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Domain Analysis and Structural characterization of Bromodomain 8 (BRD8) - Functional Implications and Importance as a Drug Target in Cancer Research

Ambili Savithri[1], Sindhu Rani J.A.[2], Asha S. Kumar[3], Anila L.[2], Maya Madhavan[4], Sabeena Mustafa[5] and Manju L.[6*]

[1]Assistant Professor, Department of Biochemistry, Sree Narayana College, Kollam (Kerala), India.
[2]Associate Professor, Department of Biochemistry, NSS College, Nilamel, Kollam (Kerala), India.
[3]Associate Professor, Department of Chemistry, Sree Narayana College, Chempazhanthy, Thiruvananthapuram (Kerala), India.
[4]Associate Professor, Department of Biochemistry, Government College for Women, Thiruvananthapuram (Kerala), India.
[5]Bioinformatician, Department of AI & Bioinformatics, King Abdullah International Medical Research Center (KAIMRC), King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), Ministry of National Guard Health Affairs (MNGHA), Riyadh, Kingdom of Saudi Arabia.
[6]Assistant Professor, Department of Botany and Biotechnology, St. Xavier's College, Thumba, Thiruvananthapuram (Kerala), India.

(Corresponding author: Manju L.)*
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ABSTRACT: Bromodomains (BRDs) are readers that bind to acetylated lysine residues in chromatin and regulate gene expression. The crystal structure of BRD8 is not elucidated yet. As BRDs play a significant role in cancer, understanding their functional implications is important for drug discovery. The theoretical model is the only reliable technique when there is no crystal structure available. We have investigated the primary and secondary structures of BRD8 (UniProt Id: Q9H0E9|) using the ExPASy (the Expert Protein Analysis System), ProtParam and SOPMA. A model of BRD8 was determined using the template (PDB Id: 3S91) and BLASTP. The stereochemical quality of the model was validated using Ramachandran Plot (97.1% residues in the most favorable region) and ProSA-web (Z score of -5.79). PTM studies show that there are 10 functional sites present in BRD8, of which Protein kinase C and Casein Kinase II sites were abundant. Protein kinase C controls the signaling pathways in proliferation, tumorigenesis and metastasis, whereas Casein Kinase II regulates apoptosis and cell cycle in cancers. We identified three binding pockets in the model. The results from this study show the relevance of BRD8 in cancer research. Functional and structural research can help provide the basis for further studies, which is significant for the pharmaceutical industry as a whole.

Keywords: Bromodomain, BRD8, Molecular modeling, Post-translational modifications, Rampage server, SOPMA, cancer.

INTRODUCTION

Bromodomains (BRDs) are a family of evolutionarily conserved motifs identified for the first time in the early 1990s in the *brahma* gene of *Drosophila melanogaster* (Tamkun *et al*., 1992). BRDs bind the acetylated lysines in histone tails, the recognition of the acetyl group being decisive for the recruitment of other chromatin factors and transcriptional machinery, and thereby regulating gene transcription. The bromodomain (BRD) is a conserved 110 amino acid structural motif composed of four α-helices (αZ, αA, αB, and αC) that comprise a left-handed bundle and forms a central hydrophobic pocket (Mujtaba *et al*., 2007). A detailed look at all the available BRD structures reveal that they all contain the characteristic left-handed bundle so formed by the four α-helices which is termed the BRD fold (Zeng *et al*., 2002). Two loop regions (ZA and BC), the diversity of which make the overall sequence similarity between bromodomain modules low, connect the α-helices and form a surface that interacts with acetylated lysines in nucleosomal histones (Dhalluin *et al*., 1999). The reading of such acetylation on histones by BRD proteins is a key event in transcriptional activation. Studies show that, proteins which contain BRDs are often deregulated in cancer. Structure-based alignments have classified human BRDs into eight families. Among this, bromodomain8 (BRD8) has been identified as one of the potential drug targets in colorectal cancer in recent years. Small molecule inhibitors targeting bromodomains can compete for binding to acetylated histones. In humans, 61 BRDs have been identified so far which are found to

be present in 46 diverse multi-domain proteins that regulate transcription (Zaware *et al*., 2019). The bromodomain proteins cluster into eight major BRD families (I–VIII) (Filippakopoulos *et al*., 2012)with BET proteins being the foremost studied group. The Bromo- and Extra-Terminal domain (BET) family belonging to subfamily II of BRDs includes bromodomain containing proteins BRD2, BRD3, BRD4 and the testis-specific BRDT which act as epigenetic readers and are characterized by two tandem N-terminal BRD regions (BD1 and BD2) followed by an extra terminal domain (ET) and a C-terminal domain (CTD).

Epigenetic mechanisms, especially DNA methylation and histone modifications, are dynamic processes that regulate the gene expression transcriptional program in normal and diseased states. BET proteins are located in the nucleus and regulate many cellular activities including gene transcription, DNA replication, cell-cycle progression, and therefore, participate in tumor development, infections, autoimmunity and inflammation. The most comprehensively characterized BET member is BRD4 which was identified in 1988 through studies on mammals as a co-activator protein involved in gene transcription(Bo Huang *et al*., 2009). Both BRD4 and BRD2 play an important role in transcription elongation by recruiting the positive transcription elongation factor complex (p-TEFb) through its BRDs to acetylated chromatin(Yang *et al*., 2005). BET proteins are highly involved in cancer, directly regulating the expression of certain cancer related genes, such as *c-MYC* (Delmore *et al*., 2011) and *NF*-κβ-dependent genes (Zou *et al*., 2014). Several HATs also contain bromodomains that are simultaneously "writers" and "readers" of acetyl groups such as the p300/CBP-associated factor PCAF (also known as KAT2B) that acetylates histones H3 and H4 (Schiltz *et al*., 1999) regulating the expression of several genes like insulin and some transcription factors including p53. Recently, BET proteins are also found to regulate melanocyte differentiation through interactions with MITF (Trivedi *et al*., 2020). The first identified inhibitors of the BET bromodomain family are (+)-JQ1 reported by the Structural Genomics Consortium (SGC) and the Dana-Faber Cancer Institute (Filippakopoulos *et al*., 2010) and I-BET762 reported by GlaxoSmithKline (GSK) (Mirguet *et al*., 2013; Nicodeme *et al*., 2010). Alarge number of studies are showing the efficacy of JQ1 in many cancers such as glioblastoma,(Cheng *et al*., 2013) hepatocellular carcinoma (Hong *et al*., 2016; Li *et al*., 2016), colon cancer (Hu *et al*., 2015), lung cancer (Shimamura *et al*., 2013) and breast cancer (Pérez-Salvia *et al*., 2017).

Apart from cancer, bromodomains are key transcriptional regulators in diabetes (Fu *et al*., 2014 , Nicholas, Andrieu, Strissel, Nikolajczyk, & Denis, 2017), inflammation (Maksylewicz *et al*., 2019)and cardiovascular diseases (Schooling *et al*., 2019) and are considered potentially druggable to treat these disorders. New data support the novel hypothesis that abnormalities in signalling through BRD2 or other bromodomain proteins underlie human predisposition to elevated body mass index and altered insulin sensitivity in adults. Despite the conservation of the overall BRD fold, the surface and loop regions of BRDs are highly diverse, suggesting that inhibitors with high specificity can be designed (Filippakopoulos *et al*., 2012). This feature, along with the involvement of BET proteins in the pathology of a wide range of diseases makes BRDs attractive targets for the design of pharmacologically active molecules that compete with protein interactions mediated by these modules.

Even though recent studies have highlighted the role of BRDs in various biological processes and their association with disease, the functions of many human BRD proteins, such as BRD8, are not well characterized. It is found that, acetylated proteins including histones, provides molecular signals which are bound by acetyl-lysine binders such as bromodomain (BRD) proteins (Chiu *et al*., 2017). The human BRD8 gene is expressed predominantly as two main isoforms. When compared to isoform 1 (102.8 kDa), isoform 2 is larger (135.4 kDa). Both isoforms are subunits of the p400/Tip60 chromatin remodeler/Histone Acetyl Transferase (HAT) complex comprising at least of 16 subunits, including p400 and Tip60 (Doyon *et al*., 2004). BRD8 appears to be involved in the regulation of cancer cell proliferation and the response to chemotherapeutic compounds, which destabilize the cytoskeleton or impede proteosomal function (Yamada *et al*., 2009). BRD8 is a potential chemosensitizing target for spindle poisons in colorectal cancer therapy (Yamada *et al*., 2009). It has also been found that cellular depletion of BRD8 leads to maintenance of genome stability through a mechanism of p53 dependent apoptosis (Lashgari *et al*., 2018).

Bromodomain and extra-terminal inhibitors (BETi) have emerged as a class of inhibitors in order to facilitate the development of anticancer compounds. Responses to hypoxia are implicated in triple negative breast cancer (TNBC). In a research, Motta *et al* showed that, hypoxia-induced genes were modulated by the inhibitor, JQ1 and showed anti-tumor activity (da Motta *et al*., 2017). Their work put forth a model which explains the role of BETi and epigenetics studies in cancer. This study also indicated inhibited xenograft vascularization in invitro and *in vivo* in TNBC cases.

Because the mechanism of epigenetic reprogramming is the main component in breast cancer progression and metastasis, the epigenetic readers are considered a bonafide oncogenic feature. The activation of these readers remains poorly understood in a wide variety of epigenetic pathway driven cancers (Flavahan *et al*., 2017). Bromodomain protein ZMYND8 interacts with HIF-1alpha and HIF-2alpha can carry out enhancing signaling mechanism by increasing recruitment of BRD4 and release of RNA polymerase II in breast cancer cells. It is found that HIF activation occurs upon acetylation of ZMYND8 at lysines 1007 and 1034 by p300 in breast cancer progression and metastasis (Chen *et al*., 2018).

The availability of structural model of a protein is one of the keys for understanding biological processes at a molecular level. However, very little is known about

the structure and role of BRD8 proteins. Generally, two techniques- X-ray crystallography and NMR (Nuclear Magnetic Resonance) are used for the identification of the three-dimensional structure of a protein, both of which are time-consuming and expensive. In this scenario, bioinformatics resources and databases can be utilised. Protein secondary structure prediction is an important area in bioinformatics. Machine learning based algorithms have proved to generate structures of various proteins so far. In this regard, available approach should be followed to predict and validate the *in silico* 3D structure of proteins based on computational methods (Dorn *et al*., 2014). A protein's 3D structure can be predicted based on its amino acid composition, a method known as structure prediction. Bioinformatics based sequence similarity searches, multiple sequence alignments; template identification etc. can be used to predict protein structure using bioinformatics. The development of data bases, identification, and validation of biomarkers that aid in identifying phenotypes for early disease diagnosis, as well as monitoring of the development of the disease and how well it responds to treatment, as well as predictors for improving of patient quality of life, all depend largely on bioinformatics methods (Beg *et al*., 2021; Piyusha Sharma, 2022; Wu *et al*., 2012). Data curation using a bioinformatics data base technique is currently used to pinpoint possible targeted candidate proteins for breast cancer studies Data curation using a bioinformatics data base technique is currently used to pinpoint possible targeted candidate proteins for breast cancer studies (Piyusha Sharma, 2022b). In this paper, we present the homology model of BRD8 protein using bioinformatics analysis. Homology or comparative modeling is known to be one of the best and extensively used methods where alignment of known protein structures with minimum 35 % similarity will be selected as templates to develop the theoretical models (Fiser, 2010). Recent studies show that bromodomain BRD8 is having a role in interaction with histone H4 or transcriptional regulation and also for the protein stability illustrating the importance of bromodomain as a therapeutic target (Fujisawa *et al*., 2017; Yamaguchi *et al*., 2023; Yu *et al*., 2020).

MATERIALS AND METHODS

Sequence retrieval and primary analysis. As first step, the query sequence of BRD8(accession id: Q9H0E9) consisting of 1235amino acid residues was retrieved from Uniprot KB database (The UniProt Consortium, 2016). In the second step, the physicochemical properties of the query, Q9H0E9 were calculated using ExpasyProtParam tool. ProtParam in Expasy Proteomics Server computes various physicochemical properties of protein sequences (Gasteiger *et al*., 2003). We computed properties such as theoretical Isoelectric Point (pI), Molecular weight, Total number of positive and negative residues, Atomic Composition, Extinction Coefficient, Instability Index, Aliphatic Index and Grand Average Hydropathy (GRAVY).

Secondary Structure analysis. Self-optimized prediction method (SOPM) helps to improve the success rate in predicting the secondary structure of proteins. So, we have selected Expasy SOPMA server for this purpose. We have submitted the query protein sequence in FASTA format to the SOPMA server. SOPMA is fast and reliable in order to predict secondary structure of proteins (Geourjon *et al*., 1995) based on the homology method. SOPMA generates secondary structure components of sequences such as percentage of α-helix, β-sheets, turns, random coils and extended strands.

Post translational modification (PTM) studies. BRD8 sequence was analyzed in PROSITE server to determine the possible Hits of PTM and domain architecture. This step is essential to provide additional information about functionally or structurally critical amino acid residues. PROSITE is used for the sequence annotation of domain features of UniProtKB/Swiss-Prot entries (Sigrist *et al*., 2010).

Molecular modeling and validation studies. Molecular modelling study of BRD8 sequence was carried out using SWISS-MODEL web server. The query sequence was submitted to the SWISS-MODEL online server, which is a completely automated server accessible via the ExPASy webserver, or from the program Deep View (Swiss PDB-Viewer) (Gasteiger *et al*., 2003; Guex *et al*., 1997). The theoretical model of BRD8 was subjected to Rampage web server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) to analyse its stability and reliability. The correctness of backbone conformation of the model is based on the feature of Psi and Phi angle orientation (Lovell *et al*., 2003). The overall quality score of the model was further calculated by ProSA-Web program (Protein Structure Analysis) Web server. ProSA-Web is a tool used to evaluate 3D models of protein structures to detect any possible errors (Wiederstein *et al*., 2007).

Active Site analysis. In order to determine the role of predicted model in ligand binding studies by docking, active site prediction was performed. The validated model was submitted to the online server, Metapocket(https://projects.biotec.tu-dresden.de/metapocket/) and analysed the results.

RESULTS AND DISCUSSION

This section describes the results of the analysis of BRD8 by bioinformatics approaches. In general, bioinformatics prediction methods obtain information on amino acid conservation through alignment with homologous and distantly related sequences (Pearson, 2013; Trembley *et al*., 2009). The physicochemical characteristics of proteins, including molecule size, net charge, amino acid content, and structure, are some of the components that affect the way they function. Various researches successfully investigate the physical and chemical characteristics of proteins, which has contributed significantly to the functional characterisation studies of proteins (Ghandehari *et al*., 2015; Roy *et al*., 2011; Ubaid *et al*., 2022). Our hypothesis is based on the fact that bioinformatics and drug discovery studies play an important role in

determining and predicting potential drug compounds which can be developed into novel therapeutic components. Computational and molecular biology techniques help to understand the molecular basis of cancer progression and treatment responses which facilitates the development of effective therapeutics (Dimitrakopoulos *et al*., 2017). In order to accomplish our goal of understanding the role of BRD8 as a druggable target in cancer, we started the analysis from its sequence level. Thus, we propose a method to construct a suitable model for query sequence of BRD8 and its applicability in drug designing studies. Since no crystal structure information for BRD8 is available till now, our aim was to construct and validate a model for BRD8. The most common criteria considered in many bioinformatics programs for predicting the functional effect of an amino acid substitution are amino acid sequence conservation across multiple species, physicochemical properties of the amino acids involved, database annotations, and potential protein structural changes (Seifi *et al*., 2018). The physicochemical properties of BRD8 protein are summarised in Table 1.

Table 1: Physico-chemical properties of BRD8 sequence.

Sr. No.	Primary structure component	Value
1.	Number of amino acids	1235
2.	Molecular weight:	135335.83
	Theoretical pI:	4.52
3.	Total number of negatively charged residues (Asp + Glu):	226
4.	Total number of positively charged residues (Arg + Lys):	112
5.	Atomic composition	
	Carbon C	5856
	Hydrogen H	9291
	Nitrogen N	1597
	Oxygen O	1975
	Sulfur S	52
6.	Formula:	$C_{5856}H_{9291}N_{1597}O_{1975}S_{52}$
7.	Total number of atoms:	18771
8.	Ext. coefficient	89225
9.	The instability index	56.02
10.	N terminal residue	MET
11.	The estimated half-life	30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).
12.	Aliphatic index:	75.26
13.	Grand average of hydropathicity (GRAVY):	0.532

Our results show that it is possible to use the sequence based data of proteins to yield important insights to understand the various mechanisms involved in cancer. As described in Table 1, the primary sequence analysis results of BRD8 protein suggested that the average molecular weight was135335.83 Da. The extinction coefficient of BRD8 protein was 89225. Total number of negatively charged residues was found to be 226. The computed isoelectric point (pI) was determined as 4.52 which indicates it is an acidic protein. As discussed in Table.1, the instability index value for the BRD8 protein showed as 56.02. If instability index value is below 40 then the protein is predicted as stable and if the value is above 40 it may exist as unstable (Idicula-Thomas *et al*., 2005). Therefore, BRD8 was found to be unstable. In primary analysis, it is found that N-terminal amino acid residue is Methionine. The N-terminal amino acid residue Methionine can acts as the main determinant of N-terminal degradation signal (Baumann, 2014; Kim *et al*., 2014). The initiator N-terminal (Nt-) Met of nascent polypeptides is co-translationally and irreversibly excised by ribosome-bound Metaminopeptidases (MetAPs) if it includes a penultimate residue with a small and uncharged side chain [Ala (A), Gly (G), Ser (S), Cys (C), Thr (T), Pro (P), or Val (V)] (Giglione *et al*., 2015). Targeting by the N-end rule pathway requires the generation of a destabilizing residue at the amino terminus of a proteolytic substrate and alanine, the second residue of BRD8 is a destabilizing residue (Gonda *et al*., 1989). This accounts for the unstable nature of BRD8, once it is processed by MetAPs exposing alanine at the N-terminus.

Aliphatic index was calculated as 75.26. The Grand Average hydropathy (GRAVY) indices was identified as 0.532. The molecular weight of a protein has a key role in its biochemical characterization (Sá-Moura *et al*., 2013) and is an analytical parameter in biochemistry (Jardine, 1990). The aliphatic index denotes the relative volume occupied by aliphatic side chains, which are comprised of alanine, valine, isoleucine and leucine, which contribute to the thermostability of protein (Ikai, 1980). In the present study, BRD8 protein showed an aliphatic index of 75.26 which indicates a fairly high thermal stability. GRAVY value of a protein is the sum total of hydropathy nature of all amino acids, divided by the number of residues in the sequence (Kyte *et al*., 1982). GRAVY) indices of BRD8 was shown a positive

value which indicates that BRD8 protein is hydrophobic in nature (Chang *et al*., 2013).

The extinction coefficient of BRD8 protein was also analysed. Protein concentration and extinction coefficients studies help in the quantitative analysis of protein-protein interaction and protein-ligand interactions in solution. Total number of negatively charged residues was high compared to the number of positively charged residues. This is in accordance with the earlier observations that there is a strong evolutionary pressure against super positively charged proteins (Requião *et al*., 2017). The computed isoelectric point (pI) of BRD8 protein shows its acidic nature. It is found that approximate pI values of proteins can be easily measured by its amino acid composition (Sillero *et al*., 1989). PI values are useful while developing buffer systems for purification of the recombinant proteins (Adhikari *et al*., 2010).

In the secondary structure prediction of BRD8 protein using SOPMA, there was a dominance of random coils, followed by alpha helix over other secondary structures. (Fig. 1). Random coils play a major role in giving flexibility and conformational changes of protein (Craveur *et al*., 2015). This structural feature reiterates the various roles of BRD8 as a regulator of many cellular processes.

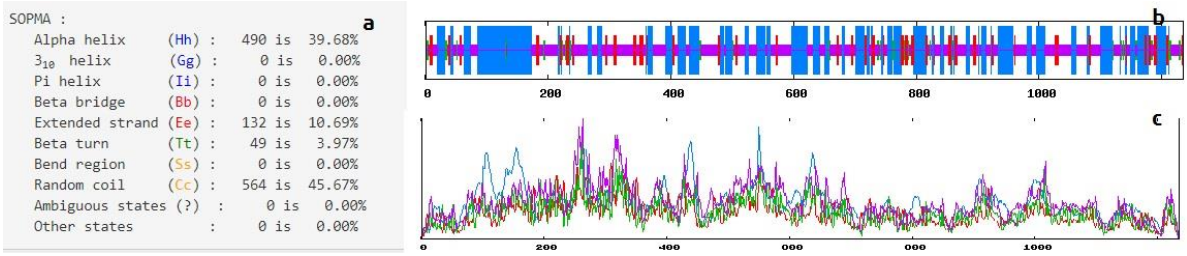

Fig. 1. Secondary structure of BRD8 predicted using SOPMA. Fig 1a shows the different types of secondary structure elements and percentage values. Fig 1b and c shows the graphical display of the occurrence of secondary structure elements in the sequence of BRD8.

The mechanism of transcriptional regulation of BRD8 with respect to ligand binding remains poorly understood. Therefore, multiple strategies, including motif and domain analysis were followed to recognize binding sites to enable the further studies of docking and dynamics. Computational Biology application efforts have now provided biologists with the information of large number of databases of sequences and structures. Similarly, protein domain architecture studies are very important tool in deciphering its functionality. Detection of protein domain and architecture is a valuable tool in knowing the atomic structure and biochemical function (Bagowski *et al*., 2010). Catalytic domains that code for specific posttranslational modifications (PTMs), such as kinases and acetyl transferases, are called writers because they give clues about the proteins which they act on (Lee *et al*., 2016). These factors allow us to study the behaviour of residues and their role in PTMs and motif functionality.

According to PTM prediction results, there were 10 hits for PTMs which resulted as patterns. These sites were predicted as critical biochemical events required for BRD8 regulation in cancer (Table 2). Myb-like domain profile shows a key role in growth, differentiation and apoptosis of cells (Oh *et al*., 1999). It is found that, MVP (Major Vault Protein) profile is found in 78% of 61 human cancer cell lines (Lara *et al*., 2011). Nuclear localization signal in a transcriptional regulator protein involved in cancer (Okazaki *et al*., 2012). cAMP is important for various metabolic processes and has shown activity in many cellular functions (Shabb, 2001). cGMP-dependent protein kinase phosphorylation site exhibits physiological functions in the mammalian system (Wolfertstetter *et al*., 2013). Tyrosine phosphorylation is also an important mechanism of signal transduction and regulation in eukaryotes (Hunter, 2009). Colorectal cancer metastasis induced by nuclear TYRO3 receptor tyrosine kinase has been found to be eradicated by inhibition of BRD3 activity (Hsu *et al*., 2023).

Alterations in glycosylation lead to changes in cell growth, survival and eventually metastasis in cancer (Stowell *et al*., 2015). Among all these sites identified, Casein Kinase II (CK2) phosphorylation sites were identified as the abundant ones. CK2 phosphorylates a variety of target proteins with numerous functions involved in cell cycle regulation, cell growth, proliferation, transcription, translation and apoptosis by influencing multitude of pathways involved in tumorigenesis (Nuñez de Villavicencio-Diaz *et al*., 2017). Being actively and co-ordinately involved in a lot of pathological conditions, the discovery of a large number of CK2 sites in BRD8 is promising since CK2 substrates are good candidates for inhibitor studies. Protein Kinase C signalling is found to be involved in cell cycle progression, tumorigenesis and metastatic dissemination which makes PKC a promising target for cancer therapy (Garg *et al*., 2014; Isakov, 2018). Hence identification of numerous PKC phosphorylation sites in BRD8 in the present study opens concrete opportunities to rationally design inhibitors with anti-cancer potential. Protein N-myristoylation, a co-translational lipidic modification specific to the alpha-amino group of an N-terminal glycine residue, catalysed by myristoyl transferases is found to be involved in regulating cellular signaling pathways in several biological processes especially in carcinogenesis (Yuan *et al*., 2020) and more recently immune function (Udenwobele *et al*., 2017). Given the potential applications of protein N-myristoylation in translational medicine, we hope the identification of N-myristoylation sites in BRD8 could help in development of inhibitors with therapeutic potential.

Table 2: Post translational modifications of BRD8.

Sr. No.	Site details
1.	PS50090 **MYB_LIKE** *Myb-like domain profile* **18 - 72:** **score** = 4.879 PWSIREKLCLASSVMRSGDQNWVSVSRAIkpfaepgrppDWFSQKHCASQYSE LL
2.	PS51224 **MVP** *MVP repeat profile* **37 - 98:** **score** = 4.979 QNWVSVSRAIKPfaepgrPPDWFSQKHCAsQYSELLETTETPKRKRGEKGEVVETVEDVI VR
3.	PS50014 **BROMODOMAIN_2 s** *Bromodomain profile* **724 - 794:** **score** = 19.178 ANHRYANVFLQPVTDDIAPGYHSIVQRPMDLSTIKKNIENGLIRSTAEFQRDIMLMFQNA VMYNSSDHDVY **1120 - 1190:** **score** = 19.771 ASHRFSSPFLKPVSERQAPGYKDVVKRPMDLTSLKRNLSKGRIRTMAQFLRDLMLMFQNA VMYNDSDHHVY
4.	PS50079 **NLS_BP** *Bipartite nuclear localization signal profile* **109 - 124:** **score** = 4.000 KKVIKETQER-YRRLKR **148 - 162:** **score** = 3.000 KKKLEEEEAE--VKRKA
5.	PS50313 **GLU_RICH** *Glutamic acid-rich region profile*
6.	PS00005 **PKC_PHOSPHO_SITE** *Protein kinase C phosphorylation site* **5 - 7:** TgK **20 - 22:** SiR **60 - 62:** SqK **77 - 79:** TpK **147 - 149:** TkK **366 - 368:** SiK **479 - 481:** TvK **610 - 612:** SlK **756 - 758:** TiK **826 - 828:** SaK **829 - 831:** SlR **835 - 837:** StR **836 - 838:** TrK **842 - 844:** SeK **1001 - 1003:** SaK **1090 - 1092:** SsK **1121 - 1123:** ShR **1133 - 1135:** SeR **1152 - 1154:** SlK
7.	PS00004 **CAMP_PHOSPHO_SITE** *cAMP- and cGMP-dependent protein kinase phosphorylation site* **98 - 101:** RKlT **160 - 163:** RKaT **939 - 942:** RKaS **1214 - 1217:** RKgS
8.	PS60007 **TYR_PHOSPHO_SITE_2** *Tyrosine kinase phosphorylation site 2* **160 - 167:** RkatDaaY
9.	PS00008 **MYRISTYL** *N-myristoylation site*

	214 - 219:	GVneSE
	527 - 532:	GVvpAT
	549 - 554:	GStaAG
	616 - 621:	GTifGS
	635 - 640:	GVseAA
	764 - 769:	GLirST
	930 - 935:	GSeeSQ
10.	PS00001 **ASN_GLYCOSYLATION** *N-glycosylation site*	
	216 - 219:	NESE
	228 - 231:	NSTG
	675 - 678:	NATL
	787 - 790:	NSSD
	875 - 878:	NDSE
	965 - 968:	NESS
	1156 - 1159:	NLSK
	1183 - 1186:	NDSD

As the next phase in our study, we performed the molecular modelling for the BRD8 sequence. Molecular modeling techniques can build and refine the model for the target which can subsequently be used for docking studies for determining the possible lead molecules as reported by earlier researches (Pimentel *et al*., 2013). Molecular modeling, a subset of computational chemistry, concentrates on predicting the behaviour of individual molecules within a chemical system. Molecular modeling is essential for understanding the structure-function relationship of molecules (Pimentel *et al*., 2013). Recent major advances in computational chemistry tools provide an alternative and approximate approach for obtaining the three-dimensional structure of the compounds (Sliwoski *et al*., 2013). Computational chemistry may be defined as the application of mathematical and theoretical principles to the solution of chemical problems. Advancements in computer storage capacity and processor performance helped modelling, big data analysis and pharmacogenomics to emerge as rapidly evolving and expanding fields (Khan *et al*., 2014). Similarly, theoretical calculations and geometrical optimization of the molecular complexes also enable the calculation of the bond lengths, bond angles, and total energy of molecules (Siddappa *et al*., 2014). We used SWISS-MODEL workspace which is an integrated Web based modelling server (Bordoli *et al*., 2009).

In this system, for a given target protein, a library of protein structures will be searched to identify suitable templates to develop a model. A suitable model was generated based on the template PDB ID: 3S91 (Crystal Structure of the first bromodomain of human BRD3 in complex with the inhibitor JQ) and predicted as a reliable model as per the psi-phi orientation. The validated model structure was visualized via BIOVIA DS visualizer (Fig. 2)

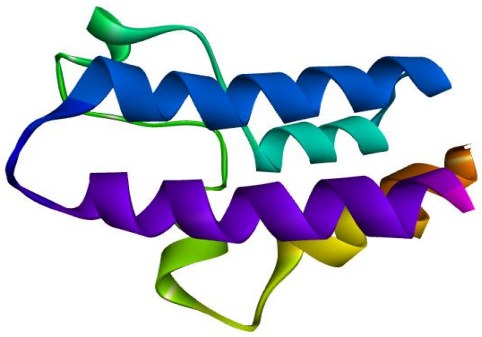

Fig. 2. Visualization of bromodomain 8 protein (BRD8) model using BIOVIADS visualizer.

When Ramachandran plot of BRD8 was analyed, it is found that 97.1% of amino acidsaree located in the highly favored region and 2.9 % in the favored region and no amino acids present in the unfavored region (Fig. 3). According to the concept of Ramachandran Plot, a good quality model should have >90% residues in favored region. Residues of the model located nearby the right-handed helices in the Ramachandran plot come under allowed and favored regions (Kurczynska *et al*., 2018). During the evaluation of the model, Rampage program showed 97.1% residues in the most favorable region, 2.9 % in the favored region and not favoured region didn't occupy any residues (Fig. 3).

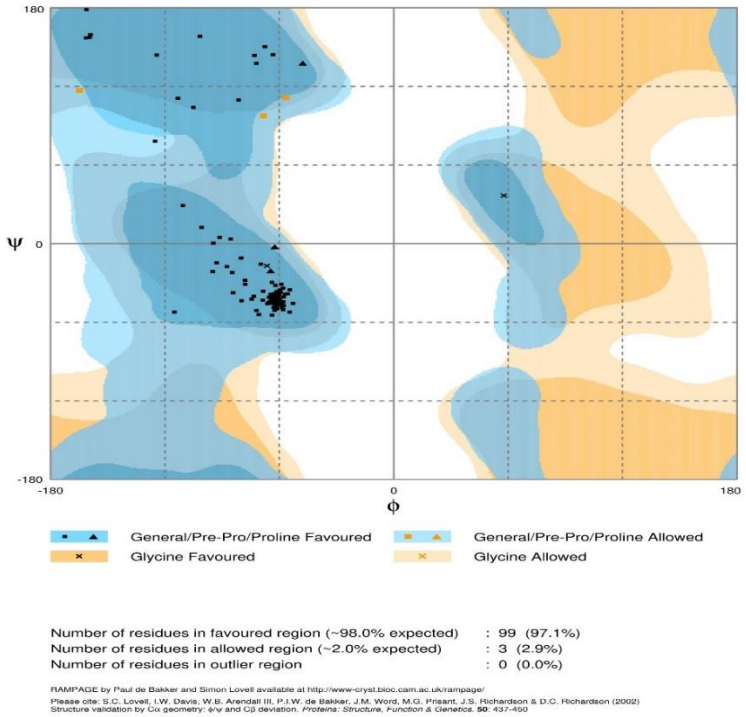

Fig. 3. Ramachandran plot analysis obtained from Rampage program. The analysis showed 97.1% of amino acids located in the region highly favored, 2.9 % in the favored region and no amino acids in the unfavored region.

In another step of validation, using ProSA-web program the overall model quality was found reliable with a Z-score value of -5.79 (Fig. 4). ProSA-web helps to refine and validate the experimental protein structures. The errors in the structures will be calculated based on energy plots and visualise in a three-dimensional manner (Wiederstein *et al*., 2007).

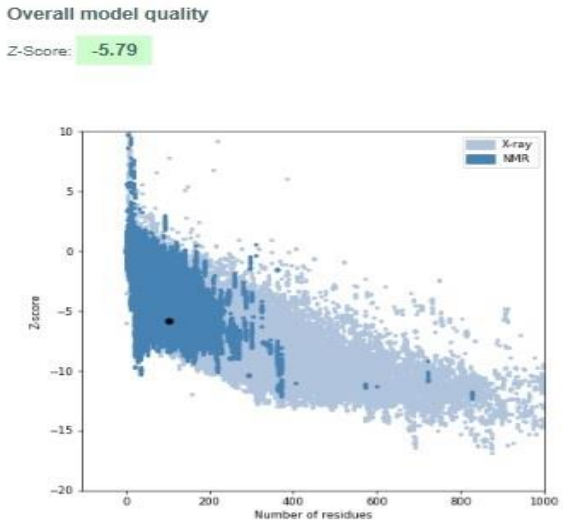

Fig. 4. Validation of the predicted BRD8 model using ProSA-Web program. The overall model quality Z-score of -5.79 is shown.

The identification of binding sites is an important step in structure-based drug design, as it helps to understand the functional sites and their mechanism of action (Lionta *et al*., 2014). This information on active sites might be helpful to evaluate the binding mode of BRD receptor and small molecules. The result from this study will serve as a toolset for bromodomain target validation in the near future. Binding sites on a receptor as well as the specific amino acid residues involved in it are significant in predicting physicochemical properties needed for a protein to perform its function. Many computational methods for the prediction of ligand-binding sites have been developed in the recent decades (Xie Zhong-Ru *et al*., 2015; Zhao *et al*., 2020). Here we used a consensus method known as MetaPocket, in which active sites will be predicted using four methods. In this method LIGSITE(cs), PASS, Q-SiteFinder, and SURFNET are combined together to improve the prediction success rate.

Table 3: Binding pocket prediction report from MetaPocket server.

Sr. No.	MetaPocket running report	Details
1.	Available methods:	PASS11(PAS,LigsiteCS(LCS), Q_SiteFinder (QSF,GHECOM(GHE) POCASA(PCS,Fpocket(FPK, SURFNET(SFN,ConCavity(CON)
2.	Success methods:	CON,FPK,GHE,LCS,PAS,SFN
3.	Failed methods:	QSF,PCS
4.	Total running time and time for each method	51.267 s PAS-1.420, LCS-1.006, FPK- 0.326, SFN-0.666, GHE -2.337, CON-2.339
5.	Base methods (time)	PAS,LCS,FPK,SFN,GHE,CON (4.123 s)
6.	metaPocket clusters and details	
	Cluster 1: Total z-score: 10.77	6 pocket sites: ['GHE-1', 'SFN-1', 'LCS-1', 'PAS-2', 'FPK-2', 'CON-1']
	Cluster 2: Total z-score: 7.61 of	5 pocket sites: ['FPK-1', 'PAS-1', 'SFN-2', 'LCS-2', 'GHE-2']
	Cluster 3: Total z-score:1.21	1 pocket sites: ['SFN-3']
	Cluster 4: Total z-score: 0.02	['FPK-3']
	Cluster 5: Total z-score: -0.42	['LCS-3', 'GHE-3']
	Cluster 6: Total z-score: -1.12	['PAS-3']

Table 4: Prediction of binding sites of BRD8 by using Metapocket server.

Sr. No.	The potential 3 ligand binding sites in BRD8 protein:
1.	HEADER binding site ID: 1 RESI PHE_A^732^ MET_A^752^ ASP_A^753^ MET_A^779^ VAL_A^731^ RESI GLN_A^734^ PHE_A^780^ ALA_A^783^ ASN_A^782^ THR_A^737^ RESI VAL_A^736^ ALA_A^797^ TYR_A^744^ PRO_A^735^ VAL_A^793^ RESI GLN_A^781^ PRO_A^751^ ASN_A^730^ ASN_A^787^ ASP_A^738^ RESI TYR_A^786^ HIS_A^791^ ILE_A^740^ ASP_A^739^ ALA_A^741^ RESI HIS_A^745^ PRO_A^742^
2.	HEADER binding site ID: 2 RESI ARG_A^721^ ALA_A^722^ ALA_A^723^ LEU_A^718^ ALA_A^724^ RESI VAL_A^719^ MET_A^800^ ASP_A^803^ VAL_A^804^ GLN_A^807^ RESI LYS_A^714^ ALA_A^715^ ILE_A^776^ ILE_A^808^ PHE_A^772^ RESI ILE_A^711^ GLN_A^773^ THR_A^769^ GLN_A^707^ LYS_A^710^ RESI GLN_A^810^ GLN_A^809^ ALA_A^708^
3.	HEADER binding site ID: 3 RESI GLU_A^771^ ARG_A^774^ ASP_A^775^ LEU_A^778^

The comparison results show that MetaPocket improves the success rate from approximately 70 to 75% at the top 1 prediction (Bingding Huang, 2009). MetaPocket has finished finding the top 3 pockets in the submitted model in PDB format (Job Id: 1575074389_42). The server took 55.390 seconds to generate results. After clustering, the top three sites from the base methods, namely PASS11 (PAS), LigsiteCS (LCS), GHECOM (GHE), Fpocket (FPK), SURFNET (SFN) and ConCavity (CON) were used to predict all the six metapocket clusters. MetaPocket running report is shown in Table 3.

The first MetaPocket site consists of 6 pocket sites with a total Z score of 10.77 from 'GHE-1', 'SFN-1', 'LCS-1', 'PAS-2', 'FPK-2' and 'CON-1'. The second MetaPocket site consists of 5 pocket sites with a total Z score of 7.61 from 'FPK-1', 'PAS-1', 'SFN-2', 'LCS-2' and 'GHE-2'. Third MetaPocket site consists of 1 pocket site with a total Z score of 1.21 from 'SFN-3'. Fourth MetaPocket site consists of 1 pocket site with a total Z score of 0.02 from 'FPK-3'. Fifth MetaPocket site consists of 2 pocket sites with a total Z score of -0.42 'LCS-3' and 'GHE-3' and Sixth MetaPocket site consists of 1 pocket site with a total Z score of -1.12 from 'PAS-3'. Three header binding sites with potential binding site residues of the receptor are shown in Table 4. Studies show that identifying potential inhibitors by finding active site of proteins and inhibiting their involvement in the cancer pathway is very helpful in the *in silico* drug target discovery process. A recent study reviewed the most current developments in BRD inhibitors, which are potential therapeutic molecules for treating a number of cancers (Liu *et al*., 2023).

CONCLUSIONS

In line with the fundamental biological concept of "Structure implies the Function", we felt that it is the need of the hour to do a structural characterisation of BRD8 since no such structural models are currently found in the repository. We constructed a three-dimensional structural model of BRD8 using molecular modelling and the quality and reliability of the constructed model was assessed satisfactorily. Further, we could predict the binding pockets present in the validated model. One limitation of this study is that the role of identified residues should be validated using *in*

silico drug binding and dynamics studies which requires sophisticated software facilities. Screening of combinatorial libraries of small molecules or natural compounds is warranted to shortlist a set of compounds before investigating them using wetlab studies.

FUTURE SCOPE

This is the first ever report of structural characterisation of BRD8 by homology modelling, which we hope could lead to further studies on docking and design of inhibitors with therapeutic potential. The results from this study may help researchers to combine and highlight the biological mechanism that underpin different classes of bromodomain proteins and initiate various clinical trials and drug screening approaches to improve the therapeutic strategies for cancer. Based on our results, potential BRD8 inhibitors with higher predicted activity and binding affinity can be evaluated by screening large combinatorial library of molecules. These results provide us a strong understanding for the discovery and optimization of novel and potent inhibitor compounds against BRD8 mediated cancers.

Conflict of Interest. None.

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