Section B

Biochemical Studies of Thiosemicarbazones
Biochemical Studies of Thiosemicarbazones

In this Section, the biochemical studies of thiosemicarbazones and modeling studies of ribonucleotide reductase (RNR) to curtail the action of DNA over the growth in the cell are discussed. An analysis on thiosemicarbazone derivatives to use as drugs for RNR inhibition is also dealt in detail.

The anticancer studies of six different piperidine thiosemicarbazones against Hepatocellular carcinoma (HCC) was carried out. For this study, we monitored the drug action towards the HCC by two important biological markers, namely serum marker enzymes and some antioxidant enzyme levels during the treatment. The results provide ample information regarding this study.

The antibacterial and antifungal studies of thiosemicarbazone derivatives were carried out in this study. For this, the agar well diffusion method was used and the results are interpreted with NCCLS standard antibiotic break points. The results obtained against these organisms and the structure activity relationship of these compounds are presented.

The modeling studies to curtail the action of RNR using two different approaches are discussed. The human RNR-R2 protein structure was predicted based on the sequence and used for the modeling study. The docking study was performed with two substrates, namely flavin and phenosafranine. The radical scavenger mechanism with these two structures is proposed and compared with the metal scavengers (thiosemicarbazones).
Anticancer Studies against *Hepatocellular Carcinoma* (HCC) using Piperidine Thiosemicarbazone Derivatives

In this Chapter the anticancer activities of the following piperidine thiosemicarbazone derivatives are studied for liver cancer. The results of this study are correlated with its three-dimensional structure and the structure-activity relationship will be proposed.

![Chemical Structures]

**NMIDPT**  
**MDPT**  
**MBCPT**  
**DPABT**  
**NDPABT**  
**ECDPT**
IUPAC name of the compounds used for HCC treatment

N-Methyl-t-3-isopropyl-r-2, c-6-diphenylpiperidin-4-one thiosemicarbazone [NMIDPT]
3-t-Methyl-r-2, c-6-diphenylpiperidin-4-thiosemicarbazone [MDPT]
3-t-Methyl-r-2, c-6-bis (4-chlorophenyl) piperidin-4-one thiosemicarbazone [MBCPT]
2, 6-Diphenyl azabicyclo [3.3.1] nonan-9-one thiosemicarbazone [DPABT]
N-Nitroso-2, 6-diphenyl azabicyclo [3.3.1] nonan-9-one thiosemicarbazone [NDPABT]
3-t-Ethoxy carbonyl- r-2, c-6-diphenylpiperidin-4-thiosemicarbazone [ECDPT]

4.1 Introduction

*Hepatocellular carcinoma* (HCC), sometimes called *hepatoma*, is the most common form of primary liver malignancy (Johnson, 1997) and treated as one among the 10 most common tumours (Sherman, 1995) in the world. HCC is most responsible for over one million deaths annually worldwide. It occurs more often in men than women in the age group of people around 50 to 60 years old. It occurs with great frequency in Asian and African countries and is becoming more common in United States as a complication of chronic Hepatitis C viral infection. Approximately 30,000 cases are diagnosed yearly in the United States, liver cancer is generally not suspected until it attains an advanced stage.

The casual relationship between HCC and cirrhosis is clear, when majority of the cases of HCC occurring in patients with cirrhotic liver disease. They develop both in epithelial and mesenchymal tissues, but the epithelial tumors spread more frequently to the liver. Cirrhosis of the liver (due to a viral infection, alcohol, toxin exposure or a genetic defect) also increases the likelihood of *hepatocellular carcinoma*, presumably because of chronic inflammation in the liver. Chronic hepatitis B or C viral infection appears to be the most important risk factor for HCC (Wallner et al., 1994). In addition, over 70% of HCC patients in Western countries have underlying liver cirrhosis (Bain et al., 1997). The risk of developing HCC varies according to the cause of the cirrhosis
itself. In a large series of 10,376 malignant neoplasms, Pickren and his coworkers (1982) reported that the metastatic tumor in the liver is 41 times more frequent than the primary hepatic tumor.

Common signs and symptoms include bloating, abdominal pain, fever, weight loss, decreased appetite and nausea. Frequently, the diagnosis of cancer is not considered until these symptoms persist or until a person develops an enlarging abdominal mass or fluid in the abdomen.

4.1.1 Overview of Liver

Somewhat larger than the size of a football and weighing about 2-3 pounds, the liver is a vital organ located in the upper right-hand side of the abdomen. It performs numerous functions for the body: converting nutrients derived from food into essential blood components, storing vitamins and minerals, regulating blood clotting, producing proteins and enzymes, maintaining hormone balances, and metabolizing and detoxifying substances that would otherwise be harmful to the body. The liver also makes factors that help the human immune system fight infection, removes bacteria from the blood, and makes bile, which is essential for digestion.
4.1.2 HCC induction

Exposure of man to preformed diethylnitrosamine (DENA) occurs through the diet in certain occupational setting such as the use of tobacco product, cosmetics, pharmaceutical products and agriculture chemicals (Hecht, 1997). Several investigations have provided convincing evidence that DENA causes a wide range of tumors in all animal species so far tested, the compounds are considered to be a potential health hazards to men (Leoppy, 1994). DENA, an environmental carcinogen primarily induces the liver. It is widely accepted that metabolic activation of nitrosamine by cytochrome p450 enzymes to reactive electrophiles is recurred for that cytotoxic, mutagenic and carcinogenic activity (Arecher, 1989). Because of its relatively simple metabolic pathway and potent carcinogenic activity, DENA has found applications as an experimental model in the field of carcinogenesis and in chemoprevention (Lee and Lee, 1999).

4.1.3 Mechanism of cancer formation by DENA

Free radicals have been implicated in cellular injury and hepatic carcinogenesis in rats in the absence of any known or added carcinogen (Cerutti and Trump, 1992). So the induction of tumour in a given organ by nitrosamine is determined by mutagenic events induced by unrepaired miscoding DNA lesions during DNA replication.

Diethylnitrosamine (DENA) induced HCC, resulted in the formation of free radicals, which then interact with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation, one of the reactions set as a consequence of the formation of free radicals in cells and tissues. Reactive oxygen species and other free radicals are known to be the mediators of phenotypic and genotypic changes that lead to mutation and to neoplasia (Cerutti and Trump, 1992). The imbalance in the antioxidant defense
mechanism can influence the sensitivity of cell to free radicals. It is well known that induction of superoxide radicals and related oxygen species cause cell damage, which have been found to be involved in the formation of malignancy (Guner et al., 1996; Marttila et al., 1988; McCord, 1993).

Reactive oxygen free radicals have also been known to damage tissues through lipid peroxidation (LP) (Guio et al., 1996). Protection of cellular structures from damages by free radicals can be accomplished through enzymatic and non-enzymatic defense mechanism (Durak, 1994). The antioxidant enzymes are of vital importance under this environment in an organism defense against oxidative stress (Guner et al., 1996). The most important one is Superoxide dismutase (SOD) whose main function is to remove O₂ radicals.

4.1.4 Biology and thiosemicarbazones

The relationship between metal ions and cancer is intriguing and contentious. French and FreedLander (1958) observed a common feature between metal complexes and antitumour agents, i.e., the ability to function as chelating agents. Subsequently French and Blanz (1966) prepared tridentate thiosemicarbazone derivatives and found that all the tumor inhibitors potentially act as N-N-S type ligands. In general, the tridentate N, S donor ligands of substituted thiosemicarbazones and thiosemicarbazides are attributed to their ability to chelate and form as metal complexes (Sorkin et al., 1952; Cymerman, et al., 1955). These metal complexes, namely the drugs act either as chelating agents of the Fe atoms of the active site in the enzyme mentioned or by destroying the tyrosine radical present in a subunit of this protein and stop the catalytic activity of DNA synthesis (Thelander and Gräslund, 1983). Brockman and coworkers (1956) reported for the first time that a thiosemicarbazone (i.e., 2-formylypyridine TSC)
possesses antitumour activity against L1210 leukemic cells. Since then intense research has been conducted in this field, mainly focusing on the biological evaluation of heterocyclic TSC's.

Owing to the interesting features of thiosemicarbazones, a number of piperidine thiosemicarbazone derivatives have been synthesized and tested against the HCC induced rats in the laboratory of Biochemistry, University of Madras. The results of these compounds are analyzed using biochemical assays such as serum marker and antioxidant enzyme levels in the biological system.

4.1.5 Serum enzymes related to liver function

Tumor markers are the molecules important for the caring of the patients having cancer. These soluble molecules in the blood are usually glycoproteins detected by monoclonal antibodies. Each tumor marker has a variable profile, namely useful for screening, diagnosis and prognosis, assessing response to therapy, and monitoring for cancer recurrence. In monitoring patients for disease recurrence, tumor marker levels should be determined only when there is a potential for meaningful treatment.

Clinical detection of liver damage is possible when the right pattern of plasma enzyme elevates. These elevations represent hepatocellular leakage and damage, but give no indication for the cause of damage or the degree of liver function impairment. The induction of cancer chemically produce some differences in enzyme specificity and sensitivity when compared to the normal.

Transaminases (aminotransferases)

Aminotransferases has the biological role of retaining the amino group during amino acid breakdown and found in many tissues including heart, liver, kidney and
muscle. They are released during acute tissue destruction (i.e., hepatic necrosis). It is classified according to its biological behaviors:

i) Alanine aminotransferase (ALT) or Serum Glutamic Pyruvate Transaminase (SGPT).

ii) Aspartate aminotransferase (AST) or Serum Glutamic Oxaloacetic Transaminase (SGOT).

Liver damages are commonly diagnosed by the enzyme test found in the liver cells, such as ALT, AST, and bilirubin (a yellow pigment removed from the body by liver). Many things could harm liver and create disease, such as infection by viruses and drinking of too much alcohol. In rare cases even some medicines can damage the liver. In any hepatocellular damage process, the levels ALT/AST increase more than 3-fold in liver cell swelling and some duct obstruction features. Any duct obstruction which is severe and of long enough duration, will lead to a secondary hepatocellular damage process.

**Alkaline Phosphatase (ALP)**

ALP levels are high for a person having liver disease and the person’s bile ducts are somehow blocked. It means the bone or liver has been damaged due to cancer. Often, ALP is high for persons who have cancer that has spread to the liver or the bones.

**Lactate dehydrogenase (LDH or LD)**

Lactate dehydrogenase (LDH) is sometimes called as Lactic acid dehydrogenase (LDH). Its function is to catalyze the reversible reaction of lactate to pyruvate and it is found in many sources of tissues. This is a blood test to measure the LDH level and important to evaluate damages in the tissues.
4.1.6 Antioxidants

Free radicals

Free radicals are unstable and highly reactive looking for stable electron partners. These free radicals are most harmful to molecules like DNA, RNA, membrane lipids and lipoproteins or enzymes. The end results of the free radicals are poor cell function (disease), control of cell death (apoptosis) or even tissue death (necrosis). The free radicals can also be implicated into numerous human diseases including cancer, heart disease, lung disease, eye disorders, inflammatory disease, rheumatoid arthritis and the aging process. Free radicals are normally formed during cellular metabolism exposure to UV light, gamma radiation, environmental pollutants and cigarette smoking.

Antioxidant means "against oxidation" and are capable of removing free radicals from such molecules RO, ROO, O$_2^-$ and H$_2$O$_2$. These are effective because they give up their own electrons to free radicals. Antioxidants are our body's front line defense against free radicals and react to hinder the process of oxidation (Dekkers et al., 1996). During this reaction the antioxidant sacrifices itself by becoming oxidized. However, antioxidant supply is not unlimited as one antioxidant molecule can only react with a single free radical. Therefore, there is a constant need to replenish antioxidant resources.

Antioxidant defenses

The discovery that oxygen-derived free radicals contribute to the pathogenesis of several disease processes and mediate chemical toxicities in the cellular antioxidant enzymes (Halliwell and Gutteridge, 1985). These protective enzymes include the selenoenzyme, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase. Much of the evidence implicating free radicals in oxidative tissue injury comes from studies in several organs including the liver, where exogenously added SOD, catalase, or
both prevented peroxidative injury during ischaemia and reperfusion (Jolly et al., 1984; Arthur et al., 1985; Itoh and Guth, 1985; Kontos, 1985; Ytrehus et al., 1987). GSH is a more efficient metabolizer of $\text{H}_2\text{O}_2$ than catalase.

**Action of antioxidants**

1. Chain breaking reactions, e.g. $\alpha$-tocopherol which acts in lipid phase to trap "ROD" radical.
2. Reducing the concentration of reactive oxygen species e.g. glutathione.
3. Scavenging initiating radicals e.g. superoxide dismutase which acts in aqueous phase to trap superoxide free radicals.
4. Chelating the transition metal catalysts.

In addition, the trace elements such as selenium (Se), manganese (Mn), copper (Cu), and zinc (Zn) also play important roles as nutritional antioxidant cofactors. Selenium is a cofactor for the enzyme glutathione peroxidase, and manganese, copper, and zinc are cofactors for SOD (Halliwell, 1995).

**Catalase enzyme**

Catalase is a homotetrameric ferriheme-containing enzyme, which decomposes $\text{H}_2\text{O}_2$ to water and oxygen molecule (Krinsky, 1992). Catalase has a relatively minor role in the catabolism at low rates of $2\text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$ generation but important as the rate of $\text{H}_2\text{O}_2$ production is enhanced (Jones et al., 1981).

**Glutathione peroxidase (GPx)**

Glutathione peroxidase (GPx) is a selenium-dependent enzyme dispersed in whole cytoplasm, which decomposes $\text{H}_2\text{O}_2$ and various hydro- and lipid peroxides (Kinnula et al.1995). Selenium is essential for protein synthesis and enzymatic activity of GPx. Severe selenium deficiency may cause liver necrosis and degenerative heart disease (Buettner, 1998). GPx requires reduced glutathione as a co-substrate, and the role
of glutathione reductase is to restore glutathione from an oxidized to a reduced form (Kinnula et al., 1995). Glutathione is also an important substrate for GST and acts as quenching free radicals (Halliwell et al., 1992, Fridovich 1998).

The glutathione redox cycle is a central mechanism for reduction of intracellular hydroperoxides. Glutathione peroxidase reduces $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ by oxidizing glutathione (GSH) (Equation A). Rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase (Equation B).

$$\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow \text{GSSG} + 2 \text{H}_2\text{O} \text{ (equation A)}$$

$$\text{GSSG} + \text{NADPH} + H^+ \rightarrow 2 \text{GSH} + \text{NADP}^+ \text{ (equation B)}$$

**Glutathione (GSH)**

In addition to its role as a substrate in GSH redox cycle, GSH is also a scavenger of hydroxyl radicals and singlet oxygen. It is capable of either directly scavenging radicals or enzymatically scavenging via glutathione peroxidase, as described previously.

**Superoxide dismutase (SOD)**

An extracellular form of SOD is a secretory homotetrameric glycoprotein with a high affinity for heparin sulfate (Tibell et al. 1993; Fridovich, 1998). Their function is to scavenge superoxide released from the cell surfaces, and the regulation of nitric oxide bioavailability is thought to be an important part of its function (Oury et al. 1996; Fridovich 1998). SOD also exhibits antioxidant activity by reducing $\text{O}_2^-$.  

$$2\text{O}_2^- + 2H^+ + \text{SOD} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$
Glutathione-S-transferases (GST)

GSTs are considered to be important detoxification enzymes; they catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophiles (xenobiotic substrates). Some of the GSTs have selenium-independent GSH peroxidase activities. Their main function is to eliminate various hydroperoxides, but not hydrogen peroxide (Buettner 1998).

4.1.7 Possible role of antioxidant enzymes in the resistance of tumors

A number of drugs are used to treat various malignancies but their clinical efficacy and use are often limited by the development of drug resistance and side-effects (Yen et al. 1996). The toxicity of the drugs is usually attributed to the formation of free radicals, and drug resistance may also be partly due to the enhanced antioxidant capacity of tumor cells (Sinha and Mimnaugh, 1990).

Anticancer drugs induce the activity of glutathione-related enzymes (GST, GPx, glutathione reductase and catalase) and the activation of these different mechanisms is associated with drug resistance (deVries et al., 1989; Hao et al., 1994; Cheng et al., 1997). Apart from the antioxidant function, the importance of glutathione and related enzymes, especially the GST family, lies in the detoxification of several drugs (Tew, 1994).

4.2 Material and methods

Dimethyl sulfoxide (DMSO) and diethylnitrosamine (DENA) were purchased from SD fine Chemicals, Chennai and Sigma Chemicals, USA. All chemicals and reagents used were of analytical grade. All the experiments were carried out as per the guidelines stipulated by Institutional Animals Ethics Committee (IAEC). For synthesis purpose, the required chemicals were purchased from SISCO Chemicals, Chennai, India.
Wistar strain male *albino* rats weighing 120–150g were purchased from TANUVAS_LAMU, Madavaram. All the animals used for the present study were fed normal pelleted (rat chow) diet and water was given *ad libitum*. The rats are classified into four groups as described below:

**Group I:** Normal control (Control rats given 0.1 ml of 10% DMSO)

**Group II:** Drug control (Control rats given 0.1ml of 10% DMSO + TSC 20mg/kg body Wt.)

**Group III:** HCC induced (Rats induced for HCC) (Phenobarbital 0.05% in diet +DENA 200mg/kg body Wt. through i.p)

**Group IV:** Drug treated (rats induced for HCC and treated with TSC 20mg/ kg body Wt. in 10% DMSO)

The hepatocarcinogenic regimen consisting of DENA and Phenobarbital was selected as described by Yoshiji *et al.*, (1991).

### 4.2.1 Preparation of Tissue Homogenate

Hyperplastic nodules and non-nodules surrounding liver tissues were obtained from all groups treated with DENA. The white hyperplastic nodules were easily identified from the reduced brown tissue. The nodules were eventually divided into three categories in accordance with their size and total area of liver parenchyma occupied (1mm, 1-3mm and >3mm) as described by Moreno *et al.*, (1991) from different carcinogen fed rats. The liver tissues were homogenized in a motor driven Teflon-coated homogenizer in ice-cold 0.1mole tris-HCl buffer (pH= 7.0.) to give 10% homogenate. It was centrifuged at 12,000 rpm for 30 minutes in a refrigerated high-speed centrifuge. The supernatant fluid was used for assay of antioxidant enzymes and marker enzymes.
4.2.2 Biochemical assays

The biochemical assays such as serum marker level [AST and ALT (Mohur et al., 1999), ALP (Garen and Levinthal, 1960), LDH (Nieland, 1995)] and antioxidant levels [GPX (Rotruck et al., 1973), GST (Baars et al., 1978), GR (Doubler and Hunderson, 1981), CAT (Beers and Sizer, 1952), SOD (Misra and Fridovich, 1972), LPO (Uchiyama and Mihara, 1978) and GSH (Pettersson and Lazarow, 1955)] were performed by different methods. The results of statistical analysis are expressed as mean ± SD and student’s t-test was used for statistical significance.

4.3 Results and discussion

The synthesized piperidine thiosemicarbazone (TSC) derivatives were used for the treatment of hepatocellular carcinoma induced rats. These drugs were applied for the HCC induced rats for six months duration as oral dosage. During cytotoxic treatment, serum marker and antioxidant levels were analyzed for liver damage. These biological assays were carried out for all the four group animals.

4.3.1 Effect of TSCs on serum marker enzymes

Blood levels of liver components are used to estimate hepatotoxicity of DENA. Abnormal levels of marker enzymes indicate the damage to hepatic cells (Wolf, 1999) and an increase in serum levels of hepatic enzymes are reported in the present study with DENA. Similar observations have been reported earlier. Effective control of serum marker enzymes by TSC seems to offer protection and maintains functional integrity of the cells.

The activity of serum marker enzymes of clinical interest such as AST, ALT, ALP and LDH for all the compounds are shown in Figs. 4.1a, b, c, d, e and f. This study
Fig. 4.1 Serum marker enzyme [ALT, AST, ALP, LDH] levels (IU/dl) of four group of animals
was carried out for all four group animals mentioned in materials and methods section. The study reveals that significant increase of serum marker enzymes in Group III animal of carcinoma induced rats (p<0.001) is noted for all six compounds. These abnormal levels of serum marker enzymes strongly support that liver damage has occurred in Group III animals. Also this liver damages were observed by significant bloatings in the liver. This increase in serum levels were inhibited (P<0.001) by TSC post treatment in Group IV animals and the swelling of liver was significantly reduced in Group IV animals after TSCs treatment. These observations show that the treated drugs (TSCs) inhibit the HCC burgeoning in Group IV animals. The serum levels in I and IV Group animals seem to be more or less similar. These similarities prove that DENA induced HCC was considerably inhibited by thiosemicarbazone drugs.

4.3.2 Structure activity relationship of piperidine thiosemicarbazones

The structure activity relationship for the treated piperidine thiosemicarbazones was analyzed and the normalized serum level is presented in Table 4.1. This normalized percentage of inhibition was done for Group III and IV animals. For the normalization, we have taken only the mean values without including standard deviations. From this inhibition range, the structural activity relationships of these treated compounds were identified. The graphical representation is shown in Fig. 4.2.

<table>
<thead>
<tr>
<th></th>
<th>NMIDPT</th>
<th>MDPT</th>
<th>MBCPT</th>
<th>NDPABT</th>
<th>DPABT</th>
<th>ECDPT</th>
</tr>
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<tbody>
<tr>
<td>ALT</td>
<td>84.56</td>
<td>68.46</td>
<td>87.18</td>
<td>91.39</td>
<td>87.75</td>
<td>96.45</td>
</tr>
<tr>
<td>AST</td>
<td>47.12</td>
<td>41.21</td>
<td>54.83</td>
<td>57.39</td>
<td>53.1</td>
<td>60.84</td>
</tr>
<tr>
<td>ALP</td>
<td>95.61</td>
<td>89.89</td>
<td>93.36</td>
<td>96.82</td>
<td>94.37</td>
<td>98.62</td>
</tr>
<tr>
<td>LDH</td>
<td>97.5</td>
<td>92.87</td>
<td>93.41</td>
<td>97.94</td>
<td>94.52</td>
<td>98.62</td>
</tr>
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</table>
Fig 4.2 Overall activity relationship between the treated drugs against HCC
The study of Fig. 4.2 reveals that all six TSC compounds show similar activities but slight variations are observed in the plots. These variations may be due to the different group substituted in the molecules. These groups may play the roles in increase or decrease the anticancer activity of TSC compounds. From the study of the graphical representation it is observed that the compound ECDPT is more active than others. This may be due to the presence of carboxylic ester group in the molecule. The carbonyl oxygen in this derivative may help to bind with metal ion atom as shown in Fig 4.3 and stop catalytic process of RNRs DNA synthesis. This molecule takes stable octahedral geometry with metal ions and it would suppress the cancerized cells.

![Chemical structure](image)

**Fig 4.3 Possible complex formation of ECDPT**

The compound NDPABT has significant activity against HCC and this may be due to the presence of NO group in the molecule. This tumor suppressing activity of NO substituted compound may be due to the radical–radical coupling reaction with the organic free radical of ribonucleotide reductase, an enzyme essential for DNA synthesis, and the radical NO (Lepoivre et al., 1994). The induction of NOS-II in some cell lines also stimulates the expression of the tumor suppressor gene p53 prior to apoptosis.
(Messmer et al., 1994; Brüne et al., 1997), so that nitric oxide (NO) inhibits tumor cell replication and also inhibits ribonucleotide reductase.

The absence of nitroso group compound DPABT shows less activity than NDPABT. These two compounds NDPABT and DPABT show more activity than other three drugs NMIDPT, MDPT and MBCPT. Both these compounds NDPABT and DPABT of bicyclic ring system may increase reductive capacity and reactivity of the thiosemicarbazone moiety and also increase the hydrophobic interaction with the biological molecules.

The drug NMIDPT shows more activity when compared to MDPT and MBCPT molecules. The presence of isopropyl group in 3rd position of NMIDPT may induce the action of thiosemicarbazone moiety against cancer growth. Other two compounds namely MDPT and MBCPT shows similar activity. But compound MBCPT shows slightly higher activity than MDPT. This is due to the presence chlorine substitution in both phenyl rings which may induce the anticancer activity against HCC cell. Overall the structural activity of these piperidine thiosemicarbazone derivatives is depicted as below:

ECDPT > NDPABT > DPABT > NMIDPT > MBCPT > MDPT

4.4 Effect of TSCs on Antioxidant Enzymes

The activity of GPx, CAT and SOD are shown in Figs 4.4a, b, c, d, e and f. The significant decrease in the activity of antioxidant enzymes is noticed in Group III carcinoma induced rats. This decrease was due to the inhibition of thiosemicarbazone (TSC) treated rats in Group IV animals of all six drugs. GST plays a key role in the metabolism of carcinogens, mutagens and in different anticancer drugs. This enzyme is functionally involved in the development of HCC and useful to find the anticancer drug
GPX (µg of glutathione consumed/min./mg protein) : GR (nM of NADPH consumed/min./mg protein)
CAT(µM of H2O2 decomposed/mg protein/min.) : SOD (Units of activity/mg protein)
LPO (nM of MDA formed/mg protein/hr) : GSH (µg/mg protein)

Fig. 4.4 Antioxidant enzyme levels of four group of animals
sensitive to such tumors (Murrey et al., 1993). The activity of GST (Fig 4.5) was gradually increased in DENA induced rats as compared to control values in all TSC treated animal. The increase in activity of GST is inhibited by TSC treated rats.

Therefore, the depressant of hepatic CAT activity and SOD (Fig 4.4) in HCC bearing rats was observed which is supported by other studies too (Guner et al., 1996; Geeta and Ganeshsundar, 1996; Reiners et al., 1991). This shows that hepatic antioxidant defense system of the HCC bearing rats may be impaired through the production of superoxide radicals, oxy-radicals and peroxide radicals. This is possible by the perturbation of the hepatic cell membrane due to its enhanced lipid peroxidation (LPO) in the carcinoma cells (Geeta and Ganeshsundar, 1996; Capel and Thormaly, 1983) as well as in hepatic microsomal membrane.

The administration of TSC during the development of HCC antagonizes the activity of hepatic antioxidant enzymes along with the induction hepatic microsomal LPO (Figs 4.4) and restore the original state to their (enzymes) respective control values suggesting that TSC may effectively scavenge OH-, ROO- and O. This TSC also play a potential antioxidant activity against the oxidative damage to the hepatic cellular system caused by the development of tumor cells.

4.5 Conclusion

The anticancer study of thiosemicarbazones (TSCs) against hepatocellular carcinoma (HCC) was done and the drug actions were monitored by two different biochemical enzyme assays, namely serum biomarker and antioxidant enzyme levels in HCC bearing rats. The abnormal serum levels of Group III animals became normal level in group IV animals. This indicates that the TSC compounds inhibit the action of HCC in rats and suppress the tumor growth. This drug action is also confirmed by antioxidant
Fig. 4.5 Antioxidant-GST enzyme levels for all the compounds during the animal treatment
enzyme levels during the treatment and support the anticancer activity of these compounds.

The present study reveals that significant changes are noticed in TSC treated animals against HCC. The induction of cell damage in HCC bearing rats caused by generation of $O_2^-$ species may be inhibited by TSC due to its effective scavenging property against oxidative damage of the biological membrane. Therefore from the experimental observations it could be suggested that the drug TSC has a definite pharmacological role against DENA induced oxidative stress in HCC through its membrane stabilizing action which in turn helps in normalizing the abnormal cell behaviour.

Also this study reveals that the compound ECDPT is more active against HCC than the other five compounds. The NDPABT is also significantly active than other compounds but comparatively lesser than that of ECDPT. This is due to the influence of substitutions which change the nature of the activity.
Chapter 5

Antimicrobial Studies of Thiosemicarbazone Derivatives

This chapter deals with the study of antimicrobial activities of some thiosemicarbazone derivatives. Different thiosemicarbazone derivatives such as piperidine, azabicyclic, phenyl and carboxylate piperidine were synthesized and used for biochemical studies against human pathogens, namely bacterial and fungal microorganisms. The thiosemicarbazones used for the biochemical studies are shown below:

i) Piperidine thiosemicarbazone derivatives

P1: 3-t-Methyl-r-2, c-6-diphenylpiperidin-4-thiosemicarbazone [MDPT]

P2: N-Methyl-t-3-isopropyl-r-2, c-6-diphenylpiperidin-4-one thiosemicarbazone [NMIDPT]

P3: 3-t-Methyl-r-2,c-6-bis (4-methoxyphenyl) piperidin-4-one thiosemicarbazone [MBMPT]

P4: 3-t-Methyl-r-2,c-6-bis(3,4,5-trimethoxyphenyl)piperidin-4-onethiosemicarbazone [MBTPT]

P5: 3-t-Methyl-r-2, c-6-bis (4-chlorophenyl) piperidin-4-one thiosemicarbazone [MBCPT]
ii) Azabicyclic thiosemicarbazone derivatives

A1: N-Nitroso-2, 6-diphenyl azabicyclo [3.3.1] nonan-9-one thiosemicarbazone [NDPABT]
A2: 2, 6-Diphenyl azabicyclo [3.3.1] nonan-9-one thiosemicarbazone [DPABT]

iii) Carboxylic thiosemicarbazone derivatives

C1: 3-t-Methoxy carbonyl- r-2, c-6-diphenylpiperidin-4-thiosemicarbazone [MCDPT]
C3: 3-t-Ethoxy carbonyl- r-2, c-6-diphenylpiperidin-4-thiosemicarbazone [ECDPT]
iv) Phenyl thiosemicarbazone derivatives

B1: 4-Chloro benzaldehyde thiosemicarbazone [CBT]
B2: 4-Hydroxy benzaldehyde thiosemicarbazone [HBT]
B3: 4-Hydroxy-2-methoxy benzaldehyde thiosemicarbazone [HMBT]
B4: 4-N,N-Dimethyl benzaldehyde thiosemicarbazone [DMBT]

5.1 Anti-bacterial studies

Bacteria are single-celled microorganisms that lack a nuclear membrane, metabolically active and divide by binary fission. They are mainly responsible for major cause of diseases; appear to be relatively simple forms of life, but sophisticated and highly adaptable. Many bacteria multiply at rapid rates, and different species utilize variety of hydrocarbon substrates for this purpose. These organisms exist widely in both parasitic and free-living forms. They are ubiquitous and capable of adapting to the changing environments by selection of spontaneous mutants. Today, most bacterial diseases of humans and their etiologic agents have been identified, although important
variants continue to evolve and sometimes emerge, e.g., Legionnaire's Disease, tuberculosis and toxic shock syndrome.

In developing countries, a variety of bacterial infections often exert a devastating effect on the health of the inhabitants. Malnutrition, parasitic infections, and poor sanitation are some of the factors contributing to the increased susceptibility of these individuals to bacterial pathogens. The World Health Organization (WHO) has estimated that each year, 3 million people die of tuberculosis and 25,000 die of typhoid. Diarrhoea diseases are the second leading cause of death in the world (after cardiovascular diseases) due to bacteria, killing 5 million people annually. Pathogenic bacteria constitute only a small proportion of bacterial species; many nonpathogenic bacteria are beneficial to humans (i.e. intestinal flora produce vitamin K) and participate in essential processes such as nitrogen fixation, waste breakdown, food production, drug preparation, and environmental bioremediation.

In recent years, medical scientists have concentrated on the study of pathogenic mechanisms and host defenses. Understanding the host-parasite relationships involving specific pathogens require familiarity with the fundamental characteristics of the bacterium, the host, and their interactions. Microorganisms damage the host; host defense mechanisms; source and distribution of pathogens (epidemiology); principles of diagnosis; and mechanisms of action of antimicrobial drugs.

5.1.1 Types of Bacteria

Bacterial pathogens are classified into two classes according to their structure and activity such as Gram-positive and Gram-negative and its morphological structure as follows:
Morphological description of Gram-positive and gram-negative bacteria

5.1.1.1 Gram-positive bacteria

*Gram-positive* bacteria have a thicker layer of peptidoglycan in their cell walls, made of a protein-sugar complex that takes on the purple color during gram staining. They cause many human diseases, including scarlet fever, toxic shock syndrome, and pneumonia. Many of these bacteria produce toxins, which are poisons to our bodies. Toxins can be deadly; a single gram of the toxin produced by *Clostridium botulinum* (*botulism*) could kill more than one million people. In our present study, we used two gram-positive bacteria for the activity study, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

*a) Staphylococcus aureus*

*Staphylococcus aureus*, resembles like grapes and found on human skin and mucosa (lining of mouth, nose etc). *S. aureus* enters the body via a cut or an abrasion and cause illnesses such as skin and wound infections, urinary track infections, pneumonia and bacteraemia (blood stream infection). It can also cause food poisoning due to the
growth of strains and produce toxins in foods and results severe vomiting with diarrhoea and abdominal pain.

b) *Bacillus subtilis*

*Bacillus subtilis* is a rod-shaped endospore-forming aerobic bacterium. It shares many common features with *E. coli*, but it differs in its metabolism and gene regulatory mechanisms. One feature that has attracted in *B. subtilis* is its ability to differentiate and form endospores.

5.1.1.2 Gram-negative bacteria

*Gram-negative* bacteria have an extra layer of lipid on the outside of the cell wall and appear pink after gram staining. The extra lipid layer stops the purple stain from entering the cell wall. Some of these bacteria are involved in photosynthesis but differ from plants by producing sulfur instead of oxygen. In our studies, we have seven gram-negative bacterium which are explained below.

a) *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* causes nosocomial infections (Wiblin, 1997), life-threatening infections in immunocompromised persons (Pollack, 1995; Kluymans, 1997; Gordon *et al.*, 1998; Fergie *et al.*, 1994; Bergen and Shelhamer, 1996), and chronic infections in cystic fibrosis patients. This is the common and lethal pathogens responsible for ventilator-associated pneumonia in incubated patients (Dunn and Wunderink, 1995; Brewer *et al.*, 1996).
b) *Escherichia coli*

*Escherichia coli* (*E. coli*), normally inhabits in the intestine of humans and animals. This may give rise to infections in wounds, urinary track, biliary track and abdominal cavity. This organism may cause septicemia, neonatal meningitis, infantile gastroenteritis, tourist diarrhoea, and hemorrhagic diarrhoea.

c) *Proteus* infection

*Proteus* can cause urinary tract and hospital-acquired infections. *Proteus* is unique, motile and does not form regular colonies. Instead, *Proteus* forms a "swarming colonies" when plated on non-inhibitory media. The important members of this genus are *Proteus mirabilis and vulgaris* which are responsible for urinary tract infections.

d) *Salmonella typhi*

*Salmonella* is a rod-shaped, non-spore forming bacteria widely found in animals (especially in poultry and swine). It spreads through environmental sources like water, soil, insects, factory surfaces, kitchen surfaces, animal feces and raw sea foods. *S. typhi* and the *paratyphi* bacteria normally cause *septicemia* and produce typhoid or typhoid-like fever (Arora et al., 1992; Zavala et al., 1991) in humans.

e) *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is a rod shaped aerobic bacteria that possess a prominent polysaccharide capsule and a well-known opportunistic pathogen that causes nosocomial infections.
5.1.2 *in vitro* Antimicrobial Sensitivity Determination Test

5.1.2.1 Antimicrobial drugs and Tested compounds

Standard antibacterial agents were used for the comparative studies (Table 5.1). The disc concentration levels are under National Committee for Clinical Laboratory Standards (NCCLS) column. The antibacterial discs were obtained from HiMedia Laboratories Pvt. Limited, Mumbai, India.

5.1.2.2 Stock solution preparation of test (thiosemicarbazone) compounds

Synthesized thiosemicarbazones (TSCs) were dissolved in dimethylsulfoxide (DMSO) at the concentration of 7,500μg/12mL as stock solutions. From these stock solutions 100μL (62.5μg/μl) and 150μL (93.7μg/μl) were immediately dispensed into the agar wells of culture inoculated (MHA) plates using sterilized micropipette.

5.1.2.3 Isolation of clinical bacteria

The most clinically important drug resistant organisms were isolated and identified by standard methods. These are all stored in the Department of Clinical Medical Microbiology, Apollo Hospitals, Chennai, India. Ten clinically important bacterial pathogens such as two gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and eight gram-negative (*Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi 'A', Salmonella paratyphi 'H', Klebsiella pneumoniae, *Proteus mirabilis*, *Proteus vulgaris* and *Escherichia coli*) strains were selected for this *in vitro* study. All strains were chosen on the basis of recent clinical isolates between the year 2004 and 2005. Isolates were stored frozen in skim milk 50% glycerol at −70°C.
5.1.2.4 Preparation of bacterial inoculum

The inoculum was prepared using gram +ve and gram -ve bacterial pathogens from a 24 hours old culture on Brain heart infusion agar. With a sterile loop, the tops of four to five colonies were transferred to a tube containing 5mL of Mueller Hinton broth or Brain Heart infusion broth. The tube was incubated at 35°C for 24 hours. The turbidity of the culture suspension was adjusted with broth or a sterile saline solution (0.85–0.9%). The density of these cultures were adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and finally the inoculum size of approximately 5x 10^5 CFU/mL.

5.1.2.5 Antibacterial Susceptibility Testing

Disk diffusion method (NCCLS M2 – A4) and Well diffusion method

The disk diffusion test (Bauer et al., 1966; Ericsson and Sherris, 1971; NCCLS, 1990) was performed using Mueller Hinton Agar (MHA) [Casein acid hydrolysate: 17.5g; Beef Heart infusion: 2g; Starch soluble 1.5g; (pH 7.3 ± 0.2) Agar 17g; H₂O 1000mL]. The medium was mixed thoroughly and heated on water bath till it dissolved completely. [The well diffusion test (Bennet et al., 1966; Janssen et al., 1987; Magaldi et al., 2004) was performed using Casitone agar (Bacto-casitone : 9g; yeast extract: 5g; tri-sodium citrate: 10g; glucose: 20g; bactoagar: 15g; phosphate buffer: KH₂PO₄: 1g; Na₂HPO₄: 1g (pH 6.6); H₂O 1000mL)]. Then this medium was autoclaved at 15lbs pressure (121°C) for 15 minutes and the sterilized medium was immediately cooled to 50-55°C. The cooled medium was poured into sterile Petri plates to a uniform depth of 4mm; this is equivalent to approximately 25mL in a 90mm plate. Once the medium had solidified then the culture was inoculated on the medium. Within 15 minutes of adjusting the density of the inoculum, a sterile cotton swab was dipped into the standardized bacterial suspension or inoculated with 1mL of the organism suspension. The sterile
swab was used to streak on the surface of the MHA medium to ensure that an even
distribution of the inoculum [for well diffusion method: Sterilized 9mm cork borer was
used to make the agar wells, 100μL of the diluted TSC’s solutions were placed into each of
wells and also put the DMSO as a control into one well]. The plates were allowed to be
undisturbed for 3 to 5 minutes to absorb excess moisture. The selected antibiotic disks
were placed on the inoculated plates and pressed firmly into the agar with the sterile
forceps to ensure the complete contact with the agar. The plates were incubated at 35-
37°C for 24 hours. NCCLS disc diffusion and MIC break points were used for the
interpretative break points (Table 5.1).

5.1.2.6 Antimicrobial Susceptibility Test – Disc diffusion method

The results of antimicrobial susceptibility test with various antibiotics against
human pathogenic microorganisms are presented in Table 5.2.

The antibiotic cefotaxime was found to be active against *Escherichia coli* since
the zone of inhibition was less than 14mm. All the human pathogenic bacteria showed
resistance to the tetracycline. *Bacillus subtilis*, *Salamonella paratyphi ‘H’* and *proteous
mirabilis* were sensitive to the antibiotic of ciprofloxacin.

*S. typhi*, *S. paratyphi ‘A’, S. paratyphi ‘H’, Pseudomonas aeruginosa* and *Proteus
mirabilis* were highly resistant to the antibiotic chloramphenicol, whereas other
remaining bacteria were sensitive to the antibiotic chloramphenicol. From the
susceptibility results, *S. aureus*, *P. vulgaris* and *E. coli* were found to be highly sensitive
compared to other organisms.

The percentage of inhibition was found to be good in *S. aureus* compared to
*E.coli, B. subtilis*, and *P. vulgaris* when tested with the antibiotic amikacin. In the
sensitivity test *B. subtilis* and *P. vulgaris* showed moderate activity to amikacin. Other
**Table 5.1 Antimicrobial agents used for susceptibility tests**

**NCCLS M2-A7- Disc Diffusion Method**

*Zone Diameter interpretive standards and Equivalent Minimal Inhibitory Concentration (MIC) NCCLS Breakpoints*

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Symbol</th>
<th>Disc content</th>
<th>Zone diameter nearest to whole mm</th>
<th>Equivalent MIC Breakpoints (µL/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resistant (mm or less)</td>
<td>Intermediate (mm)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Ak</td>
<td>30 µg</td>
<td>14</td>
<td>15-16</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>Ck</td>
<td>30 µg</td>
<td>14</td>
<td>15-19</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Ce</td>
<td>30 µg</td>
<td>14</td>
<td>15-22</td>
</tr>
<tr>
<td>Chloramphenical</td>
<td>C</td>
<td>30 µg</td>
<td>12</td>
<td>13-17</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Cf</td>
<td>5 µg</td>
<td>15</td>
<td>16-20</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>G</td>
<td>10 µg</td>
<td>12</td>
<td>13-14</td>
</tr>
<tr>
<td>Methicillin</td>
<td>M</td>
<td>30 µg</td>
<td>9</td>
<td>10-13</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>Of</td>
<td>5 µg</td>
<td>12</td>
<td>13-15</td>
</tr>
<tr>
<td>Peflozacin</td>
<td>Pf</td>
<td>30 µg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S</td>
<td>10 µg</td>
<td>11</td>
<td>12-14</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>T</td>
<td>30 µg</td>
<td>14</td>
<td>15-18</td>
</tr>
<tr>
<td>Trimazine</td>
<td>Cm-Co</td>
<td>25 µg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Va</td>
<td>30 µg</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Antifungal Agents**

<table>
<thead>
<tr>
<th></th>
<th>Symbol</th>
<th>Disc content</th>
<th>Zone diameter nearest to whole mm</th>
<th>Equivalent MIC Breakpoints (µL/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuloconazole</td>
<td>Fu</td>
<td>10 µg</td>
<td>12</td>
<td>13-18</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>It</td>
<td>10 µg</td>
<td>12</td>
<td>13-18</td>
</tr>
<tr>
<td>Antibacterial Activity</td>
<td>Antibiotic Disk diffusion method</td>
<td>Diameter of zone of inhibition in mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------------------</td>
<td>-------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ce 30μg T 30μg Cf 5μg C 30μg Ak 30μg Cm 25μg Va 30μg G 10μg M 30μg Ck 30μg Of 5μg S 10μg Pf 30μg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>13.0 10.0 - 32.0 21.5 - 14.5 26.0 31.5 30.5 18.0 22.0 -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>13.0 11.5 17.0 18.0 16.0 17.0 14.0 15.0 12.5 16.0 17.5 10.0 13.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>- - - - - - - - - 8.0 - - 6.0 - -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td>7.0 12.0 8.0 15.0 7.0 13.0 - 7.0 - - 12.0 - 9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. typhi</strong></td>
<td>9.5 - 11.5 - 11.5 - - 12.5 - 20.5 11.0 - 9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. paratyphi ‘A’</strong></td>
<td>9.0 - 10.0 10.0 10.0 10.0 - - - - 12.5 12.0 7.0 9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. paratyphi ‘H’</strong></td>
<td>11.0 - 16.0 - - 11.0 - 11.0 - 17.0 16.0 9.0 14.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. mirabilis</strong></td>
<td>12.5 - 20.0 - 13.5 - - 9.0 - 17.0 17.5 - 15.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. vulgaris</strong></td>
<td>- - - 23.5 14.0 - - 11.0 - 17.0 19.0 - -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>19.0 11.0 - 23.5 19.0 - - 21.0 - 18.5 13.5 - -</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ce- Cefotaxime, T- Tetracycline, Cf- Ciprofloxacan, C- Chloramphenical,  Ak- Amikacin, Cm- Trimazine, Va- Vanomycin, G- Gentamycin, M- Methicillin, Ck- Ceftizoxime, Of- Oflaxacin, S- Streptomycin and Pf- Peflozacan
bacterial species were resistant to Amikacin. The antibiotic co-trimoxazole was highly resistant to tested pathogens except *B. subtilis*.

Moderate sensitivity to the antibiotic gentamycin was observed into the *Salmonella typhi*, *S. paratyphi-*‘H’ and *P. vulgaris*, whereas *Bacillus subtilis*, *E.coli*, and *S. aureus* showed sensitive in the susceptibility test against gentamycin. While other three strains *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis* were resistant to the antibiotic gentamycin.

Other than *B. subtilis* and *S. aureus*, all other bacteria were highly resistant to the antibiotic methicillin and showed poor activity. Only the *gram-positive* bacterium *S. aureus* showed higher sensitive to methicillin. The antibiotic ofloxacin showed good activity against all the tested pathogens except *Pseudomonas aeruginosa*, *K. pneumoniae*, *Salmonella typhi*, *S. paratyphi-*‘A’ and *E. coli*. Significant activity was observed against *S. aureus* and *P. vulgaris* for ofloxacin.

**5.1.3 Activity of thiosemicarbazones against human bacterial pathogens**

In order to establish the structure activity relationships, a number of thiosemicarbazone derivatives were tested for antimicrobial activity against various clinical strains of bacteria that are highly resistant to the antibiotic. For comparison thirteen antibiotics recommended by NCCLS, namely *Amikacin (Ak)*, *ceftizoxime (Ck)*, *Cefotaxime (Ce)*, *Chloramphenical (C)*, *Ciprofloxacin (Cf)*, *Gentamycin (G)*, *Meticillin (M)*, *Ofloxacin (Of)*, *Peflozacin (Pf)*, *Streptomycin (S)*, *Tetracycline (T)*, *Trimazine (Cm-Co)* and *Vanomycin (Va)* were also tested against drug resistant clinical strains. However NCCLS disc diffusion break points were used for the interpretative break points (Table 5.1). For thiosemicarbazones we have selected two optimum concentrations 62.5μg/μL and 93.7μg/μL in DMSO to study the structure activity relationship. The inhibition zone
of the antibacterial activity of thiosemicarbazones at concentration of 62.5μg/μL is shown in Fig 5.1.

i) Azabicyclic thiosemicarbazones [A1 and A2]

From the biological assay, compounds A1 and A2 were screened for antibacterial activity against two gram positive and eight gram negative clinical bacterial strains using Well Diffusion method (WD). The results are shown in Fig 5.2 and presented in Table 5.3. Both A1 and A2 showed poor activity against the tested bacterial pathogens in the concentration of 62.5μg/μL. Whereas the increase in concentration to to 93.7μg/μL, the compounds A1 and A2 increased their activity against E. coli, S. paratyphi 'A', and P. mirabilis. Organism E. coli is sensitive to both A1 and A2 when compared to other bacterial pathogens. Compound A1 showed moderate activity against S. aureus, P. aeruginosa, S. paratyphi 'A' and K. pneumoniae. The compound A2 is more active against the P. aeruginosa and P. mirabilis, but other pathogens showed only moderate activity.

ii) Phenyl thiosemicarbazones [B1, B2, B3 and B4]

Phenyl thiosemicarbazones were subjected to antimicrobial study to test the activity of these compounds (Fig 5.2). All except B4 showed activity against S. paratyphi 'H' and this organism is highly sensitive to B1 and B3 at all concentrations. B. subtilis and E. coli are highly resistive to B2 and B3. Compound B1 shows good activity against S. aureus and S. paratyphi 'A'. All the four compounds showed significant activity against all other strains of bacteria at the concentration of 62.5μg/μL. Compound B4 is more active against the organisms P. mirabilis and E. coli.
Fig. 5.1 Photographs showing the inhibition zone at the concentration of 62.5 μg/ml of thiosemicarbazones for different bacteria.
Fig 5.2 Anti-bacterial activity of thiosemicarbazone derivatives (a) piperidine; (b) azabicyclic; (c) phenyl and (d) carboxylate
Table 5.3 Anti-bacterial activity at 150μl of thiosemicarbazone derivatives

<table>
<thead>
<tr>
<th>Clinical bacterial test isolates from (AMH)*</th>
<th>Activity at the concentration of 150μl of Thiosemicarbazones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>*Apollo Main Hospital (AMH)- Culture collection</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>3.04</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>2.53</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>2.78</td>
</tr>
<tr>
<td>S. typhi</td>
<td>2.09</td>
</tr>
<tr>
<td>S. paratyphi ‘A’</td>
<td>3.16</td>
</tr>
<tr>
<td>S. paratyphi ‘H’</td>
<td>2.25</td>
</tr>
<tr>
<td>K. pneumoniaiae</td>
<td>2.42</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>3.74</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>2.22</td>
</tr>
<tr>
<td>E. coli</td>
<td>5.60</td>
</tr>
</tbody>
</table>
iii) **Carboxylate piperidine thiosemicarbazones [C1 and C3]**

Two different carboxylate piperidine thiosemicarbazones were synthesized and structural activity against the ten human bacterial pathogens was studied. The tested results against the antibacterial activity for the pathogens are shown in Fig. 5.2. The compound C3 is more active against *Salmonella typhi*. Both C1 and C3 compounds show more or less similar activity against *K. pneumoniae*, *S. typhi ‘A’*, *S. typhi ‘H’*, *P. mirabilis* and *E. coli*. Other organisms showed poor activity to these compounds.

iv) **Piperidine thiosemicarbazones [P1, P2, P3, P4 and P5]**

Five different piperidine thiosemicarbazones were synthesized and screened for *in-vitro* in the microbiological study. Two gram positive and eight gram negative bacteria were used for activity studies. The results are shown in Fig 5.2. The study reveals for all the organisms poor activity against these compounds. The compound P3 is active against *P. aeruginosa*. Organisms *S. paratyphi ‘H’* and *E. coli* are sensitive to compound P5. The organism *P. mirabilis* is highly resistive to all P1, P2, P3 and P5, but P5 shows moderate activity at higher concentrations. All the compounds showed moderate activity against the organisms *S. aureus*, *B. subtilis*, *S. typhi*, *K. pneumoniae* and *P. vulgaris*. It is also noted that the organisms are sensitive when the drug concentration increases.

v) **Overall activity of thiosemicarbazones**

All the derivatives of thiosemicarbazones are subjected to *in-vitro* anti-bacterial studies by well diffusion method and the results are shown in Fig 5.3. The study concludes that compound C3 is more sensitive to *S. typhi*, *S. paratyphi ‘A’* and *S. paratyphi ‘H’*. Compound C1 is also sensitive to organisms, namely *S. paratyphi ‘H’*, *K.*
Fig 5.3 Overall anti-bacterial activities of different thiosemicarbazone derivatives
*pneumoniae, P. mirabilis* and *E. coli*. Both C1 and C3 show moderate activity against *S. aureus* but C3 shows significant activity to *B. subtilis*.

The observation of higher activity of these compounds (C1 and C3) may be attributed to the fact that the presence of methyl and ethyl carboxylate groups adjacent to thiosemicarbazone moiety. These groups make extra coordination in the binding site of biomolecules. Both these compounds are more active in the equatorial substitution of carboxylate groups than axial substitution.

![Equatorial substitution](image1) ![Axial substitution](image2)

Because only the equatorial orientation shows the ideal geometry in metal complexation during biological reactions and curtails the bacterial growth. This favourable conformation is not possible in axial substitutions.

In the same way the compounds B1, B2 and B3 are more active against *S. typhi, S. paratyphi ‘H’* and *S. paratyphi ‘A’* organisms. The compounds B2 and B3 are highly resistive to two organisms, namely *B. subtilis* and *E. coli*. Compound B1 is more active against *S. aureus* and *E. coli* but *B. subtilis, P. aeruginosa* and *K. pneumoniae* show moderate activity. Here compounds B1 and B3 show good activity when compared to the other two. This may be due to the inductive effect of the chlorine atom substituted at para position of phenyl ring and the smearing of the electron cloud on the imine nitrogen (Chattopadhyay *et al.*, 1987). In B3, the methoxy group in ortho position of phenyl rings
binds with the metal ions along with the thiosemicarbazone and curtails the catalytic action (Chattopadhyay et al., 1987; 1988).

In the azabicyclic thiosemicarbazones, all the bacterial pathogens other than \textit{P. aeruginosa} and \textit{E. coli} do not show any significant activity on A1 and A2. Out of these two A1 is more active than A2 and this may be due to the nitroso group present in the molecule.

Piperidine thiosemicarbazones P1, P2, P3, P4 and P5 show very poor activity against all the bacterial pathogens. \textit{P. mirabilis} is highly resistive to all drugs except P5. But all the compounds showed significant activity when the concentration is increased to 100\(\mu\text{g/\mu L}\). Compound P5 is more active than others at this concentration. This may be due to the inductive effect of the chlorine atom present in phenyl rings.
5.2 Antifungal Studies of Thiosemicarbazones

Fungi are eukaryotic single cell organisms and their DNA is enclosed in a nucleus. Fungi are not involved in photosynthesis and take its food from living or dead organic matter by special digestive enzymes to break down the complex nutrients into simpler one. Fungi disperse themselves by releasing spores, usually windblown. Some fungi are friendly in nature and used to make several antibiotics to fight against bacterial infections. These antibiotics are natural compounds produced by the fungi to compete against bacteria for nutrients and space. We use Saccharomyces cerevisiae, baker's yeast, used to make bread and to brew beer. Fungi break down dead plants and animals and keep the world tidier. A fungal infection on the skin is called as 'mycoses' and it is mild one. However, in sick or immune suppressed people, fungi can cause serious disease. Because of some nasty fungi, fatal diseases are unavoidable for plants, animals and people.

5.2.1 Candida

Candida is the name for a group of yeasts that commonly infect the skin and it refers to the white colour of the organisms in culture. Candida infection is known as 'candidiasis', 'candidosis' or 'moniliasis'. For most people, the presence of this yeast on the skin is not visible, but for some people it grows actively and causes a red, itchy, scaling rash. The yeast likes warm moist areas, and grows in skin fold areas like under breast, stomach, and arm pits.

An increase in the incidence of nosocomial Candida infections was first noted in 1980s, particularly in surgical patients but also in other high-risk patient populations (Kam and Lin, 2002; Rex et al., 1995). Candida is a natural part of our gut flora and it is present in our intestinal track, skin, and genito-urinary tract. The other species of the
genus *Candida*, such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. lusitaniae*, have been found to cause an increasing incidence of mycoses.

### 5.2.1.1 Reason for Candida infections

The *candida* infections arise due to the use of antibiotics, oral contraceptives, steroid medications, and sugar consumption. These drugs can destroy the bacteria in the intestines that keep *candida* growth and it produce harmful toxins in the intestinal lining, which can then allow normally, excluded larger molecules to be absorbed through the lining and into the bloodstream. These large molecules could be partially digested foods which the body doesn't recognize and the immune system reacts against them. It may be that many of the symptoms associated with *Candida* could be due to the action of such toxins rather than the yeast itself.

Most common symptoms for *Candida* infections are as follows: thrush, white coating on tongue, diarrhea / constipation, bloating / excess gas, rectal itching, recurrent cystitis, headaches, depression & mood swings, slow metabolism, menstrual cramps & irregularities lethargy & 'foggy-headedness', fatigue, acne, etc.

### 5.2.1.2 Candida albicans

*Candida albicans*, the major human pathogen (Samaranayake and Holmstrup, 1989) of the genus *Candida*, is a common organism and present universally. Because of the use of antibiotics, steroids or other immunosuppressive agents in diabetes mellitus, local disorders of the gastrointestinal tract, AIDS, cancer chemotherapy or organ transplantation can enhance the risk of fatal consequences of *candida* infections. The role of secreted aspartic proteases of *C. albicans* in experimental and clinical *candidiases* has been demonstrated (Schaller et al., 2000; Schaller et al., 1999; Borg-von et al., 1998). Secreted aspartic proteases degrade a number of cellular substrates, including proteins.
related to immunological and structural defences, such as IgG heavy chains, α2-macroglobulin, C3 protein, β-lactoglobulin, lactoperoxidase, collagen and fibronectin (Douglas, 1988; Ray and Payne, 1990; Cutler, 1991; Ruchel et al., 1992; White and Agabian, 1995; Kaminishi, 1995).

5.2.1.3 *Candida tropicalis*

*Candida tropicalis* is one of the most common non-albicans species identified, accounting for up to 25% of all *Candida* isolates. *C. tropicalis* is the third most common *Candida* species isolated from patients in worldwide and these isolates are resistant to fluconazole drug (de Bedout et al., 2003).

5.2.2 Azole treatment to *Candida*

The azole antifungal agent fluconazole is a widely used compound to treat OPC. In recent years, patients with AIDS who have recurrent OPC and who are receiving prolonged fluconazole therapy, treatment failures are raising due to the emergence of fluconazole-resistant strains (Rex et al., 1995; Ghannoum et al., 1996).

5.2.2.1 Mechanism and action of azole derivatives

*Fluconazole* is fungistatic in action and works by inhibiting fungal cytochrome P-450 sterol C14-α-demethylation, thus obstructing the conversion of lanosterol to ergosterol (Goa and Barradell, 1995); which leads to the inhibition of membrane sterol synthesis and therefore preventing fungal cell replication. Another effect is that 14α-methyl sterols may increase *fluconazole* fungistatic effects. It is selectively toxic in human cells because it curtails the production of cholesterol and ergosterol (Neal, 1997). The mechanism of azole action to stop the sterol synthesis is shown in following scheme.
**IUPAC name:**

**Fluconazole:** 2-(2, 4-Difluorophenyl)-1, 3-bis (1H-1, 2, 4-triazol-1-yl)-2-propanol

**Itraconazole:** 4-[4-[4-[2-(2, 4-Dichlorophenyl)-1, 3-dioxolan-4-yl] methoxy] phenyl]-1-piperazinyl] phenyl]-2, 3-dihydro-2-(1-methyl propyl)-3H-1, 2, 4-triazole-3-one.

---

**Scheme:** Mechanism of azole action against fungal organism

5.2.3 *in vitro* Antifungal Sensitivity Determination Test (Antifungal drugs)

Standard antifungal agents, fluconazole 10μg and itraconazole 10μg discs were used for the comparative studies. The disc content levels are under National Committee
for Clinical Laboratory Standards (NCCLS) column. The antifungal discs were obtained from HiMedia Laboratories Pvt. Limited, Mumbai, India.

5.2.3.1 Clinical yeast isolates

A total of 61 clinical isolates of *Candida spp.* were obtained from the Department of Clinical Microbiology, Apollo Hospitals, Chennai, India. All these are isolated from the samples obtained during the years 2004 and 2005 and identified by standard methods. 24 *Candida albicans* isolates were obtained from blood samples. More than 17 *Candida tropicalis* and 16 *Candida albicans* isolates were obtained from wound sites. 3 isolates strains of *Candida tropicalis* were collected from urine samples. Additional reference including one American Type Culture Collection strain *Candida albicans* (ATCC 90028) was used. Isolates were stored as glycerol cell suspensions stocks frozen at -70°C until they were used. Stored clinical isolate cultures were revived in the Sabouraud dextrose broth or Brain heart infusion broth incubated at 35 °C for 24 hours and then subcultured onto Sabouraud dextrose agar medium.

5.2.3.2 Preparation of fungal inoculum

The inoculum used was prepared using the yeasts from a 24 hours old culture on Sabouraud dextrose agar. With a sterile loop, the tops of four to five colonies were transferred to a tube containing 5mL of Mueller Hinton broth or Brain Heart infusion broth. The tube was incubated at 35 °C for 24 hours. The turbidity of the culture suspension was adjusted with broth or a sterile saline solution (0.85 – 0.9%). The culture density was adjusted with spectrophotometer at 530nm to obtain a final concentration to match that of a 0.5 McFarland standard and finally inoculum size approximately (0.5- 2.5 X 10³ CFU/mL).
5.2.4 Antifungal Susceptibility Testing

5.2.4.1 Disk diffusion Method (NCCLS M2 – A4)

The plates for Disk and Well diffusion methods were prepared as per the procedure described for antibacterial activity test. Then selected antifungal agents fluconazole 10μg and itraconazole 10μg disks were placed on the inoculated plates and pressed firmly into the agar with the sterile forceps to ensure the complete contact with the agar [For Well Diffusion method: Sterilized 9mm cork borer was used to make the agar wells, 100μL of the diluted thiosemicarbazones (TSC) stock solutions were placed into each well and also added DMSO as a control into one well]. The plates were incubated at 35°C for 24 hours. NCCLS disc diffusion and MIC break points were used for the interpretative break points.

The antifungal activity test against two clinical Candida species, namely C. albicans and C. tropicalis is presented in Table 5.4. A study of the table reveals that fluconazole was found to be active against C. tropicalis and highly sensitive to the drug. But C. albicans was highly resistant to fluconazole. Owing to the noted discrepancy, an alternative drug itraconazole was tested against C. albicans and C. tropicalis and found to be inhibited significantly by the drug. From the zone of inhibition, C. tropicalis is highly sensitive to the antifungal agent itraconazole but moderately inhibit the species C. albicans.

5.2.5 Activity of thiosemicarbazones against human fungal pathogens

The results of thiosemicarbazone derivatives against two important human fungal pathogens C. albicans and C. tropicalis are listed in Table 5.5. The inhibition zone of the antifungal activity of thiosemicarbazone derivatives is shown Fig 5.4. The results obtained for different thiosemicarbazone derivatives are as follows:
Table 5.4 NCCLS approved antimicrobial for testing human fungal pathogens

<table>
<thead>
<tr>
<th>Antifungal activity</th>
<th>Antibiotic Disk diffusion method</th>
<th>Diameter of zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fuloconazole (10 µg)</td>
<td>Itraconozole (10 µg)</td>
</tr>
<tr>
<td>C. albicans</td>
<td>-</td>
<td>10.0</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>33.0</td>
<td>18.0</td>
</tr>
</tbody>
</table>

The percentage of inhibition zone was calculated by using following formula

\[
\% \text{ of inhibition zone} = \left( \frac{(a) \text{ Area of inhibition zone} \times (radius)^2}{(b) \text{ Area of the petric plate} \times (radius)^2} \right) \times 100
\]
**Table 5.5** Anti-fungal activity at the concentration of 150µl of Thiosemicarbazones

<table>
<thead>
<tr>
<th>Clinical bacterial test isolates from (AMH)*</th>
<th>A1</th>
<th>A2</th>
<th>C1</th>
<th>C3</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>14.52</td>
<td>6.19</td>
<td>8.67</td>
<td>5.09</td>
<td>12.88</td>
<td>5.71</td>
<td>4.00</td>
<td>7.41</td>
<td>7.11</td>
<td>6.53</td>
<td>7.47</td>
<td>4.00</td>
<td>9.68</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>2.25</td>
<td>2.60</td>
<td>7.11</td>
<td>9.34</td>
<td>14.87</td>
<td>3.57</td>
<td>6.42</td>
<td>4.94</td>
<td>3.78</td>
<td>2.78</td>
<td>2.32</td>
<td>2.67</td>
<td>6.25</td>
</tr>
</tbody>
</table>

*Apollo Main Hospital (AMH)- Culture collection*
Fig 5.4 Photographs showing the inhibition zone at the concentration of 63.5 μg/μl of thiosemicarbazones for the fungi
i) Piperidine thiosemicarbazones

Both *candida* species of *C. albicans* and *C. tropicalis*, human pathogens are used for the antifungal activity with piperidine thiosemicarbazones. The results of antifungal activity of these five piperidine thiosemicarbazones are shown in Fig. 5.5a. The study reveals that all the five compounds show more activity against *C. albicans* than *C. tropicalis*. Particularly the compound P5 shows higher activity than others and *Candida albicans* is more sensitive to piperidine thiosemicarbazones. *C. tropicalis* is found to be more sensitive to the compound P5.

ii) Azabicyclic and carboxylic thiosemicarbazones

The antifungal activity of both azabicyclic and carboxylate thiosemicarbazones is shown in Fig. 5.5b. From the results, compound A1 shows significant activity against *Candida* species. *Candida albicans* is sensitive to the tested drug at the concentration of 31.2μg/μL whereas A1 does not show any activity against *Candida tropicalis*. When the concentration is increased to 62.5μg/μL, the drug A1 shows more activity against *C. albicans*, but in *C. tropicalis* less activity is noted at this concentration. From the above results it is to be concluded that *C. albicans* show more sensitivity to tested drug A1. But in carboxylic thiosemicarbazones both compounds C1 and C3 are sensitive to the organisms *C. albicans* and *C. tropicalis*.

iii) Phenyl thiosemicarbazones

A study of Fig. 5.5c reveals the following facts. Compound B1 shows more activity against the *candida* species *C. albicans* and *C. tropicalis*. Compound B4 showed moderate activity against the *C. albicans* but compound B3 shows better activity against *C. tropicalis*.
Fig 5.5 Antifungal activity of thiosemicarbazone derivatives (a) piperidine; (b) azabicyclic and carboxylate; (c) phenyl and (d) overall.
iv) Overall antifungal activity of thiosemicarbazones

Overall antifungal activity of thiosemicarbazones is shown in Fig. 5.5d. All the thiosemicarbazones are highly active against *C. albicans* than *C. tropicalis*. Here the compound A1 is more active against *C. albicans*. Compound B1 is more active to both these organisms. Comparatively all these derivatives are more active against *C. albicans*. But the compounds C3, B1 and B3 are more active against *C. tropicalis* than *C. albicans*.

5.3 Conclusion

Different thiosemicarbazones were used in the above studies for antibacterial and antifungal activity by *in-vitro* studies. For antibacterial activity ten organisms such as gram +ve and gram –ve strain were used. From this study it is found that all thiosemicarbazones show significant activity. The organisms *S. typhi*, *S. paratyphi ‘A’* and *S. paratyphi ‘H’*, are highly sensitive to the carboxylate piperidine thiosemicarbazones (C3 & C1). The phenyl thiosemicarbazone compounds B1 & B3 are also more active against these organisms.

The antifungal study of thiosemicarbazone derivatives shows significant activity against *Candida* clinical strains but more active against *C. albicans* compare than *C. tropicalis*. The compound B1 is very good activity against both strains *C. albicans* and *C. tropicalis*. Interestingly, *C. albicans* is highly sensitive for compound A1.
6.1 Introduction

Ribonucleotide reductase (RNR) is a ubiquitous cytosolic enzyme in the cell, responsible for converting ribonucleotides into deoxyribonucleotides, the eventual substrates for DNA polymerase (Lewis et al., 1978; Reichard, 1993; Wright, 1989), and also repair DNA in all living cells (Eklund et al., 2001). In mammalian cells, this enzyme contains two dissimilar protein components, R1 and R2, which are encoded by two different genes located on different chromosomes (Tonin et al., 1987). Protein R1 is a homodimeric structure, with a molecular mass of 168 kDa, and has substrate and allosteric effector sites that control enzyme activity and substrate specificity (Wright, 1989; Wright et al., 1990). Protein R2 is a homodimer, with a molecular mass of 88 kDa, and forms two equivalent dinuclear iron centers that stabilize a tyrosyl free radical required for the initiation of electron transformation during catalysis (Wright et al., 1990). R1 and R2 proteins interact at their C-terminal ends to form an active holoenzyme (Wright et al., 1990; Davis et al., 1994).

The overall function of RNR 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 performed with the help of organic free radicals, which are stored by the enzyme until required for catalysis. RNR reduces all the four main ribonucleotides to the corresponding deoxyribonucleotides and thus indispensable for the survival of all living organisms. Since the reduction of a ribonucleotide to a
deoxyribonucleotide is a chemically ‘difficult’ reaction and requires the involvement of organic free radicals (Reichard and Ehrenberg, 1983), this enzyme is considered to be very important. The cartoon diagram of the RNR enzyme is shown in Fig 6.1.

6.1.1 Different classes of RNR

RNR contains two important components such as a radical generator and a reductase. The production of the radical by the generator and its storage are the first step of the reaction. Surprisingly the radical generators of all types of RNRs are not the same, whereas the reductase component is more or less similar. The RNR enzymes isolated so far have been classified into three types (class I, II and III; Fig. 6.2) based on the oxygen dependence and metal cofactors involved in the generation of the catalytically essential free radicals (Reichard, 1993; Jordan and Reichard, 1998). Class I can further be subdivided into two classes Ia and Ib, based on differences in amino acid sequences (Jordan and Reichard, 1998). Class Ia is found in eukaryotes and in some bacteria and viruses, whereas class Ib is not found in eukaryotes.

Mammals, E. coli and DNA-viruses employ class I reductases, which is an iron-containing protein and produce the catalytically essential tyrosyl free radical. In these reductases, the quaternary structure of the holoenzyme is \( \alpha_2 \beta_2 \). The \( \alpha_2 \) dimer, called protein R1, contains the active sites and binding sites for allosteric effectors, while the \( \beta_2 \) dimer called R2, contains one dinuclear iron center and one stable tyrosyl radical per monomer. These two subunits are very essential for the enzymatic activity.
Fig 6.1 Representation of R1 and R2 - RNR complex

Fig 6.2 Tree depicting the main classes of RNR
6.1.2 Functional aspects of RNR enzyme

The best characterized reductases are those in class I, particularly RNR enzymes (Stubbe, 1990a; 1990b; Eriksson and Sjöberg, 1989). The working model for the nucleotide reduction process is presented in Fig 6.3. Catalysis is initiated by hydrogen atom abstraction from the 3’-position of the substrate by the thyl radical generated by one of the metallo cofactors (Ito et al., 1994; Stubbe et al., 1983). Theoretical studies have suggested that the subsequent elimination of the 2α-hydroxyl group as water occurs simultaneously with deprotonation of the 3’-hydroxyl group (Zipse, 1985). The overall structural activity of radical transition is shown in Fig 6.3.

6.1.2.1 Formation and function of Tyrosyl Radical

An important observation made by Atkin and his coworkers (1973) is that the incubation of apo-R2 with Fe(II) and O₂ in the presence of ascorbate led to the formation of the diferric tyrosyl radical cofactor (Fig.6.4). The diferrous cluster reacts with O₂ to produce a putative short-lived peroxo intermediate which is reduced to a paramagnetic intermediate designated by compound X (Fig. 6.5). This peroxo intermediate X is then converted into the diferric cluster by oxidation of Tyr122 to the radical form.

The one electron reduction of tyrosyl radical with the dinuclear iron cofactor to tyrosine resulted in complete loss of one electron to mediate nucleotide reduction (Ehrenberg and Reichard, 1972). The hypothesis putforth here is that the function of the tyrosyl radical in R2 is to generate a thyl radical on Cyc439 of R1 which then initiates the nucleotide reduction process (Stubbe, 1990c; Mao et al., 1992).
Fig. 6.3: A model of the overall mechanism of the radical transition for *E. coli* (Stubbe, 2003).
Fig. 6.4 Schematic representation of formation of active Fe(III) and Tyrosine radical from Apo-R2 protein

Fig 6.5 Tyrosine radical formation pathway
6.1.3 RNR in cancer and disease

Ribonucleotide reductase is an essential enzyme of all living cells (Stubbe and van der Donk, 1998; Reichard, 1993; Sjöberg, 1997), which catalyzes the reduction of the four common ribonucleoside diphosphates to the corresponding deoxyribonucleotides. Thus cell division makes it a potential target for designing drug to inhibit cell growth, applications in cancer therapy, the production of anti-malaria and trypanosome drugs, antibiotics and anti-viral agents against those viruses that have their own RNRs.

An increased interest in RNR as a target for cancer therapy is seen ever since the a human ribonucleotide reductase of a new type was identified which is regulated by p53 (Lozano and Elledge, 2000; Nakano et al., 2000; Tanaka et al., 2000). The p53 protein actively suppresses tumour formation but on mutation several forms of cancer are developed. As much as over 80% of the human tumours have been found to contain mutations in p53 or in the pathway that directly regulates it. Mammalian RNR-R2 is located in the cytoplasm and regulated by the cell cycle. The new R2 gene product is called p53R2 and found to be located in the nucleus. The p53 binds to a sequence in the first intron of p53R2 gene and is required for directly activating its transcription.

Large amounts of deoxyribonucleotides are required in proliferating cells during the S-phase of the cell cycle where the duplication of the genome takes place. The level of RNR in mammalian cells is therefore closely linked with the cell cycle and growth control mechanism. Enzyme activity and the R1 and R2 mRNAs reach maximal levels during S-phase (Engström et al., 1985; Eriksson et al., 1984). The R1 protein has a half-life of about 20 hours and production is in excess during the cell cycle. The R2 protein, in contrast, shows an S-phase specific expression and possess shorter half-life of about 3
hours, probably due to a controlled degradation (Chabes and Thelander, 2000). A second R2 subunit, called p53R2, has been identified in human and mouse cells (Tanaka et al., 2000).

It was reported recently that p53 enzyme binds both R2 and p53R2 subunits in resting cells but upon exposure to UV irradiation, they dissociate from p53 and bind to R1. Perhaps the regulation of RNR activity by p53 is more complex than activation of p53R2 (Xue et al., 2003).

The review by Eklund et al. (2001) discusses the possibility of specific inhibitors to the normal RNR. Their argument is based on the fact that cancers often have the mutations in the p53 pathway and unable to make p53R2. As all cells are dependent on the supply of deoxyribonucleotides, cancer cells would die if the normal RNR were inhibited, whereas normal cells with p53 could survive on the deoxyribonucleotide pool supplied by p53R2. This appears to be a good strategy but unfortunately the picture seems to be more complicated.

An increased level of RNR produce more deoxyribonucleotides, and thus drive the cells through DNA synthesis. In a hypoxic environment where the activity of RNR is hampered due to lack of oxygen, the level of RNR regulates the production of deoxyribonucleotides and drive the cells faster through DNA synthesis. This implies that the replication machinery in the cells is able to detect the level of deoxyribonucleotides, when exogenous deoxynucleotides are supplied (Kashlan et al., 2002).

A new strategy to kill cancer cells would be to specifically inhibit the normal RNR-R2 which is crucial to 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. The DNA damage-response pathway is largely conserved from yeast to humans, and the yeast system is suggested to be a good analogous system for studies of ribonucleotide reductase and its dependence of the cell cycle (Lozano and Elledge, 2000; Santocanale and Diffley, 1998; Tanaka et al., 2000).

6.1.3.1 Ribonucleotide reductase (RNR) inhibitors

Since ribonucleotide reductase (RNR) activity is necessary for DNA replication and inhibition of this enzyme will inhibits cell division (Szekeres et al., 1997). Therefore an understanding of the molecular mechanism of RNR is essential for the design of new cytostatic drugs. The inhibitor must be a radical scavenger to destroy the tyrosyl radical or 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. These drugs are slow and relatively unspecific (Stubbe, 1990a). However, other compounds such as flavin (Fontecave et al., 1989) and phenosafranine (Sahlin et al., 1989) are available to reduce the radical activity.

The tyrosyl radical in protein R2 is closely linked to the Fe(III) center. One can obtain a form of the enzyme that lacks the radical but maintains the iron center (met R2). On the contrary, a protein which lacks the iron center but to maintaining the radical, is not possible. The removal of Fe(III) to obtain apoR2 results in the simultaneous loss of the radical. But readdition of Fe(II) ions to apoR2 in the presence of O₂ regenerates both Fe(III) center and Tyr radical (Petersson et al., 1980). From results it was concluded that the Fe(III) center is required for both stabilization and generation of the tyrosyl radical.
Flavins have been recognized as being capable of both one- and two-electron transfer processes, and play a pivotal role in coupling the two-electron oxidation of most organic substrates to one-electron transfers of the respiratory chain. In addition, they are now known as versatile compounds that can function as electrophiles and nucleophiles, with covalent intermediates of flavin. Because of their chemical versatility, flavins are involved as a host in the biological phenomena. 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 centres through their free radical states. With this capacity, they frequently form parts of multi redox-centre enzymes (Massey, 2000).

In this chapter, the structure of human RNR-R2 protein was predicted using homology modelling to understand the interactions with its inhibitors such as flavin and phenosafarine through docking. Further, the mechanism of action for both inhibitors was proposed. In addition, the iron chelation mechanism of thiosemicarbazone derivatives was also proposed.

6.2 Methodology

6.2.1 Structure prediction of human R2 protein

6.2.1.1 Sequence analysis

It was mentioned earlier that the radical generator is not the same for all RNRs. The six well known eukaryotic sequences of radical generator R2 proteins are analyzed by multiple sequence alignment with mouse R2 protein using CLUSTAL W program (Chenna et al., 2003; Fig. 6.6). The secondary structure elements and the sequence numbers are shown on the top for the human enzyme and below the conserved sequence line E. coli R2. 100% conserved residues are shown in red colour. Appreciable sequence
Fig. 6.6 Multiple sequence alignment of different RNR-R2 enzymes. The 100% conserved residues are shown in red background whereas residues which have more than 70% conservation score is given at the bottom of the alignment. Residues with similar properties and have more than 70% conservation is shown in red and the regions are represented in blue box. The secondary structures helix is indicated at the top of the alignment.
identity is observed throughout the eukaryotic group. The yeast sequence has been truncated in the N-terminus. The sequence line has capital letters for the conserved residues and lowercase letters to indicate the identity in all eukaryotic species. The accession numbers for R2 sequences are as follows: mouse, P11157 (Thelander and Berg, 1986); human, P31350 (Pavloff et al., 1992); E. coli, P69924 (Carlson et al., 1984); and yeast, P09938 (Elledge, and Davis, 1987).

From the multiple sequence alignment, all the sequences of mouse, human, P53R2 and Yeast are highly conserved and show appreciable high sequence identity except for E. coli. The P53R2 and E. coli RNRs sequences have been truncated in the N-terminus. The dinuclear binding residues are conserved in all sequences.

6.2.1.2 Homology modeled structure of human R2 Protein

The human R2 protein (Swiss-prot No. P31350) was taken for the study. The pairwise alignment between the human R2 and mouse R2 (Fig 6.7) protein was carried out using CLASTAL W program (Chenna et al., 2003) and the results show that they are structurally similar to each other. As high as 95% sequence identity is noted between human R2 and mouse R2 and expected similar structural reactivity. In fact the active site residues are 100% conserved between them. The residue Tyr189 is a radical generator in human R2 instead of Tyr177 in mouse, so the known 3D structure of mouse (PDB id code:1xsm) was taken as a template for the homology modeling study. The online server Swiss-model (Schwede et al., 2003) software tool is used. Out of 389 amino acid residues 288 were taken for constructing the structure.
Fig 6.7 Pair wise alignment of mouse RNR-R2 (PDB ID:1xsm) and Human RNR-R2. The 100% conserved residues are shown in red background.
6.2.2 Docking of flavin and phenosafranine molecules with modeled human R2

Docking is a term used to find a “best” matching between two molecules: a receptor and a ligand. The molecular docking problem can be defined as follows: given the atomic coordinates of two molecules, predict their “correct” bound association. In its most general form, no additional data are provided but in practice, additional biochemical information may be given, in particular knowledge of binding sites (Halperin et al., 2002). Docking program has two key parts, a search of the conformational degree of freedom and scoring or evaluation function. The search algorithm searches the potential energy landscape with sufficient details to find the global energy minimum. The scoring function is made realistic by giving importance to the experimentally determined complex which assesses both the steric and the chemical complementarity (Brooijmans and Kuntz, 2003).

Radical scavenger from R2 protein is very essential for inhibiting the RNR activity and DNA replication. From the literature (Eklund et al. 2001; Fontecave et al., 1989), the potential radical scavenger flavin and phenosafranine molecules are built using Insight program (Biosym, 1995) and the energy minimized structure coordinates are used for the tyrosine radical scavenger to study on human RNR-R2 protein using AutoDock program. The possible radical scavenger mechanism is as follows (Fig 6.8):

![Flavin](image1.png) ![Phenosafranine](image2.png)

Fig 6.8 Structures of flavin and phenosafranine
Protein R2
(Tyr Radical + 2Fe\(^{3+}\)) + Flavin (red)
or Phenosafranine (red) → Protein R2
(Tyr + 2Fe\(^{2+}\))

AutoDock program version 3.0.5 [web site http://www.scripps.edu/pub/olson-web/doc/autodock/; (Morris et al., 1998)] is used for this docking. This program utilizes Lamarckian Genetic Algorithm for conformational search and energy evaluation using grid-based molecular affinity potential. Water molecules are not used for docking studies as it makes the analysis complicated. Twenty-five best conformation of the protein ligand complex are retrieved and the one that supports the biochemical studies is chosen. The hydrogen bonding and non-bonded contacts for the complexes are derived using the program HBPLUS (McDonald and Thornton, 1994) and the pictorial representations are drawn using the program LIGPLOT (Wallace, 1995).

6.3 Results and Discussion

6.3.1 Structural features of the predicted human RNR-R2

The human R2 structure belongs to all-helical class and the 288 residues in the core structure is determined. The dinuclear iron is at the center of the helical region of the molecule. The structure contains thirteen helices, of which eight long helices (αA to αH) form a bundle (Fig 6.9). These helices contribute about three quarters of the protein molecule and shows rmsd value of 0.45Å (for Swiss-model) with mouse R2 (PDB ID:1xsm). Three of the five shorter helices (α3 to α5) orient perpendicular to the long helices in the bundle, and the other two are almost parallel to long helices, named as α1 to α2. The long hydrophobic helix αE is surrounded by six other helices. The structural differences observed are only in the loop and terminal regions (Fig 6.10). The Ramachandran plot for the model structure is shown in Fig 6.11.
Fig. 6.9 Cartoon structures of the model of Human RNR-R2

Fig. 6.10 Superimposition of mouse RNR-R2 (red) and the modeled structures (blue)
Fig 6.11 Ramachandran plot for the modeled human RNR-R2. Here Green, Cyan and Red represents the helix, turns & random coils, respectively. The lines in the plots indicate preferred areas. Orthogonal crosses indicate normal residues; diagonal crosses indicate glycines and open squares indicate prolines.

Fig 6.12 Some specific interactions observed in the modeled human RNR-R2 enzyme. It is conserved in all eukaryotes RNR proteins.
Some specific interactions observed in certain residues of the mouse R2 protein are conserved in most eukaryotic R2’s but differ from those in E.coli; Asn157 in this protein binds between Phe162 and Phe241. Glu165, which is conserved in most eukaryotes, is salt linked to the conserved Arg237. In αD, Trp222 and Trp226 pack perpendicular to each other. The kind of interactions observed in mouse RNR-R2 protein (Kauppi et al., 1996) also holds good in the predicted human RNR model (Fig. 6.12).

6.3.2 Docking study of radical scavenger

The flexible docking was performed for the modeled human RNR-R2 enzyme and the flavin structure. Out of twenty five conformations of the complex structures constructed, the best one was chosen based on biochemical studies (Fig 6.13).

Docking of flavin and phenosafranine molecules with modeled human R2 enzyme structure was performed and their interactions were calculated. The flavin molecule is stabilized via seven strong hydrogen bonds as shown in Fig 6.13. The residues Tyr189, Asn272, Ser276 and Ile275 are involved in hydrogen bonding with inhibitor molecule. In addition, some hydrophobic contacts Leu268, 144, 192, Ile140, Val143 and Ser271 are useful for the stability. The other hydrophobic contacts are found with the alkyl groups of inhibitor molecule. In this model both the adjacent methyl groups in the inhibitor are making hydrophobic contacts with the Ser150.

Docking of phenosafranine molecule with modeled human R2 enzyme structure was performed and their interactions were noted. This complex enzyme and inhibitor molecule are stabilized through hydrogen bondings as shown in Fig 6.14. The hydrogen bonds are contributed by the residues Glu279 and Tyr189 with reduced phenosafranine radical scavenger. In addition, Val154 and 244, Ser150, Glu245 and Asp151 are making
Fig 6.13 (a) Oxidized flavin binds to the active site of human RNR-R2 modeled structure; (b) LIGPLOT diagram showing the various interactions between oxidized flavin and human RNR-R2 protein.
Fig 6.14 (a) Oxidized phenosafranine binds to the active site of human RNR-R2 modeled structure; (b) LIGPLOT diagram showing the various interactions between oxidized phenosafranine and human RNR-R2 protein.
hydrophobic contacts with the ligand molecule. These hydrophobic forces are attributed due to N, N-dimethyl group in the ligand molecule.

6.3.2.1 Flavin action

Upon anaerobic incubation of R2 protein with reduced flavin and the reduction of the tyrosyl radical result in the formation of metR2. This reaction occurs in the absence of ferrous ions. In their presence, the Fe (III) center is reduced to Fe(II), and reduced R2 becomes the final product. Admission of oxygen then results in the reformation of active R2. This cyclic process is summarized in Fig.6.15.

The enzyme provides the reduced flavin which under anaerobic conditions transforms R2 into metR2 by scavenging the tyrosyl radical. In a second step, together with Fe (II) ions, the flavin reductase catalyzes the formation of reduced R2, with its $Fe^{2+}$ center. Oxygen then closes the circle by reforming the radical $Fe^{3+}$ center of protein R2. In the presence of oxygen, the tyrosyl radical is formed; in its absence the radical is removed (Fontecave et al., 1989).

![Fig 6.15 Relationship between active and inactive forms of ribonucleotide reductase (RNR)]
Mechanism of radical scavenging by flavin molecule [Fig 6.16]

In anaerobic condition, in the first step, the oxido oxygen atom of iron(III) cluster scavenge the hydrogen radical from the acidic hydrogen of flavin 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., 1987a; 1987b). According to this model a form of R2 containing reduced iron ("reduced R2") should be an intermediate in the reaction.

Simultaneously the unstable flavin radical (IIa & IIb) hydrogen bonded [flavin N-H (of uracil moiety)] with oxygen atom of oxido-Fe(III) and serve as a driving force for the oxido-Fe(III) bond cleavage.

To attain the stable aromatic nature, the IIb flavin radical eliminates one more electron from the piperazine ring and form the yellow color oxidized flavin(IV) in the second step. Hydroxyl Fe(III) reacts with second hydrogen radical and form one more reduced Fe(II) and water (Fig 6.16).
Mechanism

![Diagram showing the mechanism of flavin molecule](image)

**Fig 6.16** Possible mechanism of radical scavenging by flavin molecule

### 6.3.2.2 Action of phenosafranine molecule (Fig 6.17)

In the anaerobic environment with the presence phenosafranine (I), the reduction of both the free radical and the iron center takes place. Phenosafranine cation first loses one of its protons and forms the neutral phenosafranine (II) and hydroxyl Fe-(III). One Fe(III) gets reduced to Fe(II) at first step.

In the second step, dimethyl amine nitrogen donates a pair of electrons to make aromaticity in the molecule and the piperazine moiety expels the hydrogen anion from N-H. This anion reacts with HO-Fe(II)-R to form water (IV) and reduced to Fe(II). After the radical reduction of Fe center, air was introduced into the sample and a partial regeneration of both the iron center and the radical occurs.
Mechanism:

Fig 6.17 Possible mechanism of radical scavenging by phenosafranine molecule

6.3.3 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 in anti-proliferative therapy or in combination with other drugs. An early effect due to iron chelators in the cell is that RNR activity decreases with the accumulation of apo-R2 and therefore, DNA synthesis and cell proliferation are curtailed.
6.3.3.1 Drug action of thiosemicarbazones

The drug which belongs to the group of $\alpha$-(N)-heterocyclic thiosemicarbazones, are the most potent inhibitors of mammalian RNR known till date. These compounds are metal chelating agents with exceptionally strong affinity for iron (Sartorelli et al., 1977). Experimental evidence suggests that the iron chelation of the drug is the active form of the inhibitor (Saryan et al., 1979; Preidecker et al., 1980). The study reveals that the thiosemicarbazone derivatives inhibit the enzyme by destroying the free radical. This is because the radical structure is more exposed in the mammalian reductase (Kjøller Larsen et al., 1982).

Heterocyclic thiosemicarbazones are proposed to inhibit RNR 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 reduce the drug (Fe$^{3+}$) to drug (Fe$^{2+}$) which is a necessary process for drug action according to the proposed mechanism. In addition, in the presence of iron and oxygen, they also support regeneration of the R2 specific tyrosine free radical once it has been scavenged (Gräslund et al., 1982), and thus can help to counteract the drug action.

Thiosemicarbazones are the most potent chelating inhibitors known for mammalian RNR proteins. The chelator drugs act directly to destroy the tyrosyl free radical of the R2 protein as was demonstrated for the iron-complexed form of the potent RNR inhibitor thiosemicarbazones (Thelander and Gräslund, 1983; Finch et al, 2000). According to the above suggestions, the drug thiosemicarbazones do not directly chelate the Fe$^{3+}$ ions in the active site of RNR instead they first scavenge the free radicals and reduce the metal ion from Fe(III) to Fe(II). Thereafter only thiosemicarbazones will
chelate the \( \text{Fe}^{2+} \) ions and increase the concentration of the apo-R2 level. The possible \textit{mono} and \textit{bis} chelated complexes are shown in Fig 6.18.

6.4 Conclusion

The human RNR-R2 structure was modeled based on the sequence available. Docking study was performed for this modeled structure with the radical scavengers flavin and phenosafranine. The possible mechanism involved during the process of radical scavenger from RNR-R2 was explained. From this study we conclude that the radical scavenging process is possible only in an anaerobic condition and not possible in medicine therapy for treatment to inhibit the cell replication of cancer. So, the metal scavengers such as thiosemicarbazones are more suitable drugs for cancer treatment to curtail the action of RNR enzyme and stop catalytic activity.
Fig 6.18 Formation of two different metal complexes with Fe(II) and thiosemicarbazone: (a) Tetrahedral complex $[\text{Fe}^{2+}(\text{TSC})]\text{Cl}_2$ and (b) Octahedral complex $[\text{Fe}^{2+}(\text{TSC})_2]\text{Cl}_2$. 