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ROLE OF AZASERINE IN INHIBITION OF GLUTAMINE FRUCTOSE AMIDO TRANSFERASE, GAMMA GLUTAMYL TRANSFERASE AND MUSCARINIC ACETYLCHOLINE RECEPTOR ACTIVITY: An In-silico approach

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***Abstract*: Azaserine is an analogue of glutamine and a drug used for treatment of pancreatic cancer. Our goal was to understand the antagonistic role of azaserine and the underlying mechanism of drug interactions against certain target molecules viz, membrane bound protein -Gamma glutamyl transferase, rate limiting enzyme-Glutamine fructose amido transferase, and muscarinic acetylcholine enzyme (M3) receptors. Protein-Ligand interaction has a significant role in structure-based drug studies. ADMET screened compound showed good solubility, absorption at (3 level). The internalization of the drug is moderate in intestine and cannot enter blood brain barrier. The rate of degradation and elimination of the drug is notable faster. Also, screened compound is free from noxious effect of hepatotoxicity, followed by no evidence of interacting with liver enzymes. Finally, the compound after docking showed interactions with the target proteins and binding energy levels were significant indicating the inhibitory role of azaserine. Our studies therefore reveal the mechanism of interaction at molecular levels and strong binding ability of drug to target molecules to inhibit their activity. Role of azaserine as a M3 antagonist is a model to understand mechanisms to prevent cancer cell growth and metastasis, which needs further investigation.**

***Keywords:* Docking, Azaserine, Binding energy, Gamma glutamyl transferase, Glutamine fructose amido transferase, muscarinic acetylcholine enzyme (M3) receptors.**

I. INTRODUCTION

Azaserine is a drug primarily used for treatment of pancreatic cancer. Chemically it is an analogue of glutamine and can irreversibly inhibit glutamine utilizing enzymes and therefore impairs glutamine metabolism (Moore and Le-page, 1957). Azaserine can be categorized as genotoxic (DNA damage), cytotoxic (generates free radicals and damages lipids and proteins), antitumour (antibiotic), antineoplastic (cancer drug), antimetabolite (interferes with hexosamine biosynthetic pathway and gamma glutamyl cycle), influences DNA by forming adducts with DNA and inhibits DNA formation (Henderson et al.,1957), cell cycle specific drug (inhibits purine and pyrimidine formation at G phase of cell cycle) and kills cancer cells by first order kinetics. As a chemotherapeutic, it is also used for research on cancer in animal models. It produces deleterious side effects in humans which complicated its use as a chemotherapeutic agent (Ellison and Karnofsky, 1960). Glutamine fructose amido transferase (GFAT) (EC. 2.6.1.16), a rate limiting enzyme for glucosamine

synthesis, enzyme converts glucose-6 phosphate into glucosamine-6 phosphate. Recently Hexosamine Biosynthetic Pathway (HBP) has been implicated in pathogenesis of several diseases including cancer, diabetes, arthritis, chrone's diseases. HBP is vital for synthesis of UDP GlcNAc (Rajapakse et al, 2009). The enzyme GGT (EC 2.3.2.2) is a glycoprotein located on the outer surface of the cell membrane, critical to cellular detoxification and amino acid transport (Keillor et al., 2005). GGT also uses glutathione as an acyl donor substrate and transfers its glutamyl moiety to the acceptor substrate such as amino acid with higher affinity to L- cysteine and L- glycine and release cysteinyl glycine across the cell membrane (Allison, 1981). GGT has primary role in GSH catabolism and aids cystine /cysteine balance to maintain redox state. Muscarinic receptors play a key role in regulating pancreatic endocrine and exocrine function (Gautam et al, 2005, 2006; Williams 2006), muscarinic effects on pancreatic carcinoma are less known. In normal pancreas, M3 receptors play a role in regulating insulin and glucagon secretion (Gautam et al. 2006; Gromada and Hughes, 2006), while M1 and M3 receptors are involved in acinar secretion (Gautam et al, 2005). Most epithelial and endothelial cells exhibit a cholinergic autocrine loop in which acetylcholine acts as a growth factor to potentiate cell growth. Cancers derived from these tissues similarly express a cholinergic autocrine loop, ACh secreted by the cancer or neighboring cells interacts with M3 muscarinic receptors expressed on the cancer cells to stimulate tumor growth and metastasis. Primary proliferative pathways involve MAPK and Akt activation. The ability of M3 antagonists to inhibit tumor growth has clearly been demonstrated for lung and colon cancer. The role of muscarinic receptors to stimulate growth as well as metastasis has been shown for pancreatic, breast, ovarian, prostate and brain cancers, suggesting that M3 antagonists will also inhibit growth of these tumors as well. There are very few studies undertaken to understand the mechanistic approach for azaserine. Therefore, this study is undertaken to evaluate the same using in-silico approach.

II. METHODOLOGY

Retrieval of target enzyme structures

Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) is the most commonly used database for obtaining the 3D the structure of proteins (Rose et al, 2016; Usha et al, 2017). In the current study GFAT, GGT and M3 Muscarinic Acetylcholine of Homo sapiens origin, which is recognized as the target of azaserine was retrieved from PDB(2ZJ3), (4ZBK) and (4DAJ). The criteria for selection of the indicated structure were based on Resolution and R value as previously as reported by Middha et al, 2016.

Ligand retrieval

The Azaserine (CID: 460129) was retrieved from the PubChem database. The ligand molecule was subjected to initial screening of drug likeness using Lipinski filter (http://www.scfbio-iitd.res.in/software/drugdesign/ lipinski.jsp). The rule of 5 complies majorly on: molecular mass, cLogP, hydrogen acceptor and donor (Usha et al, 2017, Lipinski, 2004). The ligand accepting the rule of 5 were subjected to further analysis.

Prominent binding site prediction

The active site of the GFAT, GGT and M3 Muscarinic Acetylcholine was identified using cavity-based method from receptor cavities and coordinates were retrieved using binding pockets calculation using Discovery studio 3.5 software (Usha et al, 2017).

Docking

To initiate docking study, selected 3D structure of the retrieved ligands from PubChem database were in SDF format, first converted to PDB format, then optimization was continued using Discovery Studio 3.5. The receptors or target proteins (2ZJ3), (4ZBK) and (4DAJ): Structures of the GFAT, GGT and M3 Muscarinic Acetylcholine Receptor were prepared by removing he atoms, water molecules and CHARMm force field was applied using simulation tool/DS 3.5 (Lounnas et al, 2013). Virtually Screened compound after ADMET were subjected to docking process, LibDock is a kind of docking algorithm which finds hotspots (A group of polar and apolar probes) using grid placed into (-13.119X, 7.776Y, -49.41 Z) 3D direction of the receptor active site. Significantly, it uses hotspot to map and align the ligand conformations for favorable interactions, any poses of ligand which results bad clashes gets removed. Finally, pose optimization was done using Broyden-Fletcher-Goldfarb-Shanno (BFGS) and top scoring ligand poses are ranked and retained (Diller, 2001; Prashanthkumar et al., 2019).

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III. RESULTS AND DISCUSSION

HBP is a key pathway in response to oncogenic stress (Gitenay et al, 2014). Cancer progression is accompanied by increase in glucose and glutamine metabolism providing carbon and nitrogen required in downstream anabolic pathways. Stimulation of HBP in cancer cells regulates metabolism and redox potential which may be exploited to target cancer cells (Rahman et al, 2013). However, many nuclear and cytoplasmic proteins are dynamically modified by increased O-linked GlcNaC and UDPN-acetyl glucosamine. Azaserine binds to the GFAT protein and binding energy is equal to 1.03 indicative of a strong affinity and interaction between them, this results in metabolic inactivation and inhibition of GFAT. Gamma-glutamyl transpeptidase (GGT) belong to the N-terminal nucleophile hydrolase superfamily, enzymes that cleave the gamma glutamyl amide bond of glutathione to give cysteinyl-glycine. The released gamma-glutamyl group can be transferred to water (hydrolysis) or to amino acids or short peptides (transpeptidation). GGT plays a key role in the gamma-glutamyl cycle by regulating the cellular levels of the antioxidant molecule glutathione, hence it is a critical enzyme in maintaining cellular redox homeostasis. GGT is upregulated during inflammation and in several human tumors, Azaserine inhibits GGT irreversibly (Hsu, 1980), our results have shown that GGT is inhibited by azaserine, the docking results show a high binding energy of -2.0 which implies a strong interaction and degradation of the target molecule thereby inhibiting its activity. There are earlier reports of the structure of Muscarinic acetylcholine M3 receptors and indicated their presence in smooth muscles, endocrine glands, exocrine glands, lungs, pancreas and the brain. Cancers derived from epithelial and endothelial cells express muscarinic acetylcholine receptors (mAChR) and activation of the G protein linked muscarinic receptors (M1, M3 and M5) leads to increased cell proliferation. Cancer cells also secrete acetylcholine (ACh) which stimulates cell growth, thus ACh acts as an autocrine growth factor. In certain cancers that do not synthesize ACh, muscarinic receptor activation can be provided from neuronal or endocrine sources. It can also be due to the constitutive activity of muscarinic receptors. Constitutive activity is well established for G-protein coupled receptors in general (Kenakin,2004) and has been specifically demonstrated for the M3 muscarinic receptor (Casarosa et al, 2010). The ability of muscarinic activation to stimulate cancer growth clearly suggests that muscarinic antagonists will have the potential to inhibit cancer growth. 50% of pancreatic adenocarcinomas expressed choline acetyltransferase (Sekhon et al, 2002) Therefore, based on muscarinic receptor expression by pancreatic carcinomas, there is potential for autocrine stimulation. Proliferation is stimulated by several mechanisms. Activation of M3 receptors leads to increased intracellular calcium which in turn leads to activation of Akt and MAPK (Song et al, 2003; 2007). Effects of muscarinic antagonists on pancreatic carcinoma growth have not been studied much. In this context, our studies on mechanism of azaserine binding to the M3 receptors and further inhibition of M3 activity is the key to understand the role of M3 antagonists in cancer treatment.

Bioactivity of the molecules was predicated using molinspiration software (Figure 1). The smile structure of the molecule was found to be (C(C(=O)O)N)OC(=C[N+]#N)[O-].

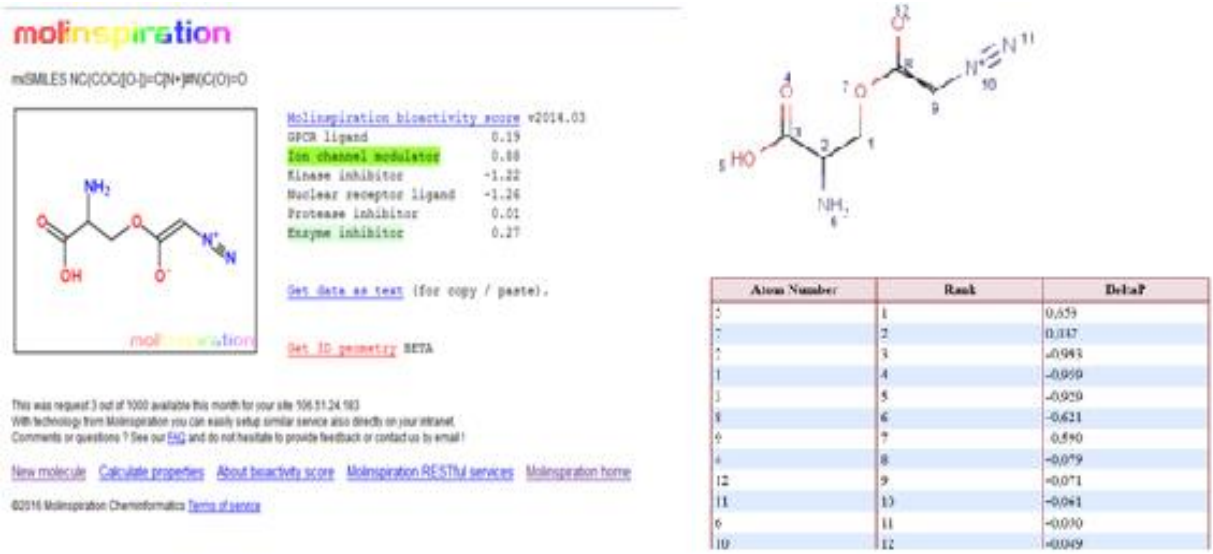

Fig 1: Activity of Azaserine.

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MOLINSPIRATION predictions show the drug to be an ion channel modulator

SOMP is a web-service for in silico prediction of site of metabolism (SOMP)

Prediction of sites of metabolism for drug-like compounds for (five major human) cytochrome P450s: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Also in the training set were included the sites of glucoronidation, catalyzed by UGT. A set of all possible SoLAs (structures with one labeled atom) with the appropriate LMNA descriptors is generated for a new compound under the prediction of sites of metabolism (SOMs). The results of prediction of SOMs for new compounds are created on the basis of the prediction results of all SoLAs generated for a compound (Fig.1).

SOMP metabolism predictions;

The drug gets metabolized at the mitochondria, the atoms susceptible include atom no 5 and 7 ranked as 1 and 2.

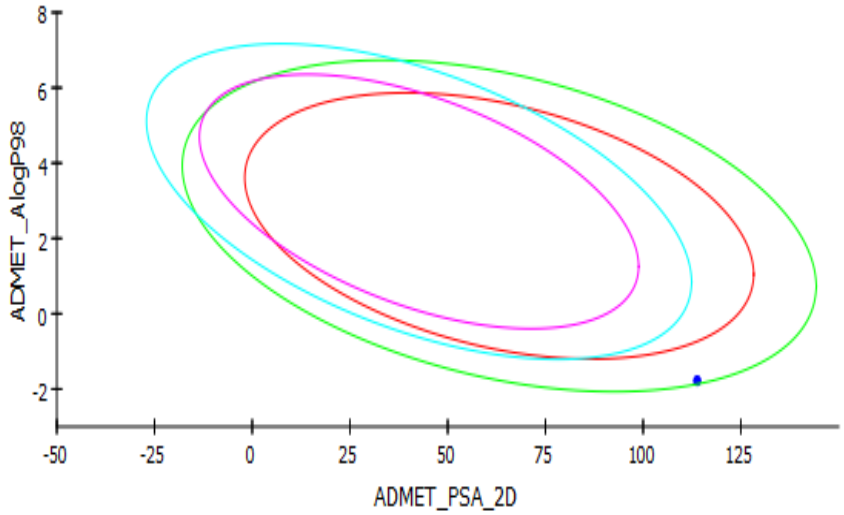

Fig 2. Standard levels of ADMET descriptors from Discovery studio

ADMET Descriptors, 2D polar surface area (PSA_2D) in A2 for each compound is plotted against their corresponding calculated atom-type partition coefficient (ALogP98). The area covered by the ellipse is a prophecy of excellent absorption without any violation of ADMET properties. Ellipses indicate the absorption model at 95% and 99% confidence limit to the Blood Brain Barrier (BBB) and Intestinal Absorption models (Fig 2, Table 1).

TABLE I: Standard levels of ADMET descriptors

Aq. Solubility & Drug Likeness		**BBB**		**CYP450**		**Hepatotoxicity**		**Intestinal Absorption**	
level	Intensity	Level	intensity	Level	Value	level	value	Level	Value
0	Extremely low	0	Very high	0	Non-inhibitor	0	Non-toxic	0	Good
1	No, very low	1	High	1	Inhibitor	1	toxic	1	moderate
2	Yes, low	2	Medium	**PPB**				2	Poor
3	Yes, good	3	Low	Level	% of binding			3	Very poor
5	No, too soluble			0	<90%				
				1	>90%				
6	Unknown			2	>95%				

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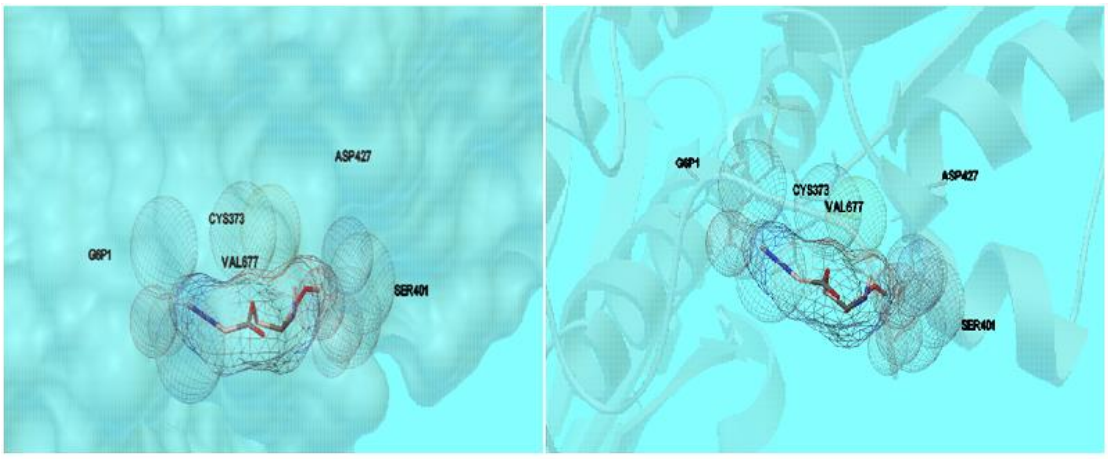
TABLE II : ADMET prediction

ADMET SOLUBILITY	0.523
Alog P98	1
BBB levels	4
CYP2D6	-7
ADMETCYPD6	False
Hepatotoxicity	-8.635
ADMETPSA2D	113.822
NTP	non carcinogenic

The standard levels of ADMET descriptors from Discovery studio shows the internalization of the drug is moderate at intestine and cannot enter blood brain barrier, its solubility being good the rate of degradation and elimination may be faster. The drug is predicted to be non-carcinogenic, non- mutant and non- toxic to liver (Table 2, 3)

TABLE III: Toxicity predictions using TOPKAT software

TOPKAT	non toxic
AMES	non mutagenic
TOLERENCE DOSE	0.551072g/kg body wt
LC50	0.603289mg/cm3/hr
CHRONIC TOXICITY	0.0266g/kg body wt
EFFECTIVE EC50	98.2884mg/L



GFAT (PDB ID : 2ZJ3) protein Interaction with Azaserine

Fig. 3 Interaction of GFAT protein (PDB: 2ZJ3) with Azaserine.

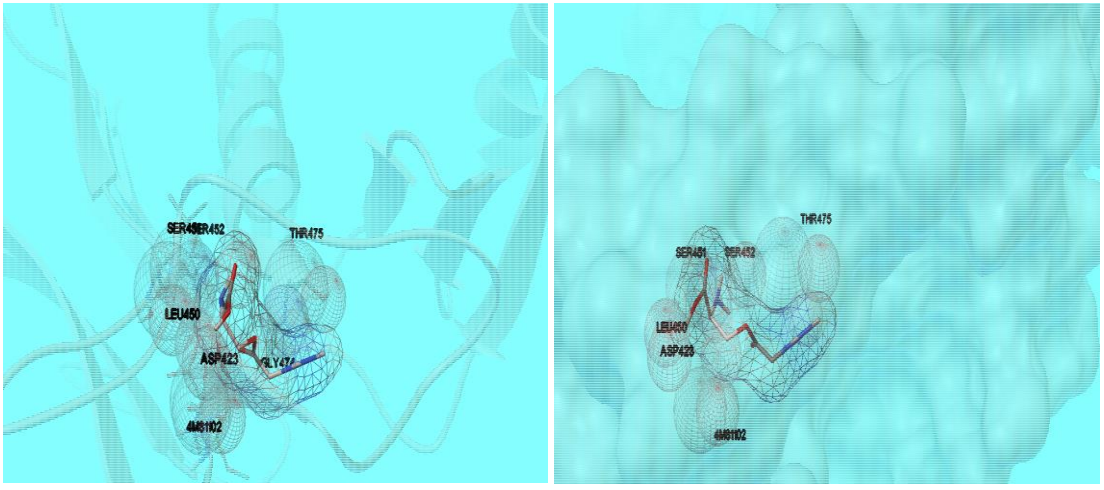


Fig 4: Interaction of GGT protein (PDB: 4ZBK) with Azaserine

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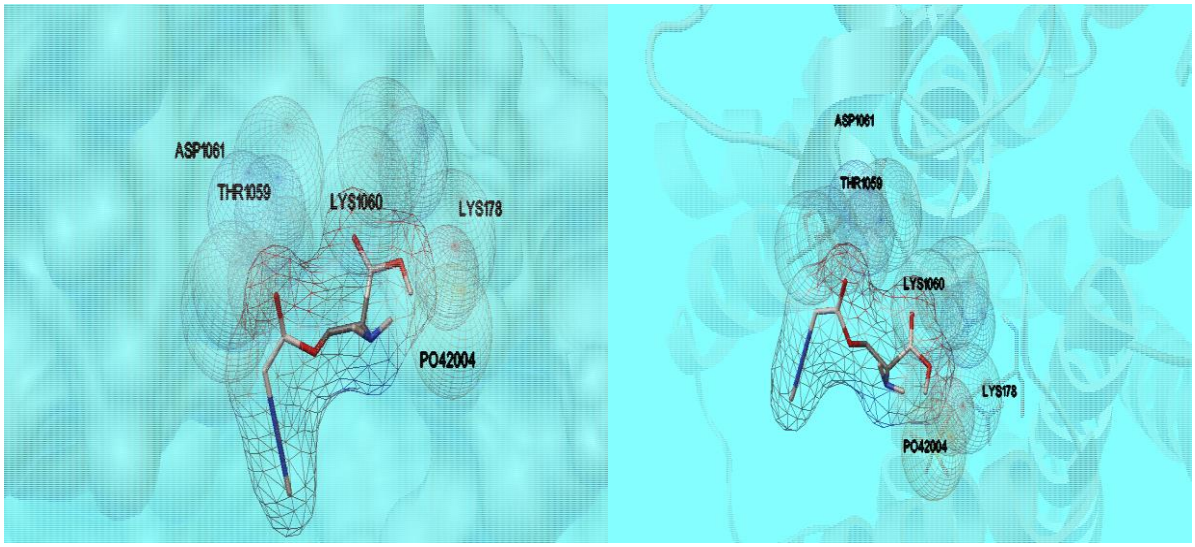

Fig 5: Interaction of M3 protein (PDB: 4DAJ) with Azaserine

IV. CONCLUSION

The present study gives an insight into the mechanism of azaserine as a drug in inhibition of target proteins viz GFAT, GGT and M3 receptors. Azaserine inhibits GFAT and GGT impairing metabolic pathways can be a mechanism to kill cancer cells however, Azaserine shows effective interaction with M3 receptor and acts as an antagonist to inhibit M3 aided cell proliferation in cancer cells, these requires further investigations.

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