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MOLECULAR MODELLING AND DRUG DESIGNING OF CASPASE ASSOCIATED RING PROTEIN 2 INVOLVED IN CANCER

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ABSTRACT

Cancer is a disease in which cells grow and divide without respect to normal limits, invasive, and sometimes spread to other locations in the body. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited in their growth and don't invade or metastasize. Caspase Associated Ring Protein 2 (CARP2) plays an important role by acting as anti apoptoptic protein which leads to Cancer. In this work CARP2 structure was generated using MODELLER7V7 software using 1YO2 as template. With the aid of the molecular mechanics and molecular dynamics methods, the final model is obtained and further assessed by procheck and verify 3D graph programs, which showed that the final refined model is reliable. After identifying active site, Pyrimido[1,2-b]indazoles as a base molecule different derivatives were designed as this molecule has inhibitory effect on the CARP2. These molecules were docked to the active site of the CARP2. Docking results indicate that molecule 19 has high binding activity with CARP2. Our investigation may be helpful for further experiemental analysis.

Key words: Cancer, Drug designing, Docking, Modelling and Pyrimido[1,2-b]indazole.

INTRODUCTION

The most significant research advance in Cancer biology may be the discovery of the role of apoptosis in Cancer. Apoptosis is a fundamental biological process by which cells die in a well-controlled or programmed manner. This process when stopped leads to cancer. Because apoptosis plays a critical role in cancer development and in the cellular response to anticancer agents, the significance of proteins affecting apoptosis in cancer is obviously an important area of current investigation. Among the apoptosis regulators Caspase associated Ring proteins are important. Caspase 8/10-associated RING domain proteins (CARP) are a recently characterized protein family with a ubiquitin protein ligase encoded in their RING domain [1]. Once the extrinsic cell death pathway is initiated and transduces a death signal these are cleaved by active caspases 8 and 10. CARP proteins contain distinctively target apical death effector domain-of caspases 8 and 10 [1]. CARP proteins, especially the functional domains, are also markedly conserved across different organisms [1]. The CARP proteins have three highly conserved functional domains, CID (Caspase-Interacting Domain), FYVE (Fab1p, YOTB, Vac1P, and EEA1) [2], and RING (Really Interesting New Gene) [3]. A point mutation of a catalytically essential histidine to an alanine in the RING finger domain removes CARP1 and CARP2 E3 ligase activity [1]. The cysteine and histidine residues of CARP RING domains completely align with the RING consensus sequence [4]. The characteristic residues of RING domains are conserved in both CARP1 and CARP2 and to a large extent with the

RING domains of MDM2 [5], c-Cbl [6], and tumor necrosis factor-R-associated factor 2 [7]. Despite their many similarities, CARP1 and CARP2 are also functionally distinct. Chemotherapeutic agents cause DNA damage, which stabilizes p53 through post-translational modifications [8]. The basal level of p53 is maintained at very low levels due to the tight regulation of p53 by the E3 ligase MDM2 [9]. Other negative regulators of p53 that rely on RING domains include COP1 [10], Topors [11], ICP0 [12], Pirh2 [13], and E6/E6-AP [14]. Because CARP silencing sensitizes cells to DNA damage and may regulate tumor-suppressive events downstream of p53. CARP1 and CARP2 that are overexpressed in cancer have oncogenic activity and are capable of eliminating stress-induced p53 protein. Here we modeled CARP2 protein to identify better inhibitor through docking studies. This derivative may act as good inhibitor as it showed best docking result with CARP2 protein.

MATERIALS AND METHODS

3D model building

The initial model of caspase-8 and-10 associated ring finger protein (CARP2) was built by using homology-modeling methods and the MODELLER software. The sequence of CARP2 (Q8WZ73) was obtained from UNIPROT database. The query sequence from Homo sapiens was submitted to Dial setrver for CARP2 prediction. The predicted domain was searched to find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) program [15,16] against PDB (Protein Databank). Sequence that showed maximum identity with high score and less e-value were aligned using Needleman and Wunsch algorithm[17] and was used as a reference structure to build a 3D model for CARP2. The 3D model of a protein is obtained by optimization of the molecular pdf such that the model violates the input restraints as little as possible. The molecular pdf is derived as a combination of pdfs restraining individual spatial features of the whole molecule. The optimization procedure is a variable target function method that applies the conjugate gradients algorithm to positions of all non-hydrogen atoms. The co-ordinates for the structurally conserved regions (SCRs) for CARP2 were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm. The structure having the least modeller objective function, obtained from the modeller was improved by molecular dynamics and equilibration methods.

Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved. The final structure obtained was analyzed by Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) [18] and environment profile using ERRAT graph (Structure Evaluation server) [19]. This model was used for the identification of active site and for docking of the substrate with the enzyme.

Active site Identification

Active site of CARP2 was identified using CASTp server [20]. A new program, CASTp, for automatically locating and measuring protein pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory. CASTp identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings.

Docking method

The ligand, including all hydrogen atoms, were built and optimsed with chemsketch software suite. Extremely Fast Rigid Exhaustive Docking (FRED) version 2.1 was used for docking studies (OpenEye Scientific Software, Santa Fe, NM). It

is an implementation of multiconformer docking, meaning that a conformational search of the ligand is first carried out, and all relevant low-energy conformations are then rigidly placed in the binding site. This two-step process allows only the remaining six rotational and translational degrees of freedom for the rigid conformer to be considered. The FRED process uses a series of shape-based filters, and the default scoring function is based on Gaussian shape fitting.

RESULTS AND DISCUSSIONS

Homology Modeling of CARP2 (Q8WZ73)

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one reference proteins 1YO2 A (Chain A) has a high level of sequence identity and the identity of the reference protein with the Q8WZ73_CARP2 domain is 44%. Structurally conserved regions (SCRs) for the model and the template were determined by superimposition of the two structures and multiple sequence alignment.

```
domain          MWATCCNWFCLDGQPEEVPPPQGARMQAYSNPGYSSFPSPTGLEPSCKSCGAHFANTARK 60
1YO2            ------------------------------------------PSCKSCGAHFANTARK 16
                                                          ***************

domain          QTCLDCKKNFCMTCSSQVGNGPRLCLLCQRFRATAFQREELMKMKVKDLRDYLSLHDIST 120
1YO2            QTCLDCKKNFCMTCSSQ----PRLCLLCQRFRATAFQREELMKMKVKDLRDYLSLHDIST 72
                ******************    ************************************

domain          EMCREKEELVLLVLGQQPVISQEDRTRASTLSPDFPEQQAFLTQPHSSMVPPTSPNLPSS 180
1YO2            EMCREKEELVLLVLGQQPV---------------------------------------- 91
                ********************

domain          SAQATSVPPAQVQENQQANGHVSQDQEEPVYLESVARVPAEDETQSIDSEDSFVPGRRAS 240
1YO2            ------------------------------------------------------------

domain          LSDLTDLEDIEGLTVRQLKEILARNFVNYKG 271
1YO2            -------------------------------
```

Fig 1: alignment of CARP2 with template 1YO2

In the following study, we have chosen 1YO2 A as a reference structure for modeling Q8WZ73 domain. Coordinates from the reference protein (1YO2 A) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. In the modeler we got 20 PDB files out of which we selected a least energy protein. All side chains of the model protein were set by rotamers. The final stable structure of the CARP2 protein obtained is shown in Figure 2.

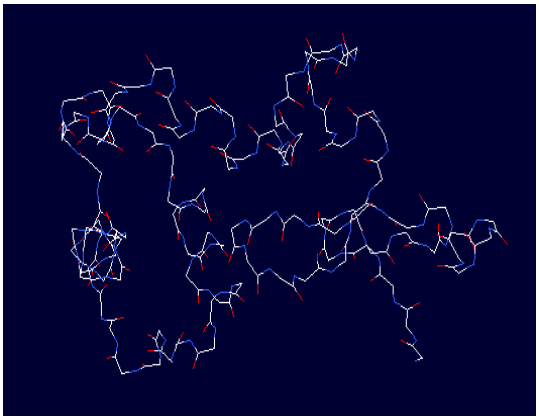


Fig 2: 3D structure of CARP2 generated by Modeller7v7

By the help of SPDBV it is evident that CARP2 domain has 4 helices and 2 sheets and it is shown in the Figure 3.

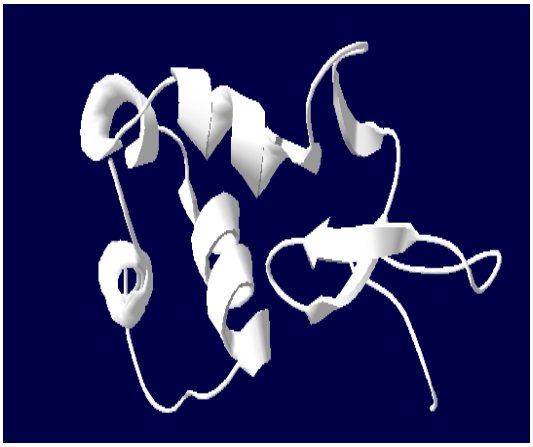
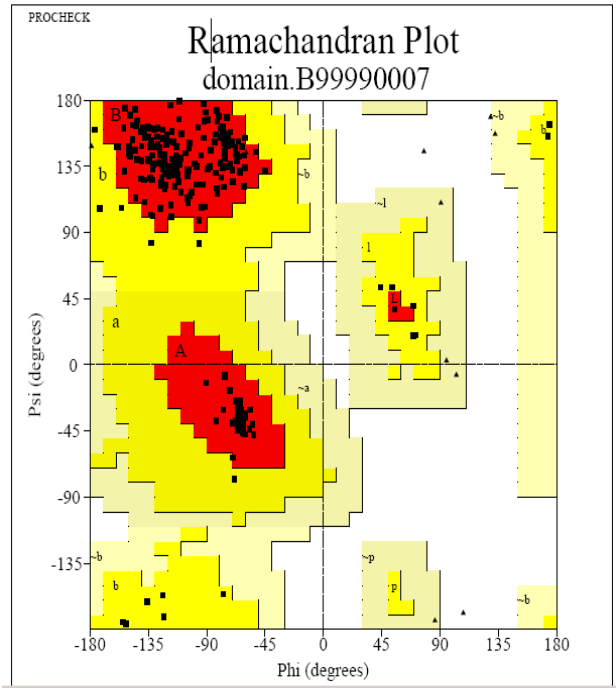


Fig 3: Protein with helices and sheets

Validation of Domain

After the refinement process, validation of the model was carried out using Ramachandran plot calculations computed with the PROCHECK program. The π and ψ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized in Table 1. Altogether 91.9% of the residues of CARP2 was in favored and allowed regions.



Plot statistics

Residues in most favoured regions [A,B,L]	217	91.9%
Residues in additional allowed regions [a,b,l,p]	19	8.1%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	236	100.0%
Number of end-residues (excl. Gly and Pro)	1	
Number of glycine residues (shown as triangles)	12	
Number of proline residues	22	
Total number of residues	271	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Figure 4: Ramachandran Plot

Superimposition of 1Y02 A with CARP2 domain

The structural superimposition of C trace of template CARP2 is shown in Figure. The weighted root mean square deviation of C α trace between the template and final refined models 0.24A^{o}. This final refined model was used for the identification of active site and for docking of the substrate with the domain CARP2.

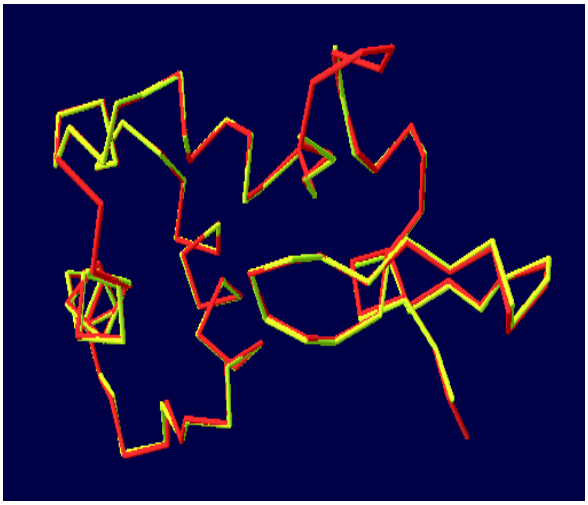


Fig 5: Super imposition of CARP2 and 1YO2 A.

Active site Identification of CARP2

After the final model was built, the possible binding sites of CARP2 was searched based on the structural comparison of template and also with CASTp server and was shown in Figure 5. Since, CARP2 and the 1YO2 A are well

conserved in both sequence and structure; their biological function should be identical. It was found that secondary structures are highly conserved and the residues., LEU 64, ASP 65, LYS 67, CYS 88, PHE 91, ARG 92, PHE 96, TYR 112, HIS 116, GLN 136, GLN 137, PRO 138, ILE 140.

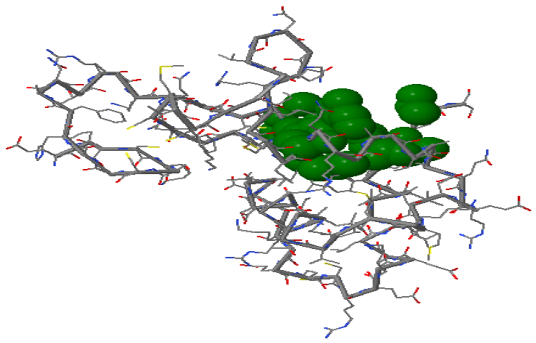
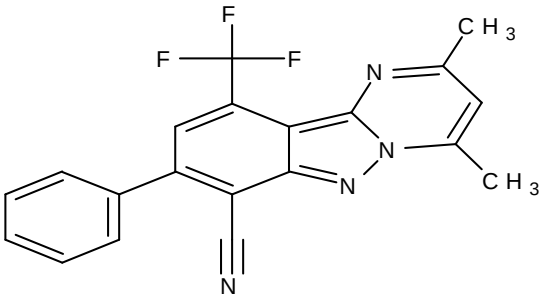


Figure 6: active site of CARP2

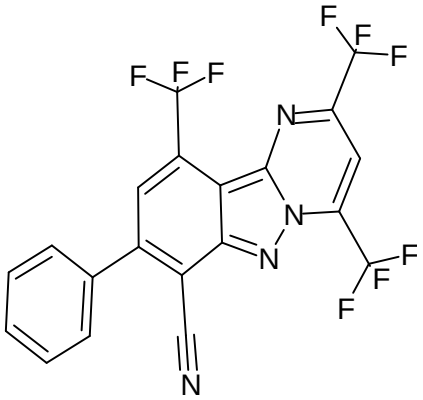
The Ligand (inhibitor) molecules used for Docking studies

MOLECULE 1



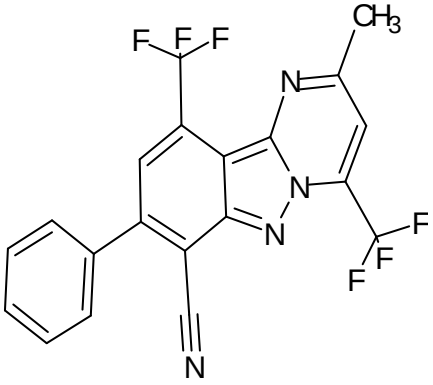
2,4-dimethyl-8-phenyl-10-(trifluoromethylpyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 2



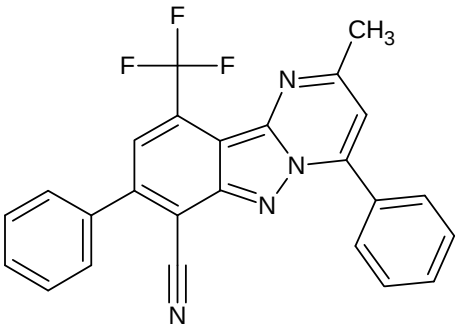
8-phenyl-2,4,10-tris (trifluoromethyl) pyrimido [1,2-*b*] indazole-7-carbonitrile

MOLECULE 3



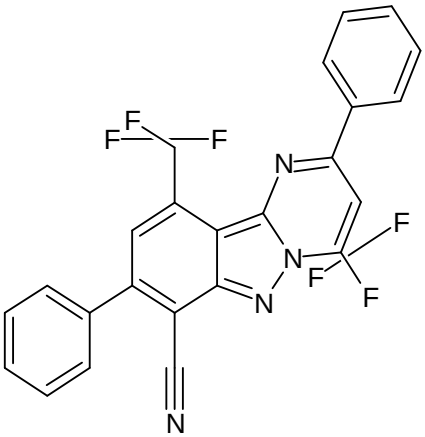
2-methyl-8-phenyl-4,10-bis(trifluoromethyl) pyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 4



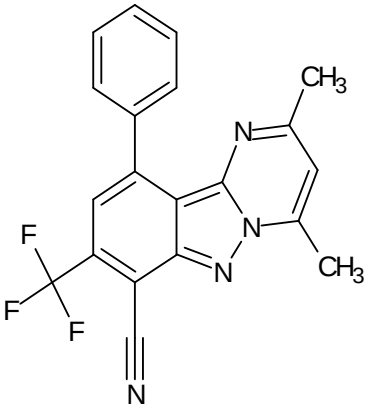
2-methyl-4,8-diphenyl-10(trifluoromethyl) pyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 5



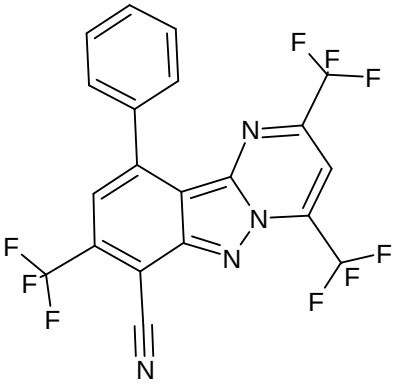
4-fluoro-10-(fluoromethyl)-2,8-diphenylpyrimido [1,2-*b*]indazole-7-carbonitrile- fluorine (1:2)

MOLECULE 6



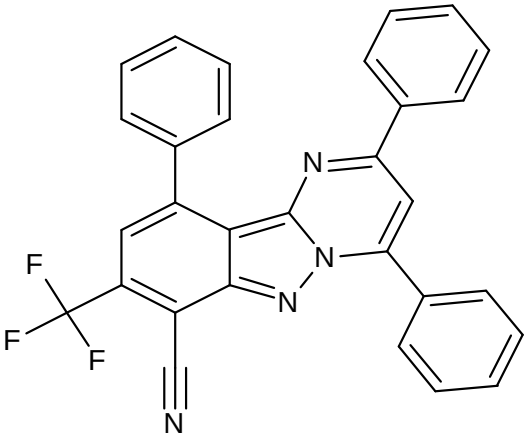
2,4-dimethyl-10-phenyl-8-(trifluoromethyl) pyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 7



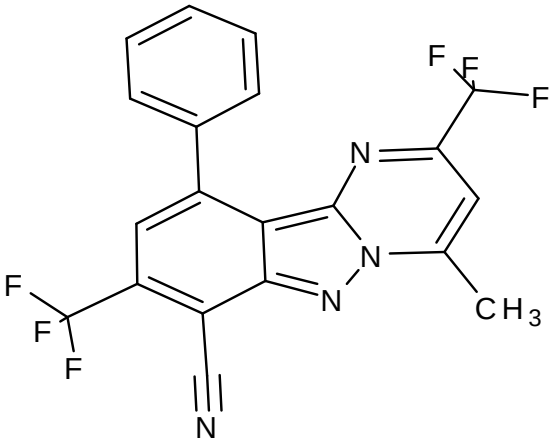
10-phenyl-2,4,8-tris(trifluoromethyl)pyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 8



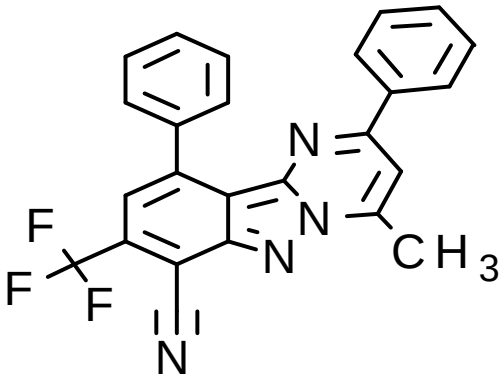
Triphenyl-(trifluoromethyl)BLAHcarbonitrile

MOLECULE 9



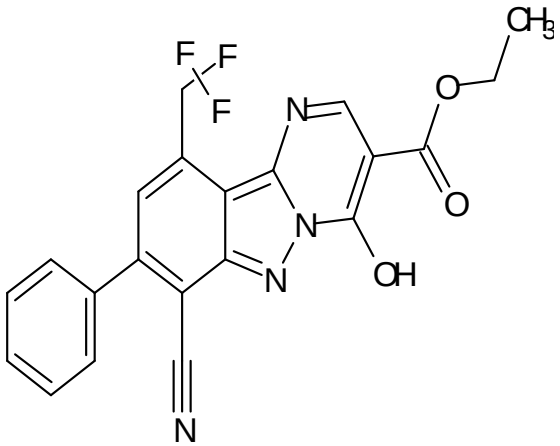
4-methyl-10-phenyl-2,8-bis(trifluoromethyl) pyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 10



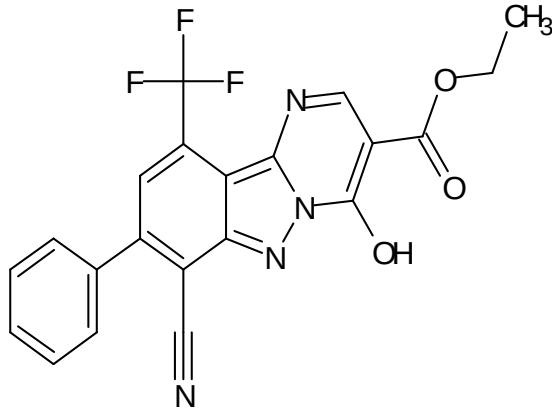
4-methyl-2,10-diphenyl-8-(trifluoromethyl) pyrimido[1,2-*b*]indazole-7-carbonitrile

MOLECULE 11



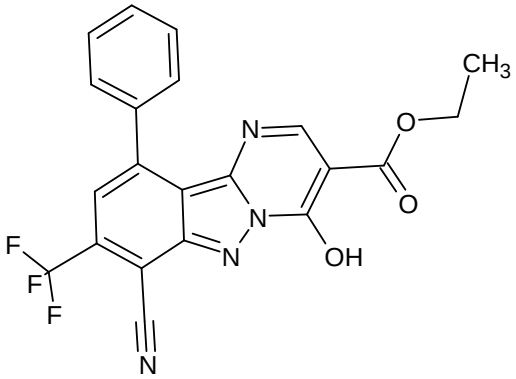
ethyl 7-cyano-10-(fluoromethyl)-4- hydroxy-8-phenylpyrimido [1,2-*b*]indazole-3-carboxylate - fluorine (1:1)

MOLECULE 12



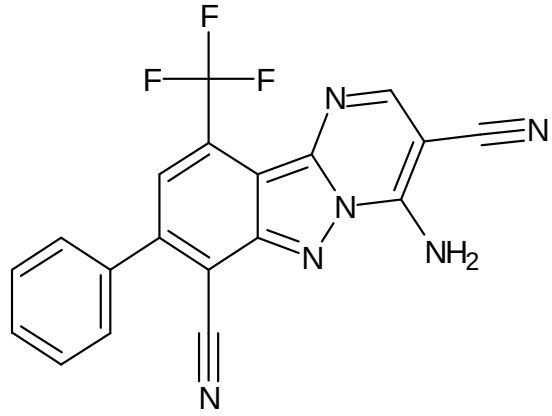
ethyl7-cyano-4-hydroxy-8-phenyl-10-(trifluoromethyl) pyrimido[1,2-*b*]indazole-3-carboxylate

MOLECULE 13



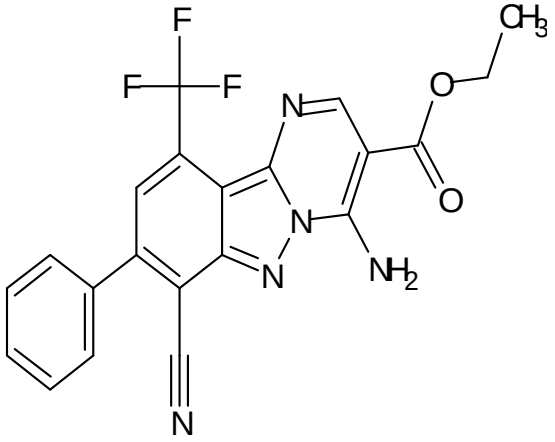
ethyl 7-cyano-4-hydroxy-10-phenyl-8-(trifluoromethyl) pyrimido[1,2-*b*]indazole -3-carboxylate

MOLECULE 14



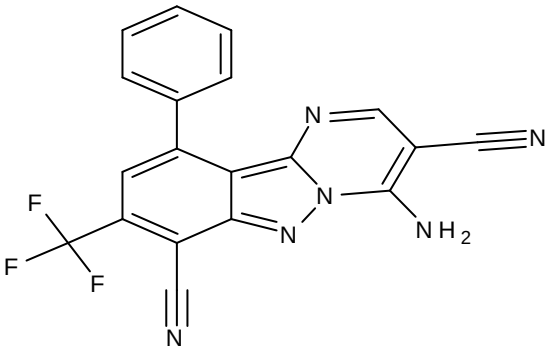
4-amino-8-phenyl-10-(trifluoromethyl)pyrimido[1,2-b]indazole-3,7-dicarbonitrile

MOLECULE 15



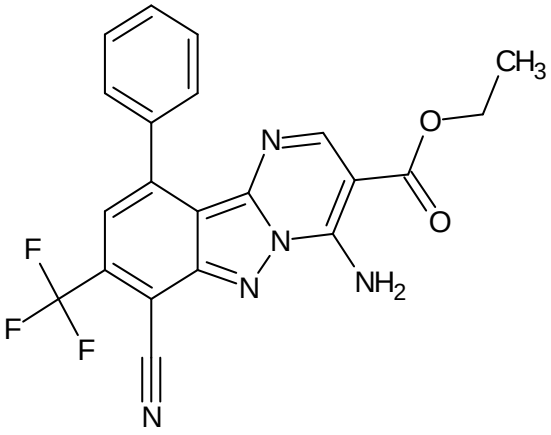
ethyl 4-amino-7-cyano-8-phenyl-10-(trifluoromethyl) pyrimido[1,2-b]indazole-3-carboxylate

MOLECULE 16



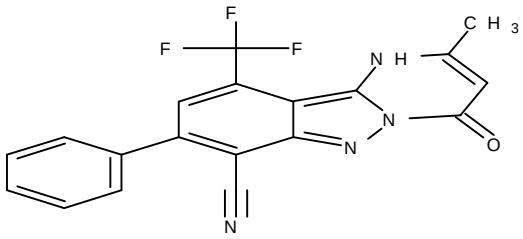
4-amino-10-phenyl-8-(trifluoromethyl)pyrimido[1,2-*b*]indazole-3,7-dicarbonitrile

MOLECULE 17



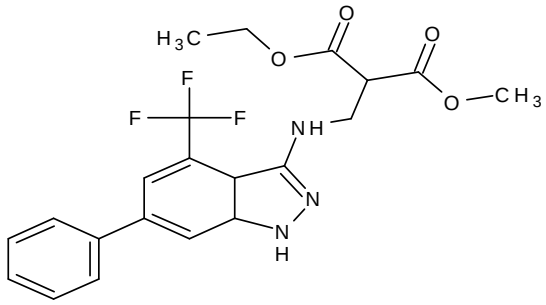
ethyl 4-amino-7-cyano-10-phenyl-8-(trifluoromethyl) pyrimido[1,2-*b*]indazole-3-carboxylate

MOLECULE 18



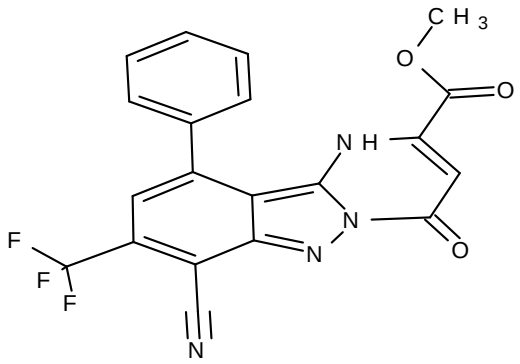
2-methyl-4-oxo-8-phenyl-10-(trifluoromethyl)-1,4-dihydropyrimido[1,2-b]indazole-7-carbonitrile

MOLECULE 19



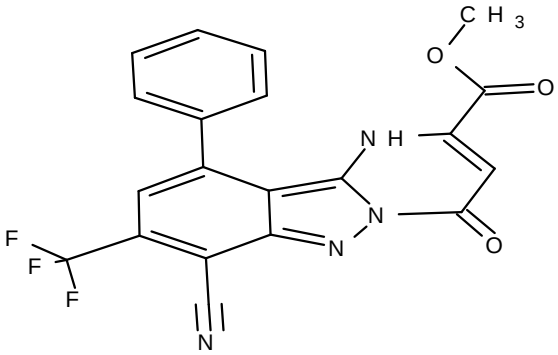
Ethyl methyl 2-[[[6-phenyl-4(trifluoromethyl) -3a,7a-dihydro-1H-indazole-3-yl]amino] ethyl]propanedioate

MOLECULE 20



methyl 7-cyano-4-oxo-10-phenyl-8-(trifluoromethyl)-1,4-dihydropyrimido[1,2-*b*]indazole-2-carboxylate

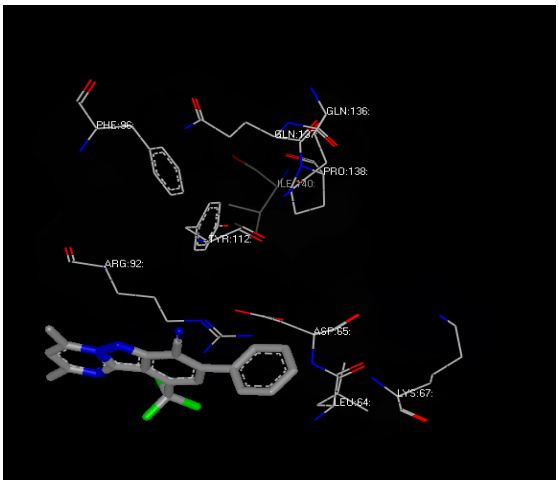
MOLECULE 21



Methyl 7-cyano-4-oxo-10-phenyl-8-(trifluoromethyl)-1,4-dihydropyrimido[1,2-*b*]indazole-2-carboxylate

DOCKING RESULTS

DOCKING MOLECULE 1



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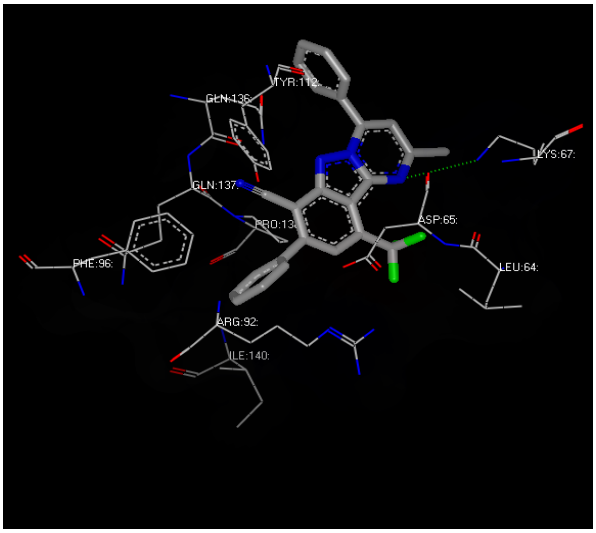

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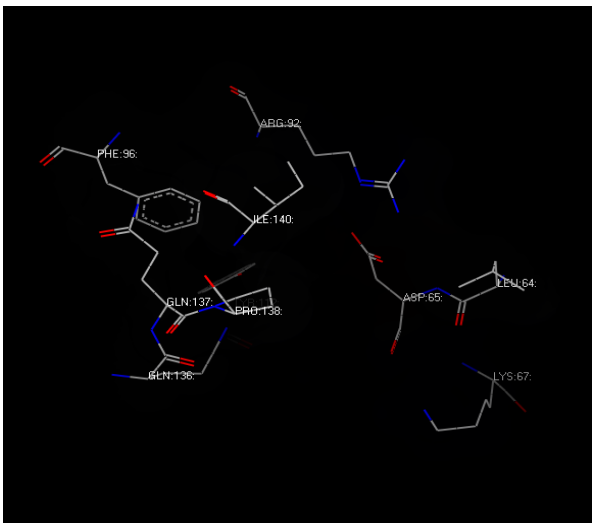

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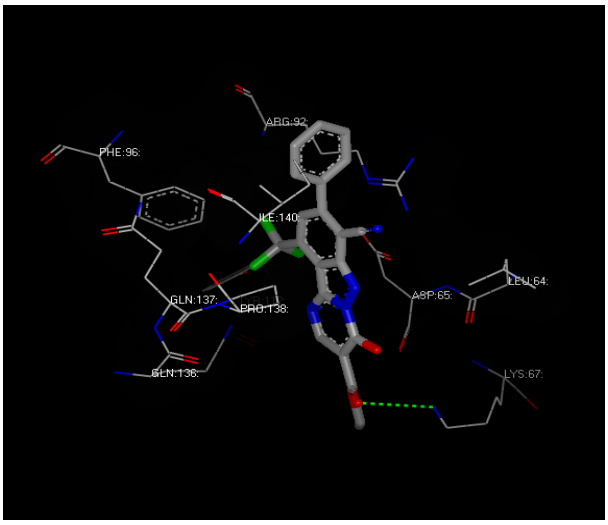

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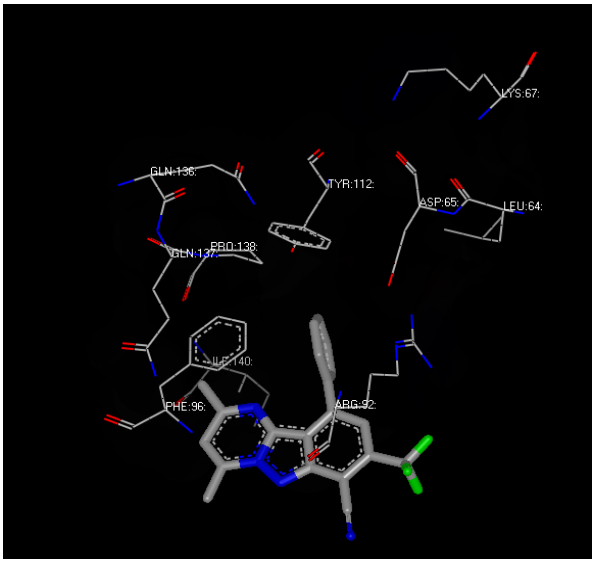
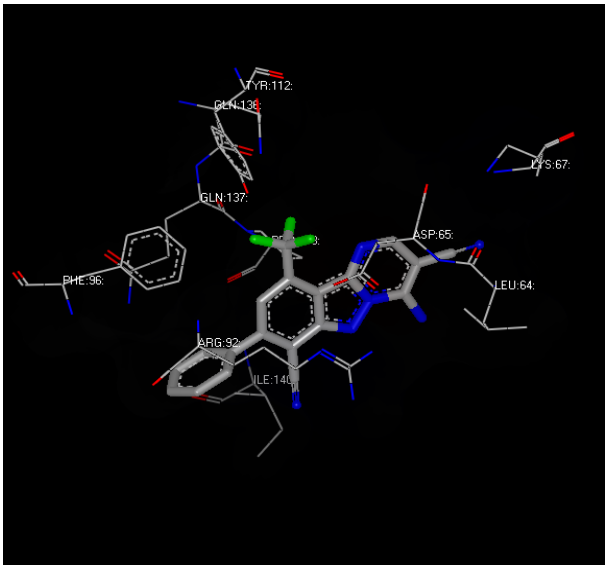


DOCKING MOLECULE 15



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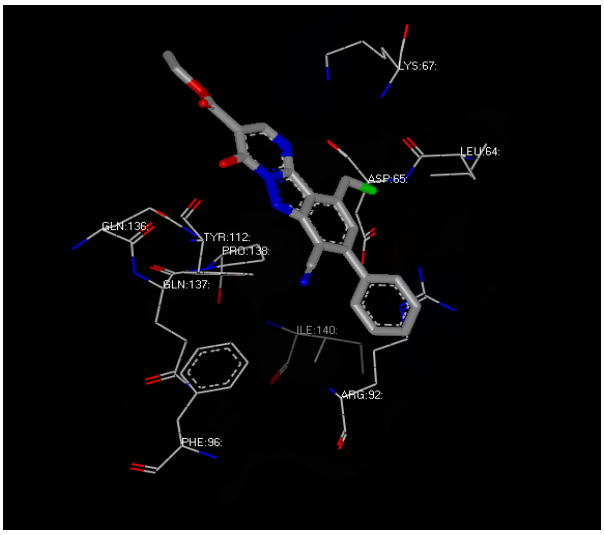

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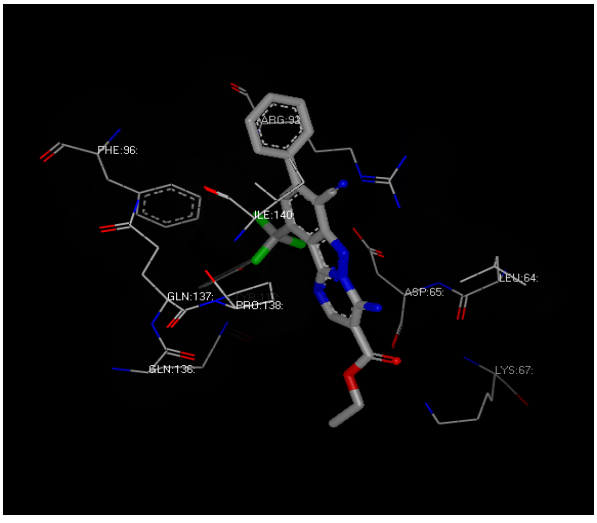

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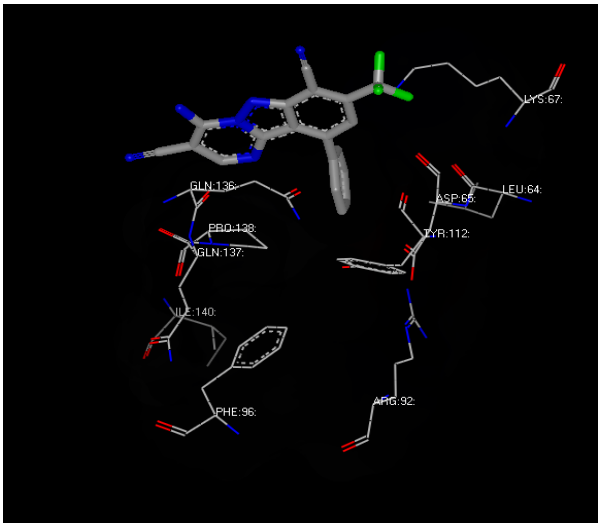

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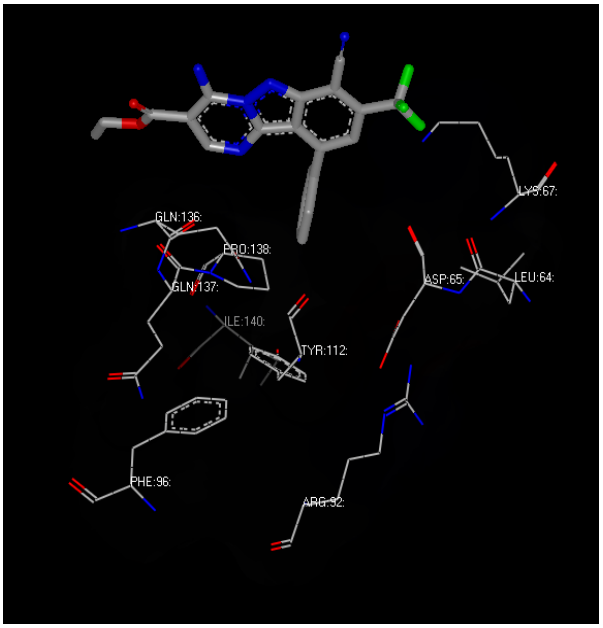

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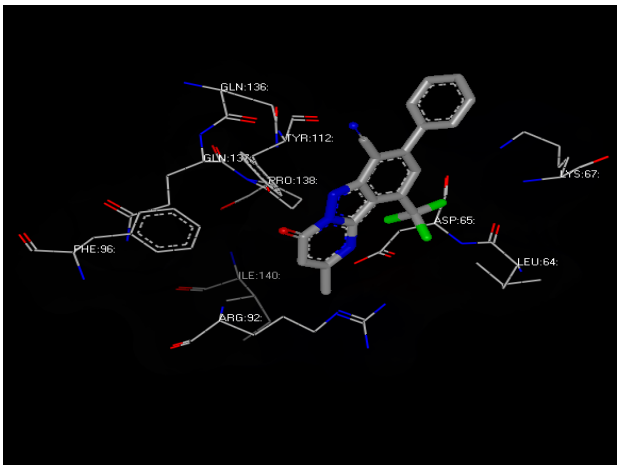

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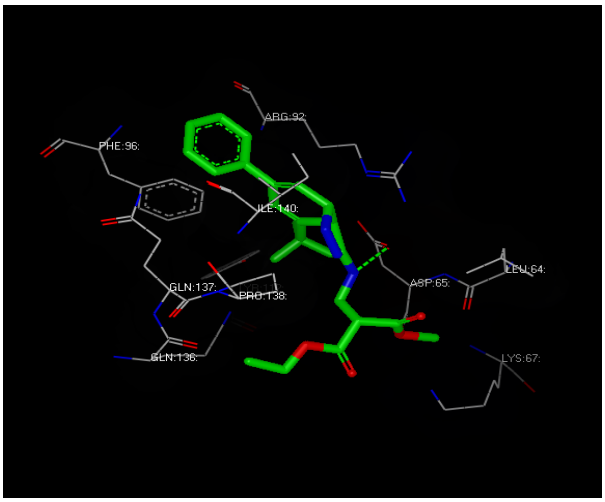
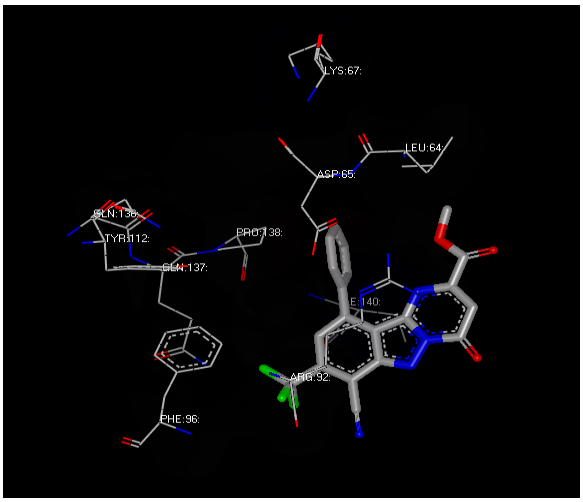


DOCKING MOLECULE 21



Docking of inhibitors with the active site of CARP2

Docking of the curcumin given in Figure 6 with Sortase was performed using FRED v 2.1, which is based on Rigid Body Shape-Fitting (Open Eye Scientific Software, Santa Fe, NM). This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a co crystalized ligand by

4 Å (add box parameter of FRED). This dimension was considered here appropriate to allow, for instance, compounds larger than the co crystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds used for docking was converted in 3D with OMEGA (same protocol as above) (Open Eye Scientific Software, Santa Fe, NM). To this set, the substrate (generation of multi conformer with Omega) corresponding to the modeled protein were added.

MOLECULE NUMBER	CHEMGUASS	CHEMSCORE	PLP	SCREEN SCORE	CONSENSUS	TOTAL SCORE
1	-46.19	-0.57	-26.73	-78.40	19	-132.89
5	-45.96	16.22	9.45	-17.71	48	10.00
6	-47.63	13.22	7.53	-35.46	44	-18.34
7	-43.72	4.61	-8.58	-73.28	41	-79.97
12	-43.28	-3.22	-28.21	-67.68	22	-120.39
13	-49.39	10.62	-10.33	-68.83	27	-90.93
15	-45.66	5.04	-20.08	-99.27	21	-138.97
16	-50.19	10.07	-16.84	-85.52	22	-120.48
17	-43.35	1.10	-21.87	-79.37	29	-114.49
18	-37.76	0.68	-19.01	-57.43	42	-71.52
19	-45.45	-3.88	-35.37	-97.06	13	-168.76
20	-57.37	16.88	-7.62	-54.54	32	-70.65
21	-41.00	0.06	-22.99	-81.44	30	-115.37

Table 2: the total energies of Chemguass score, Chemscore, PLP score and Shapeguass score of the best docked conformations of CARP2.

Conclusion

CARP2 is a member of a recently identified family of transcription factors that activate gene transcription in response to a number of different cytokines in Homo sapiens. In this work, we have constructed a 3D model of Q8WZ73 domain, using the MODELLER software and obtained a refined model after energy minimization. The final refined model was further assessed by ERRAT and PROCHECK program, and the results show that this model is reliable. The stable structure of Q8WZ73 is further used for docking with modified ligand molecules. Docking results indicate that conserved amino-acid residues CARP2 main play an important role in maintaining a functional conformation and are directly involved in donor substrate binding. The interaction between the domain and the inhibitors proposed in this study are useful for understanding the potential mechanism of domain and the inhibitor binding. As is well known, hydrogen bonds play important role for the structure and function of biological molecules. In this study it was found that, LEU 64, ASP 65, LYS 67, CYS 88, PHE 91, ARG 92, PHE 96, TYR 112, HIS 116, GLN 136, GLN 137, PRO 138, ILE 140 are important for strong hydrogen bonding interaction with the inhibitors. To the best of our knowledge LEU 64, ASP 65, LYS 67, ARG 92, PHE 96, TYR 112, GLN 136, GLN 137, PRO 138, ILE 140 are conserved in this domain and may be important for structural integrity or maintaining the hydrophobicity of the inhibitor-binding pocket. The molecule 19 showed best docking results with target protein.

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