ISOLATION AND CHARACTERIZATION OF LEISHMANIAL ADENINE AMINOHYDROLASE AS A DRUG TARGET

Submitted by

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A dissertation as a requisite aimed at the completion of M. Phil in Biotechnology

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CERTIFICATE

This thesis in present form submitted by Firdous Butt and accepted by the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University in the completion of the requirement for a degree of Master of Philosophy in Biotechnology.

External Examiner: ----------

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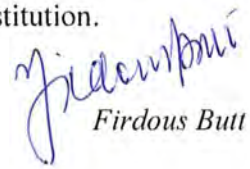
Dated: ----------

DEDICATION

I dedicate my endeavors to
Almighty ALLAH
for empowering me to persevere successfully
at achieving dearest GOAL.

DECLARATION

I hereby declare that the presented work in this thesis is original and no one has ever offered exposition under this title from Quaid-i-Azam University or any other institution.



Firdous Butt

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LIST OF ABBREVIATION

OW	Old world
NW	New World
HGPRT:	Hypoxanthine-Guanine Phosphoribosyltransferases
XPRT	Xanthine Phosphoribosyltransferases
IMP	Inosine Monophosphate
IMPDH	Inosine Mono-phospho-dehydrogenase
GMP	Guanine Monophosphate
APRT	Adenine Phosphoribosyltransferases
AMP	Adenine monophosphate
AHH	Adenine aminohydrolase
ADA	Adenosine Deaminase

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Thankful to all those who helped in the completion of M. Phil, provided material in addition to granting insightful vision that facilitated me in having good grasp on the subject matter.

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It was pleasing to be a part of Infectious Diseases Lab for allowing workspace for duly job.

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Firdous Butt

ABSTRACT

Currently available drugs for *Leishmaniasis* are insufficient in providing promising results. Pentavalent Antimonial does offer first-line drug therapy, however emergence of drug resistant strains have reduced the efficacy of marketed drugs. On the other hand, second-line drug therapy is less effective due to toxicity and high prices, and therefore encouraging new methods of exploring drug targets. Recently discovered enzyme Adenine Aminohydrolase (AHH), counterpart of human purine salvage pathway, is the crucial enzyme that de-aminates Adenine as a substrate in *Leishmania spp*, which is important for adenine utilization as a purine and a nitrogen source. Characterization in lower species supported AHH as a plausible drug target. In the current study, we have confirmed the presence of Adenine Aminohydrolase in given Pakistani *Leishmanial strain* source. The knowledge of functional partners of targeted enzyme pathway and its mode of kinetic action are important in developing robust enzyme assay and in approximating K_m and V_{max} values for enzyme identification. Enzyme exhibits K_m value equal to $14.78 \pm 5\mu M$ and V_{max} value of $0.2098 \pm 0.01\mu M/min/mg$ demonstrating catalytic efficiency (K_{cat}/ K_m) of $1.27 \times 10^7 M^{-1}sec^{-1}$. Insilico- molecular docking software GOLD probed compound [$C_{26}H_{15}N_3O_7S$;benzyl amino group] i.e. (*2-(2-(5-methylfuran-2yl)-5-nitro-1H-benzo[d]imidazole-1-ylsulfonyl) anthracene-9, 10-dione* with high score calculating sound binding affinity of -6.48 Kcal/mole having hydrophobic interactions formed in majority among all the ligand-protein i.e. 2ICS and compound interactions, thus most likely inhibit its activity at non- competitive site. In vitro kinetic analysis estimates IC_{50} value of $0.8106\mu g/ml$ showing mixed non-competitive inhibition validating molecular docking results.

INTRODUCTION

Leishmaniasis is a vector borne infectious disease found endemic in tropical and subtropical regions, transmitted by obligate intramacrophage protozoan of genus Leishmania spp to humans and animals by the bite of infected female phlebotomine genus Old world and Lutzomyia genus New World sand flies that function as vectors (Androula and Helena, 2010). According to The WHO, studies there are 1.5 to 2 million cases of Leishmaniasis worldwide annually in ninety-eight countries, of which half a million are visceral Leishmaniasis. Recent evidence has also shown that Leishmania-HIV co-infections are becoming a major health problem in affected region (http://www.who.int).

It exists in different forms; however, the most common forms are Cutaneous Leishmaniasis that result in skin sores, and Visceral Leishmaniasis affecting several internal organs (usually spleen, liver, and bone marrow) (http://www.cdc.gov).

Climate and environmental changes has caused expansion of the geographic range of the vectors and *Leishmaniasis* transmission and adaptability in the future. (http://www.cdc.gov). Owing to limited resources invested in diagnosis, treatment and control, and its strong association with poverty, the ever-increasing rate of occurrences in different continents stretching out from Northern Argentina to Southern Texas, and its advancements from rural to urban areas by human-to-human has developed interest in *Leishmaniasis* (Desjeux, 2001).

The Leishmaniasis is a cluster of human diseases classified with respect to its own location, clinical presentation, and immune response of the host. The complexity is due to its epizoology; about 21 out of 30 disease causing species of sand- flies infecting human (Nisha *et al.*, 2012). There is also a range of mammalian *spp i.e.* Rodents and canids (foxes, wolves, dogs, Jackals, and coyotes) implicated as reservoir host (Otranto, 2013), whereas humans are incidental hosts of infection (Ashford, 1997).

Out of seven families of protozoan phylum, disease causing are the Sacromastigophora and Apicomplexa. Leishmania and Trypansoma genera belong to order unicellular Kinetoplastida that branched off from zoo-flagellate of family Sacromastigophora.

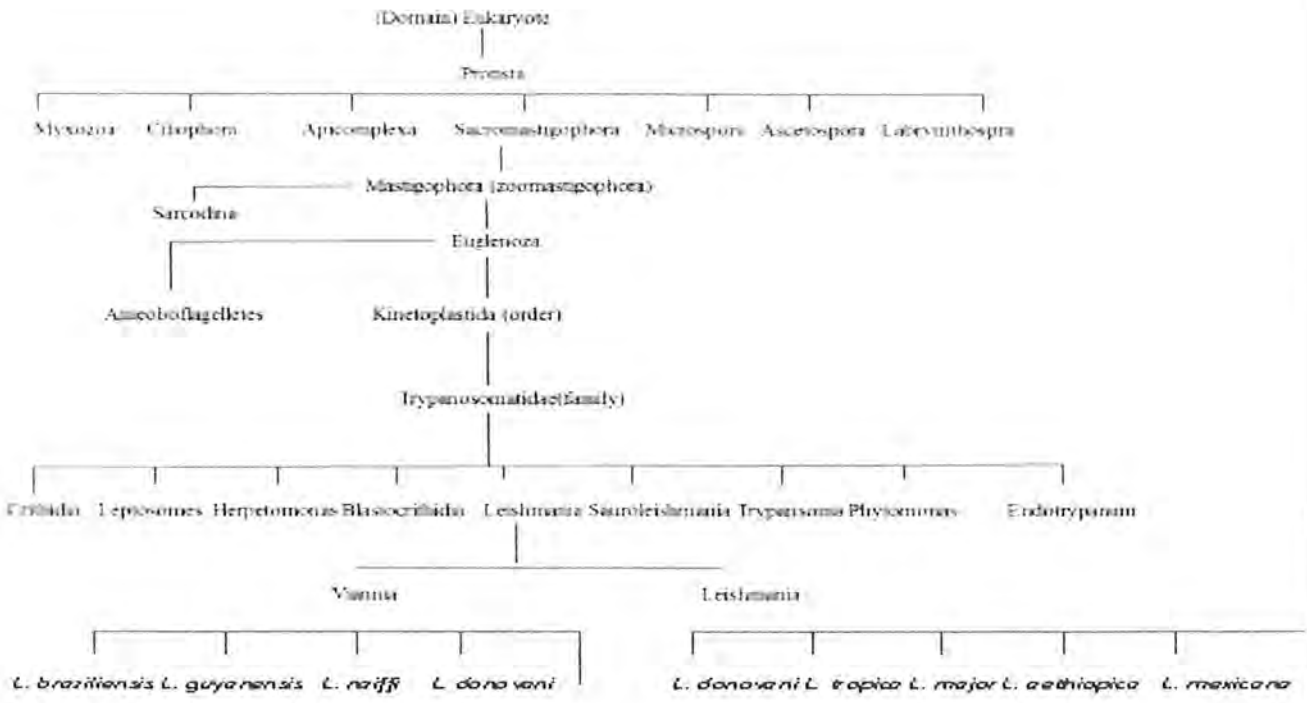


Fig1. Tentative phylogeny of Phylum Protozoa

Leishmania manifests two phenotypes called as "amastigotes" and "promastigotes" during the course of their developmental stages. Amastigotes are ovoid, non-motile, and intracellular while promastigotes are elongated, motile and extracellular. Their life cycle commences from the bite of infected female phlebotomine (92-3mm long) by injecting promastigotes through their proboscis during blood meals.

Macrophages and granulocytes (PML) phagocytize promastigotes adhered by complement serum protein (C3b, IgG etc.) as first line of defense. In the phagosome parasite retracts flagella and transforms into amastigotes, resists oxidative degradation by the production of acid phosphotases by parasite spp and its mode of action. On fed upon by sand fly during their blood meal from the infected part of skin, Amastigotes regain motility and transform back to promastigotes as they approach the midgut of the sand fly (http://www.dpd.cdc.gov/dpdx).

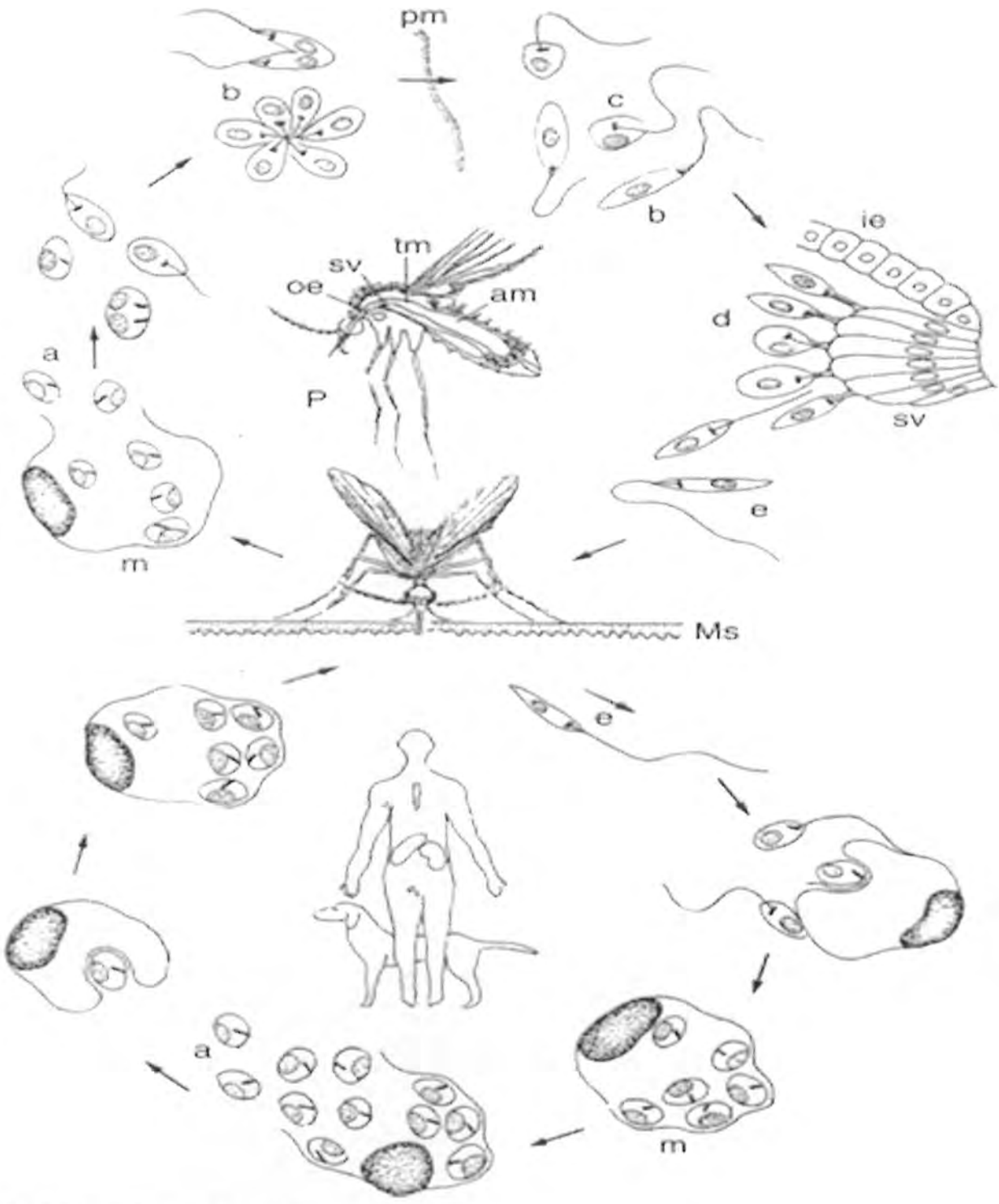


Fig 2. Life cycle of Leishmaniasis

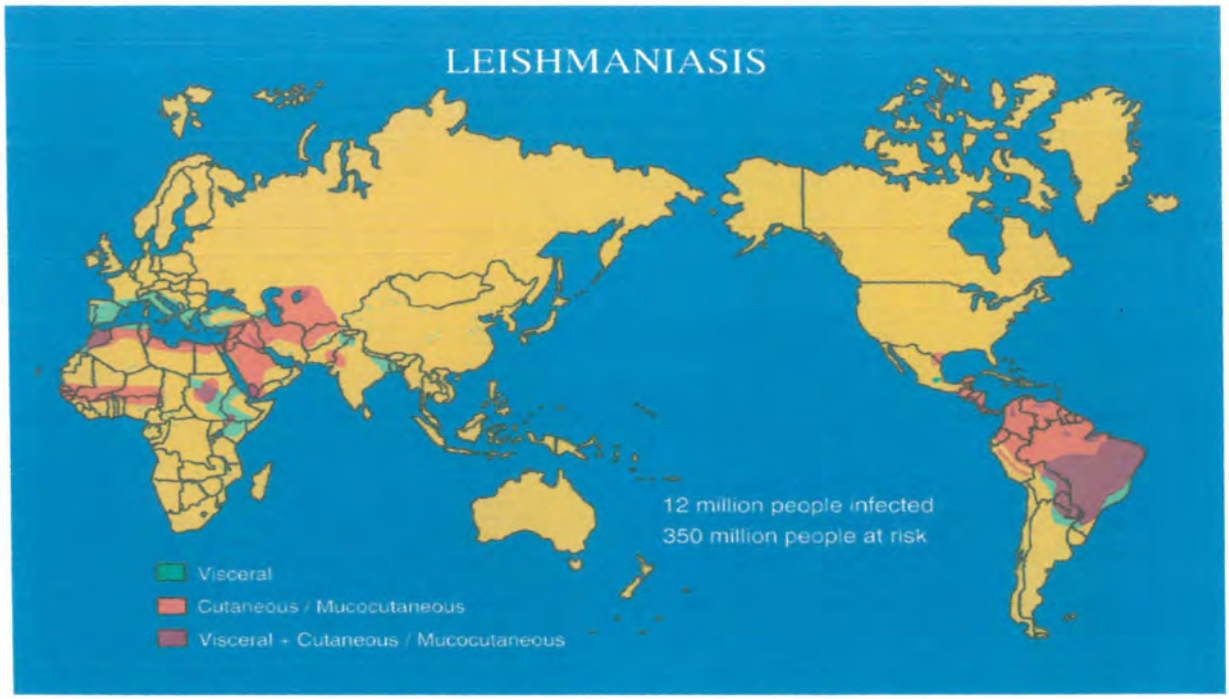


Fig 3a. Geographical Distribution of (NW i.e. S.America) & (OW i.e. Africa,Europe,Asia) Leishmanial strains. CL found along the equatorial regions moving down to Southren parts as VL and emerging as Mucocutaneous on the outskirts of epedemic regions.

Fig 3b . Map showing distribution of cutaneous leishmaniasis in Pakistan and surrounding countries of South Asia.

In India and Pakistan various endemic belts *L. tropica* and *L. major* cause simple cutaneous leishmaniasis disease and man is the most common reservoir.

In Baluchistan, Sibi, Zhob, Loralai, Pishin, and Kohlu (Yasinzai M. *et al.*, 2005–2007) disease presented in form of nodules, plaques, ulcers and/or scarring. Jacobabad, Larkana, and Dadu districts of Sindh province (Bhutto *et al.*, 1996–2001) reported dry papular type dry ulcerative type, wet ulcerative type clinical cases. In Khyber Pakhtunkhawa (Rowland *et al.*, 1997), disease clinically presented as typical "oriental sore" in Timergara and Dir. In Punjab, cases of lupoid leishmaniasis, sporotrichoid, paronychial, psoriasiform were reported in Khushab district (Anwar *et al.*, 2004), Multan (Ayub *et al.*, 1999-2000), Rawalpindi, Sargodha, and Muzaffarabad (Bariand Rahman, 2002–2006).

Leishmaniasis exemplify the Th1/Th2 paradigm; complexity and diversity of this disease requires biochemical, molecular, bio-geographical, entomological, mammalogical and ecological studies based on Molecular approaches used in epidemiological studies for resolution of genetic differences between Leishmania parasites species and strain levels to address key epidemiological and population genetic questions.

Sodium stibogluconate (Pentostam), Meglumine antimonate (Glucantime) have been the mainstay of antileishmanial therapy. Amphotericin B and Pentamidine, the traditional parenteral alternatives to Antimony prescribed as second-line status modified with the lipid formulation for use in some settings. Miltefosine and Paromomycin has shown great efficacy to treat Leishmaniasis but their high cost and therapeutic complications limit their usefulness.

Enzymes chosen as a drug target must be essential for the survival of the parasite and a counterpart of the mammalian host to allow selective inhibition in drug discovery plan.

Ergosterol biosynthesis, parasite specific proteases and reductases, purine salvage and phospholipid biosynthesis exploited as drug targets. Adenine aminohydrolase one of the functional partners of purine salvage pathway detected as a human counterpart and considered as promising drug target.

LITERATURE REVIEW

The identification of ever-evolving Leishmanial strains required review on classification. Leishmania genus categorized into Old world (OW) and New world strains (NW) supporting the idea of occurrences of same species in two different continents. Genealogical and geological data states Palearctic origin of (NW) Leishmania from (OW) Leishmania in the early Cenozoic (27 million years ago) and lately dispersion to the Nearctic (North America) in Eocene which extends down to the Neotropical (South America) in Pliocene after the formation of Panamanian bridge 3 million years ago (Thomaz-Soccol et al, 1993). Diane McMahon-Pratt (1989) justifies that the absence of GP46/M-2 gene in NW strains supported the division of Leishmania into subgenus Viannia and further down into complexes conveys periplyarian mode of development in gut of sand fly. From reptiles to murid rodents and their immediate ancestors were important mammalian reservoirs promoting adaptation of co-evolving vectors to new host accelerates the speciation among Leishmania. These different species are morphologically indistinguishable, separated by Isoenzyme analysis (Francine Pratlong, 2009), molecular method e.g. RFLP-PCR, DNA for ribosomal ITS1 and hsp70 helps identifying VL from PKDL and coinfections (Montalvo *et al.*, 2010).

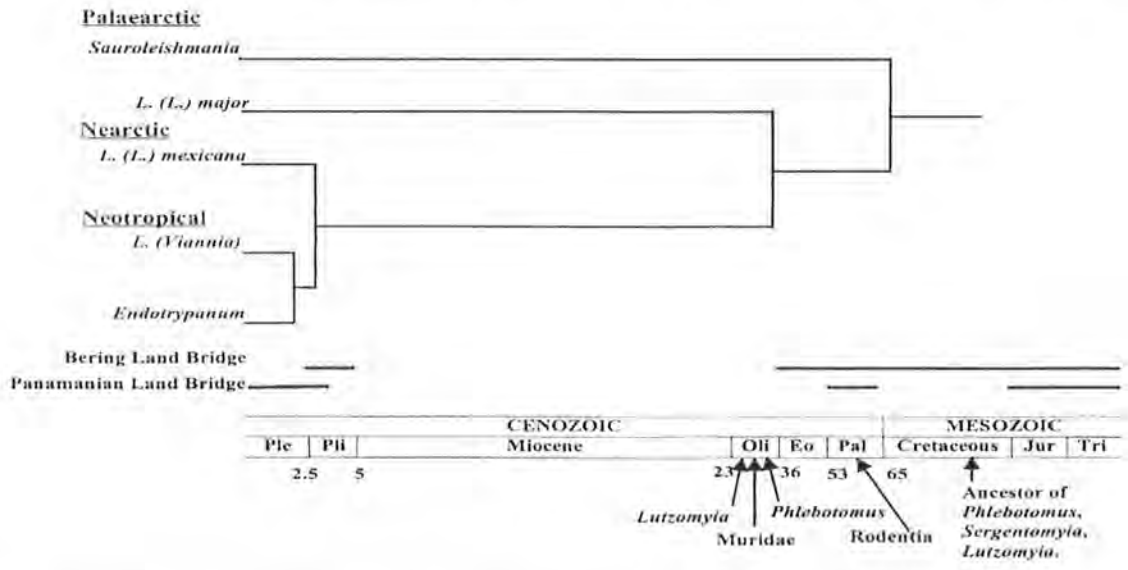


Fig 4. Genealogical &Geological record of the Sauroleishmania/Leishmania Endotrypanum clade on the fossil records of mammals (Nowak, 1991) and sand flies (Lewis, 1982) & (Kerr, 2000)

CUTANEOUS LEISHMANIASIS

Cutaneous Leishmaniasis sited in 70 countries and about 90% cases found in Afghanistan, Brazil, Pakistan, Peru, Saudi Arabia, and Syria (Desjeux, 2004)

In clinical presentations, histological details of lesion type observed in course of infection present the degree of severance caused by disease. Following incubation period, skin lesion emerges as furuncle papule that on accumulation forms nodule, which either persists as nodule or form ulceration with raised border and cavity in the center of the wound. Some have more than one primary lesions appear as diffused lesions defined as satellite lesions, other appears along the axis of lesion, and lymph nodes termed Sporotrichoid-like nodular lymphangitis. Leishmaniasis recidivans, usually a chronic form caused by *L. tropica* (OW and anthroponotic) reappears in the center or periphery of a healed lesion of Cutaneous Leishmaniasis.

Table1: Old and New world CL strains of subgenus of Leishmania and its location

Strains	Leishmania	Viannia	Location	Type of Infection
Old World	L. tropica L. major L. aethiopica L. infantum		Central, West Asia (Anthroponotic) North Africa, Central Asia, West Asia Kenya, Ethiopia	Anthroponotic, Cutaneous, Oriental sore Zoonotic, Cutaneous, Oriental sore
New world	L. mexicana L. infantum L. venezuelensis L. amazonensis L. pifanoi	V. brazilliensis V. guyanensis V. panamensis V. shawi V. naiffi V. lainsoni V. lindenbergi V. peruviana V. colombiensis	South America	

Fig 5 CLINICAL PRESENTATION OF LEISHMANIASIS (Shiraz J K& Muneeb S, 2005)

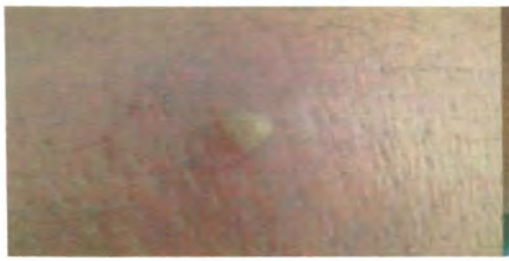


Fig 5a.Red boil nodule at the site of inoculation

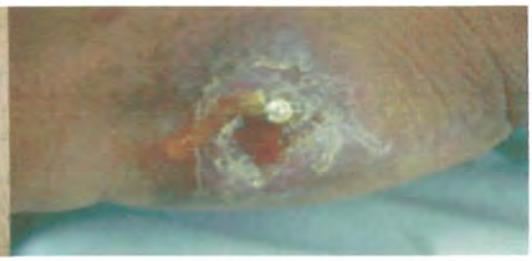


Fig 5b. Cutaneous Leishmaniasis

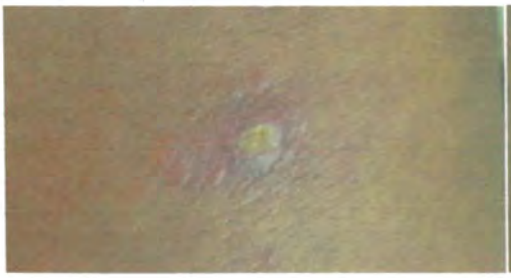


Fig 5c.Initial brownish nodule (urban)

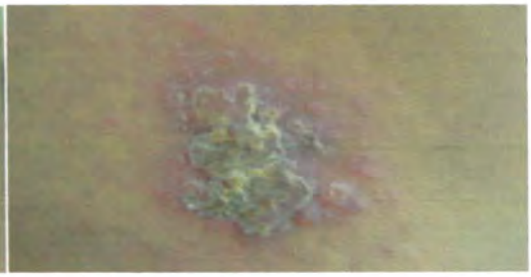


Fig 5d. Plaque lesion of dry urban

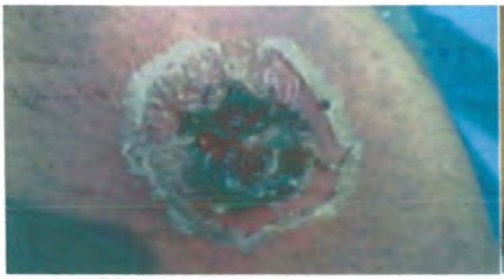


Fig 5e. Crusty plagued with central underlying ulcer

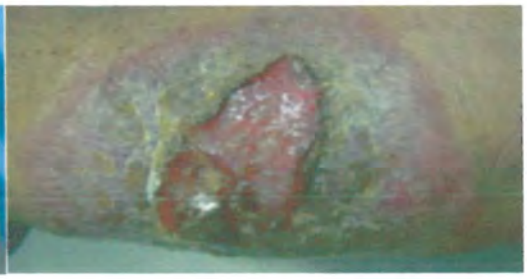
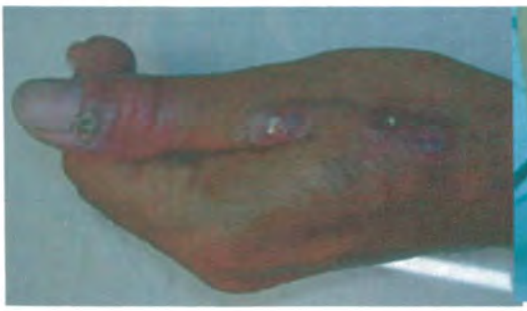
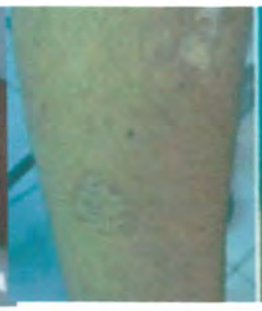


Fig 5f. Crusty, scaly with exposed ulcerated plaque



Sporotrichoid pattern



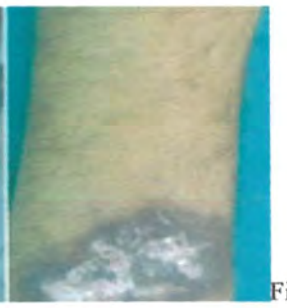


Fig 5g. Fig 5h. Cribriform scarring peripheral Hyperpigmentation

MUCOCUTANEOUS LEISHMANIASIS

1-10% of CL changes to Mucosal Leishmaniasis, which after 1-5 years lead to disfiguring and life threatening disease, termed as Mucocutaneous Leishmaniasis. ML is dreaded metastatic complication of new world Cutaneous Leishmaniasis of subgenus Viannia. It begins with nasal inflammation followed by ulceration of the nasal mucosa and perforation of septum involving nasal, soft palate, pharynx, or larynx. They initiate both Th1 and Th2 response.

Fig 6a. Mucocutaneous Leishmaniasis

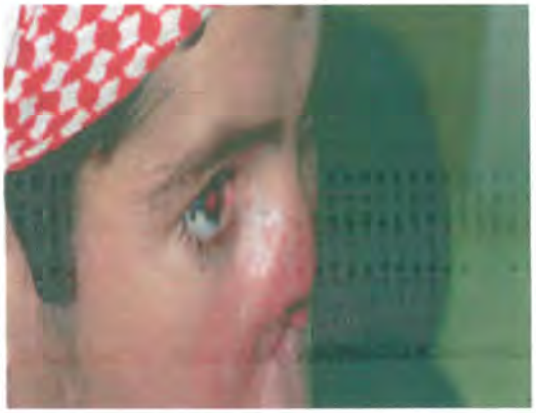


Fig 6b. Disfigurement

Table2. Profile of Mucocutaneous Leishmaniasis

Strains	Leishmania	Viannia	Location	Type of infection
Old World	*L tropica* *L major* *L infantum*			Anthroponotic, Cutaneous, Oriental sore
New World	*L amazonesis*	*L. brazilliensis* *L. panamensis*		Zoonotic, Cutaneous, Oriental sore

VISCERAL LEISHMANIASIS

Visceral Leishmaniasis is asymptomatic or subclinical from acute to chronic spread by *L.donovani* (NW) complex in India and Sudan and *L. infantum* (OW) in Mediterranean (Henry et al, 2005) and Middle East, *L. chagasi* (NW) in South America.

After incubation period of two months, patients shows chronic irregular fever, malaise, anorexia, cough, diarrhea, weight loss, and complications that may lead to enlarged spleen and liver causing anemia; chronic granulomatous infiltration of the skin, i.e. Patchy Hyperpigmentation appears after cure. The lesion regresses within 8-12 months, heals ulcer leaving a scar (Alvar *et al*, 1997). Visceral Leishmaniasis coexists with HIV as an opportunistic infection.

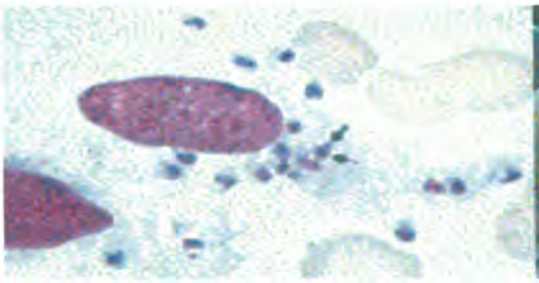


Fig 7a.Histology; amastigotes

Fig 7b. Patient of visceral Leishmaniasis

Table3. Profile of Visceral leishmaniasis

Strains	Leishmania	Viannia	Location	Type of Infection
Old World	*L. donovani* *L. infantum*		Mediterranean Basin , Central and West Asia India, East Africa	Zoonotic, Visceral Leishmaniasis Anthroponotic, Visceral, PKDL
New World	*L. chagasi*			

IMMUNOLOGY

Immune system of infected mammals exemplifies the T_{h1}/T_{h2} paradigm and the effect of disease evaluates the extent of the production of T-cell cytokine responses in early stage of infection. T-cell derived cytokine responses in humans are more complex and less polarized than they are in mice and vary in their immune responses with respect to species (Umakant *et al*, 2009). For instance in mice, Interferon (IFNγ), IL-2 producing T_h1 and Natural Killer (NK) cells mediate resistance, whereas expansion of IL-4 producing T_{h2} cells promotes antibody response. The prolong effect of immune process raises T_{h2} cells that releases IL-10 (suppressors). IL-10 suppresses IL-1 consequently reducing Interferon (INFγ), which plays primary role of enhancing nitric oxide synthase transcription factors and synergic cytokines such as tumor necrosis factor (TNFβ). However, the presence of IL-1 and IL-6 enhances Tc and mast cells production for clearance. Most of the strains of mice (C57BL6, C3H, and CBA) resemble self-limiting Cutaneous Leishmaniasis (CL) in humans when exposed to *L. major* described by (Belkaid *et al.*,2001) by suppressing healing response in persistent infections. Cutaneous Leishmanial strains i.e. *L. tropica* and *L.major* produce cell-mediated immunity responsible of delayed type hypersensitivity, and rather, weak antibody response.

L. tropica persists and results in *L. recidivans* indicating resistivity of parasite to high immunological response.

Visceral causing Leishmanial strains i.e. *L. donovani* and *L.infantum* lack delayed type hypersensitivity; nonetheless, symptoms might appear after cure expressing IL-2, IL-12 and IFNγ. Visceral infection increased humoral response induced by polyclonal Ig B that generates Ig G and Ig M.

L. brazilliensis demonstrates regulatory T cells and *L. guyanensis* $CD4^+$, $CD25^+$ regulatory T cells stimulating TGF β productions (Bourreau *et al*, 2009).

Susceptible /Resistant murine models determine the validity of production and the defensive roles of IL10, IL4, IL12 and $CD4^+$,$CD25^+$ in immunity.

Studies in human beings indicate a role of HLA molecules in LCL and TNFα in mucosal leishmaniasis (Lara *et al.*, 1991).

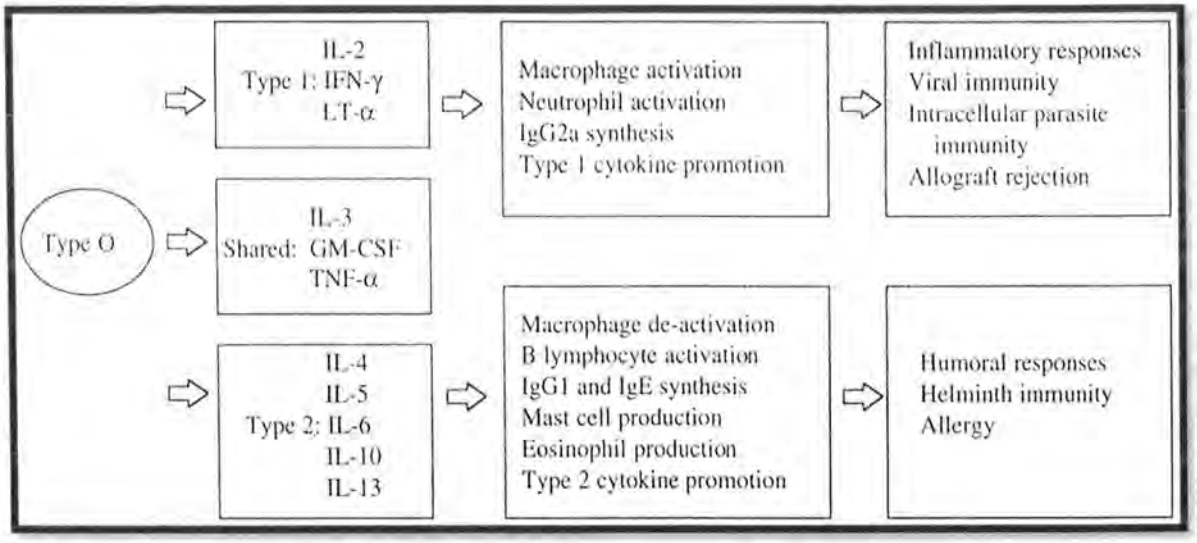


Fig 8. Cytokines partaking in immunological response against Leishmania

TREATMENT

There is limited number of effective therapeutic treatments against Leishmaniasis that immediately need novel and affordable treatments in most of the regions of the developing world.

Sodium stibogluconate (Pentostam), Meglumine antimonate (Glucantime) have been the mainstays of antileishmanial therapy. Beside highly effective, their disadvantages include: topical mode of administration; long duration of therapy and partial effectiveness and serious side effects that causes fatigue, body aches, electrocardiographic abnormalities, raised aminotransferase levels, and chemical pancreatitis.

In resistant strains overexpression of energy dependent ABC (Nucleotide binding proteins cassettes) transporters, glutathione and trypanothione synthesis resist the adverse effect of antimonial drug by efflux mechanism.

Transfection of the MRPA gene, transporter ABC family, showed sodium stibogluconate resistance which could be reverted by using the glutathione biosynthesis-specific inhibitor Buthionine Sulfoximine.

Amphotericin B and Pentamidine, the traditional parenteral alternatives to Antimony prescribed as second-line status modified with the lipid formulation for use in some settings. Amphotericin B resistant promastigotes showed a significant change in plasma

membrane sterol profile by gas chromatography-mass spectrometry, ergosterol being replaced by a precursor, cholesta-5, 7, 24-trien-3β-ol.

Miltefosine and Paromomycin has shown great efficacy to treat Leishmaniasis but their high cost and therapeutic complications limit their usefulness.

WHY ENZYME AS A DRUG TARGET

Enzyme is a biological catalyst serves as a survival kit for the gearing up of cellular processes of organisms, encouraging the exploration of selective enzymatic pathways in disease causing organisms as drug targets.

The pathways that are rigorously studied are Ergosterol biosynthesis, parasite specific proteases and reductases, purine salvage and phospholipid biosynthesis. Provided the target must be essential for the survival of the parasite and a counterpart in the mammalian host to allow selective inhibition.

2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)-Phenylcarbamoylsulfanyl] propionic Acid as irreversible inhibitor of glutathione reductases used against malarial and cancer therapies and derivatives as a Novel Class of Glutathione Reductase Inhibitors] (Seinfeld, 2005).

DISCOVERY OF ADENINE AMINOHYDROLASE

Enzyme Adenine aminohydrolase AAH (EC 3.5.4.2) found recently in Leishmania is absent in Human purine salvage pathway and therefore considered a valuable breakthrough for drug development.

ROLE OF ADENINE AMINOHYDROLASE IN PURINE SALVAGE PATHWAY (ANALYSIS)

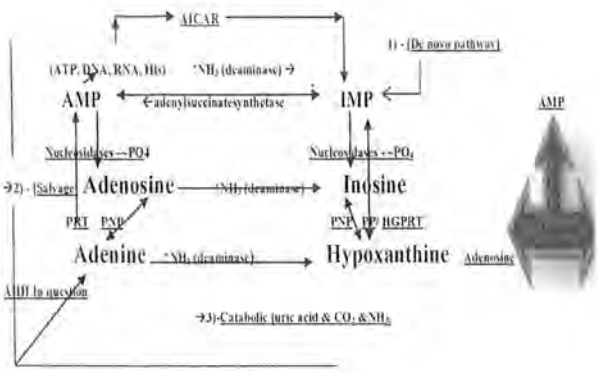


Fig 9: Generalized Purine salvage pathway (modified)

↑ **PO4 (Phosphorylase)** ←> **↑sugar (transferase)** →↑NH_3→C=O→↑CO_2 ->

Legends: PRT (Phosphoribosyltransferases) **–PNP** (Purine nucleoside phosphorylase)-

HGPRT (Hypoxanthine-guanine Phosphoribosyltransferases) **Ade** (adenylate succinylsynthase

AHH (adenine aminohydrolase) **ADA** (Adenosine aminohydrolase)

Leishmania cannot synthesize purine molecule de novo, assessed as the distinctive difference that sets it apart from its mammalian counterpart and considered as major breakthrough for drug discovery plan. Like most parasitic protozoan, genus Leishmania is an obligate auxotroph of purines and depends on its host for purine requirement (Datta, 2008). Purine is one of the four nucleobases present in nuclear chromatin network, neuromodulators, energy carrier molecules i.e. NADP, ATP, RNA, Histidines etc. Generally, purine metabolism includes three related pathways that involve synthesis by de novo pathway, recovery of free bases by salvage pathway, and purine degradative pathway.

De novo pathway forms Inosine monophosphate (IMP) that serves as a precursor of Adenosine and Guanine (pyrimidine). Purine salvage pathway is an ongoing process that recovers free adenine, adenosine, guanine, and hypoxanthine to useful form (Charles Grisham, 2003) by Posphoribosyltranferases i.e. Hypoxanthine-Guanine Phosphoribosyltransferases (HGPRT) catalyzes Hypoxanthine primed by pyrophophotase to form or restore IMP and GMP respectively releasing pyrophosphate (PPi). Another transferase, Adenine phosphoribosyltranferase (APRT) catalyzes adenine to form AMP, catalyzed by Nucleosidases to form Adenosine. For that reason, HGPRT and APRT studied intensively for their significant roles in purine salvage pathway in bacterial and fungal strains.

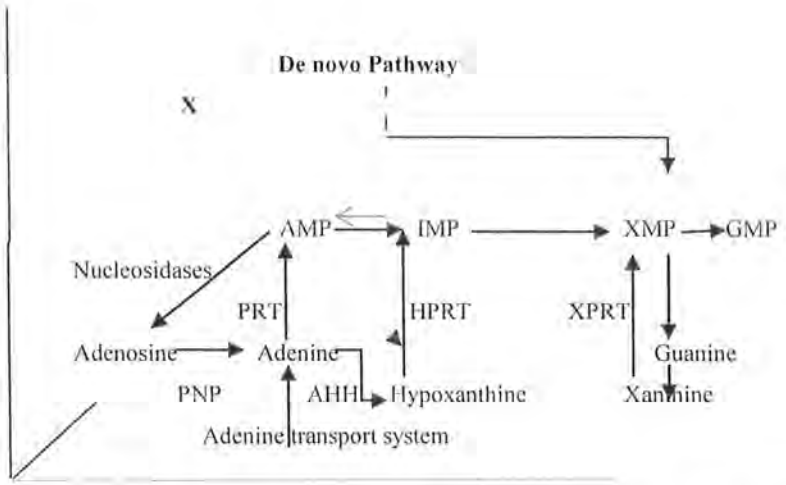


Fig 10. Purine salvage pathway and regulatory issues (Modified, Boitz *et al,* 2012)

Recent studies on Purine salvage pathway of Leishmania have shown accommodation of de novo synthesis of IMP by utilizing phosphoribosyltransferase enzymes i.e. Hypoxanthine-Guanine Phosphoribosyltransferases (HGPRT) and Adenine Phosphoribosyltransferases (APRT) (Boitza & Buddy Ullman, 2006). Adenosine kinase another enzyme in leishmanial pathway forms AMP from Adenosine. Therefore, above-mentioned enzymes studied extensively to ascertain their essential roles.

Conditionally lethal mutant deficient of HGPRT and XPRT were isolated and characterized, revealed inability to catalyze 6-oxypurine (hypoxanthine) in the presence of adenine, adenosine and AAH inhibitor. AHH inhibitor used to block further adenine degradation and to learn its crucial role in Leishmania (Zarella-Boitz 2004). APRT, another functional partner of purine salvage pathway, catalyzes Adenine to form AMP, restoring two inert pathways for organisms' survival. In addition, presence of APRT showed its importance for specific substrate and Leishmanial survival even in the absence of AHH and HGPRT/XPRT confirmed by western blotting in Fig (11). The HGPRT/XPRT mutant were allowed to infect macrophages from Murine bone marrow found to be five percent effectual than wild type parasites and could not sustain an infection (Ullman *et al*, 2006).

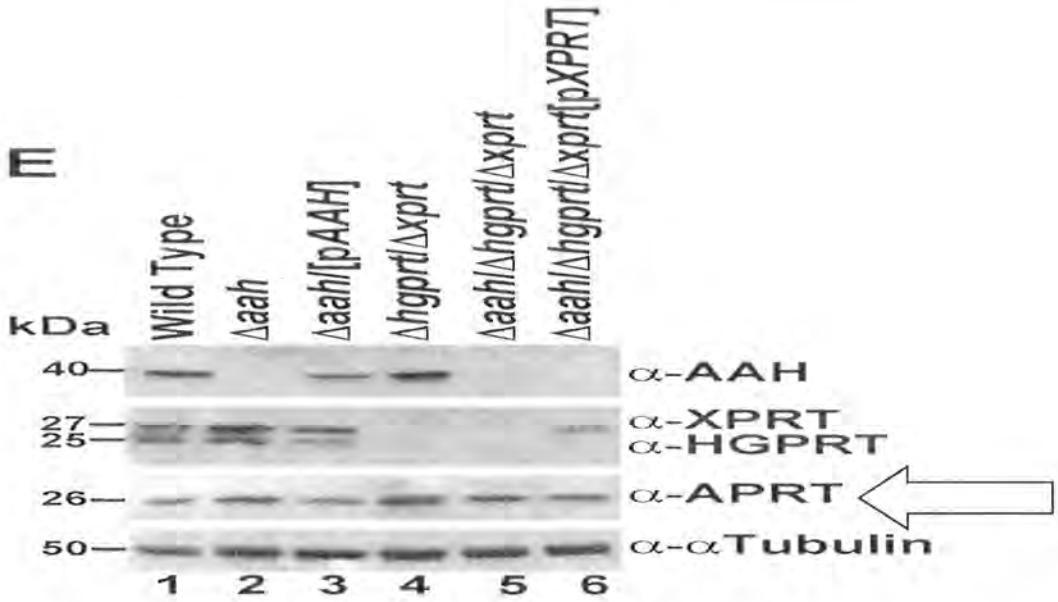


Fig 11. Presence of APRT (lane # 5) in relation to its survival in Δaah/Δhgprt/Δxprt knock out variances

This significant variation within protozoans favors purine salvage pathway to derive purine base from its mammalian host or nutritional culture media by specific nucleobase and nucleoside transporter systems that differ widely from one organism to another in their choices of bases (Looker *et al*, 1983).

S. pombe and *S. cerevisiae* requires either AAH or APRT activity. Alfonzo J.D.*et al* (1995) cloned and sequenced the APRT1 gene of *S. cerevisiae* encoding Adenine Phosphoribosyltransferases. Minimum doubling time taken by purine auxotroph deficient in APRT and AAH, cultured on medium having either ammonium or proline as a nitrogen source confirm the importance of APRT and AHH. In proline-based medium studies, AAH and APRT permitted equal utilization of exogenous adenine. In ammonium-based medium findings, the absence of APRT increased the minimum doubling time by 50%, which shows significance of AHH over APRT since AHH utilized both Ammonia and proline as its nutritional source.

Nygaard P (1996) illustrated *B. subtilis* specific transport systems utilizing adenine, guanine, and hypoxanthine and their Nucleoside derivatives as sole purine sources for nucleotide synthesis. De novo Purine synthesis shut down indicated efficient

interconversion pathways without impeding deamination of adenine for nitrogen recovery demonstrating natural selection for survival for the fittest.

In other experiments, level of the enzyme found to be reduced when exogenous guanosine served as the purine source compared to glutamine when served as the nitrogen source. These experiments show compensatory pathways backing up organism survival under stress mode experienced by insufficient energy acquiring reserves thus giving way to evolutionary processes to amend concurrent pathways.

In Purine degradative pathway, a part of purine metabolism, hydrolyzes Nucleosides by Nucleosidases secured from the nutrition. Purine nucleoside phosphorylase (PNP) removes sugar group from Inosine to form hypoxanthine found in higher organism.Other pathways that stem out of degradative pathway involve Adenosine/AMP deaminase catalyzing zinc dependent irreversible deamination of Inosine nucleosides to ammonia directly releasing ammonia in pro- and eukaryotic organisms. AHH is one such pathway recently procured form hypoxanthine not found in Mammals closely related to Adenosine deaminases. Impairment of these enzymes may cause serious disorders. In humans, deficiency of Adenosine deaminase implicates lack of immune response against infectious diseases like AIDS, Anemia, Lymphomas, and Leukemia etc., which approved this enzyme for first gene therapy trial.

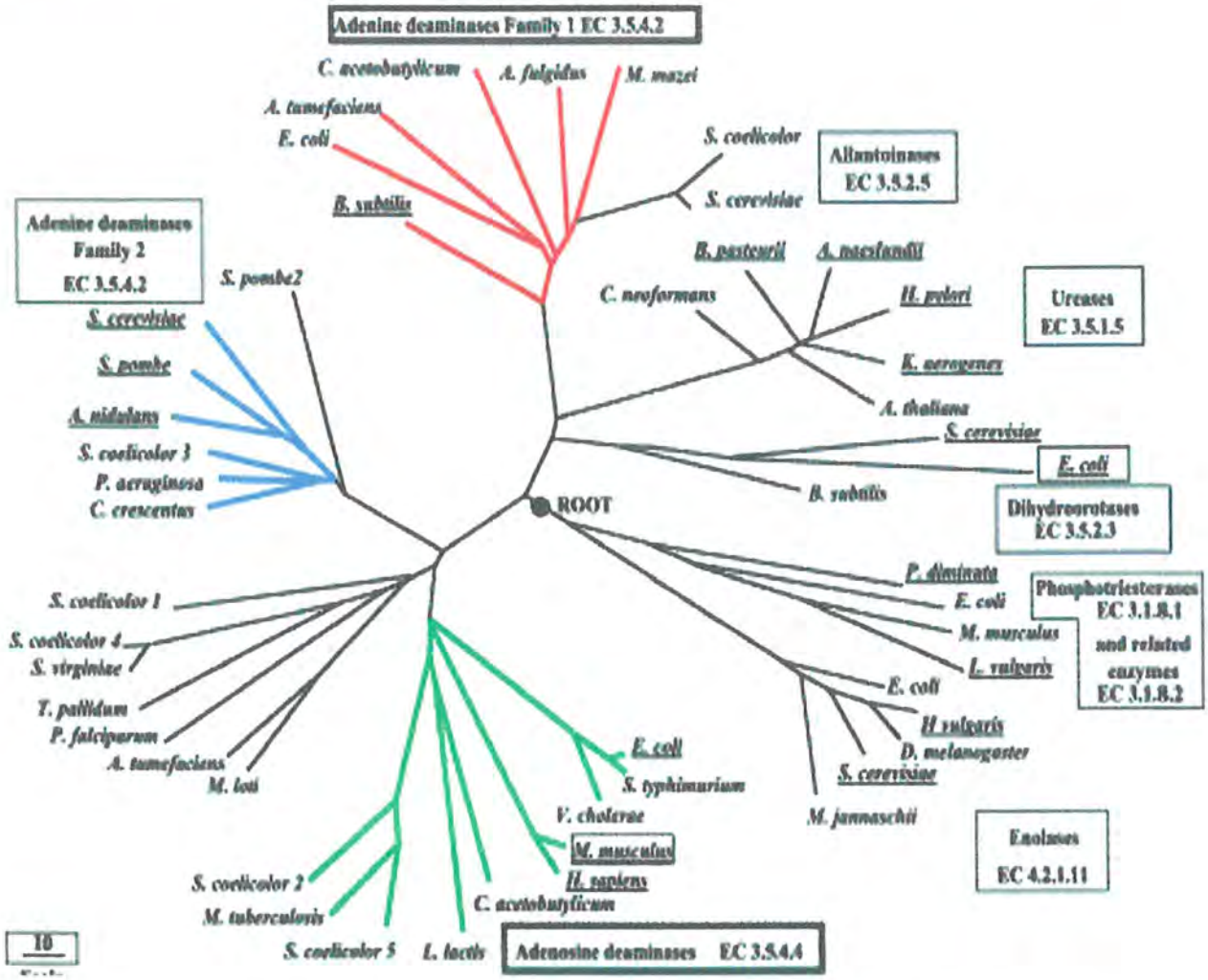


Fig12 Phylogenetic relationship of species belonging to Hydrolase family by the Rooted Tree program of the DARWIN package (Ribard *.et al*, 2003)

A rooted tree was constructed using the RootedTree program of the DARWIN package 54 to draw evolutionary relationships of adenine deaminases, adenosine deaminases and the metalloenzyme hydrolases family shown in (Fig 20) i.e. urease alpha, adenosine deaminase, phosphotriesterase, dihydroorotases, Allantoinases, hydantoinases, AMP, adenine and cytosine deaminases, imidazolonepropionase, aryldialkylphosphatase, chlorohydrolases, formylmethanofuran dehydrogenases. The root is indicated by a dimensionless branch. Red cluster, bacterial adenine deaminase family (family 1). Two sequences from archea are found in this group (Archaeoglobus fulgidus and Methanosarcina mazei). Blue cluster, fungal adenine deaminases (family 2) and related

bacterial enzymes. Green cluster, known and putative adenosine deaminases. The branch lengths are in PAM units.

Purine salvage pathway studied in *Aspergillus nidulans, Azobacter aurescens, Enterococcus faecalis, Candida utilis, Schizosaccharomyces pombe, Saccharomyces Cerevisiae, Crithidia fasciculata, and Bacillus subtilis*. Among protozoans, *Leishmania* species displayed presence of AHH; whereas *Trypanosoma cruzi* and *Trypanosoma brucei* lack AHH.

STRUCTURE

Aminohydrolase Superfamily (cl00281) of metallo-dependent hydrolases shows conversation in their topology i.e. 3-dimensional fold α/β_8 (TIM barrel) homologous to Triose phosphate isomerase and in the configuration of their active sites (Jeronimo M. B. *et al*, 2011). Their active site has a conserved metal binding site, involving four histidines and one aspartic acid residue demosntrated by Multiple alignment of superfamily cl 00281 (Fig 25) (Marchler Bauer *et al*, 2013). Phylogenetic diagram displaying list of Hydrolase enzymes as mentioned above in (Fig 22)

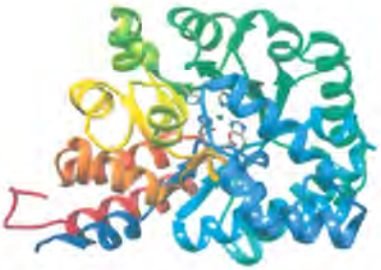


Fig13a Leishmanial TIM

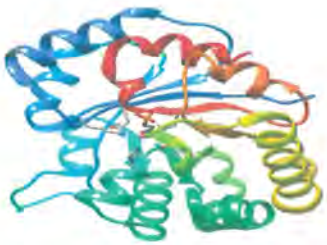


Fig13b Triose Phosphate Isomerase (TIM)

Fungal Adenine deaminases share a relatively high sequence homology with both prokaryotic and eukaryotic ADA and AHH that constitute a known family of α/β barrel enzymes scaffold. Sharing of subunits from different ancestral enzymes favorably in constituting Adenine deaminase active site can be learned from the following multiple sequences alignments comparisons shown in Fig (24 &25)(Ribard *et al*, 2003).

CATALYTIC ACTIVITY OF ADENINE AMINOHYDROLASE

Adenine deaminase (AdeC) [E 3.5.4.2] is the systemic name of one of the classes of Hydrolases that catalyzes irreversible cleavage of cyclic amines by acting on C-N bond other than peptide bond deaminating adenine to form hypoxanthine and ammonia in one of the purine salvage pathways (**http://www.chem.qmul.ac.uk**)

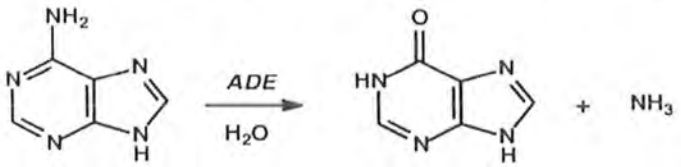


Fig 14. Hydrolysis of Adenine into Hypoxanthine and Ammonia

Specificity for substrate

Radiometric assay confirmed that AHH only use Adenine as its substrate by adding excessive amount of AHH in the presence of adenosine for 2h course of reaction and adenine as a control to verify enzyme is functionally active acquired from ().

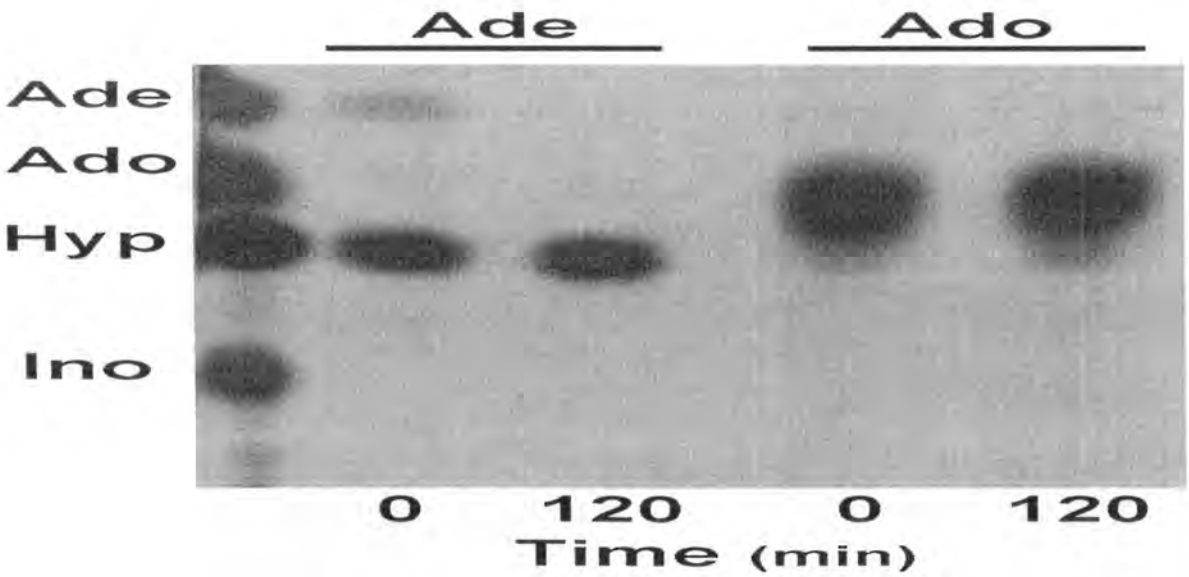


Fig 15. Standards are shown on the left of each chromatogram: (Ade), adenine; (Hyp), hypoxanthine; (Ado), adenosine; (Ino), inosine.

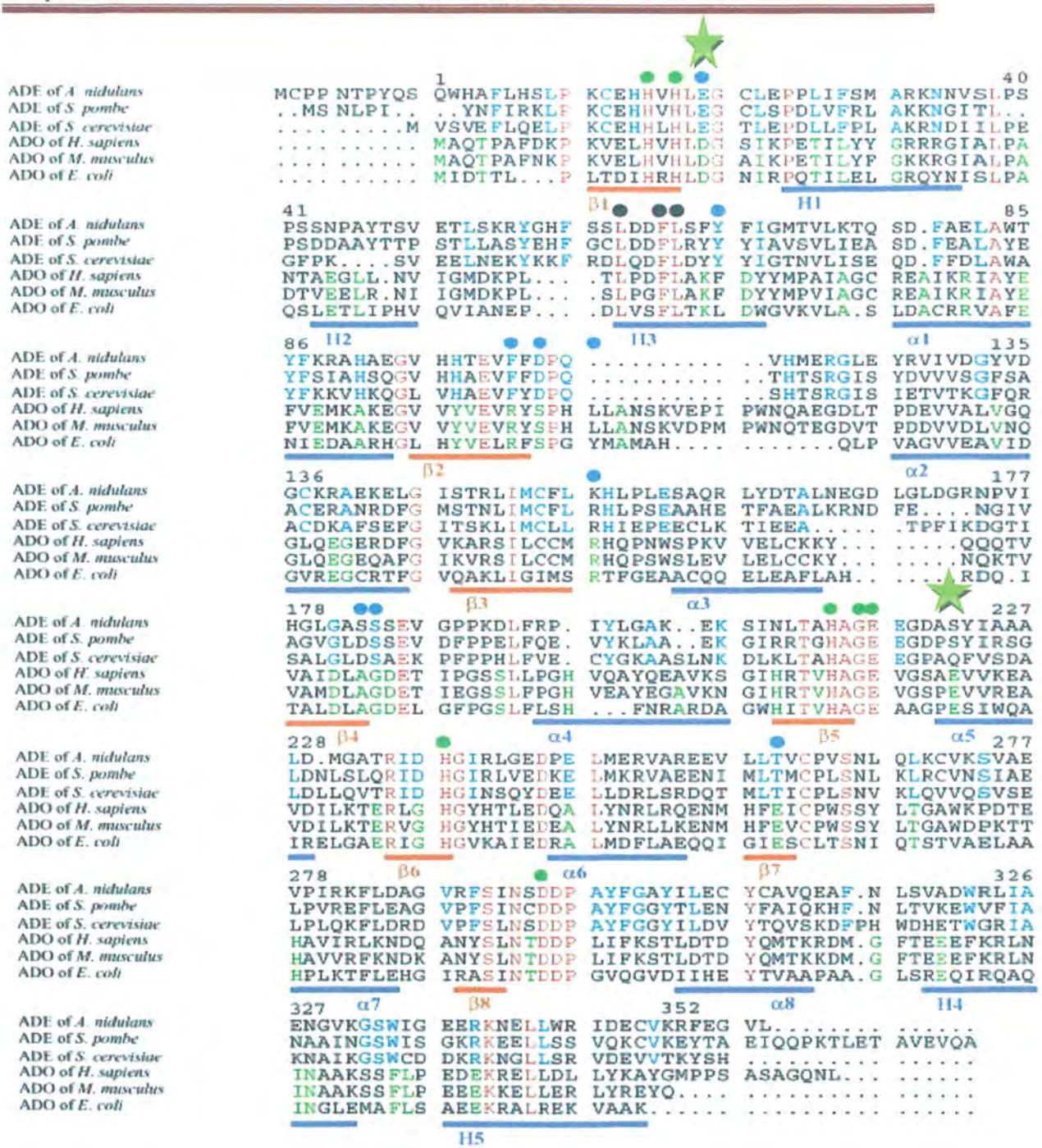


Fig 16.Sequence alignment of (ADE) Adenine deaminase (ADO) Adenosine deaminase. $(\alpha/\beta)_5$ barrel minus H1- H5 (Ribard et *al*, 2003)

Residues shown in red universally conserved, blue conserved among the adenine deaminases only; in green conserved among the adenosine deaminases only. Residues of mice adenosine deaminase shown participate in catalytic mechanism in green dot above the sequences.

The course of evolution of these enzymes is evident through the structural similarities but functionally they appear to catalyze adenine and adenosine respectively due to the residual dissimilarities showing at active site residue Glu $^{20,217\alpha5}$ location that constitutes catalytic region as shown in above multiple sequence alignments, considered as mutagenic site (Fig 26).

Insilico - catalytic Activity (X-ray Crystallography & NMR derived)

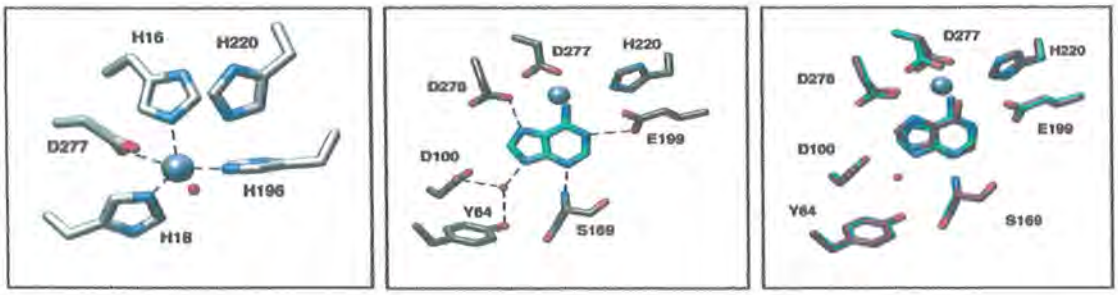


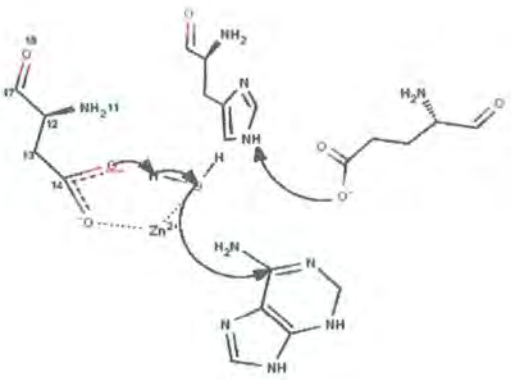
Fig17. Ligand binding site of 3PAO.pdb; pseudomonas aeruginosa (Alissa Goble et al, 2011)

Active site is five percent of the total volume of the enzyme. Protein folds itself to form an eight-stranded parallel α/β barrel with a deep pocket at the β-barrel C-terminal end. Active site Leishmania is bounded by nearly 4 histidines His^{20}-His^{22}-His^{213}-His^{237} that makes enzyme to act as an electrophile, and two aspartates to induce a nucleophile in its activation state under the influence of divalent metal ion that constitute a chelate complex in conjunction with a water molecule, at pH=~7.

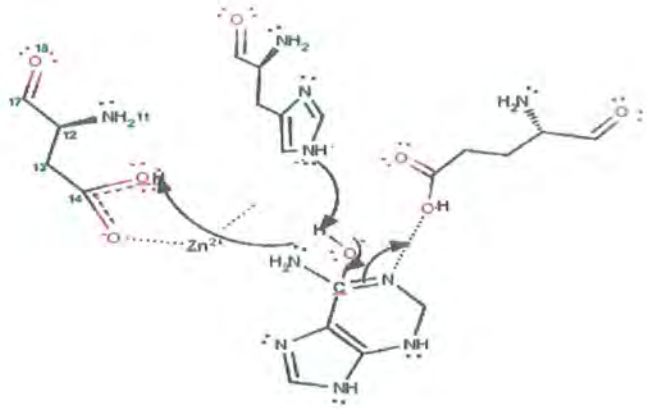
In activation state, concentration of Adenine equals to its K_m, hits the active site resulting in conformational changes energetically favorable for enzyme-substrate binding i.e. His^{220}, $Asp^{100-\ 277-278}$, Glu^{216}, Ser^{185}, and Tyr^{64} build hydrogen bonds for proper orientation of substrate as shown in fig (18).

Asp Zn^{2+} [H2O or (: OH) (H^+)] His Chelate Complex at pH=7

CATALYTIC REACTION MECHANISM

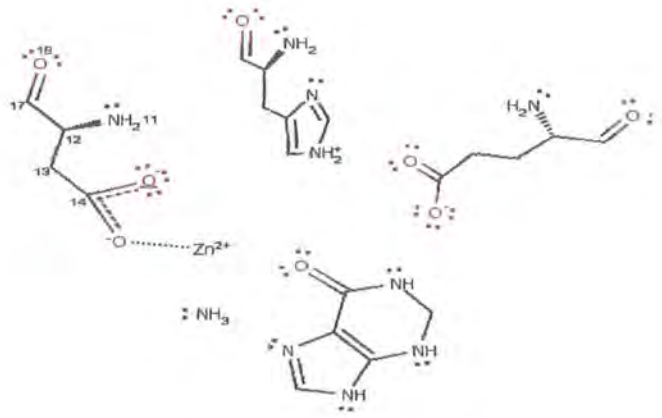


Charged Aspartate in the presence of Zn^{++} in enzyme's active site abstracts proton from water molecule to form Strong: Nu or: OH– anion



Strong: Nu or: OH– anion attacks the ring at positionC_6 attached to $N_6$with lone pair of e acts as reactive center for a nucleophilic addition, that collapses tetrahedral structure of C_6 releasingNH_3

Hydroxyl group on C_6 of ringconjugated with N_1 having lone pair of electrons in its orbital suggests unstable arrangement mimicking enolate effect. Acidic environment assist in removal of hydrogen off hydroxyl group to form carbonyl and N_1 to share its lone pair to form hydrogen bond, giving rise to hypoxanthine as irreversible product.



Removal of hydrogen allows oxygen to shares its valence electrons by forming pi bond givingrise to C=O at C_6 allowing N_1 to form bond with hydrogen, thus giving rise to hypoxanthine as an irreversible product

Sequestration of metal to accelerate or decelerate catalytic activity shows metal ions in specific concentrations are, in some cases, essential for activity.

SUBCELLULAR LOCALIZATION OF ENZYME IN THE ORGANISM

Peroxisome targeting signals (PTS) sequences, important in transporting and signaling, detected at N-terminal in *Leishmanial* strains except in *L. major* and *L. brazilliensis* depicts conserved sites in few *Leishmanial* strains. Findings showed that HGPRT and XPRT sequester within glycosome, a peroxisome like sub cellular microbody, while APRT, cystolic marker enzyme, occupy cystolic location (Zarella-Boitz *et al.*, 2004). Localization of IMPDH and AHH revealed both sediments in sucrose medium with APRT along with organelle pellet during glycosome differential centrifugation; protocol (Sambrook *et al.*, 1989). From this study, one might not find peroxisomal targeting site (PTS) playing functional role in AHH, so far.

INHIBITORS

Standard inhibitors used are coformycin and deoxycoformycin for both ADA and AHH. Apart from their distinctive roles, they hydrolyze 6-chlorosubstituted derivatives in same fashion. ADA can also convert other purine compounds, such as 2-amino-6-chloropurine riboside, 6-methoxypurine riboside, 6-methylaminopurine ribonucleoside, as well as deoxyribose derivatives, AMP, ADP, ATP and cAMP.

Cytokinins are adenine derivatives with an isoprenoid side chain, play an essential role in plant development. Plant enzymes catalyze the transfer of the isopentenyl moiety from DMAPP preferentially to ATP and to ADP. The isopentenylated side chain hydroxylated to form zeatin-type cytokinins. An alternative pathway, in which a hydroxylated side chain is directly added to the N_6 position of the adenine moiety, has also been suggested as potent inhibitor of AHH (Kaki Moto, 2003). Interestingly, the yeast enzymes also hydrolyzed N_6-substituted adenines from cytokinins, a group of plant hormones, cleaving them to inosine and the corresponding side chain amine. The hydrolytic cleavage of synthetic cytokinin 2, 6-di-substituted analogues used in cancer therapy i.e. olomoucine, roscovitine and bohemine subsequently shown for a reference sample of human ADA1.

ADA1, however, showed a different reaction mechanism than the yeast enzymes, hydrolyzing the compounds to an adenine derivative and a side chain alcohol. The reaction products identified using reference compounds on HPLC coupled to UV and Q-TOF (quadrupole-time-of-flight) detectors. (Hana POSPÍŠILOVÁ*, 2008)

OBJECTIVE

The objective of the thesis is to isolate Adenine Aminohydrolase and study its mode of kinetic action for the development of robust enzyme assay for the application of inhibitors. Usage of Knowledge based homology structuring in the characterization of enzyme structure and its catalytic sites for sound understanding of reaction mechanism. The residual activity of enzyme assess by qualitative and quantitative methods for proving data.

Materials & Methods

3.1 SAMPLE COLLECTION

Leishmania tropica (Kwh23) sample collected from seven years old patient in Peshawar Hospital suffering from Cutaneous Leishmaniasis.

3.1.1 ISOLATION OF LEISHMANIA STRAIN

Isolation of leishmanial strains done by removing organism from infected people by employing documented sampling methods either by transferring to animals for in-vivo investigation or in culture for in-vitro cultivation of infectious agents.

3.2 MAINTENANCE OF CULTURE (In-vitro cultivation)

All the promastigotes cultures of local Pakistani Leishmania strains maintained in blood agar based bi-phasic Evan's modified Tobie's medium supplemented with RPMI-1640 with 25mM TES at 25^0C (Danish Saleheen, 2004). Prepared media added into inoculum taken up by preformed culture medium at log phase reached in a time of 5-7 days.

3.2.1 SUB-CULTURING

Purpose
Sub culturing of Organisms for further investigation

Method

- Add inoculum into media taken up by preformed culture medium before they reach death phase in a sterile environment.
- Incubate them at 25°C and keep shaking them intermittently.

3.2.2 MEDIA PREPARATION

Purpose
Maintenance of culture by regular sub-culturing providing minimal essential medium

Material

- Earle's salts
- Laminar flow Hood
- Pipette

Energy sources

Medium 199 includes Earle's salts, L-Glutamine, and Sodium bicarbonate Cat.no.31100-019 –Lot No. 491675(GIBCO) (Invitrogen Corporation).

Table1. Preparation of 1 L 199 Medium

Reagent	Amount/gm
Earle's salts(L-glutamine 15.1gm of powder/L) 25mM (6.5 gm) of HEPES 2.2gm/L of sodium bicarbonate Penicillin Streptomycin 10% Heat-inactivated fetal Calf serum	• 12 gm of medium 199 • 0.2 gm penicillin • 0.2gm of Streptomycin • 2.2gm of Sodium Bicarbonate • 6.5gm of HEPES in 1L of dH_2O adjust pH at 7.4 and filter it. *Sterility test

Method

MEM 199 added in dH_2O (pH=7.4), filtered using filtration pump by passing it through membrane. Medium kept in incubator to ensure sterility for 24 hrs. FBS filtered before adding into medium. Performed parasites inoculated following recommended parameters.

3.2.3 MICROSCOPY

3.2.3 *a* VIABILITY TEST

Principle

The cell culture of Leishmania in mid log phase was assessed for its viability by using visualization method. Trypan Blue has a greater affinity for serum proteins than for cellular protein, therefore the protein-free medium or salt solution (HBSS) added prior to counting for visualization of viable organism (http://www.sigmaaldrich.com/)

Purpose

To count viable organisms for Bioassays LD_{50}

Material

- HBBS
- Trypan blue
- Eppendorff tube
- Microscope
- Hemocytometer

Table2.Preparation of staining solution for specimen observation

Reagents	Preparation
Hanks' Balanced SALTS (HBBS) Product: H 2513 4%Trypan Blue (w/v).Product Nos.T8154,T 6146 and Z 35,962-9(H7901) Cell suspension	Mix 0.5 ml of 0.4% Trypan Blue solution(w/v) with 0.3ml of HBSS and 0.2ml of cell suspension, centrifuge it and allow dye fastening Formula • Cell. sq x dilution factor (5)x 10^4(1 square=0.1mm^3 or 10^{-4}cm, thus four squares make 1cm^3x original volume cell/ml)

Method

A cell suspension, prepared in a balanced salt Solution (e.g. Hanks= Balanced Salts [HBSS], transferred to 0.5 ml of 0.4% Trypan Blue solution (w/v) in a test tube. 0.3 ml of HBSS and 0.2 ml of the cell suspension (dilution factor = 5) added and mixed thoroughly. Mixture incubated for 5 minutes. 15µl of cell suspension pipetted out on the slide for cell counting.

3.3 DEVELOPMENT OF ENZYME ASSAY

The assay method based on a published protocol for Adenine deaminase

3.3.1 EXTRACTION OF TARGETED ENZYME FOR DETERMINING RESIDUAL ACTIVITY

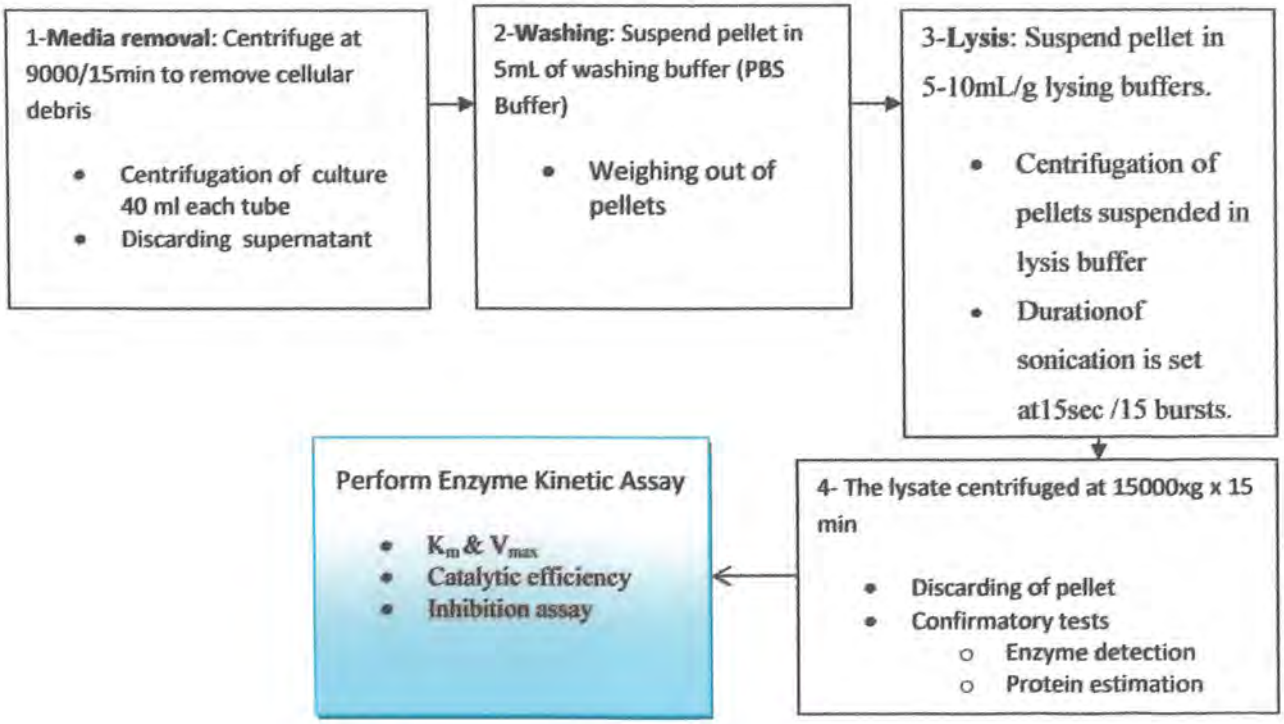
Purpose

To extract enzyme form sample for its detection and characterization using appropriate buffers

Material

- Sonicator (VirSonic300--VIRTIS)
- Beakers
- Ice
- Stop Watch
- Washing Buffer
- Lysis Buffer
- Eppendorff

Flow chart showing steps involved in studying enzymatic catalytic activity



3.3.1 BUFFER PREPARATIONS

3.3.1*a* Washing Buffer

Purpose:
To wash out debris of the sample

Material:

- Monobasic-$KH_2PO_{4(mol\ wt=136g/mol)}$
- Dibasic-K_2HPO_4 (mol wt. =174g/mol)
- NaCl (mol wt.=58.88g/mol)
- dH_2O
- Analytical balance
- Reagent bottles

Table 3: Preparation of 1Litre of 1X 0.1M Phosphate Buffer Saline (PBS)

Buffer	Reagents	Preparation
PBS	$KH_2PO_4.2H_2O_{(mol\ wt.=156g/mol)}$ $Na_2HPO_{4(mol\ wt.=141.96g/mol)}$ $NaCl_{(mol\ wt.=58.44g/mol)}$ $KCl_{(mol.wt=75g/mol)}$	• Start with 800 / 400ml of distilled water: • Add 8g/ 4.0g NaCl • Add 0.2g/ 0.1g KCl. • Add 1.44g/0.72g Na2HPO4. • Add 0.24g/0.12g KH2PO4. • Adjust the pH to 7.2 using HCl. • Add distilled water to a total volume of 1 liter/500ml.

Table 4. Preparation of 100mL of 1M PO4 Buffer pH=7.4

Buffer	Reagents	Preparation
PO_4Buffer	Monobasic-$KH_2PO_{4(mol\ wt.=136g/mol)}$	• Add 11gm in 80.2mL 13.6/100x80.2=11gm • Add 3.4gm in 19.8mL to make 1M of dibasic 17.4/100x19.8=3.4gm
	Dibasic-$K_2HPO_{4(mol\ wt.=174g/mol)}$	

3.3.1*b* LYSIS BUFFER

Purpose

To solubilize lipid content and inhibit proteases and phosphates from further degradation

Material

- Tris-HCL
- NaCl
- EDTA
- Glycerol
- Bile salt

Table 5. Preparation of 1 X Lysis buffer 50mL used in sonication

Reagents	Concentration	Amount/g
Tris-HCl	50 mM Tris-HCl pH 7.0	0.3029
NaCl	100 mM NaCl	0.292
EDTA	1 mM DTT(78.13g/mol) (for intracellular proteins)or EDTA	0.0187
5% Glycerol	5% glycerol or cysteine (possibly)	2.5mL

Table 6. Concentration of detergents for chemical Lysis added in Lysis buffer

Detergent	Concentration	Amount/g
Bile salt	10mM	0.2153

3.3.2 STEPS INVOLVED IN PREPARATION OF STUDYING ENZYME ACTIVITY

Cell Harvesting

Cellular debris removed from culture media in log phase by centrifugation at 9000 x g/15min. The medium decanted and discarded carefully, keeping pellet undisturbed at the bottom. The pellet suspended in washing buffer after discarding medium. Treated sample can be stored at -20 ^{o}C for a week or -80^{o}C for long-term storage.

LYSIS

Pellet suspended in 7-10mL/g of Lysis buffer (stored at -4^{0}C) and sonicated in glass beaker placed on ice for 15sec /15 bursts on the setting of 8 using(VirSonic300--VIRTIS). Lysate centrifuged at 15000 x g x 15 min and then filtered by low protein binding syringe filter of 0.45-µM pore size.

3.4 CONFIRMATORY TESTS

3.4.1 Enzyme Activity Assay

3.4.2 Protein Estimation

3.4.1 ENZYME ASSAY

Principle

Detection of enzyme of interest by the addition of specific substrate under standard conditions

Purpose

To detect catalytic activity of enzyme and assess its percentage recovery in purification processes and its characterization at different pH, ionic strength and temperature, suitable stabilizing agents to avoid denaturation of protein and enhance its activity for enzyme assay development

Material

- Aligent Spectrophotometer
- Adenine Free Base $_{(mol.wt=135.1g/mol)}$ cat:194606 Lot:7559C (MP BIOMEDICALS Inc.)
- Cellular Extract
- Phosphate Buffer Saline(PBS) pH=7
- Cuvette (2mL)
- Micro-pipette

Table 7. Substrate Preparation

Reagent	Preparation
5mM[Adenine]$_{(mol\ wt=135.1g/mol)}$	5mM adenine solution was prepared by adding 0.06755g/100ml in phosphate buffer

Method

0.6755g Adenine added in 100 ml of PO4 buffer to make 5mM of substrate stock solution which could be further diluted to desire concentration using $C_1V_1=C_2V_2$

3.4.1*a* EFFECT OF SUBSTRATE

Purpose

To find out the K_m and V_{max} values of the enzyme

Method

UV/vis Spectrophotometer used for product detection in sample at wavelength 292 nm. The reaction carried out in 2ml cuvette at 25℃, on the addition of adenine ranging from 20µl--200µl in PBS mixed with sonicated sample. Solution vortexed and acquired absorbance readings plotted on XY Graph as Abs (activity) Vs substrate concentration to find out K_m and V_{max} values.

3.4.1*b* EFFECT OF ENZYME

Purpose

To determine enzyme concentration to get precise curve

Method

Absorbance values plotted by varying volume of sample keeping constant substrate concentration as progression curve that displays linear velocity increase. Volume of sample chosen that demonstrate enzyme assays' characteristic non-linear graph.

3.4.1*c* ENZYME INHIBITION

Purpose

To find out IC_{50}

Method

Substrate concentration at Km from the standard curve graph incubated with set range of inhibitor concentrations for 30 minutes. Absorbance values analyzed to find out Ki and inhibitor binding sites by Graph prism pad software using Michelson Menten equations.

3.4.2 PROTEIN ESTIMATION

PRINCIPLE

Protein-dye complex used to estimate the amount of protein in given samples by means of spectrophotometer.

Purpose

To estimate the yield production of protein in purification process

Material

- Na $CO_{3(mol\ wt.=106g/mol)}$ in dH_2O
- $NaOH_{(mol\ wt.=40g/mol)}$
- $CuSO_4\ 5H_2O_{(mol.wt=249g/mol)}$
- KNa $C_4H_4O_6 4H_2O$(Sodium Potassium Tartrate)$_{(mol\ wt.=282.2g/mol}$
- (1N)Folin-Ciocalteau solution
- BSA Standard - 1 mg/ mL

Table 8. Preparation of solution used in protein estimation

Reagent	**Preparation**
Solution A	KNaTartrate=1g $NaCO_3$=1g NaOH=2g Mix in 100mL water
Solution B	$CuSO_4$.$5H_2O$=0.75g Mix in 50mL water
Solution C	Sol (A)50: Sol (B)1i.e. 49:1

METHOD

[Run triplicate determination for all samples.]

Eleven sets of three 16 x 150 mm test tubes set up in rack. BSA [0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µl] added to these tubes. 2 mL of solution D added to each test tube and then incubated for 10 minutes at room temperature. 0.2 mL of dilute Folin-phenol mixed in solution to each tube by using vortex. Mixture incubated at room temperature

for 30 minutes. Absorbance determined for each sample at 650 nm and graph plotted absorbance *vs.* mg protein to acquire standard curve. Triplicate assays for all "unknowns" were set up. Complex forming reagent is prepared immediately before use by mixing three stock solutions A, B and C

3.5 COMPUTATIONAL TOOLS USED IN ENZYME STUDY

3.5.1 INSILICO-MOLECULAR DOCKING

A computational tool for predicting the ligand-macromolecule complex interactions in the field of computer aided drug designing using free energy scoring system exploring all available degrees of freedom in the system (AUTO DOCK version 4.2).

3.5.1*a* ACTIVE SITE PREDICTION

Pocket finder web serve detected the binding pocket of 2ICS by scanning a probe radius 1.6 Å alongside gridlines of grid resolution 0.9 Å surrounding the protein proposing 10 possible binding sites.

3.5.1*b* Ligand preparation

Ninety-four inhibitors selected and minimized using default parameters of Chimera 1.5.3.2

3.5.1*c* Molecular docking studies using gold dock package (with additional software HERMES visualizer; GOLDMINE)

- GOLD features a Wizard for docking setup

3.5.1*d* OPEN HERMES

- Select protein>Load protein>open>Next

3.5.1*e* PROTEIN SETUP

- >Add all hydrogen atoms&>delete water in protein explorer >delete ligand in protein set window>extract>save in *ligand*.MOL2 ; necessary to define the correct ionization and tautomeric states of residues such as Asp, Glu and His

3.5.1*f* DEFINE THE BINDING SITE

- >options given assign residues that are in the active site region within a 5Å radius by restricting them to solvent-accessible surface.>View>Next

3.5.1*g* CONFIGURATION TEMPLATES

- Load docking protocol recommended>Next

3.5.1 *h* LIGAND SETUP

- Add and open saved *ligand.* MOL2 file

3.5.1 *i* GA RUN

- Dockings to be performed on each ligand is specified

3.5.1*j* FITNESS FUNCTION

- GOLD offers a choice of fitness functions: Gold Score, ChemScore, Astex Statistical Potential (ASP), CHEMPLP, Piecewise Linear Potential (PLP) and User Defined Score

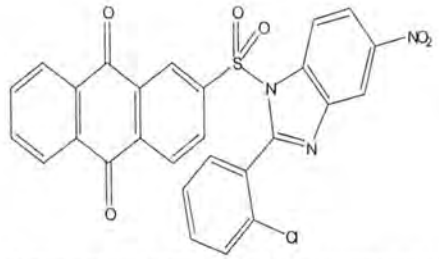
3.5.1*k* RUN GOLD

- Select Output Options under Global Options
- File Format Options, Ensure that the Save rank files, Save ligand log files and Save initialized ligand files

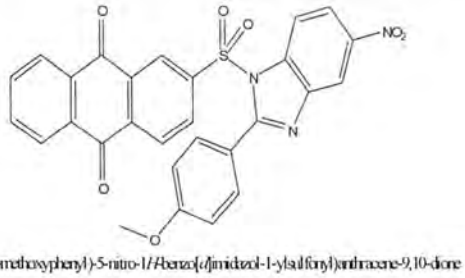
3.5.1. *l* ANALYSIS OF OUTPUT

Following above steps, molecular Docking Study carried out with 94 potent inhibitor structures to understand the binding mode of 2ICS with inhibitors with the default

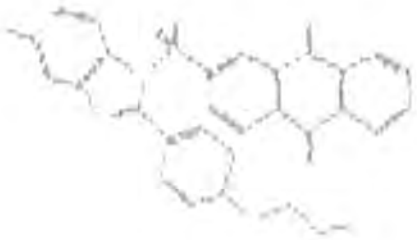
parameter of the GOLD. The compressed unified pdb of top 10 results selected for further studies.

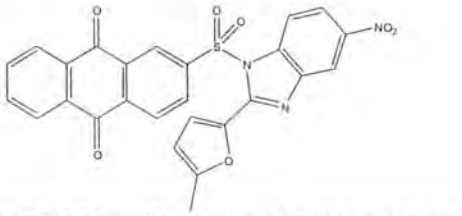


2-(2-(2-chlorophenyl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione

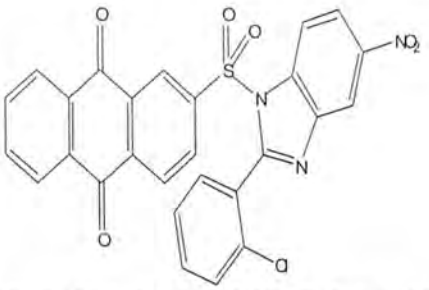


2-(2-(4-methoxyphenyl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione



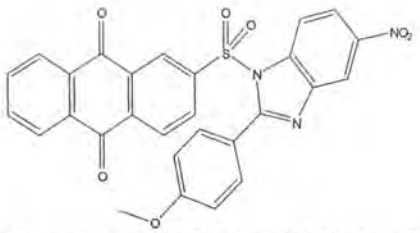


2-(2-(5-methylfuran-2-yl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione



2-(2-(2-chlorophenyl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione

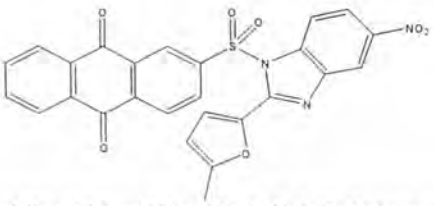
$C_{28}H_{17}N_3O_7S$



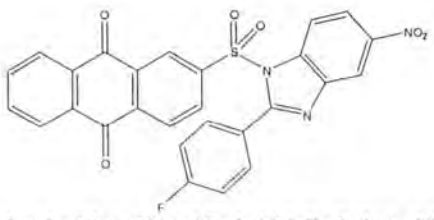
2-(2-(4-methoxyphenyl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione

$C_{26}H_{15}N_3O_7S$

$C_{27}H_{14}FN_3O_6S$



2-(2-(5-methylfuran-2-yl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione



2-(2-(4-fluorophenyl)-5-nitro-1*H*-benzo[*d*]imidazol-1-ylsulfonyl)anthracene-9,10-dione

3.5.1 NCBI

NCBI build innovative algorithms (BLAST, PSI-BLAST, VAST, and COGs), novel research approaches (text neighboring) and fundamental resources (PubChem and CDD).

3.5.2 CHIMERA

Chimera is a highly extensible program for interactive visualization and analysis of molecular structures and related data, including density maps, supramolecular assemblies, sequence alignments, docking results, trajectories, and conformational ensembles.

3.5.3 PIR

The Protein Information Resource (PIR) is an integrated public bioinformatics resource to support genomic, proteomic and systems biology research and scientific studies (Wu et al., 2003).

3.5.4 STRING

STRING is a database of known and predicted protein interactions. The database currently covers 5'214'234 proteins from 1133 organisms.

3.5.5 SIGNAL 4.0

Signal 4.1 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms

3.5.6 PHYRE

Phyre and **Phyre2** (**P**rotein **H**omology/Analog **Y** **R**ecognition **Engine**; pronounced as 'fire') are web-based services for protein structure prediction

Results

4.1 VIABILITY TEST

Culture without Adenine yielded $3.1x10^8$ cell/mL and with addition of 10mM Adenine $6.7x10^8$cell/mL on cell count.

4.2 ENZYME ASSAY

4.2.1 SONICATION RESULTS

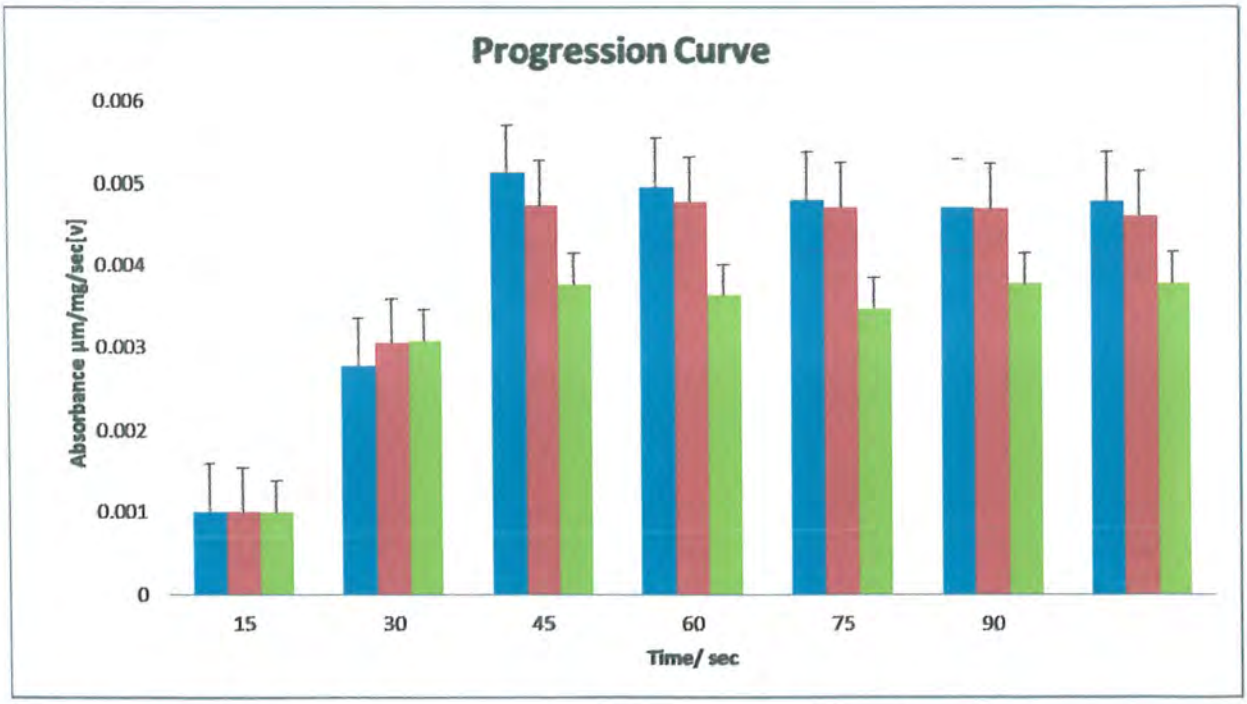


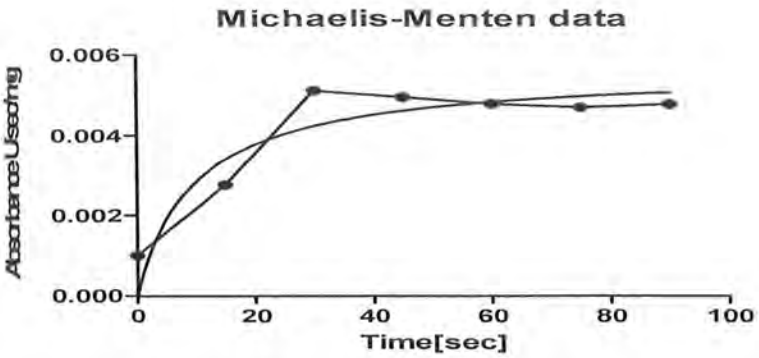
Fig18. Progression curve of the Sonicated extract treated at range of different time durations absorbance taken after every 15 sec as reported in published paper by Excel

Table1. Effect of Time on Enzyme activity

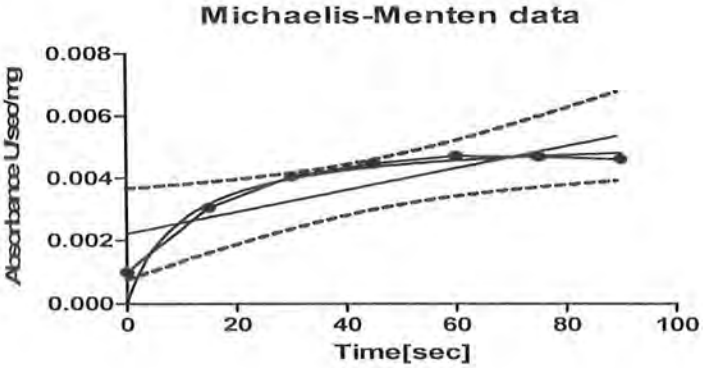
5mMAdenine(µl)	Enzyme(µl)	Absorbance(Exp1)	Absorbance(Exp2)	Absorbance(Exp 3)	Time
2	40	0.001	0.001	0.001	0
4	40	0.00277	0.00305	0.00307	15
8	40	0.00512	0.00472	0.00376	30
16	40	0.00496	0.00477	0.00363	45
32	40	0.00479	0.00471	0.00347	60
64	40	0.0047	0.00469	0.00377	75
128	40	0.00478	0.0046	0.00378	90

Graphs generated by GraphPad PrismTM V 5.0 (GraphPad software, Inc., San Diego, USA)

1 Graph of 10/15 sec



2 Graph of 15/15 sec



3 Graph of 20/15 sec

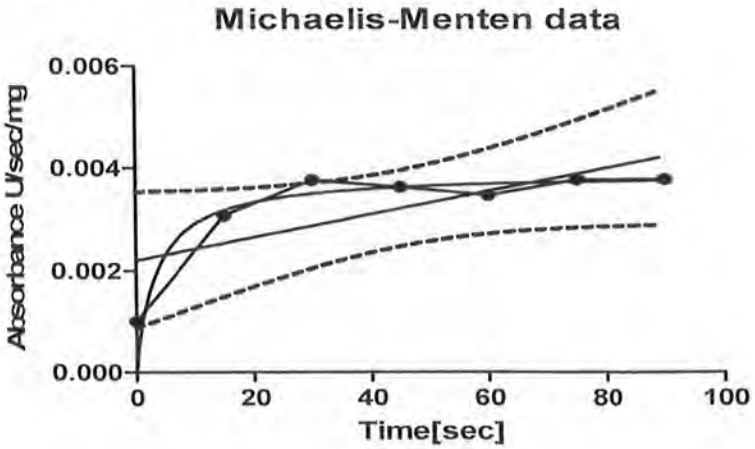


Table 2. Statistical Analysis

Michaelis-Menten	Sample 1	Sample 2	Sample 3
Best-fit values			
Vmax	0.005631	0.005357	0.003904
Km	9.834	10.38	3.447
Std. Error			
Vmax	0.0007902	0.0005408	0.0004024
Km	7.155	5.249	3.918
95% Confidence Intervals			
Vmax	0.003600 to 0.007663	0.003967 to 0.006748	0.002869 to 0.004938
Km	0.0 to 28.23	0.0 to 23.87	0.0 to 13.52
Goodness of Fit			
Degrees of Freedom	5	5	5
R^2	0.8305	0.9022	0.814
Number of points			
Analyzed	7	7	7

RESULTS

15 sec per 15 cycles burst displays estimated best-fit value of 0.9022[R^2 (estimation of Non-linear regression-Michelson Menten)] and seems appropriate sonication time duration for sample treatment. From the progression curve, we also observed maximum activity at 45 seconds. From the results above, we can safely move on to our next step of enzyme assay development. Enzyme Assay performed on published protocol as a preliminary test for detection.

4.3 QUALITATIVE ANALYSIS

PROTEIN STANDARD CURVE

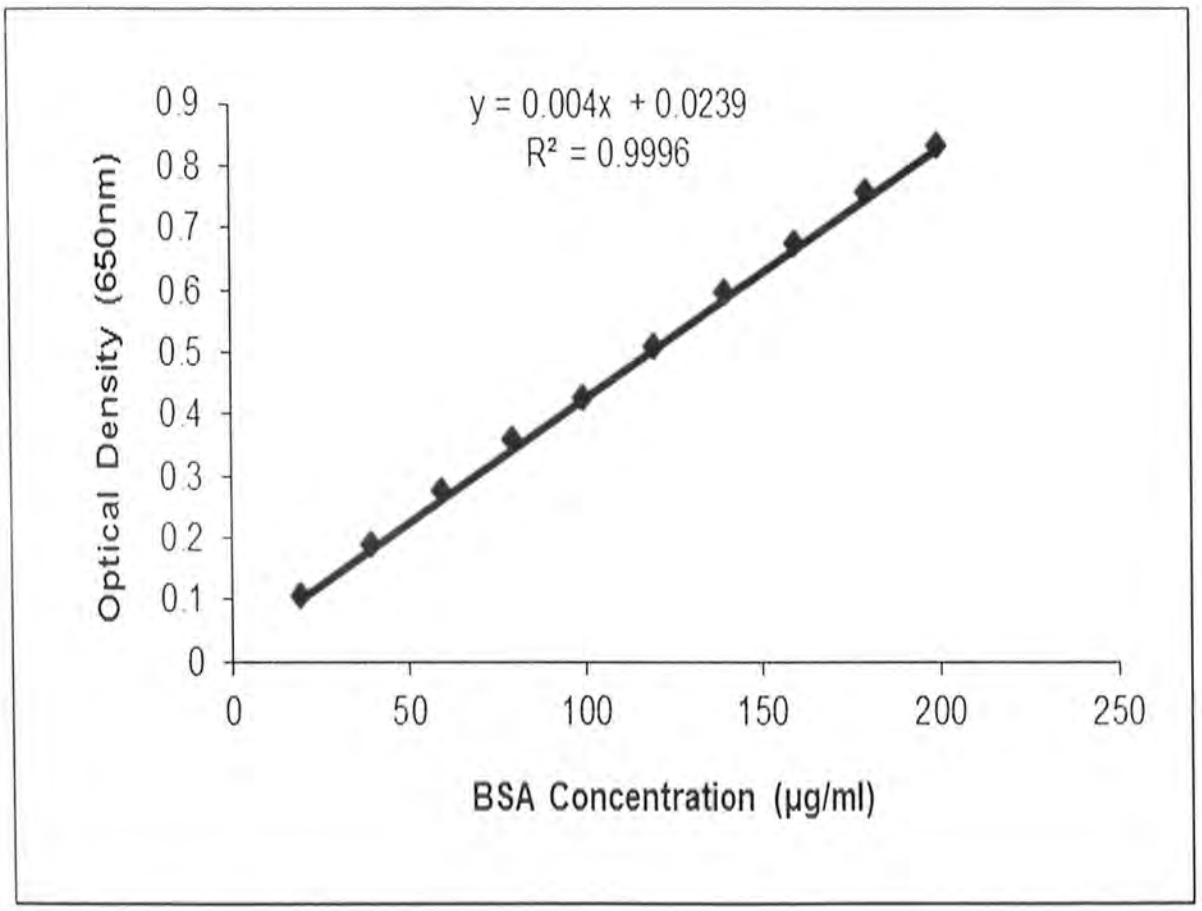


Fig19. Protein Standard Curve

Table 3. Dilutions of known protein; 1 M BSA (5mg/5ml) $[C_1]$ to generate Protein standard curve to estimate unknown sample ranging: (10µg----1000µg) at 650nm

BSA(µL) $V_1=C_2xV_2/C_1$	Water(µL)V_2	Sample concentration(µg/ml)C_2	Solution C (ml)for 10 min	Folin-Reagent(µL) for 30 min	Absorbance (nm)
10	990	0.01	2	200	0.0507
20	980	0.02	2	200	0.10134
40	960	0.04	2	200	0.18224
60	940	0.06	2	200	0.2704
80	920	0.08	2	200	0.35447
100	900	0.1	2	200	0.41904
120	880	0.12	2	200	0.50545
140	860	0.14	2	200	0.58943
160	840	0.16	2	200	0.66884
180	820	0.18	2	200	0.75186
200	800	0.2	2	200	0.82565
100 (UK-sample)	900	0.016	2	200	0.1601

RESULT

Pellet weighed **500mg** suspended in 5mL of lysis buffer for sonication. Using Lowery method, **100µl** of sonicated extract showed value of **16µg** protein approximated from protein standard graph.

5mL of our total sample indicates **0.8 mg** of protein.

4.4 QUANTITATIVE ANALYSIS

4.4.1 ENZYME ASSAY

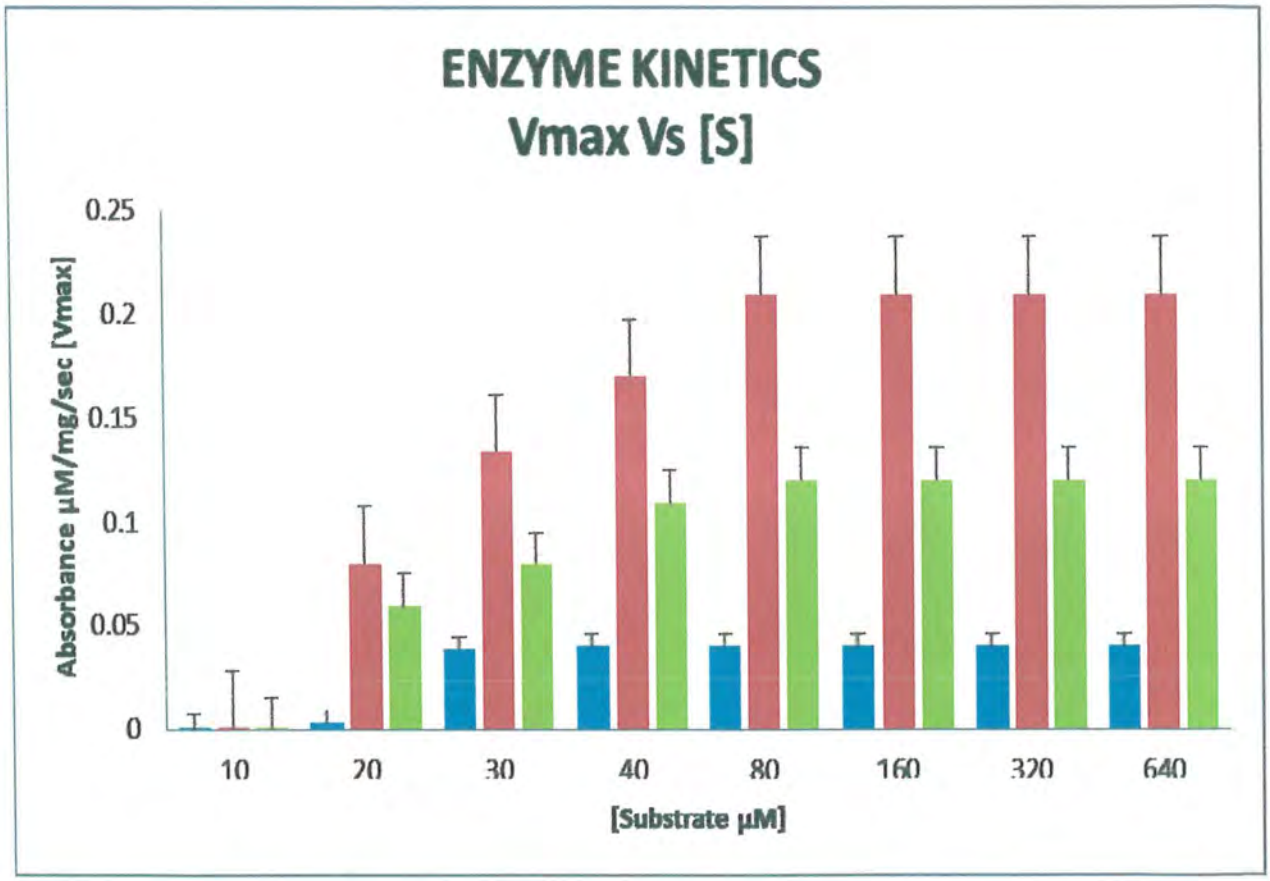


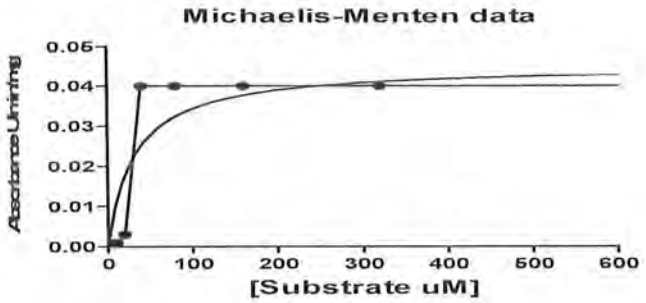
Fig 20. Effect of additives on Enzyme Assay

Table 4. Development of Substrate standard curve by employing Enzyme Assay

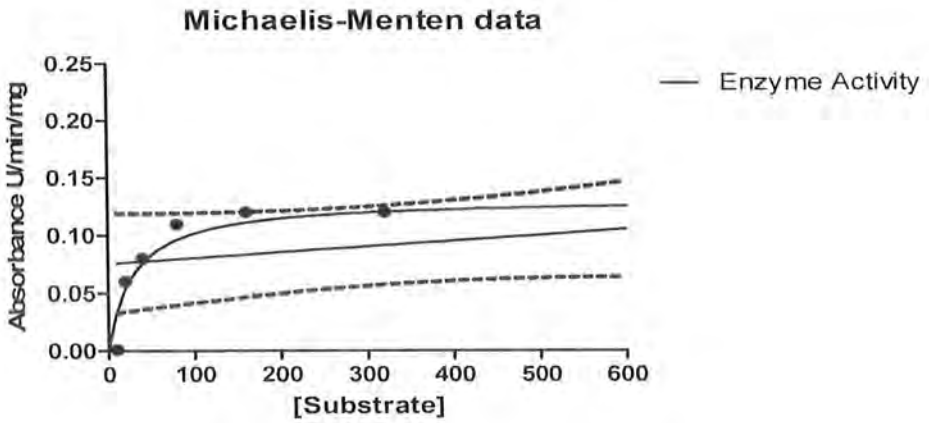
5mMAdenine(µl)	Enzyme(µl)volume	(Exp.1)Absorbance	(Exp.2)Absorbance	(Exp.3)Absorbance
4	40	0.001	0.001	0.0001
8	40	0.003	0.08	0.06
16	40	0.039	0.134	0.08
32	40	0.04	0.17	0.1095
64	40	0.04	0.2098	0.12
128	40	0.04	0.21	0.12
256	40	0.04	0.21	0.12
512	40	0.04	0.21	0.12

GraphPad PrismTM V 5.0 (GraphPad software, Inc., San Diego, USA) analyzed data applying non-linear regression curve fit for Adenine aminohydrolase.

Graph 1



Graph 2



Graph 3

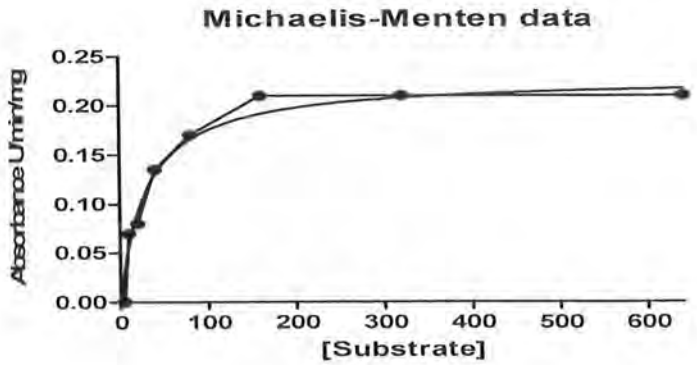


Table5. Statistical Analysis

Michaelis-Menten	Sample 1	Sample 2	Sample 3
Best-fit values			
Vmax	0.04514	0.1316	0.2318
Km	30.34	29.62	14.78
Std. Error			
Vmax	0.006487	0.01006	0.01624
Km	20.73	10.3	4.484
95% Confidence Intervals			
Vmax	0.02927 to 0.06101	0.1070 to 0.1563	0.1920 to .2715
Km	0.0 to 83.06	4.412 to 54.82	3.802 to 25.75
Goodness of Fit			
Degrees of Freedom	**6**	6	6
R^2	**0.7269**	0.8856	0.9182
Analyzed	8	8	8

RESULT

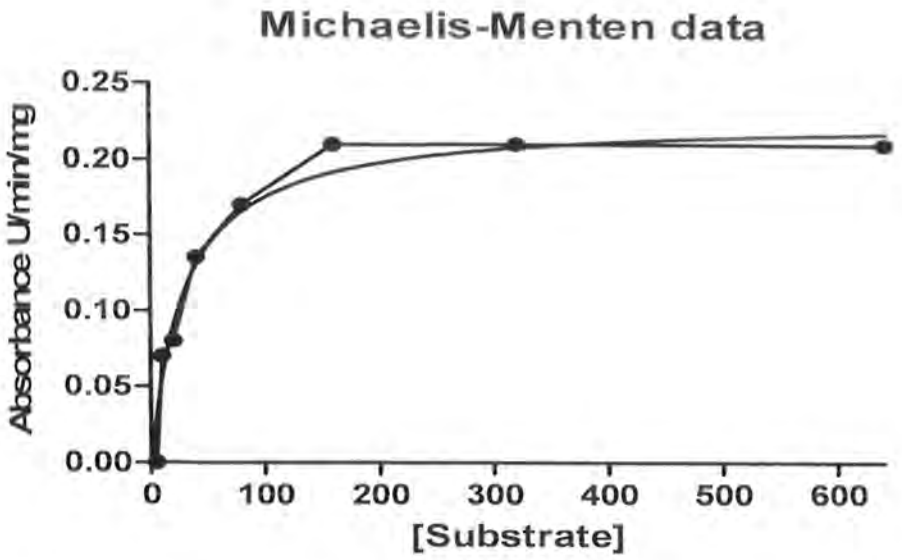
Sample: Leishmania tropica (Kwh23)

Additives used during Enzyme assay development have shown influence on the observed X and Y values.

*Best-Fit = R approximately estimates slope ($\hat{b}$) and intercept (b1): $y^{\wedge} = \hat{b} + b1x$

$R^2 = 0.9608$ with 95% confidence intervals V_{max} mean lies between 0.2025 and 0.2522, K_m mean between 3.802 to 25.75 supporting addition of additives for robust enzyme assay. Graph 3 showed estimated Goodness of Best Fit .

4.4.2 QUANTITATIVE ANALYSIS



- 500mg pellet suspended in 5mL of lysis buffer for sonication.
- Using Lowery method, 100µl of sonicated sample indicated absorbance 0.01601 µg/µl demonstrated 16µg protein from standard curve
- 5mL sample estimated to have **0.08 mg** of protein.

CALCULATIONS:

ENZYME UNIT of enzyme defined as amount of enzyme that catalyzes the formation of 1µmole of product in one minute.

Enzyme unit activity= Volume of assay x Substrate µmol/min/ml

Volume Assay of 2ml used to find out enzymatic activity in 40µl of sample by the addition of 15µl of substrate, the value that corresponds with K_m taken from standard curve.

Enzyme units: 0.4100 µmol/ml/min or 0.4 U

Specific activity=enzyme units / (vol of extract. in µl x (protein conc. in mg per mL / 1000x0.08mg) =U/mg

30ul of sample demonstrated 0.4U activity in the given Assay (i.e. 0.4U / 0.04ml x 0.08mg)

SPECIFIC ACTIVITY = 125U/mg of enzymatic activity present in the given sample

Turnover/sec=125 x 40000/60000

⇨ **COMPOSITION:**

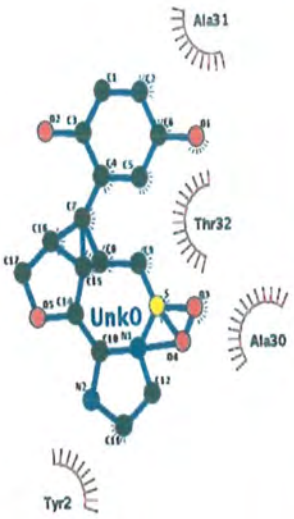
Number of residues = 362 Molecular weight = 40858.27

Mol.wt of adenine aminohydrolase = 40,000(http://pir.georgetown.edu) verification

TURNOVER (K_{cat}) = 187.3/sec Aminohydrolase catalyze 188 molecules of substrate per second.

Catalytic efficiency=K_{cat}/K_m=187.3 / 15 μM sec = $1.2x10^7 M^{-1}sec^{-1}$

4.5 RESULTS OF INSILICO DOCKING BY GOLD (2DLIGPLOT)



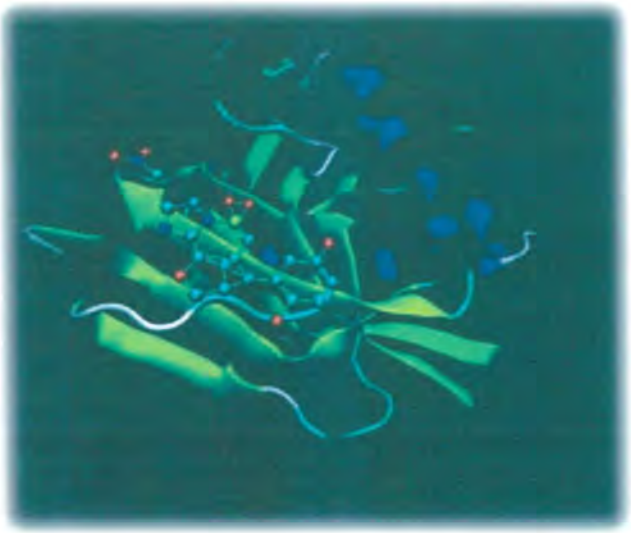


Fig 21. 2D Ligplot on interacting residues Fig 22.Insilico docking of 31.pdb drug

Table 6. Binding score

Compounds	X-score Binding Energy (Kcal/mol)	Gold score
15.pdb	**-6.29**	**36.21**
31.pdb	**-6.48**	**34.88**
2.pdb	-6.37	34.04
8.pdb	-6.18	34.03
11.pdb	-6.24	33.97
24.pdb	-6.24	33.9
19.pdb	-6.52	33.76
4.pdb	-6.50	33.75
3.pdb	-6.22	33.74
26.pdb	-6.28	33.03

4.6 INHIBITION ASSAY

4.6.1 DETERMINATION OF K_M AND V_{MAX} VALUE BY PLOTTING INCREMENTAL ADDITION OF SUBSTRATE

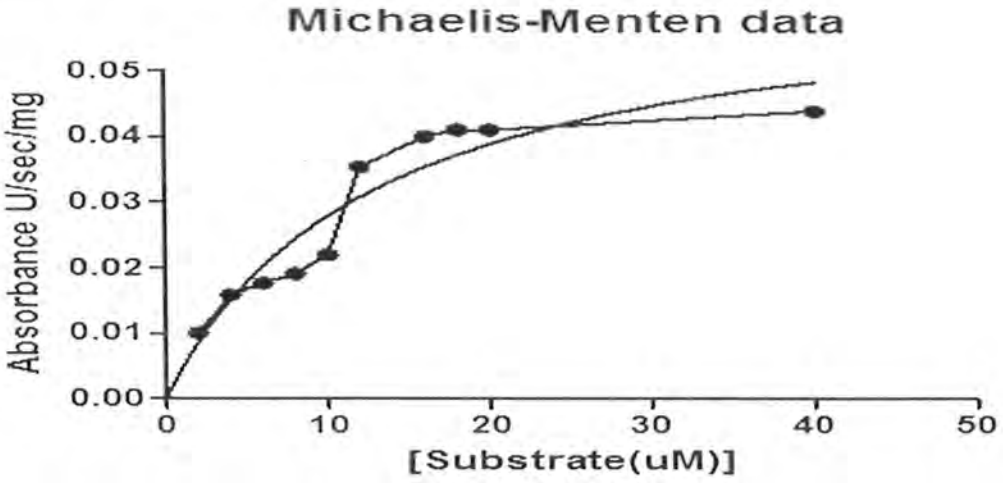


Table 7. Statistical Analysis

Michaelis-Menten	
Best-fit values	
Vmax	**0.06418**
Km	**13.06**
Std. Error	
Vmax	0.009295
Km	4.137
95% Confidence Intervals	
Vmax	0.04274 to 0.08561
Km	3.521 to 22.60
Goodness of Fit	
Degrees of Freedom	8
R^2	0.8967
Absolute Sum of Squares	0.0001533

RESULT

K_m 13.06 µM , the value showing maximum substrate concentration during reaction, thus chosen for drug or inhibitor application.

4.6.2 INHIBITION ASSAY

Dose Vs Response simulations giving IC_{50}

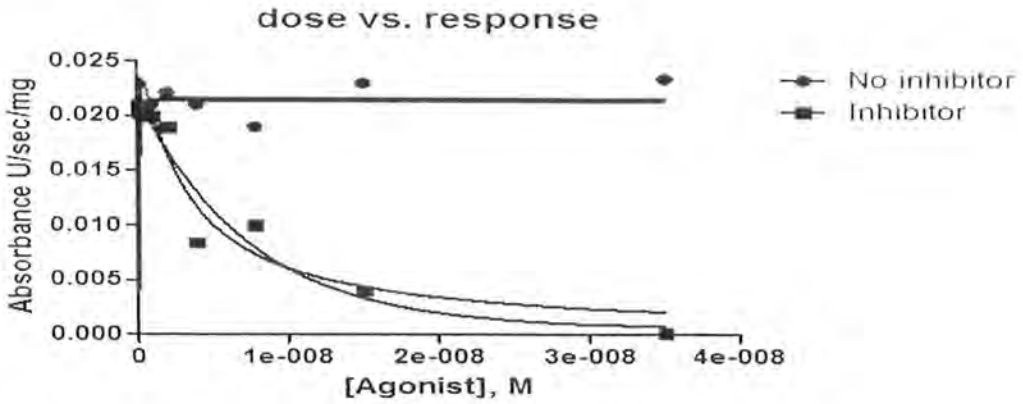


Table 8. Statistical Analysis

Substrate inhibition	No Inhibitor	Inhibitor
Best-fit values		
Vmax	0.02166	**0.02868**
Km	8.670e-012	**3.310e-011**
Ki	~ 7.781e+007	**2.683e-009**
Std. Error		
Vmax	0.0007607	0.007509
Km	1.071e-011	3.954e-011
Ki	~	1.540e-009
95% Confidence Intervals		
Vmax	0.01955 to 0.02377	0.007833 to 0.04952
Km	0.0 to 3.840e-011	0.0 to 1.429e-010
Ki	(Very wide)	0.0 to 6.959e-009
Goodness of Fit		
Degrees of Freedom	4	4
R^2	0.1580	0.9304
Absolute Sum of Squares	1.270e-005	2.897e-005
Sy.x	0.001782	0.002691

log(agonist) vs. response	Interrupted
Best-fit values	
Bottom	-0.009771
Top	0.04659
LogEC50	-0.09121
EC50	0.8106
Span	0.05636

RESULT

IC_{50} value is 0.8106 µg/ml increase value of V_{max} and K_m during reaction shows mixed non-competitive inhibition.

Catalytic site homology

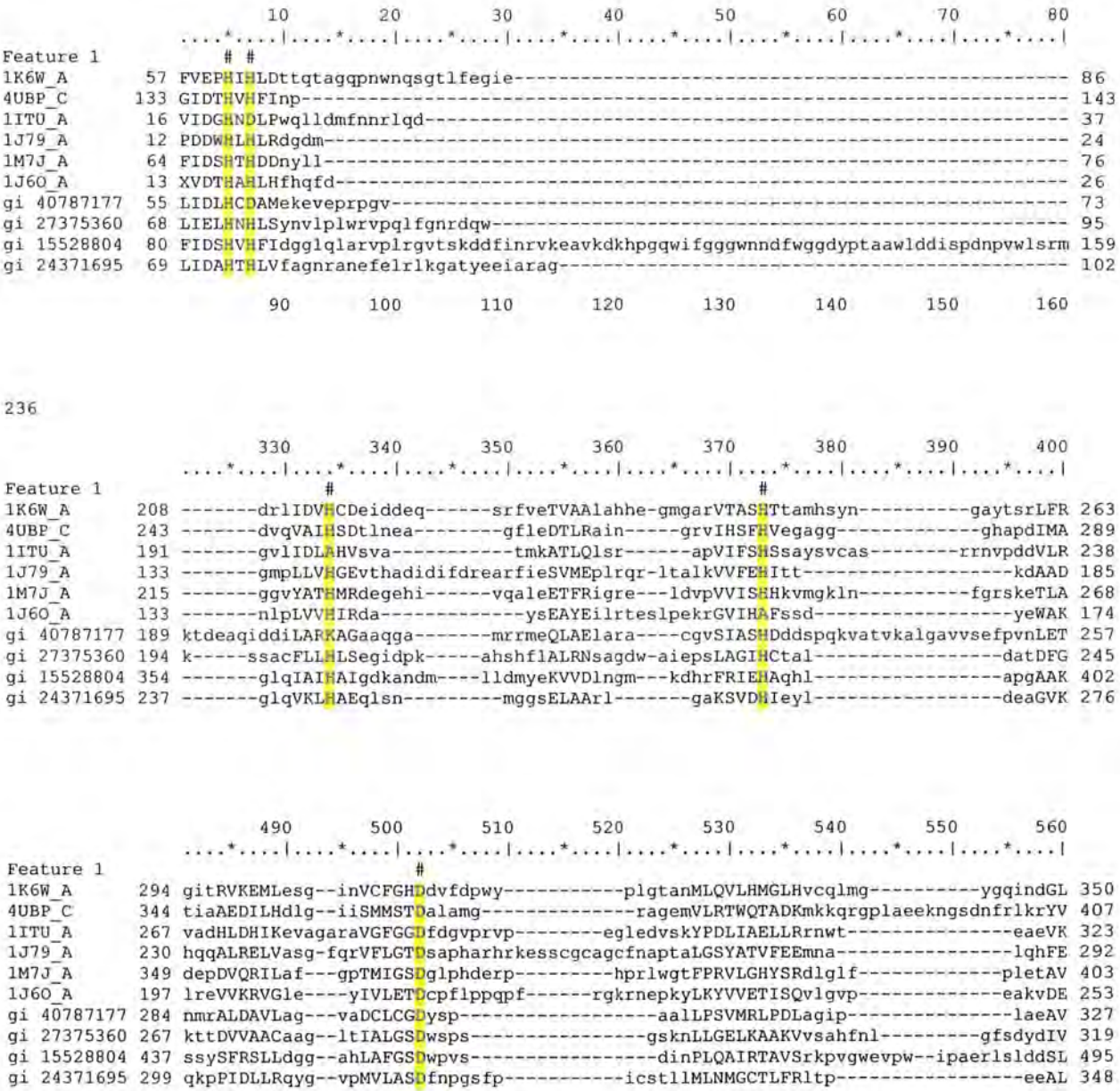


Fig23. Multiple alignment of superfamily cl 00281 (Marchler Bauer *et al*, 2013)

RESULT

Catalytic sites of Hydrolase family [cl 00281] constitute 4 histidine residues and one aspartate.

2.4.2 HOMOLOGY BASED structure of ADENINE AMINOHYDROLASE by Phyre

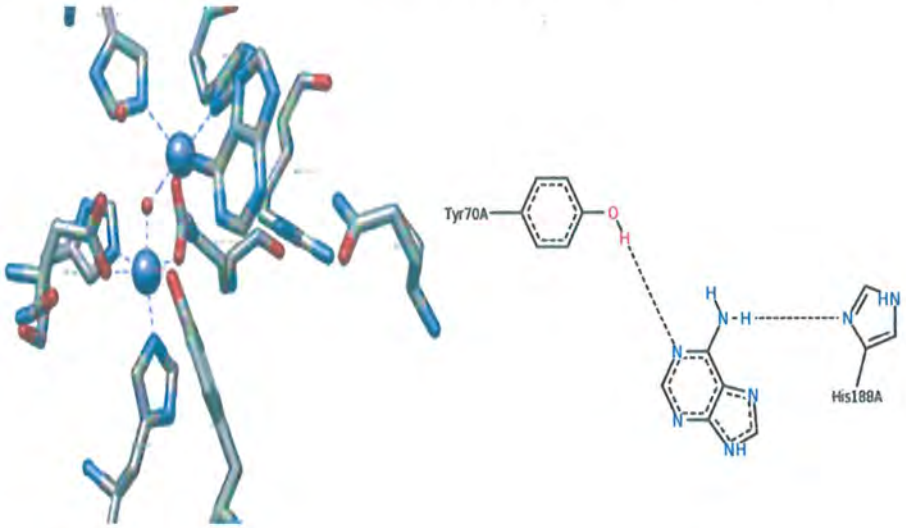


Fig24. Adenine attached to AHH; 2ICS.pdb (binuclear) (Pettersen EF. et al, 2004)

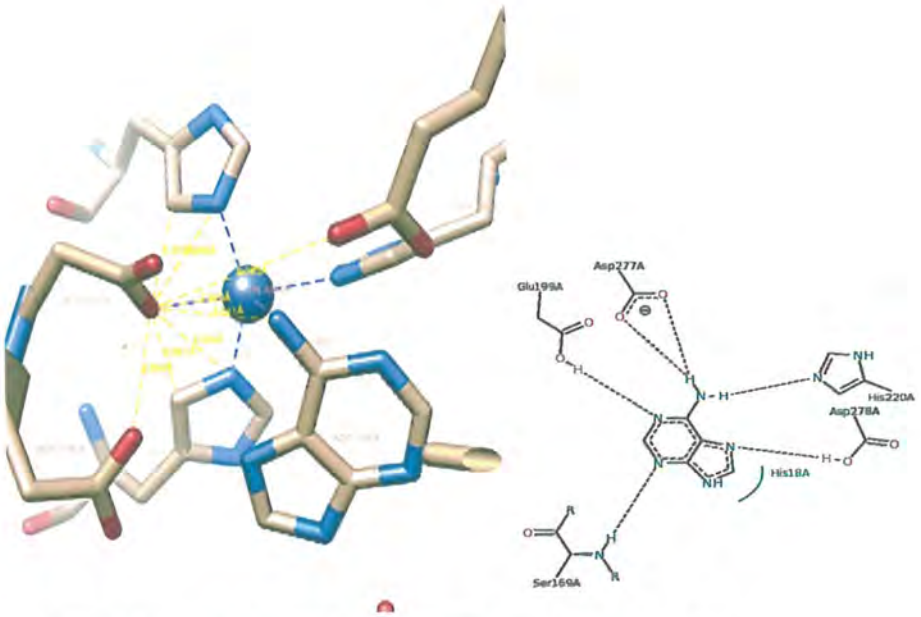


Fig25 Adenine attached to AHH; 3pao.pdb (mononuclear) (pettersenef. et al, 2004)

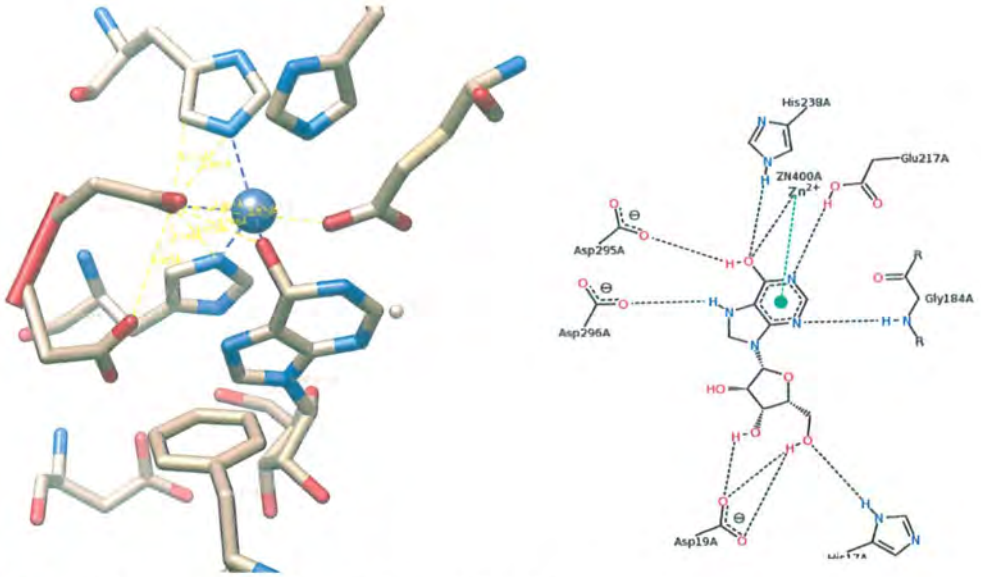


Figure 26: Attached adenosine to ADA; 2pan.pdb (Pettersen EF. et al, 2004)

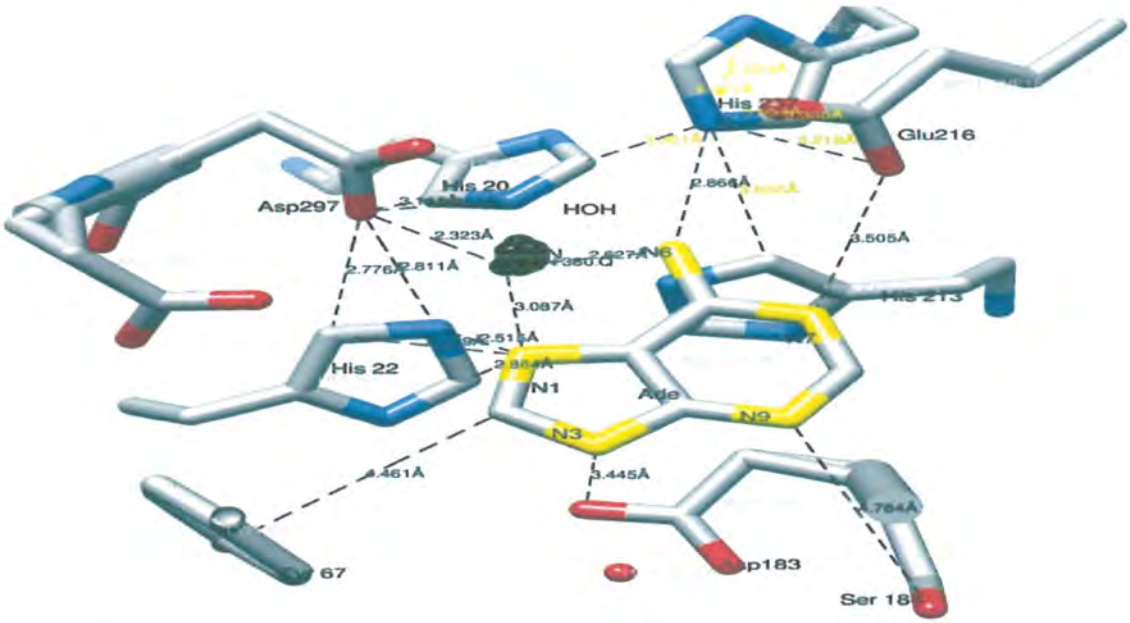


Fig28 Phyre modeled leishmanial AHH; microenvironment of catalytic site visualized by chimera; distances of nearby residues

RESULTS

#2ICS, Tyr^{70} and His^{188} [Active site]

Adenine binding site

Asp^{59},His^{60},His^{62},Phe^{64},Lys^{66},Ala^{68},Leu^{69},[illegible],Tyr^{71},Ala^{89},Gly^{90},Tyr^{91},Tyr^{92},
Asn^{96}, Leu^{114}, Asn^{116}, Trp^{120},
Ile^{122}, Val^{123}, Ala^{124}, Gln^{125}, Ile^{153}, Ala^{155}, Arg^{156}, Ser^{158}, Arg^{159}, Tyr^{160}, Val^{161},
Leu^{185},Val^{187},His^{188},Ile^{189},
Gly^{190},Ser^{191},Val^{208},Tyr^{210},His^{211},Lys^{216},Asp^{243},Gly^{247},Tyr^{248},Asp^{249},Ser^{250},
[illegible] Tyr^{274}, Arg^{276}, Asn^{277}, Phe^{315}

#1ADD, Asp$^{19, 296, 297}$, His$^{12, 238}$, Glu^{217}, Gly^{138} [Active Site]

His17,Asp19, Leu58, Phe61, Leu62, Phe65, Asp66, Met69, Tyr102, Ser103, Leu106, Trp117, Cys153, Met155, Arg156, His157, Gln158, Trp161, Ala183, [illegible], Asp185, Glu186, His214, [illegible], His238, Thr269, Asp296, Asp297, Phe300

 # 3PAO, Asp$^{277, 278}$, His^{220}, , Glu^{179,} , Ser^{169}

His18, Leu56, Phe59, Leu60, Tyr63, Tyr64, Asp100, Arg141, Asp168, [illegible], Glu171, His196, Glu199, His220, Leu251, Asp277,Asp278, Phe282

Comments:

Yellow highlighted residues are common among hydrolase enzyme; green residues are active sites of both adenine and adenosine whereas blue colored residues assign specificity to the enzyme for its substrate. 2ICS chosen for in-silico as it catalyzes specifically adenine rather than other experimentally derived structures that have receptors for both adenine and adenosine, thus could cause error in inhibitor designing.

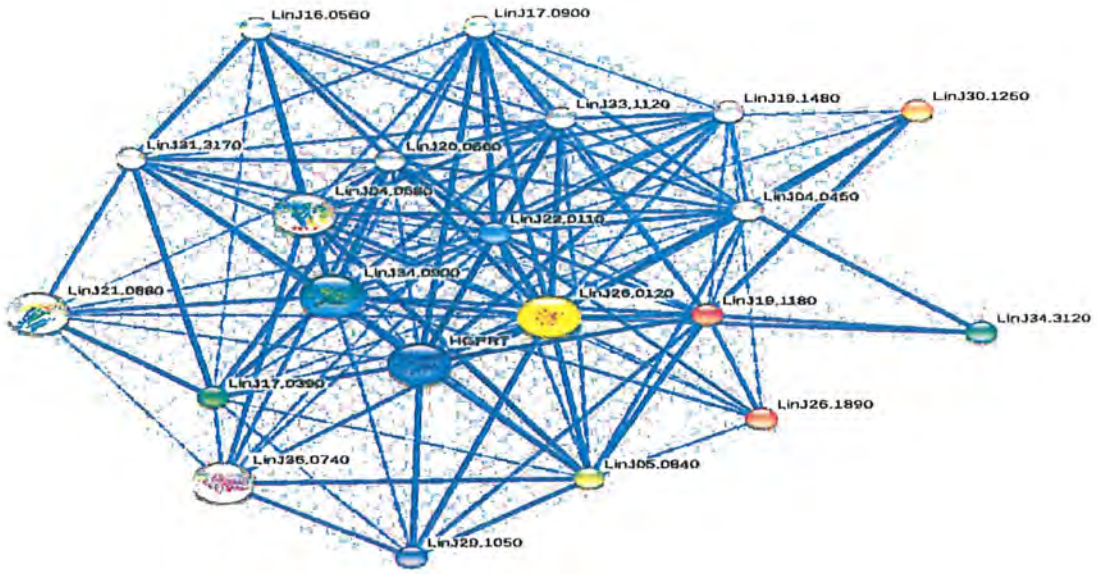


Fig28. Knowledge-based prediction of functional partners in purine salvage pathway. http://string-db.org/

Table 9. List of enzymes contributing purine salvage pathway

Predicted Functional Partners:			Score
	LinJ30.1250	adenosine kinase (345 aa)	0.956
	LinJ26.0120	adenine phosphoribosyltransferase (237 aa)	0.951
	LinJ05.0840	methylthioadenosine phosphorylase (306 aa)	0.937
	LinJ17.0390	cytidine deaminase-like protein (182 aa)	0.931
	LinJ34.3120	adenosine kinase-like protein (388 aa)	0.915
	LinJ34.0900	uracil phosphoribosyltransferase (242 aa)	0.891
	LinJ22.0110	GMP synthase (656 aa)	0.877
	HGPRT	HGPRT,hypoxanthine-guanine phosphoribosynthesis protein (211 aa)	0.793
	LinJ29.1050	guanine deaminase (454 aa)	0.751
	LinJ26.1890	hypothetical protein (326 aa)	0.750

RESULTS

Functional partners involve all enzymes that add or remove phosphate (i.e. Kinases), sugar (i.e. transferases) and amine (i.e. deaminases) groups mentioned in Table (4) and shown in Fig (19) probed by string databases showing 95% to 74% confidence score.

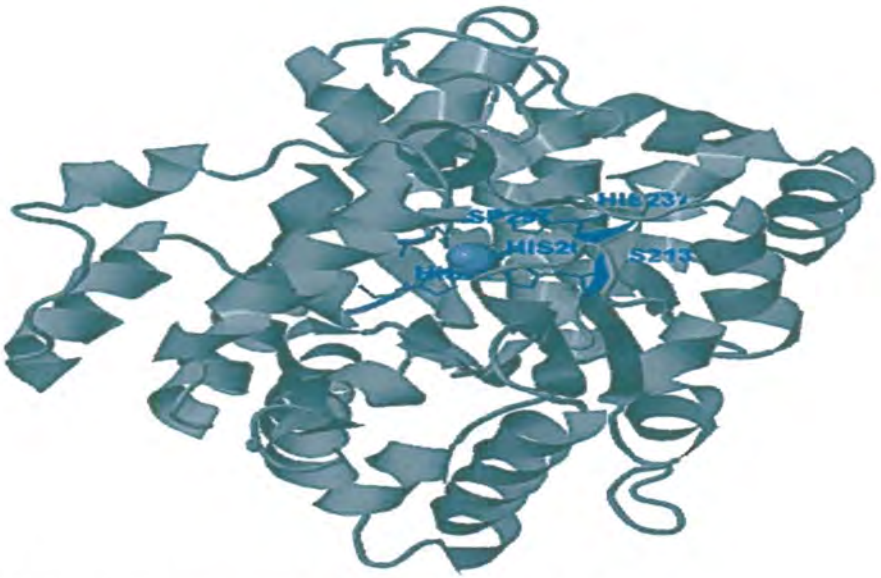


Fig 29. Homology modeling (Wass M.N et al, 2010)

Fig30 Secondary structure report of template (c3ou8B) and query sequence

RESULTS

Phyre homologues detected by PSI-BLAST, predicting secondary structure and disorder with Psi-pred and Diso-pred, constructing and scanning a hidden MARKOV MODEL (HMM) with experimentally solved structures. Modelling performed insertions and deletions using a loop library, a fitting procedure (cyclic coordinate descent) and a set of empirical energy terms. Amino acid side chains oriented using a rotamer library from ROLAND DUNBRACK'S LABORATORY and optommization by using fast graph-based approach (R3).Protein is a whole chain taken from the PDBc3ou8B_, the crystal structure of adenosine deaminase from *pseudomonas aeruginosa* at resolution 2.51 Å. Homology modeling Intensive mode turned up a model of protein with confidence in the model: Confidence in the model: 349 residues (96%) modelled at >90% accuracy (Kelley LA, 2009)

2.4 KNOWLEDGEBASED CHARACTERIZATION OF ADENINE AMINOHYDROLASE

MADEALLHRLIETLPKAELHVHIEGTLSPELLFELAKRNGVQIPYK[T V E E 50
VRVAYNFTDLQ[S F L D LYYEGMSVLI[T E D D FADLAYAYTRVMHEGCI[T H A E 100
PFFDPQGHLCRGI[T F R V L Y D G L M K G F RRGEAEF G I S V A L I FSFLRHLPEA 150
ECFALVRDDMHPDNGQYIRELFAAKAFVAVGLDSSEVGHPPEKFARLYRY 200
CRDELKVPFLVAHAGEEGPPGYMRDAMSMLTVDRIDHGVAARLDQALCKD 250
LREKRIPLTVCPTSNVALKVFQDRA[E G E V V M D L I L T E G L C V T I NSDDPA 300
YFGGDIRESFRILAETGRLTPLTLKHLVLNSFWSSFIAEDRKRAYEERVE 350
KVFKEVC G G G R T Y A 364

Table 10: Prosite pattern found in Leishmanial AHH

AC PS00006	Casein kinase II phosphorylation site.
PA [ST]-x(2)-[DE]	TVEE 47-50; SFLD 62-65; TEDD 76-79; THAE 97-100; TCGE 276-279
AC PS00008	N-myristoylation site.
PA G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}	GLMKGF 121-126; GISVAL 134-139; GLDSSE 181-186; GLCVTI 289-294; GGGRTY 358-363
AC PS00005	Protein kinase C phosphorylation
PA: [ST]-x-[RK]	TFR 114-116; SFR 309-311; TGR 316-318; TLK 323-325
AC PS00001	**N-glycosylation site**
PA: N-{P}-[ST]-{P}	**NFTD 56-59**

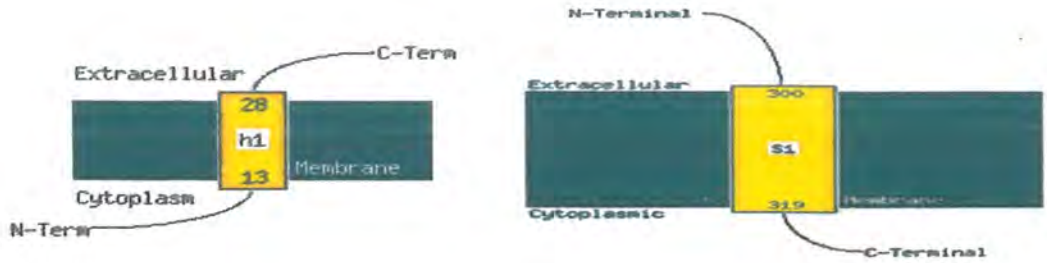


Fig 31. MEMSAT-SVM Prediction on possible occurrence of transmembrane (Nugent 2009)

RESULTS

PSI-Blast detected homologues processed by a Support Vector Machine (a powerful machine learning tool) determine transmembrane helices and their topology in the membrane using MEMSAT-SVM, which has demonstrated an average accuracy of 89% on a large test set. The extracellular and cytoplasmic sides of the membrane labeled and the beginning and end of each transmembrane helix illustrated with a number indicating the residue index. 13-28 residues at N-terminal supposed to have likelihood of pore lining helix with score -0.067572, also 300-319 residues out of membrane scoring -5.78 reflect the relative likelihood of the protein occupying different cellular localizations

DISCUSSION

Anti- parasitic therapies requires exploiting biochemical pathways and the detection of selective features of enzyme as drug targets to support drug discovery plan. Adenine aminohydrolase [E.C 3.5.4.2; Superfamily cl00281], metalloenzyme hydrolases, is the crucial enzyme that de-aminates. AHH is a mammalian counterpart, considered as a major breakthrough for drug discovery plan; utilizes Adenine as a substrate to form hypoxanthine in Leishmania spp and many other lower species. Adenine, a product of PNP, is absent in human salvage pathway that forms hypoxanthine from inosine. Three different pathways consume Adenine that either break it down to hypoxanthine or condenses it back to form AMP.

AHH presence in pre-eukaryotic organisms implies to transitory changes giving rise to emerging subsequent pathways augmenting genomic pool for the purpose of adaptation to variable environment. This pathway tends to increase the influx of adenine and subsequent reactions to carry out vital and additional functions evolving to complex organisms. In higher organism, such subsequent pathways become redundant because of self-sufficient DNA mechanism evolved by the course of time. Series of experiments conducted to learn the regulatory role of this pathway through knockout variances evaluate the role of AHH and its relationship with functional partners of purine salvage pathway in prokaryotes, fungal strains and protozoans, except in trypanosomatids. The enzyme responsible for the conversion of adenine to hypoxanthine in the various organisms regulated differently by specific nucleobase and nucleoside transporter systems (Marr, 1982).

For the Isolation and characterization of Leishmanial Adenine Aminohydrolase, Pakistani local Leishmanial strain acquired from a patient suffering from Cutaneous Leishmaniasis in Peshawar. Samples cultured in bi-phasic blood agar at 25^0C before transferring into Medium 199, supplemented with adenine for the maximum production. Culture reached log-phase in 5-7 days gave high yield and expected targeted enzyme on 10mM Adenine addition confirmed by viability counting tests and enzyme assays.

Enzyme assay development carried out in the current study confirmed the presence of Adenine aminohydrolase in given Pakistani Leishmanial strain source. The V_{max} & K_m used as parameters to evaluate non-linear graph that denotes enzyme assay. V_{max} indicates amount of enzyme partaking in reaction in the sample and K_m characterizes enzyme for identification. Phosphate buffer [pH=7.2] used for washing culture as enzyme activity found to be highest in potassium phosphate buffers, decreasing to about 75% in Tris/HCl and to 30% in MOPS (Hana POSPÍŠILOVÁ *et al*, 2008). After washing, the culture sonicated in the presence of appropriate lysis buffer to avoid use of chemical lysis, performed strictly under appropriate conditions.

Enzyme activity assays performed on Leishmanial strain tested for Adenine aminohydrolase presence by exercising Enzymes' kinetic activity at 292nm, which is molar absorption coefficient value of Hypoxanthine detection, using UV/vis spectrophotometer. Additives such as EDTA, Bile salt and β-mercaptoethanol increase the stability of enzyme for storage purposes and for relatively higher V_{max} that will definitely help to develop, design and validate kinetics of enzyme action for drug application.

Statistical analysis of graphical representation of chemical reaction validates V_{max} & K_m used as parameters to evaluate non-linear graph that denotes enzyme assay. A confidence interval criterion assumes P = zero; zero product initiates reaction forward in irreversible manner. Kinetic analysis of enzyme-catalyzed reaction states velocity of reaction (v) is the function of [S] that presents rise in v as substrate concentration increases representing first order reaction, uptil further increase in [S] becomes independent of v posing zero order reaction. Zero order reaction shows saturation point or steady state as enzymes catalytic units completely occupied leading to no further increase in product concentration indicating nonlinear regression. Sample Data analyzed by using Michaelis Menten Enzyme Kinetics equation on Graph prism

$$Y = Vmax*[X]/ (Km + [X])$$

v as Absorbance plotted against [S] on XY graph.

Y (abs) = [Enzyme activity]; *v* X= [Substrate concentration]; S

Wherein "*v* is the function of [S]"

Graphic parameters = K_m & V_{max}

To acquire a linear graph expressing initial velocity of reaction, effect of enzyme concentration studied to adjust sample size for the generation of precise graph for practical considerations. Since total enzyme concentration in a given sample might delimits product formation, excessive enzyme concentration in sample deplete substrate rapidly presenting sharp rising curve shown in Graph 1 of the quantitative analysis. Appropriate substrate range has shown influence on the observed Vmax value that gives the enzymatic activity.

Effects of substrate improved V_{max} and K_m values by applying two-fold increase in substrate concentration. Additives used during Enzyme assay development showed improvement in curve, stabilizes the ionic strength of enzymes' active site affected by release of harmful enzymes during sonication. Graph obtained shows nonlinear regression coefficient $R^2 = 0.9608$ with 95% confidence intervals V_{max} mean lies between 0.2025 and 0.2522, K_m mean between 3.802 to 25.75 supporting addition of additives for robust enzyme assay.

Based upon best-fit hyperbolic enzymatic graph one can obtain catalytic efficiency of Enzyme and perform inhibition assays to get IC_{50}. Complete knowledge of enzyme's biochemistry and kinetics is crucial to perform inhibition assays. Enzyme catalytic sites are determined from their product formed by interaction with specific substrate they catalyze (Harold Brooks, 2012).

Inhibition assays performed with set range of drug concentrations incubated with 13µL substrate concentration that favors [ES] complex indicating high product formation acquired from performed enzyme kinetic assay, for thirty minutes using UV/vis spectrophotometer for absorbance reading. XY Graphs generated by software (GraphPad PrismTM V 5.0 GraphPad software, Inc., San Diego, USA) analyzed dose Vs response simulations for IC_{50} and K_i values.

The Compound, applied as drug, $C_{26}H_{15}N_3O_7S$ (2-(2-(5-methylfuran-2yl)-5-nitro-1H-benzo[d]imidazole-1-ylsulfonyl) anthracene-9, 10-dione selected out of 90 given computer aided drug designed Sulfa derivatives compounds presented by In-silico docking. Molecular docking studies carried out to study inhibition effect of benzyl amino group on the structure of Adenine aminohydrolase suggests hydrogen-bond interactions between N-7 of the purine ring and OH of Thr^{32}, and Tyr ^{2}. $Ala^{31}$and Ala ^{30} residues found to be extending hydrophobic effect offering sound Gold score predicted as a non-competitive inhibitor. 2DLigplot analysis was performed in order to validate the in depth interaction patterns on expected allosteric sites of 2ICS which is experimentally derived structure of *Enterococcus feacelis* adenine aminohydrolase. **2ICS** PDB (Sugadev *et al*, 2006) isolated from Enterococcus faecelis, 3PAO isolated from Pesudomonas aerugenosa are experimentaly derived structures catalyze adenine occupied by different set of residual arrangement proving specificity of enzyme for its substrate. Substrate are held by specific residues that set ADA and AHH apart from eachother in function eventhough with 51% of structural similarity. Glu^{24} replaced by aspartic acid might be mutagenic site for this enzymeProsite databases matches up four prosite patterns.

Confirmed by radiometric assays, Adenine deaminase (AAH) and adenosine deaminase (ADA) exhibited specificity in catalyzing Adenine. Helices & Sheets composition percentage suggests globular structure that has capacity to turn, bend and reorientate itself. Enzyme folded up in layers of hydrophobic layers wound around core of 8 parallel βsheets forming conserved catalytic domain TIM barrel that seems arising from inwards to outward direction interspersed by αHelices giving flexibility to enzyme structure for conformational change during catalysis, involving four histidines and one aspartic acid residue, mostly found in glycolytic enzymes.

PS0001codify ASN glycosylation site [NFTD 56-59] having (N-{P}-[ST]-{P})-ref as a marker sequence apart from proline found unfavorable for glycosylation. Glycosylation enhances solubility, improve folding, facilitate secretion, and modulate antigenicity. Asparagine transferred to nascent protein catalytically in the lumen of endoplasmic reticulum. Genetic and biochemical characterization of oligosaccharide transferase (OST) complex in yeast and mammalian cells have demonstrated the importance of specific

OST subunits in protein N-glycosylation. N-linked glycosylation, a common co-translational modification in eukaryotic cells, involves the transfer of a lipid-linked oligosaccharide onto asparagine residues in a tripeptide sequence on a nascent protein in the lumen of the endoplasmic reticulum. These characterizations elucidate methodologies to control N-glycosylation site heterogeneity. (Jones et al, 2005) Pro-Asn-Gly-Thr-Ala-Val found to be glycosylated at a high rate (Bause, 1983).

Protein kinase C phosphorylation site [TFR 114-116; SFR 309-311; TGR 316-318; TLK 323-325] PA: [ST]-x-[RK] having PA [ST]-x-(2)-[DE]-ref. Protein kinase C exhibits a preference for the phosphorylation of serine or threonine residues found close to a C-terminal basic residue.

Casein Kinase 2 Phosphorylation site [TVEE 47-50; SFLD 62-65; TEDD 76-79; THAE 97-100; TCGE 276-279] having Pattern G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}-ref; Casein kinase II (CK-2) is a protein formed by serine/threonine independent of cyclic nucleotides and calcium. CK-2 phosphorylates many different proteins. Ser is favored over Thr. An acidic residue (either Asp or Glu) from the C-terminal of the phosphate acceptor site at positions +1, +2, +4, and +5 increase the phosphorylation rate. Asp is preferred to Glu as the provider of acidic determinants. A basic residue at the N-terminal of the acceptor site decreases the phosphorylation rate, while an acidic one will increase it.

N-myristoylation site [GLMKGF 121-126; GISVAL 134-139; GLDSSE 181-186; GLCVTI 289-294; GGGRTY 358-363]-ref. An appreciable number of eukaryotic proteins are acylated by the covalent addition of myristate (a C14-saturated fatty acid) at their N-terminal residue. Sequence specificity of the enzyme responsible for this modification, myristoyl CoA: protein N-myristoyl transferase (NMT) derived from the sequence of known N-myristoylated proteins and from studies, using synthetic peptides following the N-terminal preferably having glycine residue. Likelihood of occurrences of certain residues at six different residual positions determines their role.

Signal peptide [LP K] found in two of Leishmanial strains not identified by lysosomes observed by immunofluorescence. PTS2 peroxisome targeting sequence found in two strains could help in finding the location of AHH.

Signal 4.0 databases could not detect proteolytic or cleavage site detected.13-28 residues at N-terminal supposed to have likelihood of pore lining helix scored -0.067572. 300-319 residues extending out of membrane scoring -5.78 reflect the relative likelihood of the protein occupying different cellular and subcellular localizations. Results from MEMSAT-SVM suggest presence of anchoring sites within protein sequence favorable for complex structural associations for instance Kinase anchoring protein.

CONCLUSION

Best-fit values verify stability of active sites of enzyme during reaction in hyperbolic fashion on using β –Mercaptoethanol to inhibit oxidation of sulfur, EDTA to chelate proteases, lipases etc. released after Cell Lysis. Bile salt used as organic detergent to solubilize lipoprotein for clarifying protein residues of membrane and to improve protein estimation procedure for specific activity.

Interactions of the enzymes studied with substrates monitored by absorption changes in the range of 292 nm using Agilent spectrophotometer Uv/Vis. Adenine deaminase activity assayed as an increase in hypoxanthine concentration on the addition of 2-500μM adenine as substrate in 0.1 M phosphate buffer Saline (PBS) pH 7.2, at 25℃.

Molecular docking studies carried out to study inhibition effect of benzyl amino group of $C_{26}H_{15}N_3O_7S$ on the structure of Adenine aminohydrolase 2ICS.pdb suggests hydrogen-bond interactions between N-7 of the purine ring and OH of Thr^{32}, and Tyr^{2}. $Ala^{31}$and Ala^{30} residues found to be extending hydrophobic effect offering sound Gold score *i.e.* binding affinity equals -6.48 Kcal/mole.

Future Prospects:

Cloning of AHH from L. tropica cosmid library, primer designing against ORFS and its amplification, and sequenced, generating targeting constructs, creating knock out, episomal complementation, expression and purification, chromatography, Enzyme assay, Enzyme inhibition assay, Sub-cellular location procedures

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