similarity between the two sources lies in the putative conserved domains (CD), which are the functional modules of the protein. In human HMGR, CD begins at the amino acid 438 and ends at 871, whereas for yeast, it begins at 594 and ends at 1025. Based on the analysis of the expected values as well as the identities, positives, and gaps, it can be concluded that yeast can be used to conduct studies on hypercholesterolemia.

B. Molecular properties

The molecular properties of atorvastatin and its derivatives are given in Table 1. High molecular weight, high values of HBA and HBD indicate poor permeability, whereas high Log P shows poor solubility of the drug. Favorable PSA for CNS acting drug is less than 70 $Å^2$, while for non-CNS acting drug lies between 70 $Å^2$ and 120 $Å^2$.

Table 1. Molecular properties of atorvastatin and its analogues.

Molecular Properties			
	ATORVASTATIIN	Comp 1	Comp 2
MWT	558.25 (>500)	>500	>500
# HBA	5	= AT	= AT
# HBD	4	< 4	= AT
Mol Log P	5.64 (>5)	> 5	> 5
Mol PSA (Predicting absorption)	88.12 $Å^2$	< AT	= AT
Mol Vol	543.96 $Å^3$	> AT	> AT

C. Thermodynamic parameters

Some of the critical thermodynamic properties were analyzed to gain an understanding on the protein folding-defolding nature of HMGR in the presence and in the absence of inhibitors (Table 2). The deactivation rate constant of HMGR in the presence of inhibitor is slightly higher, indicating that some amount of defolding occurs in the presence of inhibitor.

egative ΔS values suggest that the reaction is thermodynamically feasible but it is slow which is characteristic of most biochemical reactions unlike chemical reactions. Higher enthalpy denotes that higher energy is required to proceed the reaction. In this case, inhibition reaction by atorvastatin requires more energy to be supplied for the reaction to proceed, compared to the other reaction without inhibition.

It has been reported that more activation energy are associated with more stable structures. It can be observed that in the presence of inhibitors, the enzyme exhibits more activation energy when compared to the reaction without any inhibitors, denoting that a more stable complex is formed with the inhibitors.

Table 2. Thermodynamic parameters in the presence and in the absence of atorvastatin.

Thermodynamic Parameters		
	Without ATORVASTATIIN	With ATORVASTATIIN
K_d (min^{-1})	0.0111	0.0115
?S (J/mol.K)	- 193	- 183
?H (KJ/mol.K)	26	35
?G (KJ/mol.K)	56	86
E (KJ/mol)	32	35

IV. CONCLUSION

Yeast has been widely used to study anti-cancerous drugs, but efforts in the cardiac arena are very less. BLASTP studies show that yeast HMGR is similar in function to the mammalian HMGR, which propels the experimental attempts with yeast. The molecular property prediction study of atorvastatin and its derivative helps to analyze properties like CNS absorption and intestinal penetration. Thermodynamic studies of the enzymatic reaction involving HMGCoA reductase, in the presence and in the absence of inhibitors show the folding/defolding of the protein when associated with inhibitors. The thermodynamic properties suggest that the reaction is feasible. The trend appears to be similar for deactivation studies performed with atorvastatin and its analogues with expected decrease in activity because of enzyme inhibition.

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Multidisplinary Approach For An Important Molecule Synthesis

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Abstract

Hypercholesterolemia and atherosclerosis are critical health risk factors, mainly caused by uncontrolled cholesterol biosynthesis. At present, statins are used for the treatment of hypercholesterolemia. One of the statins (atorvastatin) and its analogues are evaluated with the yeast system, decided on the basis of BLASTP studies. Different analogues were designed and analyzed for drug-likeness. This is followed by prediction of molecular properties of the compounds under study. The compounds were subjected to docking. Inhibition studies with the drugs revealed that all of them inhibit yeast HMGR in the competitive way. Certain thermodynamic parameters (enthalpy, entropy, Gibb's free energy, and activation energy) were estimated to understand the folding/defolding of the protein.

Key words: Biosynthesis, Thermodynamic Parameters, BLASTP Studies

I. INTRODUCTION

The 3-hydroxy-3-methylglutaryl co-enzyme A reductase (HMGR; EC 1.1.1.34; GenBank accession number M12705) is the rate-controlling enzyme which catalyzes the synthesis of mevalonate and thus controls the biosynthesis of cholesterol. HMGR is a 97 kDa integral membrane glycoprotein of the endoplasmic reticulum (ER) in the mammalian system. Hypercholesterolemia and atherosclerosis are critical health risk factors, probably caused due to uncontrolled cholesterol biosynthesis. At present, statins which inhibit HMGR are commonly used for the treatment of hypercholesterolemia. In subjects with a regular lipid metabolism, only one-third of the total body cholesterol is diet derived, whereas balance two-thirds is synthesized directly from intracellular precursors by various organs of the body [1-3]. For this reason the control of cholesterol-genesis by inhibiting its biosynthesis is an important means of lowering plasma cholesterol levels. Genetically engineered forms of the enzyme are also studied in the primitive life forms like bacteria, but certainly it requires detailed investigation for this enzyme in human system [4].

In this regard, inhibition by substrate and other inhibitors is important in drug development for hypercholesterolemia because the existing drugs act by inhibiting the key enzyme in cholesterol biosynthesis. The functional structures of proteins reflect two tendencies that are contradictory. On one hand, proteins fold to minimize their free energy. On the other hand, they organize themselves to recognize a ligand or a transition state [5]. Minimizing free energy leads to well-packed hydrophobic interiors and hydrophilic exterior [6]. The stability-function hypothesis predicts that it is usually possible to replace residues known to be important for function, reducing protein activity but concomitantly increasing stability of the folded protein [7]. Various folding and defolding take place in the protein during the process of deactivation and it is critical to understand deactivation through certain thermodynamic properties like entropy, enthalpy, and Gibb's free energy [8]. So attempts have been made to understand the stability and deactivation of the concerned enzyme by analyzing its thermodynamic properties. There are many reports on the usage of yeast in drug discovery, but this work may be the first attempt of using yeast model for hypercholesterolemia. This work shows the ease and effectiveness of an assay method to predict different inhibitions and this forms the preliminary yet important step in drug discovery.

II. MATERIALS AND METHODS

A. Chemicals

Atorvastatin calcium, with a purity of 99.4% was obtained from Biocon India Pvt Limited, Bangaluru, India. HMGCoA and NADPH were obtained from Sigma, USA. Yeast extract, peptone, dextrose, malt extract and all other chemicals with analytical grade were obtained from Sisco Research Laboratories Pvt. Ltd (Chennai, India).

B. Micro-organism and culture conditions

Saccharomyces cerevisiae MTCC 36 was obtained from the Microbial Testing Collection Center, IMTECH, Chandigarh, India. The organism was maintained in the specified YPDM medium. The organism was sub-cultured at a regular interval of two days. The slants were incubated at 30° 1° C for 24 hours and then stored at 4° 1° C until further use. These slants were used within 1 or 2

days of storage at 4° 1°C.

Approximately, 1.2 x 10^8 cells/ml from a slant culture were inoculated into the sterilized medium and incubated on a temperature controlled rotary shaker maintained at 180 rev/min and 30° 1° C for a period of 40 hours.

C. Assay of HMGCoA reductase

HMGR converts HMGCoA into mevalonate with the help of the cofactor NADPH. NADPH is oxidized to NADP concurrently, which can be monitored using UV-visible spectrophotometer at 340 nm. A change in the concentration of NADPH during the reaction is the indirect assay of the enzyme HMGR. One unit of HMGR activity is defined as the amount of enzyme that aids in the conversion of 1 micromole of NADPH to NADP per min of the reaction under the standard assay conditions. Activity is either expressed in U or U/g dry cell weight.

D. Inhibition Studies with Atorvastatin and Its Analogues

The concentration of atorvastatin and its analogues added to the reaction mixture varied between 0.002 and 0.01µg/ml. The volume of the assay mixture was maintained at 1.5 ml throughout all the reactions and the temperature maintained at 30°C. Crude extract of yeast is used as the enzyme source. Keeping the concentration of atorvastatin constant, the activity of the enzyme was estimated with different HMGCoA concentrations.

E. Thermodynamic Parameters

Certain thermodynamic parameters like enthalpy, entropy, and Gibb's free energy are important in understanding the folding and defolding of enzymes during deactivation. The transition state theory proposed by Eyring [9] was used to explain the effect of temperature on the reaction rate, as follows:

$$k_d = \frac{kT}{h} e^{\frac{\Delta S}{R}} e^{\frac{-\Delta H}{RT}}$$

Where, k = Boltzmann's constant (J/K)

h = Planck's constant (Js)

T = temperature (K)

R = gas constant (J/mol.K)

ΔS = change in entropy (J/mol.K)

ΔH = change in enthalpy (kJ/mol)

By taking logarithm of both sides of the above equation, following equations are generated:

$$\ln\left(\frac{k_d}{T}\right) = \left[\ln\left(\frac{k}{h}\right) + \frac{\Delta S}{R}\right] - \frac{\Delta H}{RT}$$

On simplification of this equation, we get,

$$\ln\left[\frac{k_d}{T}\right] = \left[\ln\left(\frac{k}{h}\right) + \frac{\Delta S}{R}\right] - \frac{\Delta H}{R}.\frac{1}{T}$$

The values of [ln[k_d/T] were calculated using the k_d values corresponding to a particular temperature. The ΔH and ΔS values were calculated from the slope and the intercept of the plot of ln[k_d/T] vs. 1/T respectively.

(a) Estimation of ΔG

Gibbs free energy of any biochemical reaction can be found out by

ΔG = ΔH - T ΔS

(b) Estimation of activation energy

The activation energy was calculated from the Arrhenius

$$k_d = k_o . e^{\frac{-E}{RT}}$$

Equation as follows:

where, k_d = Deactivation rate constant, min^{-1}

k_o = Frequency factor

E = Activation energy (kJ/mol)

R = Gas constant (J/K.mol)

T = Temperature (K)

On taking logarithm of both sides for the above equation,

$$\ln k_d = \ln k_o - \frac{E}{R}\frac{1}{T}$$

The k_d values were used for the calculation of activation energy at a particular pH within the temperature range between 55°C and 65°C.

III. RESULTS AND DISCUSSION

A. BLASTP studies

There are 888 amino acids in human HMGR, whereas yeast HMGR has 1054 amino acids. The

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