DESIGN, SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF DPP-IV *INHIBITORS* FOR ANTIDIABETIC ACTIVITY

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In partial fulfilment of the requirements for the award of degree of

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IN

PHARMACEUTICAL CHEMISTRY

Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled "**DESIGN, SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF DPP-IV INHIBITORS FOR ANTIDIABETIC ACTIVITY**" submitted by the candidate bearing register no.**26108339** in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL CHEMISTRY** by The Tamil Nadu Dr.M.G.R Medical University is a bonafide work done by her during the academic year 2011-2012 at the Department of Pharmaceutical chemistry, College of Pharmacy, Madras Medical College, Chennai-3.

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TITLE

"DESIGN, SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF DPP-IV INHIBITORS FOR ANTIDIABETIC ACTIVITY"

The Animal Ethical Committee experts screened her proposal **VIDE 25/243-CPCSEA** and have given clearance in the meeting held on 10/08/2011 at Dean's Chamber in Madras Medical College.

Signature

(Dr. JOSEPH DIXON)

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CONTENTS

INTRODUCTION

DIABETES [1]:

Diabetes mellitus represents a group of diseases of heterogenous etiology, characterised by chronic hyperglycemia, and other metabolic abnormalities, reporting from defects in insulin secretion, insulin action, or both .After a long duration of metabolic derangement specific complications of diabetes (retinopathy, nephropathy and neuropathy) may occur. Arteriosclerosis is also accelerated. Depending on the severity of the metabolic abnormality, diabetes may be asymptomatic or may be associated with symptoms like polyuria, polyphagia and polydipsia or may progress to ketoacidosis and coma.

TYPES OF DIABETES [1&2]:

The etiological classification of diabetes and related disorders of glycaemia includes:

(1) Type 1 Diabetes (2) Type 2 Diabetes (3) Those due to specific mechanisms and diseases and (4) Gestational diabetes.

Type 1 diabetes (Insulin dependent diabetes): Type 1 is characterized by destructive lesions of pancreatic cells either by an autoimmune mechanism or of unknown cause. It can occur at any age, but it is most often diagnosed in children, teens, or young adults. In this disease, the body makes little or no insulin. Daily injections of insulin are needed.

Type 2 diabetes (Non-Insulin dependent diabetes): Type 2 diabetes is characterized by combinations of decreased insulin secretion and decreased insulin sensitivity (insulin resistance) makes up most of diabetes cases. It most often occurs in adulthood, but teens and young adults are now being diagnosed with it because of high obesity rates.

Category (3) includes two subgroups; subgroup A is diabetes in which specific mutations have been identified as a cause of genetic susceptibility, while subgroup B is diabetes associated with other pathologic conditions or diseases

Gestational diabetes is glucose intolerance developed/detected at any time during pregnancy.

THE WHO report on diabetes issued in the month of August 2011 records that [3]:

1. More than 346 million people worldwide suffer from diabetes.
2. There is an emerging global epidemic of diabetes that can be traced back to rapid increase in overweight, obesity and physical inactivity.
3. Diabetes is predicted to become the seventh leading cause of death in the world by the year 2030.
4. Total deaths from diabetes are projected to rise by more than 50% in the next 10 years.
5. Type 2 diabetes accounts for around 90% of all diabetes worldwide. Reports of type 2 diabetes in children-previously rare-have increased worldwide.
6. In some countries, it accounts for almost half of newly diagnosed cases in children and adolescents.
7. Eighty percent of diabetes deaths occur in developing and under developed countries.
8. In developed countries most people with diabetes are above the age of retirement, whereas in developing countries those most frequently affected are aged between 35 and 64.
9. Diabetes is a leading cause of blindness, amputation and kidney failure.

TYPE-2 DIABETES [4&5]:

Type 2 diabetes is a lifelong (chronic) disease in which there are high levels of sugar (glucose) in the blood. Type 2 diabetes, often called non-insulin dependent diabetes, is the most common form of diabetes, affecting 90% - 95% of the 21 million people.

The pathogenesis of type 2 diabetes is multifactorial and heterogeneous in origin, involving both genetic and environmental factors. It is characterized both by defects in insulin action ('insulin resistance') and in insulin secretion from the β-cells of the islets of langerhans. Insulin resistance initially can be compensated for by increased insulin production (hyperinsulinemia), but hyperglycaemia will develop when the β-cells can no longer meet the increased insulin requirement ('β-cell failure', 'β-cell decompensation' or'β-cell exhaustion. This β-cell failure may be induced by a prolonged increase in insulin demand, and/or due to a genetic defect in β-cell function which is uncovered by insulin resistance. Other problems associated with the buildup of glucose in the blood include:

- **Dehydration** The build up of sugar in the blood can cause an increase in urination. When the kidneys lose the glucose through the urine, a large amount of water is also lost, causing dehydration.
- **Diabetic Coma (Hyperosmolar nonketotic diabetic coma)** When a person with type 2 diabetes becomes severely dehydrated and is not able to drink enough fluids to make up for the fluid losses, they may develop this life-threatening complication.
- **Damage to the body** Over time, the high glucose levels in the blood may damage the nerves and small blood vessels of the eyes, kidneys, and heart and predispose a person to atherosclerosis (hardening) of the large arteries that can cause heart attack and stroke.

CAUSES[6]:

1. Obese or overweight
2. Women who have had gestational diabetes.
3. People with family members suffering from diabetes
4. People who have metabolic syndrome (a cluster of problems that include high cholesterol, high triglycerides, low good 'HDL' cholesterol and a high bad 'LDL' cholesterol, and high blood pressure).
5. Inactive lifestyle.

SYMPTOMS[6]:

- Increased thirst.
- Increased hunger (especially after eating).
- Dry mouth.
- Nausea and occasionally vomiting.
- Frequent urination.
- Fatigue (weak, tired feeling).
- Blurred vision.
- Numbness or tingling of the hands or feet.
- Frequent infections of the skin, urinary tract or vagina.

DRUGS CURRENTLY USED IN THE TREATMENT OF DIABETES[7]:

1) **INSULIN:**

Insulin was the first medicine developed for the treatment of diabetes, and it remains the most effective therapy for treating hyperglycemia. Insulin reduces blood glucose levels by interacting with a protein on the surface of cells called the insulin receptor.

2) **ORAL HYPOGLYCEMICS:**

- **Sulfonylurea:** This family of medications includes gliclazide, glimepiride, and glyburide. These medications are widely recommended for type 2 diabetes and work by stimulating the pancreas to release insulin. However, these medications don't work for type 1 diabetes.
- **Biguanides:** These medications include metformin and work to improve insulin sensitivity and to reduce the glucose produced by the liver.
- **Acarbose:** This type of medication prolongs the absorption of carbohydrates after a meal. For these pills to work, they must be taken with or after a meal.
- **Thiazolidinediones:** This family of medications includes pioglitazone and they work to improve insulin sensitivity.
- **Meglitinides:** This family of medications includes repaglinide and nateglinide. They lower postprandial (after meals) glucose levels by stimulating the pancreas to release insulin.
- **Dipeptidyl peptidase-4 inhibitors:** This class of medications includes sitagliptin and saxagliptin. They help improve insulin release from the pancreas and decrease liver release of glucose.

- **GLP-1 analogs:** This class of medications includes liraglutide, which is a synthetic form of the hormone GLP-1. It helps the body release insulin when blood sugar levels are high, and also reduces the release of sugar from the liver. It is taken as a daily injection under the skin.

The following table compares some common anti-diabetic agents, generalizing classes although there may be substantial variation in individual drugs of each class:

Table 1a: Different Classes of Antidiabetic Drugs for Treatment of T2DM other than DPP-IV Inhibitors

DRUG CLASS	**MOLECULAR TARGET**	**SIT OF ACTION**	**ADVERSE EFFECTS**	**EXPECTED HbA1c REDUCTION (%)**
Insulin	Insulin receptor	Liver muscle	Hypoglycemia, weight gain, edema	1.5-2.5
Sulfonylurea	SU receptors	Pancreatic Beta cells	Hypoglycemia, weight gain	1.5
Biguanids	Unknown	Liver	GI problems, lactic acidosis	1.0-1.5
Acarbose	α-glucosidase	Intestine	GI problems	0.5-0.8
Thiazolidinediones	$PPAR^{\delta}$	Adipose tissue ,liver, muscles	Weight gain, edema, anaemia	0.5-1.4
$PPAR\alpha/^{\delta}$ dual agonists	PPAR $\alpha/^{\delta}$	Adipose tissue, liver, muscles	Edema ,hepatotoxicity, cardiac risk	0.5-1.3

INCRETIN THERAPIES[8&9]:

Incretins are a group of gastrointestinal hormones that cause an increase in the amount of insulin released from the beta cells of the islets of Langerhans after eating, even before blood glucose levels become elevated. They also slow the rate of absorption of nutrients into the blood stream by reducing gastric emptying and may directly reduce food intake. As expected, they also inhibit glucagon release from the alpha cells of the Islets of Langerhans. Both GLP-1 and GIP are rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4).

There are two main incretin hormones in humans, **GIP** (glucose-dependent insulinotropic peptide; also known as gastric inhibitory peptide) and **GLP-1** (glucagon-like peptide-1). Both hormones are secreted by endocrine cells that are located in the epithelium of the small intestine.

There has been a lot of interest in developing incretin-based therapies for the treatment of type 2 diabetes mellitus (T2DM). T2DM is characterized by insulin resistance, which is a decreased responsiveness of tissues to insulin, and so it may lead to a relative insulin deficiency.

DISADVANTAGES OF GLP-1[10]:

GLP-1 (7-36) amide is not very useful for treatment of type 2 diabetes mellitus, since it must be administered by continuous subcutaneous infusion. Several long-lasting analogs having insulinotropic activity have been developed, and two, exenatide (Byetta) and liraglutide (Victoza), have been approved for use in the U.S. The main disadvantage of these GLP-1 analogs is they must be administered by subcutaneous injection.

Another approach is to inhibit the enzyme that inactivates GLP-1 and GIP, DPP-4. Several DPP-4 inhibitors that can be taken orally as a tablet have been developed. One of them, Januvia (sitagliptin) was approved by the FDA on October 18, 2006.

DPP-4 INHIBITORS[10]:

DPP-IV is a serine aminopeptidase that inactivates incretins, especially GLP-1 and GIP, which are gut hormones released in response to food intake. GLP-1 has several glucoregulatory activities. As soon as it is released from the gut during meals, the incretin hormones (GLP-1 and GIP) serve as enhancers of glucose-dependent insulin release from pancreatic β-cells .As GLP-1 is rapidly eliminated (within 1 minute) because of its cleavage by DPP-IV into an active metabolite, several strategies were explored including the exogenous GLP-1, GLP-1 fusion proteins and DPP-IV resistant GLP-1 analogs for the treatment of Chronic infusion of GLP-1 to T2DM patients resulted in a significant decrease in both blood glucose and plasma HbA[1c] levels. However, the requirement of parenteral route of administration and potential for development of auto antibodies are the major drawbacks associated with this therapeutic approach. In addition sustained GLP-1 infusion induced nausea and vomiting in clinical studies. Thus, DPP-IV has emerged as a potential therapeutic target for the treatment of T2DM.

MECHANISM OF ACTION[10]:

The mechanism of action by which DPP-IV inhibitors lower blood glucose is distinct from existing class of oral glucose-lowering agents. They control elevated blood glucose by potentiating pancreatic insulin secretion, increasing circulating GLP-1, reducing glucagon secretion, and signalling the liver to reduce glucose production.

DPP-IV ENZYME: STRUCTURE, CATALYSIS REQUIREMENTS AND SUBSTRATE SPECIFICITY[10]:

DPP-IV is a 766 amino acid long amino peptidase. The crystal structure of DPP-IV revealed that it is a tetramer with each subunit comprising of two structural domains, N-terminal β propeller domain and C-terminal catalytic domain. The β propeller domain consists of eight blades with 4 anti-parallel strands and harbors an ellipsoidal and continuously open tunnel. The catalytic domain adopts a typical α/β hydrolase fold with a central eight stranded β sheet sandwiched by several α helices. The active site of the enzyme is covered by the β-propeller domain thus restricting access to the substrate. Therefore, there are two possible routes for substrate access: tunnel through the β propeller domain enter by or a side opening formed at the interface of β-propeller domain and catalytic domain.

DPP-IV is a glycosylated protease belonging to the subset of proteins that are capable of cleaving post-proline bond two amino acids downstream of the N-terminal of the protein. It has preference for X-Proline over X-Alanine, where X is any amino acid other than proline. It is generally believed that glycolysation of an integral protein is required for its enzymatic activity.

Based on the in vitro studies, a wide range of potential substrates including growth hormone-releasing hormone, bradykinin, certain chemokines, neuropeptide Y, exenatide have been identified but only few of them such as GLP-1 and GIP are reported to be the endogenous substrates. Although DPPIV has a preference for proline in the second position but GLP-1, the endogenous substrate of DPP-IV, has alanine in this position. A high expression of this enzyme is observed in kidneys, where it is localized in the glomerular basement membrane and proximal convoluted tubules.

PHYSIOLOGICAL ACTIONS OF INCRETIN HORMONES, THEIR USE AND LIMITATIONS[10]:

In the recent years, approaches targeting elevation of GLP-1 have emerged as a promising area for T2DM therapy. They stimulate glucose dependent insulin secretion, inhibit glucagon secretion, delay gastric emptying, suppress appetite, stimulate differentiation and proliferation and inhibit apoptosis of β-cells thus they increase the β-cell mass and improve peripheral glucose uptake and disposal. Extra pancreatic actions include the reduction of hepatic insulin clearance and an apparent "insulin mimetic" effect on skeletal muscle, liver and adipose tissue. But the half life of these hormones is very short (t1/2 =~1 min) as they are rapidly cleaved by circulating DPP-IV enzyme to produce an inactive product, GLP-1 (9-36aa) amide. Inhibition of circulating DPP-IV enzyme by DPP-IV inhibitors prolongs the half life of GLP-1 leading to increased levels of active endogenous GLP-1 and GIP.

SELECTIVITY ISSUES OF DPP-IV INHIBITORS[10]:

DPP-IV is a member of a family of serine peptidases that includes other related members like quiescent cell prolinedipeptidase (QPP/DPP2), DPP6, DPP8, DPP9 and DPP10. The catalytic regions of these enzymes show similarity to each other and therefore a DPP-IV inhibitor may also inhibit the related enzymes in body to a certain extent. For example, the DPP-IV inhibitor Val-boro-pro appears to be relatively nonselective for DPP-IV as it may also inhibit FAP, DPP8, DPP9 and DPP2. Functions of other family members and their clinical importance are unclear at present. Lankaset. al. [113] found that administration of DPP8/9 inhibitor produced alopecia, thrombocytopenia, reticulocytpenia, enlarged spleen, multiorgan histopathological changes and mortality in rats and GI toxicities in dogs.

QPP inhibitor was found to produce reticulo cytopenia in rats, but selective DPP-IV inhibitor exhibited no toxicities in these species. Moreover DPP8/9 inhibitor attenuated T-cell activation in human in vitro models. Based on the above preclinical study data, it was perceived that selective inhibitors of DPP-IV may be a safe clinical candidate for T2DM.

The degree of selectivity towards DPP-IV inhibition for a compound to become pharmacologically safe drug is estimated and summarized below:

Table 1.b Potency of DPP-IV Inhibitors Against Closely Associated Enzymes

Compound	IC 50 (nM)				
	DPPIV	DPP8	DPP9	DPP2	FAP
Sitaglipitin	18	48000	>10,000	>100000	>100000
Vildaglipitin	3.5(Ki=3)	Ki=810	Ki=95	>500000	NA
BI1356 (Ondero)	1.0	>40000	>10000	>100000	89
Aloglipitn	7.0	>100000	>100000	>100000	>100000
Saxaglipitn	3.37	244	104	>30000	NA
Meloglipitn(GRC 8200)	1.61	NA	NA	NA	NA

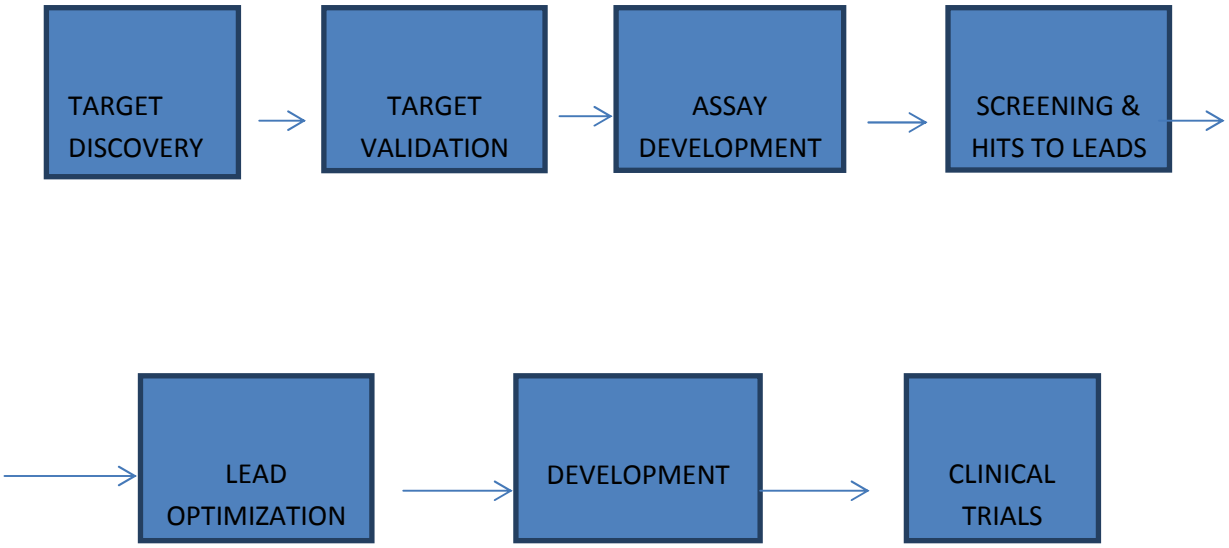
CURRENT OPINION ON DPP-IV INHIBITORS VS GLP-1 ANALOGS[10]:

Both DPP-IV and GLP-1 based therapeutic approaches have been approved by FDA for T2DM. Current evidience suggests that both DPP-IV and GLP-1 analogs may exhibit beneficial actions on the pancreatic islets, acting to preserve β-cell mass *via* opposing effects on proliferation and apoptosis. However , DPP-IV inhibitors are small, orally bio available low-molecular weight compounds but they are without any inherent anti-diabetic activity of their own and hence their therapeutic effect is reliant on enhancing

the activity of GLP-1 and GIP whereas all GLP-1 analogs are based on naturally occurring, relatively large peptide with inherent anti-diabetic activity. Currently there are no small-molecule GLP-1 mimetic drugs available for oral administration and thus they must be given parenterally. Researchers in many industries and academic labs are working on development of oral small molecule agonists of GLP-1 receptor to overcome the existing limitation (injectable and less stable) of GLP-1 based therapy. Although GLP-1 based therapy offers physiological benefits, but it is also associated with adverse events like hypoglycaemia, nausea etc as observed in human clinical trials. Further, modified GLP-1 peptide based analogs are potentially immunogenic. However, none of the compounds under investigation were reported to elicit any antibody response and thus the potential side effects of GLP-1 mimetics need to be evaluated further in long term studies. The anti hyperglycaemic effects of DPP IV inhibitors is glucose dependent meaning that the stimulation of insulin release by these compounds depends upon elevated ambient blood glucose levels and hence there may not be a potential risk of hypoglycaemia. However, long-acting DPP-IV resistant GLP-1 analogs may cause hypoglycaemia and thus dose regimen to be administered should be monitored carefully. Although both GLP-1 and DPP-IV inhibitor based therapies have been approved to be clinically efficacious as monotherapy but DPP-IV combination therapy (with TZD's, SU or biguanides) may be a safer therapeutic approach. GLP-1 analog based therapy causes reduction in body weight, which has not been observed in DPP-IV inhibition based therapy. Considering all the above effects, DPP-IV inhibitor possess more advantages (orally bioavailable, small molecular weight, low hypoglycaemic risk, weight neutral etc)over GLP-1 analogs, but the long-term effects of DPP-IV inhibitors in T2DM patients need to be explored because of its role in regulation of other physiological hormones, chemokines and many other substrates.

THE DRUG DISCOVERY PROCESS[12]:

Drug discovery is the mission of pharmaceutical research to take the path from understanding the disease to bring a safe and effective new treatment to patients. Drug discovery and development is an intense, lengthy and an interdisciplinary endeavour. Drug discovery is mostly portrayed as a linear, consecutive process that starts with target and lead discovery, followed by lead optimization and pre-clinical *in vitro* and *in vivo* studies to determine if such compounds satisfy a number of pre-set criteria for initiating clinical development. The multiple stages of drug discovery process are:



TARGET IDENTIFICATION AND VALIDATION:

Target identification involves choosing a molecule to target with the drug. A target is generally a single molecule, such as gene or protein, which is involved in a particular disease. The target should be selected in such a way that it could potentially interact with and be affected by the drug molecule.

LEAD IDENTIFICATION:

Lead identification is the search for the molecule or lead compound that may act on the target to alter the disease course. The ways to find a lead compound are as follows:

De novo:

De novo design refers to a computer assisted molecular design that supports drug discovery by suggesting novel chemotypes and compound modifications for lead structure optimization.

High-throughput Screening:

This process is the most common way that leads are usually found. Advances in robotics and computational power allow researchers to test hundreds of thousands of compounds against the target to identify any that might be promising. Based on the results, several lead compounds are usually selected for further study.

Biotechnology:

This field involves genetically engineering living systems to produce disease-fighting biological molecules.

LEAD OPTIMIZATION:

Lead compounds that survive the initial screening are then “optimized,” or altered to make them more effective and safer. By changing the structure of a compound, its properties could be altered. For example, they can make it less likely to interact with other chemical pathways in the body, thus reducing the potential for side effects. Hundreds of different variations or “analogues” of the initial leads are made and tested. Teams of

biologists and chemists work together closely: The biologists test the effects of analogues on biological systems while the chemists take this information to make additional alterations that are then retested by the biologists. The resulting compound is the candidate drug.

New techniques such as magnetic resonance imaging, X-ray crystallography, along with powerful computer modelling techniques helps us to visualise the target in three dimensions and design potential drugs to more powerfully bind to the parts of the target where they can be most effective.

DRUG DESIGN [13]:

For the pharmaceutical industry, the number of years to bring a drug from discovery to market is approximately 12-14 years and costing up to $1.2 - $1.4 billion dollars. Traditionally, drugs were discovered by synthesizing compounds in a time-consuming multi-step processes against a battery of *in vivo* biological screens and further investigating the promising candidates for their pharmacokinetic properties, metabolism and potential toxicity. Such a development process has resulted in high attrition rates with failures attributed to poor pharmacokinetics (39%), lack of efficacy (30%), animal toxicity (11%), adverse effects in humans (10%) and various commercial and miscellaneous factors. Today, the process of drug discovery has been revolutionized with the advent of genomics, proteomics, bioinformatics and efficient technologies like, combinatorial chemistry, high throughput screening (HTS), virtual screening, *de novo* design, *in vitro*, *in silico* ADMET screening and structure-based drug design.

There are two major types of drug design.

1. LIGAND BASED DRUG DESIGN
2. STRUCTURE BASED DRUG DESIGN

IN-SILICO DRUG DESIGN:

In-silico methods can help in identifying drug targets via bioinformatic tools. They can also be used to analyse the target structures for possible binding/active sites, generate candidate molecules, check for drug likeness, dock these molecules with the target, rank them according to their binding affinities, further optimise the molecules to improve binding characteristics.

The use of computers and computational methods permeates all aspects of drug discovery today and forms the core of structure-based drug design. High Performance computing, data management software and internet are facilitating the access of huge amount of data generated and transforming the massive complex biological data into a workable knowledge in modern day drug discovery process. The use of complementary, experimental and informatics techniques increases the chance of success in many stages of drug discovery process, from the identification of novel targets and elucidation of their functions to the discovery and development of lead compounds with desired properties. Computational tools offer the advantage of delivering new drug candidates more quickly and at lower cost. Major roles of computation in drug discovery are;

1. Virtual screening and de novo design
2. *Insilico* ADME/T production
3. Advanced methods for determining protein ligand binding

LIGAND-BASED RUG DESIGN:

Ligand based approaches commonly consider two or three dimensional chemistry, shape, electrostatic and interaction points (e.g. Pharmacophore modelling) to assess similarity.

STRUCTURE BASED DRUG DESIGN (SBDD) [13]:

Structure based design attempts to use the three dimensional (3D) protein structure to predict which ligands will bind to the target. Structure-based approaches, of which the best known is docking, require a protein structure or homology model as a starting point. SBDD is an iterative process, in which macromolecular crystallography has been the predominant technique used to elucidate the 3D of drug targets. Although both nucleic acids and proteins are potential drug targets, by far the majority of such targets are proteins. Given that many proteins undergo considerable conformational change upon ligand binding, it is important to design drugs based on the crystallographic structures of protein-ligand complexes.

Crystallography has been successfully used in the de novo design of drugs, but its most important use has been, and will continue to be, in lead optimization .It is important to note that what is being optimized is the affinity and specificity of compounds to their drug target.

Lead optimization is a multi-step process that can be summarized as follow:

1. Expression and purification of the protein of interest. Crystallisation of the protein in the presence of a ligand, which can be a non-hydrolysable substrate or can come from a biochemical or a cell-based screen.

2. Ligands can be low affinity compound fragments or scaffold. They are generally a collection of basic chemical building blocks, each with a molecular weight of less than 200 Daltons. If the screen identifies several promising ligands, each with a unique scaffold, determine the structures of the drug target with as many of these as possible.
3. One or more ligands have been determined and refined, analysis of each structure will reveal sites on the ligand that can be optimized to enhance potency to the drug target. This can be accomplished by redesigning the ligand with greater hydrophobic, hydrogen-bonding and electrostatic complementary to the molecular target. A high affinity lead makes the drug design process simple and intuitive.
4. After the ligands have been designed they should be chemically synthesized. It is prudent to synthesise five to ten compounds around the proposed ligand to obtain structure-activity relationship (SAR) data.
5. Once the synthesised compounds are purified, they are tested in a relevant biochemical or cell-based assay to determine whether or not the design was successful.

PRE-CLINICAL TESTING:

In-vitro and In-vivo tests are carried out before the candidate drug could be administered in humans. In vitro tests are experiments conducted in the lab, usually carried out in test tubes and beakers and in vivo studies are those in living cell cultures and animal models.

CLINICAL TRIAL DESIGN:

An incredible amount of thought goes into the design of each clinical trial. To provide the highest level of confidence in the validity of results, many drug trials are placebo controlled, randomized and double-blinded.

• **Placebo-controlled:** Some subjects will receive the new drug candidate and others will receive a placebo. (In some instances, the drug candidate may be tested against another treatment rather than a placebo.)

• **Randomized**: Each of the study subjects in the trial is assigned randomly to one of the treatments.

• **Double-blinded**: Neither the researchers nor the subjects know which treatment is being delivered until the study is over. This method of testing provides the best evidence of any direct relationship between the test compound and its effect on disease because it minimizes human error.

THE DEVELOPMENT PROCESS:

INVESTIGATIONAL NEW DRUG (IND) APPLICATION AND SAFETY:

Before any clinical trial can begin, the researchers must file an Investigational New Drug (IND) application with the FDA. The application includes the results of the preclinical work, the candidate drug's chemical structure and how it is thought to work in the body, a listing of any side effects and manufacturing information. The IND also provides a detailed clinical trial plan that outlines how, where and by whom the studies will be performed.

The FDA reviews the application to make sure people participating in the clinical trials will not be exposed to unreasonable risks.

In addition to the IND application, all clinical trials must be reviewed and approved by the Institutional Review Board (IRB) at the institutions where the trials will take place. This process includes the development of appropriate informed consent, which will be required of all clinical trial participants.

CLINICAL TRIALS:

PHASE: 1

In this phase the drug is tested on 20 to 100 healthy volunteers. The main goal of this phase is to discover if the drug is safe with humans. The pharmacokinetics of the drug are analysed and the safe dosing range is determined and moved on to the next stage of development.

PHASE: 2

In Phase 2 trials the candidate drug's effectiveness is evaluated in about 100 to 500 patients with the disease or condition under study, and examine the possible short-term side effects (adverse events) and risks associated with the drug. If the drug seems to be promising in this stage it is been taken to the next level.

PHASE: 3

In Phase 3 trials researchers study the drug candidate in a larger number (about 1,000-5,000) of patients to generate statistically significant data about safety, efficacy and the overall benefit-risk relationship of the drug. This phase of research is key in

determining whether the drug is safe and effective. It also provides the basis for labelling instructions to help ensure proper use of the drug.

ONGOING STUDIES AND PHASE 4 TRIALS:

Research on a new medicine continues even after approval. As a much larger number of patients begin to use the drug, companies must continue to monitor it carefully and submit periodic reports, including cases of adverse effects to FDA.

REVIEW OF LITERATURE:

Review of literature was carried out on the basis of the categories docking, synthesis and anti-diabetic activity. In this study high docking scores were obtained for the purine nucleus and hence the literature survey was also proceeded.

DRUG DESIGN:

Nam Sook Kang., et al[14]., (2007) described the docking-based 3D- QSAR study for selectivity of DPP4, DPP8 and DPP-9 inhibitors.

Ying- Duo Goa., et al.[15],(2007) revealed a novel, potent, and selective pyrrolopyrimidine DPP-4 inhibitors by modeling assisted rational design .

Jennifer E.Kowalchick., et al.[16],(2007) described the design, Synthesis, and biological evaluation of triazolopiperazine-based-β-amino amides as potent, orally active dipeptidyl peptidase(IV) inhbitors.

J.W.Corbett., et al.[17],(2007) explained the design and synthesis of potent amido- and benzyl-substituted cis-3-amino-4-(2-cyanopyrrolidide)pyrrolidinyl DPP-IV inhibitors.

Bradley J. Backes., et al.[18], (2007) done a job with Pyrrolidine-constrained phenethylamines: The design of potent, selective, and pharmacologically efficacious dipeptidyl peptidase IV (DPP4) inhibitors from a lead-like screening hit.

Takashi Kondo., et al.[19],(2008) explained design and synthesis of DPP-IV inhibitors lacking the electrophilic nitrile group.

Mutasem.O.Taha., et al.[20],(2008) revealed the Discovery of DPP IV Inhibitors by Pharmacophore Modeling and QSAR Analysis followed by in *silico* Screening

U.Saquib., et al.[21], (2009) using 3-D QSAR carried out studies on triazolopiperazine amide inhibitors of dipeptidyl peptidase IV as anti-diabetic agents.

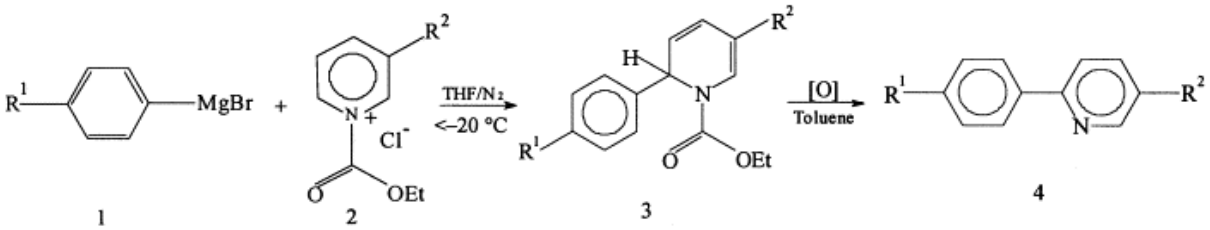
Atulkumar et.al. [22], Design and Synthesis of 3,5-diaryl isoxazole derivatives as novel class of anti-hyperglycemic and lipid lowering agents". Bioorganic and Medicinal Chemistry 17 (2009) pg.no: 5285-5292

Huang Jae Kim., et al.[23], (2011) explains the discovery of DA-1229: A potent, long acting dipeptidyl peptidase-4 inhibitor for the treatment of type-2 diabetes.

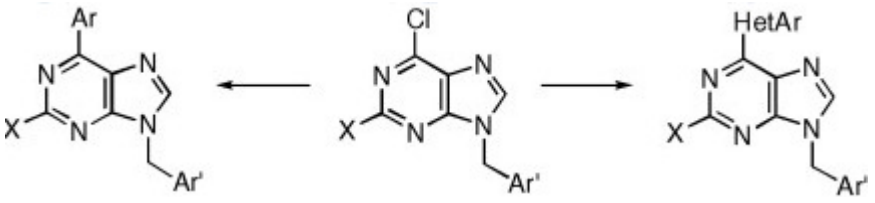
SYNTHESIS:

Young-Tae Chang.,et.al.[24],(1999) described the Synthesis and application of functionally diverse 2,6,9-trisubstituted purine libraries as CDK inhibitors.

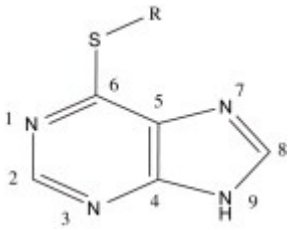
Win-Long Chia.,et.al.[25],(2001) explained the novel synthesis of liquid crystalline compounds of 5-substituted 2-(4-alkylphenyl)pyridines.



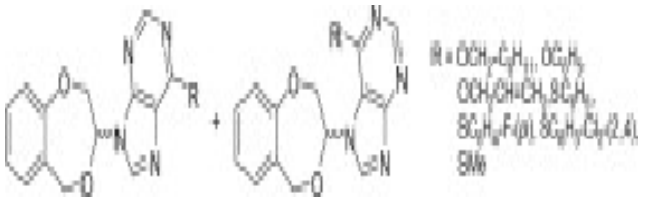
Morten Brændvang.,et.al.[26],(2005) synthesised Selective anti-tubercular purines and explained chemotherapeutic properties of 6-aryl- and 6-heteroaryl-9-benzylpurines.



Fernanda Gambogi Braga.,et.al.[27],(2007) explained the Synthesis and biological evaluation of some 6-substituted purines

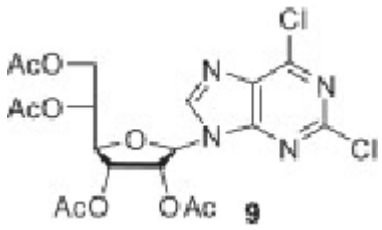


Ana Conejo-García.,et.al.[28],(2008) described the regiospecific microwave-assisted synthesis and cytotoxic activity against human breast cancer cells of (*RS*)-6-substituted-7- or 9-(2,3-dihydro-5*H*-1,4-benzodioxepin-3-yl)-7*H*- or 9*H*-purines



Stephen O. Ojwach; et.al.29, (2009) (Pyrazol-1-ylmethyl) pyridine palladium complexes: Synthesis, molecular structures, and activation of small molecules

Pedro Besada; et.al.30,(2010) explained the synthesis and cytostatic activity of purine nucleosides derivatives of allo furanose



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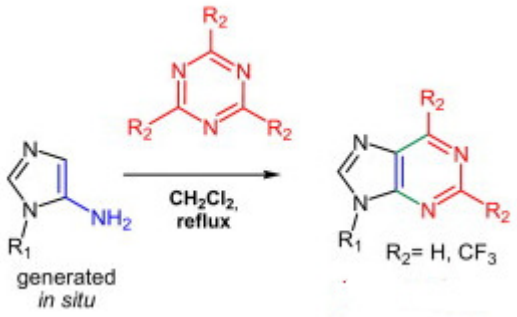
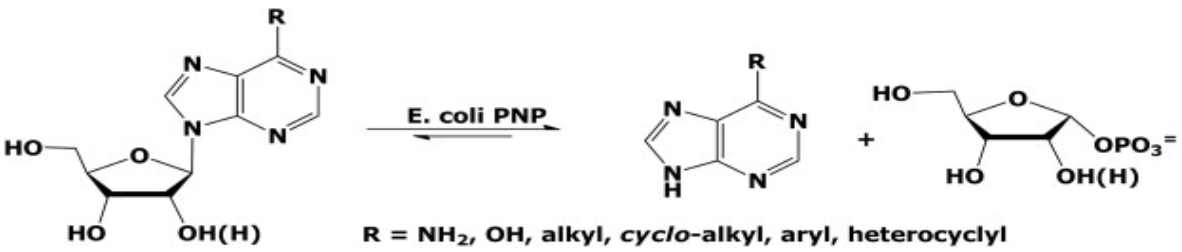
P. Sadanandam., et.al.31, (2011) synthesised and characterization of 9-methyl-2-morpholin-4-yl-8-substituted phenyl-1*H*-purine derivatives using polyphosphoric acid (PPA) as an efficient catalyst.

Viktor O. Iaroshenko.,et.al.[32],(2011) described the efficient synthesis of purines by inverse electron-demand Diels–Alder reactions of 1-substituted-1*H*-imidazol-5-amines with 1,3,5-triazines



Abdalla E.A. Hassan., et.al.33, (2012) synthesised and evaluated the substrate activity of C-6 substituted purine ribosides with *E. Coli* purine nucleoside phosphorylase: Palladium mediated cross-coupling of organo zinc halides with 6-chloropurine nucleosides



ANTI-DIABETIC ACTIVITY:

Vincent Marks et.al.[34],(1959) An improved glucose-oxidase method for determining blood C.S.F and urine glucose level.

Vincent Marks et.al.[35], (1965) Rapid Stick Method for determining Blood glucose concentration

J.S.Cheanet.al.[36], (1974) A Rapid and Simple blood sugar determination using the Ames Reflectance meter And Dextrostix system. A Preliminary Report.

Debro.T.Bustick et.al.[37],(1975)Quantitative determination of blood glucose using enzyme induced chemiluminescence of luminol.

V.T. Innanen., et.al.38,(1991) Hypoglycemia is effectively evaluated at the bedside by the ames glucometer

Ulf Hannestad et.al.[39], (1997) Accurate and precise isotopic dilution mass spectrometry method for determining glucose in whole blood.

Vincenzo Calderone., et.al.[40], (2009) NO-glibenclamide derivatives: Prototypes of a new class of nitric oxide-releasing anti-diabetic drugs.

AIM AND OBJECTIVES:

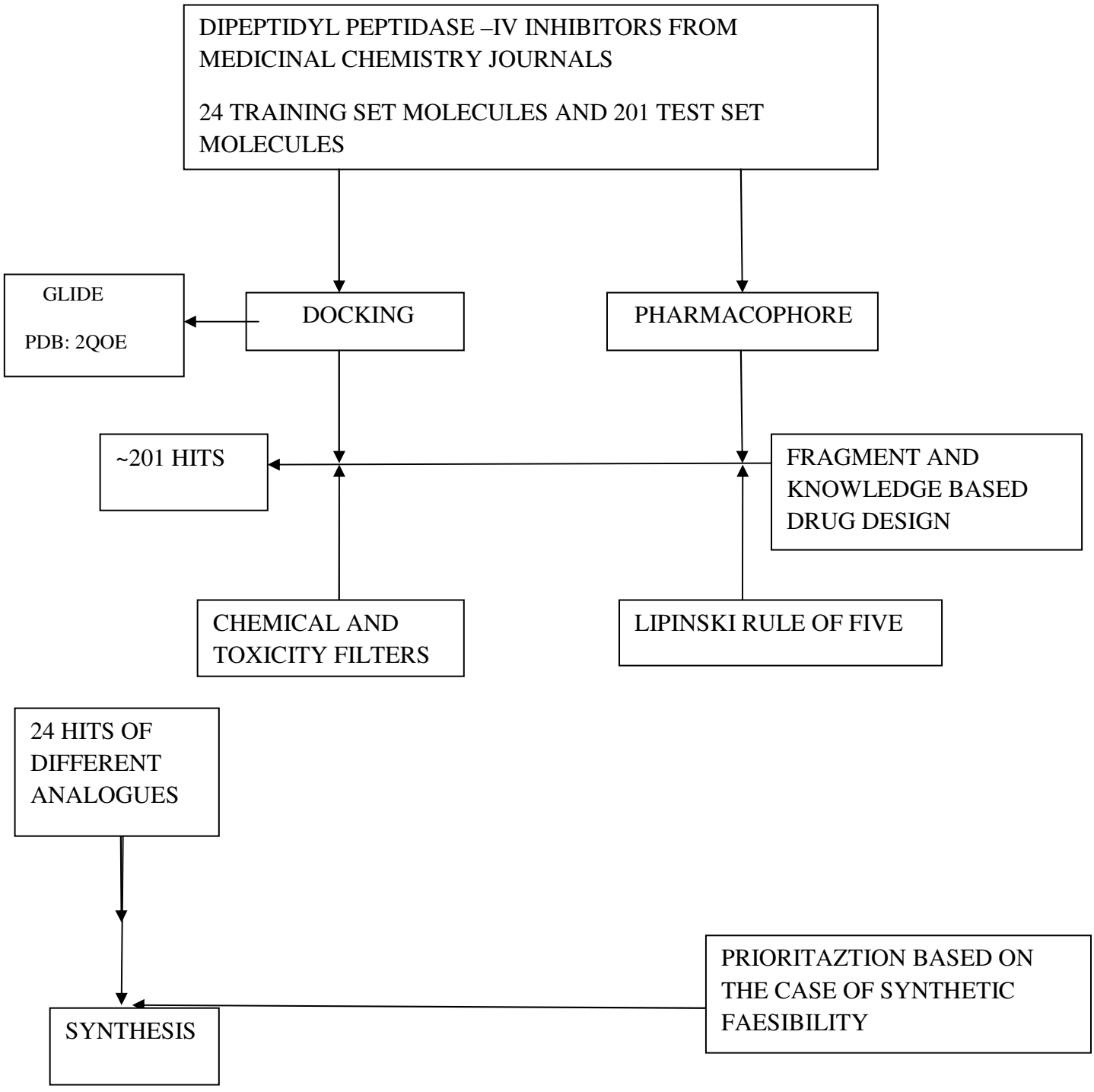
AIM:

To develop novel, potent, selective and orally active inhibitors of Dipeptidyl Peptidase IV with Anti-Diabetic activity.

OBJECTIVES:

1. Identification of common Pharmacophore features responsible for inhibiting DPP-IV using Hiphop module of Catalyst® software 4.11 from Accelrys.
2. Devlopment and validation of quantitative Pharmacophore hypothesis for series of DPP-IV receptor using Hypogen/Hyporefine module of Catalyst® software 4.11 from Accelrys.
3. Generation of 10,000 scaffolds from the drug using scaffold hopping technique.
4. Prediction of activity for designed molecules using the Hyporefine model and to identify novel and potent DPP IV receptor using Lipinski rule of five.
5. The potent receptor inhibitors attained as results may be used as lead for drug development.
6. From the lead molecule, the derivatives of the compounds which has higher score value were synthesised.
7. Characterization of the synthesized compounds by UV, Infrared spectroscopy, Nuclear Magnetic Resonance spectroscopy and Mass Spectroscopy.
8. In vivo anti diabetic activity of synthesised compounds.

The present study was conducted according to the following design



4.1 MATERIALS AND METHODS:

4.1 DRUG DESIGN:

Drug design is the process in which drugs are designed at the atomic level to interact with targets associated with a particular disease process. The designing process is based on the knowledge of the biological target. Here two softwares were used namely Catalyst ® and Glide®. Catalyst ® for Pharmacophore modelling and Glide® for docking studies.

PHARMACOPHORE STUDIES[41]: (Catalyst ®)

A Pharmacophore is a representation of generalized molecular features including three dimensional structures (3D) (hydrophobic groups, charged/ionisable groups, hydrogen bond donors/acceptors), two dimensional (2D) (substructures), and one dimensional (1D) (physical or biological) properties that are considered to be responsible for a desired biological activity.

Catalyst ® develops 3D models called hypotheses from a collection of molecules possessing a range of diversity in both structures and activities. These hypotheses could be used as queries to search 3D databases to retrieve structures that fit the hypothesis, or as models to predict the activities of novel compounds. Catalyst ® specifies hypotheses in terms of chemical features that are likely to be important for binding to the active site.

Cerius^2 [42]:

Cerius^2 is a part of Catalyst ® .Cerius^2 has a variety of force fields available. The default force field is the universal force field (UFF). Cerius^2 offers abilities for modelling materials structure properties, and processes with applications in catalysis, crystallisation

and polymer science.Cerius[2] is a suite of molecular modelling and simulation package for smaller molecules.

CATALYST ®:

4.1.1 PHARMACOPHORE MODEL GENERATION[41]:

Catalyst ® attempts to compute a model from training set data that correlates estimated activities with measured activities. The model is a collection of chemical features distributed in 3D space that is intended to represent groups in a molecule that participate in important binding interactions between drugs and their receptors. Estimated activities are computed by comparing how well the chemical features in the model(hypothesis).The proficiency of molecules to adjust their conformations in order to fit a receptor better is accommodated by considering the molecule as collection of energetically reasonable conformations(conformation models) during the analysis. The steps involved in Pharmacophore model generation are given below:

DEVELOPING A 3D QSAR PHARMACOPHORE MODEL IN CATALYST ® [41]:

The objective here is to develop an automated method for selecting a training set that can be used for Catalyst ® hypothesis generation from a large collection of compounds. Training set selection from a given SAR data is the first step in deriving a predictive QSAR model. The quality of the resultant model is highly dependent upon the molecules which are used to derive the model; therefore, selection of these compounds must be done very carefully.

Guidelines for 3D QSAR Model Generation in Catalyst ® [41]:

3D QSAR (HypoGen) model generation within Catalyst ® requires the following guidelines in order to select molecules for hypothesis generation:

1. At least 16 compounds to assure statistical significance of the Pharmacophore model
2. Activity range of the compounds should span at least 4 orders of magnitude
3. Each order of magnitude should be represented by at least three compounds
4. The most active and inactive compounds should be included
5. Two compounds with similar structures must differ in activity by magnitude to be included, otherwise the most active of the two must be taken.
6. Two compounds with similar activities must be structurally distinct in order to be included, otherwise the most active of the two must be taken.
7. No redundant information should be included

3D fingerprint for a compound is defined as the collection of all possible combinations of three features or four features fingerprints in three dimensions for all conformers. Each multiplet is characterised by a set of feature types and the corresponding inter-feature distances.

. Shape descriptors are calculated for all multi-conformer compounds using the Catalyst® functionality "Catshape". The shape descriptors consists of volume descriptors (mean, median) and x, y and z components of principal axes (min,max,mean,range and median).

A principle component analysis is then performed on the descriptors (MDS coordinates derived from 3D fingerprint, shape and activity) to reduce the high dimensionality descriptor data into principle components. To visualize the compounds in three dimensions, the first three principle components are plotted. The last step is to select a diverse set of compounds to be used a training set for developing hypogen module in Catalyst ®.

HYPOGEN-GENERATE HYPOTHESIS[42]:

With the input of full range of training set compounds from inactive to active, the hypogen algorithm can generate hypothesis with features common against active molecules and missing from inactive molecules

Hypothesis is generated in three main steps:

1: Constructive Phase

2. Substractive Phase

3. Optimization Phase

CONSTRUCTIVE PHASE:

Hypothesis common against the active compounds are identified in the constructive step. These compounds are determined by performing a simple calculation based on the activity and uncertainty as a matter of fact the activity of most active compound is multiplied by the uncertainty (which is set equal to 3 by default in the software) to the uncertainty and this result in,"B" which is compared to A. If B is smaller than A then the compound is included in the most active set, if not the procedure stops.

For the identification of Actives:

(Most Active Compound* Uncertainty) – (CompoundX/ Uncertainity) >0

In the constructive step the actives must fit at least the minimum features.

SUBTRACTIVE STEP:

The inactive compounds are identified in this phase using

Log (Compound X)-log (Most Active compound)>3.5

If more than half doesn't fit with pharmacophoric features, the inactives are identified and removed.

OPTIMIZATION STEP:

SIMULATED ANNEALING:

The optimization of quantitative Pharmacophoric models were done by simulated annealing. The best score hypothesis were estimated for its quality and complexity and the top 10 were selected.

HYPOREFINE:

This process permits consideration of exclusion volumes in Pharmacophore-baed 3D QSAR optimization. The result is to obtain better model predictivity where biological activity is determined by considerations of molecular shape.

EXCLUSION VOLUME

An excluded volume can be added to a hypothesis(or to a template molecule) to specify one or more spherical spaces that must not contain any atoms or bonds.An exclusion volume can represent a region in space that might impinge sterically on a receptor .An exclusion volume can be interpreted as a geometrical constraint.

COMPARE/FIT:

This provides the ability to fit compounds and hypotheses, and determine their degrees of similarity, both geometrically and functionally. In a database search, COMPARE fits the original hypothesis onto the hit molecules obtained from the search and a score are calculated according to the geometrical fit.

DATA ANALYSIS:

COST ANALYSIS:

The Hypogen module in the Catalyst ® performs two important theoretical cost calculations (represented in bit units) that determine the success of any Pharmacophore hypothesis.

FIXED COST:

Fixed cost (also termed as ideal cost) represents the simplest model that fits all data perfectly

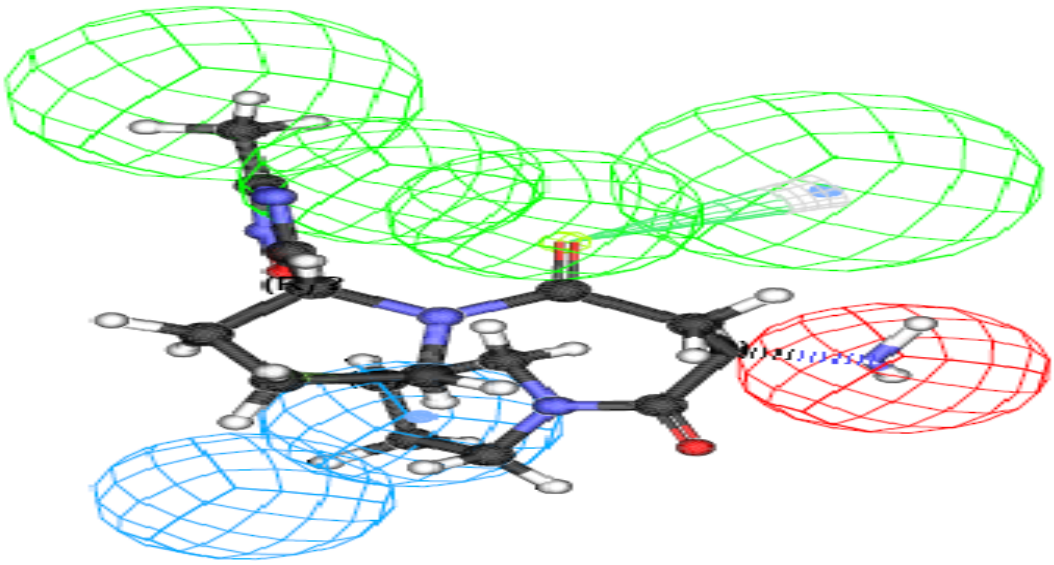
NULL COST:

Null cost (also termed as the no correlation cost) which represents the highest cost of a Pharmacophore with no features and estimates activity to be the average of the activity data of the training set molecules.

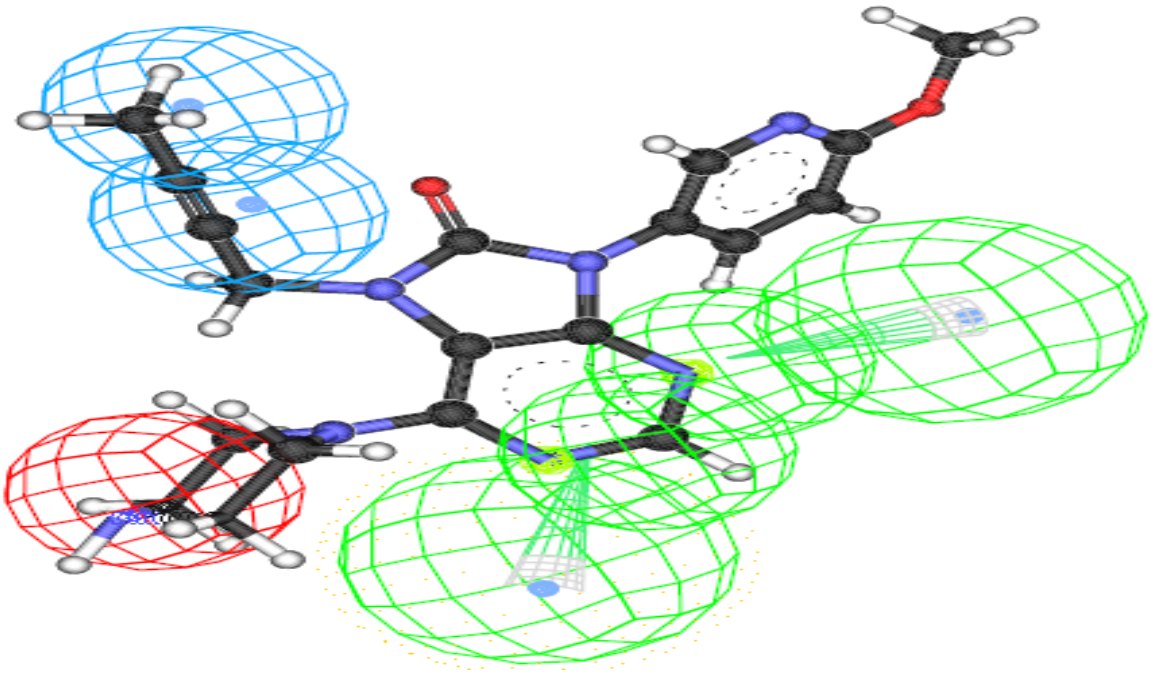
A meaningful Pharmacophore hypothesis may result when the difference between the null cost and the fixed cost is large. The total cost (Pharmacophore cost) of the Pharmacophore hypothesis should be close to fixed cost to provide any useful data's.

The other parameters determining the quality of the Pharmacophore hypothesis are the configuration cost or the entropy cost which should be <17 and the error cost which is dependent on the root mean square differences between the estimated and the actual activities of the training set molecules. The Root Mean Square Deviation (RMSD) represents the quality of the correlation between the estimated and the actual activity data. The best Pharmacophore model has the highest cost difference, lowest RMSD and best correlation coefficient

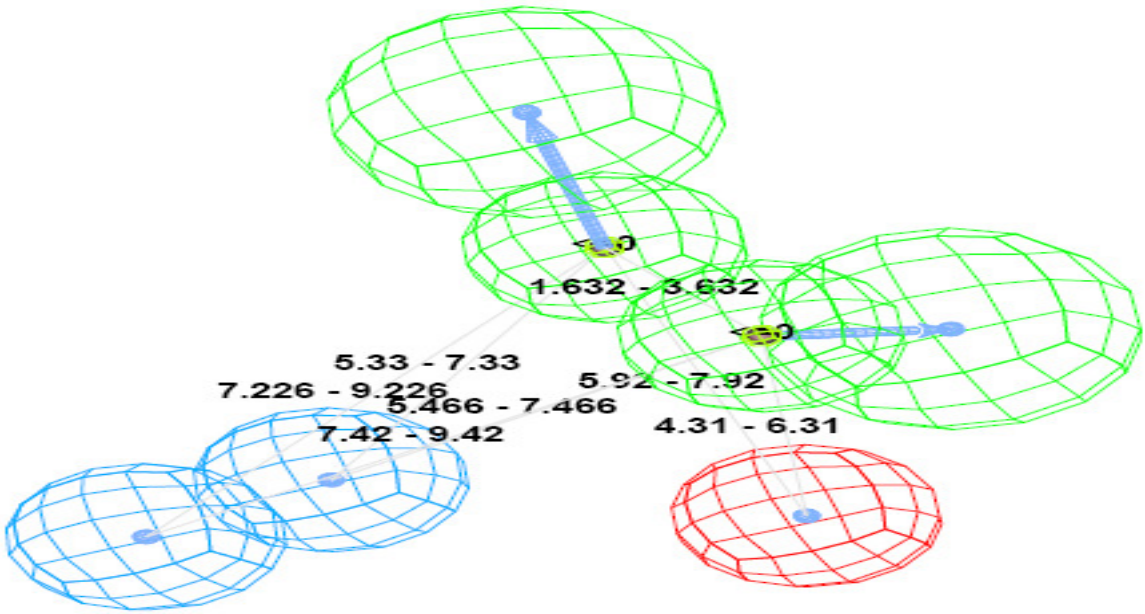
Pharmacophore were computed and the top-10 hypotheses were exported. Results of Pharmacophore hypotheses are presented in Table-5a. Top 10 hypotheses were output by Catalyst ® with cost values, RMSD and Pharmacophore features as listed in the table 5a. The top ranked one, OutHypo-1 consists of two hydrophobic Aliphatic and one Positive ionisable features. The quality of the generated Pharmacophore hypotheses was evaluated by considering the cost functions calculated by Hypogen module during hypotheses generation. In detail, the null cost and fixed cost of the 10 top scored hypotheses were equal to 147.217 and 94.635 and the configuration cost was 12.784.Hypo1 is the best Pharmacophore hypotheses in this study, as it is characterized by the lowest total cost(116.3),the highest cost difference between the null and total hypotheses cost(52.582),the lowest RMSD(1.34256) and the best correlation coefficient(0.821441).The values of the Hypo1 could indicate high predictability of the quantitative Pharmacophore model and a certain rationality for further analysis Pharmacophore model.



5(a) Pharmacophore model aligned with the most inactive molecule



5(b) Pharmacophore model aligned with the most active molecule



5(c) Phrmacophore model for DPP-4 inhibitor with its distance constraints

Training set 24 molecules contains low, moderate and highly active molecules with IC_{50} values

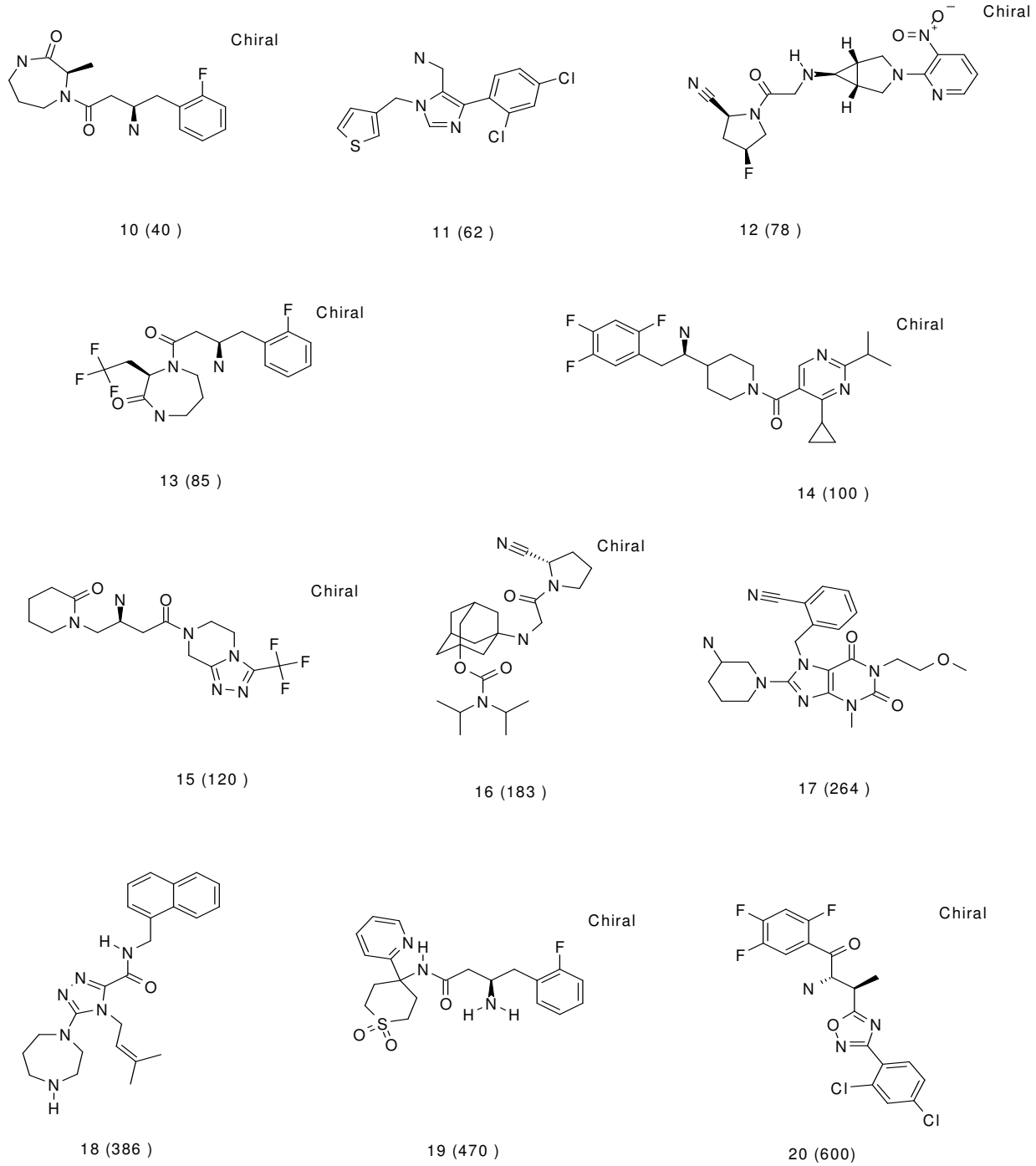


Fig 5(d) Training set 24 molecules

Table: 5a Results of Pharmacophore hypothesis generated using training set against DPP-IV inhibitors

HYPO. NO	TOTAL COST	COST DIFFERENCE	RMSD	Corr.	FEATURE					MAX.FIT
1.	116.3	21.665	1.34256	0.821441	HBA	HBA	HYA	HYA	PI	10.3979
2.	124.539	29.904	1.57805	0.742033	HBA	HYA	HYA	PI		8.23945
3.	126.11	31.475	1.61807	0.72638	HBA	HBA	HYA	PI		8.40509
4.	128.464	33.829	1.65385	0.712549	HBA	HYA	HYA	PI	RA	7.8591
5.	128.48	33.845	1.67482	0.702937	HBD	HYA	HYA	PI		7.26669
6.	128.658	34.023	1.68091	0.700307	HBA	HBA	HYA	PI		8.58388
7.	128.805	34.17	1.66784	0.706499	HBA	HYA	HYA	PI	RA	8.10273
8.	129.119	34.484	1.68114	0.70153	HBA	HYA	PI	RA		9.28727
9.	129.4	34.675	1.69018	0.696601	HBD	HYA	HYA	RA		6.81174
10.	129.669	35.034	1.70863	0.687834	HBA	HBA	HY	PI		7.95771

*Null cost ; Fixed cost; Configuration cost-12.784.All cost units are in bits. *HBA - Hydrogen Bond Acceptor, HYA- Hydrophobic Aliphatic, PI-Positive ionisable, RA-Ring Aromatic

Table: 5b Experimental and predicted IC_{50} values for 24 training set molecules along with other details such as error values and fitness scores.

COMPOUND NO	IC50		ERROR VALUE	FIT VALUE	ACTIVITY SCALE		MAPPED FEATURES				
	EXP.	EST.			EXP.	EST.	HBA	HBA	HYA	HYA	PI
1	0.089	0.086	-1	8.99	+++	+++	9	7	25	13	19
2	2	2.6	+1.3	7.50	++	+++	5	8	28	18	23
3	4	18	+4.4	6.68	+++	+++	*	9	28	16	26
4	5.6	6.2	-1.1	7.17	+++	+++	1	10	*	17	12
5	7.4	11	+1.4	6.90	+++	+++	4	24	*	12	7
6	8.7	98	+11	5.93	+++	+++	*	8	*	11	9
7	12	14	+1.1	6.79	+++	+++	*	17	29	15	17
8	17	140	+8.7	5.76	+++	+++	19	*	8	22	2
9	20	55	+2.7	6.18	++	++	12	*	*	26	20
10	40	220	+5.6	5.58	++	++	*	*	18	16	11
11	62	81	+1.3	5.87	++	++	*	12	*	18	17
12	78	140	+1.7	5.79	++	++	21	19	22	*	*
13	85	110	+1.3	5.87	++	++	10	*	*	5	13
14	100	180	+1.8	5.67	++	++	2	7	*	24	*

15	120	100	-1.2	5.92	++	++	5	*	*	25	18
16	180	96	-1.9	5.94	++	++	12	*	*	26	4
17	260	130	-2	5.81	+	+	12	*	*	20	22
18	390	78	-4.9	6.03	+	+	*	13	*	28	12
19	470	110	-2.2	5.87	+	+	7	6	*	*	18
20	600	520	-1.2	5.21	+	+	9	*	27	*	10
21	870	3800	+4.4	4.34	+	+	11	*	*	5	13
22	2200	150	-14	5.75	+	+	*	14	20	*	23
23	4400	1800	-2.4	4.67	+	+	8	1	10	*	*
24	8800	110	-77	5.86	+	+	15	*	*	17	12

- '+' indicates that the predicted IC_{50} is higher than the experimental IC_{50} ;'-' indicates that the predicted IC_{50} is lower than the experimental $IC_{50,}$ a value of 1 indicates predicted IC_{50} is equal to the experimental $IC_{50.}$
- Fit value indicates how well the features in the Pharmacophore overlap the chemical features in the molecule.
- Activity scale: c<20nM=+++(highly active); IC_{50} 20-200nM=++(moderately active); IC_{50}>200nM=+(low active)

PHARMACOPHORE VALIDATION[42]:

The main purpose of validating a quantitative model is to determine whether our model is able to identify active structures and forecast their activity accurately.

TEST SET PREDICTION[42]:

The predictive power of the Hypo1 was validated with 24 test set compounds. All the compounds were imported into spreadsheet of hypothesis generation workbench and activities were estimated. Table 5b lists the Hypo-1 activity values of test set compounds. Most of the compounds in the test set were predicted correctly for their biological activity. A correlation coefficient of 0.821441 shows a good correlation between the actual and estimated activities.

FISCHERS RANDOMISATION METHOD[42]:

Fischers randomization method was used to evaluate the statistical relevance of the model. In this test, using Cat Scramble program, the experimental activities in the training set was scrambled randomly, and the resulting training set was used for a Hypogen run. All the parameters was adopted from the initial Hypogen calculation. This procedure was repeated 19 times. None of the outcome Hypotheses has lowest cost score than the initial hypothesis. Accordingly this result indicates that there is a 99% chance for the best Hypothesis to present a true correlation in the training set activity data.

DECOY SET[42]:

Goodness of Hit[44]:

The Guner-henry (GH) scoring method was used to assess the quality of Pharmacophore models. The GH score is a measure for qualifying the precision of hits and the recall of actives mined from a database consisting of known actives and in-actives. It is considered as relevant metric, as it takes into account with the percent yield of actives in a database (%Y), and the percent ratio of actives in the hit list (%A, precision). The GH score ranges from 0, which indicates the null model, to 1, which indicates the ideal model.

Enrichment factor:

Enrichment factor indicates the quality of the model and high efficiency of the screening test.

The Hypo-1 was further validated for the ability to pick DPP4 inhibitors in a known database. Output of GH score calculation is presented on table 5c.For this validation experiment the database of compounds containing 201 inhibitors of DPP4 was taken. This database was screened with Hypo-1, 77 molecules was retrieved as hits (H_t).Among these molecules were from known actives (H_a). The calculated Goodness of hit (GH) score and Enrichment factor for Hypo-1 were 0.89 and 2.69 respectively. The false positives and false negatives are 4 and 10.Thus it retrieved 64 of actives from the database predicting validity of the model.

Table: 5c

Statistical parameter from screening test set molecules

No	PARAMETER	VALUES
1	Total compounds in database (D)	201
2	Total Number of actives in database	64
3	Total Hits(H_t)	70
4	Active Hits(H_a)	60
5	%Yield of Actives	85.71
6	%Ratio of actives in the Hit list	93.75

7	Enrichment factor or Enhancement(E)	2.69
8	False Negatives	4
9	False Positives	10
10	GH score(Goodness of Hit list)	0.81

5.1.2 **DOCKING STUDIES (Glide)** [45]**:**

DOCKING AND SCORING METHODS[45]**:**

Docking procedure aims to identify the correct binding poses within the binding site of the protein while the scoring function aims to predict binding affinity of ligand for the protein binding region. The scoring function serves three purposes.

1) For ranking the conformations generated by the docking, search for one ligand interacting with a given protein, this aspect is essential to detect the best binding mode.
2) For ranking different ligands with respect to binding to one protein i.e. prioritizing ligands according to their affinity, this aspect is essential in virtual screening.
3) For ranking one or different ligands with respect to their binding affinity to different proteins their aspect is essential for the consideration of specificity and selectivity.

STEPS IN DOCKING:

PROTEIN PREPARATION [45]:

A typical PDB file consists only of heavy metals, can contain waters, cofactors and metal ions and can be multimeric. The structure generally has no information on bond orders, topologies, or formal atomic charges. Terminal groups can also be misaligned, because the X-ray structure analysis cannot easily distinguish between O and NH_2 ion. Ionization and tautomeric states are usually unassigned. Glide calculations use an all atom force-field for accurate energy evaluation. Thus, Glide requires bond orders and ionization states to be properly assigned and performs better when side chains are reoriented when necessary and steric clashes are relieved.

The steps by which protein preparation was carried out:

1. The ligand/protein cocrystallised structure, from PDB was imported into maestro. The preparation component of a protein preparation facility requires an identified ligand.

2. The protein-ligand complex is identified for its form as dimer or other multimer containing duplicate binding sites and duplicate chains that are redundant, remove redundant binding

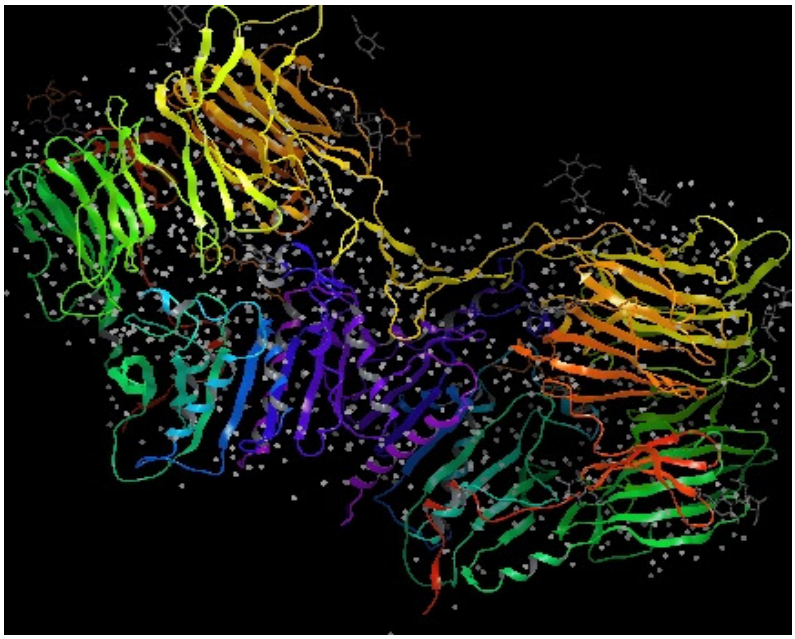
sites and the associated chains by picking and deleting molecules or chains.

3. The waters that bridge between the ligand and protein are retained and all the other waters (except those coordinated to metals) are deleted. If waters are added then hydrogen will be automatically added and the orientations of water molecules are checked once again.

4. The protein, metal ions and cofactors are adjusted. Structures that are missing residues near the active sites should be repaired. Covalent bonds from metal ions to the protein should be changed to zero-order bonds, and the formal charges on the metal and the ligating groups should be adjusted to appropriate values.

5. The ligand bond orders and formal charges are adjusted. Glide models such interactions as vanderwaals plus electrostatic interactions.

6. A restrained minimization of the protein structure reorients side chain hydroxyl groups and alleviates potential steric clashes. The minimization is restrained to the input protein coordinates by a user-selected RMSD tolerance.



5(e) Crystal structure of Dipeptidyl Peptidase IV inhibitor: 2QOE

LIGAND PREPARATION[45]:

Ligand preparation is designed to prepare high quality, 3D structures for large numbers of drug like molecules. The structures that are docked must have actual ligand structures and should meet the following conditions:

1. They must be three dimensional.

2. They must have realistic bond lengths and bond angles.

3. They must each consist of a single molecule that has no covalent bonds to the receptor, with no accompanying fragments, such as counter ions and solvent molecules.

4. They must have all their hydrogens.

5. They must have an appropriate protonation state for physiological pH values (around 7).

The LigPrep process consists of a series of steps that perform conversions, apply corrections to the structures, eliminate unwanted structures, and optimize the structures. The simplest use of LigPrep produces a single low-energy 3D structure with correct chiralities for each successfully processed input structure. LigPrep can also produce a number of structures from each input structure with various ionization states, tautomers, stereochemistries, and ring conformations, and eliminate molecules using various criteria including molecular weight or specified numbers and types of functional groups present.

RECEPTOR GRID GENERATION[45]:

The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. For receptors that adopt more than one conformation on binding, grids must be prepared for each conformation to ensure that possible actives are not missed.

The receptor grid defines the receptor structure by excluding any co-crystallised ligand that might be present, determine the position and the size of the active site as it will be represented by receptor grids, set up glide constraints, and set up flexible hydroxyl groups.

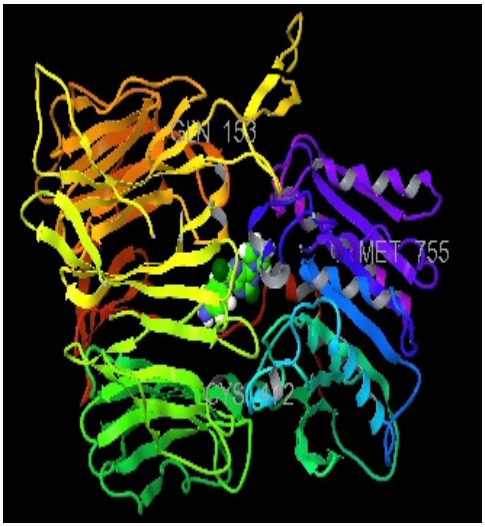
Receptor grid generation requires a prepared structure: an all-atom structure with appropriate bond orders and formal charges.

LIGAND DOCKING[45]:

Glide ligand docking jobs require a set of previously calculated receptor grids and one or more ligand structures. The ligand structures must satisfy the conditions listed above. Information on setting up grid generation jobs is given above. Preparation of the ligands before docking is strongly recommended. LigPrep or macro Model can be used to prepare ligands. If a correct lewis strucuture cannot be generated for a ligand, it is skipped by the docking job.

DOCKING OF DPP-IV[45]:

Crystal structure of DPP-4 (PDB code-2QOE) was used for the study. The 3D structures was downloaded from the Protein Data Bank (PDB) and loaded to the Maestro® workbench. The hydrogen atoms were added to the proteins and further minimization was performed using protein preparation wizard. Structure based docking studies were carried out using GLIDE on DPP4 inhibitors to the 3D structure of DPP4 and generated 10 best docking poses. During the docking, Glide initially performs a complete systematic search of the conformation, orientation, and position of a compound in the defined binding site and eliminates unwanted poses using scoring and energy optimization. The best poses were selected based on the scoring functions and quality of pose orientation within the active site amino acids. Molecules selected for synthesis were examined from this docking score which is having high dock value.



5(f) 5(g)

5 (f),(g) Docked molecule in the cocrystal of DPP-4 receptor in ribbon form

COCRYSTAL AND DOCKING[45]:

Cocrystal and docking is the conformational comparison of these two ligands having similar binding modes at the DPP IV binding site.

Crystal structure of DPP4 (Pdb code: 2 QOE) was downloaded from the Protein data bank into the maestro workspace. The protein was split by molecule and generates the cocrystal. The cocrystal alone was subjected to Ligprep. The output file was further docked and generates docked conformations.

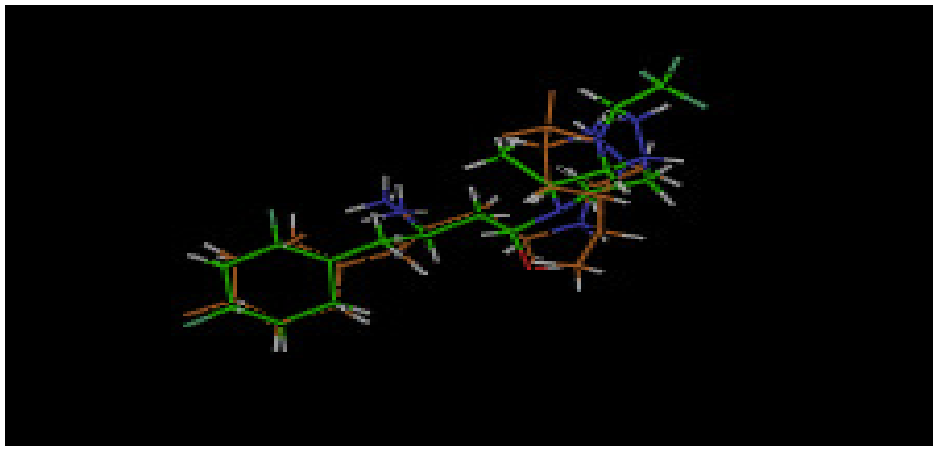
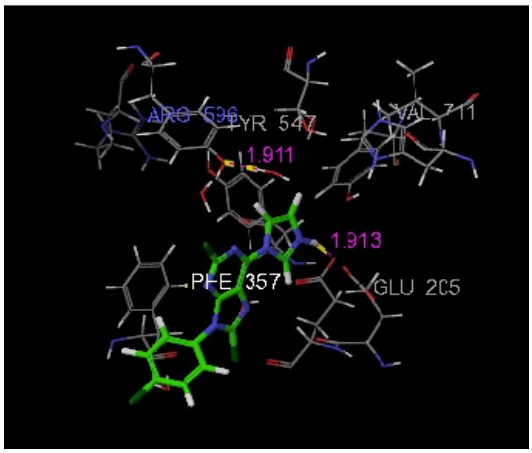


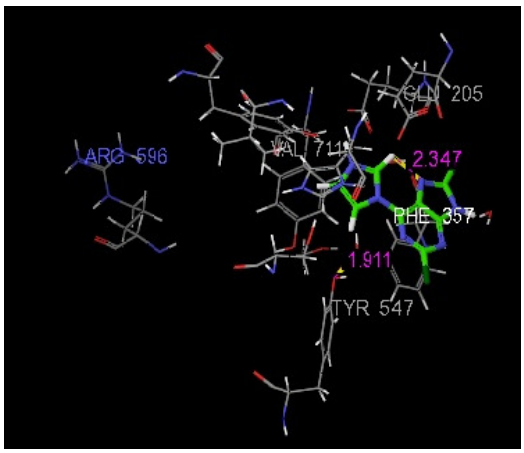
Fig: 5(h) Cocrystal and docked orientation

INTERACTIONS[45]:

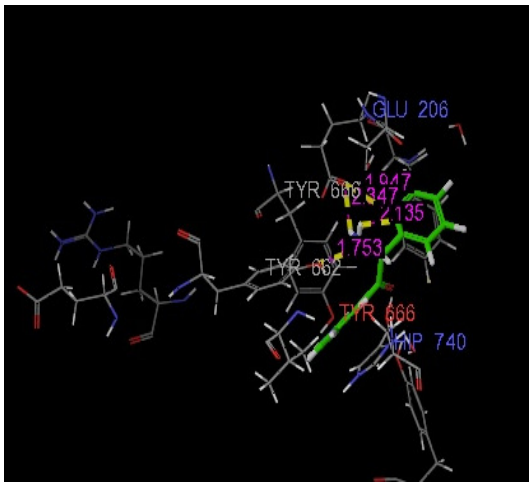
Binding mode of compound SR A, SR B, SR C and SR D in the active site of DPP-4. Carbon atoms of the protein and the ligand are indicated in gray. Each dotted line indicates a hydrogen bond.



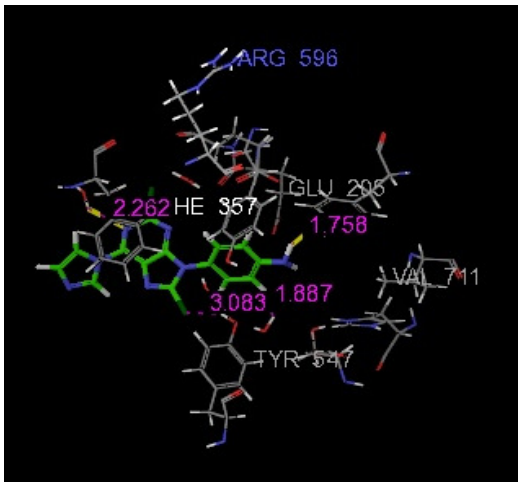
5(i)



5(j)



5(k)



5(l)

5(i) Interaction between protein and ligand SR A

5(l) Interaction between protein and ligand SR B

5(j) Interaction between protein and ligand SR C

5(k) Interaction between protein and ligand SR D

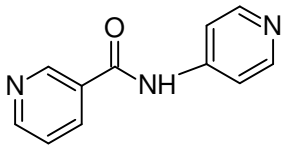
XP DOCKING[45]:

Ligand	G Score	Lipophilic Evdw	PhobEn	PhobEn HB	PhobEn PairHB	H bond	Elect	Site map	Pi Cat	ClBr	Low Mw
1	-10.34	-3.71	-1.22	-1.5	0	-1.36	-0.82	-0.03	0	0	-0.19
2	-6.36	-3.97	-1.55	0	0	-0.9	-0.11	-0.4	0	0	-0.38
3	-6.16	-4.08	-1.7	0	0	-0.35	-0.13	-0.26	0	0	-0.13
4	-6.04	-4.67	-1.20	0	0	0	-0.23	-0.31	0	0	-0.19

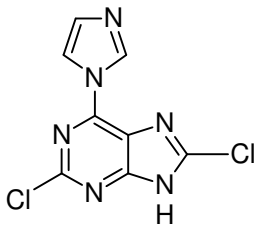
XP TERM	DESCRIPTION
G score	Total Glide Score; sum of XP terms
Lipophilic evdW	Lipophilic term derived from hydrophobic grid potential and fraction of he total protein ligandvdW energy
PhobEn	Hydrophobic enclosure reward
PhobEnHB	Reward for hydrophobically packed H-bond
PhobEnPairHB	Reward for hydrophobically packed correlated H-bonds
Hbond	Chem Score H-bond pair term
Electro	Eletrostatic rewards
SiteMap	Sitemap ligand/receptor non-H bonding

	polar/hydrophobic and hydrophobic/hydrophilic complementarity terms
LowMW	Reward for ligands with low molecular weight
Penalties	Polar atom burial and desolvation penalties, and penalty for intra-ligand contacts
HBPenal	Penalty for ligands with large hydrophobic contacts and low H-bond scores
PhobicPenal	Penalty for exposed hydrophobic ligand groups
RotPenal	Rotatable bond penalty

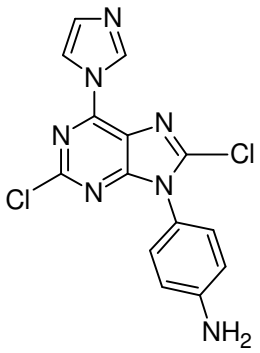
SYNTHESISED MOLECULES WITH DOCKING SCORE:



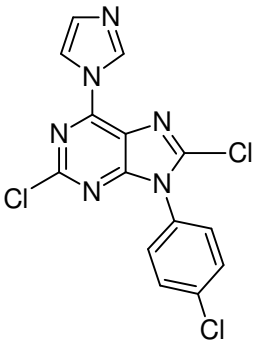
-10.34



-6.36



-6.16



-6.07

DRUG LIKENESS SREENING:

DRUG-LIKE PROPERTIES:

The properties which can differentiate drugs from other chemicals can be considered as drug like properties. Drug-likeness is a qualitative concept used in drug design for how drug like substance is to be an effective drug, a substance must be characterised by optimal solubility to both water and fat. Orally administered drugs must pass through the intestinal lining and be transported in aqueous blood, then penetrate the lipid cellular membrane to reach the inside of a cell. The model compound for cellular membrane is octanol, so the logarithm of the octanol/water partition coefficient, known as logP is used to estimate solubility.

Since the drug is transported in an aqueous media like blood and intracellular fluid, it has to be sufficiently water-soluble. Solubility in water can be estimated from the number of hydrogen bond donors vs. the alkyl side chains in the molecule. Low water solubility translates to slow absorption and action. Too many hydrogen bond donors, on the other hand, leads to low fat solubility, so the drug cannot penetrate the cell wall.

The lower the molecular weights, the better molecule .A total of 80% of the drugs have molecular weight under 450 dalton.

LIPINSKIS RULE[47]:

Lipinski's rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most medication drugs are relatively small and lipophilic molecules. As a rule of thumb, orally absorbed drugs tend to obey Lipinski's rule of five. The rule of five was derived from the analysis of compounds from the World Drugs Index database, aimed at identifying features that were important in making a drug orally active.

The factors concerned involved numbers that are multiples of 5:

1. A molecular weight less than 500
2. No more than 5 hydrogen bond donor groups
3. No more than 10 hydrogen bond acceptor groups
4. A calculated log P value less than +5 (log P is a measure of a drug's hydrophobicity)

The rule describes molecular properties important for a drugs pharmacokinetics, including Absorption, Distribution, Metabolism and Excretion. It is to evaluate drug

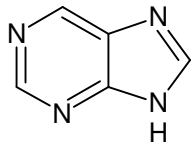
likeness or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug.

COMPOUND	Log P	Mol.Wt	TPSA	nOHNH	nON	No. of rotatable bonds.	No. Of violations
SRI A	1.72	$C_{14}H_7Cl_3N_6$	61.436	0	6	2	0
SRI B	0.118	$C_{14}H_{11}Cl_2N_7$	87.459	2	7	2	0
SRI C	1.929	$C_{12}H_{10}N_{20}$	41.99	1	3	2	0
SRI D	0.276	$C_8H_4C_{l2}N_6$	67.664	2	6	1	0

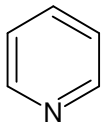
SYNTHESIS:

PURINES AND PYRIDINES [48, 49]

A purine is a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring of molecular formula $C_5H_4N_4$. Purines including substituted purines and their tautomers, are the most widely distributed kind of nitrogen-containing heterocycle in nature. Purines and pyrimidines make up the two groups of nitrogenous bases, including the two groups of nucleotide bases.

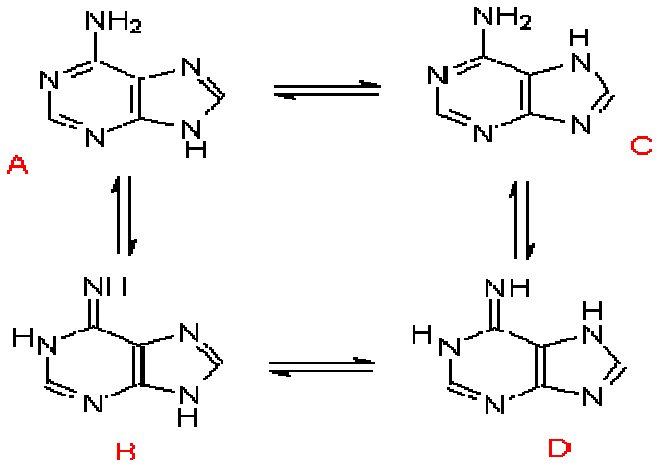


Pyridine is a basic heterocyclic compound containing one nitrogen atom and five carbon atoms in its molecules with molecular formula C_5H_5N. It is used as a solvent and waterproofing agent and in the manufacture of various drugs and vitamins.



TAUTOMERS OF PURINE:

Purine can exist in four tautomeric forms in which the hydrogen atom is joined to different nitrogen atoms: N-1, N-3, N-7, and N-9. In the first two the aromaticity of the pyrimidine ring is lost, the ring now being virtually equivalent to the far less stable (and more reactive) ortho-quinonoid structure. In practice, purine appears to behave completely as the tautomers of N-7 H and N-9 H.

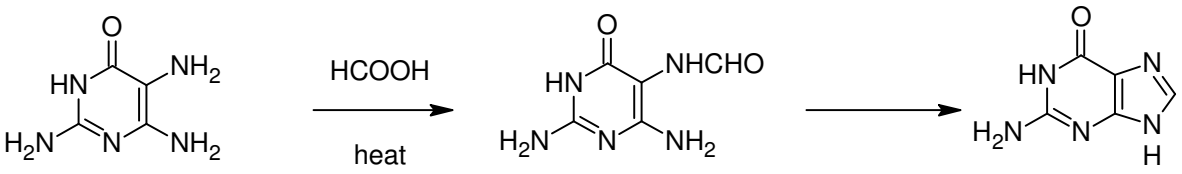


SYNTHESIS OF PURINES:

The purine ring system is a fusion of two aromatic heterocycles, pyrimidine and imidazole. The starting point for the ring synthesis is substituted pyrimidine or imidazole from which the second ring can be constructed by a cyclisation process. Both of these methods have been used for the synthesis of purines.

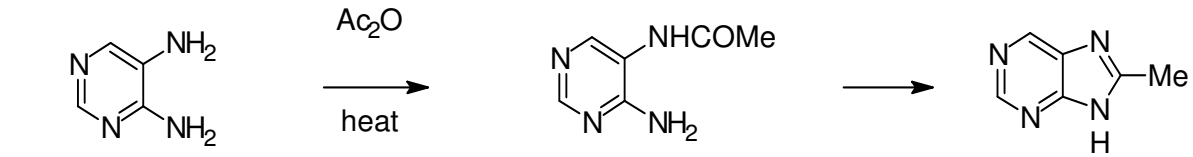
METHOD A:

The most common method is the Traube's synthesis. This is a versatile method because of the variety of substituents possible in both components. Formic acid was originally used as the cyclisation reagent; its reaction with 2, 5, 6-triaminopyrimidin-4-one gave guanine by way of the intermediate, N-formyl derivative.

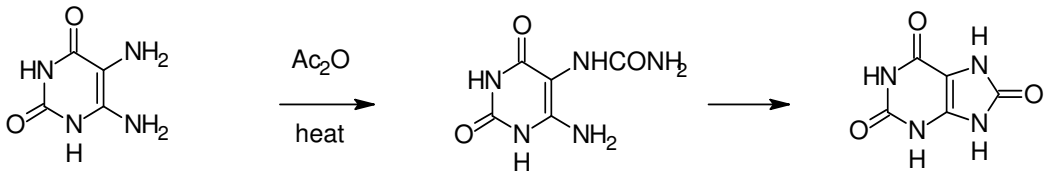


Formamide, formamidine, ethyl orthoformate and similar electrophilic reagents can be used in place of formic acid in this type of synthesis.8-substituted purines can be

constructed by using a different type of acylating agent such as acetic anhydride. When the diamino pyrimidines are heated with urea, 8-oxopurines are formed.



pyrimidine-4,5-diamine *N*-(4-aminopyrimidin-5-yl)acetamide 8-methyl-9*H*-purine

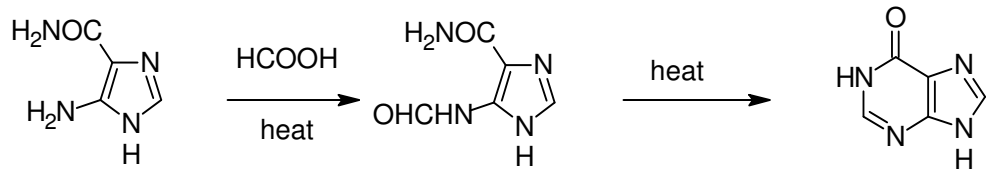


5,6-diaminopyrimidine-2,4(1*H*,3*H*)-dione 7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione

METHOD B:

The synthesis of purines by route (b) is a useful alternative to that based on diaminopyrimidines. 5-Aminoimidazole-4-carboxamide and related compounds are commonly used as precursors.

For example, the carboxamide can be formylated with formic acid and the formamide is cyclised on heating.This method of synthesis is closely related to the biological route to purines: 5-aminoimidazole-4-carboxamide occurs naturally (as ribonucleotide) and it is the precursor of the purine nucleotides.



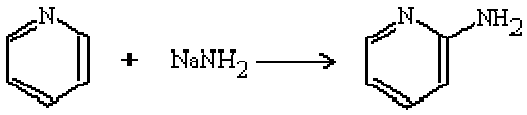
5-amino-1*H*-imidazole-4-carboxamide

1,9-dihydro-6*H*-purin-6-one

SYNTHESIS OF PYRIDINES AND RELATED COMPOUNDS:

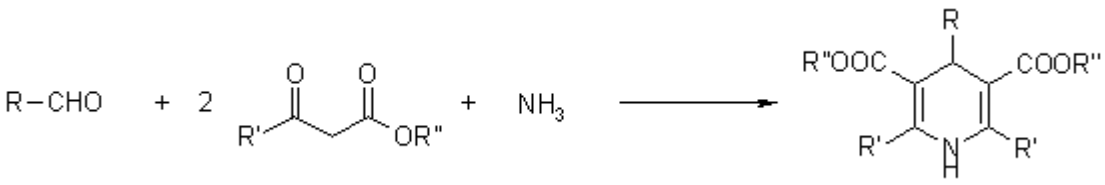
CHICHIBABIN SYNTHESIS:

The Chichibabin pyridine synthesis is a condensation reaction of aldehydes, ketones and α,β unsaturated carbonyl compounds, or any of the combination of the above, in ammonia or ammonia derivatives.



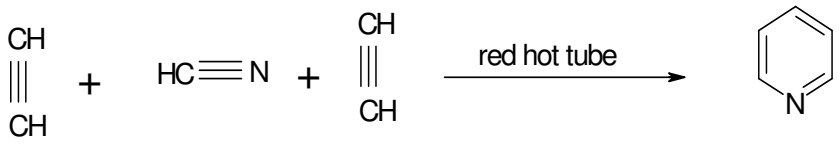
HANTZSCH PYRIDINE SYNTHESIS:

This reaction allows the preparation of dihydropyridine derivatives by condensation of an aldehyde with two equivalents of a β-ketoester in the presence of ammonia. Subsequent oxidation (or dehydrogenation) gives pyridine-3, 5-dicarboxylates, which may also be decarboxylated to yield the corresponding pyridines.



BONNEMANN CYCLIZATION:

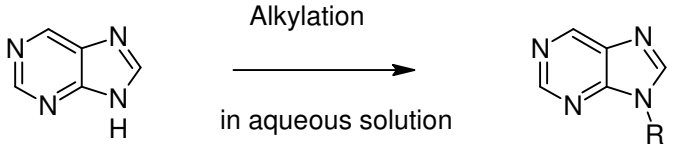
The trimerization of a part of a nitrile molecule and two parts of acetylene activated either by heat or light into pyridine is called Bonnemann cyclization. While the thermal activation requires high pressures and temperatures, the photo induced cyclo addition proceeds at ambient conditions with $CoCp_2$ (cod) (Cp = cyclopentadienyl, cod = 1, 5-cyclooctadiene) as a catalyst, and can be performed even in water.



REACTIONS OF PURINES:

1. Purines can be N-alkylated and N-oxidized but the site of reaction is dependent upon the substituent's present and the reagent.

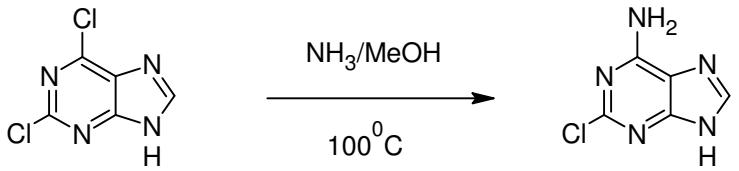
Purine is converted into 9-methylpurine by reaction with dimethyl sulphate in aqueous solution



9*H*-purine N⁻Alkylated purine

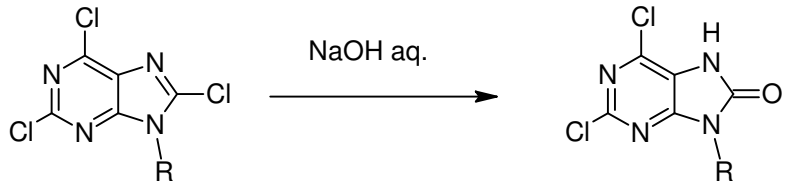
2. Reaction of oxoderivatives with phosphorous oxychloride gives chloropurines.Chlorine is more easily displaced at C-6 than at C-2.

2,6-dichloropurine gives 6-amino-2-chloro purine with methanolic ammonia at 100°C.



2,6-dichloro-9*H*-purine 2-chloro-9*H*-purin-6-amine

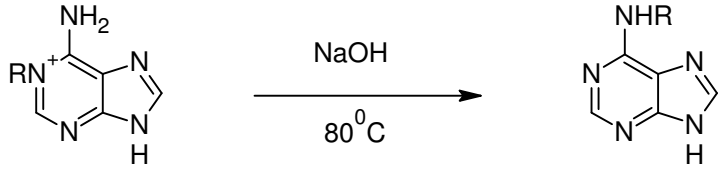
3. With 2,6,8-trichloropurine, the chloride at C-8 is the least easily displaced in alkaline media because the proton at the adjacent nitrogen atom is removed by the base.



Examples of selective displacement of chloride

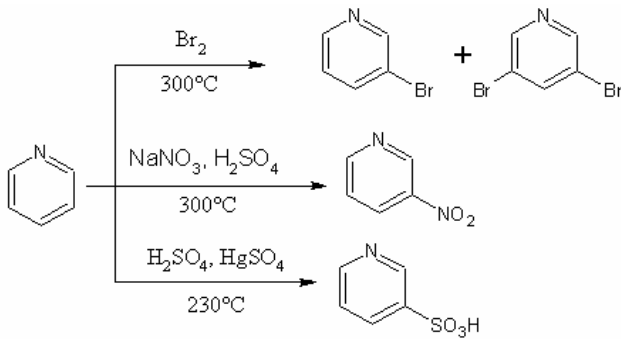
4. The six-membered ring system of purines also undergoes some of the other reactions such as photoaddition reactions across N-1 and C-6 and nucleophilc ring cleavage.

An example of nucleophilic cleavage is the Dimroth rearrangement of a 1-alkylated adenine.



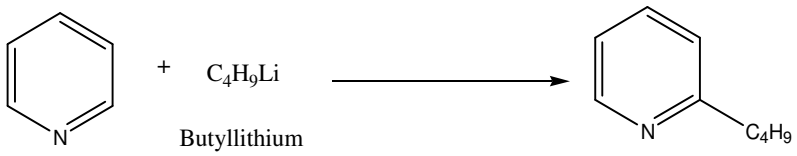
REACTIONS OF PYRIDINES:

1. ELETROPHILIC SUBSTITUTION:



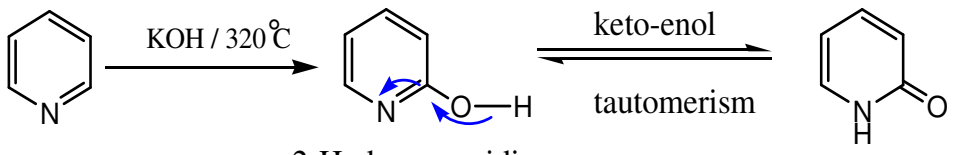
2. NUCLEOPHILC SUBSTITUTION REACTIONS:

i) Reaction with Organometallic compounds Lithium Reagents:



2-Butylpyridine

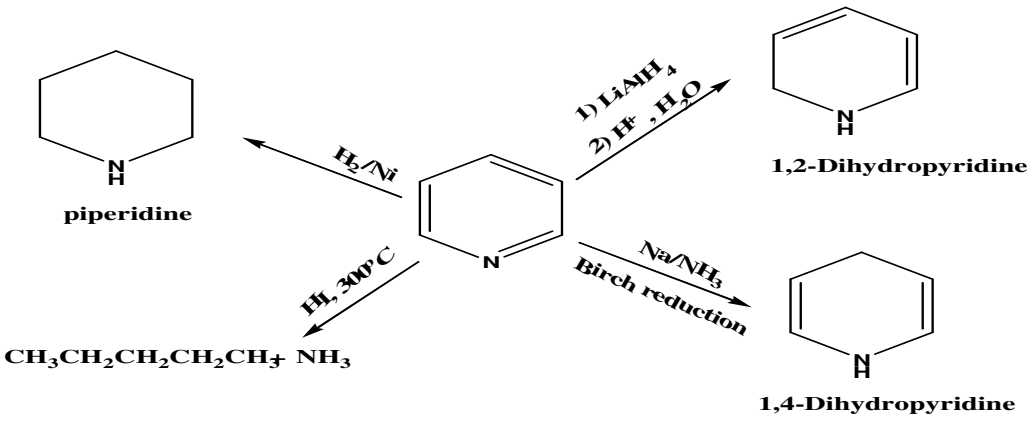
Reaction with Potassium Hydroxide:



2-Hydroxy pyridine

2-Pyridone

REDUCTION REACTIONS:



GENERAL SYNTHETIC PROCEDURE: [47, 48, 49]

Ligand based and structured based docking studies led to the identification of 25 molecules with high glide score and interactions. Four molecules that were synthetically feasible were selected and synthesised and labelled as SR A, SR B, SR C and SR D

2,8-dichloro-9-(4-chlorophenyl)-6-(1*H*-imidazol-1-yl)-9*H*-purine- SR A

4-[2,8-dichloro-6-(1*H*-imidazol-1-yl)-9*H*-purin-9-yl]aniline- SR B

2,8-dichloro-6-(1*H*-imidazol-1-yl)-9*H*-purine- SR C

N-(pyridin-4-yl) pyridine-3-carboxamide- SR D

SYNTHETIC PROCEDURE FOR PURINE:

There have been numerous investigations on the synthesis of purine ring which is the base component of nucleic acids, major methods of the purine synthesis being as follows:

(a) Cyclization of 4,5-diaminopyrimidine with anhydrous formic acid in the carbon monoxideatmosphere

1)(b) Reaction of tris(formylamino) methane with phthalimidacetonitrile at high temperature

2)(c) Reaction of aminoacetonitrile with formamide

3)(d) Reaction of formamide and glycine or glycineamide with phosphorus oxychloride in a sealed tube.

These synthetic methods have various handicaps such as the low yield of product and complex procedures.

PROCEDURE FOR THE SYNTHESIS OF COMPOUND SR C, SR A and SR B:

STEP 1:

Synthesis of 2, 6, 8-trichloro-9*H*-purine:

CHLORINATION PROCEDURE:

Phosphorus oxychloride and a nitrogenous base were added to uric acid in the molar ratio of 10:3:1 respectively. The phosphorus oxychloride was freshly distilled, the amine was dried over potassium hydroxide, while the uric acid was dried over phosphorus pentoxide prior to use. The reaction mixture was refluxed with the exclusion of moisture and with stirring for 20-24 hours. Distillation of the resultant solution under reduced pressure to about half volume yielded a syrupy reaction mixture.

ISOLATION PROCEDURE:

The syrupy reaction mixture was poured slowly over the crushed ice. Ice was added periodically to the hydrolysis mixture to maintain a few suspended pieces in the solute, thus assuring low temperatures at all times; the tar was triturated frequently to hasten the conversion to a more granular solid. Approximately after one hour the mixture and the

aqueous filtrate were extracted with six portions of ether which were then used to extract the solid.

The ether extracts were combined and evaporated to dryness yielding a solid residue which was extracted with minimum of boiling 3N ammonium hydroxide and filtered. **The insoluble portion was fraction 1 c.** Upon cooling the filtrate, ammonium salt of 2, 6, 8-trichloropurine was yielded as a mass of fine needles. Neutralization of the mother liquor precipitated a small amount of crude 2, 6, 8-trichioropurine.

STEP: 2

Synthesis of 2, 8-dichloro-6-(1*H*-imidazol-1-yl)-9*H*-purine (SR C):

The 6-chloro function of 2, 6-dichloropurine can be region selectively replaced by imidazole in DMF at 65 ^{o}C to give the product. It is well known that the 6-chloride is much more readily replaced than the 2-Cl group.

2,6-Dichloropurine (2 mmol) and imidazole (12.1 mmol) were dissolved in freshly distilled DMF (36 mL), and the mixture was stirred at 65^{o}C for ~20h. Volatiles were evaporated, and the residue was washed with a large amount of CH_2Cl_2 to give SR C (66%).

STEP:3 FROM SR C-SYNTHESIS OF SR-A:

Synthesis of 2, 8-dichloro-9-(4-chlorophenyl)-6-(1*H*-imidazol-1-yl)-9*H*-purine:

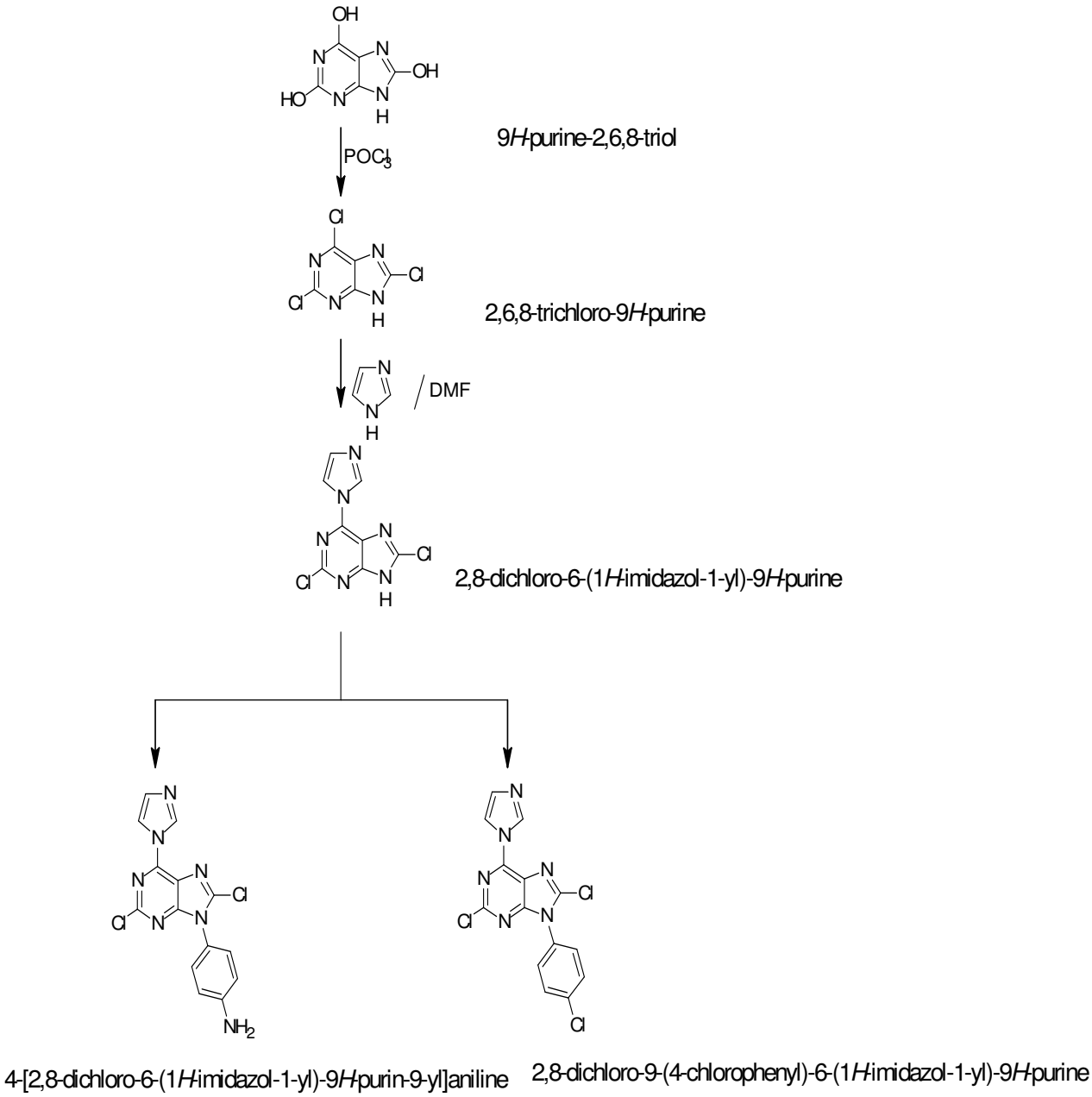
A mixture of 0.22 mol of 2,8-dichloro-6-(1*H*-imidazol-1-yl)-9*H*-purine, 0.22 mol of anhydrous potassium carbonate, and 0.20 mol of 1,4-dichloro benzene in 400 ml of Dimethyl formamide were stirred at ambient temperature for 64 hours. The reaction

mixture was poured into ice water with stirring and the solid that precipitated was collected by filtration.

FROM SR C -SYNTHESIS OF SR-B:

Synthesis of 2, 8-dichloro-6-(1*H*-imidazol-1-yl)-9*H*-purine:

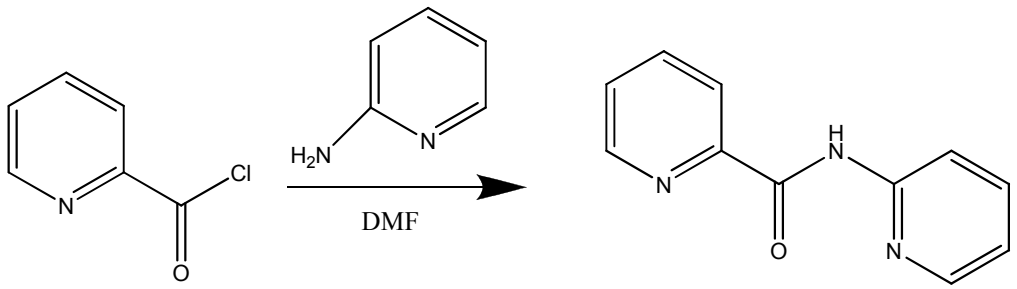
A mixture of 0.22 mol of 8-dichloro-6-(1*H*-imidazol-1-yl)-9*H*-purine, 0.22 mol of anhydrous potassium carbonate, and 0.20 mol of 4-chloro aniline in 400 ml of Dimethyl formamide was stirred at ambient temperature for 64 hours. The reaction mixture was poured into ice water with stirring and the solid that precipitated was collected by filtration.



Synthesis of *N*-(pyridin-4-yl) pyridine-3-carboxamide: [54]

2-amino pyridine in pyridine solvent showed that the reaction with pyridine-2-carbonyl chloride gives derivatives by direct reaction on the exocyclic nitrogen. 25ml pyridine-2-carbonyl chloride was added drop wise to 2-amino pyridine and dissolved in pyridine solvent. The reaction is maintained at lower temperatures and was accelerated by addition

of few drops of DMF. The resulting reaction mixture was poured into cold water and white crystals were formed and isolated. It was recrystallized.



pyridine-2-carbonyl chloride *N*-(pyridin-2-yl)pyridine-2-carboxamide

CHARACTERISATION:

INSTRUMENTATION:

The techniques employed for the characterization of the synthesised compounds were UV spectra, IR spectra, ^{1}H-NMR spectra and elemental analysis .

UV SPECTROSCOPY:

UV-Visible spectroscopy involves the measurement of amount of UV radiation absorbed by a substance in the solution. The wavelength between 200-400nm is considered to be UV radiations or UV region. Colored compounds absorb in visible range (i.e.) 400-800nm.The UV spectrum of an organic compound helps

- To make the research procedure easy and accurate
- To determine the concentration of drug, compounds in the media
- To determine impurity
- For quantitative analysis of compounds
- To determine geometrical isomer

INFRA RED SPECTROSCOPY:

The vibrational spectrum of a molecule is considered to be a unique physical property and is characteristic of the molecule. As such, the infrared spectrum can be used as a fingerprint for identification by the comparison of the spectrum from an ‘‘unknown’’ with previously recorded reference spectra. This is the basis of computer-based spectral searching. In the absence of a suitable reference database, it is possible to effect a basic interpretation of the spectrum from first principles, leading to characterization, and possibly even identification of an unknown sample. This first principle approach is based on the fact that structural features of the molecule, whether they are the backbone of the molecule or the functional groups attached to the molecule, produce characteristic and

reproducible absorptions in the spectrum. This information can indicate whether there is backbone to the structure and, if so, whether the backbone consists of linear or branched chains. Next it is possible to determine if there is unsaturation and/or aromatic rings in the structure. Finally, it is possible to deduce whether specific functional groups are present. If detected, local orientations of the group and its local environment and/or location in the structure are determined.

^{1}H - NMR MAGNETIC RESONANCE SPECTROSCOPY:

Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy is a powerful method used in the determination of the structure of unknown organic compounds. The ^{1}H NMR spectrum of an organic compound provides information concerning:

1) Number of Signals --- indicates how many "different kinds" of protons are present.

2) Positions of the Signals --- indicates something about magnetic (electronic) environment of protons.

3) Intensities (areas) of the Signals --- proportional to number of protons present.

4) Splitting of a Signal into Several Peaks --- indicates the number of nearby nuclei having magnetic moments (usually protons, sometimes fluorine).

Proton NMR Spectra were recorded using solvent Deutrated methanol on BRUKER. Advance III 500 NMR spectrometer. Chemical shifts are reported in parts per million ($^{\delta}$ppm).

MASS SPECTROMETRY:

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining the masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structure of molecules.

It determines molecular mass by manipulating flight path of molecular ion (or fragments) in magnetic field. The mass spectra of an organic compound provide information concerning:

M : reveals mass of molecule consisting of lowest mass isotopes

M+1: intensity / 1.1% give approximate number of carbons

M+2: intensity reveals presence of S, Cl or Br

The compounds were found to have

1. **Ultraviolet spectroscopy (UV) by Shimadzu UV-Spectrophotometer.**

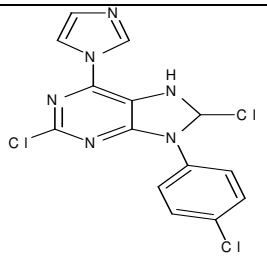
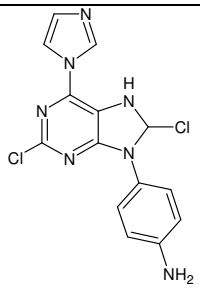
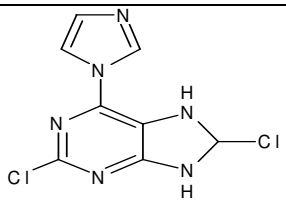
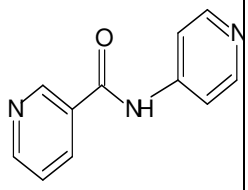
2. Infrared spectroscopy (IR) by Perkin-Elmer Spectrometer usin KBr pellets.

3. Nuclear Magnetic Resonance spectroscopy (1HNMR) by 500MHZ JEOL using DMSO.

4. Mass spectroscopic (MS) by JEOL GC mate.

PHYSICAL DATA:

Synthesis and identification of purine and pyridine derivatives:

COMPOUND NO.	SR A	SR B	SR C	SR D
PRODUCT				
RECRYSTALLISATION	Ethanol	Ethanol	Ethanol	Ethanol
YIELD(%)	75	70	78	80
Melting Point	222^0C	210^0C	230^0C	192^0C
TLC:				
Stationary Phase	Precoated silica gel GF plates	Precoated silica gel GF plates	Precoated silica gel GF plates	Precoated silica gel GF plates
Mobile Phase	Methanol/Chloroform	Methanol/Chloroform	Methanol/Chloroform	Methanol/Chloroform
Location	UV Chamber	UV Chamber	UV Chamber	UV Chamber
UV SPECTROSCOY:				
	289nm	295nm	291nm	279nm
IR SPECTROSCPY:				
	3000-3100cm^{-1}(C-H), 1389cm^{-1}(C=C),1100 cm^{-1}(C-N), 3400cm^{-1}(Ar-NH_2)N-H, 1300cm^{-1}Ar-	3000-3100cm^{-1}(C-H),1389cm^{-1}(C=C),1011cm^{-1}(C-N),3411cm^{-1}(Ar-NH_2)N-H,	3070cm^{-1}(C-H), 1304cm^{-1}(C=C), 3448cm^{-1}(N-H), 1674 cm-^{1}(C=O), 1065cm^{-1}(C-N), 1582cm^{-1}(C=N).	3008cm^{-1}(C-H),1404cm^{-1}(C=C),1018cm^{-1}(C-N),1589 cm^{-1}(C=N),741cm^{-1}(C-Cl),1738 cm^{-1}

	NH_2(C-N).	1300cm^{-1}Ar-NH_2(C-N).		^{1}(C=O)
NMR				
	δ6.8 (d,2H,Aromatic C-H),7.1(d,2H,Aromatic C-H),7.6(d,2H,heteroaromatic C-H),8.5(s,1H,heteroaromatic C-H)-(Aromatic)	δ6.8 (d,2H,Aromatic C-H),7.1(d,2H,Aromatic C-H),7.6(d,2H,heteroaromatic C-H),8.5(s,1H,heteroaromatic C-H)-(Aromatic),10.5 (s,2H,-NH_2)	6.9(s,1H,Aromatic C-H),7.1(s 2H,Hetroaromatic),8.3(s,1H,Aromatic C-H)	7.5(m,8H,heteroaromatic C-H),8.6(s,1H,-NH)
MASS SPECTROSCOPY				
	367.71(M^{+2}), (2%) 365.61(M^+)(5%),63.22(B) (100%)	348.43(M^{+2}) (2%), 346.17(M^+)(7%),60.05(B) (100%)	198.38(M^+)(12%) ,106.6(B) (100%)	235.01(M^+)(6%), 60.3(B) (100%)

ACUTE TOXICITY[54]:

Acute toxicity is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours.

Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose and studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans.

This acute toxicity study was designed as per the OECD Guidelines for Testing of Chemicals, Acute Oral Toxicity (Acute Toxic Class Method),Guideline 423.

PRINCIPLE OF THE TEST:

Acute toxicity testing determines the toxicity of a chemical or drug substances after single administration.

Today, acute toxicity testing focuses on levels on acute tolerance, nature of acute toxicity symptoms in the sub-lethal range, and dose levels which cause mortality in two animals i.e. quality has replaced quantity.

It is the principle of the test that, based on a stepwise procedure with the use of a minimum

number of animals per step, sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using four animals of a single sex (normally females).

Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.;

- no further testing is needed,
- dosing of three additional animals, with the same dose
- Dosing of three additional animals at the next higher or the next lower dose level.

DESCRIPTION OF THE METHOD:

EXPERIMENTAL ANIMALS:

Healthy adult female Albino mice (4nos.) were used in research. Females were weighing between 20-25mg, for all the four animals food, but not water withheld overnight prior to dosing.

SELECTION OF DOSE LEVELS & ADMINISTRATION OF DOSES:

Purine and Pyridine derivatives, being novel synthetic compounds, the mortality was unlikely at the highest starting dose level (300mg/kg body weight). Hence a limit test at one dose level of 300mg/kg body weight was conducted in all the four animals.

HOUSING AND LIGHTING CONDITIONS:

The temperature in the experimental animal room should be 22ºC (+ 3ºC). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but
the number of animals per cage must not interfere with clear observations of each animal.

PROCEDURE:

ADMINISTRATION OF DOSES:

The test substance is administered in a single dose by 19 guage using an oral tube.

TESTING PROCEDURES:

The test compounds should be administered to animal to identify doses causing no adverse effects and doses causing major (life-threatening) toxicity. The use of vehicle control groups should be considered. For compounds with low toxicity, maximum feasible dose should be administered.

Studies should be conducted in at least two mammalian species, including a non rodent species when reasonable. The objectives of acute studies can usually be achieved in rodents using small groups of animals (for instance, three to five rodents per sex per dose). Where non rodent species are appropriate for investigation, use of fewer animals may be considered. Any data providing information on acute effects in non rodent species, including preliminary dose-range finding data for repeat-dose toxicity studies, may be acceptable.

OBSERVATION

The animals were observed individually after dosing once during the first 30 minutes, periodically for the first 24 hours , with special attention given during the first 4 hours & daily there-after, for a total of 14 days. The following clinical observation were made and recorded.

- **Toxic Signs**

All mice were observed for any toxic signs.

All the rats were observed for any pre terminal deaths.

- **Body Weight**

Individual body weights were recorded for all the animals once in a week.

- **Cage Side Observation**

The faeces colour, faeces consistency, changes in skin & fur, eyes & mucous membrane (nasal) of the animal were observed once in a week.

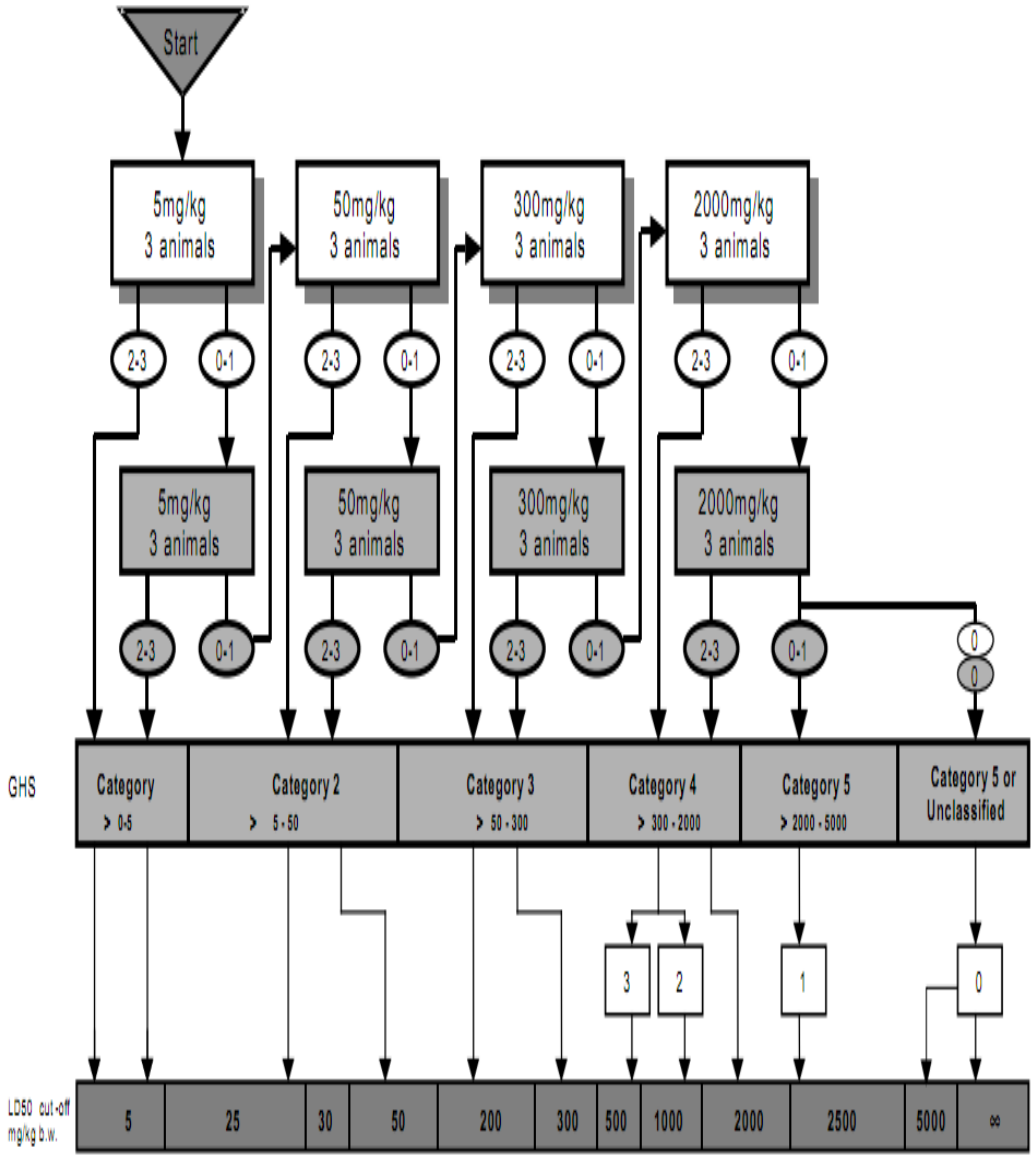
- **Physical examination**

Physical examination included changes in,

- Respiratory System
- Cardiovascular system(hear rate)
- Salivation, lacrimation, piloerection, urinary incontinence and defecation.
- Central nervous system:

  Ptosis, drowsiness, gait, eye prominence, eyelid closure, convulsions, biting,staub's test, motor incoordination, writhing, stereotypy, aggression, righting reflex, pinna reflex, corneal reflex,tremors and convulsions.

ANNEX 2a: TEST PROCEDURE WITH A STARTING DOSE OF 5 MG/KG BODY WEIGHT



ANTI-DIABETIC ACTIVITY[55]:

IN VIVO ANIMAL MODELS OF DIABETES MELLITUS:

Diabetes can be induced by pharmacologic, surgical or genetic manipulations in several animal species. In vivo is the experimentation using a whole, living organism as opposed to partial or dead organism. Animal testing and clinical trials are two forms of clinical research. In vivo testing is often employed because it is better suited for observing the overall effects of an experiment on a living subject.

PHARMACOLOGICAL INDUCTION OF DIABETES:

Streptazotocin and alloxan are the most frequently used drugs for the induction of Diabetes mellitus. Both drugs exert their diabetogenic action when they are administered parentrally, intravenously, intraperitoneally or subcutaneously. The dose of these agents required for inducing diabetes depends on the animal species, route of administration and nutritional status. According to the administered dose of these agents syndromes similar to either type 1, type 2 diabetes mellitus or glucose tolerance can be induced.

The cytotoxic action of these diabetogenic agents is mediated by reactive oxygen species, but both the drugs differ in their mechanism of action.

Alloxan and the product of its reduction dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide with a simultaneous massive increase in cytosolic calcium concentration, which causes rapid destruction of pancreatic β cells. The range of diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic and may cause the loss of many animals. This loss is likely to stem from kidney tubular cell necrotic toxicity, in particular when too high doses of alloxan are administered. The most frequently used intravenous dose of alloxan in rats is 65mg/kg, but when it is administered intraperitoneally or subcutaneously its effective dose must be higher. For instance, an intraperitoneal dose

below 150mg/kg may be sufficient for inducing diabetes in the animal species. In mice doses vary among 100-200 mg/kg by intravenous route.

The destruction of pancreatic β cells by alloxan is associated with a huge release of insulin which makes animal more sensitive to severe hypoglycaemia that may be lethal. Thus following the treatment with alloxan, animals are fed with glucose solution (5%) for 12-24 hrs.Afterwards an increase of glucose levels is observed in comparison to control animals due to insulin deficiency. It is also reported that fasted animals are more susceptible to alloxan effects and increased blood glucose in fed animals provides partial protection. The experimental protocols recommend that administration of alloxan must be done in fasting period (8-12hrs), followed by addition of glucose solution to avoid hypoglycaemia.

EXPERIMENTAL DESIGN:

Animals : Swiss albino mice.

Sex : Either sex.

Weight : 25-30g.

Groups: 6, six animals each.

Group 1: Diabetic control – animals receiving alloxan at the dose of 60 mg/kg b.w.

Group 2: Synthetic compound 1

Group 3: synthetic compound 2

Group 4: synthetic compound 3

Group 5: synthetic compound 4

Group 6: Animals receiving Glibenclamide at the dose of 3mg/kg b.w.

Induction of experimental diabetes

Diabetes is induced in mice by intraperitoneal (i.p.) injection of alloxan at a dose of 60mg/kg body weight, dissolved in saline. Diabetes is confirmed by the determination of fasting blood glucose concentration on the third day post administration of alloxan.

Evaluation of Anti-Diabetic Activity:

Blood glucose and urine glucose levels are measured on day 1, 4, 7, 10 and 15 of the study.

1. ESTIMATION OF BLOOD GLUCOSE

METHOD OF BLOOD COLLECTION:

Blood sample for glucose estimation was collected from mice tail tip. In a well restrained mice, the tail was embedded in hot water 45^0C and about 1mm of it s end was cut and the drop of blood was collected directly on the strip placed in the glucometer (One Touch Horizon, Life Scan Inc.Milpitas USA, Johnson and Johnson Company).

2. ESTIMATION OF URINE GLUCOSE

Glucose will overflow into the urine only when the blood glucose level is high, that is, too high for the kidneys to stop it spilling over into the urine. In most people, blood glucose levels above 10 mmol of glucose per litre of plasma will cause glucose to appear in the urine. This level is called the 'renal threshold' for glucose.

PROCEDURE

Fresh urine was collected by slightly pressing the tail and back of the rat. Glucose and ketonein urine was checked using keto-diastix strips

RESULTS AND DISCUSSION

The results of the Drug design, Synthesis of compounds and Evaluation of compounds are discussed below:

1) DRUG DESIGN

In the Hypogen studies 10 hypotheses were generated using the most diverse 24 molecules from the training set (Fig.5d) and validated using 201 test molecules. Pharmacophore were computed and the top-10 hypotheses were exported. Results of Pharmacophore hypotheses are presented in Table 5a. The top ranked one, OutHypo-1 consists of two hydrophobic Aliphatic and one Positive ionisable features. The quality of the generated Pharmacophore hypotheses was evaluated by considering the cost functions calculated by Hypogen module during hypotheses generation. The cost values, correlation(r) and root mean square deviations (R.M.S.D) values are given in Table 5a. Hypo1 is the best Pharmacophore hypotheses in this study, as it is characterized by the lowest total cost (116.3) the highest cost difference between the null and total hypotheses cost(52.582),the lowest RMSD(1.34256) and the best correlation coefficient(0.821441). It is evident that as error, weight and configuration component are very low, the total Pharmacophore cost is also low and close to the fixed cost. Also, as total cost is less than the null cost, this model accounts for all the Pharmacophore features and has a good predictability power. Fig.5c shows the best Pharmacophore features with their geometric parameters. Fig 5b and 5a represents the best Pharmacophore model aligned with the most active and inactive molecules with IC_{50} of 0.089nM and 8800nM respectively.In the Fig 5b

the Pharmacophore features are mapped well to the active molecule. On the other hand in Fig 5a the feature of the could not fit well since it is a low active molecule.

In the training set, out of eight highly active molecules eight are correctly predicted as highly active, out of eight medium active molecules eight were predicted well, and in the eight inactive molecules, eight were predicted to be less activity. It was inferred that there is less difference between the experimental and predicted IC_{50} values and thus the error values are less and the fit value gives a gives a good measure of how precisely the defined features in the best Pharmacophore fit well with each molecule.

2) SYNTHESIS AND CHARACTERISATION

The synthesised compounds were recrystallized and identified by TLC. The melting points of the products were found. The characterisation was carried out using sophisticated instruments like UV, IR, NMR, MASS spectroscopy.

UV SPECTROSCOPY

Electronic Transitions observed in synthesised compounds:

The synthesized compounds SR A-SR D show R, K&B bands. ($n \rightarrow \prod^*$)

The R-band due to presence of Oxygen and Nitrogen. ($\prod \rightarrow \prod^*$)

The K-band is due to conjugated system

The B-band is due to the presence of aromatic and hetero aromatic systems.

Presence of auxochrome like OH, NR2, groups in the synthesized compounds are responsible for the high intensity absorption and bathochromic shifts.

IR SPECTROSCOPY:

The IR Spectra have been used to identify the synthesised compounds.

The presence of impurities like the starting raw materials uric acid, 1, 4-dichloro benzene and 4-chloro aniline were ruled out.

The synthesised compounds show the following specific characteristic stretching and bending vibration.

3400-3100 cm^{-1} stretching vibration of N-H group.

3100-3000 cm^{-1} stretching vibration of Aromatic ring.

Formation of the purine nucleus has been confirmed by the following characteristic regions:

1400-1000 cm^{-1} bending vibration of C-N group.

Presence of impurities:

The presence of starting materials as impurity was ruled out by observing the following characteristic stretching region:

Absence of stretching in the region of 3650-3584 cm^{-1} indicates the absence of OH group.

NMR SPECTROSCOPY:

The ^{1}H NMR Spectral data of all the synthesised compounds were in conformity with the structure assigned. All the compounds showed peak in the region 6-9ppm proving the presence of Aromatic protons. The peak in the region of 8.6, 7.2 observed the presence of specific protons NH&CH.A common peak appears in all spectra in the region of 1 to 2 ppm. This implies the solvent peak.

MASS SPECTROSCOPY:

The entire synthesised compounds exhibited molecular ion peak (M+) of varying intensities ascertains the molecular weights of the compounds. Compound SR A, SR B and SR C shows M+2 peak due to the presence of Cl.

All the above data confirmed the assigned structure of the synthesised compounds.

PHARMACOLOGICAL EVALUATION:

ACUTE TOXICITY:

Acute oral toxicity was performed according to the OECD guidelines 423A method.

This method has been designed to evaluate the substance at fixed doses and provides information both for hazard assessment and substance to be ranked for hazard classification purposes.

The synthesised compounds were administered initially at a starting dose of 5mg/kg body weight in 1% CMC (p.o) and observed 14 days mortality due to acute toxicity.

Careful observation was made at least twice a day for the effect on CNS, ANS, motor activity, salivation, skin coloration and other general signs of toxicity were also observed and recorded.

Since no sign of toxicity was observed at 50mg/kg body weight to the group of animals, the LD_{50} value of the title compounds (SR A-SR D) expected to exceed 50mg/kg body weight and represented as class 2.

From the toxicity studies the data revealed that all the synthesised compounds proved to be non toxic at tested dose level and well tolerated by the experimental animals as their LD 50 cut –off values > 50mg/kg body weight.

Pre-terminal deaths:

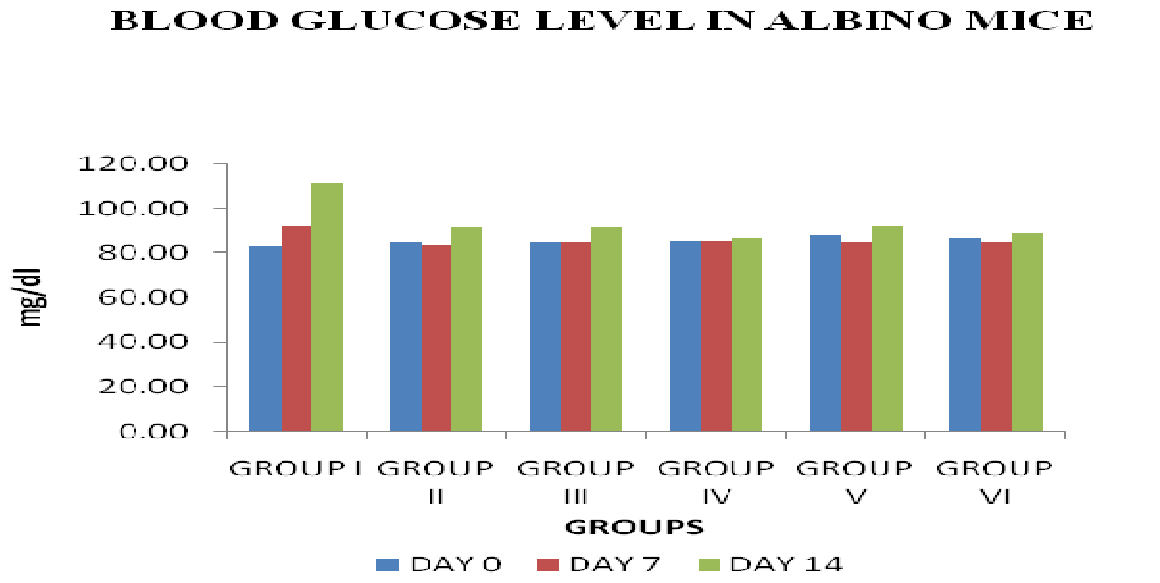
S. No	PARAMETER	4hr	24hr	48hr	7day	14day
1	Salivation	Normal	Normal	Normal	Normal	Normal
2	Lacrymation	Normal	Normal	Normal	Normal	Normal
3	Respiratory rate	Normal	Normal	Normal	Normal	Normal
4	Colour of the eye`	Normal	Normal	Normal	Normal	Normal
5	Inflammation	Absent	Absent	Absent	Absent	Absent
6	Jumping	Absent	Absent	Absent	Absent	Absent
7	Grooming	Absent	Absent	Absent	Absent	Absent
8	Gnawing	Absent	Absent	Absent	Absent	Absent
9	Straubs Tail effect	Absent	Absent	Absent	Absent	Absent
10	Fur colour	Normal	Normal	Normal	Normal	Normal
11.	Aggression	Absent	Absent	Absent	Absent	Absent
12	Anxiety	Absent	Absent	Absent	Absent	Absent
13	Colour of faces	Normal	Normal	Normal	Normal	Normal
14	Urination	Normal	Normal	Normal	Normal	Normal

ANTI-DIABETIC ACTIVITY:

Effects of various compounds on glucose levels:

GROUPS	DAY 0	DAY 7	DAY 14
GROUP I	83.00±1.41	92.25±2.06*	111.50±5.20*
GROUP II	85.25±2.63	83.75±1.71	91.75±6.18
GROUP III	84.50±1.29	85.25±1.71	91.75±4.19
GROUP IV	85.75±2.75	85.75±1.26	86.50±3.70
GROUP V	88.25±2.99	84.75±.50	92.25±5.91
GROUP VI	86.75±3.77	85.25±2.22	88.50±4.51

VALUES ARE EXPRESSED AS MEAN ± SD, N=6, *= P<0.05



GROUP I – ALLOXAN
GROUP II – COMPOUND I WITH ALLOXAN
GROUP III – COMPOUND II WITH ALLOXAN
GROUP IV – COMPOUND III WITH ALLOXAN
GROUP V – COMPOUND IV WITH ALLOXAN
GROUP VI – GLIBENCLAMIDE WITH ALLOXAN

SUMMARY AND CONCLUSION:

- Twenty five molecules which passed Lipinski's rule were docked against DPP-4.
- The molecule containing purine and pyridine nucleus was selected on the basis of synthetic feasibility.

- Four compounds with top scores were synthesized, purified, characterized using UV, IR and NMR spectroscopy and Mass spectrometry.
- These four compounds were subjected to acute toxicity studies to fix the LD_{50}. The LD_{50} value of the title compounds (SR A- SR D) was expected to exceed 50mg/kg.
- All the synthesised compound so were subjected to, in*vivo* experiment to determine antidiabetic activity and were found to decrease the blood glucose levels, but they did not fare better than the standard drug glibenclamide.

FUTURE:

The synthesized compounds showed good anti-diabetic activity. Hence the anti-diabetic study deserves for further investigations like MTT Assay using different cell lines. Based upon the literature, purine and pyridine have wide pharmacological activity, so the synthesized compounds deserves for further investigation against new therapeutic targets.

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