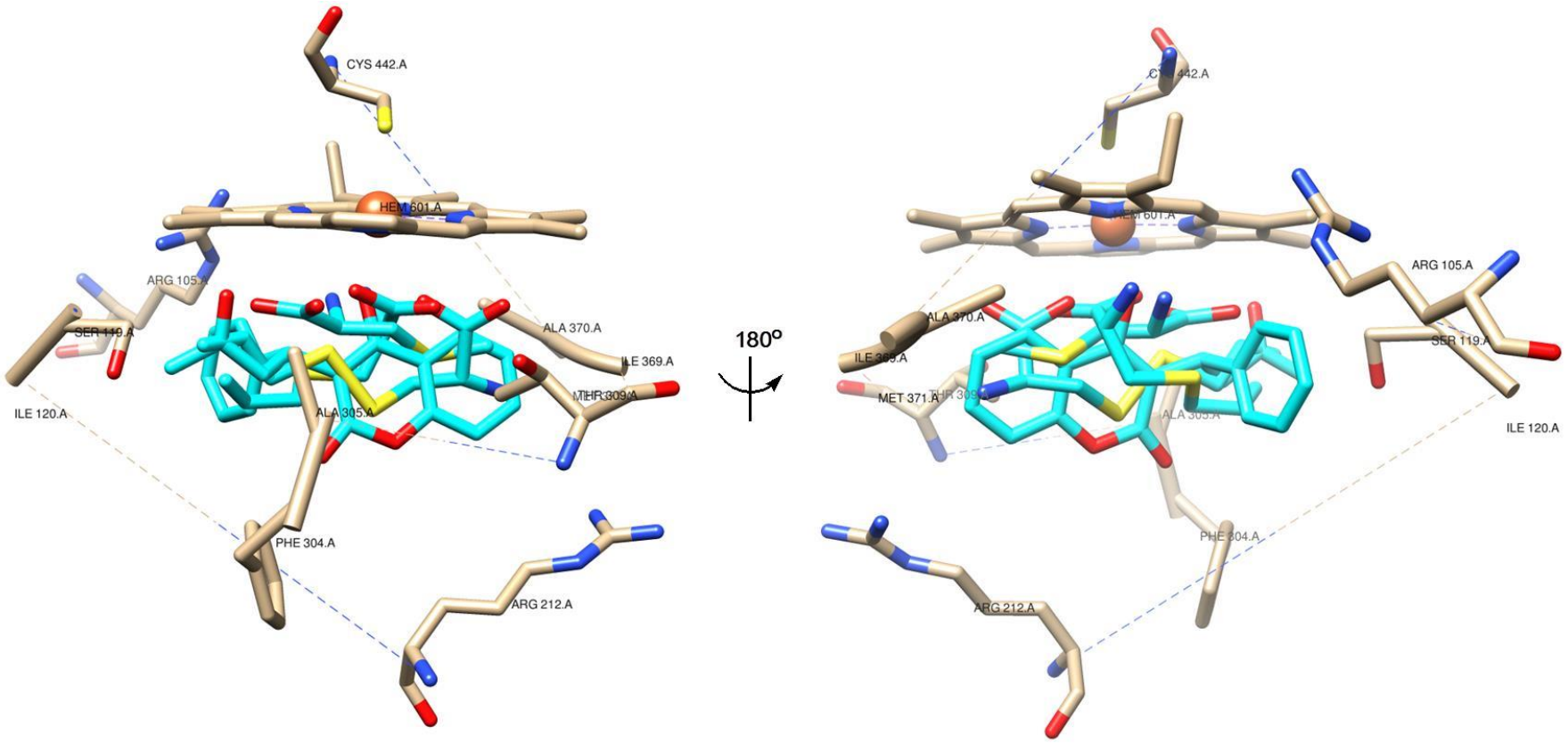

Figure 4. Binding poses of S-allyl, methyl cysteine derivatives and *(R)*-warfarin in active site of human CYP3A4 (PDB code: 4D75) calculated by Igemdock 2.1 and visualization using Chimera (v. 1.10.1).

CONCLUSION

These findings indicate that the consume of garlic should be monitored in patients receiving warfarin therapy and health professionals as well as patients must be aware of this interaction. *In silico* study suggests the interaction of SAC, SMC and SAMC with CYP3A4 promoting inhibition of warfarin metabolization. Consequently, there is a possibility to increase of prolonged warfarin effect, potentializing bleeding risk. However, *in silico* studies are not allow to evaluate dose and/or combined garlic compounds may cause adverse effects. Also, studies with platelet aggregation and cyclo-oxygenase enzymes would be necessary to complement the understanding on the biological effect of garlic compounds, mainly on warfarin therapy.

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