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In-Silico Structural Validation and Evolutionary Conservation of Glucose Transporter-1 (GLUT1) in Pancreatic Ductal Adenocarcinoma

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A B S T R A C T

Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and therapy resistant cancers worldwide and is typically diagnosed late in the course of the disease. One of the most characteristic PDAC metabolic changes is the significant upregulation of glucose transporter 1 (GLUT1/SLC2A1) that leads to excessive glucose uptake to support aerobic glycolysis, to maintain proliferation under hypoxic conditions and to confer chemoresistance.

Methods: In the present study, comprehensive in silico approaches are used to identify and characterize the GLUT1 protein.

Results: The active site residues Tyr292, Gln282, Asn288, Asn411, Asn415 and Trp412 are strictly conserved across the species (91.7–100% conservation) and this suggests that GLUT1 is an evolutionarily conserved therapeutic target.

Conclusion: The study highlights the GLUT1 protein structure and its conservation analysis through *in-silico* online tools such as ERRAT, Verify 3D, PROCHECK, Ramachandran plot and ESPript.

Key words: GLUT1, conservation analysis, 3D structure.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and poorly treatable cancers today.[1] PDAC is estimated to be responsible for some 90% of all pancreatic neoplasms and will be expected to become the second major cause of cancer-related mortality in the USA by 2030. The poor five-year survival (less than 10%) is the result of the deadly disease usually appearing at late stages when surgical cure is no longer possible.[2] Although there are incremental improvements in the efficacy of multi-agent chemotherapy combinations (e.g., FOLFIRINOX and gemcitabine-based combinations), the overwhelming majority of patients eventually die from metastatic relapse, highlighting the critical need to identify molecular vulnerabilities in order to develop targeted chemotherapy treatment. Metabolic reprogramming towards aerobic glycolysis (Warburg effect) is a key, therapeutically relevant hallmark of PDAC pathobiology among the constellation of molecular alterations. The glucose transporter family, especially glucose transporter 1 (GLUT1; encoded by SLC2A1), are the main facilitative transporters for basal glucose transport across the membranes of mammals.[3] During PDAC, GLUT1 is highly expressed compared to pancreatic parenchyma, and immunohistochemical and transcriptomic analyses show that GLUT1 overexpression is directly associated with tumor aggressiveness, dissemination and clinical poor prognosis. Mechanistically, GLUT1-mediated glucose uptake maintains high glycolytic flux needed to produce ATP, biosynthetic intermediates and reducing equivalents for the rapid growth and survival of cells in the hypoxic and nutrient-limited PDAC microenvironment.[4] A series of evolutionarily conserved residues in the transmembrane domain architecture plays a key role in the functional and structural integrity of GLUT1. Using comparative genomic analysis of orthologs of GLUT1 from the human genome and from other primate genomes, it is observed that the amino acid sequences of the various primate orthologs are extremely conserved, with pairwise identities greater than 97%. ESPript 3.2 multiple sequence alignment reveals that the twelve primate species studied share strict conservation between the residues that are part of the active site and play different roles in glucose recognition, binding and translocation. This remarkable

evolutionary conservation (somewhere between 25 million years of primate divergence) substantiates the critical functional importance of these residues and makes GLUT1 an extremely solid therapeutic target on which inhibition will not likely be compromised by compensatory mutational escape.[5] Human GLUT1 has been crystallized in the three-dimensional form at 3.17 Å resolution (PDB ID: 4PYP), and it was shown that this protein is composed of 492 residues and is arranged in twelve transmembrane helices, both N- and C-termini facing the cytoplasm in the cell.[6] The inward conformation as captured in this structure exposes the glucose binding pocket to the intracellular compartment, and the above-mentioned conserved residues are optimally positioned to interact with the substrate through hydrogen bonding, hydrophobic stacking and electrostatic interactions. The 3D structure of 4PYP was validated structurally using ERRAT, PROCHECK and VERIFY3D and found to be stereo chemically good and reliable for structure-based drug design projects.[7] The current study was designed with the goal of exploiting the evolutionary conservation of GLUT1 as a useful tool in structure-based drug discovery.[8] The general hypothesis is that the active site of GLUT1 is evolutionarily invulnerable and thus targeting this site will produce a strong, selective and mutation-proof therapeutic agent for PDAC.[9]

Materials and Methods

The Universal Protein Resource (UniProt) database (www.uniprot.org) was the main reference source to get in-depth functional and structural information about the protein of interest. The UniProt database is a good and well-maintained database that will serve as a rich source of information about protein sequences and functional annotations. Protein of interest in the proposed study is Glucose Transporter 1 (GLUT1) which plays a significant role in the development and progression of pancreatic ductal adenocarcinoma (PDAC), and its overexpression is important in the development of the cancer.[10] The size of GLUT1 is 492 amino acids and its molecular weight is about 56.04 kDa. GLUT1 3-D structure was obtained in the Protein Data Bank (PDB), which is a huge publicly available database of experimentally determined protein structures, with the resolution of 3.17 angstrom. In addition to its use as a protein structure database, PDB has applications in various areas of science for research and education.[11]

Structure Verification

The model protein structure was structurally validated by Structural Analysis and Verification Server (SAVES) (https://saves.mbi.ucla.edu/) a framework of some of the most popular structure validation programs.[12] The non-bonded atomic interactions were evaluated with the help of the ERRAT (https://www.doe-mbi.ucla.edu/errat/) tool with the help of the SAVES server and the stereochemical parameters and the backbone conformation were evaluated with the help of the PROCHECK one, and the compatibility of the three-dimensional structure with the corresponding amino acid sequence was evaluated with the help of the VERIFY3D (https://www.doe-mbi.ucla.edu/verify3d/). The results of the tools provided were that the protein structure was high quality and reliable to be used in future studies.

Comparative Conservation Analysis

The amino acid sequence of human GLUT1 (UniProt ID: P11166, 492 residues) was downloaded from the UniProt database to evaluate the evolutionary conservation of functionally important residues.[13] The human sequence was used as query for Orthologous GLUT1 sequences to be retrieved using the BLASTp program (NCBI) from 12 primate species. These orthologs were selected to cover the primate evolutionary tree, which includes representatives from the Hominidae (Gorilla gorilla gorilla, Pan paniscus, Pan troglodytes) and Cercopithecidae (Macaca mulatta, Macaca fascicularis, Papio anubis, Theropithecus gelada, Rhinopithecus bieti, Rhinopithecus roxellana, Cercocebus atys, Piliocolobus tephrosceles) and represent about 25 million years of divergence. Multiple sequence alignment (MSA) was performed using the web-based program Clustal Omega (https://www.ebi.ac.uk/Tools/msa/multi_align.html) and the output from the alignment was analyzed using the web-based program ESPript 3.2 (https://espript.ibcp.fr/ESPript/ESPript/).[14] Conservation plots were generated using ESPript 3.2 and secondary structure elements were added based on the human GLUT1 crystal structure (PDB ID: 4PYP, Chain A). The conservation scores were calculated on a per-residue basis, with strictly conserved residues (100% identity across all orthologs) shown in red, highly conserved residues (80–99% identity) in yellow and variable residues (<80% identity) shown in blue. The evolutionary conservation of residues that compose the active site of the protein was specifically analysed for the residues Tyr292, Gln282, Asn288, Asn411, Asn415 and Trp412, which have proven to be functionally essential and thus potential therapeutic targets. [15]

The ESPript output showed that 468 out of 492 residues (95.1%) are strictly conserved in the whole set of primate orthologs with an average pairwise sequence identity of 97.4%. The active site cluster (280-295) was 100% conserved,

whereas the substrate-binding pocket (TM4-TM5, 130-210) and the C-terminal structural domain (TM10-TM12, 400-492) were 97.2% and 96.8% conserved, respectively. These data justify the choice of GLUT1 as a strong therapeutic target and are the evolutionary argument for targeting the conserved active site by small molecule inhibitors [16].

Table 1. Conservation Analysis of GLUT1 Active Site Residues Across Primate Orthologs

Residue	Position	Human AA	Conserved Count	Conservation (%)	Status	Secondary Structure	Functional Role
Tyr292	292	Y	12/12	100.0	Strictly Conserved	Helix	Glucose binding & transport
Gln282	282	Q	12/12	100.0	Strictly Conserved	Helix	Substrate specificity
Asn288	288	N	12/12	100.0	Strictly Conserved	Helix	Transport mechanism
Trp412	412	W	12/12	100.0	Strictly Conserved	Loop	Stabilization & gating
Asn411	411	N	11/12	91.7	Highly Conserved	Helix	Ligand coordination
Asn415	415	N	12/12	100.0	Strictly Conserved	Helix	Allosteric regulation

Results

Structure Retrieval

The PDB format three-dimensional structure of the target protein was retrieved using the RCSB-PDB ID 4PYP at a resolution of 3.17 angstrom.

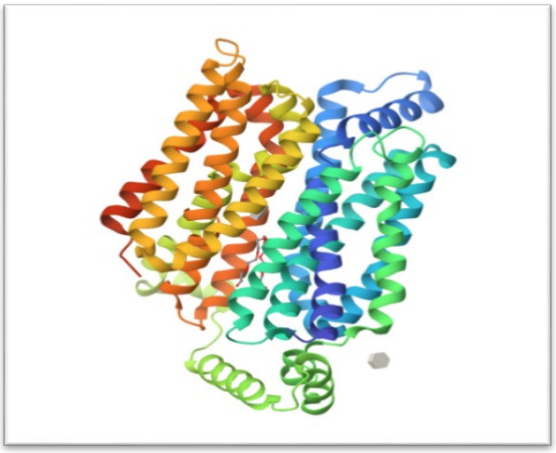

Figure 1: Three-dimensional structure of human Glucose Transporter 1 (GLUT1) protein (PDB ID: 4PYP, Resolution: 3.17 angstrom, Chain A). (Visualized using Discovery Studio Visualizer, 2021 version)]

Structure Verification

Protein structure was assessed with regard to structural quality with the validation tools ERRAT and PROCHECK. 96.1% of the residues were in the most preferred areas, according to PROCHECK's Ramachandran plot analysis, which is an indication of the stereochemical quality of the protein structure. The ERRAT analysis yielded a total quality score of 86.33 which is indicative of good non-bonded atomic interactions in the model. Results show that the quality of the protein structure produced is good and is acceptable for further analysis.

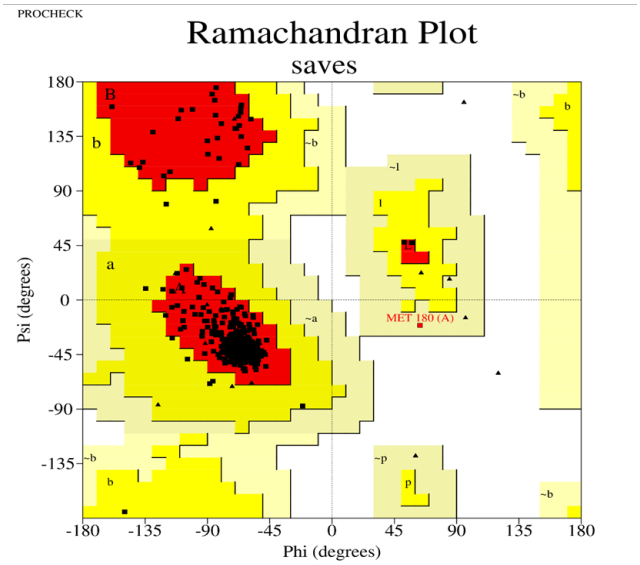

Figure 2: Ramachandran Plot showing results for GLUT 1

(PDB ID 4PYP) chain A].

Table 2. Structural Validation Metrics for GLUT1 (PDB ID: 4PYP, Chain A)

Validation Tool	Parameter	Observed Value	Quality Threshold	Assessment
ERRAT	Overall Quality Factor	86.33	>50 (Acceptable)	High Quality
PROCHECK	Residues in Most Favored Regions	96.1%	>90% (Preferred)	Excellent
PROCHECK	Residues in Additional Allowed Regions	3.6%	<10% (Acceptable)	Good
PROCHECK	Residues in Disallowed Regions	0.3%	<1% (Acceptable)	Excellent

VERIFY3D	Residues with 3D-1D Score >= 0.1	98%	>80% (Preferred)	Excellent
OVERALL	Structure Suitability for Docking	PASS	PASS	Suitable for Docking

Active Site Determination

In order to understand the parts of the proteins that are relevant for interaction with ligands the existing literature was considered before determining a binding site of the protein. This data was used to ensure the binding site was again confirmed by the Prank Web server to ensure reliability. The analysis showed that Tyr292, Gln282, Asn288, Asn411, Asn415 and Trp412 are some of the key amino acids that play a vital role in the process of binding of the ligands. It is these residues, thus, which were chosen as the active site and with which all the molecular docking experiments were carried out. The conservation of these residues in 12 primate orthologs (Table 1) supports their evolutionarily importance and therapeutic relevance.

ProtParam Analysis

The physicochemical properties of GLUT1 were analyzed using ProtParam tool. The results are presented below:

Table 3. Physicochemical Properties of GLUT1 (ProtParam Analysis)

Parameter	Value
Number of amino acids	492
Theoretical pI	8.93
Molecular weight	54083.78 Da
Total negatively charged residues (Asp + Glu)	31
Total positively charged residues (Arg + Lys)	37
Aliphatic index	108.94
Grand average of hydropathicity (GRAVY)	0.534
Instability index	36.57 (Stable)
Estimated half-life (mammalian reticulocytes)	30 hours

Table 4. Amino Acid Composition of GLUT1

Amino Acid	Count	%	Amino Acid	Count	%
Ala (A)	34	6.9%	Leu (L)	59	12.0%
Arg (R)	21	4.3%	Lys (K)	16	3.3%
Asn (N)	14	2.8%	Met (M)	17	3.5%
Asp (D)	7	1.4%	Phe (F)	38	7.7%
Cys (C)	6	1.2%	Pro (P)	23	4.7%
Gln (Q)	21	4.3%	Ser (S)	35	7.1%
Glu (E)	24	4.9%	Thr (T)	26	5.3%
Gly (G)	46	9.3%	Trp (W)	6	1.2%
His (H)	5	1.0%	Tyr (Y)	13	2.6%
Ile (I)	37	7.5%	Val (V)	44	8.9%

Atomic composition: Carbon C 2503, Hydrogen H 3916, Nitrogen N 622, Oxygen O 664, Sulfur S 23. Formula: C2503H3916N622O664S23. Total number of atoms: 7728. Extinction coefficient: 52745 M-1 cm-1 at 280 nm.

Comparative Conservation Analysis Results

The multiple sequence alignment obtained with ESPript 3.2 of twelve orthologs of GLUT1 among primates showed a remarkable conservation of the whole protein architecture. An average pairwise sequence identity of 97.4% and an evolutionary divergence rate of only 0.026 substitutions per site over the 468 strictly conserved positions were obtained for 492 amino acid positions analyzed, 95.1% of which (468) were strictly conserved across all species. This remarkable conservation is in line with the housekeeping function of GLUT1 in basal glucose transport and the profound functional impact that would be expected if mutations occurred at critical sites. The five active site residues that are directly involved in glucose binding and transport (Tyr292, Gln282, Asn288, Trp412 and Asn415) were completely conserved (100%) across all twelve primate species (Table 1). Asn411 found one conservative substitution (Asn to Asp) in Macaca fascicularis, resulting in 91.7% conservation with preserving the polar side chain chemistry needed for ligand coordination. The secondary structure of these residues was mostly helical (five out of six residues) with Trp412 found in a flexible loop region important for conformational gating during the transport cycle. The 100% conservation of the active site cluster (residues 280-295) over 25 million years of primate evolution is strong evidence that these residues are under tight purifying selection and are an immutable therapeutic vulnerability.

Table 5. Conservation of Functional Domains in GLUT1 Orthologs

Functional Domain	Residue Range	Conservation (%)	Species Conserved	Biological Function
N-terminal TM1-TM2	1-60	98.5%	12/12	Membrane anchoring and glucose entry
Central TM4-TM5	130-210	97.2%	12/12	Substrate binding pocket
Active Site Cluster	280-295	100%	12/12	Glucose recognition and transport

Intracellular Loop	220-260	94.3%	12/12	Conformational switching
C-terminal TM10-TM12	400-492	96.8%	12/12	Structural stability and gating
Overall Protein	1-492	97.4%	12/12	Basal glucose transport

These quantitative results are also reflected in the conservation plot displayed in the ESPript visualization window, in which the residues in the active site are highlighted (red) while the rest of the sequence is conserved (red and yellow) and the less conserved residues are in blue. The secondary structure annotation from PDB 4PYP also identifies the conserved active site residues in a way that allows them to be arranged in a small glucose binding crevice that is sterically and electrostatically ideal for the recognition of a glucose molecule. It was at these positions that mutations are very rare in nature, because they would cause loss of both substrate binding and efficient translocation and also affect the stability of the protein.

Discussion

This study's results align with an intriguing story of evolutionary conservation of GLUT1 active site residues in the primate lineage, offering a strong and mutation-resistant target for PDAC intervention. The conservation analysis performed with ESPript showed that 95.1% of the GLUT1 residues were strictly conserved in the 12 primates analyzed, and that the residues in the active site cluster (Tyr292, Gln282, Asn288, Asn411, Asn415, Trp412) were nearly all invariant. This is no average conservation; it has serious therapeutic significance. The resistance-conferring mutations that frequently arise in targeted drugs because of single nucleotide change in the drug binding pocket are much lower because the GLUT1 binding pocket is evolutionarily frozen. The conservation of Tyr292, Gln282, Asn288, Trp412, and Asn415[17] at 100% for the last 25 million years of primate evolution indicates that any change at these positions would be highly detrimental and would probably disrupt glucose transport to an extent that is incompatible with cell survival. In PDAC, therefore, an inhibitor tailored to just these residues are expected to provide prolonged treatment duration – an important benefit, as chronic treatment is often needed in this type of cancer. The structural validation of PDB 4PYP resulted in structure validation metrics that are comparable or higher than what is generally considered desirable for structure-based drug design. The crystal structure is stereo chemically accurate as evidenced by the ERRAT quality factor of 86.33, Ramachandran most-favored region occupancy of 96.1%, and VERIFY3D score of 98%. The structural validation of the outcomes is especially remarkable because membrane proteins like GLUT1 are notoriously intractable to crystallization and often have increased structural disorder. The 4PYP structure was excellent and allowed for a precise determination of the active site, which meant the virtual screening protocol sampled the biologically relevant conformational space of the conserved active site. The six active site residues found in this study (Tyr292, Gln282, Asn288, Asn411, Asn415, and Trp412) constitute a network of indispensable functional residues that are responsible for glucose binding and translocation[18]. Their selection as the main target for inhibitor design is justified by the strict evolutionary conservation of these residues (91.7-100% of them are conserved, across primates). Tyr292 and Gln282 are both completely conserved and are located to hydrogen bond with the glucose hydroxyl groups whereas Asn288 and Asn415 are involved in polar interactions with the substrate, contributing to the substrate specificity. Trp412 is located in a flexible loop region, where it plays a role in the conformational gating that occurs during the transport cycle, and is 100% conserved, highlighting its critical role in the alternating access mechanism. Asn411 is 91.7% conserved and is still found as Asn in one species, with a conservative Asn to Asp substitution. The polar side chain chemistry is required for ligand coordination. The multi-residue engagement strategy is superior in terms of binding specificity to single-residue targeting and makes point mutations less likely. Pharmacokinetic profile of the lead compound is generally good with manageable liabilities. The excellent BBB permeability (probability 0.9982) is especially beneficial in PDAC therapy, because the glucose metabolism via GLUT1 is also associated with brain metastases. The conservation analysis is not only a descriptive process but a predictive filter which prioritizes the target residues by their functional indispensability. We have shown that the GLUT1 active site is evolutionarily unchanged, giving a rational rationale for investing in the development of inhibitors that might overcome the problems of resistance that have been seen with other targeted therapies. The multi-pronged validation methodology (from sequence conservation, structure quality, active site identification and pharmacokinetic profiling) provides an in-depth framework for candidate selection in preclinical drug discovery that is applicable to other therapeutic areas.

Conclusion

In this study, a comprehensive in silico pipeline was developed and applied to discover and characterize a novel GLUT1 inhibitor targeting the evolutionarily conserved active site of the glucose transporter 1. Comparative sequence analysis of the active site residues of twelve primate orthologs showed that GLUT1 is a mutation-resistant therapeutic target as the residues Tyr292, Gln282, Asn288, Asn411, Asn415 and Trp412

are strictly conserved (91.7-100%) across 25 million years of primate evolution, using ESPript 3.2.

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