

In Silico Design and SAR Study of Dibenzyl Trisulfide Analogues for Improved CYP1A1 Inhibition

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Dibenzyl trisulfide (DTS) is a natural compound with potential cancer-preventive properties occurring in *Petiveria alliacea* L., an ethnomedicinal plant native to the Americas. Previous studies revealed its inhibitory activity toward cytochrome P450 (CYP)1 enzymes, key in the activation of environmental pollutants. Accordingly, the aim of this study was to design novel DTS analogues, aimed at improving not only inhibitory activity, but also specificity toward CYP1A1. This was achieved by targeting interactions with CYP1A1 residues of identified importance.

Three-dimensional structures for the novel analogues were subjected to molecular docking with several CYP isoforms, before being ranked in terms of binding affinity to CYP1A1. With three hydrogen bond donors, two hydrogen bond acceptors, a molecular mass of 361 Da, and a log P of 3.72, the most promising DTS analogue obeys Lipinski's rule of five. Following synthesis and in vitro validation of its CYP1A1-inhibitory properties, this compound may be useful in future cancer-preventive approaches.

Introduction

Cytochrome P450 (CYP) enzymes are a superfamily of enzymes capable of metabolizing a plethora of foreign chemicals (xenobiotics), including over 80% of clinically used drugs.^[1] They catalyze a vast range of reactions including hydroxylation, oxidation, and the conversion of various organic compounds. The CYP1 family of enzymes, containing the members CYP1A1, CYP1A2, and CYP1B1, is known to metabolize procarcinogens found in many environmental pollutants.^[2,3] These include benzo[a]pyrene, which is typically found in cigarette smoke. Catalysis by CYP1 enzymes often activates these entities into oxygenated radicals that are capable of DNA binding, ultimately leading to mutations that initiate tumorigenesis.^[4,5] In fact, CYP1 enzymes were revealed to be overexpressed in breast cancer cells with respect to surrounding tissue.^[6] Thus, CYP1 inhibitors can have an effect on this cancer-initiating process, leading to their identified role in chemoprevention.^[7,8]

Numerous natural products have been evaluated as inhibitors of CYP1 enzymes. Herbal medicines, representing a potent source of these natural products, are commonly used around the world. For example, dibenzyl trisulfide (DTS) is a natural compound with potential cancer-preventive properties occurring in *Petiveria alliacea*,^[9–11] an ethnomedicinal plant native to the Americas. It is colloquially known as guinea hen weed in


Jamaica,^[12] an island country where it was reported that 73% of the population engages in self-medication with the use of ethnomedicines.^[13] Extracts of *P. alliacea*, as well as its primary active compound DTS, were found to substantially inhibit tumor growth.^[14–17] The inhibitory activity of DTS toward key CYP enzymes responsible for drug metabolism has been evaluated, including CYPs 1A2, 2C19, and 3A4.^[18] Furthermore, a previous in vitro, in vivo, and in silico study revealed its ability to reversibly and competitively inhibit the CYP1A family of enzymes, binding in the active site in close proximity to the heme group, thus showing potential in chemoprevention.^[19]

Of the three CYP1 enzymes, CYP1A1 has been studied the most, and inhibiting its activity has been strongly associated with chemoprevention, including in breast cancer.^[20,21] Considering the similarity of CYP1A enzyme binding pockets,^[22,23] off-target effects can be an issue when attempting to selectively inhibit a specific isoform. CYP1A2 is involved in the metabolism of numerous therapeutics and, thus, identifying specific inhibitors of CYP1A1 is of paramount importance to avoid drug interactions with those subject to CYP1A2 catalysis. CYPs 1A1 and 1B1 (responsible for the metabolism of polycyclic aromatic hydrocarbons) are notoriously inhibited by the same compounds owing to their similarly narrow and long active site, despite CYP1A2 (responsible for the metabolism of aromatic amines), with a more compact active site, sharing greater amino-acid sequence identity with CYP1A1.^[22,24]

In silico pharmacology represents an important component of the drug discovery pipeline. Considering the time-, cost-, and labor-intensive nature of conventional drug discovery strategies,^[1,2] in silico methods present the potential to accelerate the identification of lead compounds of pharmacological interest. Thus, computer-aided drug design entails the discovery and optimization of small molecules using a suite of computational tools, one of which is molecular docking.^[3] Molecular docking enables predicting the binding conformation of ligands (e.g., small molecules and drugs) to an identified binding pocket (e.g., the active site of an enzyme). In

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combination with an evaluation of structure–activity relationships (SAR),^[4] a small molecule can be rapidly modified *in silico* to determine its optimal functionality and size.

Accordingly, the aim of this study was to design novel DTS analogues primarily by functional modifications of its benzyl rings, in an attempt to improve not only its inhibitory activity, but also its specificity toward CYP1A1 with respect to other CYP1 enzymes, as revealed through molecular docking studies and SAR analyses. The novel DTS analogues were designed through functional modifications of the two benzyl rings, focusing on the targeting of CYP1A1 residues of identified importance (see Figure 1). Furthermore, an elongation of the chain, attempting to take advantage of CYP1A1's narrow and long active site, was also evaluated.

Results and Discussion

Molecular Docking of DTS Analogues to CYP1A1

According to a previous molecular docking study evaluating CYP1A1 and DTS,^[5] their binding is mediated by several hydrophobic interactions, primarily π – π stacking interactions with phenylalanine residues conserved across the CYP family (Phe-123, Phe-224, and Phe-258 in CYP1A1). Thus, to improve both inhibitory activity and specificity, the goal of functionally modifying DTS was to supplement these interactions whilst targeting other conserved and non-conserved residues in the CYP1A1 active site (see Figure 1).

Thus, following the design and generation of structural coordinates for a series of novel DTS analogues as described in the Experimental Section, they were subjected to molecular docking into the CYP1A1 active site. The results are presented

in Table 1, indicating the compound IDs, functional modifications, structures, and binding affinity to CYP1A1.

Any modifications resulting in an elongation of the molecule through the addition of a ring (e.g., a 1,3-benzothiazole ring, a 2,1,3-benzoxadiazole ring, or a 1,3-thiazolo[5,4-*b*]pyridine ring) typically resulted in a slight improvement in binding affinity to CYP1A1, whereas modification of a benzyl ring to a pyrazine ring resulted in a reduction in binding affinity. On the other hand, substantial improvements in binding affinity were obtained upon elongation of the chain connecting the two benzyl rings to a prop-2-en-1-yl chain on one or both sides of the three sulfur atoms.

The addition of an electronegative (4-fluoro) group to one or both benzyl rings also substantially improved binding affinity, in line with the efficacy in inhibiting tumor growth observed for fluorapacin,^[6] an anticancer drug candidate. This effect was synergistic with modifications resulting in an elongation of the molecule.

Lastly, although the addition of a hydrogen-donating group (3-hydroxyl) and/or a hydrogen-accepting group (5-amino) only moderately improved binding affinity, these modifications were hypothesized to lead to an improvement in CYP1A1 specificity by directly targeting hydrogen-bonding interactions with Asn-222 (hydrogen acceptor) and Ser-122 (hydrogen donor), respectively, residues which are not conserved in CYPs 1A2 and 1B1, respectively.

Molecular Docking of Shortlisted DTS Analogues to CYP Isoforms

Upon completion of the molecular docking evaluation of the novel DTS analogues with CYP1A1, the six compounds with the three highest binding affinities were shortlisted for further evaluation with other CYP isoforms commonly involved in drug metabolism (CYPs 1A2, 1B1, 2C9, 2C19, 2D6, and 3A4; see Table 2). As previously outlined, these favorable modifications involved combinations of functional group additions, ring extensions, and linker chain extensions: Compound 1-5 (4-fluoro group + 2,1,3-benzoxadiazole ring), Compound 1-7 (4-fluoro group + prop-2-en-1-yl chain), Compound 4-7 (1,3-benzothiazole ring + prop-2-en-1-yl chain), Compound 5-7 (2,1,3-benzoxadiazole ring + prop-2-en-1-yl chain), Compound 7 (prop-2-en-1-yl chain), and Compound 7-7 (2 × prop-2-en-1-yl chain).

In addition, modifications deliberately introduced to improve CYP1A1 specificity with regard to other CYP1 isoforms were evaluated in comparison with CYPs 1A2 and 1B1 to confirm their effect. These favorable modifications involved the addition of 3-hydroxy and 5-amino groups (Compound 8-9) to opposite benzyl rings, in an attempt to target hydrogen-bonding interactions with Asn-222 and Ser-122, respectively. To incorporate the improved binding affinity due to molecule elongation, these modifications were also combined with extension of the linker extension to a prop-2-en-1-yl chain on one or both sides of the sulfur molecules (Compounds 7-8-9 and 7-7-8-9).

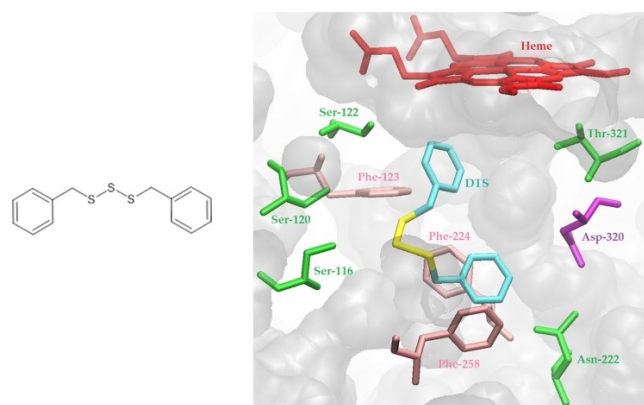


Figure 1. Left: Chemical structure of dibenzyl trisulfide (DTS), revealing its two benzyl rings linked by a chain featuring three S atoms. Both structural elements were targeted by functional modifications in an attempt to improve its inhibitory activity and specificity toward CYP1A1. Right: Amino-acid residues in the CYP1A1 active site identified as important for binding or with potential for targeting are highlighted in the best theoretical binding pose achieved for CYP1A1 (surface representation (gray), along with heme in stick representation (red)) and DTS (stick representation, colored according to atom type: C—cyan; S—yellow). Nonpolar aromatic residues (pink), acidic residues (purple), and polar uncharged residues (green) are shown in stick representation.

Table 1. Compilation of DTS analogues, highlighting their functional modifications, structures, and binding affinity to CYP1A1. Analogues showing equal or superior affinity compared to DTS are highlighted in bold.

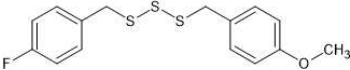
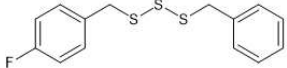
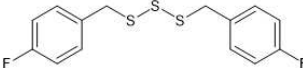
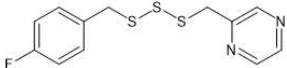
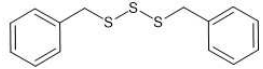
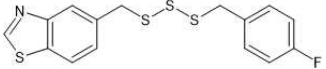
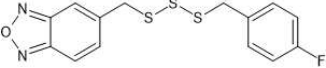
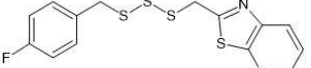
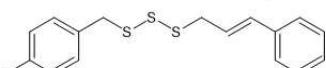
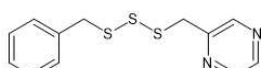
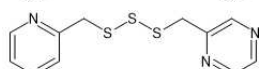
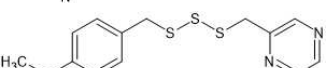
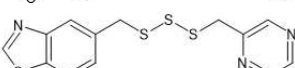
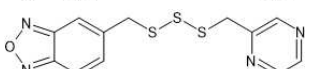
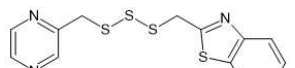
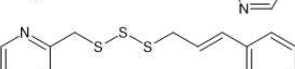
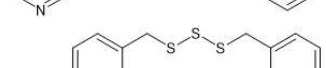
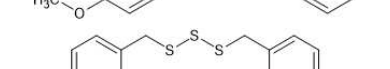
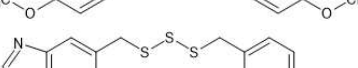
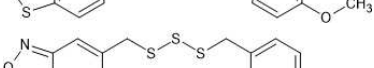
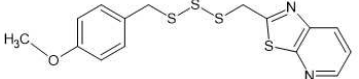
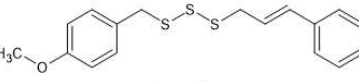
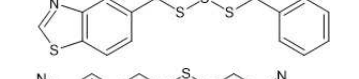
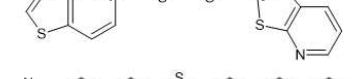
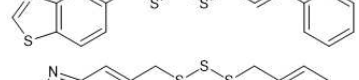
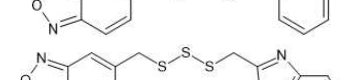
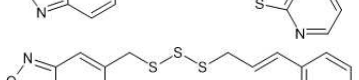
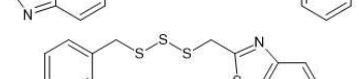
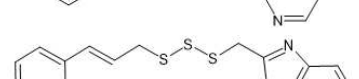
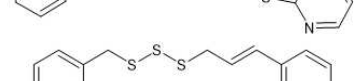
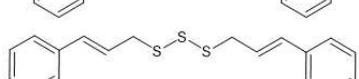
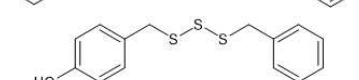
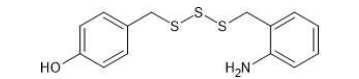
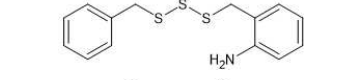
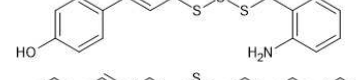
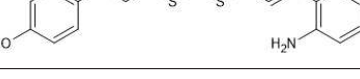
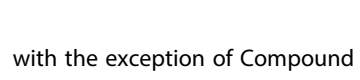
Compound ID	Functional Modification	Structure	Binding Affinity to CYP1A1 (kJ·mol ⁻¹)
0 (DTS)	None		-40.17
1	4-Fluoro group		-41.00
1-1	2×4-fluoro group		-42.26
1-2	4-Fluoro group + pyrazine ring		-36.82
1-3	4-Fluoro group + 4-methoxy group		-41.42
1-4	4-Fluoro group + 1,3-benzothiazole ring		-42.26
1-5	4-Fluoro group + 2,1,3-benzoxadiazole ring		-42.68
1-6	4-Fluoro group + 1,3-thiazolo[5,4-b]pyridine ring		-42.26
1-7	4-Fluoro group + prop-2-en-1-yl chain		-42.68
2	Pyrazine ring		-35.15
2-2	2×pyrazine ring		-28.45
2-3	Pyrazine ring + 4-methoxy group		-33.89
2-4	Pyrazine ring + 1,3-benzothiazole ring		-35.15
2-5	Pyrazine ring + 2,1,3-benzoxadiazole ring		-36.40
2-6	Pyrazine ring + 1,3-thiazolo[5,4-b]pyridine ring		-34.73
2-7	Pyrazine ring + prop-2-en-1-yl chain		-36.40
3	4-Methoxy group		-40.17
3-3	2×4-methoxy group		-38.91
3-4	4-Methoxy group + 1,3-benzothiazole ring		-39.33
3-5	4-Methoxy group + 2,1,3-benzoxadiazole ring		-39.75

Table 1. continued			
Compound ID	Functional Modification	Structure	Binding Affinity to CYP1A1 (kJ·mol ⁻¹)
3-6	4-Methoxy group + 1,3-thiazolo[5,4- <i>b</i>]pyridine ring		-39.75
3-7	4-Methoxy group + prop-2-en-1-yl chain		-41.42
4	1,3-Benzothiazole ring		-41.42
4-6	1,3-Benzothiazole ring + 1,3-thiazolo[5,4- <i>b</i>]pyridine ring		-39.33
4-7	1,3-Benzothiazole ring + prop-2-en-1-yl chain		-43.10
5	2,1,3-Benzoxadiazole ring		-41.84
5-6	2,1,3-Benzoxadiazole ring + 1,3-thiazolo[5,4- <i>b</i>]pyridine ring		-41.00
5-7	2,1,3-Benzoxadiazole ring + prop-2-en-1-yl chain		-43.10
6	1,3-Thiazolo[5,4- <i>b</i>]pyridine ring		-41.00
6-7	1,3-Thiazolo[5,4- <i>b</i>]pyridine ring + prop-2-en-1-yl chain		-41.42
7	Prop-2-en-1-yl chain		-43.93
7-7	2 × prop-2-en-1-yl chain		-43.93
8	3-Hydroxyl group		-40.58
8-9	3-Hydroxyl group + 5-amino group		-41.00
9	5-Amino group		-40.17
7-8-9	Prop-2-en-1-yl chain + 3-hydroxyl group + 5-amino group		-42.26
7-7-8-9	2 × prop-2-en-1-yl chain + 3-hydroxyl group + 5-amino group		-43.10

As presented in Table 2, DTS exhibited overall good binding affinity toward most CYP isoforms; however, the most favorable interactions were obtained with the CYP1 family of enzymes. The functional modifications proposed to improve inhibition of CYP1A1, as evaluated in the shortlisted compounds, also typically improved binding affinity toward CYP1A2 and CYP1B1,

with the exception of Compound 7-7, which resulted in slightly decreased affinity.

As outlined in the Introduction, the selective inhibition of a specific member of the CYP1A family is considered difficult due to the similarity of their active sites. Accordingly, additional modifications intended to increase specificity toward CYP1A1 were investigated. The addition of 3-hydroxyl and 5-amino

Table 2. Comparison of the binding affinity of DTS and selected analogues to various CYP isoforms commonly involved in drug metabolism.

CYP Isoform	Binding Affinity (kJ·mol ⁻¹)									
	DTS	1-5	1-7	4-7	5-7	7	7-7	8-9	7-8-9	7-7-8-9
1A1	-40.17	-42.68	-42.68	-43.10	-43.10	-43.93	-43.93	-40.17	-39.75	-43.10
1A2	-38.49	-41.00	-39.33	-38.91	-41.00	-39.33	-38.07	-36.40	-35.56	-36.40
1B1	-39.75	-42.26	42.26	-38.49	-39.75	-41.00	-38.49	-38.91	-39.75	-37.66
2C9	-29.71	-33.05	-29.71	-30.12	-30.12	-28.45	-31.38			
2C19	-26.36	-36.82	-31.38	-30.54	-31.38	-30.96	-29.29			
2D6	-32.64	-35.15	-34.73	-35.56	-35.15	-33.89	-35.98			
3A4	-24.27	-29.71	-27.20	-29.29	-31.38	-24.27	-26.78			

groups indeed reduced binding affinity toward CYP1A2 and CYP1B1, without compromising the binding affinity toward CYP1A1. Upon incorporating the favorable effects of chain elongation, this effect was insignificant when only one prop-2-en-1-yl chain extension was introduced; however, when introducing this modification to both sides of the triple sulfur moiety (a modification shown above to slightly increase specificity to CYP1A1 with respect to CYP1A2 and CYP1B1), a marked improvement was noted.

Selection of Most Promising Analogue for CYP1A1 Inhibition

When evaluating Compound 7-7-8-9, not only was a higher binding affinity to CYP1A1 regained, but this was also accompanied by relatively substantial reductions in binding affinity to CYPs 1A2 and 1B1. Therefore, this novel DTS analogue achieved the objective of improving the inhibitory activity of DTS, whilst improving its specificity toward CYP1A1. An evaluation of the binding mode of CYP1A1 with Compound 7-7-8-9 is shown in Figure 2.

As presented in Figure 2, Compound 7-7-8-9 took up a similar position to DTS in the CYP1A1 binding pocket, close to the heme group (3.5 Å). The previously identified edge-to-face π - π stacking interaction with Phe-123 and offset π - π stacking interactions with Phe-224 and Phe-258 were retained.^[5] However, novel interactions could also be identified. As hypothesized, hydrogen-bonding interactions were successfully obtained between the newly introduced 3-hydroxyl group and Asn-222, as well as between the newly introduced 5-amino group and Ser-122. In addition, a previously unseen hydrogen-bonding interaction between Ser-116 and a sulfur atom in the linker chain was revealed. Overall, there were also a greater number of residues involved in hydrophobic interactions with the novel DTS analogue, due to its increased occupancy of the CYP1A1 active site.

Taken altogether, elongation of the DTS molecule in tandem with the introduction of hydrogen-donating/accepting functional groups to its benzyl rings enabled increased binding affinity and improved specificity toward CYP1A1, taking advantage of its long and narrow binding pocket,^[7,8] as well as the position of non-conserved polar amino-acid residues in its active site. With three hydrogen bond donors, two hydrogen bond acceptors, a molecular mass of 361 Da, and a log*P* of 3.72, the proposed novel DTS analogue obeys Lipinski's rule of five.^[9]

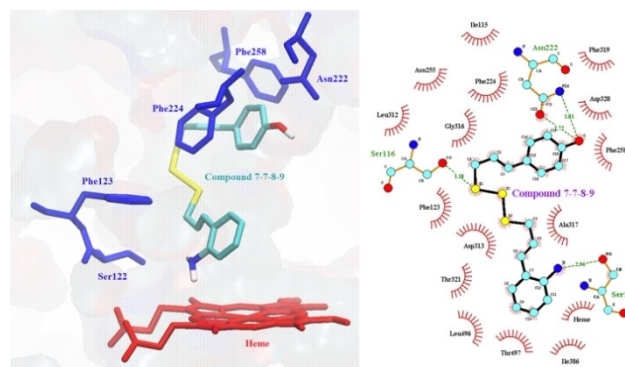


Figure 2. Left: Best theoretical binding pose achieved for compound 7-7-8-9 with CYP1A1. The protein is shown in surface representation, with residues identified as key for binding and specificity (Ser-122, Phe-123, Asn-222, Phe-224, and Phe-258) shown in stick representation (blue), as well as the heme group (red). Compound 7-7-8-9 is also shown in stick representation, colored according to atom type (C-cyan; S-yellow; O-red; N-blue; H-white). Compound 7-7-8-9 is shown to engage in an edge-to-face π - π stacking interaction with Phe-123, as well as in offset π - π stacking interactions with Phe-224 and Phe-258. Additionally, hydrogen-bonding interactions exist between Ser-122 and the novel 5-amino group, as well as between Asn-222 and the novel 3-hydroxyl group. Right: Potential interactions between compound 7-7-8-9 and CYP1A1 according to LigPlot+. Compound 7-7-8-9 is colored as in Figure 2A; all residues participating in hydrophobic interactions (red arcs) and hydrogen-bonding interactions (green dashed lines) are schematically displayed.

Therefore, following its synthesis and in vitro validation of its CYP1A1-inhibitory properties with respect to other CYP1 family members, Compound 7-7-8-9 may be useful as a drug candidate for cancer-preventive approaches in the future.

Conclusion

This paper presented the design and generation of a novel DTS analogue, Compound 7-7-8-9, featuring an elongation of the linker either side of the three sulfur atoms to a prop-2-en-1-yl chain, as well as the introduction of 3-hydroxyl and 5-amino groups to opposite benzyl rings. DTS, a natural product derived from *Petiveria alliacea*, has cancer-preventative properties and is a direct competitive inhibitor of the CYP1 family of enzymes. Through enhanced hydrogen-bonding and π - π stacking potential, this novel analogue not only exhibited improved binding affinity toward CYP1A1, but also increased specificity, whereby its binding affinity toward CYP1A2 and CYP1B1 was reduced. Thus, following its synthesis and in vitro validation, this

compound, via inhibiting CYP1A1 activity in carcinogen formation, may represent a promising candidate for cancer-preventive approaches in the future.

Experimental Section

Structures of DTS Analogues and CYP Isoforms

Initial 2D structures for all DTS analogues outlined in Table 1 were drawn in ACD/ChemSketch software version 2020.2.0.^[31] Analogue generation involved the addition of novel functional groups to or ring modifications of the two benzyl rings of DTS, as well as an elongation of the connecting chain between rings. The aim was to take advantage of CYP1A1's long and narrow binding pocket, as well as to target non-conserved polar residues located in the active site. Accordingly, functional modifications included a 4-fluoro group (prefix 1), a pyrazine ring (prefix 2), a 4-methoxy group (prefix 3), a 1,3-benzothiazole ring (prefix 4), a 2,1,3-benzoxadiazole ring (prefix 5), a 1,3-thiazolo[5,4-*b*]pyridine ring (prefix 6), elongation to prop-2-en-1-yl connecting chain (prefix 7), a 3-hydroxyl group (prefix 8), and a 5-amino group (prefix 9). The 2D coordinates were subsequently introduced into Avogadro^[32] for 3D coordinate generation and optimization.

The 3D coordinates for various CYP isoforms commonly involved in drug metabolism were retrieved from the Protein Data Bank with the following IDs: CYP1A1, 4I8V,^[33] CYP1A2, 2HI4,^[34] CYP1B1, 3PM0,^[35] CYP2C9, 4NZ2,^[36] CYP2C19, 4GQS,^[37] CYP2D6, 3TBG,^[38] and CYP3A4, 6DAA.^[39] The structures were used as obtained with no further refinement or modification.

Molecular Docking of DTS Analogues

The binding modes of all DTS analogues were initially determined in CYP1A1 using the three-dimensional coordinates obtained as described above. Polar hydrogens were added to the protein structure using AutoDock Tools,^[40] before centering the grid box on the heme group with dimensions of 20 Å in the *x*-, *y*-, and *z*-dimensions. Next, the 3D coordinates of the analogues were also prepared using AutoDock Tools,^[40] and each analogue was subjected to automated flexible docking with CYP1A1 using AutoDock Vina,^[41] without added restrictions.

Evaluation of Docking Results

The best docking pose was selected for each analogue, and all compounds were ranked on the basis of their binding affinity with CYP1A1. Following the identification of those analogues that showed improved binding compared to DTS, the top six compounds with the three most favorable binding affinities were shortlisted for additional molecular docking evaluation with the remaining CYP isoforms. The docking was conducted as described above, yielding a second ranked list of DTS analogues. Compound 7-7-8-9, which showed very favorable affinity toward CYP1A1, as well as reduced affinity toward other CYP1 enzymes (CYP1A2 and CYP1B1), suggesting CYP1A1 specificity, was highlighted as the most promising DTS analogue. Therefore, an image of the binding mode was generated using VMD^[42] for further characterization of the compound's position in the active site. Additionally, LigPlot + v 2.2^[43] was applied with default settings to characterize the CYP1A1 interactions with Compound 7-7-8-9, identifying novel hydrogen-bonding interactions with the targeted non-conserved residues, while maintaining the hydrophobic π - π stacking interactions with phenylalanines observed in the CYP1A1-DTS binding pose. Lastly,

the physicochemical properties of Compound 7-7-8-9 were characterized to determine its suitability as a drug candidate according to Lipinski's rule of five.^[30]

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: dibenzyl trisulfide (DTS) · cancer prevention · CYP1A1 inhibition · *Petiveria alliacea* · structure-activity relationship

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Manuscript received: January 21, 2022
Revised manuscript received: April 17, 2022