Investigation of Caffeic Acid Effect on Human Cancer Cell Line and Some Enzymes

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Abstract

Caffeic acid, a prominent antioxidant compound, has garnered significant attention in research due to its multifaceted properties, which include anti-inflammatory, antiobesogenic, antithrombotic, vasodilating, and antitumor activities. This study aims to comprehensively investigate the impact of caffeic acid on various metabolic enzymes (carbonic anhydrase I, II, IX, and glutathione reductase) through both *in silico* and *in vitro* approaches. Furthermore, in vitro experiments did conducted on the AGS (gastric cancer cell) line and the HaCaT (keratinocyte normal cell) line to elucidate the effects of caffeic acid in these cellular systems.

Keywords: Caffeic acid, carbonic anhydrase, glutathione reductase, cancer.

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1. Introduction

Caffeic acid (CAA) emerges as a polyphenolic entity synthesized via the secondary metabolic pathways occurring within a diverse range of vegetables, notably encompassing olives, coffee beans, fruits, potatoes, carrots, and propolis. Moreover, this constituent assumes a pivotal role as the primary hydroxycinnamic acid derivative prevalent in the human dietary regimen (SILVA et al., 2014; TOSOVIC, 2017).

This phenolic compound (CAA), exists in a range of molecular configurations within the plant kingdom. It is encountered in its elemental state as monomers, taking the form of sugar esters, organic acid esters, glycosides, and amides. Additionally, CAA can adopt more intricate structures, such as dimers, trimers, and flavonoid derivatives. It is also capable of forming associations with proteins and other polymers located in the cellular wall of vegetables (CHEN and HO, 1997; VERMA and HANSCH, 2004; ESPÍNDOLA et al., 2019; SENTURK et al., 2022).

CAA assumes a crucial role in the defense mechanisms deployed by plants to fend off predators, pests, and infections. This compound exhibits inhibitory properties against the proliferation of insects, fungi, and bacteria (TOSOVIC, 2017). Furthermore, it aids in the safeguarding of plant leaves against the harmful effects of ultraviolet

radiation B (UV-B) (GOULD et al., 2000; ESPÍNDOLA et al., 2019; AYGUL et a., 2022).

Extensive investigations, encompassing both *in vitro* and *in vivo* experiments, have substantiated the multifarious physiological effects of CAA and its derivatives. These effects include but are not limited to antibacterial activity (GENARO-MATTOS et al., 2015), anti-inflammatory and antiviral activity (RODRIGUES et al., 2015), cardioprotective and antioxidant activity (AGUNLOYE et al., 2019), antiproliferative and anti-atherosclerotic activity (NAGAOKA et al., 2002; VERMA and HANSCH, 2004), hepatoprotective activity (YANG et al., 2013), anticancer activity (LEE et al.i 2008; MCGLYNN et al., 2015), and anti-hepatocellular carcinoma activity (LEE et al.i 2008; WON et al., 2010). Of particular significance among these properties is the highlighted anti-hepatocarcinoma activity, given the status of hepatocarcinoma (HCC) as a leading cause of cancer-related mortality worldwide (MCGLYNN et al., 2015). Consequently, further exploration concerning the chemical and pharmacological aspects of CAA is imperative to pave the way for potential new drug development and, subsequently, expand therapeutic avenues (ZHANG et al., 2017).

Carbonic anhydrases (CA, EC 4.2.1.1.) play a pivotal role in facilitating the conversion between carbon dioxide (CO_2) and bicarbonate (HCO_3^-) and the subsequent dehydration of bicarbonate, resulting in the regeneration of CO_2 within an acidic milieu (SUPURAN, 2008; FIDAN et al., 2015; YAKAN et al., 2023).

In mammals, a total of sixteen CA isozymes have been identified thus far, with notable emphasis on CA II and CA IX as highly efficient catalysts for carbon dioxide hydration (SUPURAN, 2008; SUPURAN, 2017; ARSLAN et al., 2016; ARSLAN et al., 2020). CA I and II is primarily found in erythrocytes, but also exhibits distribution in numerous secretory tissues of the gastrointestinal tract, kidneys, lungs, eyes, central nervous system, and more. Conversely, CA IX represents a tumor-associated isoform (SUPURAN, 2008; ABDEL-AZIZ et al., 2015; DIZDAROGLU et al., 2020; YAKAN et al., 2023). Moreover, several other CA isoforms have been identified in diverse tissues, actively participating in vital biological processes including acid-base homeostasis, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis, and electrolyte secretion. The multitude of CA isozymes involved in these intricate processes hold significant therapeutic potential as they emerge as promising targets for modulation, whether through inhibition or activation, in the treatment of various disorders such as edema, glaucoma, obesity, cancer, and epilepsy (SUPURAN, 2008; SUPURAN, 2017; URCAR et al., 2016; YAKAN et al., 2023).

Glutathione reductase (GR), an enzyme categorized as EC 1.6.4.2, plays a crucial role in maintaining the equilibrium of the intracellular redox system. Its primary function involves facilitating the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) through the utilization of nicotinamide adenine dinucleotide phosphate (NADPH) molecules (KARPLUS et al., 1989; COBAN et al., 2007; URCAR et al., 2016). Extensive research has been conducted to explore the inhibition of the GR enzyme using various compounds. The findings of these investigations have consistently indicated a reduction in the GSH/GSSG ratio and an elevation in the $NAD(P)H/NAD(P)^+$ ratio (SENTURK et al., 2008; COUTO et al., 2016; KOCAOGLU et al., 2019; USTUNDAG et al., 2022). Although some studies have demonstrated that GR inhibition does not influence the generation of free radicals or the expression of other enzymes involved in GSH biosynthesis, it is worth noting that the potent activity of the GR enzyme in cancer cells contributes to their resistance against several chemotherapeutic drugs. Increased intracellular GSH levels offer a significant survival advantage for tumor cells, particularly in lung, breast, larynx, colon, and bone marrow cancers. Consequently, the inhibition of the GR enzyme presents a promising avenue for anticancer treatment, considering the potential of such substances (BALENDIRAN et al., 2004; SENTURK et al., 2009; TRAVERSO et al., 2013; SANTACROCE et al., 2023).

In this study, the potential of CAA to inhibit human CA I, II, IX and GR enzymes was tried to be determined. For this purpose, both theoretical (embedding) and experimental experiments were carried out. It also aimed to explore the potential therapeutic effects of CAA on gastric cancer cells and keratinocyte normal cells.

2. Materials and Methods

Chemicals

hCA I (C4396), hCA II (C6165), and hCA IX (CA 9, human recombinant (SRP6483) were procured from Sigma-Aldrich company. All other chemicals and solvents were purchased from Merck (Darmstadt, Germany).

Measurement of Glutathione Reductase Activity

To assess the enzymatic activity of GR, a spectrophotometric method is employed, which involves monitoring the decrease in NADPH levels when the substrate GSSG is present. This reduction in NADPH concentration is measured specifically at a wavelength of 340 nm (BEUTLER, 1984; USTUNDAG et al., 2022).

Measurement of Carbonic Anhydarase Activity

The inhibitory activity of CAA on hCA I, hCA II, and hCA IX were determined according to the esterase method (VERPOORTE et al., 1967; ARSLAN et al. 2020). In inhibitory studies, p-nitrophenyl acetate was employed as the substrate.

Inhibitory Effect Determination of IC_{50} Values of CAA

With inhibitor studies, the activity of CAA, whose solutions were prepared, were added to the cuvette in different concentrations and their activities were measured. CAA were plotted as % Activity- [I], IC_{50} values were calculated from the equation of the curve.

In silico Studies

In silico placement studies were conducted to investigate interactions between CAA and amino acid residues around the active site of the CA I, II, and IX enzyme. All pdb files were obtained from rcsb.org. The AutoDockTools1 program (version 1.5.7) was used for the preparation of all ligands and enzymes. Autodock Vina2 program (version 1.1.2) was used for all docking experiments, the entire surface of each enzyme was investigated, the exhaustiveness value was set as 32, the energy_range value was assigned as 5, and the best 5 results were asked to be listed. Twelve trials of each molecule were made for each enzyme, and the highest scoring conformation of these was aligned with the receptor protein in the PyMOL-oss3 program (version 2.4.1) (SANNER, 1999; TROTT and OLSON, 2010). The evaluation of the interactions was carried out in the Biovia Discovery Studio Visualizer4 (version 21.1.0.2029) program. Docking scores are summarized in Table 1, Figure 2, 3, and 4.

AGS and HaCaT Cell Line Culture Studies

AGS gastric cancer and HaCaT keratinocyte normal cell line attached to the base of the cell culture layer were passaged when their cell density reached 70-80%. Cells were washed with 10 ml of PBS and treated with 4 ml of 0.25% Trypsin/EDTA solution (Gibco) for 2-3 minutes in the incubator to lift them off the flask base and the separation of cells was also observed under the microscope. Trypsin activity was stopped by adding 2 ml of FBS. The cells were then transferred to a 15 ml centrifuge tube and centrifuged at 1800 rpm for 10 minutes at room temperature. The supernatant was completely discarded and the cell pellet was dissolved in 10 ml of DMEM-LG. The mixture was then centrifuged again at 1800 rpm for 10 minutes at room temperature. The supernatant was discarded again, and the cell pellet was dissolved in the appropriate volume of DMEM-LG, and the cells were stained with Trypan blue and counted with a hemocytometer. Depending on the number of cells, cells were seeded into new cell culture dishes in DMEM-LG containing 10% FBS and 0.1 ml/ml Primocin, and the dishes were placed in an incubator at 37°C containing 5% CO_2. The MCF-7 cell line was passaged again when they reached 70-80% density (STRIEDINGER et al., 2021).

Cell Viability Test (MTS)

The passaged cells were counted and seeded in two separate 96-well plates with 1x104 cells in each well. After 24 hours of incubation, CAA was given to the cells in the wells at certain concentrations (500, 250, 100, 50, 25 μM). Each concentration was run in 3 replicates. Cell viability test (MTS) was performed after 24 hours. Absorption was measured in the ELISA reader at 450 nm. The graph was drawn according to the resulting absorption values (KOCANCI et al., 2017).

3. *Results and Discussion*

GR, an antioxidant enzyme, carries out a vital function by regulating the redox metabolism of GSH within the cellular framework of numerous organisms. Its involvement in the modulation of GSH redox hemostasis contributes to the synthesis of deoxyribonucleotides. Moreover, peroxide plays a critical role in the detoxification of 2-oxoaldehydes and xenobiotics. The viability of rapidly dividing cells and those resilient to oxidative stress is significantly reliant on the replenishment of GSH. Consequently, the identification of potential GR inhibitors assumes paramount importance in the advancement of antitumor and antiparasitic pharmaceutical agents (KARPLUS et al., 1989; BOEHME et al., 2000; USTUNDAG et al., 2022).

In investigations concerning the human erythrocyte GR enzyme, which serves as a model for drug trials, it has been ascertained that nitro aromatic compounds containing a quinoline moiety, commonly employed as antisitulant and anticancer medications, exert an influence on the activity of GR enzyme (GRELLIER et al., 2001). Furthermore, diverse substances encompassing certain drugs, metal ions, and nitro groups have been discovered to possess inhibitory properties against GR enzymes derived from various sources (GRELLIER et al., 2001; COBAN et al., 2007; CAKMAK et al., 2011; KOCAOGLU et al., 2019).

In trials for human CA I, II, IX and GR enzymes with CAA. The IC_{50} value for hCA I was determined as 10.26 µM, for hCA II as 9.14 µM, for hCA IX as 8.96 µM and for GR as 25.84 µM. Acetazolamide (AZA) was used as the reference for the tested CA enzymes, and N,N-bis(2-chloroethyl)-N-nitrosourea (BCNU) was used for the GR. AZA was observed as 36.2 µM for hCA I, 0.37 µM for hCA II and 0.93 micromolar for hCA IX. For GR it was observed as 465 µM for BCNU.

The utilization of CA inhibitors in the management of several conditions, such as cancer, glaucoma, and obesity, is well-established. Consequently, the quest for innovative and potent molecular frameworks for the therapeutic intervention of these ailments assumes a noteworthy strategy (ISIK et al., 2015; SUPURAN, 2017; SENTURK et al., 2022).

Three methods were used to determine the solubility of the molecule in water in Swiss-ADME. These are the ESOL, Ali and SILICOS-IT methods. Caffeic acid was estimated to be water soluble according to three methods. In pharmacokinetic estimates, gastrointestinal (GI) absorption was predicted to be high. In the drug similarity part, it shows that the CAA may be active in four of the analyzes of proprietary chemical collections from five different major pharmaceutical companies. According to the estimates of Medicinal Chemistry; It should be used with caution in human therapy as it gives a warning for PAINS (pan assay interference compounds).

Bcolor analysis (to be toxic by default, chemically reactive, metabolically unstable or having properties responsible for poor pharmacokinetics) has given two warnings. In this case, direct use in the body should be well adjusted. There is no similar molecule.

Systemic acceptability score is good.

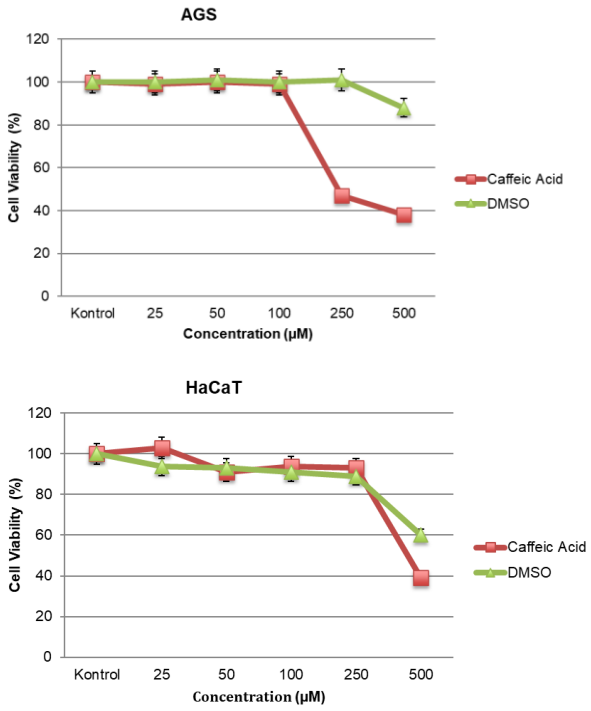


Figure 1. Cell viability result graph of AGS and HaCaT cells treated with 500, 250, 100, 50, 25 µM caffeic acid.

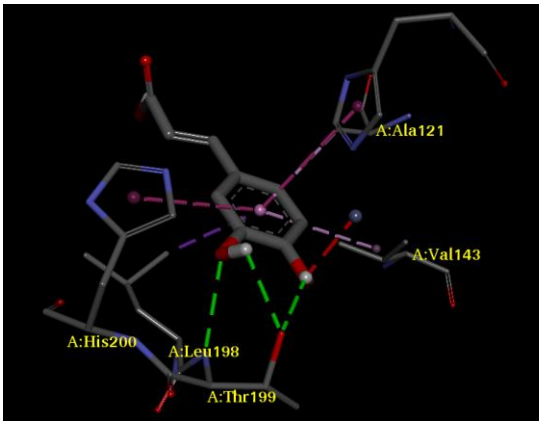
The passaged cells were counted and seeded in two separate 96-well plates with 1x104 cells in each well. After 24 hours of incubation, CAA was given to the cells in the wells at certain concentrations (500, 250,

100, 50, 25 µM). Each concentration was run in 3 replicates. Cell viability test (MTS) was performed after 24 hours. Absorption was measured in the ELISA reader at 450 nm. According to the result in Figure 1, in gastric cancer (AGS) cell line, CAA reduced cell viability to 47% and 38%, respectively, at 250 and 500 µM concentrations. As the dose increased, the toxic effect on the gastric cancer cell line also increased. These substances were also tested on a normal human keratinocyte cell line (HaCaT). According to the graph, CAA showed toxic effect only at the highest dose (500 µM), reducing cell viability to 39%.

Using SwissADME, we attempted to calculate physicochemical descriptors as well as predict ADME parameters, pharmacokinetic properties, drug-like nature and medicinal chemistry friendliness of one or more small molecules to support drug discovery (Figure 5).

Table 1. Docking scores and K_i values of caffeic acid for tested enzymes.

Enzyme (PDB ID)	Affinity (kcal/mol)	K_i value (µM)
hCA I (2CAB)	-6,2	5.12
hCA II (3KS3)	-6,5	4.86
hCA IX (6FE2)	-7,2	4.25



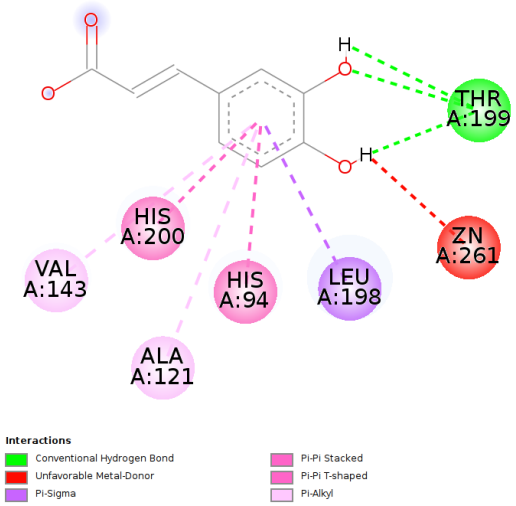
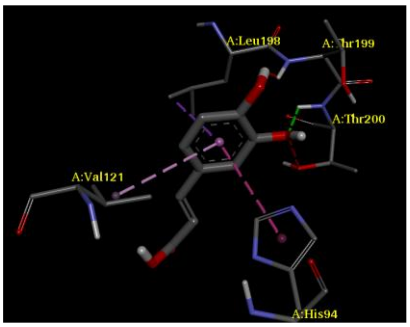


Figure 2. 3D and 2D docking binding models of caffeic acid with hCA I enzyme, respectively.



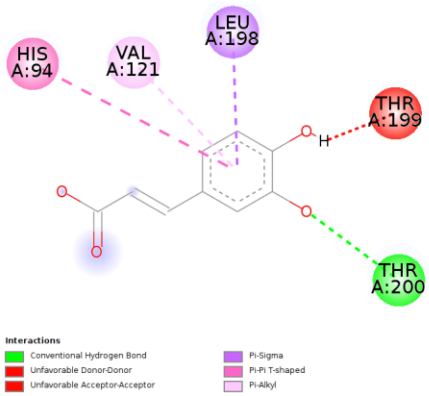
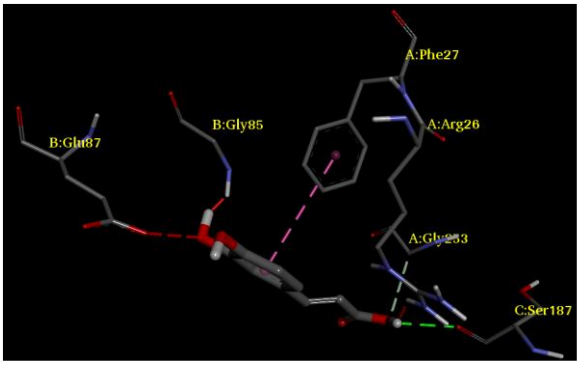


Figure 3. 3D and 2D docking binding models of caffeic acid with hCA II enzyme, respectively.



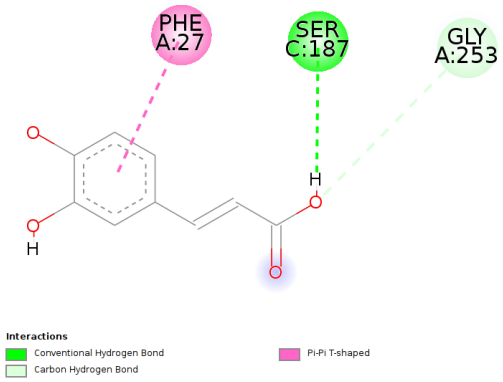
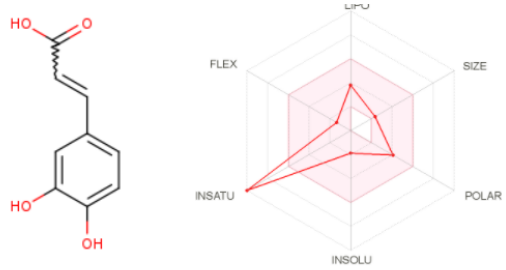


Figure 4. 3D and 2D docking binding models of caffeic acid with hCA IX enzyme, respectively.



SMILES OC(=O)/C=C/c1ccc(c(c1)O)O

Physicochemical Properties	
Formula	C9H8O4
Molecular weight	180.16 g/mol
Num. heavy atoms	13
Num. arom. heavy atoms	6
Fraction Csp3	0.00
Num. rotatable bonds	2
Num. H-bond acceptors	4
Num. H-bond donors	3
Molar Refractivity	47.16
TPSA	77.76 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	0.97
Log $P_{o/w}$ (XLOGP3)	1.15
Log $P_{o/w}$ (WLOGP)	1.09
Log $P_{o/w}$ (MLOGP)	0.70
Log $P_{o/w}$ (SILICOS-IT)	0.75
Consensus Log $P_{o/w}$	0.93
Water Solubility	
Log *S* (ESOL)	-1.89
Solubility	2.32e+00 mg/ml ; 1.29e-02 mol/l
Class	Very soluble
Log *S* (Ali)	-2.38
Solubility	7.55e-01 mg/ml ; 4.19e-03 mol/l
Class	Soluble
Log *S* (SILICOS-IT)	-0.71
Solubility	3.51e+01 mg/ml ; 1.95e-01 mol/l
Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	No
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-6.58 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	No; 1 violation: MW<200
Bioavailability Score	0.56
Medicinal Chemistry	
PAINS	1 alert: catechol_A
Brenk	2 alerts: catechol, michael_acceptor_1
Leadlikeness	No; 1 violation: MW<250
Synthetic accessibility	1.81

Figure 5. SwissADME result for caffeic acid.

4. Conclusion

In this investigation, the primary objective is to employ a dual methodology involving in silico and in vitro analyses. The *in silico* assessment will provide valuable insights into the potential interactions of CAA with specific metabolic enzymes. Through this computational approach, the molecular interactions and binding affinities between caffeic acid and target enzymes will be evaluated, offering a preliminary understanding of the possible effects.

Overall, this study seeks to shed light on the in silico and *in vitro* effects of CAA on metabolic enzymes, while simultaneously exploring its potential therapeutic implications in AGS gastric cancer cells and HaCaT keratinocyte normal cells. Through the integration of computational and experimental approaches, this investigation strives to contribute to our understanding of the molecular mechanisms underlying CAA's multifaceted properties and its potential application in cancer therapy.

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