

Genetic Engineering of Suicide Genes to Improve Therapeutic Efficacies

A Thesis

**Submitted in Partial Fulfillment
For the award of the degree of**

Doctor of Philosophy

By

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December 2013

*Dedicated to My Beloved,
Parents and Sisters*



DECLARATION

I do hereby declare that the matter elucidated in the present thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the guidance of Prof. Siddhartha Sankar Ghosh. This work has not been submitted elsewhere for any degree, diploma etc. of any other university or institute to the best of my knowledge and belief.

December 2013.

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CERTIFICATE

This is to certify that the thesis entitled “**Genetic Engineering of Suicide Genes to Improve Therapeutic Efficacies**” being submitted by **Ms. V. Kohila** for the award of the degree of **Doctor of Philosophy** is an authentic record of the results attained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India. The results presented in this thesis have not been submitted to any other University or Institute for the award of any degree.

Prof. Siddhartha Sankar Ghosh

(Thesis Supervisor)

Acknowledgements

First of all, I would like to express my foremost gratitude to my thesis supervisor Prof. Siddhartha Sankar Ghosh for his exemplary guidance, encouragement and endless support throughout the work.

I am grateful to my doctoral committee members, Dr. Biplab Bose, Dr. Anil Mukund Limaye and Dr. Subrata K. Majumder for their time to time evaluation and critical comments. I would like to acknowledge Department of Biotechnology, Govt. of India for providing me the financial assistanceship. I am also thankful to my seniors Dr. P. Gopinath, Dr. Pallab Sanpui, Asim Da, Dr. Atul, Dr. Urmila Saxena, Mr. Saravanan and Dr. Vinod Kumar Yata for their kind assistance in my thesis work. I am obliged to our Department Technical Assistants Mr. Sharan, Mr. Nurul, Mrs. Prarthana, Mrs. Anita and Mr. Nirranjan Barah. I am thankful for their cooperation during the period of my research career.

I take this opportunity to express my heartfelt gratitude to Amit, for his cordial support, unflagging encouragement, love and care.

I revere my close association with my dearmost friends, Sumi, Puri, Priya akka, Anusha, Anupa, Sangi, Durga, Shubaash, Muthu, Saranya, Shyam anna, Vinoth...and other lovely people in IIT. Special thanks to my wonderful group members Chocks, Subhamoy, Shilpa, Upashi, Amaresh, Nidhi, Archi, Swati, Sharmi, Asif, Deepshi and trainees Manju and Preeti who bestowed me a home away from home.

My sincere thanks to my colleagues, more than that, my friends Manab, Sagarika, Ashok, Babu, Rishi, Mitun, Nandhu, Purabi Di, Pojul Da, Ankana, Sandipan, Sudeep, Thyahoo, Pallavi,.....and other friendly faces who made my working environment very healthy and cheerful.

I owe my debt of gratitude to my loving parents, sisters, grandma, Dr. Laveen and the sweetest Aditi, without them I would not have contemplated this journey. And last, but not least, my thanks to Almighty for being everything in my life.

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ABSTRACT

Gene Directed Enzyme Prodrug Therapy, also known as Suicide gene therapy (SGT) has the potential to provide specific targeting of the tumor cells thereby conquer the deteriorate effects of chemotherapy treatment of cancer. As the inefficient binding of the prodrug(s) at the active site of the key enzymes which are involved in the SGT applications, the present thesis focused mainly on the development of novel suicide gene mutants to boost up their therapeutic efficacies.

Bacterial expression vector which carries the gene codes for *E. coli* cytosine deaminase (CD) was constructed with the fused N-terminal GST tag. The single step purification of the recombinant enzyme was demonstrated and the functional activity assays with the substrates cytosine and 5-Fluorocytosine (5-FC) were examined. Further, the kinetic parameters were determined with double reciprocal plots.

Emergence of novel CD mutants having enhanced binding affinity towards the prodrug than its actual substrate cytosine was elucidated with *in silico* site-directed mutagenesis. The study offered five mutants such as F186W, F82C, S126N and R91T which displayed efficient binding towards 5-FC as compared to cytosine. The study further revealed the explicit binding of the prodrug 5-FC with the mutant S126R. The *in silico* derived mutants were constructed *in vitro*; overexpressed, purified and further examined for their catalytic efficiencies towards the substrates cytosine and 5-FC. The investigation of functional characterization of these mutants revealed the fact that the redesigned proteins S126R, F186W and F82C exhibited enhanced catalytic efficiency towards the prodrug relative to the wild-type enzyme and could act as more potent candidates for SGT. Further *in vitro* studies showed drastic decrease in cell viability with increasing concentrations of prodrug 5-FC with the mutant S126R as compared to that of wild-type CD in human cervical cancer cell line, HeLa.

In the next part, the construction of bacterial expression vector possesses the gene codes for the key enzyme *E. coli* Uracil phosphoribosyltransferase (UPRT) was demonstrated. Purification and preliminary functional assays of the recombinant enzyme with the substrates uracil and 5-Fluorouracil (5-FU) were performed. Like CD, the UPRT

enzyme too has the similar constraint in binding with the drug 5-FU than the native substrate uracil. In order to address this, an *in silico* site-directed mutagenesis study was employed on UPRT protein with the aid of homology modeling and docking studies. The docking results explored six mutants which tend to show improved binding affinity with the drug 5-FU. More significantly, the study offered the mutants M101V, I194Y and E105D which could exclusively bind only with the drug 5-FU. The ensued restructured mutants could surmount the need to administer high doses of 5-FU due to their elevated binding with the drug 5-FU.

Finally, molecular cloning, inclusion body solubilization and purification of the recombinant *E. coli* CD-UPRT fusion protein were demonstrated. Highly efficient recovery of recombinant protein from inclusion bodies was successfully achieved with an anionic detergent, sarkosyl. Affinity column purification of the N-terminal GST tagged recombinant protein was purified to near homogeneity and analyzed in Western blot with anti-GST antibody. The enzyme activity assays of the recombinant protein were performed with the substrates cytosine and the prodrug 5-FC. The kinetic parameters were revealed with the help of double reciprocal plots. The simple and efficient purification of the recombinant CD-UPRT enzyme reported in the present study could enable the enzyme to be more effective in 5-FC/CD-UPRT based suicide gene therapy applications.

In summary, the present study illustrated the construction of novel CD gene mutants and their improved kinetic prospects with their prodrug(s). The study also disclosed the novel UPRT mutants which exhibit competent binding with the drug 5-FU. Moreover, the efficient purification and activity assays of the recombinant CD-UPRT fusion protein were demonstrated.

Key words: Suicide gene therapy, CD, UPRT, site-directed mutagenesis, docking, inclusion body solubilization, sarkosyl, CD-UPRT.

ABBREVIATIONS

SGT	Suicide Gene Therapy
G-CSF	Granulocyte-colony stimulating factor
HSV	Herpes Simplex Virus
TK	Thymidine kinase
GCV	Ganciclovir
ACV	Acyclovir
CD	Cytosine deaminase
5-FC	5-Fluorocytosine
5-FU	5-Fluorouracil
UPRT	Uracil phosphoribosyltransferase
UMP	Fluorouridine monophosphate
5-FUMP	5-Fluorouridine monophosphate
PRPP	Phospho-D-ribose 1-diphosphate pentasodium salt
WT	Wild-type
PCR	Polymerase Chain Reaction
GST	Glutathione S-Transferase
IPTG	Isopropyl β -D-thiogalactopyranoside
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
CUPSAT	Cologne University Protein Stability Analysis Tool
SDM	Site directed mutagenesis
DMEM	Dulbecco's modified Eagle's medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
GTP	Guanosine 5'-triphosphate

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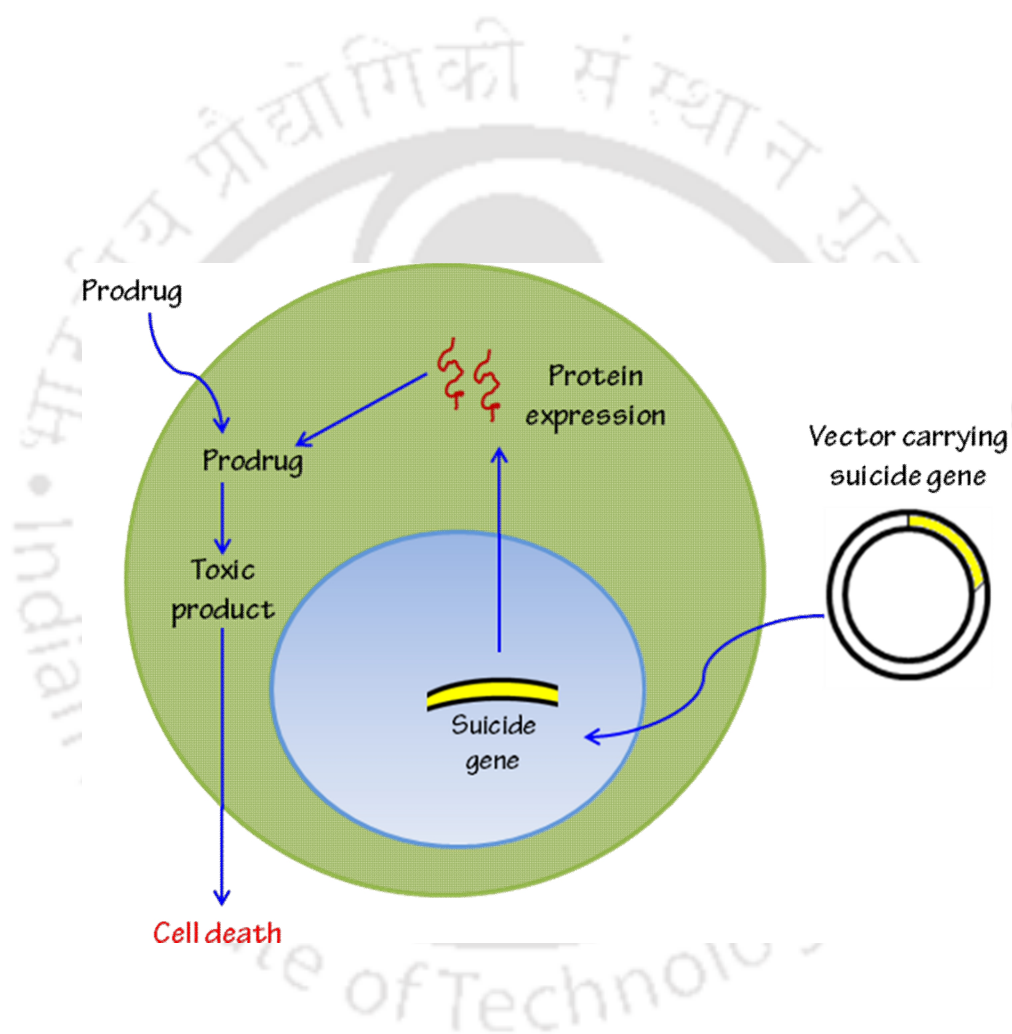
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Introduction and Literature Review

This chapter presents a brief introduction on the salient features of suicide gene therapy. The therapeutic efficacies of the suicide genes have been discussed. The chapter also gives the application of computational methodologies in the redesigning of enzymes.



Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

In recent days cancer is one of the primary causes of death and mortality. It has the characteristic features of uncontrollable cell growth and proliferation of the abnormal cells. Recent advances in cancer treatments comprise of chemotherapy, radiation, and surgery or combinations of them, which are widely used to fight against cancer. Even then, the above said therapies suffer with the major problem of nonspecific destruction of the normal cells. Along with the other cancer treatments such as surgery and radiotherapy, chemotherapy is also widely used for treating cancer but side effects including nausea (Planting et al., 1997), nephrotoxicity, prolonged myelosuppression (Lalami et al., 2012) and central nervous system disorders (Ressel et al., 2002) sometimes limits its therapeutic utility. Moreover, the emergence of drug-resistant tumor cells too hampers the effectiveness of these treatments.

In order to address the drawbacks of chemotherapy, gene therapy is an alternative technology wherein modification of a genetic component of cells has been employed for therapeutic benefits (Springer et al., 2002). Cancer gene therapy could be employed in different modes, such as transfer of a suicide gene, which converts non toxic prodrug into its toxic form (suicide gene therapy), transfer of a gene, which produces toxins (toxin gene therapy), modifying the pro-apoptotic genes, which induce apoptosis, introduction of genes code for angiogenesis inhibitors (Mulherkar et al., 2001; Scholl et al., 2003) etc. Amongst them, suicide gene therapy is more appealing because upon administration it selectively kills tumor cells and leaves the normal healthy cells unaffected. However, there are instances where prodrug competes with a natural substrate of the suicide enzyme for binding to its active site (Mahan et al., 2004). Therefore, there is enough scope to redesign such suicide genes to obtain therapeutic enzymes with altered structural motifs to render efficient binding with prodrugs. Such mutants may exhibit higher therapeutic potential when tested *in vitro*.

1.2. Recombinant DNA technology approaches for therapeutic proteins

Recombinant DNA technology has emerged as an efficient tool in last few decades where cloning, expression and purification of proteins are now possible for their therapeutic applications. Recombinant therapeutic proteins can be administered exogenously to target cells. This has been ushered in by the approval of insulin for the treatment of diabetes mellitus. A number of such therapeutic proteins have now approved for clinical testing (Leader et al., 2008). Recombinant proteins offer several advantages over small molecule drugs; they can perform complex functions, elicit reduced or negligible immune responses without interfering with normal biological functions and are highly specific in their targeting. Examples include blood coagulation factors, granulocyte- colony stimulating factor (G-CSF) (Leader et al., 2008), pancreatic lipases (Fieker et al., 2011) and monoclonal antibodies against Vascular Endothelial Growth Factor Receptor (Kabbinavar et al., 2003). Moreover, increased therapeutic efficacy of a recombinant $\text{I}\kappa\text{B}\alpha$ encapsulated in hydrogel nanocarrier has been demonstrated recently in HeLa cells (Banerjee et al., 2013). In this context, there are scopes for further improvement of therapeutic proteins by redesigning their structural moieties using recombinant DNA technology.

1.3. Genetic engineering approach to redesign gene(s) to improve functions of recombinant enzymes

The recombinant proteins that are mostly used in therapy require high substrate specificity, selectivity and thermal stability, which usually are not the native features of any natural enzymes. Hence, genetic engineering of the recombinant enzymes plays a crucial role in addressing these issues, where introduction of point mutations at specific regions might augment the functions of the natural enzymes. Salman et al., (2010) employed site-directed mutagenesis to investigate the effects of phosphorylation of Ser 348 on activity and cellular localization of human SULT2B1b, a major isoform of human cytosolic sulfotransferases2 (SULT2) family. They mutated Ser 348 to Asp and evaluated the functional consequences of the mutant SULT2B1b-S348D. The mutant has shown a ten-fold higher specific activity for dehydroepiandrosterone (DHEA) sulfation than the wild-type enzyme. Moreover, the mutant SULT2B1b-S348D displayed 60% greater thermal stability than wild-type.

Finally, the observations from the study concluded that the redesigning of the SULT2B1b enzyme based on mutagenesis studies could open up a new platform to improve the functional characterization of the enzyme.

Takahashi et al., (2012) identified and characterized novel homologues of RPE65 (isomerohydrolase in retinal pigment epithelium), RPE65c and 13-cis isomerohydrolase (13cIMH). They replaced candidate residues by site-directed mutagenesis in RPE65c and 13cIMH to study the impacts of the key residues, which determine the isomerization product specificity of RPE65. Significant alterations in the product specificities were observed with the point mutations at residues Tyr58, Phe103, and Leu133 in RPE65c. The study illustrated that the results attained could aid to understand the core molecular mechanisms of the isomerization reaction catalyzed by RPE65.

Singh et al., (2012) employed site-directed mutagenesis to reveal the hidden oxidized LDL (ox-LDL) - binding site of C-reactive protein (CRP) and to construct a CRP mutant, which could bind to ox-LDL at physiological pH. They replaced the residue Glu 42 to Gln (E42Q) and demonstrated the significant binding activity of the mutant for ox-LDL at physiological pH. Moreover, the mutant E42Q exhibited higher binding activity with ox-LDL at any given pH conditions. Conclusively, the study stated that the mutant E42Q CRP could be used as a tool to investigate the functions of CRP in inflammatory diseases where the application of wild-type CRP is limited due to requirement of acidic pH condition to exert the optimum catalytic activity.

Cheriyian et al., (2012) implemented structure-based mutagenesis combined with library screening of mutant enzymes to explore the key residues of substrate selectivity and alter the specificity of *E. coli* 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase. They found that mutations of T161S and S184L worked together to enhance the substrate specificity of the enzyme to catalyze the retro-aldol cleavage of (4S)-2-keto-4-hydroxy-4-(2'-pyridyl) butyrate (S-KHPB). The study demonstrated that the mutant proteins exhibited superior catalytic efficiencies towards the native substrate, KDPG and with the 2-keto-4-hydroxy-octonoate than the wild-type enzyme. As a result, the study suggested that targeting the active site mutations could offer an immense improvement in substrate selectivity and other characteristic features of the enzymes.

Antibody-directed enzyme prodrug therapy (ADEPT) exhibits an advantageous feature of selectively targeting cancer cells by conjugation of the prodrug-activating enzyme with tumor-specific antibody. Since most of these enzymes are not from the mammalian origin, they are likely to develop an immunoresponse, which limits their applications. In order to address this problem, Smith et al. (1997) employed the combined molecular modeling and site-directed mutagenesis approaches to develop a mutant human enzyme which catalyzes a reaction that is not generally carried out in the human body. They designed and synthesized metabolically stable prodrugs of methotrexate (MTX) and also produced a mutant of human carboxypeptidase A1, replaced at position 268 from threonine to a glycine (hCPA1-T268G). The mutant enzyme has the potential to use the stable prodrugs, which are not substrates for the wild-type enzyme. The observed superior kinetic aspects as well as the *in vitro* cytotoxic efficiencies of the hCPA1-T268G mutant enzyme relative to the wild-type strongly supported that the mutated human enzyme with a single amino acid change could act as an incredible candidate for ADEPT.

1.4. Fundamentals of Suicide Gene Therapy

Suicide gene therapy (SGT) is a promising approach for the treatment of cancer in contrast to the conventional chemotherapy as chemotherapy suffers from insufficient therapeutic index, adverse side effects and lack of specificity toward cancer cells (Montgomery et al., 1997; Watson et al., 1998; Monsuez et al., 2010; Joensuu et al., 2011; Coolbrandt et al., 2011). The technique of SGT involve transfer of a desired gene to cancer cells, where the gene either encodes an enzyme, which activates a nontoxic prodrug to toxic metabolites, leading to cell death, or the gene itself produces toxins, which kills the tumor cells (Greco et al., 2001). The former method is termed as gene-directed enzyme prodrug therapy (GDEPT), while the latter is called toxin gene therapy (Springer et al., 2000). The schematic representation of SGT technique is illustrated in **Figure 1.1**.

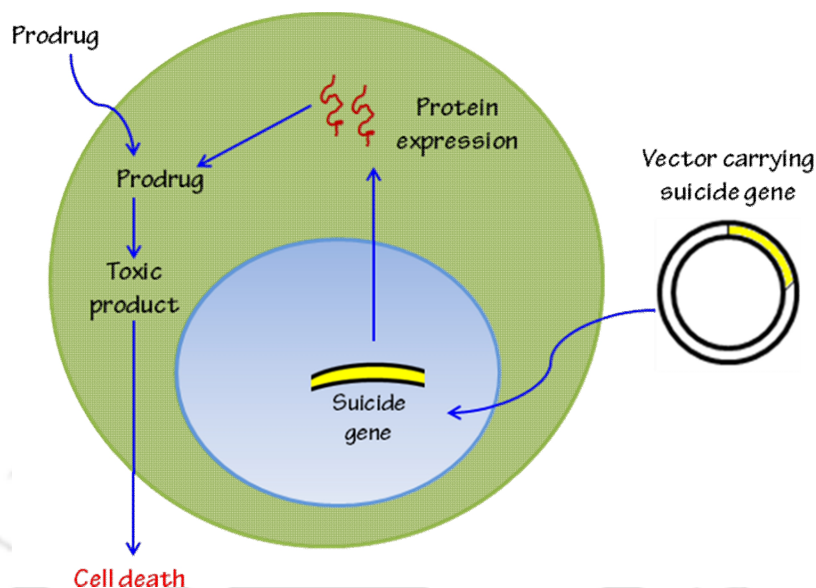


Figure 1.1. Schematic representation of GDEPT / SGT.

1.4.1. Important Suicide Gene Therapy Vectors

Gene therapy vectors are the key tools for gene delivery to achieve efficient, safe and targeted gene modifications. The success of SGT depends on the effective gene delivery vectors to exert the cytotoxic effects in the cancer cells. Therefore, there is a need for competent gene delivery systems, which include viral and non-viral vectors. In general, these gene delivery vectors should be capable to overcome the extracellular barriers such as avoiding particle clearance mechanisms, targeting specific cells or tissues and protecting the nucleic acid from degradation. Subsequently, the cellular barriers, such as cellular uptake, endosomal escape, nuclear membrane entry and gene release should also be taken into consideration (Gascon et al., 2013).

Viral based vector systems using adenoviruses, adeno-associated viruses, retroviruses, lentiviruses etc. are mostly used to deliver the gene into cancer cells owing to their salient features such as long-term gene expression, integration into host genome, wide tissue tropism etc. (Palmer et al., 2006). Viral vectors are the most effective as compared to other delivery systems, but their application is limited by their immunogenicity, oncogenicity and the small size of the gene of interest (Gascon et al., 2013).

1.4.2. Prospects of Non-Viral Vectors

Non-viral vectors emerged to circumvent some of the problems occurring with viral vectors such as endogenous virus recombination, oncogenic effects and unexpected immune response. Non-viral vector gene delivery includes gene transfection into target cells using plasmid DNA, which is profoundly a simple and safe approach that could be further improved by combining several physical techniques, such as, electroporation, gene gun, ultrasound, hydrodynamic pressure and biocompatible nanoparticles (Niidome et al., 2002). Furthermore, non-viral vectors possess significant features in terms of simplicity of use, ease of large-scale production and lack of specific immune response. Though viral vectors dominate the clinical trials, the improvement of efficacy of non-viral vectors has lead to an increased number of products entering into clinical trials.

1.4.3. Major Suicide Gene Therapy Approaches

1.4.3.1. Herpes Simplex Virus-Thymidine Kinase/Ganciclovir mediated SGT

Among the currently available GDEPT systems, the Herpes Simplex Virus-Thymidine Kinase/ganciclovir method is intensively studied. Thymidine kinase (TK) from Herpes simplex virus (HSV) is highly efficient in converting the nucleoside analogue ganciclovir (GCV) and related agents which are poor substrates for the mammalian nucleoside monophosphate kinase, to monophosphate. Phosphorylated GCV will be converted into a number of toxic metabolites, primarily triphosphate by subsequent reactions catalyzed by cellular enzymes. GCV-triphosphate will be incorporated into replicating DNA strands during cell division; thereby acting as a terminator of DNA polymerization reaction and leading to cell death (Azatian et al., 2009; Wang et al., 2004). Furthermore, the principal advantage of this SGT is bystander effect, by which non-transformed malignant cell death occurs due to the export of the produced toxic metabolites from the transfected cells via gap junction protein, such as connexin (Bi et al., 1993; Mesnil et al., 1996; Asklund et al., 2003; Misumi et al., 2003; Soubrane et al., 1996; Matuskova et al., 2010). These advantageous characteristics of HSV-TK/GCV combination make it suitable for the eradication of tumor cells which used to divide rapidly.

Soubrane et al., (1996) examined the therapeutic efficacy of the HSV-TK/GCV *in vivo* in murine malignant melanoma. After virus-free mediated transfer of HSV-TK, upon administration of GCV, the reduction in tumor weight (40-50%) was observed in treated animals as compared to the control groups.

HSV-TK/GCV therapy exhibits more cytotoxicity towards cancer cells due to the bystander effect wherein the neighboring cells near to the transfected cells also get killed in the presence of GCV. Since the triphosphorylated GCV cannot diffuse across the cell membrane owing to its larger size, the HSV-TK/GCV system depends on the gap junction intercellular communication (GJIC) which mediated by gap junction proteins, connexins to exert the cytotoxicity (Portsmouth et al., 2007). Mesnil et al., (1996) demonstrated the gap junction protein (eg. connexin-43) mediated bystander effect of HSV-TK/GCV with evidence from *in vitro* studies. They examined the contribution of gap junction proteins by co-culturing the HeLa/HSV-TK cells with nontransfected control cells and also with connexin-43 (Cx-43) gene transfected cells. More cell death was observed in presence of the Cx-43 transfected cells. Thus, the results give strong evidence that the bystander effect seen in HSV-TK gene therapy may be due to connexin-mediated GJIC.

Augmentation of Thymidine kinase: Apart from the salient features of bystander effect and greater cytotoxicity, the deteriorate effects of the prodrug GCV play a vital role in limiting this SGT application. Novel HSV-TK mutants with increased specificity for the prodrug GCV could enhance tumor cell killing and reduce prodrug-mediated toxicity as well. Hence, Kokoris et al., (1999) generated a large library of thymidine kinase mutants and screened their ability to enhance *in vitro* cell sensitivity to the prodrugs, GCV and acyclovir (ACV), a guanosine analog used as an antiherpetic drug. Amongst them, TK mutant 30 which contains six amino acid substitutions at or near the active site displayed nanomolar IC₅₀ values with GCV and ACV as compared to the wild-type TK in *in vitro* rat c6 cell prodrug sensitivity assays. Moreover, *in vivo* studies carried out with the mutant showed that the growth of mutant 30 expressing tumors is restricted by GCV at a dose at least 10-fold lower when compared with the growth of wild-type TK expressing tumors.

Furthermore, in 2002, Kokoris et al., identified seven TK variants containing multiple amino acid substitutions on the basis of activity towards GCV and ACV

based on negative selection in *E. coli* from a random mutagenesis library. Of these, three mutants SR11, SR26 and SR39 were purified and their kinetic parameters towards thymidine, GCV and ACV were determined. Mutant SR39 displayed a 14-fold decreased K_m value with the substrate GCV when compared to the wild-type enzyme. Remarkably, the mutant SR26 displayed 124-fold decreased K_m value towards ACV. Thus the results suggested that the novel HSV-TK mutants could exert their cytotoxicity even with lower concentrations of the prodrug (s) to provide specific emphasis on cancer gene therapy applications.

Another major impact of the HSV-TK is to understand the functional and mechanistic properties of this multi-substrate enzyme to define its role as a major pharmacological target in cancer gene therapy. HSV-TK has an inherent limitation of the activity of >70-fold difference in the K_m s for phosphorylation of the native substrate thymidine over the prodrug GCV. In order to overcome this, Mercer et al., (2002) have generated 16 site-specific mutants which were concentrated at conserved residues involved in nucleoside base binding, Gln125 and near sites 3 and 4 involved in catalysis and substrate binding to engineer an HSV-TK isoform that is specific for GCV as the preferred substrate. The preferred substrate binding affinities of each mutant enzyme were compared with wild-type HSV-1 TK. Out of 16 different HSV-1 TK mutants generated, Q7530 TK and N7530 TK have been characterized as GCV-specific kinases. But, more significantly the former one possessed a lower K_m for GCV than that of thymidine. Further the expression studies with the mutant Q7530 TK in tumor cells showed comparable metabolism and improved sensitivity to GCV over wild-type HSV-1 TK.

1.4.3.2. 5-Fluorocytosine/Cytosine deaminase mediated SGT

Cytosine deaminase (CD) of the bacterial origin (EC 3.5.4.1), a key enzyme involved in the pyrimidine salvage pathway catalyzes the deamination of cytosine to uracil. The enzyme also has the tendency to convert the nontoxic prodrug 5-Fluorocytosine (5-FC) to toxic drug 5-Fluorouracil (5-FU) which trigger cancer cell death (Kaliberov et al., 2007).

Ireton et al., (2001) have solved the X-ray crystal structure of *E. coli* CD in the presence and absence of a bound mechanism-based inhibitor. The crystal structure of

bacterial cytosine deaminase revealed the active site residues (310-320 amino acids), which undergo a conformational change upon substrate binding and making a number of contacts to the pyrimidine ring. The resolved structure of CD led to the redesigning of the enzyme to improve its catalytic efficiency towards the prodrug 5-FC.

Augmentation of Cytosine deaminase: Despite of the advantageous features of SGT, the insufficient binding of the prodrugs at the active site of the enzyme still exists as a major obstacle. 5-FC/CD based system can prevail over the requisition of gap junction proteins to exert the bystander effect as the toxic product 5-Fluorouracil (5-FU) can diffuse freely through the plasma membrane owing to its small size (Kuriyama et al., 1995). However, the binding affinity of the prodrug 5-FC at the enzyme active site is very poor as compared with the natural substrate cytosine (Kievit et al., 1999), which makes the system inefficient.

Mahan et al., (2004) carried out the random and alanine scanning mutagenesis studies on CD and revealed the fact that the mutant D314A demonstrated a dramatic decreased activity towards cytosine (17-fold) as well as a slight increase in activity towards 5-FC (2-fold), thereby acting as a superior suicide gene in the prospect of gene therapy applications.

Kaliberov et al., (2007) developed an adenoviral vector which carries a cytosine deaminase mutant D314A (AdbCD-D314A), having higher binding affinity for the prodrug 5-FC than cytosine as compared to the wild-type CD. The study further demonstrated that AdbCD-D314A infection resulted in increased prodrug mediated cell killing as compared to wild-type bCD (AdbCDwt). Moreover, *in vitro* studies on glioma cells showed a significant increase in cytotoxicity following AdbCD-D314A administration and radiation treatment as compared to AdbCDwt. *In vivo* studies showed significant inhibition of tumor growth of glioma xenografts by the combination of AdbCD-D314A/5-FC with ionizing radiation and compared with either agent alone, and with AdbCDwt/5-FC plus radiation. Hence, the results indicated that combined treatment with this novel mutant enzyme and radiation could provide an attractive platform for potential cancer therapeutics.

Fuchita et al., (2009) described an expanded random mutagenesis and selection experiment that yielded novel CD enzyme variants, which showed significantly improved sensitization towards the prodrug. Of these, three mutant

constructs (1246, D314E/F316L/D317G; 1525, V152A/F316C/D317G; 1779, V315L/F316V/D317G) were characterized for kinetic analyses, *in vitro* assays for 5-FC sensitization, bystander effects and formation of 5-FU metabolites. All three enzyme variants displayed 18- to 19-fold increased substrate preference towards 5-FC, a remarkable reduction in IC₅₀ values and enhanced bystander effect as compared to wild-type CD. *In vivo* studies with xenograft tumor model showed that the enzyme mutant 1525 was shown to prevent tumor growth at much less concentrations of 5-FC when compared with tumor cells expressing wild-type CD. In addition to that, the crystallographic analyses of the constructs further demonstrated the reason behind the improved activity of these mutants towards 5-FC.

1.4.3.3. Potential therapeutic applications of Mutant enzymes

Most of the enzymes have been mutated to show comparatively better or new potential therapeutic applications. Mutation studies carried out on CD revealed that the resultant CD variant (1525) displayed superior 5-FC kinetics, cell killing, bystander effect and tumor growth restriction activities. Moreover, the study showed that the mutant 1525 may be used in a variety of different applications including for restenosis, noninvasive tumor imaging, and in negative selection systems (Fuchita et al., 2009).

1.4.3.4. 5-FU/Uracil phosphoribosyltransferases mediated SGT

Uracil phosphoribosyltransferases (UPRT) (EC 2.4.2.9) enzyme belonging to the class I phosphoribosyltransferases family and has been purified and characterized from various sources such as *T. gondii* (Carter et al., 1997), Baker's yeast (Natalini et al., 1979), *Acholeplasma laidlawii* (Mcivor et al., 1983), *Sulfolobus shibatae* (Linde et al., 1996), *Candida albicans* (Alloush et al., 1994) etc. The enzyme catalyzes the formation of UMP and 5-Fluorouridinemonophosphate (5-FUMP) from the substrates uracil and 5-FU respectively. Among the known UPRT enzymes, *T. gondii* UPRT is deeply studied and characterized. The multiple sequence alignment of all the known UPRT enzymes displayed the presence of four highly conserved regions, such as Region I (residues 76-80, involved in PRPP binding), Region II (flexible loop residues 103-110), Region III (residues 130-140, PRPP binding motif) and Region IV

(hood residues 192-208, involved in pyrimidine substrate binding). The crystallographic structure of *T. gondii* UPRT revealed that the conserved residues Arg 137, Met 166, Ala 168, Tyr 228, Ileu 229, Asp 235 and Phe 236 are playing a vital role in the substrate binding and stacking interactions; thereby participating in the catalytic activity of the enzyme (Schumacher et al., 1998).

Lundegaard et al., (1999) observed the subtle deviations present over the consensus PRPP binding site that all uracil phosphoribosyltransferases have a proline residue at a position where other phosphoribosyltransferases and the PRPP synthases have aspartate. They changed the proline residue to an aspartate to investigate the role of this unusual proline (Pro 131 in the *E. coli* UPRT) for catalytic activity. The kinetic prospects of the wild-type and P131D mutant enzyme concluded that the proline present in the PRPP binding site of UPRT is critical for binding of uracil to the enzyme-PRPP complex and for the catalytic rate.

To examine the increased sensitivity of cancer cells towards 5-FU, Kawamura et al., (2000) retrovirally transduced a murine colon carcinoma cell line, with the UPRT gene and tested *in vivo* antitumoral effect of 5-FU in syngeneic immunocompetent mice. The results have concluded that expression of the UPRT gene in tumor cells followed by 5-FU administration is a promising strategy for cancer gene therapy, but potentiation of the bystander effect is an essential requirement for its therapeutic application.

Yata et al., (2012) carried out the molecular docking studies on *E. coli* UPRT to elucidate the key residues interacting with the substrates uracil and 5-FU. The study showed that the ligand uracil interacted with Arg 103, Ala 134, Tyr 192, Ile 193, Gly 198, Asp 199, Ala 200, and Met 132 residues of UPRT. Moreover, the study revealed the major interactions between the ligand 5-FU with Tyr 192, Ile 193, Gly 196, Leu 197, Gly 198, Asp 199, and Met 132 residues of UPRT. Altogether these findings could open up the rational designing of the UPRT enzyme.

Augmentation of Uracil phosphoribosyltransferases: 5-FU/UPRT mediated SGT application also suffers with the obstruction of weak binding affinity of the prodrug 5-FU at the active site of the enzyme, which probably arises from the relatively fixed stereochemical constraints of the substrate-binding pocket (Schumacher et al., 1998).

Since 5-FU can passively diffuse across cell membranes, the transfer of 5-FU does not depend on gap junctions like the HSV-TK system. However, UPRT catalyzes the conversion of 5-FU to 5-FUMP and because 5-FUMP cannot passively diffuse cell membrane like 5-FU, the bystander effect becomes dependent on gap junctions. To overcome the diminished bystander effect and the poor conversion rate of 5-FU to 5-FUMP, from a regio-specific mutagenesis study was conducted. Three UPRT enzyme variant constructs such as 3662 (three substitutions at I194N, P195S, and G196S), 3802 (three substitutions at I194V, D202Y and F205L) and 4312 (six substitutions at Y192S, I194V, D202Q, K203N, F205L, and T207I) displaying enhanced activity towards 5-FU were identified. The kinetic studies of these mutants suggested that the mutant 3662 displayed very poor activity (130-fold decrease in k_{cat}/K_m) when 5-FU was used as substrate. The two other mutants, 3802 and 4312, demonstrated a modest improvement (1.7-and 1.3-fold increases in k_{cat}/K_m) in activity towards 5-FU when compared to wild-type enzyme (Ardiani, Dissertation, 2009).

1.4.3.5. 5-FC/CD-UPRT mediated SGT

The major problems associated with gene therapy approach are the low level of gene transfer and the existence of certain 5-FC resistant cancer cell types (Tiraby et al., 1998). A hybrid cytosine deaminase- Uracil phosphoribosyltransferase gene (CD-UPRT) was developed by co-expressing the CD gene and UPRT gene to render the cancer cells more sensitive to 5-FC in order to facilitate the bystander effect (Tiraby et al., 1998; Gopinath et al., 2008; Xing et al., 2009). In this case, prodrug 5-FC will be metabolized into 5-FU by the CD enzyme and the 5-FU formed will get anabolized into its further toxic phosphorylated metabolites with the fused UPRT enzyme which further trigger the apoptosis of cancer cells. The sequential steps involved in the mechanism of 5-FC mediated CD-UPRT cell killing is illustrated in **Figure 1.2**.

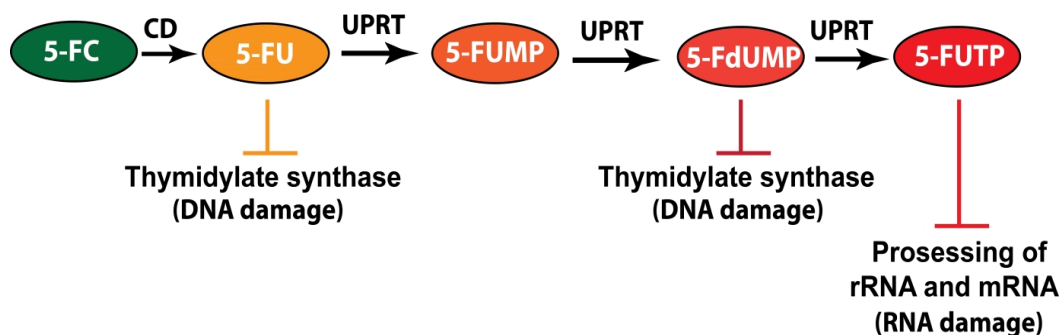


Figure 1.2. Schematic showing the action mechanism of the bifunctional CD-UPRT enzyme leading to the conversion of the non toxic prodrug 5-FC to 5-FU and other toxic metabolites.

The therapeutic implications of the bifunctional CD-UPRT based suicide gene therapy have been well demonstrated previously in numerous studies. Chung-Faye et al., (2001) developed a replication-deficient adenovirus containing a bifunctional fusion gene, CD-UPRT (AdCD-UPRT) to enhance the efficacy of 5-FC/CD system. A strong bystander effect was observed *in vitro*, wherein about 70% of tumor cells were killed by 5-FC when only 10% of cells expressed the hybrid CD-UPRT gene. Moreover, *in vivo* studies performed on athymic mice with colon cancer with intratumoral AdCD-UPRT treatment showed significant reduction in tumor growth rates compared to untreated controls, while no change was observed in mice which treated solely with AdCD/5-FC.

In 2008, Gopinath et al. investigated the mechanism of cell death by the bifunctional 5-FC/CD-UPRT suicide system in both cancer and non-cancer cells and determined the optimum 5-FC concentration led the cancer cells to undergo apoptosis *in vitro*. Furthermore, the 5-FC/CD-UPRT mediated apoptosis was potentiated with addition of the conventional anticancer agent curcumin and the synergistic induction of apoptotic pathway in the combination treatment was also demonstrated.

Gopinath et al., (2008) have used green fluorescent protein (GFP) as a noninvasive probe to monitor the therapeutic effect of CD-UPRT with the BHK21 cell line expressing both CD-UPRT and GFP from two separate transcripts. Further, the study demonstrated for the first time that the molecular level changes occurred in the pro-apoptotic and anti-apoptotic genes upon 5-FC/CD-UPRT mediated cell death.

Tanaka et al., (2009) demonstrated that the combined CD and UPRT genes sensitizes HeLa cells more towards 5-FC and displays heightened bystander effect which was not mediated by gap junction proteins. However, the partial contribution of gap junction proteins was observed when very low percentages of CD-UPRT cells were present to exert the cytotoxic effects against cancer cells.

Xing et al., (2009) observed that CD-UPRT expressing cells were sensitized more towards 5-FC and 5-FU than CD-expressing cells alone. Further, enhanced radiosensitizing effect of 5-FC was achieved with CD-UPRT expression relative to that achieved by CD expression alone. Altogether, the results suggested that CD-UPRT/5-FC strategy could be more effective than CD/5-FC in the prospect of cancer therapeutics.

Augmentation of Cytosine deaminase - Uracil phosphoribosyl transferase:

Previous studies demonstrated that the fusion of CD with UPRT (CD-UPRT) along with 5-FC exhibits heightened tumor killing activity than CD alone. Therefore, to further enhance the cytotoxic effects, Jhonson et al., (2011) incorporated the previously identified CD mutant 1525 along with the UPRT (1525/UPRT) and evaluated its tumor cell killing and bystander effects *in vitro*. The study revealed that 1525/UPRT showed only a slight increase in relative specificity towards the prodrug 5-FC and exhibited no improvement in bystander killing effect.

Moreover, site-directed mutagenesis was employed to introduce the UPRT mutants 3662, 3802, and 4312 into the 1525/UPRT fusion construct. *In vitro* studies with rat C6 glioma cells showed no improvement in tumor killing for 5FC-mediated toxicity. Previous kinetic studies suggested that the UPRT mutant 3662 displayed weak activity towards 5-FU. A higher IC₅₀ value at concentration of 5mM 5-FC was observed for tumor cells expressing fusion construct 1525/3662. Though the kinetic analyses suggested that the mutants 3802 and 4312 appeared to have a moderate shift in substrate preferences towards 5-FU, *in vitro* studies showed no improvement in 5-FC-mediated toxicity when the tumor cells transfected with 1525/3802 and 1525/4312. Towards the end, further crystallographic analyses of these mutant constructs and rational redesigning of the fused CD-UPRT enzyme could provide the superior candidates for potential SGT applications (Ardiani, Dissertation, 2009).

1.5. Expression of Suicide Genes

Suicide genes were cloned in bacterial systems for expression studies and purified to near homogeneity to analyze their kinetic parameters such as K_m , V_{max} and catalytic efficiency, etc. (Mahan et al., 2004). Further, in order to examine the cytotoxic effects, suicide genes were initially cloned into mammalian vector systems and later transfected into cancer cell lines to perform comparative studies (Fuchita et al., 2009).

1.6. 5-FU, as a chemotherapeutic drug

The antimetabolite drug 5-FU is used for more than two decades in the chemotherapy treatment of malignancies like head and neck cancer (Budach et al., 2006; Posner et al., 2007), colorectal cancer (Nitti et al., 1997; Wiebke et al., 2003; Hsu et al., 2011; Xie et al., 2012), metastatic oesophageal cancer (Bleiberg et al., 1997; Taieb et al., 2002), gastric adenocarcinoma (Zhang et al., 2011), etc. 5-FU used to act alone or synergistically in combination with other chemotherapeutic agents such as cisplatin (Hitt et al., 2002; Budach et al., 2006), docetaxel (Posner et al., 2007), oxaliplatin (Zhang et al., 2011), leucovorin (Taieb et al., 2002; Hsu et al., 2011), Nedaplatin (Uchida et al., 1998). 5-FU exhibits its cytotoxic effects primarily on the S-phase of the cancer cells by irreversibly inhibiting the thymidylate synthase enzyme; thereby arresting the cells in premitosis stage (Wiebke et al., 2003).

Adverse effects: Though the drug 5-FU acts as an effective chemotherapeutic drug, the side effects exhibited by the drug still remains a critical concern. A phase II study carried out on the advanced squamous cell oesophageal cancer demonstrated that the combination of 5-FU/Cisplatin (CDDP) was possibly more active than CDDP alone (Bleiberg et al., 1997). However, the adverse effects shown by the combination of drugs drawn a conclusion of no standard treatment can be suggested for the patients with advanced squamous cell oesophageal carcinoma. Even if the central nervous system is less prone to the toxic effects arise from chemotherapeutic drugs due to their less permeability across the blood brain barrier, there are reports available to indicate the 5-FU induced neurologic complications (Soussain et al., 2009). One of the case study reported that the patient developed acute superior mesenteric ischaemia less than 48 hours after receiving the chemotherapy regimens 5-FU, cisplatin and vincristine (Allerton, 1996). The study also stated that 5-FU is probably responsible

for the event either alone or in synergy with cisplatin rather than vincristine based on the temporal relationship from the time of administration of the individual drugs to the onset of symptoms. Taken together, the studies insisting the requisite of alternative therapies to surmount the harmful effects of the drug 5-FU.

1.7. Applications of Bioinformatics tools in the genetic engineering of enzymes

Computational approaches such as homology modeling and molecular docking are more implemented in recent days to alter the characteristic features, such as thermal stability, catalytic activity, etc. of the enzymes.

Yoon et al., (2003) applied the molecular docking to develop the prodrug analogues of irinotecan (CPT-11), a well known anti-cancer compound to increase its therapeutic index for enzyme/prodrug gene therapy applications.

Pavlova et al., (2009) identified the key residues in access tunnels which connect the buried active site with bulk solvent of the enzyme haloalkane dehalogenase from *Rhodococcus rhodochrous*. Further, they employed site-directed mutagenesis which generated the mutants with up to 32-fold higher activity than wild-type enzyme in degrading the toxic anthropogenic compound 1,2,3-trichloropropane (TCP).

Docking studies have also been employed to identify the potential anti-cancer targets. Grinter et al., (2011) followed the inverse docking method to identify potential direct targets of PRIMA-1 (2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one), known for its ability to restore mutant p53's tumor suppressor function, thereby leading to apoptosis in several kinds of tumor cells. Docking studies revealed the oxidosqualene cyclase (OSC) enzyme as a possible anti-cancer target. The experimental studies further shown that the PRIMA-1 and Ro 48-8071 (OSC inhibitor), significantly reduced the viability of breast cancer cells with respect to normal mammary cells.

1.8. Key Areas and Scopes

Depending on the literature review carried out in the field of therapeutic implications of SGT, the key areas were perceived and are given below:

- ❖ Implications of computational approaches for the development of novel suicide gene mutants.
- ❖ Exploring the functional characteristics of the mutant proteins.
- ❖ Deciphering the structural alterations that lead to the improved therapeutics of the mutant proteins.
- ❖ Investigation of high therapeutic efficacies of the mutants.

1.9. The Present Work

1.9.1. Objectives:

- ❖ To perform *in silico* mutagenesis to explore novel *E. coli* cytosine demainase (CD) mutants which could have greater binding affinity with the prodrug 5-FC as compared to the wild-type enzyme.
- ❖ To generate novel CD mutants by *in vitro* site-directed mutagenesis and further investigate their elevated relative specificities with the prodrug.
- ❖ To construct a mammalian expression vector with the wild-type and CD variant to investigate their comparative heightened *in vitro* cytotoxic effects.
- ❖ To develop novel *E. coli* uracil phosphoribosyltransferase (UPRT) mutants via *in silico* mutagenesis to improve the 5-FU/UPRT based cancer therapeutic applications.
- ❖ To construct a bacterial expression vector with the fusion gene CD-UPRT to reveal the kinetic parameters with its substrates.

1.9.2. Significance and Salient Features of the Present Thesis Work

The significance and salient features of the present study are given in below.

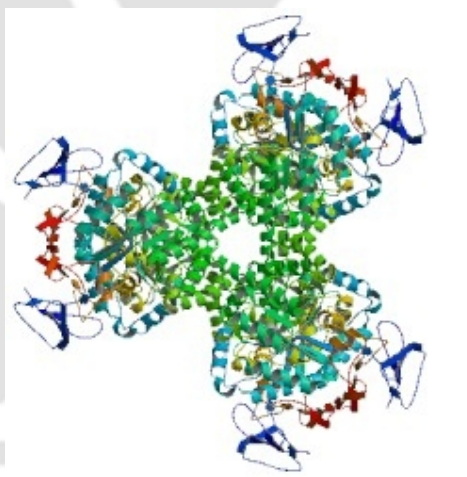
- ❖ Gene transfection into target cells using plasmid DNA is a simple and safe approach. The current thesis is focused on development of plasmid based suicide genes and their mutants for easy expression, purification and bioassay.
- ❖ The detailed literature on the therapeutic impacts of suicide genes, inefficient binding of the prodrug(s), adverse effects of the drug 5-FU, computational

methodologies followed to improve the characteristic features of the enzymes have been assessed.

- ❖ Bacterial expression vector carrying the suicide gene CD was constructed and the functional assays over the substrates were performed to find out the kinetic parameters and the catalytic efficiencies of the recombinant enzyme.
- ❖ CD mutants were found based on the *in silico* biology.
- ❖ Novel CD mutants were created by site-directed mutagenesis.
- ❖ The eminent relative specificities of the CD mutants with the prodrug 5-FC as compared with the wild-type enzyme were experimentally validated.
- ❖ Mammalian expression vectors carrying the wild-type and CD variant S126R were constructed and enhanced *in vitro* cytotoxic effects of the mutant S126R as compared to the wild-type enzyme have been documented.
- ❖ Bacterial expression vector carrying the UPRT gene was constructed and the functional activity assays towards the substrates have been demonstrated.
- ❖ *In silico* approach on UPRT was carried out to define novel UPRT mutants with greater binding affinity with the drug 5-FU.
- ❖ Bacterial expression vector construct with the hybrid CD-UPRT gene has been shown.
- ❖ An efficient sarkosyl solubilization of the recombinant CD-UPRT fusion enzyme was demonstrated.
- ❖ The functional activities of the recombinant CD-UPRT enzyme were assessed.

Cloning, Expression, Purification and Functional Activity assays of *E. coli* Cytosine Deaminase

The chapter demonstrates the cloning, expression and purification of E. coli cytosine deaminase. In addition to that, the functional activity assays of the enzyme towards its substrates and the kinetic values determined were also presented.



Hexameric assembly of *E. coli* CD (PDB code: 1K6W)

Chapter 2

CLONING, EXPRESSION, PURIFICATION AND FUNCTIONAL ACTIVITY ASSAYS OF *E. COLI* CYTOSINE DEAMINASE

2.1. Introduction

Cytosine deaminase (CD) catalyzes the deamination of cytosine to uracil and ammonia, thereby playing an important role in the pyrimidine salvage pathway. *E. coli* CD categorized to the amidohydrolase superfamily by evolutionary constraints (Holm et al., 1997) requires a single divalent metal ion Fe^{2+} for maximum activity (Porter et al., 1993). The CD enzyme also has the potential to deaminate the nontoxic prodrug 5-FC to 5-FU. The 5-FU itself and after its subsequent anabolism to 5-FdUMP by cellular enzymes restricts DNA replication by inhibiting the thymidylate synthase enzyme, causing cell death (Huber et al., 1993; Kievit et al., 1999). CD is encoded intrinsically by bacteria and yeasts, but mammalian cells do not have the gene codes for this enzyme (Nishiyama et al., 1985) which enables the non-transfected normal cells to escape from cell death after treatment with the prodrug.

The increased sensitization of many of the cancer cell types towards the prodrug 5-FC as a consequence of the expression of CD enzyme was well demonstrated in several studies. For example, WiDr, a human colorectal carcinoma cell line which was transfected with CD gene (WiDr/CD), the growth rate of the WiDr cells was not altered due to the expression of CD gene. But expression of CD enhanced the sensitivity of the cells to the prodrug 5-FC by decreasing the inhibitory concentration for the prodrug in case of WiDr/CD cells as compared to the parental WiDr cells. The increased sensitivity of WiDr/CD cells to 5-FC was resulted from the efficient conversion of 5-FC to 5-FU and subsequent 5-FU anabolites which was mediated by the cytosine deaminase (Huber et al., 1993).

2.2. Outline of the Research Work

- 1) The gene encoding *E. coli* cytosine deaminase was cloned in bacterial expression vector pGEX-4T2 along with N-terminal GST tag.
- 2) The gene was overexpressed in *E. coli* BL21 (DE3) host system.
- 3) The protein was affinity purified in a single step with the help of fused GST tag.
- 4) Western blot was performed with the anti-GST antibody to confirm the GST tagged protein of interest.
- 5) The CD activity assays were carried out with the substrates cytosine and 5-FC to determine the kinetic parameters K_m , V_{max} and catalytic efficiencies (K_{cat}/K_m) towards the substrates.

2.3. Experimental Section

2.3.1. Materials

pORF codA::upp plasmid was purchased from *Invivogen*. Oligonucleotides used to amplify the CD gene, Glutathione-agarose beads, L-glutathione-reduced and Bradford reagent were procured from Sigma Aldrich Pvt. Ltd., USA. Restriction enzymes used for cloning were purchased from New England Biolabs (Beverly, MA).

2.3.2. Bacterial strains

E. coli DH5 alpha was used for cloning and *E. coli* BL21 (DE3) was used for the expression studies of the wild-type (WT) enzyme.

2.3.3. Construction of the WT CD vector

The cytosine deaminase gene was PCR amplified from pORF codA::upp (*Invivogen*) vector using the primers 5' CGCGGATCCATGGTGTCTGAATAACGC3' and 5'CCGCTCGAGTGACGCCATTAGCTCCGCTG 3' with Bam HI and Xho I overhangs under the conditions of denaturation at 94 °C for 30 s, annealing at 55 °C for 1min, extension at 72 °C for 1min. The amplified CD gene (1.3 Kb) was cloned in pGEMT-Easy vector (*Promega*) and further subcloned into pGEX-4T2, a bacterial expression vector with N-terminal GST tag and designated as pGEX-CD. **Figure 2.1** denotes the schematic of the cloning of CD gene in the pGEX-4T2 vector.

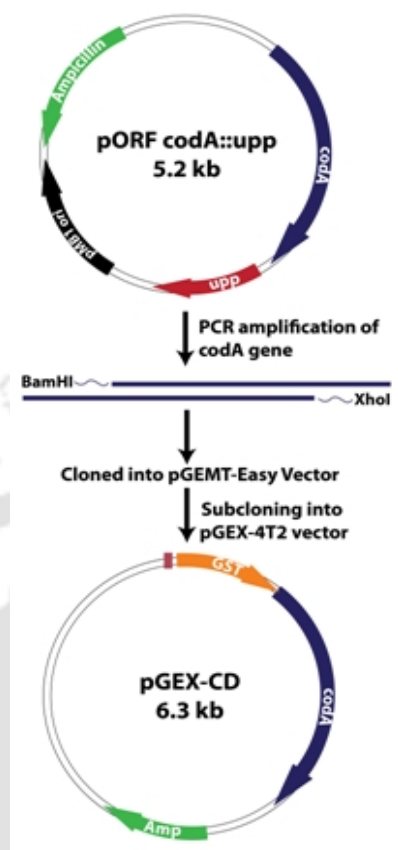


Figure 2.1. Schematic representation of the different steps used for cloning of CD.

2.3.4. Expression of WT GST-CD

WT GST-CD enzyme was overexpressed in the *E. coli* strain BL21 (DE3) *tdk*⁻. A 50 mL culture of LB medium was inoculated with 3mL primary culture and induced for overnight with 0.5 mM IPTG at 37 °C. The cells were harvested at 6000 rpm for 10 min at 4 °C, and the cell pellet was resuspended with pre chilled 1× PBS (pH 7.4) and disrupted twice at constant cell disruptor at 20 kpsi. The disrupted sample was incubated with 1% triton ×100 for 30-40 min on ice. Then the sample was centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant which contains the soluble fraction of the protein of interest was collected and passed through a 0.45μ membrane filter.

2.3.5. Purification of WT GST-CD

The WT GST-CD enzyme was purified near homogeneity by affinity column chromatography using immobilized glutathione-agarose beads. In short, 5 mL of filtered lysate was added to 2 mL of beads and allowed to mix on a rocker for 30 min

in chilled condition to obtain the maximum binding of protein of interest to the resin. After draining out the flow through, the column was washed at least with ten column volumes of wash buffer (1× PBS, pH 7.4) to remove the non-specific proteins. Then the purified protein was eluted with 5 mL of elution buffer (10 mM glutathione reduced in 50 mM Tris-HCl, pH 9.5). The purified protein was dialyzed in dialysis buffer (1× PBS, pH 7.4) at 4 °C for overnight. After dialysis, the protein samples were collected and kept at -20 °C. Protein concentration of the WT GST-CD enzyme was determined using Bradford method using BSA as standard. Purified protein fractions were analyzed using SDS-PAGE, and silver staining was performed to visualize the purified fractions.

2.3.6. Western blot

Purified protein fractions of the GST-CD were run on 12% SDS-PAGE and then blotted onto PVDF membrane for 3 hours at constant 25 V. After blotting, the membrane was blocked for overnight with 5% milk solution which is made up of PBS-T (1× PBS along with 0.01% of Tween 20) at 4 °C. Then the membrane was incubated with anti-GST antibody for 1 hour and further treated with horse-radish peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature. The protein bands were developed using the substrate 3, 3'-Diaminobenzidine (DAB) along with the catalyst H₂O₂.

2.3.7. Enzymatic assay of the WT GST-CD protein with cytosine

CD assays were carried out with varying substrate concentrations. Enzyme assays were performed using the spectrophotometer based method (Ipata et al., 1978). A main stock of 25 mM cytosine was made in 1× PBS, pH 7.4. The degradation of substrate cytosine was monitored at 286 nm and the readings were taken immediately after collecting the sample every 2 min for a total of 18 min. The procedure was repeated using the same amount of enzyme for every cytosine concentration. Lineweaver-Burk plot was used to determine K_m value for the WT CD enzyme. The catalytic efficiency (K_{cat}/K_m) was also determined for WT CD. Assays were performed three to five times for each substrate concentration.

2.3.8. Enzymatic assay of the WT GST-CD with 5-FC

Kinetic values for the WT GST-CD was obtained with 5-FC substituted for cytosine as the substrate. A stock of 25 mM 5-FC was made in sterile water. The reaction mixture containing the desired concentration of 5-FC and water was placed at 37 °C. The reaction was started immediately after addition of the enzyme. A small 100 µL aliquots of the reaction were collected every 2 min over a 18 min time period and immediately quenched in 0.1 M HCl. Readings were then taken at 290 and 255 nm and the concentrations of 5-FC and 5-FU (in mM) were calculated using the formula $[5\text{-FC}] = 0.119(A_{290}) - 0.025(A_{255})$ and $[5\text{-FU}] = 0.185(A_{255}) - 0.049(A_{290})$ (Hayden et al., 1998). Assays were repeated three to five times. Similar to cytosine the kinetic parameters were determined using the double reciprocal plots.

2.4. Results and Discussion

2.4.1. Clone confirmation of the WT CD gene

The cloned WT CD gene was confirmed by restriction digestion using the Bam HI and Xho I restriction enzymes (**Figure 2.2**) and sequencing analysis. The released fragment of the CD gene corresponding to 1.3 kb size can easily be identified in **Figure 2.2**.

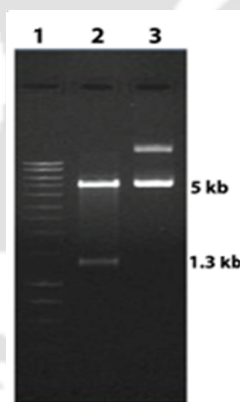


Figure 2.2. Clone confirmation by restriction digestion of pGEX-CD vector. Lane 1 represents the DNA HyperLadder, Lane 2 shows the Bam HI/Xho I digested pGEX-CD showing the vector backbone of 5 kb and the insert of 1.3 kb, and Lane 3 is the uncut pGEX-CD vector.

2.4.2. Expression of the recombinant WT GST-CD protein

The recombinant WT CD enzyme was overexpressed with the fused N-terminal GST tag and has the molecular weight of 75 kDa. The overexpressed GST-CD protein and

the control GST protein (molecular mass of 26 kDa) were appeared as dense bands after overnight induction with 0.5 mM IPTG, which can be clearly seen from the **Figure 2.3**.

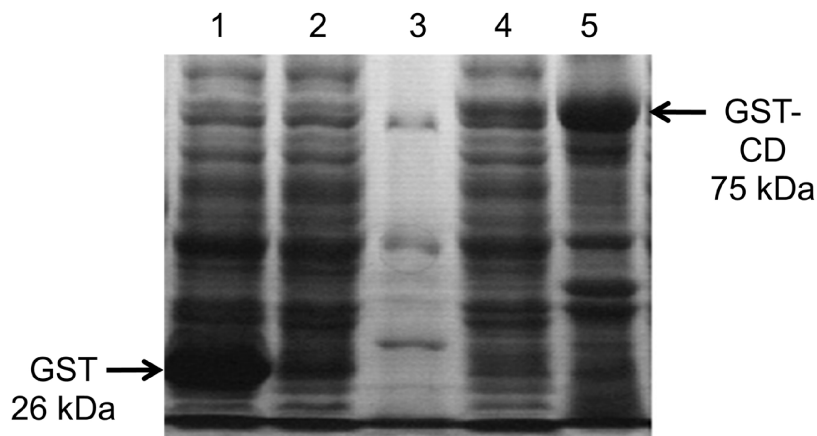


Figure 2.3. SDS-PAGE analysis of the overexpressed recombinant WT GST-CD protein. Lane 1 is the BL21 transformed pGEX-4T2 vector after induction with 0.5 mM IPTG. Lane 2 indicates the IPTG induced *E. coli* BL21 host. Lane 3 is the protein molecular weight marker. Lane 4 is the insoluble fraction of the BL21 transformed pGEX-CD construct after induction with 0.5 mM IPTG. Lane 5 shows the soluble fraction of the BL21 transformed pGEX-CD construct after induction with 0.5 mM IPTG.

2.4.3. Purification of the recombinant WT GST-CD protein

The overexpressed GST-CD protein was affinity purified with the ease of fused GST tag. **Figure 2.4** indicating the single purified band of GST-CD protein.

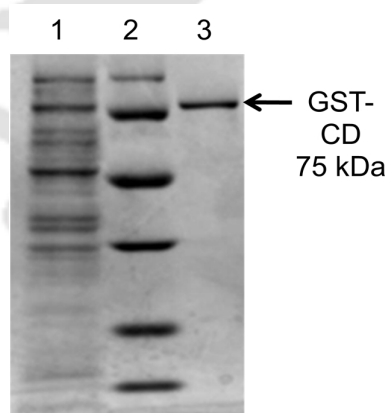


Figure 2.4. Affinity column purification of WT GST-CD protein. Lane 1 is the column flow through. Lane 2 is the protein marker. Lane 3 shows the single band of purified GST-CD protein.

2.4.4. Western blot of the WT GST- CD protein

The western blot analysis carried out with the anti-GST antibody showed single protein bands of GST-CD and control GST proteins. **Figure 2.5** illustrating the anti-GST detected GST-CD and the control GST proteins.

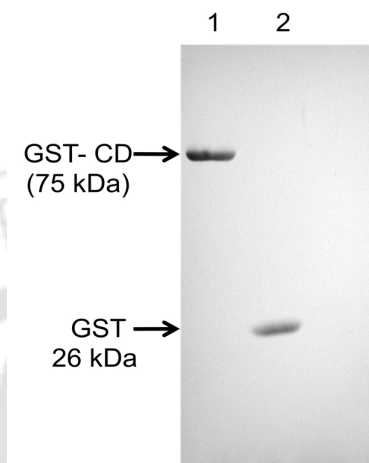


Figure. 2.5. The western blot picture showing the presence of GST tagged CD protein. Lane 1 shows the anti-GST detected band of GST-CD protein (molecular mass – 75 kDa). Lane 2 shows the control GST protein (molecular mass - 26 kDa).

2.4.5. Kinetic parameters

Kinetic data obtained for the WT GST-CD enzyme with the substrates cytosine and 5-FC are illustrated in **Table 2.1**. Kinetic parameters were determined at varying concentrations of cytosine (25 – 150 μM) as substrate. The K_m and K_{cat}/K_m of the WT GST-CD protein found out by double reciprocal plot were 0.25 ± 0.04 mM and 12.67 s⁻¹/mM respectively. Moreover, kinetic values were obtained for the recombinant protein by using different concentrations of 5-FC (1 – 6 mM) as substrate. The K_m and K_{cat}/K_m estimated by double reciprocal plot were 15.82 ± 2.37 mM and 1.32 s⁻¹/mM respectively. The WT GST-CD enzyme is highly capable of binding with the natural substrate cytosine as relative to the prodrug 5-FC which could be stated from the K_m values. In addition to that, the WT enzyme displayed the enhanced catalytic efficiencies towards cytosine as compared with 5-FC.

Table 2.1. Kinetic parameters obtained using double reciprocal plots for the recombinant WT GST-CD enzyme when assayed with cytosine and 5-FC

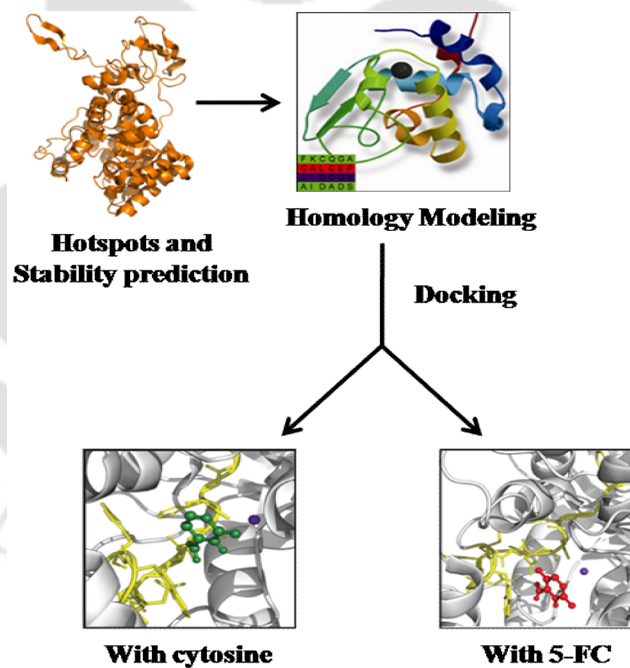
Substrate	K_m (mM)	K_{cat} / K_m ($s^{-1} \text{ mM}^{-1}$)
Cytosine	0.25 ± 0.04	12.67
5-FC	15.82 ± 2.37	1.32

2.5. Conclusion

In conclusion, the WT CD gene was cloned in bacterial expression vector, pGEX-4T2 along with N-terminal GST tag and designated as pGEX-CD. The pGEX-CD construct was transformed into *E. coli* BL21 host and overexpressed by inducing with 0.5 mM IPTG for overnight. The GST-CD protein was purified to near homogeneity in a single step using affinity column chromatography. In addition, western blot analysis was carried out for the purified GST-CD protein with anti-GST antibody. Finally, the WT CD enzyme activity assay was carried out with varying concentrations of cytosine and 5-FC and the catalytic efficiency of the enzyme towards both the substrates was calculated using double reciprocal plots.

In silico and *In vitro* Site Directed Mutagenesis (SDM) Approaches to create Novel *E. coli* Cytosine Deaminase Mutants

Generation of the novel *E.coli* Cytosine deaminase mutants by *in silico* approach is described in this chapter. The *in silico* derived mutants were found to have increased binding affinity towards the prodrug 5-fluorocytosine than their native substrate cytosine. Further, the functional characterization of *in silico* derived mutants also demonstrated. Moreover, the construction of the mammalian expression vectors with the wild-type CD and S126R mutant was shown. Towards the end, the cell viability assay with the wild-type CD and S126R mutant was also demonstrated.



Chapter 3

IN SILICO AND IN VITRO SITE DIRECTED MUTAGENESIS (SDM) APPROACHES TO CREATE NOVEL *E.COLI* CYTOSINE DEAMINASE MUTANTS

3.1. Introduction

Despite of the advantageous features such as being a non-mammalian enzyme and heightened bystander effect, CD suffers from its low specificity towards the prodrug, 5-FC, which is the analogue of its native substrate cytosine. Though, yeast cytosine deaminase (yCD) shows better affinity towards the prodrug than CD, however, the high thermostability of CD (Kievit et al., 1999) may depict the bacterial enzyme as a preference for SGT applications. In this regard, rational molecular engineering could play an important role in improving the substrate specificity of the enzyme towards the prodrug. Genetic engineering of CD to develop mutants with improved specificity was possible only after the revelation of the crystal structure of the protein by X-ray crystallography, which furnished the positions of the active site and the metal coordinating residues (Ireton et al., 2002). Alanine scanning mutagenesis studies carried out on the active site residues 310–320 of CD demonstrated that the mutant D314A had increased 5-FC sensitivity to *E. coli* GIA39 (DE3) cells and thus, it can act as a valuable candidate for the CD/5-FC SGT system (Mahan et al., 2004). Simultaneously performed random mutagenesis of CD also supported the enhanced catalytic efficiency of mutant D314A towards the prodrug (Mahan et al., 2004). Recently, the action mechanism of CD has been demonstrated with bound zinc and phosphonocytosine, a tetrahedral intermediate analogue formed during the deamination of cytosine (Hall et al., 2011). The study showed that the residues Glu 217, His 246, and Asp 313 are involved in the activation of the nucleophilic water molecule which coordinates with the metal ion to facilitate proton transfer reaction leading to deamination (Hall et al., 2011). Thus, mutation of any of these residues could decrease the catalytic efficiency of the enzyme.

Recent day's homology modeling and computational applications are focusing more on engineering the enzymes; regarding their substrate recognition and thermostability, etc. (Murphy et al., 2009; Garcia-Casado et al., 1998; Adam et al., 2008; Korkegian et al., 2005; Chakravorty et al., 2011). Therefore, the *in silico* based approaches could be employed to generate novel CD mutants having increased specificity towards the prodrug, which can serve as better candidates for potential SGT applications.

3.2. Outline of the Research Work

1) The mutagenesis hotspot residues were identified over the CD protein structure and the stability of the protein upon introduction of point mutations was verified using CUPSAT web server.

2) The stable mutants obtained were modeled using MODELLER 9.9, a homology modeling program.

3) The modeled CD mutants were docked with the ligands cytosine and 5-FC using similar docking parameters.

4) The docking results were validated based on the binding free energy scores obtained, as well as the interactions between the ligands and the mutant proteins.

5) The docking studies revealed five mutants which are more capable of binding towards the prodrug 5-FC than the cytosine; thereby, could act as superior candidates in the perspective of SGT applications.

6) The potentiality of the *in silico* derived novel CD mutants was validated by *in vitro* SDM.

7) The WT and the CD mutants were over expressed with N-terminal GST and purified to near homogeneity.

8) The activity assay was performed with cytosine and 5-FC for the WT and the redesigned CD mutants.

9) The kinetic parameters (K_m , V_{max}) and the catalytic efficiency (K_{cat}/K_m) of the WT and the mutants towards their substrates were determined.

10) Three of the 5 mutants showed improved specificity towards the prodrug 5-FC and can suitably be used for SGT.

11) *In vitro* cell viability assay was performed with the WT and the Mutant S126R in Human cervical cancer cell line.

12) A gradual decrease in cell viability was observed with increasing concentrations of the prodrug 5-FC with the mutant S126R as compared to the WT CD and preferably can be used for SGT.

3.3. Experimental Section

3.3.1. Identification of Hotspots of the protein

Identification of mutagenesis hotspots present in the single subunit of WT CD (426 residues) was done by Hotspot wizard (Pavelka et al., 2006). The software takes the protein PDB file (code: 1K6W) as input and gives the output in the order of mutability of functional residues (residues lining in the active site pocket) as well as the active site as hotspots.

3.3.2. Prediction of protein stability upon point mutation

Stability of the protein upon single amino acid substitutions in the hotspot residues was performed using CUPSAT (Parthiban et al., 2006). The software predicts the stability of the protein upon point mutations for already existing structures and custom structures as well (Parthiban et al., 2006). The stable mutants obtained were chosen for further modeling studies.

3.3.3. Homology modeling of mutants

Predicted stable mutants were modeled; taking WT CD (1K6W) as template with MODELLER (Eswar et al., 2006), a homology modeling program which models the proteins by satisfaction of spatial restraints. Totally 5 iterations were given to create models for each mutant. The model with lowest Discrete Optimized Protein Energy (DOPE) score was considered as the top model and chosen for docking analysis.

3.3.4. Macromolecule preparation for Docking

The crystal structure of CD (1K6W) was obtained from PDB and all the hydrogen atoms (polar and non polar hydrogen atoms) were added using AutoDock (Morris et al., 1998). After removing the water molecules from the WT structure, the mutants were modeled and used for docking. In both cases the charge of the metal ion (Fe) was set to zero.

3.3.5. Ligand preparation

The structure of the ligands cytosine and 5-FC were drawn using Java Molecular Editor (JME) (Peter, 2006) and transferred to PRODRG (Schüttelkopf et al., 2004), a small molecule topology generator to convert the ligands in required format for docking studies.

3.3.6. Docking procedure

Docking of CD with cytosine and 5-FC was performed using AutoDock 4.2.3 (Morris et al., 1998). Lamarckian Genetic Algorithm (LGA) was employed to obtain the energetically favored binding modes. Grid box with the dimensions of $116 \times 104 \times 48$ and $0.602 \text{ \AA}$ grid spacing were set to cover the entire protein. Docking parameters were fixed as 250 individual populations, 2500000 energy evaluations, and a mutation rate of 0.02 with the output level of 0. One hundred docked conformations were generated for each ligand. Similar conformations of the ligand were combined as a single cluster. Maximum root mean square tolerance of $2 \text{ \AA}$ was set. The free energy of binding score obtained from AutoDock includes the van der waals, electrostatics and desolvation energy. The results were analyzed based on known interactions between the macromolecule and the ligands as well as the free energy of binding of the ligands.

3.3.7. In vitro Site Directed Mutagenesis

Site-directed mutagenesis was used to individually introduce the following substitutions: F186W, F82C, S126N, R91T and S126R. The mutagenesis reaction was carried out with the QuikChange site-directed mutagenesis kit obtained from Stratagene (La Jolla, CA). The schematic of the mutagenesis reaction can be seen in the **Figure 3.1**. The pGEX-CD expression vector was used as template for the mutagenesis reactions. **Table 3.1** lists the synthetic oligonucleotides used for *in vitro* site-directed mutagenesis.

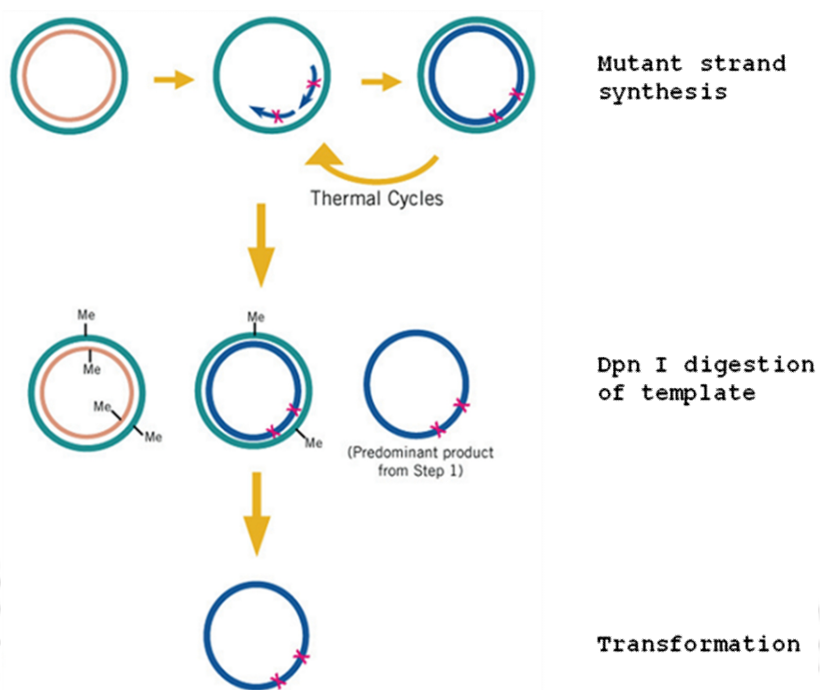


Figure 3.1. Schematic representation of the overview of the Multi Site-Directed Mutagenesis Method. (Source: Stratagene QuikChange Multi Site-Directed Mutagenesis kit manual).

Table 3.1. List of synthetic oligonucleotide primers used for site-directed mutagenesis

Mutation	Oligonucleotide Sequence
F186W	5'-GTGGGGGCGATTCCGCATTGGGAATTTACCCGTGAATACG-3'
F82C	5'-GAATCAGTCCGGCACGCTGTGTGAAGGCATTG-3'
S126N	5'-GCGTACCCATGTCGATGTTAATGATGCAACGCTAACTGCGC-3'
R91T	5'-TTGAACGCTGGGCCGAGACCAAAGCGTTATTAACCC-3'
S126R	5'-TGTGCGTACCCATGTCGATGTTAGGGATGCAACGCTA-3'

3.3.8. DNA sequencing

Sequencing was carried out to confirm the introduction of mutation sites. All the sequencing reactions were performed at Xcelris labs ltd, India.

3.3.9. Expression and Purification of WT CD and the mutants

WT CD and the mutant enzymes were over expressed in the *E. coli* strain BL21 (DE3) *tdk*⁻. Then the WT and the mutant enzymes were purified near homogeneity by affinity column chromatography using immobilized glutathione-agarose beads.

3.3.10. Enzymatic assay of the WT and mutant proteins with cytosine

CD assays were carried out with varying cytosine concentrations. Double reciprocal plot was used to determine K_m values for each mutant and WT CD enzyme. The catalytic efficiency (K_{cat}/K_m) was also determined for each mutant and WT CD.

3.3.11. Enzymatic assay of the WT and mutant proteins with 5-FC

Kinetic values for the WT and the mutants were obtained with 5-FC substituted for cytosine as the substrate.

3.3.12. Construction of the mammalian expression vectors containing WT CD and its variant S126R

The coding region of both WT CD and its variant S126R were PCR amplified from their respective pGEX-4T2 vectors using the gene specific primers 5'CGTCCATGGGAATGGTGTCTGAATAACGC3' and 5'CCTGCTAGCTTAGCTCCGCTGATACGTTT3' with NcoI and NheI overhangs under the conditions of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 1 min for 30 cycles. The PCR amplified products (1.3 Kb) were purified from gel by using agarose gel extraction and purification kit (Sigma Aldrich). The purified products were cloned in pGEMT- Easy vector. Subsequently, these genes were subcloned into mammalian expression vector pVITRO2, from which the lacZ gene fragment was excised out. For the purpose of subcloning into pVITRO2, the genes were amplified using gene specific forward and reverse primers with restriction site overhangs (NcoI and NheI respectively). The amplified products were digested with NcoI and NheI restriction enzymes and ligated into the pVITRO2 vector

backbone. The resulting plasmids were designated as pVITRO2:CD and pVITRO2:S126R respectively. The clones were confirmed by DNA sequencing analysis.

3.3.13. Transfection of WT CD and Mutant S126R into HeLa cells

Following sequence analysis, the purified recombinant plasmids containing WT CD and mutant S126R encoding genes were transfected into Human cervical cancer cells (HeLa) using electroporation, following the manufacturer's protocol. In brief, cells were seeded in 96 well plates with the cell density of 5×10^6 cells / mL and after 24 hrs of seeding, 10-15 μ g of each plasmid DNA construct were transfected into the HeLa cells by electroporation. The electroporator settings are as followed: Choose Mode: LV Mode, 500V / 99 msec; Set Voltage: 75V; Set Pulse Length: 99 msec; Set Number of Pulses: 2 Pulses; Desired Field Strength: 0.375 kV/cm. Transfected cell lines were maintained in their growth medium for further bioactivity experiments.

3.3.14. Bioactivity assay

For MTT cell viability assays, HeLa cells expressing WT CD and S126R genes were plated in 0.1 mL DMEM medium (1×10^4 cells / well) in a 96-well microtiter plate in triplicates and were treated with the prodrug 5-FC (0-25 mM) for 48 hrs. After 48 hrs, MTT solution was added to each well (50 μ g / well) and incubated at 37 °C for 2 hrs followed by the addition of 150 μ L of DMSO to solubilize the precipitated crystals. Cell viability was determined by using a plate reader measuring optical density at 550 nm.

3.4. Results and Discussion

3.4.1. In Silico identification of Hotspot residues

CD exists in the form of an enzymatically active hexamer, but it does not show any kinetic cooperativity, with all six subunits acting independently for catalytic activity (Mahan et al., 2004). The monomer of WT CD (PDB ID: 1K6W) was used in HotSpot Wizard to identify the hotspot residues. The premise of this web server is to identify the hotspots over the enzyme of interest to alter its substrate specificity, activity or enantio selectivity (Pavelka et al., 2006). The mutagenesis hotspots are

selected based on the assimilation of structural, functional and evolutionary information acquired from the established protein databases (Pavelka et al., 2006).

The mutability scores allotted to the residues of the protein CD is given in Appendix, **Table 3.A1**. The substrate binding residues Glu 217, Gln 156 were assigned the lowest mutability score by the wizard and declared as immutable residues; as mutation in these residues could result in the inactivation of enzyme due to its role in the binding of substrate molecules. Similarly, the vital metal coordinating residues His 63, His 214, His 246 and other active site residues Asp 313, Asp 314, Trp 319 were ascribed with lower mutability scores; suggesting mutation in these residues will also lead to the destabilization and inactivation of the enzyme. The highest and the average mutability scores were attributed to Tyr 162, Thr 129, Phe 286 and Leu 95, Thr 131, Phe 186, Arg 91, Ser 126, Phe 82 residues respectively and these were designated as hotspots due to the fact that its modification with other amino acids will not alter the protein functionality. **Figure 3.2** shows the ribbon model of the CD enzyme with the hotspot residues highlighted with different colors. Strikingly, all the residues identified as the hotspots were away from the active site of the enzyme and enabled us to manipulate these residues without compromising the enzyme functionality.

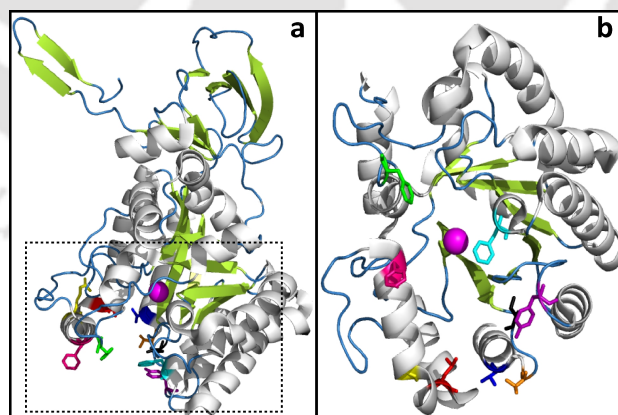


Figure 3.2. (a) Hotspot residues chosen for *in silico* mutagenesis (b) Zoomed in image of the protein for clear view of the hotspot residues. The metal ion (Fe) is shown as magenta sphere. Phe 82 (in hotpink), Arg 91 (in yellow), Leu 95 (in red), Ser 126 (in black), Thr 129 (in orange), Thr 131 (in blue), Tyr 162 (in purple), Phe 186 (in cyan), Phe 286 (in green). Both the figures were generated using PyMOL (PyMOL, Version 1.2r3pre).

3.4.2. CUPSAT validation and homology modeling

The stability of the protein upon substitution of the nine hotspot residues with different 19 possible amino acids was verified using CUPSAT. The web server predicts the difference in binding free energy of unfolding between WT and mutant proteins by utilizing specific atom and torsion angle potentials (Parthiban et al., 2006). Appendix, **Table 3.A2** summarizes the overall stability of the protein upon single amino acid substitutions in the hotspot residues. Amongst the nine hotspot residues, the protein was unstable when Tyr162 was substituted with any of the 19 possible amino acids. Hence, out of the 152 (8×19) possible substitutions, 68 mutants showed stable configuration and these 68 mutants were further used for docking. The stable mutants were modeled using MODELLER 9.9 program (Eswar et al., 2006) by taking X-ray crystal structure of CD (PDB ID: 1K6W) as the template.

3.4.3. Docking with cytosine

All the 68 mutants were docked with cytosine with the previously mentioned docking parameters and the resulting binding energy and the number of clusters is given in Appendix, **Table 3.A3**. The respective electrostatics and van der waals energy are summarized in Appendix, **Table 3.A4**. The ligand orientations, positioning of catalytic residues and hydrogen bond interactions between the ligand and the protein are shown in **Figures 3.3a-3.3c** and **Figures 3.4a-3.4c**.

Molecular docking of the mutants with cytosine revealed that the mutation of Leu to Trp at position 95 (L95W) did not allow the ligand to bind to its active site. Moreover, upon superposition of WT and L95W, structural alterations in the substrate binding residues as well as metal coordinating residues of active site (Trp319, Asp 313, Asp 314, Gln 156 and His 63) were observed (**Figure 3.3a**). Previous report on the X-ray crystal structure analysis of CD demonstrated that the metal ion and all the surrounding active site residues should be in highly ordered manner to maintain the structural integrity of the enzyme (Ireton et al., 2002) and it also demonstrated that the residue Trp319 is involved in substrate binding as well as substrate induced conformational change of the protein which lead to the closure of active site upon substrate binding (Ireton et al., 2002). Therefore, the alteration of structure of Trp319 in the L95W mutant could be responsible for inactivating the enzyme due to the

incapability of the ligand to bind to the active site of the mutant. Similar kind of structural destabilization was observed for the mutant S126R (**Figure 3.3b**).

From **Figure 3.3c**, it can be clearly seen that the catalytic residues of WT and the mutant (F82E) are nearly merging with each other in their three dimensional orientation. The minor alterations observed might not affect the mutant F82E to show similar catalytic activity relative to the WT towards cytosine which was also reflected from the same binding energy (-4.02 kcal/mol) ascribed to the mutant as that of WT.

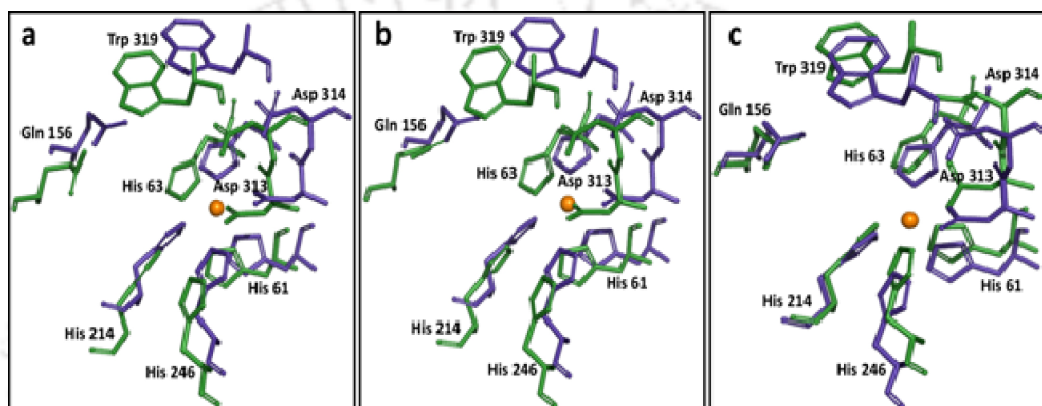


Figure 3.3. Superimposed images of (a) WT and the mutant L95W (b) WT and the mutant S126R (c) WT and the mutant F82E. In all the figures, WT and mutant residues are shown as blue and green sticks respectively. Metal ion is shown as orange sphere.

Among the evaluated 68 mutants, 13 mutants displayed increased binding energy towards cytosine than the WT (Appendix, **Table 3.A3**). Out of these 13 mutants, T129M showed the highest binding energy of -3.64 kcal/mol. As compared with the WT enzyme (**Figure 3.4c**), the binding mode of the ligand cytosine with the mutant T129M (**Figure 3.4a**) showed that the N1 and the N2 nitrogen atoms of the cytosine form hydrogen bond with O δ 2 of Asp 313 and Asp 314 residues respectively. Absence of any hydrogen bond interaction with residues participating in substrate recognition and catalytic activity of the enzyme (Gln 156, Glu 217) might be the cause for diminished activity of the enzyme towards cytosine.

Fifty-two of the 68 mutants showed decreased docking scores towards cytosine than the WT (Appendix, **Table 3.A3**). Amongst them, mutant F286S displayed the least binding free energy of -4.67 kcal/mol. The binding mode of the ligand with the protein showed the possibility of forming five hydrogen bonds with

the protein side-chain (**Figure 3.4b**) relative to that of WT (**Figure 3.4c**). The oxygen atom present in the backbone of cytosine can make hydrogen bonds with N ϵ 2 and N ϵ 1 nitrogen atoms of His 214 and Gln 156 residues in the order. The N1 nitrogen atom of cytosine can also form hydrogen bonding with O ϵ 2 oxygen atom of Glu 217. Further, there is also a possibility of formation of hydrogen bonds between the N2 nitrogen atom of the pyrimidine ring with O ϵ 1 oxygen of Glu 217 and O δ 2 oxygen atom of Asp 313. In addition, the ability of the mutant F286S to form hydrogen bonding with Gln 156 residue helps in the proper orientation of ligand in the active site. Moreover, the Glu 217 and Asp 313 residues facilitate the electrostatic interactions with the metal ion. As a consequence of the above mentioned features, mutant F286S might show increased sensitivity towards cytosine.

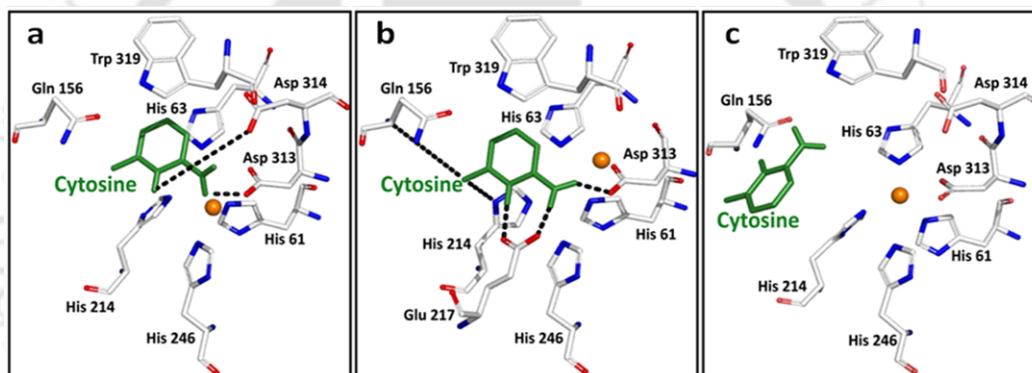


Figure 3.4. Hydrogen bond interactions and binding poses of cytosine in the mutants (a) T129M (b) F286S and (c) WT. Cytosine is indicated as green sticks. Hydrogen bonds are shown as black dashed lines and metal ion as orange sphere.

3.4.4. Docking with 5-FC

The prodrug, 5-FC was also docked with 68 mutants using the same docking parameters as set for cytosine and the resulting binding energy and the number of clusters are listed in Appendix, **Table 3.A3**. The corresponding electrostatics and van der waals energy are shown in Appendix, **Table 3.A4**. The ligand orientations, the positioning of catalytic residues and the hydrogen bond interactions between the ligand 5-FC and the CD protein are illustrated in **Figures 3.5a, 3.5b** and **Figures 3.6a-3.6c**.

The substrate binding residues of 5-FC is similar to those of cytosine. In this regard, parallel to cytosine, the ligand 5-FC also failed to bind to the active site of the mutant L95W due to structural destabilization of the mutant (**Figure 3.3a**).

F82H and F82W mutants showed same binding energy of -4.03 kcal/mol in consistent with the WT, as a consequence of which they are predicted to show similar activity towards 5-FC as that of the WT protein. Absence of any significant alterations in the active site residues in the superimposed images of the mutant and the WT further supported this observation (**Figures 3.5a, 3.5b**).

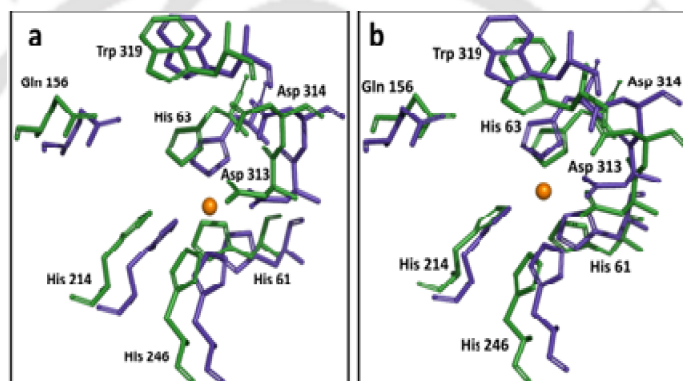


Figure 3.5. Superimposed images of (a) WT and the mutant F82H (b) WT and the mutant F82W. WT and mutant residues are shown as blue and green sticks respectively. Metal ion is shown as orange sphere.

Based on the binding free energy score, 36 mutants displayed lower affinity towards 5-FC as compared to WT (Appendix, **Table 3.A3**). Mutant F82Y was ascribed with the highest binding energy of -3.37 kcal/mol with 5-FC. The analysis of binding mode of 5-FC with the mutant showed that the N1 and N2 nitrogen atoms can be hydrogen bonded to the O δ 1 of Asp 314 and O δ 1 oxygen atom of Asp 313 residues respectively. As seen from the **Figure 3.6a**, there is a possibility of steric clash between the critical substrate binding residue Trp319 and the ligand when compared with the WT (**Figure 3.6c**) which might be responsible for the reduced activity of the protein towards 5-FC.

Twenty-nine of the 68 mutants attained lesser scores with 5-FC as compared with WT (Appendix, **Table 3.A3**). Mutant F286H obtained the least binding energy of -4.49 kcal/mol, which could be attributed to its ability to form six different

hydrogen bonds with the protein side-chain. The oxygen backbone of the 5-FC can be hydrogen bonded to N ϵ 2 nitrogen atom of the residues His 214, His 246, His 61, His 63 whereas, N1 and N2 nitrogen atoms can be bonded with O δ 2 of Asp 313 and O δ 2 atom of Asp 314 respectively (**Figure 3.6b**). Interestingly, the significant observation is that the ligand's orientation can successfully avoid the steric constraints with Trp 319 residue to that of WT (**Figure 3.6c**). The above mentioned critical hydrogen bond interactions and the absence of any steric clash with the adjacent catalytic residues might be responsible for the assignment of higher affinity thereby, making the mutant more active towards 5-FC.

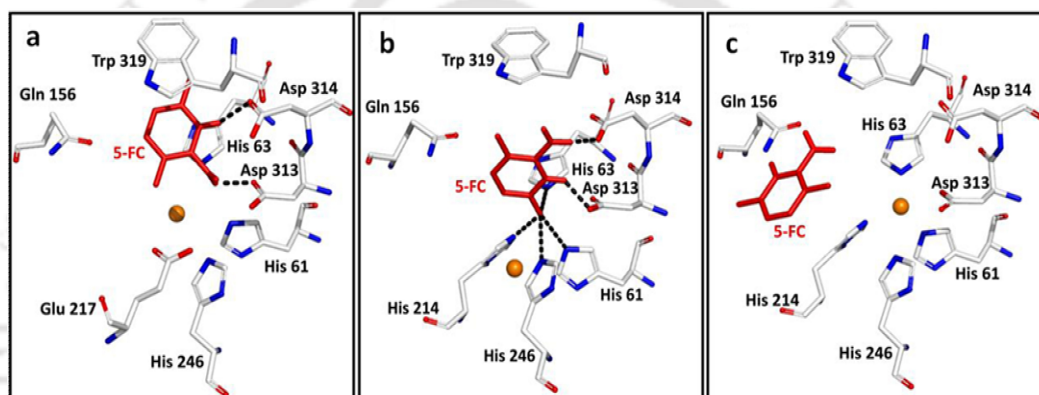


Figure 3.6. Hydrogen bond interactions and binding poses of 5-FC in the mutants: (a) F82Y (b) F286H and (c) WT. 5-FC is indicated as red sticks. Hydrogen bonds are shown as black dashed lines and metal ion as orange sphere.

3.4.5. Mutants showing increased sensitivity towards 5-FC than cytosine

The Mutants F186W, F82C, S126N and R91T achieved lesser docking scores and are predicted to show increased sensitivity towards 5-FC than cytosine. **Table 3.2** summarizes the docking scores of these mutants with both ligands.

Table 3.2. Binding free energy of the mutants towards cytosine and 5-FC as obtained from Autodock

Mutant	Binding Free Energy (kcal/mol)	
	Towards cytosine	Towards 5-FC
F186W	-4.18	-4.25
F82C	-4.2	-4.21
S126N	-4.17	-4.19
R91T	-3.92	-4.0
S126R	-	-3.6

In case of mutant F186W, both the ligands cytosine and 5-FC made similar hydrogen bonds with the protein side-chain. The N2 nitrogen atom of the ligands can be hydrogen bonded with O δ 1 of Asp 314 and the N ϵ 2 nitrogen atom of residues His 61, His 63, His 246 can form hydrogen bonding with the oxygen backbone of both the ligands (**Figures 3.7a, 3.7b**). These essential hydrogen bond interactions can facilitate the binding of the ligands in the active site of the enzyme. In addition to this, the binding poses of both ligands are identical. Though F186W can be characterized as a superior candidate with 5-FC by docking score, there is lack of distinguishable features between the binding modes of ligands. The *in vitro* evaluation of this mutant may reveal the cause for this mutant to show elevated sensitivity towards 5-FC than cytosine.

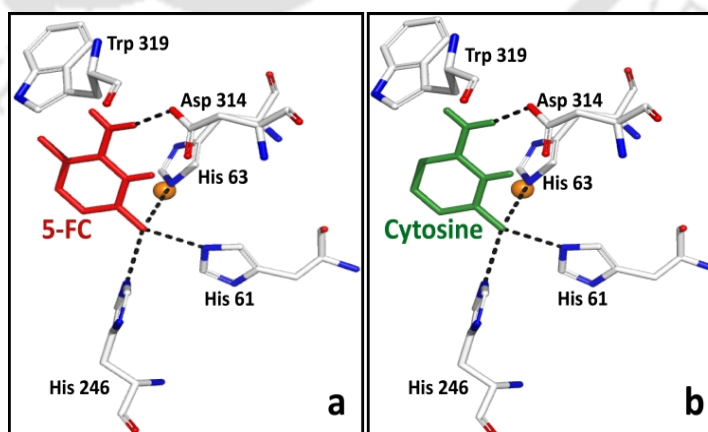


Figure 3.7. Hydrogen bond interactions and binding poses of (a) 5-FC and (b) cytosine in the mutant F186W. Cytosine and 5-FC are shown as green and red sticks respectively and metal ion as orange sphere. Hydrogen bonds are indicated with black dashed lines.

In the redesigned mutant F82C, the oxygen backbone of cytosine can make hydrogen bonds with N ϵ 2 nitrogen atom of the residues His 61, His 246 and the N2 nitrogen can be bonded to O δ 2 of Asp 314. N1 nitrogen atom of both cytosine and 5-FC is bonded with O δ 2 of Asp 313 backbone (**Figures 3.8a, 3.8b**). The oxygen of 5-FC too can form hydrogen bonds with His 61, His 246 and with the residue His 63. Additionally, N2 nitrogen of 5-FC can make hydrogen bonding with O ϵ 1 oxygen of Glu 217. It is worth noting that Glu 217 participates in the activation of the water molecule and proton transfer reactions (Hall et al., 2011) and is required for catalytic activity of the enzyme, therefore interaction with this residue may render the mutant F82C as a better candidate to convert the prodrug into toxic product.

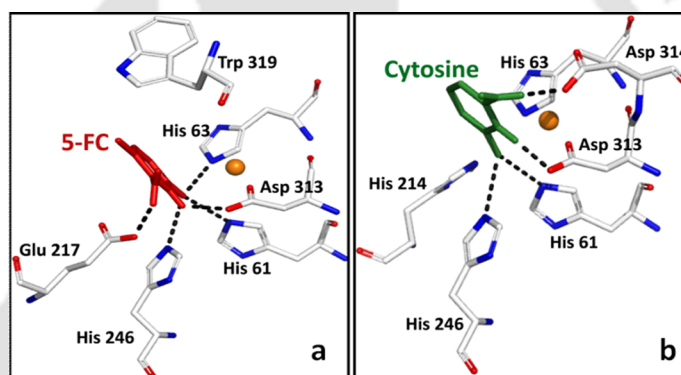


Figure 3.8. Hydrogen bond interactions and binding poses of (a) 5-FC and (b) cytosine in the mutant F82C. Cytosine and 5-FC are shown as green and red sticks respectively and metal ion as orange sphere. Hydrogen bonds are indicated with black dashed lines.

The hydrogen bonding interaction of the mutant S126N with 5-FC is shown in **Figure 3.9a**. Docking of the same mutant with cytosine (**Figure 3.9b**) showed various interactions. The N2 atom of cytosine interacted with O δ 2 of Asp 313 and with O ϵ 1 atom of Glu 217 and the O ϵ 2 atom of Glu 217 interacted with the N1 nitrogen atom of cytosine. The lack of interactions between cytosine and the vital metal coordinating residues His 61, His 63, His 214 as compared with 5-FC may diminish the activity of the redesigned enzyme towards cytosine than the WT enzyme.

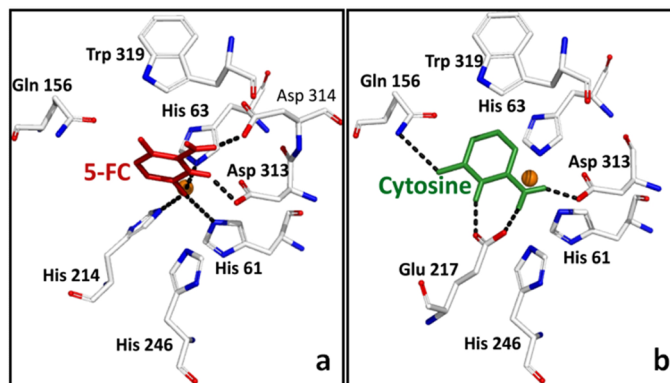


Figure 3.9. Hydrogen bond interactions and binding poses of (a) 5-FC and (b) cytosine in the mutant S126N. Cytosine and 5-FC are shown as green and red sticks respectively and metal ion as orange sphere. Hydrogen bonds are indicated with black dashed lines.

Mutant R91T was ascribed with higher binding energy towards cytosine than 5-FC. In this mutant, O δ 2 of Asp 314 could interact with N1 and N2 nitrogen atoms of 5-FC and the oxygen backbone of 5-FC with N ϵ 2 of His 63 residue through hydrogen bonding (**Figure 3.10a**). Docking pose of cytosine (**Figure 3.10b**) revealed that the oxygen backbone of cytosine can be bonded with N ϵ 2 atom of His 63, His 214, His 246 residues and the O δ 1 of Asp 314 with N2 nitrogen of cytosine via hydrogen bonds. The steric hindrance occurred between cytosine and Trp 319 may also contribute for the lesser affinity towards cytosine.

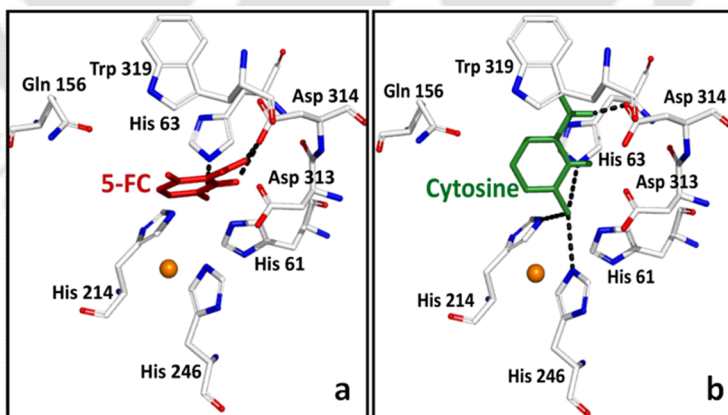


Figure 3.10. Hydrogen bond interactions and binding poses of (a) 5-FC and (b) cytosine in the mutant R91T. Cytosine and 5-FC are shown as green and red sticks respectively and metal ion as orange sphere. Hydrogen bonds are indicated with black dashed lines.

Strikingly, the substitution of serine to arginine at position 126, allowed the binding of 5-FC to the active site and not the actual substrate cytosine. The reason behind this observation might be the formation of hydrogen bonds between the oxygen atom of 5-FC and N ϵ 2 of His 214 and the N1 of 5-FC with O ϵ 1 of Glu 217. Moreover, the N2 atom of the ligand interacted with O δ 2 of Asp 314 (**Figure 3.11**). Interaction with the residue Glu 217 may facilitate the enzyme activity towards the prodrug, 5-FC because of the involvement of Glu 217 in the activation of the water molecule and proton transfer reactions (Hall et al., 2011). The *in vitro* evaluation of the mutant S126R will reveal the exact mechanisms which promote the efficient binding towards the prodrug.

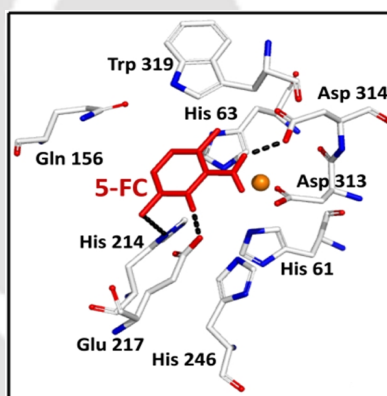


Figure 3.11. Hydrogen bond interactions and binding pose of 5-FC in the mutant S126R. 5-FC is shown as red stick and metal ion as orange sphere. Hydrogen bonds are indicated with black dashed lines.

3.4.6. Multiple Sequence Alignment

The mutant clones were confirmed both by restriction digestion and sequencing analysis. The multiple sequence alignment of the WT and the mutants is illustrated in **Figure 3.12**.

S126N_A SNNALQTIINARLPGEGLWQIHLQDGKISIDAQSGVMPITENSLDAEQ 50
S126R_A SNNALQTIINARLPGEGLWQIHLQDGKISIDAQSGVMPITENSLDAEQ 50
F186W_A SNNALQTIINARLPGEGLWQIHLQDGKISIDAQSGVMPITENSLDAEQ 50
R91T_A SNNALQTIINARLPGEGLWQIHLQDGKISIDAQSGVMPITENSLDAEQ 50
1K6W_A SNNALQTIINARLPGEGLWQIHLQDGKISIDAQSGVMPITENSLDAEQ 50
F82C_A SNNALQTIINARLPGEGLWQIHLQDGKISIDAQSGVMPITENSLDAEQ 50

S126N_A GLVIPPFEVPHIHLDTTQTAGQPNWNQSGTLFEGIERWAERKALLTHDDV 100
S126R_A GLVIPPFEVPHIHLDTTQTAGQPNWNQSGTLFEGIERWAERKALLTHDDV 100
F186W_A GLVIPPFEVPHIHLDTTQTAGQPNWNQSGTLFEGIERWAERKALLTHDDV 100
R91T_A GLVIPPFEVPHIHLDTTQTAGQPNWNQSGTLFEGIERWAETKALLTHDDV 100
1K6W_A GLVIPPFEVPHIHLDTTQTAGQPNWNQSGTLFEGIERWAERKALLTHDDV 100
F82C_A GLVIPPFEVPHIHLDTTQTAGQPNWNQSGTLCEGIERWAERKALLTHDDV 100

S126N_A KQRAWQTLKWQIANGIQHVRTHVDVSDATLTALKAMLEVKQEVAPWIDLQ 150
S126R_A KQRAWQTLKWQIANGIQHVRTHVDVSDATLTALKAMLEVKQEVAPWIDLQ 150
F186W_A KQRAWQTLKWQIANGIQHVRTHVDVSDATLTALKAMLEVKQEVAPWIDLQ 150
R91T_A KQRAWQTLKWQIANGIQHVRTHVDVSDATLTALKAMLEVKQEVAPWIDLQ 150
1K6W_A KQRAWQTLKWQIANGIQHVRTHVDVSDATLTALKAMLEVKQEVAPWIDLQ 150
F82C_A KQRAWQTLKWQIANGIQHVRTHVDVSDATLTALKAMLEVKQEVAPWIDLQ 150

S126N_A IVAFPQEGILSYPNGEALLEEALRLGADVGAIPHFEFTREYGVESLHKT 200
S126R_A IVAFPQEGILSYPNGEALLEEALRLGADVGAIPHFEFTREYGVESLHKT 200
F186W_A IVAFPQEGILSYPNGEALLEEALRLGADVGAIPHFEFTREYGVESLHKT 200
R91T_A IVAFPQEGILSYPNGEALLEEALRLGADVGAIPHFEFTREYGVESLHKT 200
1K6W_A IVAFPQEGILSYPNGEALLEEALRLGADVGAIPHFEFTREYGVESLHKT 200
F82C_A IVAFPQEGILSYPNGEALLEEALRLGADVGAIPHFEFTREYGVESLHKT 200

S126N_A FALAQKYDRLIDVHCDEIDDEQSRFVETVAALAHHEGMGARVTASHTTAM 250
S126R_A FALAQKYDRLIDVHCDEIDDEQSRFVETVAALAHHEGMGARVTASHTTAM 250
F186W_A FALAQKYDRLIDVHCDEIDDEQSRFVETVAALAHHEGMGARVTASHTTAM 250
R91T_A FALAQKYDRLIDVHCDEIDDEQSRFVETVAALAHHEGMGARVTASHTTAM 250
1K6W_A FALAQKYDRLIDVHCDEIDDEQSRFVETVAALAHHEGMGARVTASHTTAM 250
F82C_A FALAQKYDRLIDVHCDEIDDEQSRFVETVAALAHHEGMGARVTASHTTAM 250

S126N_A HSYNGAYTSRLFRLKMSGINFVANPLVNIHLQGRFDTYPKRRGITRVKE 300
S126R_A HSYNGAYTSRLFRLKMSGINFVANPLVNIHLQGRFDTYPKRRGITRVKE 300
F186W_A HSYNGAYTSRLFRLKMSGINFVANPLVNIHLQGRFDTYPKRRGITRVKE 300
R91T_A HSYNGAYTSRLFRLKMSGINFVANPLVNIHLQGRFDTYPKRRGITRVKE 300
1K6W_A HSYNGAYTSRLFRLKMSGINFVANPLVNIHLQGRFDTYPKRRGITRVKE 300
F82C_A HSYNGAYTSRLFRLKMSGINFVANPLVNIHLQGRFDTYPKRRGITRVKE 300

S126N_A MLESGINVCFGHDDVDFDPWYPLGTANMLQVLHMGLHVCQLMGYGQINDGL 350
S126R_A MLESGINVCFGHDDVDFDPWYPLGTANMLQVLHMGLHVCQLMGYGQINDGL 350
F186W_A MLESGINVCFGHDDVDFDPWYPLGTANMLQVLHMGLHVCQLMGYGQINDGL 350
R91T_A MLESGINVCFGHDDVDFDPWYPLGTANMLQVLHMGLHVCQLMGYGQINDGL 350
1K6W_A MLESGINVCFGHDDVDFDPWYPLGTANMLQVLHMGLHVCQLMGYGQINDGL 350
F82C_A MLESGINVCFGHDDVDFDPWYPLGTANMLQVLHMGLHVCQLMGYGQINDGL 350

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S126N_A NLITHHSARTLNLQDYGIAAGNSANLIILPAENGFDALRRQVPVRYSVRG 400
S126R_A NLITHHSARTLNLQDYGIAAGNSANLIILPAENGFDALRRQVPVRYSVRG 400
F186W_A NLITHHSARTLNLQDYGIAAGNSANLIILPAENGFDALRRQVPVRYSVRG 400
R91T_A  NLITHHSARTLNLQDYGIAAGNSANLIILPAENGFDALRRQVPVRYSVRG 400
1K6W_A  NLITHHSARTLNLQDYGIAAGNSANLIILPAENGFDALRRQVPVRYSVRG 400
F82C_A  NLITHHSARTLNLQDYGIAAGNSANLIILPAENGFDALRRQVPVRYSVRG 400

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S126N_A GKVIASTQPAQTTVYLEQPEAIDYKR 426
S126R_A GKVIASTQPAQTTVYLEQPEAIDYKR 426
F186W_A GKVIASTQPAQTTVYLEQPEAIDYKR 426
R91T_A  GKVIASTQPAQTTVYLEQPEAIDYKR 426
1K6W_A  GKVIASTQPAQTTVYLEQPEAIDYKR 426
F82C_A  GKVIASTQPAQTTVYLEQPEAIDYKR 426
*****

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Figure 3.12. Multiple sequence alignment of the WT CD (1K6W) and the mutants. F82C mutation is shown as red box. R91T mutation is shown as green box. S126N and S126R mutations are given in blue box. F186W mutation site is shown as black box. ClustalX-2.1(Larkin et al., 2007) program was used to generate the multiple sequence alignment.

3.4.7. Expression of the WT and mutant CD proteins

The over expression of the proteins of interest as compared to the BL21 host strain; without the gene, after induction with identical concentration of IPTG is illustrated in

Figure 3.13. The over expressed proteins appeared as thick bands and have the size of 75 kDa including N-terminal GST tag.

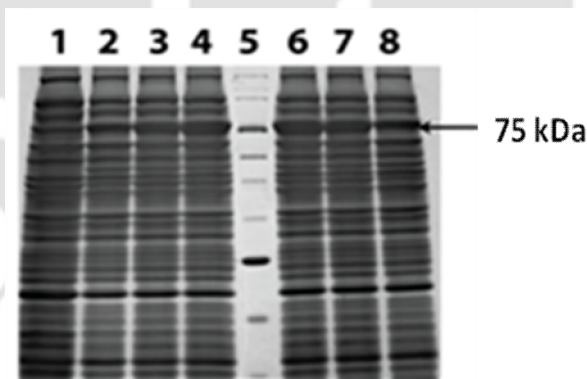


Figure 3.13. SDS-PAGE illustrating the expression of WT and mutant proteins. Lane 1 is the BL21 no insert control, Lanes 2, 3, 4 and 6, 7, 8 indicates the over expressed WT, F186W, F82C, S126N, R91T and S126R mutant proteins respectively. Lane 5 is the protein molecular weight marker.

3.4.8. Purification of the WT and the mutants

Figure 3.14 shows the single bands of purified WT and the mutant N-terminal GST

tagged enzymes corresponding to molecular weight of 75 kDa.

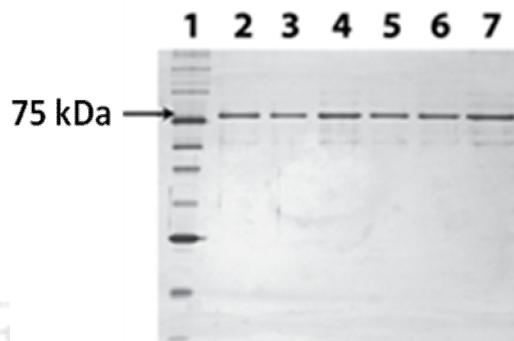


Figure 3.14. Purified fractions of the WT and mutant CD enzymes. Lane 1 is the protein marker and Lanes 2-7 indicate the purified WT CD and mutant proteins.

3.4.9. Enzyme Assay with cytosine

The kinetic parameters of the WT enzyme and the mutants towards its native substrate cytosine were determined spectrophotometrically (Ipata et al., 1978). Double reciprocal plots were used to determine the K_m value for all the mutants and the WT CD enzyme. The catalytic efficiency (K_{cat}/K_m); where $K_{cat} = V_{max}/[E]$ ($[E]$ is the total enzyme concentration) was also calculated for each of the WT and mutant CD and is represented in **Table 3.3**. As it can be seen from the **Table 3.3**, the K_m of WT CD is 0.25 mM towards cytosine which is similar to those reported previously (Mahan et al., 2004).

Table 3.3. Kinetic values obtained using double reciprocal plots for the WT and mutant enzymes when assayed with cytosine

Enzyme	Kinetic parameters towards cytosine	
	K_m (mM)	K_{cat}/K_m (s^{-1}/mM)
WT	0.25 ± 0.04	12.67
F186W	1.15 ± 0.28	5.59
F82C	ND ^a	-
S126N	0.65 ± 0.06	11.17
R91T	0.40 ± 0.07	13.06
S126R	1.55 ± 0.43	3.87

^a ND = not detectable.

The docking conformations of the ligands cytosine and 5-FC when docked with the mutants F186W, F82C, S126N, R91T and S126R were depicted in **Figures 3.7-3.11**. As seen earlier, the mutant F186W obtained a higher docking score and thus showed lower binding affinity towards cytosine. From the **Figures 3.7a** and **3.7b**, though there were no significant changes observed while docking with 5-FC and cytosine, the experimental analysis of this mutant revealed that the K_m of this mutant towards cytosine to be 1.15 mM (**Table 3.3**), which showed 4.6 fold decreased affinity against the WT enzyme. The catalytic efficiency of the mutant was also drastically reduced to 5.59 s⁻¹ /mM. This could lead the mutant to act better than the WT enzyme.

The engineered mutant F82C did not show any activity with cytosine as a substrate in the detectable range indicating weak interaction between the mutant and cytosine. The observed result can be explained from the docking poses of F82C with cytosine (**Figure 3.8b**). As already mentioned, the residue Glu 217 participates in the activation of the water molecule and proton transfer reactions (Hall et al., 2011) and is required for catalytic activity of the enzyme, therefore, the lack of any interaction of the substrate with this residue may render the mutant F82C to show diminished affinity, as observed.

As it can be clearly seen from the **Figure 3.9b**, the lack of any interactions between cytosine and the vital metal coordinating residues His 61, His 63, His 214 present over the mutant S126N may decline the activity of the mutant enzyme towards cytosine. Accordingly, the mutant S126N acquired K_m and K_{cat}/K_m values of 0.65 mM and 11.17 s⁻¹ /mM respectively. The K_m value got increased by 2.6 fold with cytosine than the WT CD and further the catalytic efficiency also reduced, indicating the decreased affinity of the mutant towards cytosine.

As per the theoretical prediction, the mutant R91T was ascribed with higher binding energy towards cytosine than 5-FC. As already revealed from the crystal structure of CD, the amino acid residue Trp 319 directly stacks against the substrate cytosine and seems to play a significant role in substrate binding; thus its presence cause for a substrate induced conformational change which leads to the shutting of active site (Ireton et al., 2002). Hence, the steric hindrance between cytosine and Trp 319 observed from the **Figure 3.10b** could contribute for the lesser binding affinity of

the mutant R91T towards cytosine. The experimental results supported the prediction and the mutant got the K_m value of 0.4 mM which is ~ 1.6 fold higher than that of WT, but it has obtained K_{cat}/K_m of $13.06 \text{ s}^{-1}/\text{mM}$. The higher catalytic efficiency than the WT towards cytosine indicates that the mutant binds with cytosine ineffectually, but once binds in the active site; it deaminates the cytosine to uracil effectively as reported previously in case of CD mutant P318A (Mahan et al., 2004).

The functional characterization of the mutant S126R disclosed that cytosine binds weakly to the active site of the enzyme, which can be clearly seen from the highest K_m value of 1.55 mM amongst all the mutants and a further reduced catalytic efficiency ($3.87 \text{ s}^{-1}/\text{mM}$). It is noteworthy, that the docking studies also revealed the incapable binding of cytosine in the active site of the enzyme. This might be due to the changes occurred in the positioning of the active site residues which needs further detailed structural analysis of the mutant.

3.4.10. Enzyme Assay with 5-FC

The enzymatic activity assay of the WT CD and the engineered mutants against the prodrug 5-FC was performed using different concentrations of 5-FC with a constant total enzyme concentration. The K_m of WT enzyme was determined to be 15.82 mM with 5-FC, which was nearly similar to those reported previously (Kievit et al., 1999). The K_{cat}/K_m of the WT enzyme towards the prodrug was calculated to be $1.32 \text{ s}^{-1}/\text{mM}$.

Though, the mutant F186W did not show any distinguishable features while docked with 5-FC and cytosine (**Figures 3.7a, 3.7b**), the binding energy was very low with 5-FC as compared with cytosine (**Table 3.2**). The docking studies showed that the mutant S126R did not allow cytosine to bind in its active site but allowed 5-FC to bind only once in the 100 docking runs performed by Autodock. The reason behind this observation could be attributed to the fact that the interaction with the residue Glu 217 may facilitate the binding of 5-FC with the mutant enzyme, because of the participation of the residue Glu 217 in the proton transfer reactions (Hall et al., 2011). *In vitro* results showed that the mutant binds inadequately with the cytosine, whereas, it binds strongly with 5-FC (**Table 3.4**).

Table 3.4. Kinetic values obtained using double reciprocal plots for the WT and mutant enzymes when assayed with 5-FC

Enzyme	Kinetic parameters towards 5-FC	
	K_m (mM)	K_{cat}/K_m (s^{-1}/mM)
WT	15.82 ± 2.37	1.32
F186W	10.96 ± 3.25	7.10
F82C	4.09 ± 1.31	2.74
S126N	4.68 ± 1.00	6.26
R91T	20.96 ± 1.06	1.37
S126R	8.96 ± 1.52	7.11

The high throughput of conversion from 5-FC to 5-FU was observed in case of mutants F186W and S126R. The K_m values of these F186W and S126R mutants were 10.96 and 8.96 mM respectively, which were lesser than that of WT. Moreover, their catalytic efficiencies towards 5-FC also showed ~5.4 fold increase than that of WT enzyme. These results attested the efficient binding of the mutants to 5-FC in their active site as indicated by the docking results.

In case of the mutant F82C, the prodrug 5-FC can make an additional hydrogen bonding with the Glu 217 residue (**Figure 3.8a**), which was lacking when cytosine was used as ligand (**Figure 3.8b**). This additional hydrogen bond interaction may render the mutant with lesser binding energy score towards 5-FC. The experimental investigation of this mutant disclosed that the enzyme binds insubstantially with the natural substrate cytosine rather than the prodrug. The absence of any measurable absorbance differences with cytosine, but higher catalytic rate towards 5-FC ($2.74 s^{-1}/mM$) in comparison to the WT CD, prevail this mutant to serve as better candidate amongst the mutants considered.

The hydrogen bonding interaction of the mutant S126N with 5-FC is shown in **Figure 3.9a**. The interactions between 5-FC and the vital metal coordinating residues His 61, His 63, His 214 as compared to cytosine could increase the activity of the designed enzyme towards 5-FC than cytosine. As mentioned in **Table 3.4**, the mutant is ~4.8 fold catalytically effective with 5-FC than WT CD.

Unlike the docked position of the ligand cytosine, the lack of steric hindrance between the 5-FC and Trp 319 residue (**Figure 3.10a**) may contribute for the higher affinity of the mutant R91T towards 5-FC. The experimental characterization of this mutant showed that the mutant has higher K_m value than the WT CD but has slightly increased catalytic efficiency (K_{cat}/K_m of 1.37) towards 5-FC.

In order to have a comparative account on the catalytic activity of the mutants with the WT CD, and to elect the most suitable enzyme for SGT, the relative specificity of the enzymes towards the prodrug as well as relative to WT CD was determined. Relative specificities were calculated using the equation (1) as obtained from the previous reports (Mahan et al., 2004; Mahan et al., 2004).

$$\frac{K_{cat} / K_m(5-FC)}{K_{cat} / K_m(\text{cytosine}) + K_{cat} / K_m(5-FC)} \quad (1)$$

Table 3.5. Relative specificity of the WT and mutant enzymes

Enzyme	Relative specificity with respect to 5-FC	Relative to WT
WT	0.094	1.0
F186W	0.556	5.95
F82C	-	-
S126N	0.36	3.82
R91T	0.095	1.01
S126R	0.65	6.88

The relative specificities of the WT and the mutants with both the substrates are given in the **Table 3.5**. Amongst the mutants, S126R showed ~7 fold increased catalytic rate with 5-FC than the WT enzyme. 6 and 4 fold elevated catalytic efficiency than the WT with 5-FC were shown by the mutant F186W and S126N respectively. The mutant R91T showed almost similar catalytic efficiency towards 5-FC compared to the WT CD. Though the mutant F82C failed to show any measurable

activity towards cytosine, the increased rate of catalysis towards the prodrug 5-FC rather than WT may depict this mutant to have potential for SGT owing to the fact that, there will be a competition between the prodrug 5-FC and intracellular cytosine.

3.4.11. Confirmation of the WT CD and S126R mutant clones

The WT CD and mutant S126R clones were confirmed by restriction digestion using the NcoI and NheI restriction enzymes and sequencing analysis. The released fragments of the WT CD and S126R genes corresponding to 1.3 kb size were identified in lane 2 of the **Figures 3.15A and 3.15B**, respectively.

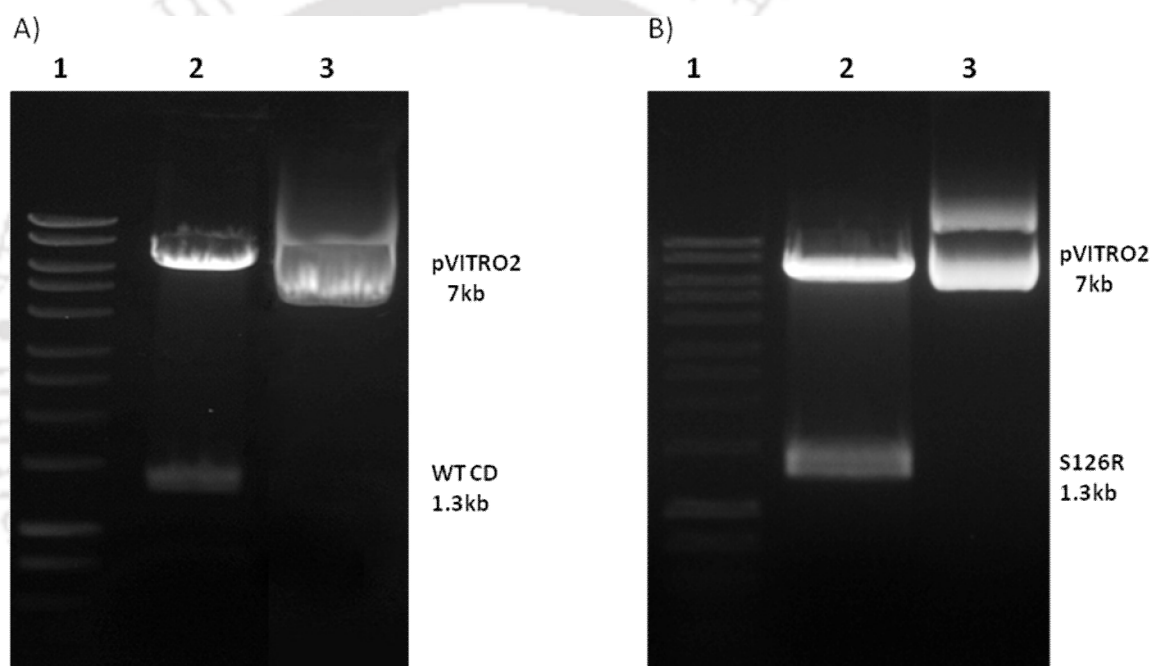


Figure 3.15. Confirmation of WT CD and S126R genes cloned in pVITRO2 vector by restriction digestion using the NcoI and NheI restriction enzymes. **(A)** Lane 1- DNA Hyperladder, Lane 2- NcoI/NheI digested pVITRO2-CD showing the vector backbone of 7 kb and insert WT CD of 1.3kb and Lane 3- Uncut pVITRO2-CD clone **(B)** Lane 1- DNA Hyperladder, Lane 2- NcoI/NheI digested pVITRO2- S126R showing the vector backbone of 7 kb and insert S126R of 1.3kb and Lane 3- Uncut pVITRO2-S126R clone.

3.4.12. Bioactivity of WT CD and Mutant S126R

To assess the effect of the WT CD and mutant S126R, the HeLa cells transfected with pVITRO2:CD and pVITRO2:S126R were subjected to *in vitro* 5-FC sensitivity assays as described above. Results from prodrug sensitivity assay showed that the S126R variant conferred increased sensitivity to 5-FC as compared to the WT CD. That is, with increasing concentrations of the prodrug, there was a gradual decrease in cell viability which can be clear seen from the **Figure 3.16**. A significant decrease in cell viability was observed at all the tested concentrations of 5-FC ($P < 0.001$). As compared to the WT CD, mutant S126R displayed remarkable response to 5-FC with decreased cell viability.

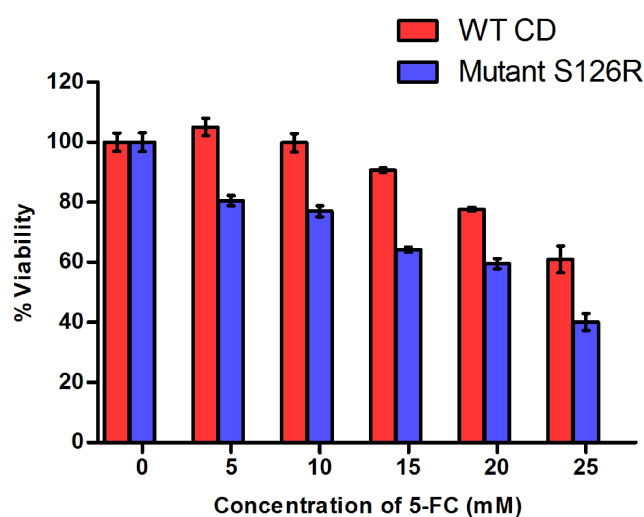


Figure 3.16. Bioactivity of WT CD and mutant S126R in HeLa cells. MTT cell viability assays were done for 5-FC sensitivity for WT CD and S126R transfected HeLa cells. Cells were seeded in a 96-well microtiter plate; after 48 hrs, 5-FC was added in the dose range 0–25 mM. Cell viabilities were determined with a plate reader measuring optical density at 550 nm following 2 hrs of MTT treatment. Statistical analysis was done by two way ANOVA using Graphpad Prism 5.1 and the values were observed to be extremely significant ($P < 0.001$).

3.5. Conclusion

Homology modeling and molecular docking studies were highly implemented to study the ligand-protein interactions. *In silico* SDM and docking of bacterial cytosine deaminase were carried out to get potent mutants for CD/5-FC suicide gene therapy for cancer. The mutagenesis hotspots present in CD were located and all the predicted

stable mutants were modeled and subsequently docked with cytosine and 5-FC. The docking results were substantiated based on the known ligand-protein interactions and also based on the binding energy scores. As a conclusion, the docking studies revealed the mutants F186W, F82C, S126N and R91T which display enhanced catalytic efficiency towards the prodrug. The most significant outcome of the docking studies is the explicit binding of the prodrug with the mutant S126R. The docking results were further validated experimentally through protein expression and enzyme assays and revealed the fact that the novel mutants S126R, F186W and F82C can act as better candidates in SGT applications; having higher relative specificity towards the prodrug 5-FC than the WT CD. Since there is a competition between cytosine and the prodrug inside the cell for the active site of CD, the incapable binding of these mutants with the cytosine adds more potency to make utility of these mutants. Further to understand the effects of the developed mutants in the mammalian expression system, the WT CD and its variant S126R were cloned and transfected into human cervical cancer cell lines. Moreover, the performed *in vitro* cell viability assay revealed that the variant S126R displayed increased sensitivity towards the prodrug in comparison to its wild-type counterpart. Towards the end, the relative inefficient binding of the mutant with cytosine than 5-FC and decreased cancer cell viability in response to 5-FC adds more insight to utilize these mutants in its possible futuristic applications.

3.6. Appendix

Table 3.A1. Mutability scores given for the residues over the CD structure (1K6W) by HotSpot Wizard

Mutability Score	Residues
1	ILeu 85, His 246, His 63, His 61, Asp 246, Gln 150, Arg 293, Arg 120, Pro 184, Thr 80, Ala 153, Leu 81, Ala 204, Gly 176, Gly 323, Val 308, Gly 115, Gly 401, Ala 358, His 214, Asp 313, Asp 212, Gln 156, Glu 227, Asp 219, Thr 354, Ser 196, Pro 155, Pro 276, His 185, Asp 178, Gly 181, Asp 65, Asp 220, Gly 158, Gly 79, Gly 284, Gly 371, Asp 317, Thr 296, Phe 154, Asp 124, Arg 285, Asp 287, Glu 217, Ala 374, Ser 245, Glu 187, His 122, Ala 233, Ser 223, Ser 397, Arg 103, Ala 172, Pro 290, Arg 292, Leu 52, Asn 279, Glu 83, Gly 294, Asn 76, Gly 367, ILeu 183, Pro 318, Arg 224, Val 152, Arg 297, Ser 78, Leu 328, Leu 282, Lys 92, Ser 252, Val 298
2	Val 180, Ala 230, Gly 305, Val 242, His 336, Thr 69, Ala 249, Gly 84, Ala 104, Gln 283, Asn 326, Trp 319, Asn 275, Thr 248, Ala 135, Ala 244, Leu 261, Tyr 366, Val 394, Glu 300, Thr 96, Thr 189, Ala 387, Asp 127, Asn 307, Tyr 257, Ala 177, Leu 64, Pro 55, Met 250, ILeu 22, Gln 117, Glu 59, Gly 311, Lys 291, Ser 161, Thr 121, Gly 237, Val 179, Ser 268, ILeu 218, Ala 381, Asp 314, Val 123, Ala 256
3	Gly 27, Ala 11, Tyr 320, Val 58, Thr 67, Gln 50, ILeu 29, ILeu 377, Trp 88, Gly 193, Met 327, Pro 56, Val 315, Leu 133, ILeu 32, Leu 130, Asn 254, Leu 277, His 312, Ala 48, Pro 321, Arg 389, Ala 325, Pro 73, Thr 324, Asp 99, Phe 57, Val 278, Glu 157, Leu 340, Cys 215, Tyr 253, Thr 247, Glu 138, Ala 405, Leu 331, Tyr 207, Leu 13, His 356, Gln 339, Ala 182, ILeu 211, Leu 203, Leu 24, Thr 66, ILeu 151, Lys 140, Phe 225, His 251, Ala 132, Asn 375, Gln 21, ILeu 353, Val 53, Gly 165, Val 139, ILeu 116
4	Val 125, Phe 82, Arg 190, Thr 243, Arg 241, Asn 10, ILeu 368, Arg 260, ILeu 62, Asp 47, Gln 6, Met 341, Gly 334, Ser 126, Leu 379, Arg 91, Val 194, Thr 228, Leu 173, Leu 168, Asp 148, Val 119, Leu 169, Met 333, Lys 299, Thr 407, Gly 71, Val 273, Arg 399, ILeu 112, Leu 197, Val 226, Leu 352, Val 330, Leu 265, Phe 272, Leu 149, Glu 191, Lys 206, Val 100, Thr 107, Glu 171, Thr 288, Asn 271, Cys 309, Lys 266, Glu 221, Gly 239, His 281, Met 301, Phe 186, Gln 111, Val 213, Ala 144, Val 398, Gly 255, Ala 274, Met 136, Val 337, Asp 386, Gly 342, Leu 5, Pro 380, ILeu 8, Ser 304

5	Phe 201, ILeu 346, Arg 87, Val 229, Leu 361, Leu 322, Ala 70, Leu 46, Leu 264, ILeu 159, Val 403, Gln 391, ILeu 404, Leu 137, Met 238, Asp 98, Asp 208, Thr 200, Ser 357, Leu 363, Leu 108, ILeu 270, Thr 360, Gln 205, Arg 263, Thr 258, Val 143, Ala 128, Arg 12, Met 267, ILeu 280, Ser 259, Thr 42, ILeu 306, Ala 240
6	Pro 60, Glu 303, Glu 86, Pro 145, Phe 310, Gly 15, Arg 395, Gln 329, Glu 90, Pro 163, Gln 141, Gln 68, Lys 134, Leu 350, Tyr 289, Ala 89, Phe 316, Leu 388, Thr 131, ILeu 54, Ala 113, Ala 231, Cys 338, Leu 376, His 235, Val 392, ILeu 9, Leu 95, Asn 74, Glu 142, Leu 335, ILeu 378, Glu 170, Asn 383
7	Leu 210, Arg 174, Pro 409, Trp 20, Leu 232, His 332, Thr 7, Phe 262, Asn 362, Arg 209, Ala 31, Gly 349, Arg 359, ILeu 295, Ala 202, Ser 36, Lys 402, Asn 114, Lys 101, Leu 175, ILeu 147, His 198, Phe 286, Gln 345
8	Thr 129, Lys 109, Ala 370, Tyr 162, Phe 188, Gln 222, Asn 351, Ser 30, Ala 93, Asp 26, His 118, Leu 160, Asn 164, Phe 385, Arg 390, Lys 28, Gln 106, Tyr 192, Pro 393
9	ILeu 422, Ala 34, His 355, Val 38, Glu 16, Thr 412, Glu 195, Glu 236, Ala 167, Asn 372, His 23, Trp 110, Gly 269, Asn 44, Asp 348, Val 414, Gln 364, Gly 384, Pro 40, Gly 18, Asp 423, Ala 369, Ser 406, Leu 19, Gln 77, Tyr 343, Ser 373, Ala 410, Ala 4, Met 39, Gly 400, Pro 14, Ser 45, Tyr 424, Gln 25, Trp 105, Gln 102, Tyr 396, His 234, Gly 344, Arg 426, Glu 420, Leu 416, Gly 51, Glu 43, Leu 302, Glu 17, Asp 33, Gln 418, Trp 75, Glu 417, Gln 72, Asp 365, Glu 382, Glu 166, Lys 425, Ala 421, Leu 94, His 97, Pro 419, Gln 408, ILeu 41, Gly 37, Glu 49, Trp 146, Thr 413, Asn 347, Lys 199, Tyr 415, Gln 411, Gln 35

Table 3.A2. Effect of the protein stability upon mutation in the hotspot residues

Residue No.	Residue	Overall stability	Substituted amino acid
162	Tyr	Stabilizing	-
		Destabilizing	Ala, Val, Leu, ILeu, Met, Thr, Cys, Asp, Glu, Asn, Gln, Ser, Gly, Arg, His, Trp, Phe, Pro, Lys
129	Thr	Stabilizing	Ser, Met, Lys, Cys, Asp
		Destabilizing	Ala, Val, Leu, ILeu, Glu, Asn, Gln, Arg, His, Pro, Trp, Tyr, Phe, Gly
286	Phe	Stabilizing	Gly, Ala, Val, Pro, Ser, Thr, Gln, Lys, Asn, Cys, Glu, Asp, Arg, His
		Destabilizing	Met, Leu, ILeu, Trp, Tyr
95	Leu	Stabilizing	Trp, Ser
		Destabilizing	Ala, Val, ILeu, Glu, Asn, Gln, Arg, His, Pro, Tyr, Phe, Gly, Met, Lys, Cys, Asp, Thr
131	Thr	Stabilizing	Ala, Leu, ILeu, Pro, Trp, Phe, Glu
		Destabilizing	Val, Lys, Asp, Arg, His, Asn, Gly, Cys, Met, Ser, Tyr, Gln
186	Phe	Stabilizing	Met, Pro, Trp, Ser, Thr, Lys, Asn, Cys, Asp, His
		Destabilizing	Ala, Val, Gly, Leu, ILeu, Arg, Tyr, Glu, Gln
91	Arg	Stabilizing	Ala, Met, Thr, Phe, Lys, Glu, Asp
		Destabilizing	Val, Leu, ILeu, Gly, Gln, Asn, Ser, Pro, His, Trp, Tyr, Cys
126	Ser	Stabilizing	Met, Pro, Gln, Lys, Asn, Glu, Asp, Arg, His Ala, Val, Gly, Leu, ILeu, Phe, Trp, Tyr, Cys,
		Destabilizing	Thr
82	Phe	Stabilizing	Ala, Val, Leu, Pro, Trp, Gln, Lys, Tyr, Cys, Glu, Asp, Arg, His
		Destabilizing	ILeu, Gly, Met, Ser, Asn, Thr

Table 3.A3. Binding energy scores and the number of clusters obtained from AutoDock for the WT and the mutants docked with cytosine and 5-FC

Mutant	Binding energy (kcal/mol)		No. of Clusters	
	With cytosine	With 5-FC	With cytosine	With 5-FC
F82A	-4	-3.92	1	3
F82C	-4.2	-4.21	11	13
F82D	-4.11	-3.91	2	2
F82E	-4.02	-3.81	6	6
F82H	-4.24	-4.03	4	1
F82K	-3.82	-3.8	1	3
F82L	-4.14	-3.98	24	10
F82P	-4.32	-3.79	6	2
F82Q	-3.96	-3.91	21	5
F82R	-4.34	-3.98	7	5
F82V	-4.58	-4.15	15	12
F82W	-4.14	-4.03	7	4
F82Y	-3.81	-3.37	2	1
R91A	-4.08	-3.98	9	3
R91D	-4.47	-3.98	10	9
R91E	-3.85	-3.87	2	7
R91F	-4.36	-3.98	19	11
R91K	-4.32	-4.11	18	6
R91M	-4.46	-4.18	16	12
R91T	-3.92	-4	1	3
L95S	-4.26	-3.99	1	4
L95W	-	-	-	-
S126D	-3.99	-3.8	3	7
S126E	-4.41	-4.07	14	15
S126H	-4.28	-4.23	16	6
S126K	-4.35	-4.11	6	1
S126M	-4.43	-4.04	8	6

Mutant	Binding energy (kcal/mol)		No. of Clusters	
	With cytosine	With 5-FC	With cytosine	With 5-FC
S126N	-4.17	-4.19	14	1
S126P	-4.05	-3.98	6	8
S126Q	-4.49	-4.24	11	8
S126R	-	-3.6	-	1
T129C	-4.3	-4.26	24	6
T129D	-4.07	-3.89	12	1
T129K	-3.87	-3.48	3	2
T129M	-3.64	-3.62	2	3
T129S	-4.05	-3.87	15	13
T131A	-4.29	-4.1	1	8
T131E	-4.35	-3.97	8	13
T131F	-4.21	-4.08	2	8
T131I	-3.74	-3.63	1	4
T131L	-4.09	-3.72	2	7
T131P	-4.41	-4.08	9	4
T131W	-3.78	-3.62	2	3
F186C	-4.46	-4.09	12	5
F186D	-4.34	-4.05	7	12
F186H	-4.53	-4.3	21	17
F186K	-4.16	-3.84	13	10
F186M	-4.26	-3.96	13	4
F186N	-4.38	-3.7	3	2
F186P	-4.47	-4.23	16	13
F186R	-4.33	-3.91	12	5
F186S	-4.28	-4.11	15	8
F186T	-4.47	-4.02	8	5
F186W	-4.18	-4.25	6	6
F286A	-4.44	-4.19	4	5
F286C	-4.44	-4.1	18	6

Mutant	Binding energy (kcal/mol)		No. of Clusters	
	With cytosine	With 5-FC	With cytosine	With 5-FC
F286D	-4.46	-4.24	17	6
F286E	-4.07	-4.02	6	4
F286G	-4.4	-4.1	8	7
F286H	-4.54	-4.49	37	4
F286K	-4.09	-4.04	9	11
F286N	-4.15	-4.07	9	7
F286P	-3.95	-3.73	14	6
F286Q	-3.99	-3.81	13	8
F286R	-4.11	-3.87	2	3
F286S	-4.67	-4.42	13	10
F286T	-4.2	-3.88	7	8
F286V	-4.52	-4.06	9	7
WT	-4.02	-4.03	19	18

Table 3.A4. The electrostatics and van der waals energy scores obtained from AutoDock for the mutants docked with cytosine and 5-FC

Mutant	Electrostatics energy (kcal/mol)		Van der waals energy (kcal/mol)	
	With cytosine	With 5-FC	With cytosine	With 5-FC
F82A	-0.36	-0.31	-3.94	-3.91
F82C	-0.29	-0.29	-4.2	-4.21
F82D	-0.35	-0.24	-4.06	-3.96
F82E	-0.4	-0.24	-3.92	-3.87
F82H	-0.4	-0.28	-4.14	-4.05
F82K	-0.46	-0.33	-3.65	-3.77
F82L	-0.47	-0.31	-3.96	-3.97
F82P	-0.51	-0.24	-4.11	-3.85
F82Q	-0.28	-0.39	-3.98	-3.82

Mutant	Electrostatics energy (kcal/mol)		Van der waals energy (kcal/mol)	
	With cytosine	With 5-FC	With cytosine	With 5-FC
F82R	-0.48	-0.25	-4.16	-4.03
F82V	-0.49	-0.3	-4.39	-4.15
F82W	-0.51	-0.13	-3.93	-4.2
F82Y	-0.16	-0.24	-3.94	-3.43
R91A	-0.3	-0.11	-4.07	-4.17
R91D	-0.46	-0.25	-4.31	-4.02
R91E	-0.11	-0.22	-4.04	-3.94
R91F	-0.44	-0.29	-4.22	-3.99
R91K	-0.44	-0.3	-4.18	-4.11
R91M	-0.51	-0.28	-4.24	-4.2
R91T	-0.28	-0.32	-3.94	-3.98
L95S	-0.14	-0.24	-4.41	-4.05
L95W	-	-	-	-
S126D	-0.26	-0.23	-4.03	-3.86
S126E	-0.44	-0.3	-4.27	-4.08
S126H	-0.41	-0.47	-4.16	-4.06
S126K	-0.5	-0.46	-4.15	-3.94
S126M	-0.56	-0.35	-4.16	-3.99
S126N	-0.49	-0.38	-3.98	-4.11
S126P	-0.49	-0.3	-3.85	-3.97
S126Q	-0.49	-0.27	-4.29	-4.27
S126R	-	-0.21	-	-3.68
T129C	-0.54	-0.39	-4.05	-4.17
T129D	-0.44	-0.46	-3.93	-3.74
T129K	-0.28	-0.27	-3.89	-3.51
T129M	-0.28	-0.26	-3.65	-3.65
T129S	-0.33	-0.24	-4.01	-3.93
T131A	-0.37	-0.25	-4.22	-4.14
T131E	-0.51	-0.36	-4.14	-3.91

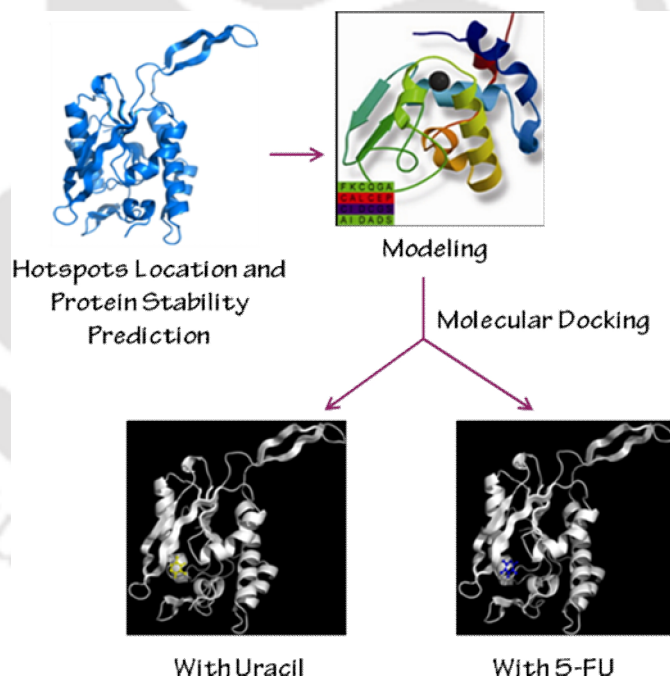
Mutant	Electrostatics energy (kcal/mol)		Van der waals energy (kcal/mol)	
	With cytosine	With 5-FC	With cytosine	With 5-FC
T131F	-0.48	-0.36	-4.02	-4.02
T131I	-0.26	-0.27	-3.78	-3.67
T131L	-0.32	-0.3	-4.07	-3.72
T131P	-0.44	-0.24	-4.27	-4.13
T131W	-0.04	-0.27	-4.04	-3.65
F186C	-0.44	-0.35	-4.32	-4.04
F186D	-0.48	-0.25	-4.16	-4.1
F186H	-0.51	-0.3	-4.31	-4.3
F186K	-0.48	-0.18	-3.97	-3.95
F186M	-0.57	-0.24	-3.99	-4.02
F186N	-0.49	-0.21	-4.19	-3.79
F186P	-0.45	-0.28	-4.32	-4.25
F186R	-0.52	-0.23	-4.11	-3.98
F186S	-0.4	-0.23	-4.18	-4.17
F186T	-0.54	-0.25	-4.23	-4.07
F186W	-0.28	-0.33	-4.19	-4.22
F286A	-0.43	-0.24	-4.3	-4.25
F286C	-0.49	-0.39	-4.25	-4.01
F286D	-0.48	-0.36	-4.27	-4.17
F286E	-0.25	-0.28	-4.11	-4.04
F286G	-0.51	-0.21	-4.19	-4.19
F286H	-0.46	-0.4	-4.38	-4.39
F286K	-0.29	-0.34	-4.1	-3.99
F286N	-0.16	-0.24	-4.29	-4.13
F286P	-0.44	-0.19	-3.81	-3.84
F286Q	-0.31	-0.24	-3.98	-3.86
F286R	-0.43	-0.23	-3.98	-3.94
F286S	-0.53	-0.3	-4.44	-4.42
F286T	-0.42	-0.15	-4.07	-4.03

Mutant	Electrostatics energy (kcal/mol)		Van der waals energy (kcal/mol)	
	With cytosine	With 5-FC	With cytosine	With 5-FC
F286V	-0.41	-0.31	-4.4	-4.04
WT	-0.05	-0.05	-4.26	-4.27



Molecular Cloning, Functional Assays and Bioinformatics studies on E. coli Uracil PhosphoRibosylTransferases (UPRT)

The molecular cloning, expression and purification of the UPRT is presented in this chapter. The chapter also describes the in silico site-directed mutagenesis studies performed over the UPRT protein.



Chapter 4

MOLECULAR CLONING, FUNCTIONAL ASSAYS AND BIOINFORMATICS STUDIES ON *E.COLI* URACIL PHOSPHORIBOSYLTRANSFERASE (UPRT)

4.1. Introduction

Suicide gene therapy is an alternative method to prevail the detrimental effects exerted by the chemotherapeutic drugs used to treat cancer owing to their nonspecific targeting of the normal cells (Morrow et al., 1989; Yeh et al., 2009; Meyers et al., 2008; Sitzia et al., 1997; Morrow et al., 1999). For example, the drug 5-Fluorouracil (5-FU) is being used to treat the patients with cancer for more than 50 years (Heggie et al., 1987). Though the drug is acting as a potent candidate; the adverse lethal effects induced by this drug remain a critical concern (Jhonson et al., 1999; Choi et al., 2001; Han et al., 2008; Ciccolini et al., 2010). *E. coli* cytosine deaminase (CD) with the administration of the prodrug 5-fluorocytosine (5-FC) is more effective because of the elevated bystander effect achieved by the diffusion of the toxic product 5-FU, which in turn overcomes the requirement of gap junction proteins (Kuriyama et al., 1995). Analogous to this system, UPRT converts the drug 5-FU into 5-FUMP, which is anabolised to further toxic metabolites to trigger cancer cell death. Adenoviral mediated transfer of UPRT gene to colon cancer cells augmented the sensitivity of cancer cells to 5-FU (Kawamura et al., 2000). Nevertheless, the UPRT/5-FU system depends on the cell-to-cell communication to exhibit the bystander effect because of the membrane impermeability of 5-FUMP (Kawamura et al., 2000). In spite of the above advantageous features, the major obstruction remains the lower binding affinity of the prodrug (s) than the natural substrates at the active site of these enzymes (Kievit et al., 1999; Schumacher et al., 1998). As a consequence, random and alanine scanning mutagenesis studies were performed over the CD protein and explored the

superior catalytic efficiency of the restructured mutant D314A (Mahan et al., 2004; Mahan et al., 2004). Thus, the development of novel UPRT mutants could lead to the improvement of therapeutic efficacies of the UPRT/5-FU based suicide gene therapy applications.

4.2. Outline of the Research Work

(1) The UPRT gene was cloned into the bacterial expression vector, pGEX-4T2.

(2) The UPRT enzyme was over expressed and purified in a single step to near homogeneity with the ease of fused N-terminal GST tag.

(3) The preliminary enzyme assay was performed with uracil and 5-FU for the UPRT enzyme.

(4) The *in silico* SDM was carried out over the UPRT protein to create novel UPRT mutants which could have higher binding affinity with the drug, 5-FU.

(5) The bioinformatics study disclosed the UPRT mutants which displayed improved binding affinity towards 5-FU as with the wild-type (WT); thereby could act as potent candidates in the prospect of suicide gene therapy applications.

4.3. Experimental Section

4.3.1. Materials

pORF codA::upp vector was procured from *Invivogen*. Oligonucleotides for the amplification of the UPRT gene, Glutathione-agarose beads, L-glutathione-reduced, Bradford reagent, uracil, 5-Fluorouracil, Guanosine 5'-triphosphate (GTP), 5-Phospho-D-ribose 1-diphosphate pentasodium salt (PRPP) were purchased from Sigma Aldrich Pvt. Ltd., USA. Restriction enzymes used for cloning were attained from New England Biolabs (Beverly, MA).

4.3.2. Bacterial strains

E. coli DH5 alpha was used as a host strain for cloning and *E. coli* BL21 (DE3) strain was used for the expression studies.

4.3.3. pGEX-UPRT vector construction

The UPRT gene was amplified from pORF codA::upp (*Invivogen*) vector using the primers 5'CGCGGATCCATGGCTAAGATCGTGG3' and 5'CCGCTCGAGGAGCTAGCGAATTTCG3' with Bam HI and Xho I overhangs. The PCR conditions applied were as follows: denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 1 min. The amplified UPRT gene fragment with the size of 630 bp was initially cloned in pGEMT-Easy vector (*Promega*) and further subcloned into a bacterial expression vector, pGEX-4T2 with N-terminal GST tag and named as pGEX-UPRT. **Figure 4.1** indicates the schematic of the steps followed for the cloning of UPRT gene in the pGEX-4T2 vector.

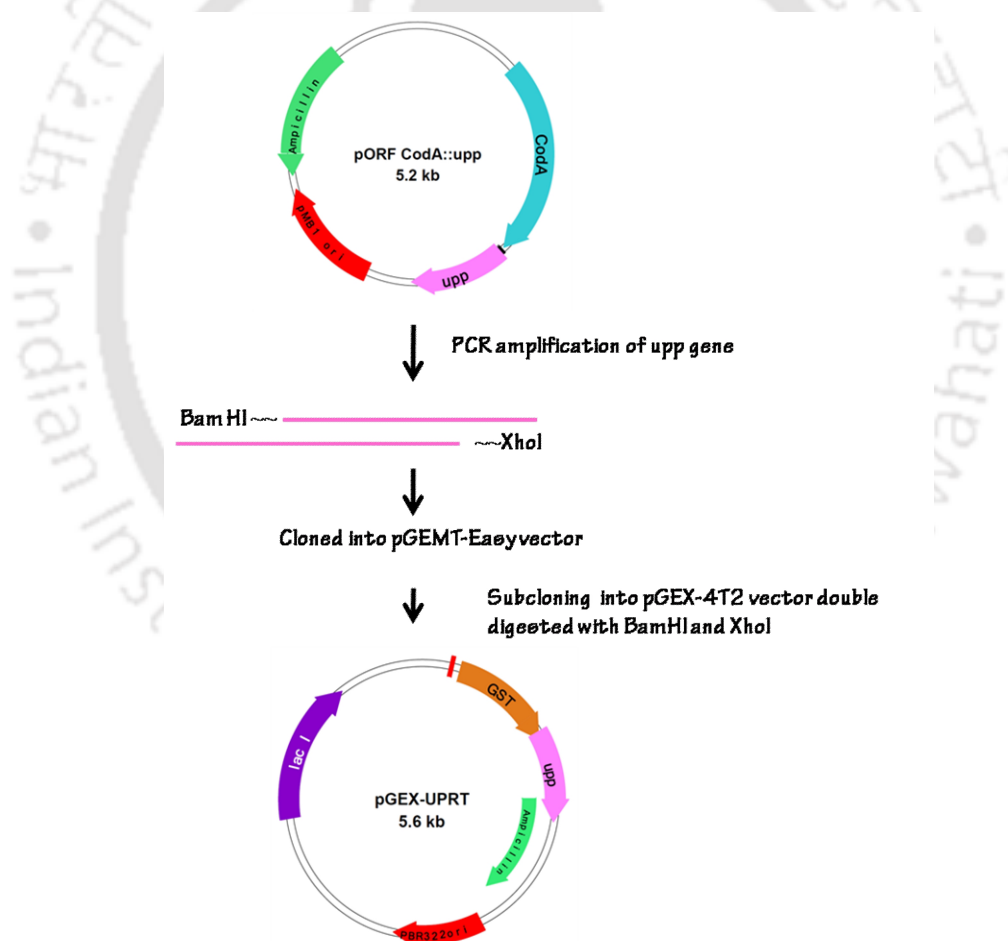


Figure 4.1. Schematic representation of the different steps involved in the cloning of UPRT.

4.3.4. Sequencing Analysis

The cloned UPRT gene was confirmed by DNA sequencing analysis prior to the expression studies.

4.3.5. Expression of the UPRT enzyme

The recombinant UPRT enzyme was over expressed in the *E. coli* strain BL21 (DE3) *tdk*⁻. A 50 mL culture of LB medium was inoculated with 1% primary culture and induced for overnight with 0.5 mM IPTG at 37°C. The culture was spun at 6000 rpm for 10 min at 4 °C, and the cell pellet got resuspended with pre chilled 1× PBS (pH 7.4) buffer. The obtained cell suspension was disrupted at 20 kpsi in a constant cell disruptor. A final concentration of 1% triton × 100 was added to the disrupted sample and incubated for 30-40 min on ice. Then the sample was spun at 15,000 rpm for 30 min at 4°C and the supernatant which contains the soluble fraction of the UPRT protein was collected. The crude was allowed to pass through a 0.45µ membrane filter to get rid of any debris or other contaminants.

4.3.6. Purification of the UPRT enzyme

The affinity column which has the immobilized glutathione-agarose beads was equilibrated with 1× PBS buffer, pH 7.4. The crude filtrate was added to the beads and incubated for 30 min to 1 h at 4°C to allow the binding of protein of interest to the resin to a greater extent. Then, the column was washed with ten column volumes of wash buffer (1× PBS, pH 7.4) to drain out the non-specific proteins. The purified protein fractions were eluted with elution buffer of 10 mM glutathione reduced in 50 mM Tris-HCl, pH 9.5. Purified fractions were examined using SDS-PAGE, and silver staining was performed to visualize the homogeneity of the purified fractions. After that, the protein was dialyzed in dialysis buffer (1× PBS, pH 7.4) at 4°C for overnight. The concentration of the purified protein was determined using Bradford method with BSA as standard.

4.3.7. Western blot

A purified fraction of the protein of interest was run in SDS-PAGE; after that, the blotting was done in a PVDF membrane. After blotting, the membrane was washed

with PBS buffer for three times. The membrane was blocked with 2% milk + 0.1% Tween-20 solution for overnight. Then, the membrane was again washed with PBS buffer for a while and incubated with primary antibody (mouse monoclonal anti-GST antibody) which was made in 2% milk solution for 2 h at room temperature. After 2 h of incubation, the membrane was washed with PBS buffer and incubated with the HRP-conjugated secondary antibody (prepared in 2% milk solution) for 2 h at room temperature. After incubation, the membrane was developed with H_2O_2 + 3, 3'-Diaminobenzidine (DAB) solution.

4.3.8. Enzymatic assay of the UPRT with uracil

UPRT enzyme assays were performed using the spectrophotometer based method (Natalini et al., 1978). A main stock of 12.5 mM uracil was prepared in $1\times$ PBS, pH 7.4. The reaction mixture which had 0.5 mM of GTP, 0.5 mM PRPP, 5% MgCl_2 , required concentration of uracil was pre incubated at 37°C ; after addition of the enzyme, the sample was collected each 1 min for a time period of 10 min. The degradation of the uracil and the formation of UMP were monitored at 265 nm and 280 nm respectively. The procedure was repeated for varying concentrations of uracil. The kinetic parameters were determined using Lineweaver-Burk plots. The catalytic efficiency (K_{cat}/K_m) towards uracil was also found out for UPRT enzyme.

4.3.9. Enzymatic assay of the UPRT with 5-FU

Kinetic values for the UPRT enzyme were obtained with 5-FU as well. A main stock of 50 mM 5-FU was made in DMSO. The working stock of 5-FU was prepared with $1\times$ PBS, pH 7.4. The reaction mixture containing the desired concentration of 5-FU, 0.5 mM GTP, 0.5 mM PRPP, 5% MgCl_2 and buffer was pre warmed at 37°C . The reaction was initiated instantaneously after addition of the enzyme. A small aliquots of the reaction mixture were collected every 1 min over a 10 min time period and the reaction was stopped using 0.1 M HCl. Absorbance readings were taken at 255 nm to monitor the degradation of 5-FU. As with uracil the kinetic parameters were calculated using the double reciprocal plots.

4.3.10. Identification of Hotspots of the UPRT

Mutagenesis hotspots present over the single subunit of UPRT (208 amino acid residues) were identified by HotSpot wizard. The web server takes the PDB file of the protein (code: 2EHJ) as input and allots the mutability scores from 1-9 to each and every residue present over the protein. Based on the protein of interest's structural, functional and evolutionary conservation, the mutagenesis hotspot residues will be located (Pavelka et al., 2009).

4.3.11. Protein stability prediction and Homology modeling

Protein stability upon point mutation in the hotspot residues was predicted using CUPSAT (Parthiban et al., 2006). The obtained stable mutants were homology modeled by taking WT UPRT (2EHJ) as a template using MODELLER (Eswar et al., 2006) program. Five iterations were set to generate models for each mutant. The model which got the lowest DOPE (Discrete Optimized Protein Energy) score was considered as the best model and used for subsequent docking analysis.

4.3.12. Macromolecule and ligand preparation

The crystal structure of UPRT (2EHJ) was acquired from PDB and the polar and non polar hydrogen atoms were added up using AutoDock (Morris et al., 1998) to set up the macromolecule for further docking studies. The structures of the ligands uracil and 5-FU were created with the Java Molecular Editor (JME) (Ertl) and assigned to PRODRG (Schüttelkopf et al., 2004) to prepare the ligands in PDB format which is an essential requirement for docking.

4.3.13. Docking parameters used

Docking studies on UPRT with the ligands uracil and 5-FU was carried out using AutoDock (Morris et al., 1998) program. Grid box with the dimensions of $112 \times 110 \times 48$ and the grid spacing of 0.561 were remaining constant throughout the docking procedure. Lamarckian Genetic Algorithm (LGA) was applied to attain the different binding modes of the ligands which are energetically favorable. An individual population of 250, energy evaluations of 2500000, and a mutation rate of 0.02 were fixed as docking parameters. A number of 100 docking runs were performed for both

the ligands and the similar conformations of the ligand were clubbed together to get a single cluster. Maximum root mean square tolerance was set as 2 Å.

4.4. Results and Discussion

4.4.1. Clone confirmation of pGEX-UPRT vector

The UPRT gene cloned into the inducible bacterial expression vector was confirmed by double digestion with the restriction enzymes Bam HI and Xho I (**Figure 4.2**) and sequencing analysis. **Figure 4.2** denoting the released fragment of the UPRT gene corresponding to 630 bp size and the digested vector backbone with the size of 5 kb.

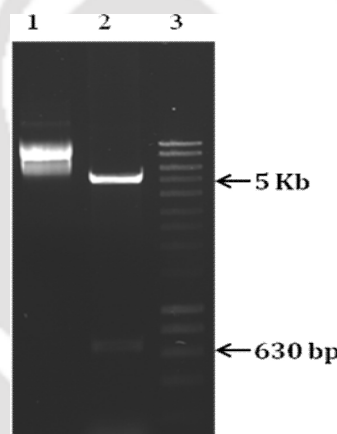


Figure 4.2. Clone confirmation of UPRT gene by restriction digestion analysis. Lane 1 denotes the undigested pGEX-UPRT vector, Lane 2 denotes the Bam HI / Xho I digested vector backbone of 5 kb and the released insert size of 630 bp, Lane 3 is the DNA HyperLadder.

4.4.2. Expression and Purification of UPRT enzyme

The overexpressed UPRT protein in the BL21 host strain was appeared as intense bands and had the size of 52 kDa. **Figure 4.3** shows the single bands of purified GST tagged UPRT and GST proteins corresponding to molecular weight of 52 kDa and 26 kDa respectively.

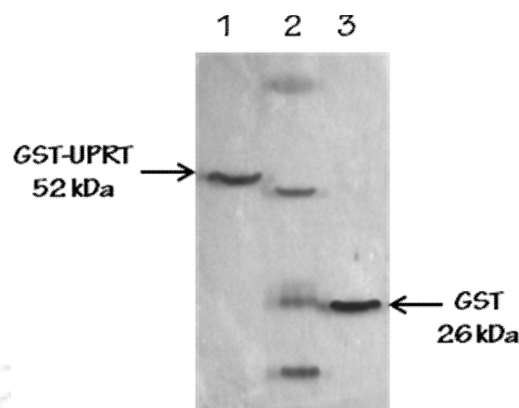


Figure 4.3. Purified fractions of the N-terminal GST tagged UPRT and GST enzymes. Lane 1 is the purified GST tagged UPRT protein, Lane 2 is the protein marker and Lane 3 is the purified GST protein.

4.4.3. Western blot analysis

Figure 4.4 indicating the anti-GST antibody detected GST tagged UPRT protein with the molecular weight of 52 kDa.

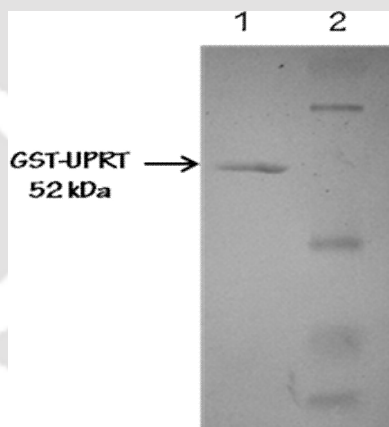


Figure 4.4. Western blot showing the presence of GST tagged UPRT enzyme. Lane 1 is the anti-GST detected GST tagged UPRT protein; Lane 2 is the prestained colorplus protein ladder.

4.4.4. UPRT assay with uracil and 5-FU as substrates

The preliminary UPRT enzyme assays were carried out with different concentrations of uracil and 5-FU based on spectroscopic methods. The kinetic parameters obtained were not exactly correlating with the previously reported values which could probably due to the lack of radio labeled substrates. Thus, the further characterization of the

recombinant UPRT enzyme is needed to attain its kinetic parameters towards its substrates uracil and 5-FU.

4.4.5. Hotspots Identification

The single subunit of WT UPRT was used as an input in HotSpot wizard. The mutability scores allocated for the residues over the UPRT protein is given in Appendix, **Table 4.A1**. The residues which are known to involve in substrate binding and stacking interactions such as Arg 78, Ala 134, Ileu 193 and Asp 199 were allotted with the lowest mutability scores and marked as immutable residues. Likewise, the conserved residues Ileu 76, Leu 77, Tyr 192, Ala 200 and Asp 202 were assigned with lower mutability scores since mutation in these residues could destabilize and inactivate the enzyme. The highest and the average mutability scores were ascribed to His 190, Ileu 194 and Glu 106, Glu 105, Lys 208, Gly 191, Met 101 residues respectively and these were chosen as hotspots, as alteration of any of these residues will not affect the catalytic activity of the enzyme. **Figure 4.5** illustrating the cartoon diagram of single subunit UPRT protein with the mutagenesis hotspot residues which are indicated with different colors.

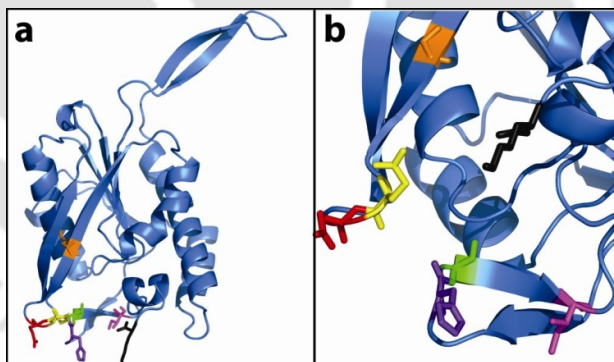


Figure 4.5. (a) Hotspot residues selected for *in silico* mutagenesis (b) Enlarged image of the protein which explicitly indicating the hotspots. Ileu 194 (in hotpink), Glu 105 (in yellow), Glu 106 (in red), Gly 191 (in green), Met 101 (in orange), His 190 (in purple), Lys 208 (in black). Both parts of the figure were made with PyMOL.

4.4.6. CUPSAT prediction and modeling

The protein stability upon substitution of the hotspot residues with rest of the 19 possible amino acids was tested using CUPSAT. The overall stability of the protein

upon single amino acid substitutions in the hotspot residues is summarized in the Appendix, **Table 4.A2**. MODELLER 9.9 was employed for the construction of the PDB structures of the stable mutants.

4.4.7. Docking of WT UPRT with uracil

The ligand uracil was docked with 54 mutants using the docking parameters mentioned previously and the resulting binding free energy scores are given in Appendix, **Table 4.A3**. Molecular docking of the mutants with uracil disclosed that there are 19 mutants which did not permit the ligand to bind to their active site. Moreover, upon superposition of WT and all of these mutants, structural alterations in the substrate binding as well as substrate stacking residues were noted. The list of mutants which lost their activity towards the substrate uracil is given in Appendix, **Table 4.A3**. As an example, only two mutants (M101A and M101G) amongst the 19 were shown in the **Figures 4.6a, 4.6b**. Previous studies on the crystal structure of *T. gondii* UPRT demonstrated that the residues Met 166, Tyr 228 (corresponding to Met 132, Tyr 192 of UPRT respectively) are involved in substrate stacking interactions and allowing the tight binding of the substrate in the active site; thus, these residues should be positioned in proper orientation to maintain the structural stability of the enzyme (Schumacher et al., 1998). The study further demonstrated that the residue Asp 235 (Asp 199 in case of UPRT) is involved in positioning of Tyr 228 to stack over the substrate. Moreover, Arg 137 (similar to Arg 103 in UPRT) contributes the positive charge around the active site; thus, facilitates the binding of negatively charged substrate, PRPP (Schumacher et al., 1998). Therefore, the dislocation of residues Tyr 192, Met 132, Arg 103 and Asp 199 in the nineteen mutants could be the plausible reason for inactivation of the enzymes owing to the inability of the ligand to bind to their active site.

The key residues for the enzymatic activity of WT UPRT and the mutants (G191K, I194D and K208V) are exactly overlapping with each another (**Figures 4.6c-4.6e**). As a consequence, these mutants could contribute similar catalytic activity as compared to the WT towards the substrate uracil.

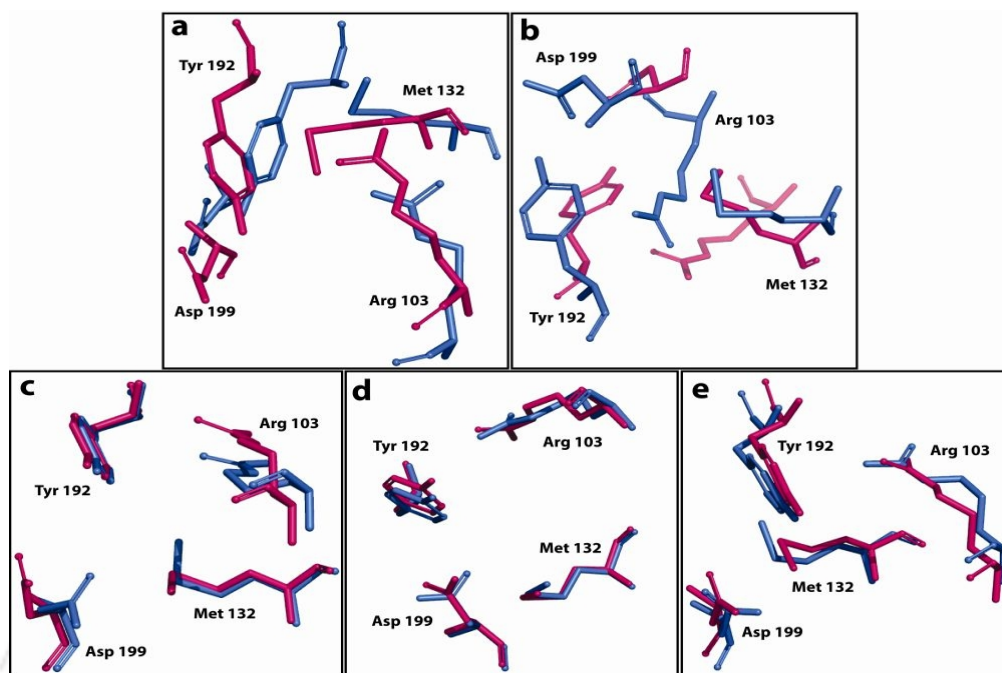


Figure 4.6. Superimposed images of WT and the mutants (a) M101A (b) M101G. (Bottom panel) Superimposed images of WT and the mutants (c) G191K, (d) I194D and (e) K208V. WT and mutant residues are signified as pink and blue sticks respectively. All parts of this figure were generated with PyMOL.

4.4.8. Docking of WT UPRT with 5-FU

The drug, 5-FU too docked with 54 mutants with the same docking parameters and the corresponding binding free energy scores are listed in Appendix, **Table 4.A3**. The substrate binding residues of 5-FU is similar to those of uracil. In this regard, parallel to uracil, 19 mutants out of 54 also failed to bind to the 5-FU in their active site (Appendix, **Table 4.A3**) because of the similar structural destabilization occurred in the mutant proteins (**Figures 4.6a, 4.6b**). As observed in case of the uracil, lack of any significant alterations in the active site residues upon superposition of WT and the mutant proteins (**Figures 4.6c-4.6e**) may attribute the mutants G191K, I194D and K208V to show similar activity towards 5-FU as with the WT protein.

4.4.9. Mutants showing enhanced binding affinity towards 5-FU than uracil

The mutants I194L, E106Y, M101I, E105Y, M101C and M101F acquired minimum docking scores and are presumed to show increased binding affinity towards 5-FU.

Table 4.1 shows the binding free energy scores acquired from AutoDock for these mutants with the ligands uracil and 5-FU.

Table 4.1. Binding free energy scores for uracil and 5-FU attained from AutoDock

Mutant	Binding Free Energy (kcal/mol)	
	Towards Uracil	Towards 5-FU
I194L	-3.92	-4.65
E106Y	-4.37	-4.41
M101I	-4.02	-4.14
E105Y	-3.8	-3.87
M101C	-3.73	-3.76
M101F	-3.71	-3.74
M101V	-	-3.76
I194Y	-	-3.55
E105D	-	-3.31

Hydrogen bond interactions attained, while the mutant I194L was docked with uracil and 5-FU are shown in **Figures 4.7a, 4.7b**. It can be seen from the **Figure 4.7a**, the oxygen atom of the Met 132 is bonded with N3 atom of the substrate uracil. From **Figure 4.7b**, the N1 of 5-FU is bonded with the oxygen atom of Met 132; the O2 atom of 5-FU making hydrogen bond with nitrogen atom of Val 139 and O4 of 5-FU is bonding with Nε2 of Arg 103. Besides interacting with Met 132, the additional hydrogen bond interactions with the conserved residues Val 139 and Arg 103 could lead the mutant I194L to show signs of acting as an incredible candidate to bind effectively with the drug 5-FU.

Identical hydrogen bond interactions were found when both the ligands were docked with the mutant E106Y (**Figures 4.7c, 4.7d**). The nitrogen atom of the Val 139 is forming hydrogen bond with O2 atom of uracil and 5-FU; In addition, oxygen atom of the Met 132 constitutes a hydrogen bond with N3 atom of both the ligands; however, the successful avoiding of the steric hindrance between the residue Ala 134

and the substrate 5-FU suggests this mutant to show improved binding affinity against 5-FU.

In case of mutant M101I, the oxygen atom of Met 132 is bonded with N3 atom of uracil; Nitrogen atom of Gly 136 contributes a hydrogen bond with O2 atom of uracil; Furthermore, the oxygen atom of Thr 135 is bonding with N1 atom of uracil. N1 of 5-FU with oxygen atom of Met 132; O2 with Nitrogen of Gly 136; N3 of 5-FU with oxygen atom of Thr 135 (**Figures 4.7e, 4.7f**).



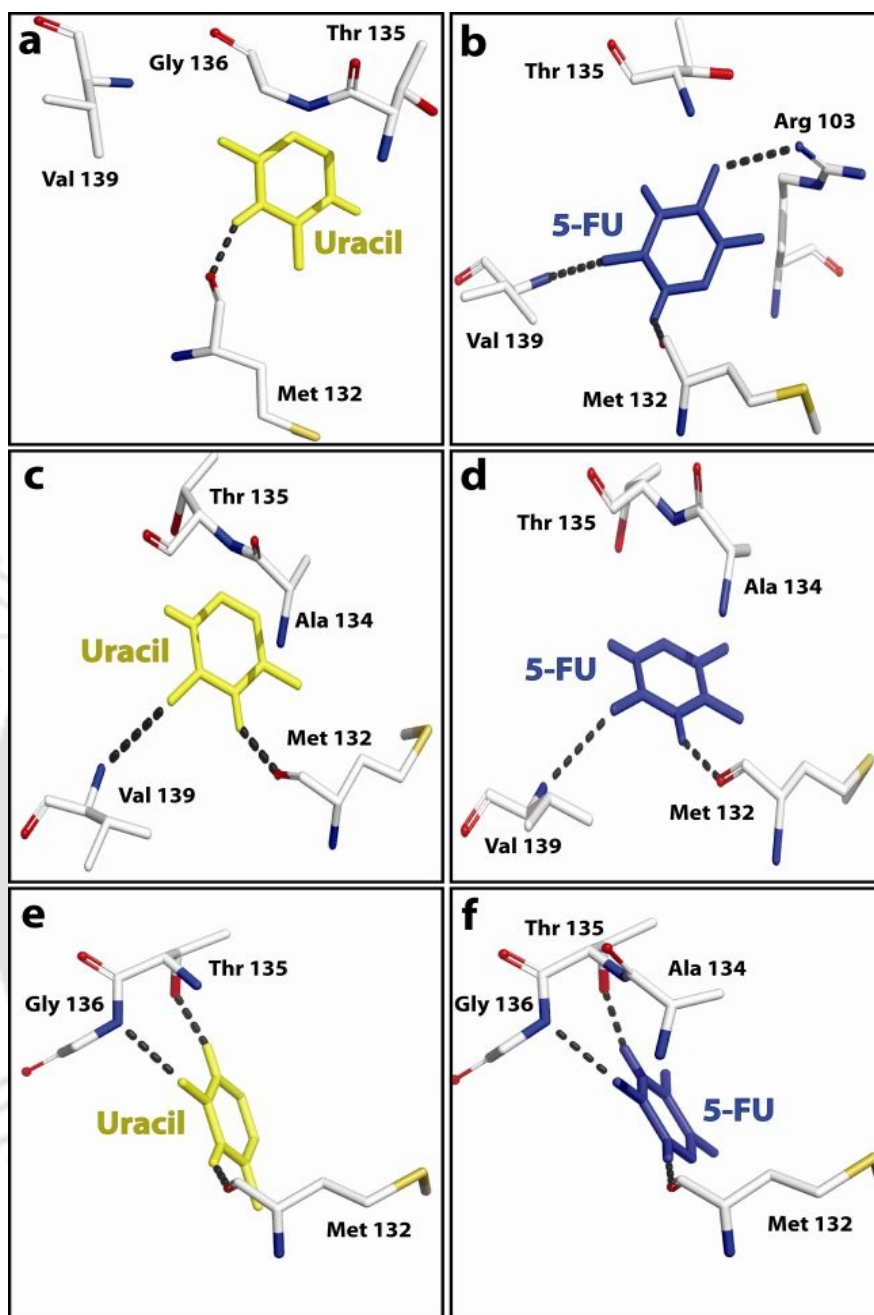


Figure 4.7. Hydrogen bond interactions and binding poses of uracil and 5-FU in (a, b) Mutant I194L, (c,d) Mutant E106Y, (e, f) Mutant M101I. Uracil and 5-FU are indicated as yellow and blue sticks respectively. Hydrogen bonds are displayed as black dashed lines. PyMOL graphics system was utilized to create this figure.

In the altered mutant E105Y, the N1 of uracil is bonding with oxygen atom of Met 132; O2 of uracil with nitrogen atom of Gly 136 (**Figure 4.8a**). Exactly similar interactions were seen while docking with 5-FU (**Figure 4.8b**). In case of the mutants M101C and M101F, the O4 of uracil is bonded with N δ of Arg 78; O2 of uracil with nitrogen of Gly 201; N1 of uracil with O μ 2 of Asp 199. The identical hydrogen bonds were noticed when 5-FU was used as a ligand (**Figures 4.8c-4.8f**).

Though the mutants M101I, E105Y, M101C and M101F can be characterized as superior candidates with 5-FU by docking scores, there is lack of apparent features between the binding modes of ligands as observed in case of the bCD mutant F186W (V. Kohila et al., 2012). The *in vitro* evaluation may reveal the cause for these mutants to show elevated binding affinity towards 5-FU than uracil.



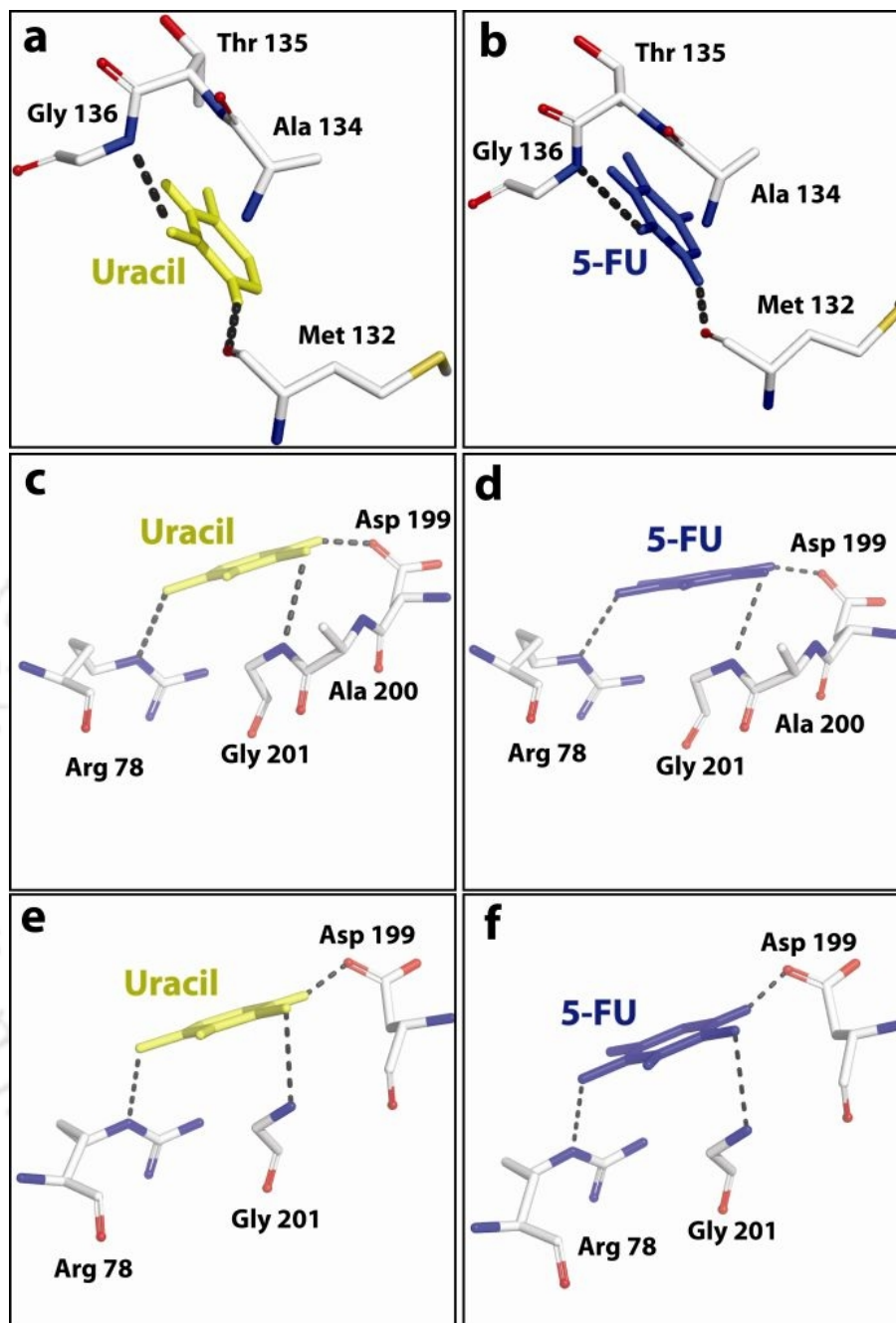


Figure 4.8. Hydrogen bond interactions and binding poses of uracil and 5-FU in (a, b) Mutant E105Y, (c, d) Mutant M101C, (e, f) Mutant M101F. Uracil and 5-FU are depicted as yellow and blue sticks respectively. Hydrogen bonds are shown as black dashed lines. PyMOL was used to generate this figure.

Remarkably, there are three mutants M101V, I194Y and E105D, which allowed only the drug 5-FU to bind at their active site and not the native substrate

uracil. It can be seen from the **Figure 4.9a** that the docking of the mutant M101V with 5-FU showed highly significant hydrogen bond interactions. The O4 atom of 5-FU is interacting with nitrogen atom of the Ala 134; N3 of 5-FU with oxygen atom of Met 132; O2 of 5-FU is bonded with nitrogen atom of Ser 138. As it can be observed from the **Figures 4.9b, 4.9c** in the reconstructed mutants I194Y and E105D, the N1 atom of 5-FU is hydrogen bonded with phenolic OH group of the Tyr 192 residue. The O4 atom of 5-FU is making hydrogen bond with nitrogen atom of Arg 103 (O4-NH) and N3 of 5-FU with CO of Arg 103. The crucial hydrogen bond interactions with the conserved residues which are involved in the substrate binding and stacking such as Ala 134, Met 132, Ser 138, Arg 103 and Tyr 192 may aid these mutants to show increased binding affinity towards the drug 5-FU. These mutants ought to be examined *in vitro* to authenticate the *in silico* findings which demonstrated the proficient binding of these mutants towards 5-FU.

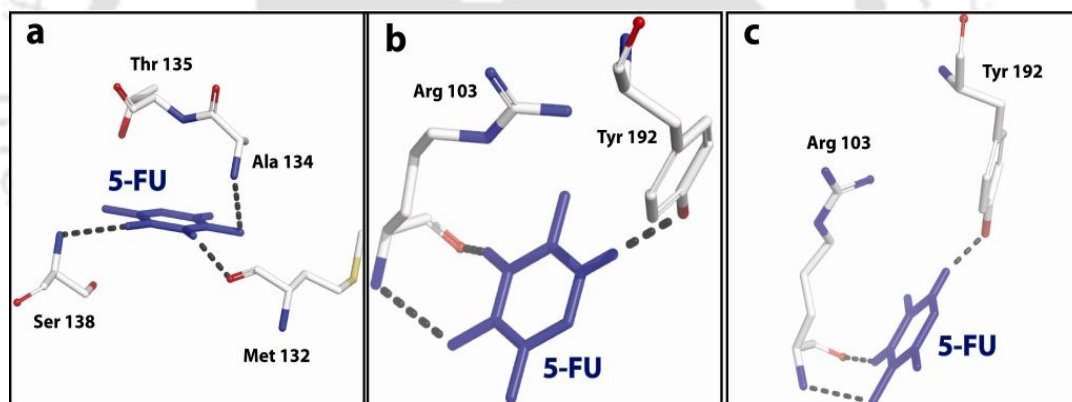


Figure 4.9. Hydrogen bond interactions and binding poses of 5-FU in (a) Mutant M101V, (b) Mutant I194Y, (c) Mutant E105D. 5-FU is exemplified as blue sticks. Hydrogen bonds are denoted as black dashed lines. PyMOL graphics system was used to draw this figure.

4.5. Conclusion

In the current study, the molecular cloning, expression, purification and preliminary enzyme assays of UPRT was demonstrated. Further, *in silico* SDM and molecular docking of UPRT was undertaken to achieve the goal of potentiating the UPRT/5-FU applications. Homology modeling and molecular docking studies were highly implemented to study the ligand-protein interactions. In this context, the emerged mutagenesis hotspots of UPRT were examined for their protein stability upon point mutation; the stable mutants were modeled and subsequently docked with uracil and 5-FU. The docking results were corroborated on the basis of the previous reports on ligand-protein interactions as well as the binding free energy scores. In conclusion, docking studies divulged the number of six mutants I194L, E106Y, M101I, E105Y, M101C and M101F which display improved binding affinity towards 5-FU. It is also worth noting that the docking studies revealed three mutants M101V, I194Y and E105D which bind exclusively with the drug 5-FU. Since the concomitant expression of bacterial CD and UPRT genes exhibits the heightened cytotoxicity, the combination of redesigned CD and UPRT mutants could sensitize the cancer cells more towards the drug; thereby leading to greater therapeutic efficiencies.

4.6. Appendix**Table 4.A1.** Mutability scores allotted to the amino acid residues present over the UPRT protein

Amino acid Residues	Mutability score allotted
Tyr 41, Pro 174, Thr 142, Leu 10, Val 176, Gly 56, Pro 75, Thr 207, Thr 107, Trp 57, Ser 138, Tyr 113, Gly 86, Gly 206, Leu 133, Leu 77, ILeu 76, Lys 147, Glu 42, Arg 29, Asp 202, His 8, Ala 200, Ala 79, Tyr 192, Phe 28, Gly 80, Gly 100, Gly 165, Gly 196, Gly 198, Gly 201, Leu 187, Leu 197, Pro 9, Pro 92, Pro 131, Pro 195, Lys 14, Glu 34, Arg 19, Arg 78, Arg 103, Arg 124, Asp 130, Asp 184, Asp 199, His 13, Met 132, Ala 94, Ala 134, ILeu 193, Thr 135	1
Lys 70, Asn 104, Gly 150, ILeu 23, Val 118, Gly 136, Leu 38, Leu 146, Ala 43, Val 98, Ala 161, Asn 188, Met 83, His 173, Ala 181, Gly 82, Gly 68, Ser 97, Lys 116, Glu 164, Pro 163, Asp 175, ILeu 154, Ala 162, Lys 203	2
Val 111, Val 74, Val 139, Leu 159, Gly 137, Thr 44, Val 99, Pro 110, ILeu 96, Ala 141, Lys 69, ILeu 143, Leu 108, Arg 95	3
Val 35, Gly 16, Ser 182, Asp 46, Asn 120, Ser 24, Val 160, Asp 85, Lys 208, ILeu 54, Val 11, Gly 191, Tyr 102, Leu 39, Met 101, Ala 45, Leu 31, ILeu 204, ILeu 128, Thr 72, Leu 178, Thr 25	4
Ser 93, Leu 157, Val 87, Glu 5, Glu 50, Lys 155, Lys 12, Phe 205, Glu 189, Leu 81, Glu 105, Val 129, Leu 88, Leu 47, Lys 26, Asn 90, Phe 114	5
Ala 172, Met 18, Val 158, Ala 126, Glu 30, Thr 180, ILeu 66, ILeu 166, Leu 15, Met 84, Pro 112, Glu 106, Thr 49, Val 91	6
ILeu 71, Glu 55, ILeu 140, ILeu 121, Cys 151, ILeu 183, Val 61, Gly 36, His 190, Leu 117, ILeu 3, ILeu 194, Ala 32	7
Lys 7, Val 73, ILeu 63, Glu 48, Asp 64, Val 52, Lys 1, Gln 21, Glu 123	8
Glu 62, Ser 119, Met 125, Val 4, Glu 170, Glu 20, Lys 171, Asp 22, Asp 122, Gln 185, Lys 51, Pro 60, Gly 186, Glu 89, Ser 37, Ser 33, Ala 167, Gln 65, Leu 145, Lys 148, Ala 149, Glu 177, Asn 58, Asp 144, Ser 152, Lys 2, Gly 59, Arg 27, Ala 168, Ser 153, Lys 67, Thr 53, Glu 109, Leu 169, Val 156, Leu 17, Tyr 179, Gln 115, Val 6, Leu 127, Thr 40	9

Table 4.A2. Protein stability prediction upon point mutation in the hotspot residues

Hotspot Residues	Overall stability	
	Stabilizing Residues	Destabilizing Residues
His 190	Gln	Val, Leu, ILeu, Lys, Met, Thr, Ser, Cys, Phe, Tyr, Trp, Gly, Glu, Ala, Asn, Asp, Arg, Pro
ILeu 194	Val, Leu, Thr, Phe, Tyr, Gly, Ala, Trp, Ser, Lys, Asn, Glu, Asp, Arg, His	Met, Cys, Gln, Pro
Glu 106	Val, Trp, Asp, Gln, Tyr, Asn, Cys	Leu, ILeu, Lys, Met, Thr, Ser, Phe, Gly, Ala, Arg, His, Pro
Glu 105	Pro, Trp, Lys, Val, Leu, ILeu, Phe, Tyr, Asn, Cys, Asp, Arg	Met, Thr, Ser, Gln, Gly, Ala, His
Lys 208	Val, Thr, Asn, Cys, Asp, His, Gly, Pro, Glu	Leu, ILeu, Met, Ser, Phe, Tyr, Trp, Gln, Ala, Arg
Gly 191	Lys	Val, Leu, ILeu, Met, Thr, Ser, Cys, Phe, Tyr, Trp, Glu, Gln, Ala, Asn, Asp, Arg, His, Pro
Met 101	Ala, ILeu, Trp, Phe, Tyr, Cys, Gly, Val, Leu	Lys, Thr, Ser, Glu, Gln, Asn, Asp, Arg, His, Pro

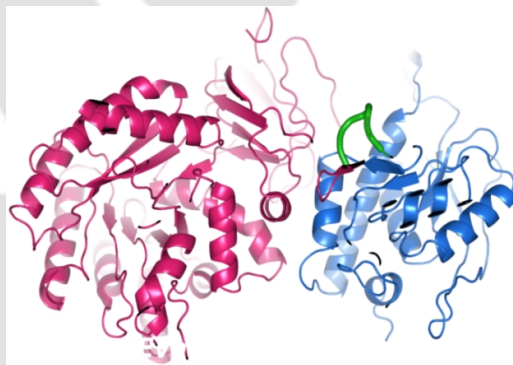
Table 4.A3. Binding free energy scores provided by AutoDock for all 54 mutants and the mutants who lost their binding affinity toward the ligands uracil and 5-FU

S.No	Mutant	Binding Free Energy (kcal/mol)		Binding Affinity	
		With Uracil	With 5-FU	With Uracil	With 5-FU
1	M101A	-	-	Lost	Lost
2	M101C	-3.73	-3.76		
3	M101F	-3.71	-3.74		
4	M101G	-	-	Lost	Lost
5	M101I	-4.02	-4.14		
6	M101L	-4.31	-4.25		
7	M101V	-	-3.76	Lost	
8	M101W	-4.03	-3.96		
9	M101Y	-3.86	-3.77		
10	E105C	-3.21	-3.14		
11	E105D	-	-3.31	Lost	
12	E105F	-4.03	-3.28		
13	E105I	-3.38	-3.32		
14	E105K	-4.2	-		Lost
15	E105L	-	-	Lost	Lost
16	E105N	-3.46	-3.34		
17	E105P	-4.23	-3.95		
18	E105R	-	-	Lost	Lost
19	E105V	-3.75	-3.62		
20	E105W	-4.05	-3.72		
21	E105Y	-3.8	-3.87		
22	E106C	-3.86	-		Lost
23	E106D	-	-	Lost	Lost
24	E106N	-3.57	-3.33		
25	E106Q	-4.29	-3.5		

S.No	Mutant	Binding Free Energy (kcal/mol)		Binding Affinity	
		With Uracil	With 5-FU	With Uracil	With 5-FU
26	E106V	-3.92	-3.45		
27	E106W	-	-	Lost	Lost
28	E106Y	-4.37	-4.41		
29	H190Q	-3.92	-3.81		
30	G191K	-3.61	-3.54		
31	I194A	-3.57	-		Lost
32	I194D	-3.68	-3.61		
33	I194E	-	-	Lost	Lost
34	I194F	-3.84	-3.74		
35	I194G	-4.25	-4.25		
36	I194H	-4.61	-4.22		
37	I194K	-	-	Lost	Lost
38	I194L	-3.92	-4.65		
39	I194N	-	-	Lost	Lost
40	I194R	-	-	Lost	Lost
41	I194S	-3.77	-3.7		
42	I194T	-	-	Lost	Lost
43	I194V	-4.23	-4.17		
44	I194W	-3.94	-3.84		
45	I194Y	-	-3.55	Lost	
46	K208C	-4.38	-3.66		
47	K208D	-	-	Lost	Lost
48	K208E	-	-	Lost	Lost
49	K208G	-	-	Lost	Lost
50	K208H	-	-	Lost	Lost
51	K208N	-	-	Lost	Lost
52	K208P	-3.93	-3.83		
53	K208T	-4.05	-3.81		
54	K208V	-3.73	-3.55		

Cloning, Expression and Purification of the bifunctional Cytosine deaminase – Uracil phosphoribosyltransferase protein

In this chapter, cloning, expression and purification of recombinant E. coli cytosine deaminase - uracil phosphoribosyltransferase fusion protein was demonstrated. Moreover, the functional activity assays and catalytic efficiencies of the recombinant enzyme towards its substrates were also reported.



I-TASSER predicted three dimensional structure of *E. coli* CD-UPRT fusion protein

Chapter 5

CLONING, EXPRESSION AND PURIFICATION OF THE BIFUNCTIONAL CYTOSINE DEAMINASE-URACIL PHOSPHORIBOSYLTRANSFERASE (CD-UPRT) FUSION PROTEIN

5.1. Introduction

Bacterial cytosine deaminase (CD) catalyzes the formation of uracil by deamination of the substrate cytosine. The uracil phosphoribosyltransferase (UPRT) enzyme further catalyzes the formation of UMP from the substrates uracil and PRPP. Thus, CD and UPRT act as important enzymes in the pyrimidine salvage pathway. The higher sensitization of the CD-UPRT transformed *E. coli* cells as compared with sole CD transformed cells towards 5-FC was demonstrated previously with an *E. coli* mutant strain, lacking the cytosine deaminase, cytosine permease and UPRT to mimic the mammalian cell environment and the functional activities of crude proteins within the transformed cell extracts were found with the substrates cytosine and uracil (Tiraby et al., 1998). In addition to that the enhanced cytotoxic effects of *E. coli* CD/5-FC (Gopinath et al., 2007; Kuriyama et al., 1995; Huber et al., 1994; Nishiyama et al., 1985) and UPRT/5-Fluorouracil (5-FU) (Kawamura et al., 2000; Kanai et al., 1998) methods have been already demonstrated in a variety of cancer cell types. Since some of the cancer cell types are resistant to the 5-FC treatment, the UPRT gene was fused with CD to achieve greater cytotoxicity (Chung-Faye et al., 2001; Khatri et al., 2006; Koyama et al., 2000; Adachi et al., 2000; Gopinath et al., 2008; De-zhi et al., 2009; Kambara et al., 2002). In such case, the CD enzyme catalyzes the formation of the toxic drug 5-FU from the deamination of the prodrug 5-FC. The drug 5-FU then further gets anabolized to its highly toxic products such as 5-FUMP, 5-FdUMP and 5-FUTP by the action of UPRT enzyme, which inhibits the DNA and RNA synthesis of the cancer cells (Gopinath et al., 2008).

Though the elevated sensitization of CD-UPRT hybrid system with the prodrug 5-FC has been previously observed in the total cell extracts of transformed

E. coli cells (Tiraby et al., 1998), the enzyme was not purified and the kinetic parameters were not examined towards the substrates. Hence, the functional activity assays of the purified CD-UPRT fusion enzyme is needed to achieve the redesigning of the enzyme for its improved kinetics towards the prodrug and further depict the enzyme as an invaluable candidate for cancer therapeutics.

5.2. Outline of the Research Work

- 1) The molecular cloning and expression of the recombinant *E. coli* CD-UPRT enzyme with the fused N-terminal GST tag was demonstrated.
- 2) The simple and efficient one step solubilization of the recombinant protein was achieved with the optimum concentration of an anionic detergent sarkosyl.
- 3) Further, the binding affinity of the GST tagged recombinant protein was enhanced by optimizing the ratio of the detergents sarkosyl and Triton $\times 100$.
- 4) Western blot was performed with the purified recombinant protein using anti-GST antibody.
- 5) In addition, the functional activity assays were carried out for the recombinant protein towards its substrates cytosine and 5-FC and the kinetic parameters were determined.

5.3. Experimental Section

5.3.1. Materials

pORF codA-upp vector was procured from *Invivogen*. Oligonucleotides used to amplify the CD-UPRT fusion gene; sarkosyl, glutathione agarose beads, glutathione-reduced and Bradford reagent were procured from Sigma Aldrich Pvt. Ltd., USA. Restriction enzymes used for the cloning experiment were purchased from NEB. PVDF membrane used for western blot analysis was purchased from Millipore. Antibody used for Western blot was obtained from Sigma Aldrich Pvt. Ltd., USA.

5.3.2. Bacterial strains and growth conditions

E. coli DH5 alpha and *E. coli* BL21 (dE3) host strains were used for the cloning and expression studies, respectively. Both strains were grown in *Luria-Bertani* broth at 37°C and 180 rpm.

5.3.3. Molecular cloning of CD-UPRT gene

The 2 kb fragment of CD-UPRT fusion gene was amplified from pORF codA::upp plasmid using the oligonucleotide sequences 5'CGCGGATCCATGGTGTCTGAATAACGC3' and 5'CCGCTCGAGGAGCTAGCGAATTTTCG3' with Bam HI and Xho I overhangs with the PCR conditions of denaturation at 94°C for 30s, annealing at 55°C for 1min, extension at 72°C for 1min. The amplified gene fragment was cloned into pGEMT-Easy vector and subcloned into pGEX-4T2, an inducible bacterial expression vector with N-terminal GST tag; and denoted as pGEX CD-UPRT. The schematic illustration for the sequential steps of cloning of CD-UPRT gene in the pGEX-4T2 vector is shown in **Figure 5.1**.

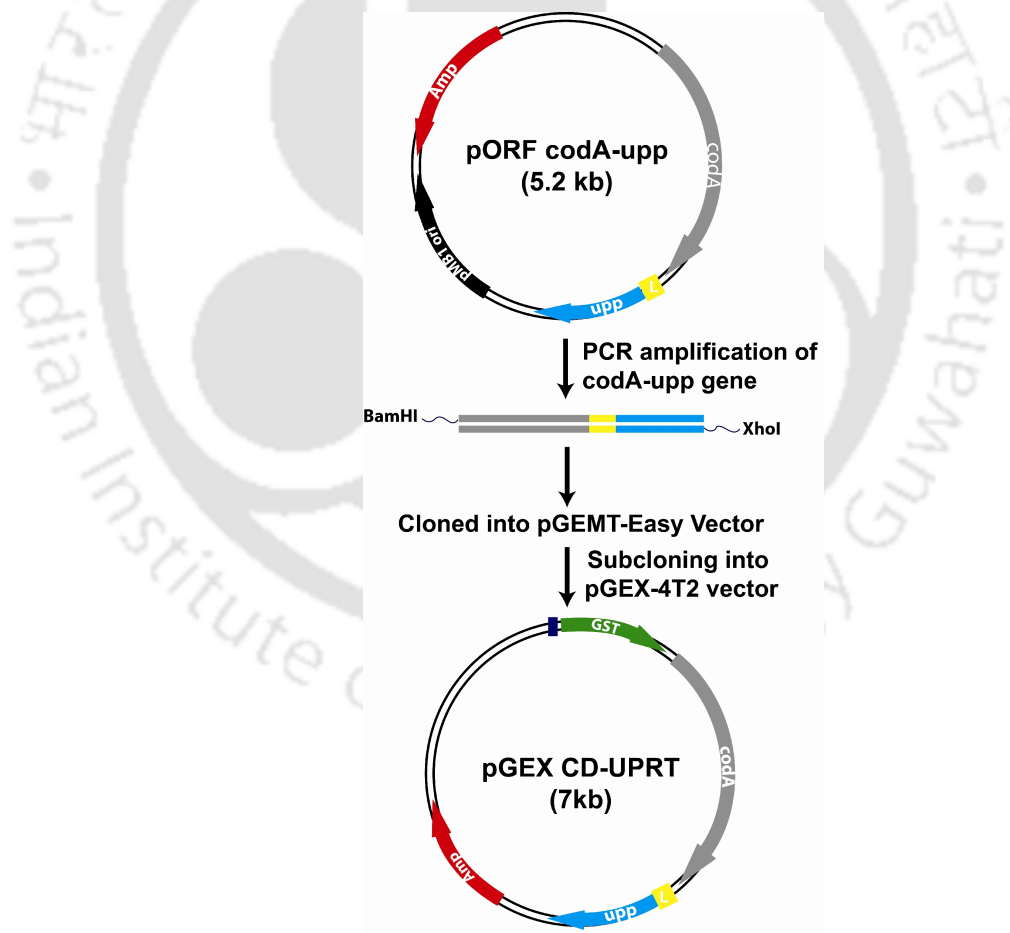


Figure 5.1. Schematic representation of the cloning steps involved in the construction of pGEX CD-UPRT vector.

5.3.4. DNA Sequencing

The CD-UPRT fusion gene was confirmed by DNA sequencing analysis. The sequencing reaction was carried out at Xcelris labs ltd, India.

5.3.5. Expression of the recombinant CD-UPRT enzyme

The recombinant fusion protein was expressed in the *E. coli* strain BL21 (DE3) *tdk* ⁻. The pGEX CD-UPRT construct was transformed into *E. coli* BL21 host strain. The BL21 transformed small scale culture (3 mL) was induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for overnight at 37 °C and sonicated for 10 min with the amplitude of 20 using sonicator (Hielscher UP200S Ultrasonic Processor). Then, the cleared lysate was centrifuged at 15,000 rpm for 20-30 min and the insoluble (pellet) and soluble (supernatant) fractions were checked in SDS-PAGE.

5.3.6. Solubilization of the inclusion bodies

N-sarkosyl, a well-known anionic detergent was used as the solubilizing reagent. The sarkosyl concentration was varied from 0.3 to 2% (Frankel et al., 1991; Burgess, 1996) to obtain the optimum concentration to be used. In brief, the cells were harvested from 50 mL culture medium at 6000 rpm for 10 min at 4°C. The cell pellet was resuspended in chilled 1× PBS (pH 7.4) and sarkosyl was added to a final concentration of 1%. The sample was mixed thoroughly and incubated in ice for 1 h. Then the cells were disrupted by sonication for 20-30 min time period with the amplitude set as 20. The supernatant containing the soluble fraction of the recombinant protein was separated by centrifugation at 15,000 rpm for 30 min at 4°C.

5.3.7. Optimization of the Sarkosyl: Triton ×100 ratio

Though the GST-tagged proteins can be solubilized more than 95% using sarkosyl, the presence of sarkosyl could hinder the binding affinity of the recombinant enzyme to the glutathione beads (Park et al., 2011). Previous studies have shown that nonionic detergents can sequester the anionic detergent sarkosyl by forming mixed micelles with sarkosyl; thus, enhancing the binding of the GST-fusion proteins to glutathione beads (McNally et al., 1991). Hence, different ratios of sarkosyl to Triton ×100 (S: T)

were applied to find out the optimum S: T ratio to achieve maximum binding of the recombinant protein with the glutathione beads.

5.3.8. Purification of the fusion protein

The recombinant protein was purified to homogeneity using affinity column containing immobilized glutathione agarose beads with ease of the fused N-terminal GST tag. In brief, the column was equilibrated with 1× PBS prior to sample loading. The crude soluble protein fraction was filtered using 0.45 µ membrane filter before loading into the column and incubated with the beads for 30-45 min. Then the beads were washed with five column volumes of 1× PBS solution. The fusion protein was eluted with the elution buffer (50 mM Tris-HCl, pH 9.5) and dialyzed at 4°C for overnight with 1× PBS and the protein sample was stored at -20°C.

5.3.9. SDS-PAGE analysis

The purified fraction of the recombinant protein was resolved with 12% SDS-PAGE to verify the intactness of the enzyme, during purification procedure and to find out the molecular weight of the protein. The gel was silver stained to examine the homogeneity of the enzyme.

5.3.10. Western blot analysis

Purified protein fractions resolved on 12% SDS-PAGE were transferred onto PVDF membrane. The membrane was blocked with 5% nonfat dried milk in PBS-T (1× PBS with 0.01% Tween 20) for overnight at 4°C with shaking; then incubated with anti-GST antibody for 1 h at room temperature. After washing with PBS-T, the membrane was incubated with horse-radish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature with gentle shaking. The protein bands were developed using the substrate DAB along with the catalyst H₂O₂.

5.3.11. Enzyme assay with cytosine and 5-FC

Enzyme assay using cytosine and 5-FC as a substrate was performed with the previously reported spectrophotometer based method with varying concentrations either of cytosine or the prodrug 5-FC (Ipata et al., 1978; Hayden et al., 1998). Sterile

water and PBS were used as buffers in case of 5-FC and cytosine, respectively. FeSO₄ with the final concentration of 10 µM was included in all the reaction mixtures. The absorbance readings were taken at 286 nm to monitor the degradation of the cytosine. The optical density readings were taken at 290 and 255 nm and the concentrations of 5-FC (in mM) were calculated using the equation 1 (Hayden et al., 1998). Assays were repeated at least five times to get the reproducible data. K_m and V_{max} were determined using the Lineweaver-Burk Plot. The turnover number of the fusion protein was calculated using equation 2. The total protein concentration [E] used in the assay was determined using the Bradford assay and was used for the calculation of K_{cat}. Finally the catalytic efficiency (K_{cat} / K_m) for each substrate was determined.

$$[5-FC] = 0.119(A_{290}) - 0.025(A_{255}) \quad 1$$

$$K_{cat} = V_{max} / [E] \quad 2$$

5.3.12. Homology modeling of CD-UPRT

The three dimensional structure of the recombinant CD-UPRT fusion protein was modeled using I-TASSER server (Zhang, 2008). The web server is an Internet service system for protein structure and function predictions. Protein 3D models were built based on multiple-threading alignments by LOMETS and iterative TASSER assembly simulations.

5.4. Results and discussion

5.4.1. Clone confirmation of the CD-UPRT fusion gene

The CD-UPRT gene was cloned in the pGEX-4T2 vector following the steps shown in **Figure 5.1**. The recombinant clone was confirmed by double digestion with Bam HI and Xho I restriction enzymes. The release of the 2 kb fragment of CD-UPRT gene can be clearly seen in the agarose gel in **Figure 5.2**.

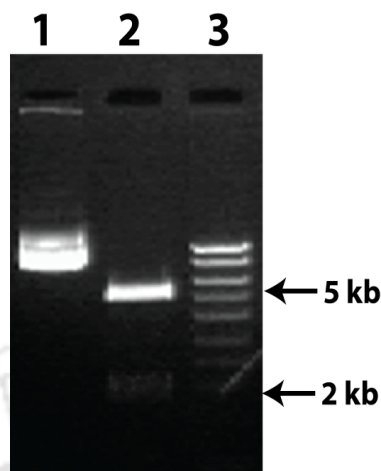


Figure 5.2. Agarose gel picture showing the restriction digestion analysis of pGEX CD-UPRT vector. Lane 1 represents the uncut pGEX CD-UPRT vector, Lane 2 shows the Bam HI/Xho I digested pGEX CD-UPRT showing the vector backbone of 5 kb and the insert of 2 kb, and Lane 3 is the DNA HyperLadder.

5.4.2. Solubilization of the inclusion body proteins

GST is commonly used as a tag protein for most of the recombinant proteins expressed in the prokaryotic system; so as to improve the solubility of the fused protein of interest. However, the tendency of some of the recombinant proteins to aggregate in the form of inclusion bodies remains as an obstacle in case of the GST tag based purification system (Frankel et al., 1991). To address this problem, many approaches have been followed to enhance the protein solubility using compounds such as urea, DTT, n-propanol, etc., (Rothel et al., 1997; Patra et al., 2000; Singh et al., 2012). Among them, sarkosyl has shown to act as a potent reagent for most of the proteins (Tao et al., 2010; Zou et al., 2009). Therefore sarkosyl was used to solubilize the CD-UPRT protein. The concentration of sarkosyl was varied from 0.3-2% to obtain the optimum concentration for the solubilization procedure. As it can be clearly seen from the **Figure 5.3**, the protein was getting solubilized with the increasing concentrations of the sarkosyl. The bands corresponding to the overexpressed CD-UPRT fusion protein started appearing in the supernatant (S) with the addition of the detergent sarkosyl. From **Figure 5.3** it is prominent that with the increase in the concentration of sarkosyl, the band intensity for the pellet (P) got decreased whereas, that in the supernatant increased indicating efficient solubilization of the CD-UPRT protein. Since the sufficient amount of soluble protein fraction was found at 1% of

sarkosyl, the same was used for rest of the experiments to minimize the deteriorate effects of sarkosyl.

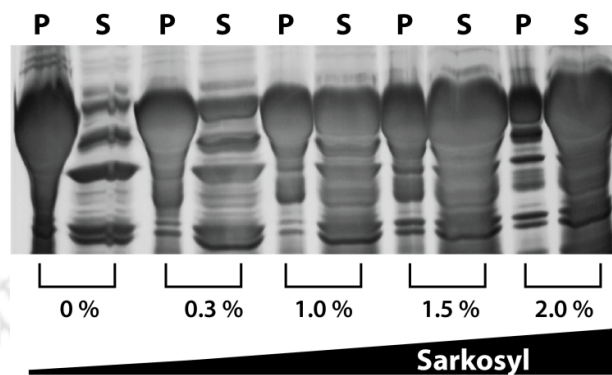


Figure 5.3. SDS-PAGE showing the solubilization of the recombinant GST tagged CD-UPRT protein using the different concentrations (0.3 – 2%) of sarkosyl. ‘S’ indicates the supernatant (soluble fraction) and ‘P’ indicates the pellet (insoluble fraction).

5.4.3. Protein purification with optimum S: T ratio

Although the protein was solubilized >95% in presence of sarkosyl, it was unable to bind with the glutathione beads hindering its further purification using the affinity chromatography column. Hence, after solubilization with the final concentration of 1% sarkosyl, the protein sample was diluted with varying amount of 1× PBS buffer to reduce the sarkosyl concentration and treated with fixed concentration of Triton ×100 (2%) (McNally et al., 1991) to achieve the different ratios of sarkosyl and Triton ×100 (S: T). Five different S:T ratios of 1:5, 1:10, 1:15, 1:20 and 1:25 were tested to determine the improvement in the binding of the GST tagged protein to the glutathione beads. **Figure 5.4** clearly indicates that the protein yield increased with the increasing S: T ratios. The S: T ratio of 1:25 was used as the optimum condition in the purification procedure.

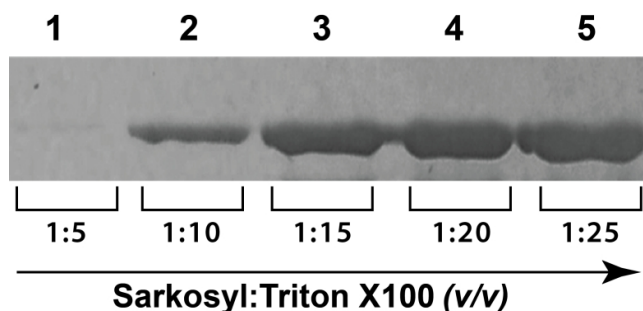


Figure 5.4. SDS-PAGE showing the increase in the binding affinity of the recombinant CD-UPRT protein with the glutathione-agarose beads using different ratios of sarkosyl and Triton $\times 100$. Lanes 1, 2, 3, 4 and 5 indicate the purified protein fractions with the S: T ratios of 1:5, 1:10, 1:15, 1:20 and 1:25 respectively.

5.4.4. Western blot analysis

The purified GST tagged CD-UPRT protein was transferred to the PVDF membrane and identified with the antibody against the GST tag. **Figure 5.5** depicted the presence of the GST tagged CD-UPRT protein with the molecular mass of 98 kDa.

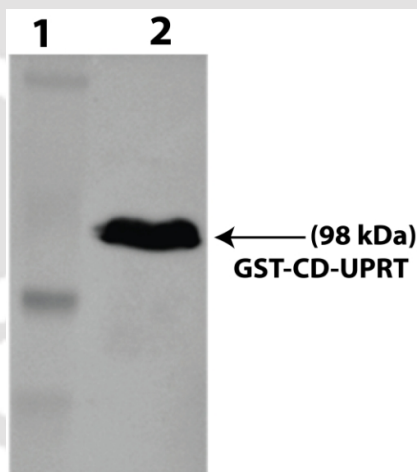


Figure 5.5. The Western blot showing the presence of GST tagged CD-UPRT fusion protein. Lane 1 represents the prestained colorplus protein ladder. Lane 2 represents the GST-CDUPRT protein fragment size of 98 kDa.

5.4.5. Kinetic parameters towards cytosine and 5-FC

Kinetic parameters of the CD-UPRT enzyme were determined at varying concentrations of cytosine (25 – 150 μM) and the prodrug 5-FC (1 – 6 mM) as substrates. The degradation of the cytosine or 5-FC was monitored to obtain the Michaelis Menten constant, K_m and the turnover number, K_{cat} using the double

reciprocal plots. The catalytic efficiency (K_{cat} / K_m) was determined using the above two parameters and is summarized in **Table 5.1**.

The total enzyme concentration $[E]$ was determined with the Bradford method, where the standard plot was made using BSA as a standard protein and was $0.036 \mu\text{M}$. The K_m of CD-UPRT protein when assayed with cytosine was found to be $0.4 \pm 0.04 \text{ mM}$. The recombinant protein attained the catalytic efficiency (K_{cat} / K_m) of $22.84 \pm 2.25 \text{ s}^{-1} / \text{mM}$ with cytosine. Further, the bifunctional CD-UPRT protein demonstrated the higher K_m value of $11.74 \pm 2.3 \text{ mM}$ towards 5-FC as compared to the native substrate cytosine. Moreover, the catalytic efficiency (K_{cat} / K_m) of the recombinant protein towards 5-FC was lower than that of cytosine and was $7.54 \pm 0.35 \text{ (s}^{-1} / \text{mM)}$.

Table 5.1. Kinetic values obtained using Lineweaver-Burk plots for the recombinant CD-UPRT fusion enzyme when assayed with cytosine and 5-FC

Substrate	$K_m \text{ (mM)}$	K_{cat} / K_m ($\text{s}^{-1} \text{ mM}^{-1}$)
Cytosine	0.4 ± 0.04	22.84 ± 2.25
5-Fluorocytosine	11.74 ± 2.3	7.54 ± 0.35

5.4.6. 3D structure of the CD-UPRT protein

Finally, three dimensional structure of the CD-UPRT fusion protein was modeled and was shown in **Figure 5.6**. The CD and UPRT proteins were fused with a linker region which can be observed from the **Figure 5.6**. I-TASSER predicted model of the CD-UPRT protein was submitted in the Protein Model Data Base (PMDB) with the id: PM0078400. The model shown here indicates that the CD and UPRT exist as two independent enzyme modules without interfering with each other, which possibly retained its functional activity.

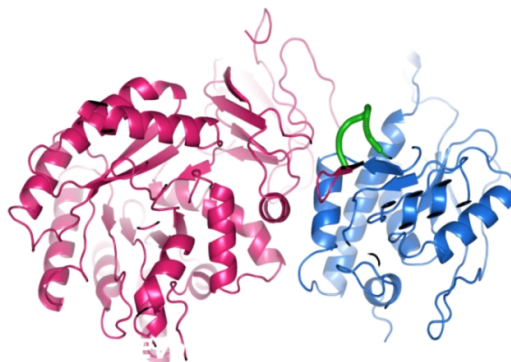


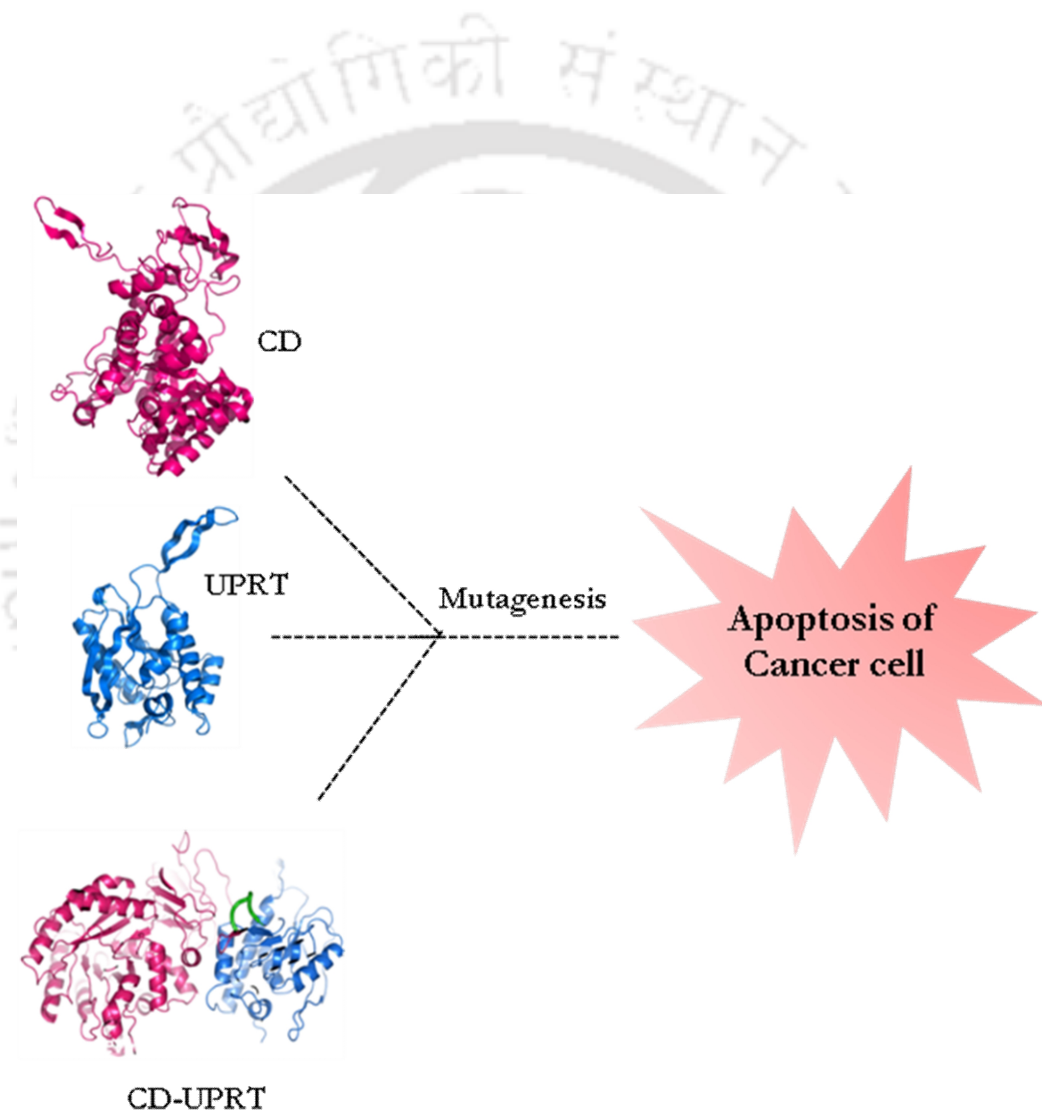
Figure 5.6. The ribbon diagram of the CD-UPRT protein modeled using I-TASSER server. The CD and UPRT proteins are indicated as pink and blue colors respectively. The linker region is shown as green color.

5.5. Conclusion

In summary, the recombinant fusion protein CD-UPRT was successfully cloned and expressed in bacterial expression system. More than 95% of the recombinant protein was solubilized in a single step using the alkyl anionic detergent sarkosyl. The binding affinity of the recombinant protein to the glutathione beads was enhanced by optimizing the ratio of the detergents sarkosyl and Triton $\times 100$. Moreover, the functional activity assays of the recombinant enzyme towards its substrates cytosine and 5-FC were carried out. This is probably the first report of purification and determination of kinetic parameters of the functional CD-UPRT hybrid enzyme. Since this hybrid enzyme plays a vital role in the prospect of suicide gene therapy applications against cancer, the simple purification and functional characterization of the enzyme could lead to the construction of its novel mutants, which are more capable of binding with the prodrug 5-FC than the natural substrate cytosine.

Concluding Remarks

An overall summary of the present work and scopes for the future work are given in this chapter.



Chapter 6

CONCLUDING REMARKS

6.1. Summary of the Present Work

The work described in the present thesis comprised of the redesigning of suicide genes based on computational predictions of hotspot mutants, development of mutants by experimental procedure and investigation of their functional capabilities to achieve greater therapeutic efficacies.

The molecular cloning of wild-type *E. coli* cytosine deaminase (CD) gene in bacterial expression system along with N-terminal GST tag has been demonstrated. The cloned CD gene was overexpressed and was purified to near homogeneity in a single step with the help of N-terminal GST tag using affinity column chromatography. Moreover, Western blot analysis carried out with the anti-GST antibody confirmed the presence of purified GST-CD protein. Finally, the wild-type CD enzyme activity assay carried out with varying concentrations of cytosine and 5-FC revealed the kinetic parameters that correlated with the previously reported values.

Further, *in silico* site-directed mutagenesis and docking of CD were employed to get highly potent CD mutants. The docking studies were validated based on the previously known ligand–protein interactions and the binding free energy scores. The docking studies revealed the mutants F186W, F82C, S126N and R91T having greater binding affinity with the prodrug 5-FC. Notably, the explicit binding of 5-FC with the mutant S126R was also observed. Subsequent experimental validation of the *in silico* derived CD mutants has shown the greater relative specificity of the novel mutants S126R, F186W and F82C with the prodrug 5-FC than the wild-type CD. Moreover, *in vitro* cell viability assay performed with the wild-type CD and the mutant S126R revealed the dramatic decrease in the cell viability with increasing concentrations of the prodrug 5-FC in case of the mutant as compared to the wild-type enzyme.

The molecular cloning, expression and purification of the recombinant GST tagged UPRT enzyme has been demonstrated. Moreover, UPRT enzyme assay was performed with uracil and 5-FU as substrates. In addition, *in silico* site-directed

mutagenesis generated UPRT mutants showed higher binding affinity towards the drug, 5-FU than uracil. The docking studies divulged six mutants I194L, E106Y, M101I, E105Y, M101C and M101F which showed greater binding affinity with the drug 5-FU than uracil. Besides that, docking studies unveiled the mutants M101V, I194Y and E105D which could bind only with the drug 5-FU. The *in silico* results acquired could lead to the development of UPRT mutants so as to increase the sensitivity of the cancer cells more towards the drug 5-FU.

Finally, the recombinant fusion protein CD-UPRT was cloned and overexpressed with N-terminal GST tag. The intact recombinant protein was effectively recovered from the inclusion bodies using sarkosyl as a solubilizing agent. The optimization of the ratio of the detergents sarkosyl and Triton $\times 100$ was done to improve the binding affinity of the recombinant protein to the glutathione beads. Moreover, enzyme assays of the recombinant protein towards its substrates cytosine and 5-FC have been disclosed the kinetic parameters towards both the substrates. The efficient purification and kinetic values determined for the CD-UPRT enzyme could open up new avenues of redesigning hybrid CD-UPRT with high therapeutic impacts in cancer gene therapy.

6.2. Scope for Future Work

- ❖ The emerged CD mutants will be explored *in vitro* as well as *in vivo* to achieve the enriched therapeutic efficacies.
- ❖ UPRT mutants can be generated and their functional characterization will be done.
- ❖ The kinetic properties determined for the CD-UPRT in the present work can lead to the development of highly potent CD-UPRT mutants.
- ❖ The structural studies (Circular dichroism, X-ray crystallography, etc.) of the mutants shall be carried out to reveal the exact structural modifications occurred which led to show enhanced binding affinity towards prodrug.

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LIST OF PUBLICATIONS

Journal Publications

- 1) **V. Kohila**, Amit Jaiswal, Siddhartha Sankar Ghosh. Rationally designed *Escherichia coli* cytosine deaminase mutants with improved specificity towards the prodrug 5-fluorocytosine for potential gene therapy applications. **Med. Chem. Commun.**, 2012, 3, 1316-1322 [Publisher: Royal Society of Chemistry].
- 2) **V. Kohila** and Siddhartha Sankar Ghosh. *In silico* engineering of *E. coli* Uracil phosphoribosyltransferase enzyme to enhance its binding affinity towards 5-Fluorouracil. (Communicated)
- 3) **V. Kohila**, Amit Jaiswal, Siddhartha Sankar Ghosh. Cloning, Expression and Purification of the bifunctional Cytosine deaminase – Uracil phosphoribosyltransferase fusion protein (CD-UPRT) from *E. coli* K12. (Communicated)

Conference Publications

- 1) **V. Kohila**, Amit Jaiswal, Siddhartha Sankar Ghosh. Redesigning of *E. coli* cytosine deaminase in the perspective of cancer therapeutics, 3rd International Conference on Stem Cells and Cancer: Proliferation, Differentiation and Apoptosis (**ICSCC 2012**), New Delhi, India. (*Poster Presentation*)
- 2) **V. Kohila** and Siddhartha Sankar Ghosh. The Emergence of *E. coli* Uracil PhosphoRibosylTransferase mutants in the prospect of cancer therapeutics: a Bioinformatics Approach, International Symposium on Bioengineering (**ISBE 2012**), IIT Guwahati, India. (*Poster Presentation*)