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RESEARCH ARTICLE

Binding studies of trans-resveratrol with superoxide dismutase (SOD1): Docking assessment and Thermoanalysis

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Abstract: The binding pursuits of trans-resveratrol (t-RSV), an amazing health supplement are investigated with an antioxidant enzyme, superoxide dismutase (SOD1). The aim of the study is to dock t-RSV on the adrenaline binding site on SOD1 in order to explore its potential to act as a safety net against amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder that affects motor neurons. In *silico* GLIDE docking methodology and in vitro microcalorimetry technique is utilized for the investigation of binding parameters of t-RSV with SOD1. The study provides useful and distinct information about the amino acids involved in the interactions at molecular level along with the nature of forces involved in binding of t-RSV with SOD1. The docking analysis using the scoring functions of Schrodinger's Glide package depicts that GLU100, PRO28, LYS23, TRP32 residues of the peptide backbone on SOD1 interact with phenolic groups of t-RSV. The information on thermodynamic parameters, *i.e.* binding constant (K_b), free energy (ΔG) and enthalpy (ΔH) generated through calorimetric titrations suggests that the reaction between t-RSV and SOD1 is spontaneous and exothermic. Both the studies are found to be in close agreement with each other based as far as the magnitude of binding constant ($K_b = 9.9 \times 10^4$) is concerned.

Keywords: trans-Resveratrol, superoxide dismutase, docking, microcalorimetry, binding constant, free energy

1 Introduction

Trans-resveratrol (3,5,4′-trihydroxy-trans-stilbene), widely regarded as a powerful dietary supplement with a multitude of health benefits, slows down the ageing process and helps keeping the body's cells, inside and out, looking young and healthy.[1] It is already reported that various species treated with resveratrol has shown lifespan extensions.[2,3] This nutraceutical helps in preventing age-related diseases such as atherosclerosis, cancer, Parkinson's disease, and Alzheimer's disease. It mainly exerts its action due to its intrinsic antioxidant property that attenuates oxidative damage to biological systems.[4–8] It works against ageing by protecting the body cells from free radicals generated during normal metabolism and damage caused by oxidative stress. Trans-resveratrol (t-RSV) either directly scavenges reactive oxygen species (ROS) or modulates the expression and activity of antioxidant enzymes such as superoxide dismutase (SOD).[9]

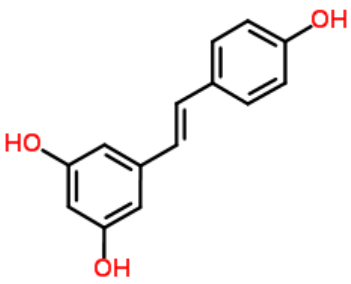

Figure 1. Molecular structure of trans-resveratrol

SOD acts as one of the essential biomarkers of ageing,[10–12] the parameters that change qualitatively or quantitatively during ageing or age related diseases.[13] These are regarded as potential key targets for studying anti-ageing effect of the drug molecules. Thus one of the ways to contribute towards quest of youth is to study the interaction of these intrinsic key targets with drug molecules having anti-ageing potential. The metalloenzyme, SOD plays a pivotal role in metabolism of

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deleterious ROS and free radicals. It removes superoxide O^{2-} radical, repairs cells and reduces the damage done to them by superoxide and oxygen free radicals. SOD catalyzes the reduction of superoxide anions to hydrogen peroxide (Figure 1). It also promotes the activity of nitric oxide (NO) by scavenging the superoxide anions and thus prevents the conversion (inactivation) of NO to peroxynitrite.[14]

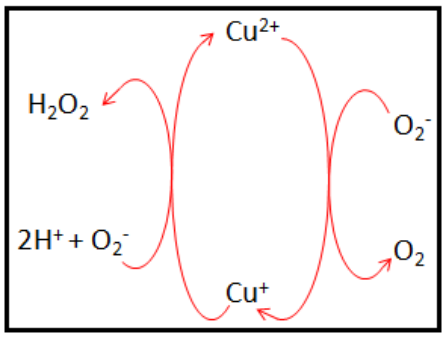

Figure 2. Catalytic cycle of SOD-1 ('ping-pong' mechanism)

The x-ray crystal structure (Figure 2) of the oxidized form of Cu, Zn-SOD from bovine erythrocytes shows a protein consisting of two identical subunits (a dimer composed of 2 chains (A and F)) held together almost entirely by hydrophobic interactions.[15]

Intervention to stabilize SOD1 dimer and inhibit aggregation is regarded as a potential therapeutic strategy in control of ageing. This approach can be successfully applied in treatment of major age-related disorders characterized primarily by selective neurodegeneration of the motor neurons leading to muscle atrophy and paralysis (fatal human neuropathy).[16] It is well established that binding of adrenaline with SOD1 dimer maintains its integrity by circumventing the dimer aggregation and thus controls amyotrophic lateral sclerosis (ALS).[17] Literature reports show that amyotrophic lateral sclerosis (ALS) is one of the rapidly progressive ageing disorders in which mutations in the gene encoding Cu/Zn SOD1 decrease protein stability. This further promotes misfolding or aggregation leading to toxicity due to oxidative damage stemming from aberrant SOD1 redox chemistry. However, this malfunctioning of superoxide dismutase (SOD1) can be prevented by binding SOD1 with ligands at the site where adrenaline is bound. The drugs or ligands binding at this site are found to be mainly useful in treatment of ALS by acting as SOD1 stabilizers as proposed in literature.[17–22] This binding site is different from well studied hydrophobic cavity created by Val7-Gly147-Val148 in the dimerization region and active site on SOD1.[23,24]

Many group of workers have successfully investigated the interactions between SOD1 and some important molecules.[13,25–28] However, the binding aspects of SOD1 with t-RSV, an innovative anti-ageing molecule, have not yet been taken into consideration. With this background in mind, it was envisaged to study the binding of t-RSV with SOD1 at a key region identified in chain A of SOD1(Figure 2) with the aim to stabilize SOD1 dimer which is useful for treatment of ALS. In this manuscript, it is tried to explore the interaction of t-RSV with SOD through in silico docking and in vitro microcalorimetry technique. The molecular docking provides useful information on the binding mode and microcalorimetry is a direct tool to generate the thermodynamic data pertaining to binding of t-RSV with SOD1.[29–31]

2 Materials and Methods

2.1 Datasets

The crystal structure of SOD1(PDB entry code 4A7U) was downloaded from the RSCB Protein Data Bank (http://www.rscb.org), and ligand structures (SDF 3D format) were obtained from the PubChem database (http://pubchem.ncbi.nlm.nih.gov).

2.2 Chemicals

Trans-resveratrol (99% purity) and Superoxide dismutase (SOD) (bovine) were procured from Alfa Aesar (Thermo Fisher Scientific). They were properly stored at cool and dry place as per their recommendations.

Phosphate buffer (pH= 7.4, 0.1 M): It was prepared as per IP' 1996 recommendations.

2.3 In silico binding study using molecular docking methodology

A robust, high- speed and accurate computational strategy was employed to predict binding mode of a t-RSV on SOD1. The Schodinger's GLIDE (grid-based ligand docking with energetics)[32–36] docking methodology, supported by Maestro 10.5 was used to locate the appropriate binding orientations and ligand conformations with respect to SOD1.

The study involves the preparation of SOD1 (4A7U) structure. It was done by cleaning up the X ray structure using the protein preparation wizard in the Schrodinger software graphical user interface Maestro. This was attained by adding protons, fixing bond orders, optimizing protonation states and hydrogen bond networks and performing a minimization under restraints. Here chain A of SOD dimer was retained and chain F was deleted for further simplification of structure. This was followed by

the generation of energy grid using the GLIDE protocol previously described.[35,36] A grid representing the properties of SOD 1(4A7U) PDB structure, *i.e.* electrostatic potential generated on each grid points, van der Waals forces *etc* was generated from the prepared structure. Further the ligand (t-RSV) was also prepared using Ligprep from the Schrdinger suite. All possible protomers (protonation states) and ionization states were enumerated generating various potential tautomers which are themselves minimized. The prepared ligand was then screened using the grid that was previously generated. During this step, the ligand poses generated pass through a series of hierarchical filters that evaluate the ligand's interaction with the protein (SOD). The ligand orientations (poses) were assigned scores, related to the intermolecular interaction energy, and ranked relatively. The best 10 poses and corresponding scores were evaluated using Glide in Standard precision mode (Glide SP) of Glide algorithm. The protocol of Prime/MM-GBSA module of Schrdinger software 2016 was applied on the minimum energy conformation state of ligand bound protein complex obtained from the Glide SP to estimate the binding free energy (ΔG_{bind}). Energy minimization for the complex using OPLS3 force field within Macro Model was performed. The results of Glide docking were incorporated in the project table as a pose viewer file which was further explored for various types of bonding and non-bonding interactions. The theoretical calculations were done as follows:

$GScore = 0.05\,vdW + 0.15\,Coul + Lipo + H_{bond} + Metal + Rewards + RotB + Site$

vdW- Van der Waals energy

Coul- Coulomb energy

Lipo- lipophilic term

H_{bond}- hydrogen- bonding term

Metal- Metal-binding term

Rewards- term including rewards and penalties for buried polar groups, hydrophobic enclosure, *etc*.

RotB- penalty for freezing rotatable bonds Site- Polar interactions in binding site.

2.4 *In vitro* binding study using micro calorimetry

Calorimetric titrations were performed on Micro reaction calorimeter-μRCSYS-001 (Thermal Hazard Technology, UK) in isothermal mode at 25^{o}C. The stock solutions of SOD (3μM) and trans-resveratrol (150 μM) were prepared in phosphate buffer, pH= 7.4. The temperature difference between the sample cell (1.5 ml capacity) containing SOD1 and reference cell (1.5 ml capacity) containing buffer was measured as heat change signal. The titration was done by adding equal volumes of (10 aliquots of 25 μl) t-RSV solution taken in 250μl syringe to the sample and reference cells. Prior to start of titration, all solutions were degassed properly and the system was properly calibrated to get a stable base line. The dilution effect and machine effects were nullified by running the control experiments. It was achieved by first titrating the t-RSV taken in the syringe with phosphate buffer taken in sample cell and then titrating the buffer taken in syringe and SOD in the sample cell. The contents of the sample cell were stirred throughout the experiment at 200 rpm to ensure thorough mixing.

3 Results

3.1 Molecular docking analysis using site marker adrenaline

The t-RSV was made to dock at adrenaline (site marker) binding site in chain A of SOD 1(figure 1). This particular site was chosen in the study as discussed earlier[9] based on the established hypothesis of stabilization of SOD1 dimer by adrenaline as a means of preventing ALS, an age-related disorder.

The GlideScore values of the 10 best energy minimized docked ligand poses were used for qualitative assessment of the binding of t-RSV to SOD1. Visual inspection of those in silico poses clearly demonstrates the existence of both hydrogen bonding and hydrophobic interactions. It is quite apparent from the docking observations that the phenolic functional groups present in t-RSV facilitates hydrogen bonding interactions with the carboxyl functional groups of glutamic acid and proline residues of the peptide backbone present in the selected cavity of SOD1 *i.e.* 3-O-H (t-RSV)--- O-C=O (GLU 100) and 5-O-H (t-RSV)---O=C (PRO 28). Besides this one of the phenolic group of t-RSV forms hydrogen bond with amino group of lysine residue at the considered site *i.e.* (LYS 23) N-H---O-H (5) (t-RSV). Figure 2 also displays the hydrophobic interaction in form of pi-pi stacking between phenyl rings of t-RSV and tryptophan residue present in the cavity on SOD1 *i.e.* 4' Hydroxyphenyl ring---TRP 32. Furthermore the relevant amino acid residues lying within within 2 A^o around the docked t-RSV are also portrayed. The docking results are represented in Figure 2 and Table 1.

The minimum energy conformation state of ligand bound protein complex with least GlideScore was further considered out of ten generated binding poses. The hydrogen bonding and hydrophobic interactions between ligands and SOD1 were observed using pose viewer file.

The binding free energy (ΔG_{bind}) was calculated using Prime-MM-GBSA module of Maestro 10.5 as fol-

lows (Equation 1) and was found out to be -40.05.

$$\Delta G_{bind} = G_{complex} - (G_{protein} + G_{ligand}) \quad (1)$$

The binding constant (K_b) for SOD- t-RSV interactions was calculated by using the following equation (Equation 2) and was found out to be 9.9×10^4 kJmol^{-1}.

$$\Delta G = -2.303RTlogK_b \quad (2)$$

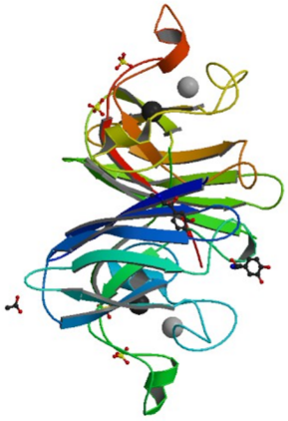

Figure 3. Crystal structure of SOD1 bound with adrenaline

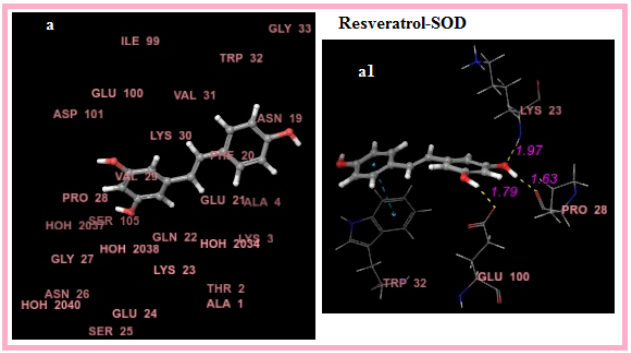

Figure 4. The Docking results. (a) represents best docked poses depicting relevant amino acid residues at SOD binding site within 2 A^o around the docked ligand (t-RSV); (a1) represents type of interaction (hydrogen bonds and stacking) between t-RSV and SOD.

3.2 Thermodynamics of t-RSV- SOD 1 interaction using microcalorimetry

This ultrasensitive technique analyses the thermal parameters associated with binding process of t-RSV with SOD 1. The hydrogen bonds and van der Waals forces are the major driving forces behind these binding events.

At each injection of ligand into the sample having 1:1 stochiometry, an equilibrium of free and bound ligand is established, and heat is released (exothermic) as a binding event. At the end of the titration, all the binding sites in the sample are occupied and heat evolved become negligible.

Interaction of t-RSV and SOD1 at equilibrium is represented as:

$$SOD + t-RSV \leftrightarrow SOD : t-RSV \quad (3)$$

The enthalpy of binding per mole of drug (ΔH^o) can be calculated using the molar concentration of SOD1–t-RSV complex (c) in solution at equilibrium and experimentally observed enthalpy of interaction (ΔH_{exp}), by using Equation 4.

$$\Delta H_{exp} = \Delta H^o x\, c \quad (4)$$

Equilibrium constant for Equation 3 is calculated as

$$K = \frac{c}{(a-c)\ (b-c)} \quad (5)$$

where 'c' is the concentration of SOD–t-RSV complex; a and b corresponds to the concentrations of reactants, *i.e.*, SOD and t-RSV, respectively.

$$c = \frac{\left[A - \sqrt{(A^2 - 4ab)}\right]}{2} \quad (6)$$

where $A = a + b + \frac{1}{K}$

$$\Delta H_{cal} = \Delta H^o \times \frac{\left[A - \sqrt{(A^2 - 4ab)}\right]}{2} \quad (7)$$

The data assessment was executed with an assumption of one-site binding model. The binding constant (K_b)[1] and ΔH were calculated using a self-consistent iterative nonlinear least-square regression program to minimize the values of (ΔH_{exp}- ΔH_{cal}).[2]

Binding parameters such as enthalpy of binding (ΔH) and binding constant (K_b) were calculated (Table 2) and binding isotherm was thus generated from the computer program prepared by us as discussed under experimental section (Figure 3). Since temperature[1] is held constant throughout, the free energy (ΔG) of the binding reaction is determined following the Equation 2 and entropy is determined as follows:

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

4 Discussion and Conclusions

Molecular docking g1ives us an insight of the preferred orientation and interaction paradigm of the drug molecule to the macromolecule. Microcalorimetry is an explicit technique to elucidate the thermodynamics of binding of a ligand to macromolecules. Both these

Table 1. Molecular docking parameters of t-RSV with SOD 1 using adrenaline as site

Docking Score	Type of interaction	Corresponding Bond length (A^o)
-5.966	(i)3O-H---O-C=O (GLU 100)	1.79
	(ii) 5O-H---O=C (PRO 28)	1.63
	(iii) (LYS 23)N-H---O-H (5)	1.97
	(iv) 4'Hydroxyphenyl ring---TRP 32 (pi-pi stacking)	-

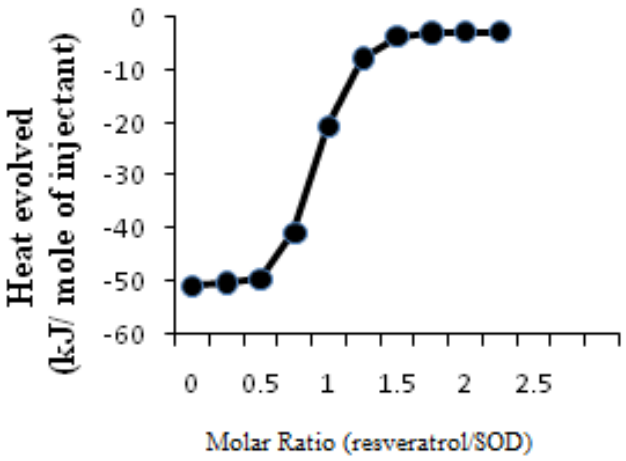

Figure 5. Binding isotherm of trans-resveratrol with SOD obtained from calorimetric measurements

methodologies work complementary to each other defining the binding criteria of t-RSV with SOD1 in the present study. Both the above mentioned results were found to complement each other with respect to binding affinity data as compiled in Table 2. The small variations in computational and experimental values may be attributed to the fact that docking was based upon the static and fixed X-ray crystal structure of protein where significant protein structural freedom is not allowed to acquire different conformations upon ligand binding.

Unlikely, the calorimetric results are based upon full freedom in the structural flexibility of the protein in phosphate buffer (pH 7.4).[37,38] Thus, structural rearrangements observed in the SOD that occurred upon ligand binding in solution, may be the plausible cause of this difference.

The negative values of free energy (ΔG) and enthalpy (ΔH) support that the binding of t-RSV to SOD is spontaneous and exothermic. The fairly good value of binding constant indicates good stability of t-RSV-SOD 1 complex. Thus binding strategy of t-RSV to SOD1 further supports the hypothesis of stabilization of SOD dimer and thus acts as an effective measure to prevent the occurrence of ALS.

Table 2. Binding parameters of t-RSV with SOD 1 obtained from docking analysis and calorimetric titrations

Binding parameter	Docking analysis (in silico study)	Calorimeteric titrations (in vitro study)
K_b (M^{-1})	9.9 x10 ^{4}	7.3x 10^4
ΔH ($kJmol^{-1}$)	-	-47.8
ΔG($kJmol^{-1}$)	-40.05	-39.17
ΔS($kJmol^{-1}K^{-1}$)	-	0.29

It has been concluded that the virtual and experimental aspects are working hand in glove with each other to figure out the binding events between the nutraceutical, t-RSV and the natural antioxidant, SOD1. The docking analysis and calorimetric results were found to complement each other in the present study. The significant magnitude of binding constant summarized in Table 2 revealed the strong binding affinity of t-RSV with ageing biomarker, SOD1. The present study is a beneficial step towards exploring the potential of ligands in stabilization of the antioxidant enzyme, SOD1 leading to control of the neurodegenerative disorders such as ALS. This information about the binding affinity and the interactions involved during complex formation of SOD1 with t-RSV would embolden future studies to postulate the various mechanisms of ageing and its control at molecular and cellular level. The knowledge generated from this study would be a favorable underpinning in the promising field of anti-ageing therapy and proteomics.

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