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Full Length Research

Modeling and proposed mechanism of two radical scavengers through docking to curtail the action of ribonucleotide reductase

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Ribonucleotide reductase (RR) is a ubiquitous cytosolic enzyme required for DNA synthesis and repair in all living cells. Therefore, the crucial role of this enzyme in cell division makes it a potential target for designing drugs that inhibit cell growth for cancer therapy. An increased interest in RR as a target for cancer therapy has been documented since the discovery that human RR is regulated by p53 enzyme and that a mutation in p53 leads to several forms of cancer. Cell proliferation stops if normal RR is inhibited. A new strategy to kill the cancer cells would be using specific inhibitors that inhibit the action of RR enzyme. The inhibitor must be a radical scavenger which destroys the tyrosyl radical or an iron metal scavenger (which affects iron center). In this view, modeling studies on human RR-R2 were done to understand its interaction with radical scavengers, flavin (FLA) and phenosafranine (PHE) through docking since they have good reductive property. Radical scavengers are active against RR enzymes at anaerobic condition and their radical scavenging mechanism has been proposed. In aerobic condition RR enzyme will reproduce the radicals and then the radical scavengers fail to act as drug. So, the metal scavengers may be better than the radical scavengers to curtail the action of RR enzyme.

Key words: Ribonucleotide reductase, tyrosyl radical, flavin, phenosafranine, p53 enzyme, human R2.

INTRODUCTION

Ribonucleotide reductase (RR) is a ubiquitous cytosolic enzyme, responsible for converting ribonucleotides into deoxyribonucleotides, the eventual substrates for DNA polymerase (Reichard, 1993) and also repair DNA in all living cells (Eklund et al., 2001). It contains two dissimilar protein components, R1 and R2 (Figure 1). The R1 subunit has homodimeric structure, with a molecular mass of ~170 kDa and has allosteric effector site that controls enzyme activity and substrate specificity (Wright et al., 1990). The R2 subunit is also a homodimer, with a molecular mass ~85 kDa, and forms two dinuclear iron centers that stabilize a tyrosyl free radical which is required for catalytic activity (Wright et al., 1990). The R1 and R2 subunits interact each other at their C-terminal ends to form an active holoenzyme (Davis et al., 1994). The overall function of RR involves the reduction of the hydroxyl group on the 2- carbon of the ribose moiety of nucleoside diphosphates and triphosphates (NDPs and NTPs). This conversion is achieved by free radicals which are stored in the dinuclear center of the enzymes until they are required for catalysis.

RR enzyme has two important components such as a radical generator (R2 subunit) and a reductase (R1 subunit). The production of radicals by R2 and its storage constitute the first step of the reaction in RR enzymes. Surprisingly the radical generators of all types of RRs are not the same, whereas the reductase component is more or less similar. The RR enzymes isolated so far have been classified into three types (class I, II and III) based on the oxygen dependence and metal cofactors involved in the generation of essential free radicals (Reichard, 1993; Jordan and Reichard, 1998). Mammalian RRs belong to class I reductases, which contain two pairs of

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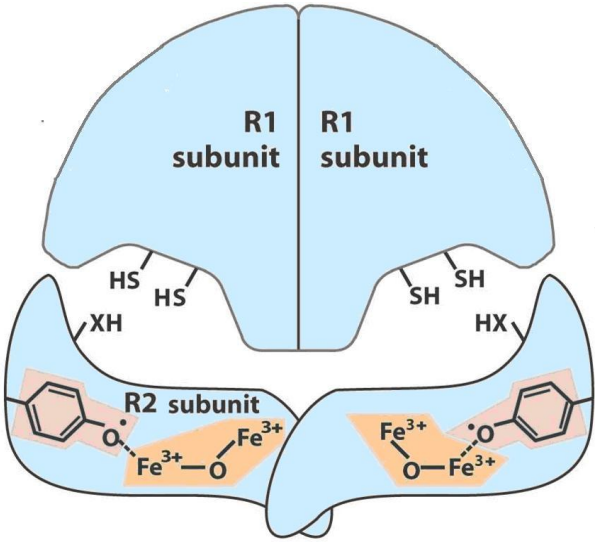


Figure 1. Schematic cartoon diagram of the RR enzyme.

iron and produce the catalytically essential tyrosyl free radical.

Ribonucleotide reductase (RR) enzyme and cancer

An increased interest in RR enzyme as a target for cancer therapy is seen ever since the *Homo sapiens* ribonucleotide reductase (HsRR) of a new type was identified which is regulated by p53 (Lozano and Elledge, 2000; Nakano et al., 2000). The p53 protein actively suppresses the tumor formation, but its mutation causes several forms of cancer. Over 80% of the human tumors are found to contain mutations in p53 (Tanaka et al., 2000). Mammalian RR-R2 is located in the cytoplasm and regulated by the cell cycle. The new R2 gene product called p53R2 is located in the nucleus. The p53 binds to the first intron of p53R2 gene and is important to activate its transcription factor directly (Priya and Shanmughavel, 2009). This gene has been identified in human and mouse cells. The review by Eklund et al. (2001) discusses the possibility of specific inhibitors to RR enzymes. Their argument is based on the fact that cancers often have mutations in the p53 pathway and unable to make p53R2. RR is necessary for DNA replication and thus inhibition of this enzyme will in turn inhibits cell division, whereas normal cells with p53 could survive on the DNA pool supplied by p53R2. A new strategy to kill cancer cells would be to specifically inhibit the RR enzyme, which is essential for cancer cells after DNA damage since they cannot induce the p53R2 due to lack of p53. In contrast, normal cells can repair their DNA damage with the help of the induced p53R2.

Since RR activity is necessary for DNA replication inhibition of this enzyme will inhibit cell division, (Szekeres et al., 1997). Therefore an understanding of the molecular mechanism of RR is essential in designing new cytostatic drugs. The inhibitor must be a radical scavenger to destroy the tyrosyl radical or an iron metal scavenger (which affects the iron center). The iron or radical site of R2 protein can react with one-electron reductants (e.g., hydroxyurea and hydroxylamine), whereby the tyrosyl radical is converted to a normal tyrosine residue (Sneeden and Loeb, 2004). These drugs are slow and relatively unspecific (Stubbe, 1990). However, other compounds such as flavin (FLA) (Fontecave et al., 1989) and phenosafranine (PHE) (Sahlin et al., 1989) are available to reduce the radical activity.

In view of inhibitor study using radical scavengers, the structure of radical generator, human RR-R2, enzyme was constructed using homology modeling to understand the interactions of drug with the active site residues. Based on the observation and reports, two different drug actions, as radical scavengers (FLA and PHE) or as metal scavengers (thiosemicarbazones) are needed to stop the catalytic activity. Our study has been concluded by finding the most appropriate drug to curtail the catalytic action of RR enzyme.

MATERIALS AND METHODS

Collection of sequences

The complete protein sequences of RR-R2 from *H. sapiens* (P31350) and other RR-R2 sequences were retrieved from NCBI (http://www.uniprot.org/uniprot). The relatedness of sequences deposited in databases was evaluated by Blast (Altschul et al., 1990) implemented via the NCBI server (http://www.ncbi.nim.nih.gov/blast) against the complete training data set. The Blast P (protein query–protein database) comparison was performed using the protein database (PDB).

Sequence alignment

Since RR-R2 radical generators vary from species to species, four well known eukaryotic sequences including mouse RR-R2 (template model), yeast RR-R2, human p53R2 and target human RR-R2 proteins (Figure 2) and one bacterial sequence (*Escherichia coli* RR-R2) were selected for multiple sequence alignment (MSA) using ClustalW program (Chenna et al., 2003). The secondary structure elements of the RR-R2 enzymes are shown on the top of the human enzyme and 100% conserved residues are shown in red colour. The Swiss-prot accession numbers for various species of R2 enzyme sequences are as follows: mouse, P11157 (Thelander and Berg, 1986); human, P31350 (Pavloff et al., 1992); *E. coli*, P69924 (Carlson et al., 1984); human, p53R2 (Q75PY9); and yeast, P09938 (Elledge and Davis, 1987). The P53R2 and *E. coli* RR-R2 sequences have been truncated in the N-termini residues. The dinuclear binding residues are conserved in all sequences including *E. coli*.

Homology modeling of human R2 enzyme

The pair wise alignment between the human RR-R2 and mouse

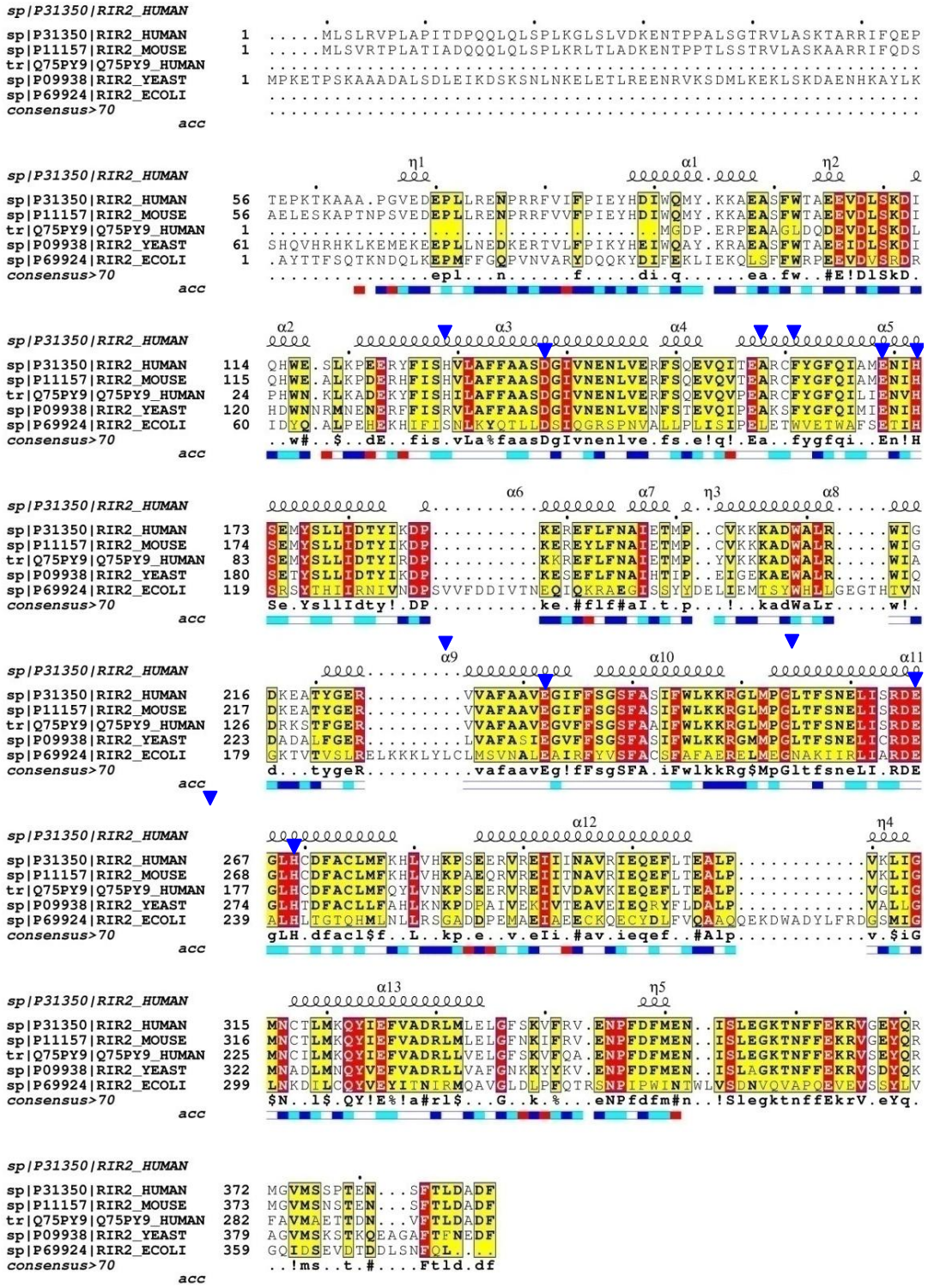


Figure 2. Multiple sequence alignment of RR-R2 structures from various species. The proteins listed from top to bottom: human RR-R2 from *Homo sapiens* (Uniprot accession number P31350); RR-R2 from mouse (P11157), p53RR-R2 from human (Q75PY9), RR-R2 from yeast (P09938) and RR-R2 from *E. coli* (P69924). Human RR-R2 secondary structure elements are shown in top of the sequence alignment. Fully 100% conserved residues shown in red box with white character and partially more than 80% conserved residues shown in yellow color with black character. All metal binding residues (100% conserved) are shown in blue color triangle box.

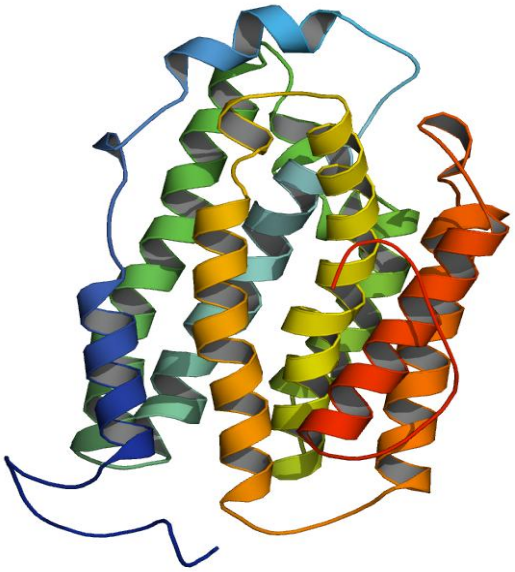


Figure 3. Modeled human RR-R2 enzyme (Swiss-model).

RR-R2 (data not shown) proteins was carried out using ClustalW program and the result showed 95% sequence identity between these two, both are expected to have similar structural reactivity. All active site residues are 100% conserved including the residues Tyr-189 (human RR-R2) and Tyr-177 (mouse RR-R2) which are important for radical generation. So, the crystal structure of mouse RR-R2 (pdb id: 1xsm) was used as a template model to generate the 3D modeled human RR-R2 structure of *H. sapiens* through online server of Swiss-model program (Schwede et al., 2003). The template model structure was directly retrieved from the PDB database (http://www.rcsb.org/pdb/). Visualization and cartoon diagrams were drawn using PyMOL (DeLano, 2002) graphics program. Evaluation and validation of the modeled structure was done using PROCHECK (Laskowski et al., 1993) and WHATIF. These programs generated Ramachandran plots of the amino acid residues in the allowed region and consider the overall G-factors. Out of 389 amino acid residues 288 were taken for constructing the structure. The homology modeled human RR-R2 structure was submitted to Protein Model Database (PMDB; http://mi.caspur.it/PMDB/) and it is assigned the accession code PM0075775.

Molecular docking study

Preparation of radical scavengers (Flavin and phenosafranine)

Tyrosine radical scavengers are very essential to inhibit DNA replication. According to the reports (Eklund et al., 2001; Fontecave et al., 1989), two potential radical scavengers, FLA and PHE, whose structures were built using the program PRODRG2 (Schuettelkopf and Aalten, 2004) and their energy-minimized structure coordinates were used for the docking studies with human RR-R2 protein. In addition, they are known as versatile compounds that can function as electrophiles and nucleophiles. Because of their chemical versatility and potential redox properties, they play a central role in aerobic metabolism through their ability to catalyze two-electron dehydrogenations of numerous substrates and to participate in one-electron transfer to various metal centers through their free radical states. With this capacity, they frequently form parts of multi redox-center enzymes (Massey, 2000).

In this study the AutoDock program [v.3.0.5] (Morris et al., 1998) was used for docking studies which utilizes a Lamarckian Genetic Algorithm for conformational searching and energy evaluation using a grid-based molecular affinity potential. Water molecules are not used for docking study as they make the analysis complicated. The distance-dependent function of the dielectric constant was used to calculate the energy maps and all other parameters have taken default values. Twenty-five best conformation of the protein ligand complex were retrieved and compounds with highest binding affinity from the best docked complexes were selected. The hydrogen bonding and non-bonded contacts for the complexes were derived using the program HBPLUS (McDonald and Thornton, 1994) and the pictorial representations are drawn using the program LIGPLOT (Wallace et al., 1995).

RESULTS AND DISCUSSION

Structural description of modeled human RR-R2 subunit

The Multiple sequence alignment results are shown in Figure 2 and it suggests that all of the sequences from mouse, human, P53R2 and yeast are highly conserved with appreciable sequence identity except *E. coli*. The dinuclear iron metal binding residues are conserved in all sequences including *E. coli*. The modeled human RR-R2 structure consists of α-helical bundle with 288 residues in the core structure. The dinuclear iron centre is located at the center of helical bundle in R2 subunits in all reported RR-R2 enzymes including template molecule, but the modeled human RR-R2 does not have dinuclear iron center. The overall human RR-R2 structure contains thirteen helices, of which eight long helices form a bundle (Figure 3).

These helices contribute about three quarters of the protein molecule and have an rmsd value of 0.45Å (for Swiss-model) compared to mouse RR-R2 (pdb id: 1xsm). Three of the five shorter helices orient perpendicularly to the long helices in the bundle and the other two are almost parallel to long helices. The long hydrophobic helix is surrounded by six other helices. The structure quality was validated by the Ramachandran map (Figure 4) using PROCHECK program. The torsion angles of 86.2% of residues are occupied within the allowed region and only 1.1% of residues are located in the disallowed region.

Docking study of radical scavengers

Flexible docking was performed for the modeled human RR-R2 enzyme with two well-known radical scavengers, FLA and PHE (Table 1) using the program Auto dock (Morris et al., 1998). Out of twenty-five conformations, one of its best conformations was chosen for the protein-

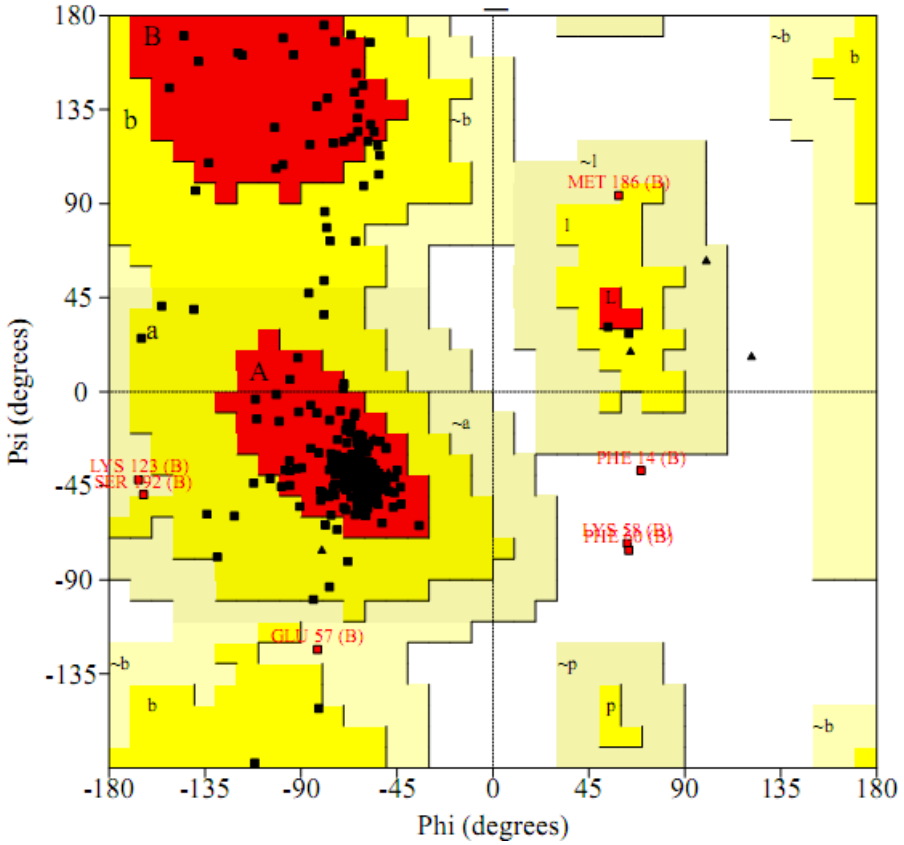
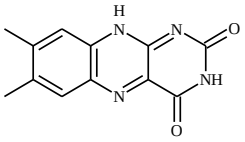
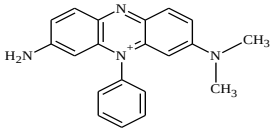


Figure 4. Ramachandran plot for the amino acids of modeled human RR-R2 enzyme occupied in favoured regions (block colour residues). Few residue's conformational angles are occupied in unfavoured region (red color residues with labeled).

Table 1. Details of drugs used for docking study.

S/N	Common name	Molecular formula	Molecular weight (g/mol)	IUPAC name	Structure
1	Flavin (FLA)	$C_{12}H_{10}N_4O_2$	243.23	7,8-dimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione	
2	Phenosafranine (PHE)	$C_{18}H_{15}N_4$	287.34	3,7-diamino-5-phenylphenazin-5-ium)	

drug interaction study from each ligand. On the basis of docking studies the interaction of radical scavengers with human RR-R2 protein, several energies calculated (docking energy, inter molecular energy, and internal energy) are given in Table 2. The docking energies of the FLA and PHE are noted as −09.37 and −07.69 kcal/mol, respectively. Highest binding affinity of the drug has been identified based on the lowest docking energy and FLA shows higher affinity than PHE to human RR-R2 protein.

The cartoon and LIGPLOT diagram of FLA and human RR-R2 docking interactions are shown in Figure 5a and b, respectively. Molecule FLA is comfortably occupied at

Table 2. The interaction energy of human RR-R2 and radical scavengers (drug) from molecular docking.

Drug	Binding energy (kcal mol-^{1})	Docked energy (kcal mol^{-1})	Intermolecular energy (kcal mol^{-1})	Internal energy (kcal mol^{-1})
FLA	-08.72	-09.37	-12.22	0.67
PHE	-07.11	-07.69	-9.83	1.38

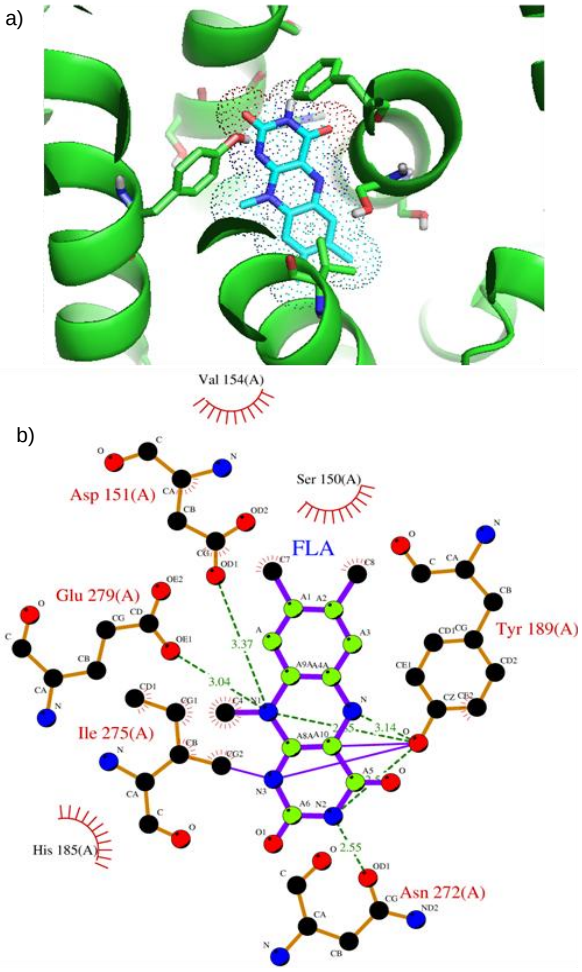


Figure 5. Modeled human RR-R2 enzyme- flavin drug interaction. (a) Cartoon diagram of the protein- drug (Cyan stick) interactions. (b) Ligplot diagram of the human RR-R2-flavin drug interactions. Hydrogen bonds are depicted in green colour dotted lines and hydrophobic interaction residues are shown in red colour.

the active site of human RR-R2 protein via six strong hydrogen bonds using the residues of Tyr-189, Asn-272, Glu-279 and Asp-151 (Table 3). In addition, some hydrophobic non-bonded interactions by the residues of His-185, Val-154 and Ser-150 also help the ligand molecule for its conformational stability. In this model, both the adjacent methyl groups in the inhibitor make hydrophobic contacts with the Ser-150. The human RR-R2 and PHE complex molecule (Figures 6a and b) is stabilized through four hydrogen bonds rises from two residues Glu-279 and Tyr-189. A few hydrophobic non-bonded interactions (Table 3) by the residues Val-154, Val-244, Ser-150, Glu-245 and Asp-151 are observed.

These hydrophobic forces are attributed due to N, N-dimethyl group in the ligand molecule. In the docking model of both these radical scavengers FLA and PHE, the reduced nitrogen atoms are directly binding to Tyr-189 and it is promising evidence that radical scavengers directly interact with the radical generator residue, Try-189. Based on these evidences radical scavenging reaction mechanism is proposed as follows using these two radical scavengers independently.

Proposed mechanism of flavin (FLA) action as radical scavenger

The FLA drug action as potential radical scavenger is shown in Scheme 1. In anaerobic condition, the oxido oxygen atom of iron (III) cluster scavenge the hydrogen radical from the acidic hydrogen of FLA and form the hydroxyl Fe (III). Another Fe (III) was reduced to Fe (II) and that the reductive step precedes oxygen requiring radical formation (Eliasson et al., 1986; Fontecave et al., 1987). According to this model a reduced R2 should be an intermediate in the reaction. Simultaneously, the unstable FLA radical model (IIa and IIb) is hydrogen bonded (FLA's N-H from uracil moiety) with oxygen atom of oxido-Fe (III) and serves as a driving force for the oxido-Fe (III) bond cleavage. To attain the stable aromatic nature, the IIb FLA radical eliminates one more electron from the piperazine ring and form oxidized FLA (IV). Hydroxyl Fe (III) reacts with second hydrogen radical and form one more reduced Fe (II) and water.

Proposed mechanism of phenosafranine action as radical scavenge

The mechanism of another radical scavenger PHE is shown in Scheme 2. At anaerobic condition, reduced PHE (I) react with oxygen center of dinuclear iron (III) and loses one of its protons and forms neutral PHE (II) and hydroxyl Fe (III). One Fe (III) gets reduced to Fe (II) in the first step. In the next step, dimethyl amine nitrogen

Table 3. Active site residues of human RR-R2 and drug interactions (hydrogen bonds and hydrophobic contacts) involved through the molecular docking.

Drug	Interaction of human RR-R2 and radical scavengers	Distance of hydrogen bonds (Å)	Hydrophobic contact residues
FLA	Tyr-189 (OH…N)	3.14	His-185 Val-154 Ser-150
	Tyr-189 (OH…N2)	2.50	
	Tyr-189 (OH…N1)	2.35	
	Asn-272 (OD1…N2)	2.55	
	Glu-279 (OE1…N1)	3.04	
	Asp-151 (OD1…N1)	3.37	
PHE	Tyr-189 (OH…N2)	3.13	Ser-150
	Tyr-189 (OH…N4)	2.88	Asp-151
	Tyr-189 (OH…N)	2.24	Val-154
	Glu-279(OE1…N4)	2.88	Val-244 Glu-245

donates electron to make aromaticity in the molecule and the piperazine moiety expels the hydrogen anion from N-H. This anion H reacts with HO-Fe (II)-R to form water (IV) and gets reduced to Fe (II).

Formation and function of tyrosyl radical

An important observation made by Atkin et al. (1973) is that the incubation of Apo RR-R2 enzyme with Fe (II) and O_2 led to the formation of diferric tyrosyl radical cofactor. The diferrous cluster reacts with O_2 to produce a putative short-lived peroxo intermediate which is reduced to a paramagnetic intermediate dinuclear ferric complex. This peroxo intermediate is then converted into the diferric cluster by oxidation of Tyr residue to the radical form. In presence of oxygen environment, diferric cluster and Tyr radicals are regenerated and then RR-R2 enzyme will be ready for catalytic process to participate in DNA synthesis. So the alternative drugs might be needed to stop the enzyme action which must be better than radical scavengers. Metal chelators will be a good choice to remove the metal ions and reduction of radicals from the enzyme.

Mechanism of Iron chelators

Chelating molecules normally play a vital role to remove or prevent incorporation of iron in the enzymes. When cells lack iron, cell proliferation stops due to specific inhibition of DNA synthesis (Fan et al., 2001; Li et al., 2001). For this reason, iron-chelators have been used as such or in combination with other drugs in anti-proliferative therapy. An early effect due to iron chelators in the cell is that RR activity decreases with the accumulation of Apo RR-R2 and therefore, DNA synthesis and cell proliferation are curtailed. The drug which belongs to the group of *a-(*N)-heterocyclic thiosemicarbazones is the most potent inhibitor of mammalian RR-R2, e.g. Triapine (Shao et al., 2006).

In recent year, Triapine's analogs have been tested against human RR enzyme, which showed a strong inhibition. The authors have demonstrated their mechanism as metal chelators having exceptionally strong affinity for iron in aerobic condition (Zhu et al., 2009; Sartorelli et al., 1977). The study reveals that the thiosemicarbazone (TSC) derivatives inhibit the enzyme by destroying the free radical. This is because the radical structure is more exposed in the mammalian reductase (KjØller et al., 1982). Heterocyclic TSCs are proposed to inhibit RR enzymes by a redox reaction involving reduced iron and oxygen which explain why the iron chelate is the active form of the drug (Moore et al., 1970; Preidecker et al., 1980). The iron chelation process reduces the Fe (III) to Fe (II) which is a necessary process for drug action according to the proposed mechanism.

Thiosemicarbazone (TSC) chelator drugs act directly to destroy the tyrosyl free radicals of RR-R2 enzyme by forming Fe (II)-TSC complex (Thelander and Gräslund, 1983; Finch et al., 2000). According to the above statement, the drug TSCs do not directly chelate the Fe (III) ions in the active site of RR-R2 enzymes, instead they first scavenger the free radicals and reduce the metal ion from Fe (III) to Fe (II). Thereafter, TSCs will chelate the Fe (II) ions and increase the concentration of the apo-RR-R2 level.

Conclusion

The human RR-R2 structure was modeled and docking

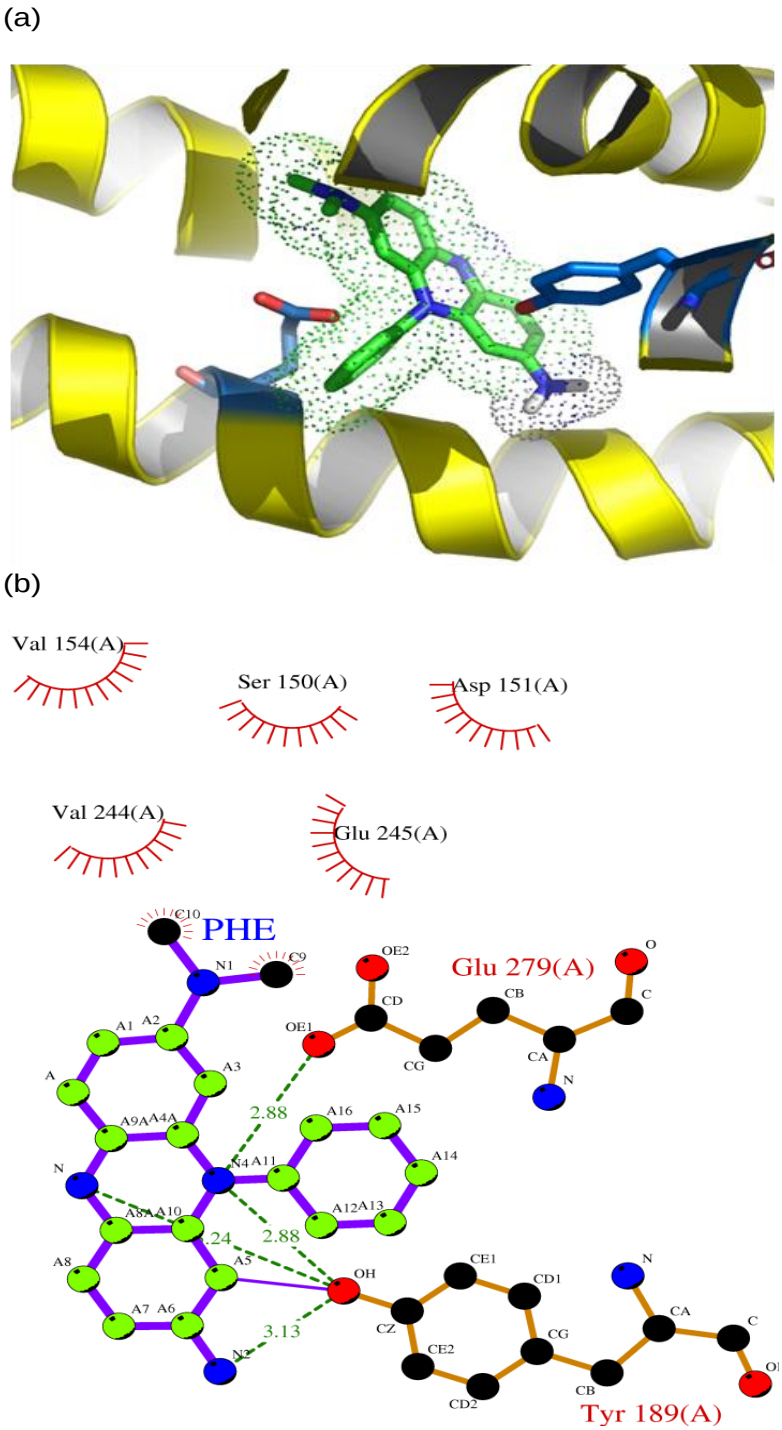
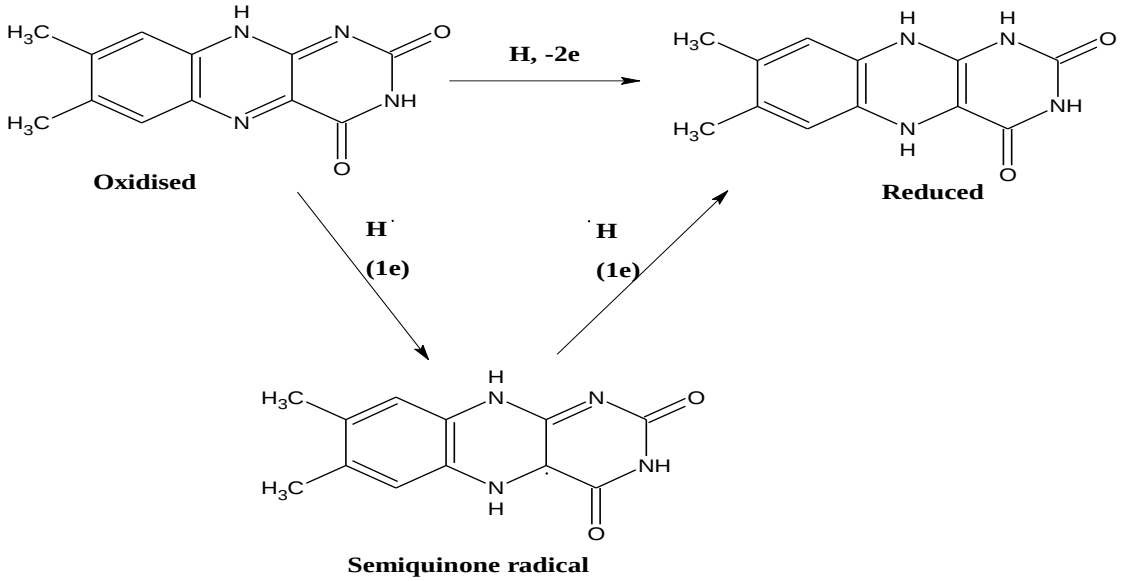
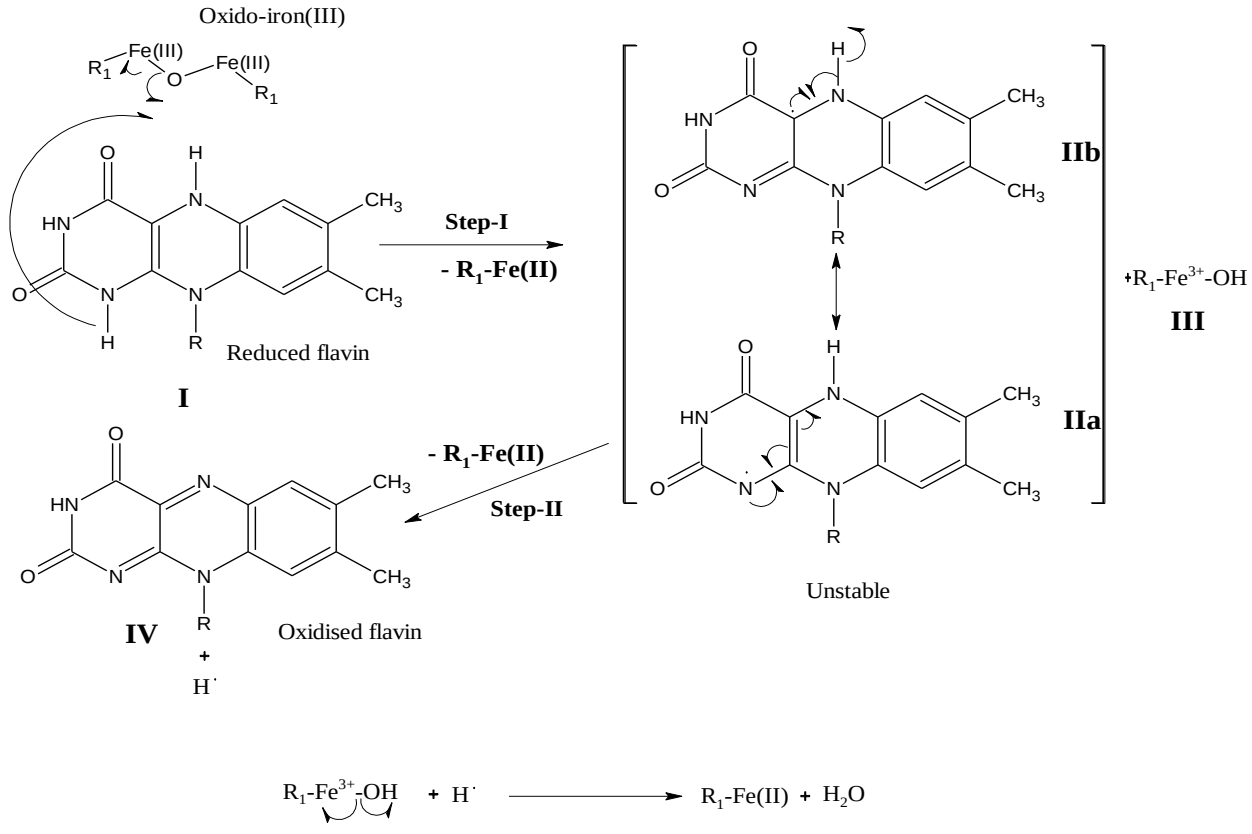


Figure 6. Modeled human RR-R2 enzyme-phenosafranine drug interactions. (a) Cartoon diagram of the protein-drug (green stick) interactions. (b) Ligplot diagram of the human RR-R2-Phenosafranine drug interactions. Hydrogen bonds are depicted in green color dotted lines and hydrophobic interaction residues are shown in red colour.

studies were performed with the radical scavengers, FLA and PHE. Their interactions with human RR-R2 were studied and the plausible scavenging mechanism has been proposed in anaerobic condition. Since attaining



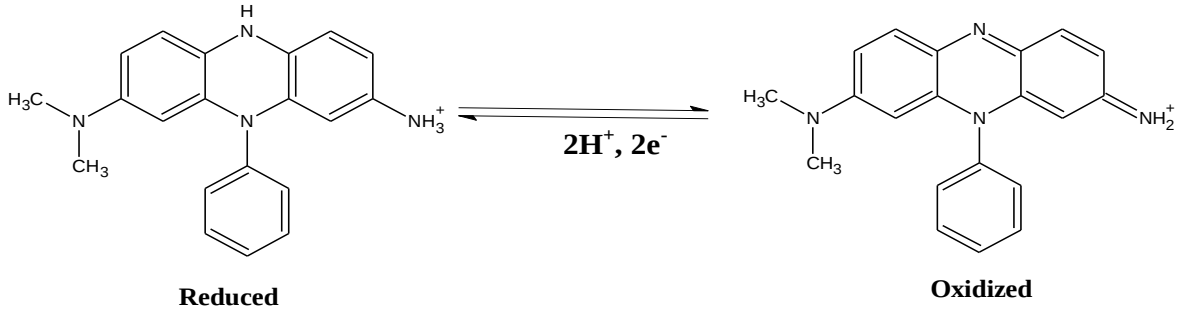
Mechanism



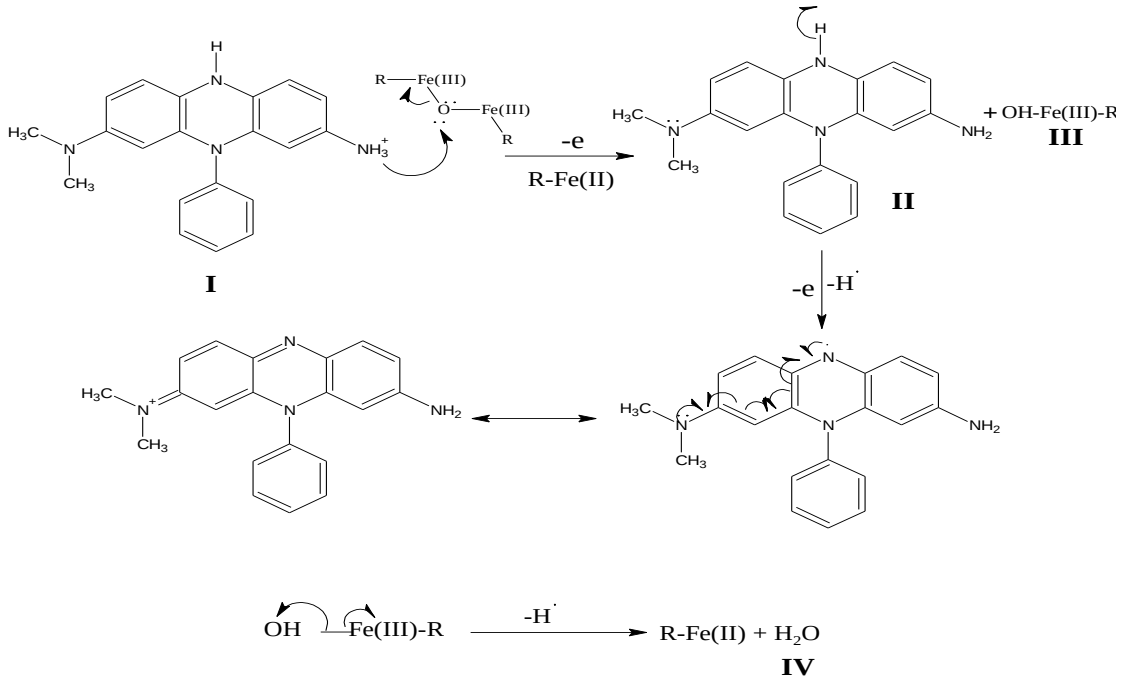
Scheme 1. Possible reaction mechanism of radial scavenger FLA molecule.

anaerobic condition is not possible, using these drugs for cancer therapy becomes difficult. So, alternate drugs are needed to inhibit the activity of the RR enzyme.

According to the various reports, metal scavengers would be more ideal drugs for RR inhibition. Since TSCs have more affinity to Fe (II) and are more effective



Mechanism:



Scheme 2. Possible mechanism of radical scavenger PHE molecule.

against RR enzyme, it may be a more convenient drug to curtail the action of RR and thus prevent cell proliferation by increasing the Apo-RR enzymes. This information about metal scavenger turns to be a reasonable and promising evidence to design selective drugs to inhibit the RR enzyme.

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