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Research Article | Araştırma Makalesi

COMBINATION THERAPY WITH SELENOUREA AND ETHACRYNIC ACID TARGETING GST INHIBITION AND REVEALS SOME APOPTOSIS-CLEAVED PROTEINS IN BREAST CANCER

SELENOUREA VE ETAKRINIK ASIT ILE KOMBINASYON TEDAVISI GST INHIBISYONUNU HEDEFLIYOR VE MEME KANSERINDE APOPTOZLA AYRILAN BAZI PROTEINLERI ORTAYA ÇIKARIYOR

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ABSTRACT

Objective: Glutathione S-transferase (GST) participates in the maintenance of cellular redox homeostasis through several mechanisms. Therefore, compounds or drug-like drugs that GSTs target are important for preclinical and clinical studies. We hypothesized that inhibition of GST by selenourea and Etacrynic acid combination sensitizes breast cancer cells to apoptotic signaling by altering redox homeostasis, leading to the identification of specific apoptosis-cleaved proteins that drive cell death pathways. **Methods:** This study was carried out to demonstrate the binding target molecular docking, cell proliferation inhibition with MTS method, and protein expression with western blot analysis of the combination therapy with selenourea and Etacrynic acid to target GST inhibition. **Results:** In the results, it was found that selenourea acts through targeting by indirect S-glutathionylation modification of cysteine residues in target proteins, although its polar covalent bond, hydrogen bond, and ionic interaction bind to other amino acids in all sub-types of GST. The combination of selenourea and etacrynic acid dose-dependently inhibited cell proliferation at $p<0.0001$ levels. Selenourea only exhibited 52% and 50% inhibition on MDA-MB-231 and MCF7 cells, respectively. In contrast, the combination of Selenourea and Etacrynic acid showed 41% and 38% inhibition on MDA-MB-231 and MCF7 cells, respectively. This combination also revealed apoptosis-cleaved proteins in estrogen-positive MCF7 cells and non-estrogenic MDA-MB-231 breast cancer cells. **Conclusions:** This work may provide a practical guide and useful insights into new therapeutics. This could be considered a very promising strategy for the development of new antineoplastic drugs. Targeting identified proteins, in combination with GST inhibition, enhances therapeutic efficacy.

Keywords: Selenourea, GST, ADME analysis, protein-ligand interaction, signal pathway analysis.

ÖZ

Amaç: Glutatyon S-transferaz (GST), çeşitli mekanizmalar aracılığıyla hücresel redoks homeostazının korunmasına katılır. Bu nedenle, GST'lerin hedef aldığı bileşikler veya ilaç benzeri ilaçlar preklinik ve klinik çalışmalar için önemlidir. GST'nin selenoüre ve Etakrinik asit kombinasyonu ile inhibisyonunun, redoks homeostazını değiştirerek meme kanseri hücrelerini apoptotik sinyale duyarlı hale getirdiğini ve hücre ölüm yollarını yönlendiren spesifik apoptozla ayrılan proteinlerin tanımlanmasına yol açtığını varsaydık. **Yöntem:** Bu çalışma, GST inhibisyonunu hedeflemek için selenoüre ve Etakrinik asit ile kombinasyon tedavisinin bağlanma hedefi moleküler yerleştirme, MTS yöntemi ile hücre proliferasyonu inhibisyonu ve western blot analizi ile protein ekspresyonunu göstermek için gerçekleştirilmiştir **Bulgular:** Sonuçlarda, selenoürenin hedef proteinlerdeki sistein kalıntılarının dolaylı S-glutatyonilasyon modifikasyonu ile hedefleme yoluyla etki ettiği, ancak polar kovalent bağı, hidrojen bağı ve iyonik etkileşiminin GST'nin tüm alt tiplerindeki diğer amino asitlere bağlandığı bulunmuştur. Selenoüre ve etakrinik asit kombinasyonu hücre proliferasyonunu $p<0.0001$ düzeyinde doza bağlı olarak inhibe etmiştir. Selenoüre, MDA-MB-231 ve MCF7 hücreleri üzerinde sırasıyla yalnızca %52 ve %50 inhibisyon sergilemiştir. Buna karşılık, Selenourea ve Etacrynic asit kombinasyonu MDA-MB-231 ve MCF7 hücreleri üzerinde sırasıyla %41 ve %38 inhibisyon göstermiştir. Bu kombinasyon aynı zamanda östrojen pozitif MCF7 hücrelerinde apoptozla parçalanmış proteinler ortaya çıkarmıştır. **Sonuç:** Bu çalışma, yeni terapötikler için pratik bir rehber ve faydalı bilgiler sağlayabilir. Bu, yeni antineoplastik ilaçların geliştirilmesi için çok umut verici bir strateji olarak düşünülebilir. GST inhibisyonu ile birlikte tanımlanmış proteinlerin hedeflenmesi, terapötik etkinliği artırır.

Anahtar Kelimeler: Selenourea, GST, ADME analizi, protein-ligand etkileşimi, sinyal yolu analizi.

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Introduction

Selenourea is an organic compound with the formula CH_4N_2Se (Figure 1) and solid white or crystalline solid molecular weight 122.02 g/mol, selenium inorganic or organic forms is rapidly absorbed from the human gut, with total bioavailability of 84% to 97%, respectively.[1,2] Since selenium-containing compounds are less toxic than other inorganic compounds, synthesis and biological evaluation of these compounds. Drug design is the inventive process of finding new medications depending on the knowledge of a biological target such as receptor agonists, antagonists, inverse agonists, or modulators; ion channel openers or blockers; enzyme activators or inhibitors. In the most basic sense, drug design involves the design of molecules that are complementary in shape and charge to the molecular target with which they interact and bind. Therefore, this study was carried out to demonstrate the binding of selenourea compound to glutathione s-transferase enzymes (GSTs) which are a superfamily of proteins found in all cellular organisms, which usually exist as multiple isoforms have many different exogenous and multifunctional enzymes that detoxify endogenous compounds.[3,4] This extensive family has been categorized into at least 13 classes. The 3 big families of proteins cytosolic based upon alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega, mitochondrial based Kappa, and microsomal GSTs like Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) superfamily. One general function of these superfamilies is the involvement in detoxication reactions for both endogenous and xenobiotic compounds.[5] Besides their molecular and catalytic role (Figure 2), GSTs may bind to a range of exogenous and endogenous compounds in a non-catalytic manner. These affect hormones, fatty acids, bilirubin, and xenobiotics.[6,7] GSTs are phase II metabolic enzymes that play a key role in drug metabolism.[8] GSTs act as the thiol group of glutathione (GSH) in the active center to the electrophilic site of a second substrate to catalyze conjugation reactions. The glutathione conjugate formed is less toxic and excreted in soluble form GSTs recognize a large number of substrates, although the common features of the substrates are that most of them are hydrophilic, and carry an electrophilic center.[9] The supramolecular structure of cytoskeletal proteins determines exposed thiol groups.[10] This effect has been associated with glutathionylation of these sites affecting protein function by protecting them from irreversible oxidation under stress conditions or inhibiting polymerization.[11,12] In this manner, maintaining cellular homeostasis and participating in various pathological processes may be associated with cancer cell survival, particularly in telomere-targeted compounds.[13] GSTs protect cancer cells from oxidative stress and may contribute to tumor progression by maintaining cellular homeostasis under oxidative conditions.[14] So, inhibition is needed there. Inhibiting GST activity disrupts the cellular defense mechanisms against oxidative stress, which results in an accumulation of reactive oxygen species (ROS). This accumulation can overwhelm cellular repair mechanisms, ultimately triggering apoptotic pathways in cancer cells.[15,16] By targeting GSTs, researchers aim to enhance the efficacy of chemotherapy and reduce cancer cell survival, particularly in drug-resistant tumors.[17,18] Therefore, it is important to determine the inhibition effect of drugs or combinations on GST enzymes. GST inhibitors, such as etacrynic acid disrupt the GSH-GST system. This leads to increased ROS accumulation, making cancer cells more prone to oxidative stress and triggering apoptosis. This study aims to investigate the therapeutic potential of combining selenourea and Etacrynic acid in targeting GST inhibition, while also identifying apoptosis-associated cleaved proteins in breast cancer cells, supported by molecular docking to predict key binding interactions.

Methods

Material

Cells and Reagents

In our study, the anticancer effects of the combination of selenourea and etacrynic acid were investigated on breast cancer cells such as estrogen-positive MCF7 (HTB-22; ATCC, USA), estrogen-negative MDA-MB-231(HTB-26; ATCC, USA), and non-cancer cells. RPMI has been supplemented with 10% fetal bovine serum (FBS), penicillin (50 IU/mL Capricorn Scientific, Germany), streptomycin (50 µg/mL, Capricorn Scientific, Germany), and 2 mM L-glutamine. The cells were incubated in a humidified incubator at 5% CO_2 at 37℃. PBS, FBS, antibiotic (Penicillin-Streptomycin), and Trypsin/EDTA solution were purchased from Sigma-Aldrich (Germany). MTS was purchased from Promega (Germany). Selenourea was purchased from Sigma-Aldrich (Germany), etacrynic acid was purchased from Sigma-Aldrich(Germany).

Used devices

In this study, Nuve marked a laminar flow cabinet, a Thermo-Scientific mark a CO_2 Incubator, and an Emax-Plus Microplate reader were used.

Method

Cell proliferation assay with MTS

The MTS assay is a colorimetric assay for assessing a cell's metabolic activity. The MTS tetrazolium compound is bio-reduced by cells into a colored formazan product that is soluble in the culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.[19] Measurement of the anti-proliferative effect using the MTS cell proliferation kit was performed in a 96-well plate using human breast cell lines. Cells were grown in the appropriate medium and then seeded in 96 well plates in a 100 µL medium. The presence of 1500 cells in each well were verified and then incubated at 37°C in a 5% CO_2 atmosphere for 24 h. At the end of the time cycle,

different concentrations from 10 to 140 µM of the test sample were added. The cells were then incubated under conditions appropriate for the cell lines ranging from 24 to 72 h. Then, 25 µL of MTS reagent was added to each well and incubated for 1 h. At the end of the incubation time, the optical density (OD) of the color was measured using a microplate reader. The absorbance was measured at a primary wavelength of 490 nm. The mean absorbance values were calculated, and the cell viability percentages were documented using the Excel Office program. Then the IC_{50} values were calculated. The best cell proliferation reducing were found 72 hours and this time, western blot analysis. The results are shown in Figure 3. Apoptosis-cleaved protein expression studies were performed according to the determined IC_{50} values. Results are shown Figure 3.

Western blotting

Western blotting was carried out as described elsewhere.[18] Western blotting was performed to evaluate the protein expression levels of E-cadherin, β-catenin, and β-actin in MCF7 and MDA-MB-231 cells. Cells were lysed using RIPA buffer (Thermo Scientific, Cat# 89901) supplemented with protease inhibitor cocktail (Abcam, Cat# ab271306), phosphatase inhibitor cocktail (Sigma-Aldrich, Cat# P0044), and 1 mM each of NaF and Na_3VO_4. After incubation on ice for 30 minutes, lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. Protein concentrations were measured by Bradford assay (Bio-Rad, Cat#5000006), and 40 µg of total protein was loaded per lane onto 8–12% SDS-polyacrylamide gels for electrophoresis at 90V, followed by transfer onto PVDF membranes (Millipore, Cat# IPVH00010). The choice of 40 µg total protein was based on prior optimization trials to achieve optimal band intensity and clarity, especially for moderately expressed proteins such as E-cadherin and β-catenin, while maintaining consistent detection of β-actin as a loading control. Membranes were blocked in 5% non-fat dry milk in TBS-T for 1 hour at room temperature and incubated overnight at 4°C with the following mouse monoclonal primary antibodies diluted 1:1000 in 2.5% milk-TBS/T: anti-E-cadherin (Cell Signaling Technology, Cat# 3195, 135 kDa), anti-β-catenin (Cell Signaling Technology, Cat# 8480, 92 kDa), and anti-β-actin (Sigma-Aldrich, Cat# A5441, 43 kDa, internal control). After washing three times with TBS-T, membranes were incubated for 1 hour at room temperature with HRP-conjugated anti-mouse IgG secondary antibodies (Cell Signaling Technology, Cat# 7076, Anti-rabbit IgG, HRP-linked Antibody #7074, dilution 1:2000 in 2.5 % milk-TBS/T). Protein bands were visualized using enhanced chemiluminescence (Bio-Rad Clarity ECL, Cat# 1705061) and imaged with the UVP ChemiDoc-It² system. Results are shown Figure 4.

Protein preparation

The methods presented in this paper are specifically based on machine learning (ML) techniques. The Protein Data Bank was used for protein preparation. Molecular docking analyses were made by using network analysis in systems to predict their binding mode and binding energy. Afterward, in silico analysis to evaluate the pharmacokinetic properties was predicted by using the ADME software program. The Protein Data Bank20 was used for molecular docking analysis. The PDB archive contains information on experimentally determined structures of proteins, nucleic acids, and complex assemblies. As a member of RCSB, PDB compiles and annotates PDB data according to agreed standards. RCS and PDB also provide a variety of tools and resources. Users can perform simple and advanced searches based on annotations related to ranking. PDB IDs and X-RAY Diffraction Resolutions 3EIN (1.13 Å), 1GSD (2.50Å), 2WJU (2.30Å), 1TDI (2.40Å), 1R4W (2.50Å), 1GTU (2.68Å), 1AQW (1.80 Å), 11GS (2.30 Å) A chain structures due to no mutation and homogenization and then all the water and buffer molecules, as well as the ions, were deleted, and all the protein subsequently, hydrogen atoms were added to the system (according to pH 7.4) using the Protein Preparation tool in the playmolecule.com data analysis program and, Auto Dock Vina (4.0).[21,22]

Ligand preparation and optimization

The molecular structures of all of the molecules were sketched using the ChemBioDraw-Ultra-v12.0 (2010) software. The chemical structures of known drugs were retrieved from the PubChem compound database, which is available at NCBI.[23]

ADME, Molecular docking and gene ontology analysis

ADME analysis was performed using the SwissADME software (version 1.0, 2023) program. The results of this analysis are shown in Figure 3. Molecular docking analyses were performed using the Kdeep/data analysis system.[24] Perform virtual screening of the compound was made using a neural-network-based predictor of bind scope and aesthetic 2D diagrams of protein-ligand interactions including hydrogen-bonds and pi-pi stacking were made by Plexview programs (version 1.0, 2023).[25] Results were shown in Figure (5-6) and signaling pathways were made using a state-of-the-art neural networks pathway map program and gene ontology analysis were made using by STRING online tool (Figure 6).[26] Results are shown Table1, Figure 5, and Figure 6.

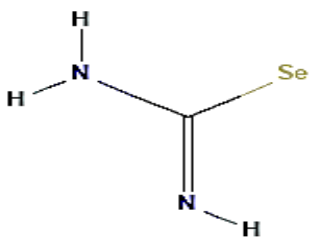


Figure 1. Chemical structure of selenourea ($H_2N–C(=NH)–Se$): A selenium analog of urea. The molecule contains a selenium atom bonded to the central carbon, replacing the oxygen typically found in urea. This compound is of interest due to its applications in coordination chemistry and as a precursor for selenium-containing biomolecules. X-ray crystallographic measurements on crystals at -100°C give average C=Se bond lengths of 1.86 Å and 1.37 Å for C-N. Both

the Se-C-N and N-C-N angles were measured at 120°, as expected for a sp2-hybridized carbon.[27] Both the shortened length of the N-C σ bond and the longer Se=C bond suggest that the lone pair is displaced on amines; the Se=C π bond electrons are attracted to the selenium atom, while the nitrogen lone pair is attracted to the carbonyl carbon.[28] The synthesis of selenium-containing heterocycles and another class of reactions take place by complexation of selenurea with transition metals and metalloids, which is attributed to the electron-donating effect of the amino groups and the consequent stabilization of the selenium-metal π bond.

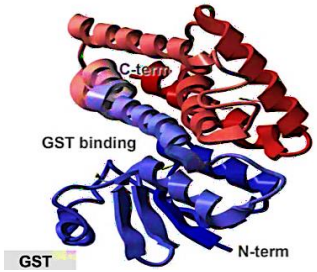


Figure 2. GST terminal structure: Structural representation of the GST (Glutathione S-Transferase) fusion protein. The N-terminal domain (N-term) is shown in blue, and the C-terminal domain (C-term) is highlighted in red. The GST-binding site is labeled and structurally positioned between the two domains, indicating the orientation and domain organization of the protein.

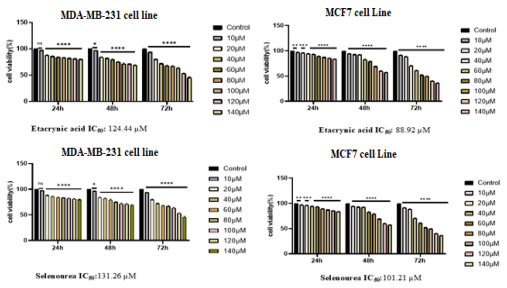
Results

Cells were treated with increasing concentrations (10–140 µM) of each compound for 24, 48, and 72 hours. Cell viability was measured using an MTS assay and is expressed as a percentage of untreated control cells. The best results found at 72 hours in both cells. For 72 hours was the best result, optimization was achieved. We found that selenourea and etacrynic acid combination reduced cell proliferation dose and time. Selenourea only segmented with 50 % inhibition exhibited at 131.26 µM, 101.21µM concentration in MCF7 and MDA-MB231 cell respectively, while it's combination with Etacrynic acid IC_{25} exhibited 41% and 38% at 35-50µM in both cells.

Western blot analyses were performed by combine with selenourea IC_{50} and Ethacrynic acid IC_{25} and IC_{50} doses at 72 hours.

Figure 3A top panels have shown Etacrynic acid treatment resulted in dose- and time-dependent cytotoxicity with IC_{50} values of 124.44 µM (MDA-MB-231) and 88.92 µM (MCF7). Bottom panels: Selenourea also reduced cell viability with IC_{50} values of 131.26 µM (MDA-MB-231) and 101.21 µM (MCF7).

A)



B)

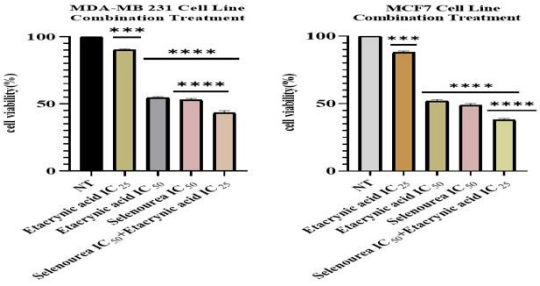


Figure 3. Cell proliferation analysis results of etacrynic acid and selenourea (A) and combination(B) on MDA-MB-231 and MCF7 breast cancer cell lines.

Considering the results of Figure 4, expression slightly increases with EIC_{50} and SIC_{50}, and even more in the combination (E+S) treatment IC_{50} in MDA-MB-231 have shown a partial reversal of Epithelial Mesenchymal Transition (EMT) link to E-cadherin, potentially linked to cell differentiation or apoptosis. Combination treatment, indicating a reduction in β-catenin signaling, which may correlate with decreased Wnt pathway activity, relevant to both proliferation and apoptosis regulation. Generally, the upregulation of E-cadherin and reduction of β-catenin in combination treatment in MDA-MB-231 cell supports a reversal of mesenchymal phenotype, which can be associated with increased susceptibility to apoptosis.

Considering the results of Figure 4, the combined E+S treatment IC_{50} in MCF7 cell have exhibited E-cadherin expression moderate increase compared to non-combination. This upregulation may reflect either enhanced cell-cell adhesion or a response to apoptotic stress (cells often upregulate adhesion molecules in early apoptosis).

Table 1 results suggest that the molecule is hydrophilic. This may limit its ability to passively diffuse through the lipid bilayer of cell membranes. However, it could still be efficiently taken up by active transport mechanisms, which is particularly relevant if the molecule targets intracellular apoptotic pathways. However, compound is well-suited for oral formulations with 84.1mg/ml so, it has excellent solubility enhances bioavailability, allowing the molecule to effectively reach intracellular compartments and exert potential pro-apoptotic effects. Moreover, no CYP enzyme inhibition (CYP1A2, CYP2C9, CYP3A4, etc.) has shown low metabolic interaction and good pharmacokinetic stability. This compound is efficiently absorbed and retained in cells and may be an anticancer agent inducing apoptosis.

Figure 6 top results have shown Reactome pathway enrichment analysis revealed significant upregulation of apoptosis-related signaling, particularly R-HSA-111465 (apoptotic cleavage of cellular proteins), indicating that the treatment triggers both extrinsic caspase activation and executioner-mediated proteolysis. These findings are consistent with western blot data showing caspase-related protein cleavage and align with ADME properties that predict effective intracellular bioavailability.

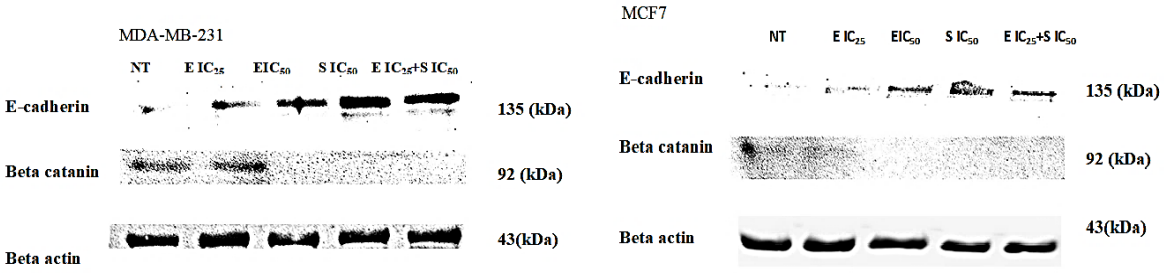


Figure 4. Western blot analysis results.

Table 1. ADME analysis

Category	Property	Abbreviation / Model	Value	Interpretation / Notes
Lipophilicity	Log P (Octanol/Water)	iLOGP	0.00	Neutral lipophilicity
	Log P	XLOGP3	-0.69	Slightly hydrophilic
	Log P	WLOGP	-0.95	Hydrophilic
	Log P	MLOGP	-1.13	Hydrophilic
	Log P	SILICOS-IT	-0.85	Hydrophilic
	Consensus Log P	—	**-0.16**	Overall indicates mild hydrophilicity
Water Solubility	Log S	ESOL	-0.16	Very soluble
	Solubility (mg/mL; mol/L)	ESOL	84.1 mg/mL ; 0.689 mol/L	Very soluble
	Log S	Ali	0.12	Soluble
	Solubility (mg/mL; mol/L)	Ali	160 mg/mL ; 1.31 mol/L	Soluble
	Log S	SILICOS-IT	0.16	Soluble
	Solubility (mg/mL; mol/L)	SILICOS-IT	176 mg/mL ; 1.44 mol/L	Soluble
	Solubility Class	—	Very soluble to soluble	Favorable for absorption
Pharmacokinetics	Gastrointestinal Absorption	GI	High	Good oral bioavailability
	Blood-Brain Barrier Permeant	BBB	No	Not CNS-active
	P-glycoprotein Substrate	P-gp	No	Not effluxed by P-gp transporter
	CYP1A2 Inhibition	CYP1A2	No	Not expected to inhibit
	CYP2C19 Inhibition	CYP2C19	No	—
	CYP2C9 Inhibition	CYP2C9	No	—
	CYP2D6 Inhibition	CYP2D6	No	—
	CYP3A4 Inhibition	CYP3A4	No	—
	Skin Permeability	Log Kp	-7.53 cm/s	Poor skin penetration
Drug-Likeness	Bioavailability Score	—	0.55	Moderate drug-likeness
	Synthetic Accessibility	—	3.15	Moderately easy to synthesize (scale: 1–10)
	Rule of Five Compliance	Lipinski, Veber	Compliant with 3 Lipinski + Veber rules	Drug-like

Log P : Logarithm of partition coefficient (lipophilicity), Log S : Logarithm of solubility, P-gp: P-glycoprotein, CYP:Cytochrome P450 enzymes, GI :Gastrointestinal, BBB:Blood-Brain Barrier, Kp: Skin permeability coefficient, ESOL, Ali, SILICOS-IT:Solubility prediction models, iLOGP, XLOGP3: Lipophilicity prediction models

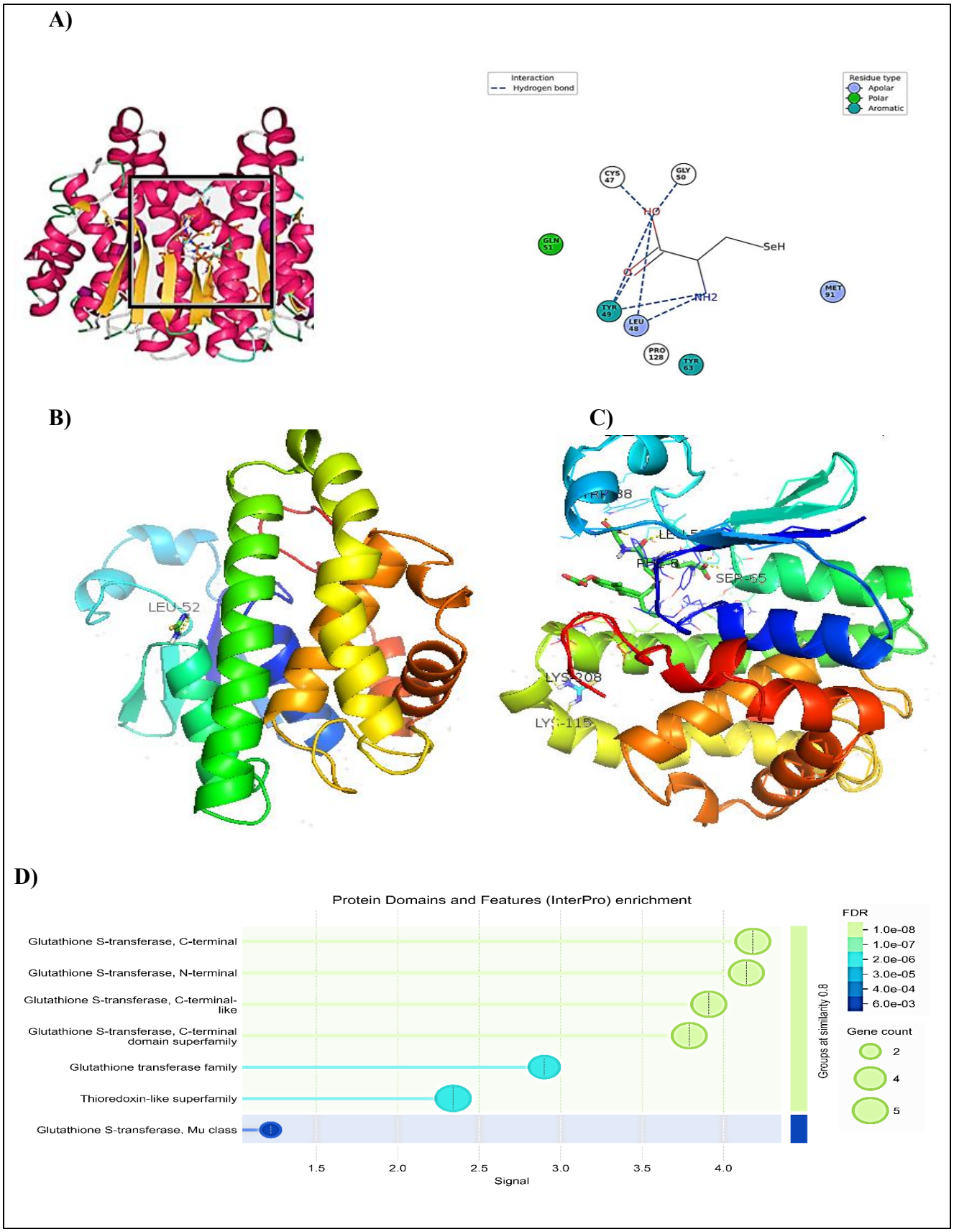


Figure 5. A: Molecular docking analysis for (PDB ID:1AQW; Glutathione S-Transferase in Complex with Glutathione) GST of selenocysteine (A) and selenourea (B) Molecular docking analysis for GST inhibition of selenourea. (C) (PDB ID:11GS; Glutathione S-transferase complexed with etacrynic acid-glutathione conjugate). (D) Protein Domains and features.

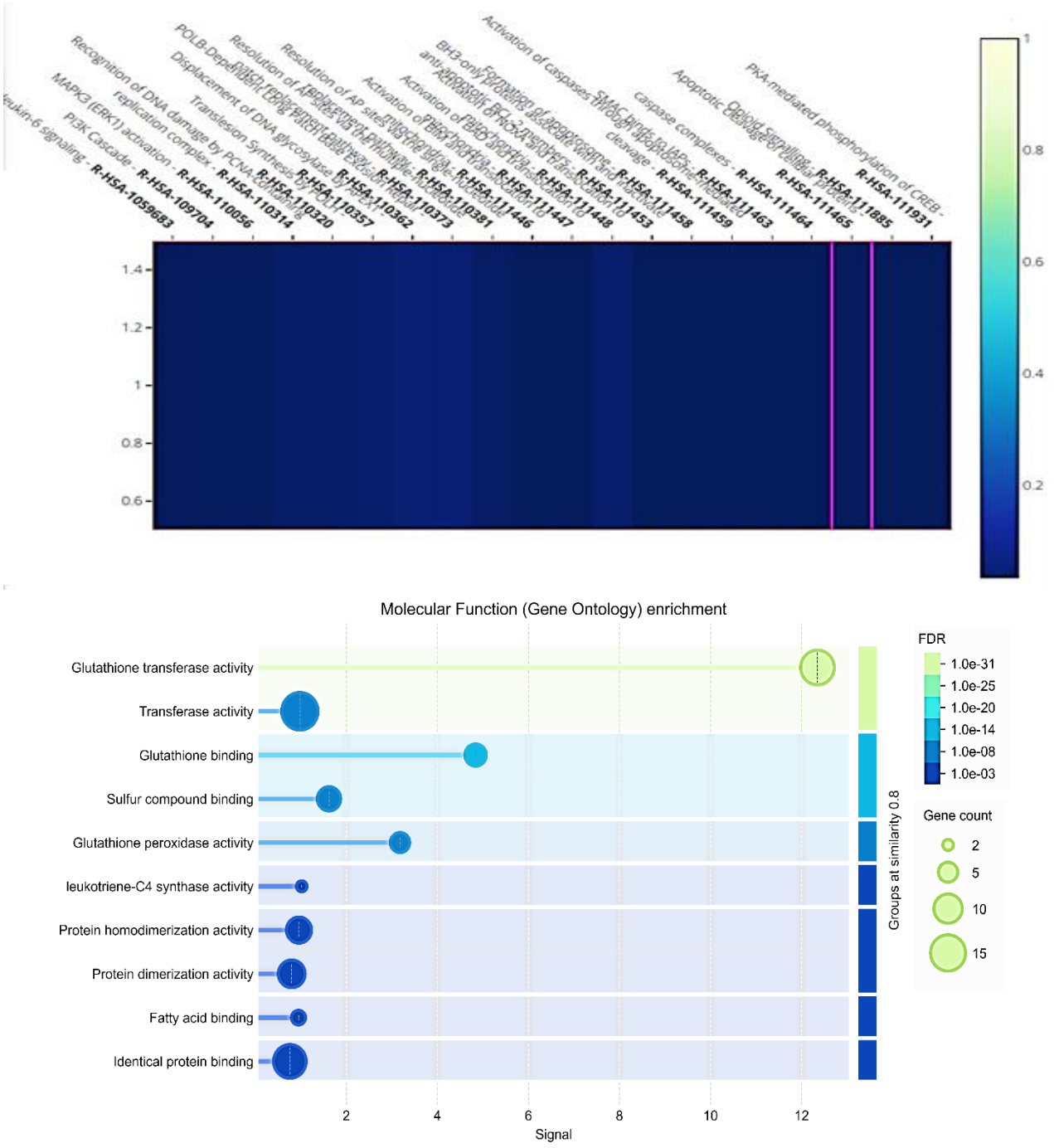


Figure 6. Molecular pathway association with self-normalizing neural networks (top panel) and gene ontology(below pane) of Selenourea

Discussion

Drug designs bind to the biomolecules with which they interact, changing their structure, shape, and charge states. Moreover, they affect many other properties, such as bioavailability, metabolic half-life, side effects, etc.[29] Computer-designed studies have been carried out recently to show the structure-activity relationship of drugs, and improving the affinity, selectivity, and stability of these protein-based therapeutics have also been developed. So, this study was conducted using computer-aid protein-ligand analysis of selenourea compound on GST family enzyme (Figure 6) Binding selectivity was defined concerning the binding of Selenourea to a substrate (protein) forming a complex with non-covalent interactions, such as hydrogen bonding or Van der Waals force. Moreover, ΔGs were found to be -3.6, -3.7, -3.1, -3.8, 3.1, and -2.8 kcal/mol for GSTA1-1, GST A2, GSTA3, GST Delta, GST K, and GSTM1, respectively. Selenourea's low energies are associated with small-molecule probability, identifying potential therapeutic compounds capable of modulating the activity of specific biological targets.

This study determined a quantitative structure-activity relationship (QSAR), in which a correlation between calculated biophysical properties of selenourea (Figure 1) and significant interactions were observed between these compounds and with the amino acids of the active site of the protein with amino acid ion charged (Figure 2). For example, the selenourea interacts with GST delta with hydrogen bonds GSH210, GLN124, ASN123, and ARG122 amino acids. (AIt was bound to hydrogen bind of GST alpha (A2) with GSH230, and LEU198 amino acids, but it was not connected to GST A1. GSTA1-1PHE97, GLN199, SER202 hydrogen bond. For GSTM1 ASP8, TYR6 amino acid binds to hydrogen bound. Finally, there could be a target between GST Kappa with SER15 MET102 hydrogen bond ARG204 GLU17 contact amino acid residues. This situation can be associated with GSTK found in mitochondria. Furthermore, selenourea was directly bound by GST with GSH201, LEU52 hydrogen bond ΔG 2.8 kcal/mol, while selenocysteine was bound by CYS47, and GLY50, LEU48, and TYR49 amino acid residue to hydrogen bind with ΔG-3.84 kcal/mol, but selenourea bind to LEU52 amino acid hydrogen bound (Figure 5). GST inhibition of selenourea with complexed with etacrynic acid-glutathione conjugate amino acid residue to hydrogen binds LYS2028, LYS2025 with ΔG-3.0 kcal/mol. A previous study showed that GSTP was subjected to phosphorylation at Thr109, Ser28, Ser154, and Ser184; O-glycosylation at Thr5 amino acids, but unknown placement to N-terminal. When these Figures (5) are compared to Figure 2, selenourea bonds to the GST enzyme N-terminal side. A study reported that the GST N-terminal domain fold is similar to cellular homeostasis, and detoxification proteins such as glutathione peroxidases and glutaredoxin.[30] The accumulation of ROS, particularly following the inhibition of Glutathione S-transferases (GSTs), can trigger apoptosis in cancer cells.[31,32] Identifying cleaved proteins such as PARP and Bid provides valuable insights into the mechanisms by which GST inhibition facilitates apoptosis and may offer potential therapeutic strategies for cancer treatment.[33]

Signaling analysis results (Figure 6) show that selenourea acts to apoptotic cleave cellular proteins (R-HSA-111465) found in the cytosol. These proteins are E-cadherin, Beta-catenin, alpha Fodrin, GAS2, FADK, alpha adducin, HIP-55, and desmoglein and they have critical roles for cell adhesion and maintenance of the cytoskeletal structure, and also these structures target caspase proteins. Moreover, it has been reported that cleavage of proteins such as APC and CIAP1 can forward stimulate apoptosis by producing proapoptotic protein.[34,35] In this study, we conducted E-Cadherin and Beta-catenin protein expression analyses (Figure 4). We found that both the IC_{50} dose of etacrynic acid and the IC_{50} concentration of selenourea, when compared to beta-actin, upregulated E-cadherin expression in the non-estrogenic MDA-MB-231 cell line. Additionally, the combination of selenourea (IC_{50}) and etacrynic acid (IC_{25}) also upregulated E-cadherin expression in these cells. When comparing Beta-catenin expression levels to beta-actin expression, individual treatments resulted in upregulation in the MDA-MB-231 cell line. However, under combination conditions, Beta-catenin expression was downregulated. Furthermore, neither the individual treatments nor the combination had any effect on expression in estrogenic MCF7 cells. In this case, the combination of selenourea (IC_{50}) and etacrynic acid (IC_{25}) also led to downregulation of expression. This effect is associated with the inhibitory action of the selenourea and etacrynic acid combination on GST activity and its selective effect on apoptotic progression.

Apoptosis and deterioration of redox homeostasis are among the main mechanisms that lead to multidrug resistance. Moreover, resistance to cancer chemotherapeutic agents in several oncogenes is mediated by the overexpression of certain GST enzymes associated with their ability to catalyze drug conjugation to GSH. So, in this study, between selenourea and glutathione-S-transferase complexed with Etacrynic acid-glutathione conjugate analysis was made for inhibitory effect. GST inhibitor, Etacrynic acid disrupted the GSH-GST system. This leads to increased ROS accumulation, making cancer cells more prone to oxidative stress and triggering apoptosis. The use of GST inhibitors to manage to prevent resistance among anticancer agents can be promised therapeutic emerging as a pro-agent. Therefore, the effect of medicinally active compounds on metabolic enzyme determination is crucial for drug design studies. Despite the limitations of the study, further *in vitro* and *in vivo* analyses of selenourea on drug metabolism enzymes will increase its biological importance. Proteomic analysis reveals a subset of apoptosis-cleaved proteins, including novel candidates, that regulate cell death in breast cancer.

In conclusion, this study demonstrates that combination therapy involving selenourea and Etacrynic acid, both acting as glutathione S-transferase (GST) inhibitors, holds significant potential in the treatment of breast cancer. By targeting GST, a key enzyme implicated in detoxification and drug resistance, this dual approach disrupts cellular redox balance, leading to the accumulation of reactive oxygen species and the induction of oxidative stress. This oxidative stress, in turn, amplifies apoptotic signaling pathways. Western blot (↑E-cadherin, ↓β-catenin), ADME analysis (good bioavailability, P-gp evasion), and functional enrichment, the data collectively supports that your treatment is effectively inducing apoptosis, likely through mitochondrial and DNA-damage-related pathways. Importantly, our findings reveal that GST inhibition facilitates the identification of apoptosis-associated cleaved proteins, suggesting a direct link between GST suppression and enhanced apoptotic machinery activation. These cleaved proteins may serve as novel biomarkers or therapeutic targets in breast cancer. The observed synergistic effect of selenourea and Etacrynic acid may be attributed to their complementary mechanisms, while both inhibit GST, they may differentially affect metabolic and signaling pathways associated with tumor cell survival.

Overall, this combinatorial strategy not only enhances the efficacy of pro-apoptotic responses in breast cancer cells but also offers insights into the molecular events downstream of GST inhibition. These findings underscore the therapeutic value of simultaneously modulating redox homeostasis and apoptosis in developing more effective breast cancer treatments.

Conflict of interest statement

The author declared no conflict of interest in the manuscript.

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