1 Re-profiling of natural inhibitor via combinatorial drug screening: 2 Brefeldin A variant design as an effective antagonist leading to EPAC2 3 structure modification and antibody design for identification

4

5 Abstract

6 Drug discovery can be impactful with re-purposing and combinatorial strategies leading
7 to potential pharmaceutical outputs. Epac2 has been a target of interest for various
8 physiological conditions where suppression is inevitable to achieve desired therapeutic
9 effect. Epac isoforms inhibition is crucial in vascular functions to prevent chronic
10 inflammation leading to hypertension and myocardial infarction. An attempt utilizing
11 brefeldin A, a natural inhibitor was subjected to substitution of 3 side chain groups with
12 43 fragments via combinatorial strategy. This resulted in generating a library of 79507
13 brefeldin A variants. High throughput virtual screening yielded 68,043 variants
14 followed by precision docking providing 117 lead like brefeldin A variants. The best
15 docked variant (3-((1R,2E,6R,10E,11aS,13S,14A)-6-(methylsulfonamido)-13-(3-
16 methylureido)-4-oxo-4,6,7,8,9,11a,12,13,14,14a-decahydro-1H-
17 cyclopenta[f][1]oxacyclotridecin-1-yl)-2,3-dihydro-1Himidazol-1-ium) has an increased
18 binding efficiency of -10.841 kcal/mol. Simulation studies up to 200ns of complex lead
19 to re-orientation of target tertiary structure resulted in RMSD change of 30.221 Å,
20 suggesting the epac2 structure modification leading to unavailability of RAS-GEF
21 domain and its interaction with Rap1b. A single domain antibody was designed to bind
22 specifically to re-structured epac2 for potential identification over the native target
23 structure. The resulting Brefeldin variant can be potentially labelled as a most effective

24 antagonist against epac2 which induces theoretically irreversible structural re-
25 conformation. This study also provides a robust *in-silico* workflow for searching of
26 chemical space, generating and screening of combination libraries and the efficient
27 utilization of known inhibitor.

28 **Keywords** EPAC2, Brefeldin A, Combinatorial drug screening, Protein design, MD
29 simulations, Mutagenesis.

30

31 **Introduction**

32 Exchange protein directly activated by cAMP2 (Epac2) is a protein coded by RAPGEF4.
33 It interacts with Rap1b via Cyclic adenosine monophosphate (cAMP) mediated
34 mechanism. cAMP binds to the cNB-DB domain inducing a conformational change to
35 epac2 structure. This change enables the activation of catalytic region for interaction
36 with Rap1b. [1]

37 Epac2/Rap1b pathway has a significant physiological role in vascular isoforms. The
38 effect of cAMP in preventing inflammation of vascular tissues particularly by interleukin
39 (IL-6) propagation has well studied [2]. Fluorescent cAMP analogue has provided
40 insights into Epac2 selective inhibitors. The work focusses on competitive binding of
41 14,400 molecules in NDB-cAMP domain [3]. Amidst, all the efforts of targeted inhibition
42 of epac2 we find many reports suggesting denaturing of target proteins. Such reports
43 raise grave questions regarding non-specific binding leading to denaturation [4]. This
44 brings us to providing a specific compound from a known derivative which causes
45 structural re-confirmation and prevent denaturation. Overall, the status of antagonist to
46 epac2 issues need to re-addressed by an initiative approach by reprofiling of known
47 inhibitors to achieve desired functionality.

48 To achieve the particular re-purposing of drugs, a robust chemical fingerprinting and
49 combinatorial screening protocols have to be standardised. This prevents non-specific
50 and cross-interactions between proteins of same family. For anticancer therapy, to
51 avoid cross talks between proteins in signalling pathway a combination drug research
52 lead to increased efficiency of therapeutics [5]. Generation and screening of
53 combination libraries is crucial to avoid generation of false positives, leading to skewed
54 results. Understanding of excluded volumes via pharmacophore studies provides in
55 depth insight on the chemical space occupied and impact of the same on binding
56 efficiency.

57 In recent studies, the antagonists have shown lack of confidence in results for *In-silico*
58 and *In-vitro* of epac2 signalling pathway [6]. The study carried out is an attempt to
59 deliver a workflow to achieve stability in in-silico study.

60 In the current study, Brefeldin A has been our choice of antagonist which can be re-
61 profiled. It is a small hydrophobic compound having impactful effects on mammalian
62 cells produced by *Penicillium brefeldianum*. It is a potential inhibitor against activation
63 of ADP-ribosylation factors and small G protein of Arf family (7).

64 All the docking experiments carried out should be followed by a simulation study for a
65 minimum of 100ns to confirm stability of binding molecule and the structural variations
66 caused to the target molecule due to stable interactions.

67 The combination of above-mentioned approach appeals to finding a better antagonist
68 that provides solution to current problem of target degradation. The major focus of the
69 study is the re-modification of the target structure and developing a single domain
70 antibody (sdAb) for detection of the target-ligand complex.

71

72 **Methods**

73 **Domain and structural analysis:** The protein structure for Epac2 is available in
 74 Protein data bank, with following id's 4FHZ, 3CF6, 4MH0 and 107F. For the current
 75 study 4FHZ (*Mus musculus*) and 3CF6 (*Homo sapeins*) have been selected based on the
 76 coverage of amino acid sequence and known protein interaction in Rap1b. Similarity,
 77 Identity and structure superimposition was carried out with 4MH0 and 107F for
 78 validation.

79 Domain analysis for epac2 was conducted using InterPro and Conserved domain
 80 database of NCBI. A schematic of complete workflow is shown in (Fig 6).

81

82 **Fig 6 Schematic representation of methodology:** The overall objective of the method
 83 designed in to re-profile the Brefeldin A (natural inhibitor) into an antagonist and
 84 achieve desired conformational changes to EPAC2 structure. Docking studies were
 85 performed for cAMP and BrefA. 3 side chain groups of BrefA were further subjected to
 86 substitution with 43 fragments, leading to a database of 79507 variants of BrefA. High
 87 throughput virtual screening (HTVS) of brefA variants, followed by Standard precision
 88 (SP) and Extra precision (XP) docking with EPAC2. The protein complexes EPAC2-
 89 cAMP, EPAC2-BrefA and EPAC2-BrefA1 (best docked variant) were subjected to
 90 simulation studies for 200ns. Laterally, the complementary determining regions (CDR)
 91 for EPAC2 were targeted for developing a single domain antibody (sdAB), which was
 92 refined by epitope mapping and mutagenesis. The aim was to have a sdAB, exhibiting
 93 preferential binding towards EPAC2-brefA1 complex over other complexes and native

94 structures. The above schematic representation was created using Draw.io
95 (<https://www.draw.io/>)

96

97

98 **Combinatorial drug screening:** The Small molecule Discovery suite 2019-3 was used
99 for Docking, R-group enumeration and virtual screening. Various tools like Protein
100 Preparation Wizard [8] Epik (9), Impact (10), LigPrep [11], SiteMap [12], Receptor grid
101 generation and Glide [13] were utilized.

102 The protein was subjected to pre-processing for defining residues, HET atoms and
103 verify valency. Under structure refinement, hydrogen bond assignment was carried out
104 for neutral pH. Structure minimization for less than 0.30 Å was conducted with OPLS3e
105 defined force field. The top ranked potential binding sites were selected with restricted
106 hydrophobicity. The in the present case, the cAMP2 binding domain was selected as a
107 binding pocket which was evaluated as top binding site. A receptor grid was defined for
108 docking of BrefeldinA and cAMP with the selected binding pocket with constraints and
109 predefined excluded volumes. LipPrep was used to generate 3D structures of ligand
110 molecules with 32 conformers for each respectively. An extra precision (XP) ligand
111 docking was performed using Glide, with a scaling factor of 0.8 and partial cut-off of
112 0.15. Epik state penalties were added to the docking score.

113 The Enumeration and Ideation tool [14] was used for preparation of custom R-Group
114 libraries. A custom R groups of 43 fragments was created and was utilized was
115 substitution with 3 side chain atoms of BrefeldinA. A total of 79,507 variants of
116 Brefeldin A were generated. This generated library was subjected to LigPrep and stored
117 as a database for further screening.

A virtual screening workflow was created. The ligand input files were selected from the database created. A pre-filter was defined to follow Lipinski's rule using QikProp [15] properties. Epac2 protein structure was selected as receptor with defined binding site developed using Sitemap and receptor grid. A docking workflow for High throughput virtual screening (HTVS) to dock flexibly, perform post-docking minimization and generates one pose per compound state. All states are retained after docking. The top 10% of compounds are carried forward for Standard precision (SP) docking. The parameters remain the same as selected for HTVS. The top 10% are carried forward for Extra precision (XP) docking. For XP, except for generating 3 poses for compound state, remainder parameters are constant. The final lead like compounds are subjected to post processing with prime MM-GBSA for accuracy in binding efficiency. The best docked compound was merged with the target protein structure and the complex was exported as .pdb file from the workspace navigator.

The 2D interaction plot was generated from ligand interaction viewer in Maestro [16]. The 2D plots for docking score vs energy was generated using Plot viewer tab in tables section of Maestro.

ADME studies: ADME studies were performed on SwissADME web server [17] by importing the compound structure, conversion to SMILES and prediction.

Simulation studies: Desmond [18] was used for performing simulations. The interaction complex was subjected to protein pre-processing and H-bond assignment with similar parameters as mentioned earlier. Simulation system was built utilizing the system builder. The solvent model selected was TIP3P, boundary conditions defined by orthorhombic box with minimized volume encapsulating the complex. The force field applied is OPLS3e. System was neutralized by adding Cl⁻ or Na⁺ ions based on the system total charge.

To perform molecular dynamics simulation, the system was imported from the workspace. Simulation time was 200ns, with trajectory recording interval set to 1ns (the optimised interval time helps in clear visualization and appropriate storage space). Ensemble class was set to NPT (defines the thermodynamic parameters of constant pressure and temperature, a variable volume allows the protein to undergo conformational change) and modelled relaxed for 10 ns before performing simulation (a relaxation protocol avoids increase in atoms velocity due to increase in temperature). Time step was set to 2 femto-seconds (fs) and temperature of 300K. A cut-off short range method with a radius of 9.0 Å (This optimized value avoids overlapping of atoms). No restraints were pre-defined.

Simulation has a temperature increase of 10K per time step after solvation of binding pocket.

Simulation interaction diagram tool was used for analysing the simulation results for change in RMSD and protein-ligand contacts. The surface depth analysis was carried out using the DEPTH server [19]. Protein structure superimposition calculation and visualisation was carried out by Pymol [20].

The stability of protein structures after completion of simulation course was evaluated by Ramachandran plot utilizing Mol-probity web server [21].

Antibody design: The Biologics suite [22] provides tools for antibody design, antigen-antibody interaction and mutagenesis. The human heavy variable chain sequence (A2NXP8) was selected as sequence for antibody design. Under antibody prediction tool, for single domain antibody the selected sequence was provided as input. A homology search was conducted for shortlisting of antibody template structure. The top most template was selected as antibody framework. The H1, H2 and H3 loops of the CDR regions were modelled and the best model was reported back.

Protein Protein docking: Protein protein docking tool was utilized with antibody mode masking the non-CDR regions. The antigen and antibody were selected for docking. Number of rotations to probe was set to 70000 and maximum poses to return set to 30.

No attractions-repulsions and distance restraints were defined. The protein interaction visualization was used for epitope mapping and interaction calculation.

The protein-protein docked complex was subjected to antibody humanization-residue mutation to improve stability of protein-protein interaction. The critical residues were subjected of substitution with reminder of amino acids to calculate affinity, SASA, pKa and complementarity.

The Residue mutation viewer was used to plot the change in delta stability results. The amino acids substitution was carried out in sequence viewer. The protein-protein docking was carried out for mutated antibody and antigen. The docking parameters and visualization was carried out as mentioned earlier.

Results and Discussion

Domain and Structural analysis

The domain analysis of the epac2 was carried out (Fig 1a). The cAMP1 ranges from 43-166 amino acid (aa), DEP ranges from 198-273 aa, cAMP2 ranges from 338-458 aa, N-terminal RAS-GEF ranges from 480-616 aa and RAS-GEF ranges from 757-934. cAMP domain is further classified into PBC ranging from 403-417 aa and hinge ranging from 433-438 aa. The cAMP binding at the PBC region triggers the hinge mechanism providing RAS-GEF domain an interacting surface for Rap1b.

Three structures (4F7Z, 4MH0 and 1O7F) of epac2 are available in PDB and 4F7Z was selected. Similarity, identity and structure superimposition was carried out with PDB id's 4MH0 and 1O7F for validation purposes of protein structure taken into consideration. 4F7Z had similarity of 98.36% and 91.17% with 4MH0 and 1O7F respectively. A structural superimposition revealed a RMSD variation of 1.21 Å and 1.68 Å with 4MH0 and 1O7F respectively. Based on the observations, we can conclude that 4F7Z has structural similarity to 4MH0 and 1O7F. Since 4F7Z has better sequence coverage it has been selected for docking studies. The sequence coverage can be visualized in (Supplementary Fig 1)

Combinatorial drug screening

Comparison of cAMP and Brefeldin A interaction with Epac2

As discussed in the previous section, regarding the hinge mechanism both cAMP (Fig 1b) and Brefeldin A (BrefA) (Fig 1c) have shown highest binding efficiency in the cAMP2 domain. We have performed extra precision docking of cAMP and BrefA with EPAC2. cAMP has a binding energy of - 10.692 kcal/mol whereas BrefA has a binding energy of -6.248 kcal/mol. cAMP forms conventional hydrogen bonds with D402, G406, A407, A415, K450 and has an attractive charge interaction with R414 (Fig 1d). Whereas BrefA exhibits hydrogen bonding with D402 and L406 (Fig 1e). It can be concluded that cAMP undergoes efficient binding due to more significant interactions in the binding pocket. A higher binding energy and stronger interactions promotes the hinge mechanism with cAMP. BrefA is a competitive binding molecule aimed to avoid cAMP binding.

Fig 1. Structural information of target and compounds, along with interactions (a)

Epac2 structure colour coded highlighting structural and catalytic domains (b)

Structure of cAMP (c) Structure of Brefeldin A (d) Interactions of cAMP in the binding

pocket, along with a 2D interaction diagram exhibiting the interaction with epac2. (e)

Interactions of BrefA in the binding pocket, along with a 2D interaction diagram

exhibiting the interaction with epac2. The above images were developed using Maestro

2019-3 (<https://www.schrodinger.com/maestro>), available free for academic usage.

Generation of combinatorial library

Three sites on the Brefeldin A molecule were selected by avoiding core atoms for

replacing it with 43 side chain fragments. The sites selected were (R1) C9 linked with -

OH (O1 and H31) with bond length of 1.423 Å (R2) C10 linked with -OH (O2 and H33)

with a bond length of 1.428 Å and (R3) C18 linked -CH3 (C20, H42, H43, H44) with a

bond length of 1.523 Å (Fig 2c). A library of 43 fragments was built which will be in

permutation be replaced with each of the selected sites. This resulted in a library of

79507 Bref A variants (Table 1) with a common core similar to of Bref A.

Fig 2. Selection of Brefeldin A side atoms, combinatorial screening and docking

plot along with Brefeldin A variant interactions (a) BrefA best variant (BrefA1) in

the binding pocket of EPAC2 (b) BrefA1, 2D interaction diagram exhibiting the

interaction with epac2 (c) 2D diagram of Brefeldin A showing core and side chain atoms

R1, R2 and R3 were replaced with 43 selected fragments in permutation and created a

combinatorial library of 79507 BrefA variants. (d) Docking score vs Energy plot for

HTVS, SP and XP. Representation via gradient colour coding of docking score to show

selection of variants via better and efficient docking score. The above images were

237 developed using Maestro 2019-3 (<https://www.schrodinger.com/maestro>), available
238 free for academic usage

239

240 **Table 1. List of side chain fragments used in Brefeldin A variants library**

241

SI no	Fragment	SI No	Fragment	SI no	Fragment
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242

1	methane	16	Ammonium	31	N,N-dimethylmethanesulfonamide
2	propane	17	methylammonium	32	benzenesulfonamide
3	tert-butane	18	dimethylammonium	33	benzenesulfonamide 2
4	trifluoromethane	19	hydrogen cyanide	34	N-methylbenzenesulfonamide
5	cyclohexane	20	Water	35	N-methylurea
6	benzene	21	Acetamide	36	N-phenylurea
7	pyridine 1	22	acetamide 2	37	morpholine
8	pyridine 2	23	acetamide 3	38	morpholinium
9	pyridine 3	24	N-methylacetamide	39	piperidinium
10	imidazole 1	25	N-methylacetamide 2	40	formate 1
11	imidazole 2	26	N,N- dimethylacetamide	41	tetrazolate
12	imidazole 3	27	Acetanilide	42	N-methylmethanesulfonamide
13	imidazolium 1	28	methanesulfonamide	43	N-methylmethanesulfonamide 2
14	imidazolium 2	29	methanesulfonamide 2		
15	imidazolium 3	30	methanesulfonamide 3		

243 Virtual screening of Combinatorial library

244 High throughput virtual screening (HTVS) of library compounds resulted in docking of
245 68,043 variants. The remainder of 11,805 variants could not be bound to target. The
246 reason behind omission is compound surface area is larger than the size of binding
247 pocket. The top 10% of the HTVS docked molecules based on binding efficiency i.e 6,804
248 compounds were subjected to standard precision (SP) docking. The top 10% of the SP
249 docked molecules i.e 680 molecules were subjected to Extra precision (XP) docking
250 with 10 best poses to be reported. The final set of 117 variants were shortlisted. The
251 threshold of selecting 117 lead like variants was their binding efficiency was > -6.248

kcal/mol (binding energy of Brefeldin A). These variants were subjected to molecular mechanics combined with poisson-boltzmann surface area (MM-GBSA) to calculate the accurate binding energy. The lead molecules docking information is provided in (Supp table 1). The docking score and energy of the docked molecules are shown in (Fig 2d). The best docked variant of Brefeldin A is Brefeldin A; [N-methylurea 1];[imidazolium 2]; [from methanesulfonamide 3]. The IUPAC name of the obtained variant is (3-((1R,2E,6R,10E,11aS,13S,14aR)-6-(methylsulfonamido)-13-(3-methylureido)-4-oxo-4,6,7,8,9,11a,12,13,14,14a-decahydro-1Hcyclopenta[f][1] oxacyclotridecin-1-yl)-2,3-dihydro-1Himidazol-1-ium). It has an increased binding efficiency of -10.841 kcal/mol. The best docked variant will be now onwards referred to as Bref A1.

The details of fragments replaced is R1 replaced with methylurea 1, R2 replaced with imidazolium 2 and R3 replaced with methanesulfonamide 3. The docked BrefA1 in the binding pocket and 2D interaction with EPAC2 is shown in (Fig 2a and Fig 2b).

Absorption Distribution Metabolism Excretion (ADME) studies of Brefeldin A1

In (Table 2), a detailed analysis of five components like water solubility, pharmacokinetics, physicochemical, drug likeness, lipophilicity and medical chemistry of Bref A1 has been reported.

Bref A1 is found to be soluble in water, with Log S values suggesting the same. druglikeness study satisfies all the parameters including the Lipinski's rule, Ghose filter and a bioavailability score of 0.55. The topological polar surface area (TPSA) is 141.83

274 Å², an expected threshold is TPSA < 140 Å². The permeability with cell surface is still
275 feasible but with low rate of absorption.

276 Blood brain barrier permeability is not possible with TPSA score >90 Å². The synthetic
277 accessibility score is 6.05 which makes the compound moderately feasible to synthesize.

278 XLogP3 value of 1.62 is within the range of -0.7 < XLogP3 > +5 ensuring favourable
279 dissolution in non-polar solvents.

280 **Table 2. Featuring various properties regarding ADME of Brefeldin A1**

Water Solubility		Pharmacokinetics	
Log S (ESOL)	-3.37	GI absorption	Low
Solubility	2.00e-01 mg/ml; 4.27e-04 mol/l	BBB permeant	No
Class	Soluble	P-gp substrate	Yes
Log S (Ali)	-4.21	CYP1A2 inhibitor	No
Solubility	2.88e-02 mg/ml ; 6.16e-05 mol/l	CYP2C19 inhibitor	No
Class	Moderately soluble	CYP2C9 inhibitor	No
Log S (SILICOS-IT)	-2	CYP2D6 inhibitor	No
Solubility	4.65e+00 mg/ml ; 9.93e-03 mol/l	CYP3A4 inhibitor	No
Class	Soluble	Log Kp (skin permeation)	-8.01 cm/s
Drug likeness		Physicochemical Properties	
Lipinski	Yes; 0 violation	Formula	C ₂₁ H ₃₄ N ₅ O ₅ S
Ghose	Yes	Molecular weight	468.59 g/mol
Veber	No; 1 violation: TPSA>140	Num. heavy atoms	32
Egan	No; 1 violation: TPSA>131.6	Num. arom. heavy atoms	0
Muegge	Yes	Fraction Csp ³	0.62
Bioavailability Score	0.55	Num. rotatable bonds	6
Medicinal Chemistry		Num. H-bond acceptors	7
Brenk	1 alert: isolated_alkene	Num. H-bond donors	4
Leadlikeness	No; 1 violation: MW>350	Molar Refractivity	129.03

Synthetic accessibility	6.05	TPSA	141.83 Å ²
Lipophilicity			
Log Po/w (iLOGP)	1.82	Log Po/w (MLOGP)	-2.93
Log Po/w (XLOGP3)	1.62	Log Po/w (SILICOS-IT)	-2.22
Log Po/w (WLOGP)	0.02	Consensus Log Po/w	-0.34

281

282 Molecular Dynamics Simulation studies

283 Simulation study was performed for three protein-ligand complexes namely epac2-
 284 cAMP, epac2-BrefA and epac2-BrefA1 (Bref A best docked variant). Each of the complex
 285 was subjected to 200ns simulation in a neutral orthorhombic environment.

286

287 **Simulation interaction analysis** An in-depth analysis of P-L contacts was conducted to
 288 confirm the stability of ligand binding efficiency. Throughout the length of the
 289 simulation, the ligands were engaged in stable interactions with target residues. The
 290 interactions of epac2-cAMP remain consistent with G404, A407, R414, A415, K450,
 291 K489. K450 exhibits hydrogen bond interaction with N1 and N2 of adenosine ring,
 292 throughout the course of simulation. Epac2-BrefA interactions are D402, L406. Both the
 293 interactions exhibit hydrogen bonds significantly mediated via water bridges. Epac2-
 294 BrefA1 have interactions with D402, L406, A 407 R414, A415 and K450. All the residues
 295 have exhibited stable hydrogen interaction, with D402 forming salt bridge in the
 296 simulation course. Consideration of hydrogen-bonding properties in drug design is
 297 important because of their strong influence on drug specificity, metabolism and
 298 adsorption. The interaction diagram (Fig 3 panel b) suggests stronger interaction in
 299 epac2-brefA1 complex with higher number of hydrogen bonds.

In (Fig 3 panel a), protein ligand interaction is summarized and stacked as bar plots suggesting “a particular type of interaction” over a total period of simulation. A score of 0.5 indicates that for 50% of total simulation time the interaction existed. A score >1.0 can exist and suggests that more than one atom is involved in interaction. The stable complex of epac2-brefA1 has D402 forming hydrogen bond, ionic bond mediated via water bridge. A G404, R414 and A415 have shown strong hydrogen bonding with minimalistic mediation of water bridge. These stable interactions have greater impact on the stable conformational change induced onto EPAC2.

Fig 3 2D Interactions and protein-ligand contact histogram for 200ns (Panel a) from left consists of epac2-cAMP, epac2-brefA and epac2-brefA1 complexes subjected to 200ns simulation. The interaction of ligands with residues exhibiting over the course of simulation are recorded. The histogram (Interaction fraction vs Residues) suggests the percentage of specific type of interaction of an amino acid with ligand. Green suggests hydrogen bonding, Blue for water bridges and pink for ionic interactions. (panel b) from left consists of 2D interaction for epac2-cAMP, epac2-brefA and epac2-brefA1 complexes respectively. The plot suggests respective interactions at the end of simulation has been shown along with percentage contribution of each interacting residue with the compound. The above images were developed using Desmond 2019-2 (https://www.deshawresearch.com/resources_desmond.html) available free for academic and non-commercial usage.

Root Mean Square Deviation (RMSD) of protein structure

Monitoring RMSD calculation for C- α atoms of all residues over the course of the simulation gives in-sights into the structural confirmation changes undergoing. Changes of the order 1-3 Å are perfectly acceptable for small and globular protein. Larger the change, higher will be the protein structure evolving the course of simulation. All the three complexes were subjected to simulation of 200ns to study the changes induced by ligand interactions.

Both epac2-cAMP and epac2-BrefA have a similar RMSD change of 5-6 Å (Fig 4a). The epac2-cAMP on contrary exhibits an initial shift which results in hinge mechanism and availability of RAS-GEF domain for interaction. On the contrary, epac2-BrefA1 showed a lot of variation. In (Fig 4b), it can be clearly visualized that from 1 to 40 ns time frame the fluctuations were ranging from 1 to 32 Å. From 40 to 100ns time frame, the protein structure evolved by a stabilized increasing trend in RMSD values. After 105 ns, the fluctuations in RMSD stabilized suggesting the protein structure attaining stable conformation.

The changes in the tertiary structure of epac2 can be compared by superimposition of simulated structure with reference structure (PDB id - 3cf6). In (Fig 4 panel c), a compare and contrast view on the change in structure is provided. Epac2-brefA superimposition is almost in alignment, as which is in terms with literature evidence. The epac2-cAMP on superimposition provides a clear picture on the conformational change induced, hinge movement and availability of RAS-GEF domain for further interaction with Rap1b. Epac2-brefA1 interaction is in no alignment with the reference structure validating the change epac2 conformation.

The changes in the protein structure at the end of simulation has been captured by calculating the residue depth for each of the complex. In (Fig 4 panel d), the distance of each residue from the centre of the structure is calculated via distance ($\text{\AA}$). Based on this calculation, the nature of a residue whether buried or exposed to surface is plotted. This change in peaks suggest change in residue position and structure. This further validates the change in RMSD and superimposition results.

Conclusion drawn is binding of BrefA1 in cAMP2 domain induces a complete re-conformation of EPAC2 structure. This change can be justified purely due to increase in binding efficiency of BrefA1 relative to cAMP. The steric effect in protein is also due substituted side chain atoms of BrefA1 and its interaction with cAMP2 domain.

The brefA1 induced changes are not leading to any breakage in protein structure and that the stability is attained in course of simulation.

Protein stability evaluation Each of the docked complex at the end of simulation was subjected to protein structure quality evaluation. Ramachandran plot scores for all residues in allowed regions are 98.5 %, 98.9% and 99.0% for epac2-cAMP, epac2-BrefA and epac2-BrefA1 respectively (Fig 4 panel e). These results suggest stable confirmation of protein tertiary structure.

Fig 4. Analysis of structural conformations exhibited by epac2 on interaction with cAMP, BrefA and BrefA1 (a) A dual axis plot of RMSD ($\text{\AA}$) vs time frame (ns) plots for epac2-cAMP and epac2-BrefA show a similar change in deviation. Epac2-cAMP in red and epac2-BrefA in green rescaled to fit and highlight the change in RMSD. (b)Epac2-BrefA1 in cyan exhibit the RMSD change versus time. The above figures were plotted using R studio 1.1.456 (<https://rstudio.com/products/rstudio/download/>) and ggplot libraries (<https://rstudio.com/products/rpackages/>), free open source licensing

available. (Panel c) From left, Epac2-cAMP, Epac2-BrefA and EPAC2-BrefA complexes respectively were subjected to superimposition over the reference structure. Green corresponds to final structures after simulation over the reference structure (3CF6) in Red. The above images were developed using pymol version 2.3.2 (<https://pymol.org/2/>), available on trial version. (Panel d) From left, Epac2-cAMP, Epac2-BrefA and EPAC2-BrefA complexes respectively the residue depth is calculated and the residue versus depth in Å from the centre of the protein is plotted. The peaks in red refer to surface residues and gradient colouring to green for buried residues. The above images were obtained from DEPTH server (<http://cospi.iiserpune.ac.in/depth/htdocs/index.html>) (Panel e) From left, Epac2-cAMP, Epac2-BrefA and EPAC2-BrefA complexes respectively the Ramachandran plot of the protein structures after simulation to check structural stability. The images were plotted using Molprobit (<http://molprobit.biochem.duke.edu/>)

Antibody modelling

Single domain antibody (sdAb) design

The single domain antibody is labelled as nanobody contain single variable antibody domain. These are designed with antigen specificity and expresses stable interactions.

Design was carried out by selecting human variable chain sequence. A best template was 1DEE_B (Fig 5a) with a score of 0.98. The antibody comprises of 118 amino acids and CDR regions of modelled antibody (Fig 5b) are highlighted.

393

394 **Benchmarking of Protein-protein docking**

395 Interaction with epac2-Rap1b complex has been studied and validated structure is
396 available is considered as a reference standard. The same two proteins were subjected
397 to Protein-protein docking. The top 10 interaction results showed similar
398 domain/cavity in epac2 protein. 5 out of 9 residues of Rap1b has similar residues
399 interaction with epac2. These results prove the accuracy and efficiency of protein
400 docking protocols. The docking results are shown in (Supplementary Fig 2).

401

402 **Epac2 interaction antibody**

403 Two set of antigen-antibody interactions are carried out compare and contrast the
404 efficiency of antibody binding.

405 **Antibody interaction with simulated epac2** Epac2 protein simulated after interaction
406 with brefA1 is subjected to interaction with Rap1b. The top 10 interactions were
407 analysed and results which showed more stability are compiled in (Supp Table 3). We
408 found that there were no clashes with during the epitope mapping studies. Hydrogen
409 bonding and salt bridge was identified between Arg 100- Asp 331 and Asp72 and Lys
410 381. Lys 75-Asp 423 showed hydrogen bonding (Supp table 2). The mentioned
411 interactions showed stability proving efficiency. (Fig 5c)

412 **Mutagenesis of Antibody** From the antibody and simulated epac2 interactions we
413 compiled the most crucial residues of antibody to perform mutagenesis study. The
414 residues S55, Y100A, R100, D72, R19 and K75 were subjected to mutagenesis by their
415 substitution with alternative 19 amino acids to verify if it leads to increase in binding
416 stability and antibody interaction. The results are fluctuations in solvability scores are
417 recorded in (Table 3). It can be observed that R19 substitution with Pro, S55 with Lys,

418 K75 with Pro and Y100A with Pro lead to increase in delta (δ) stability of antibody
419 interactions. A graphical representation of increase in stability for crucial amino acids

Amino acids	CYS	HIP	ASP	SER	GLN	LYS	ILE	PRO	MET	THR	PHE	ASN	GLY	HID	HIE	TRP	ALA	VAL	GLU	TYR	LEU
-------------	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

420 with individual amino acids in (Fig 5 panel e). These above-mentioned substitutions
421 were performed in the antibody sequence.

422

423 **Fig 5 Antibody design and Antigen-antibody docking via mutagenesis** (a) The
424 template of 1DEE_B used for modelling the framework and loops of antibody (b)
425 Modelled antibody representing H1, H1 and H3 regions highlighted in red, green and
426 purple respectively. (c) Native Epac2 interaction with single domain antibody (d) Epac2
427 interaction with mutated single domain antibody. The above images were developed
428 using Maestro 2019-3 (<https://www.schrodinger.com/maestro>), available free for
429 academic usage. (Panel e) A graphical representation of the most interactive antibody
430 residues (x axis) subjected to mutagenesis plotted against alternative amino acids (y
431 axis) with the solvability score. The antibody was substituted with residues with highest
432 solvability score. The δ solvability scores can be obtained from table 3. The above stack plot
433 was plotted using R studio 1.1.456 (<https://rstudio.com/products/rstudio/download/>)
434 and ggplot libraries (<https://rstudio.com/products/rpackages/>), free open source
435 licensing available.

436

437 **Table 3 Mutagenesis table with solvation score.**

TYR:100A	ARG:100	LYS:75	ASN:73	SER:55	ARG:19
9.25	6.35	1.82	7.38	7.69	15.43
3.78	0.31	-9.79	7.98	16.29	8.89
9.42	3.87	10.86	8.07	9.2	20.93
8.84	6.23	2.6	6.92	8.12	15.32
6.26	1.28	4.65	0.76	0.67	13.11
13.47	7.11	-3.01	13.43	41.56	16.97
2.57	4.94	4.63	-1.6	9.72	13.72
38.91	26.9	56.81	6.25	34.49	32.75
1.18	-1.27	3.24	1.92	-5.04	5.46
7.73	5.15	2.37	2.34	2.84	16.57
-1.73	0.03	2.9	3.51	6.98	16.51
7.51	9.23	5.49	7.7	5.95	18.88
11.28	4.41	-0.31	9.4	9.41	17.83
1.68	1.36	1.81	3.23	9.41	12.89
5.45	1.29	3.61	4.89	13.19	6.59
1.67	-2.42	-15.75	2.18	5.91	17.45
3.15	4.95	5.92	-1.29	38.32	16.75
-1.43	2.85	5.07	-2.22	10.02	14.75
2.3	-3.23	4.36	2.92	4.86	17.76
10.17	1.13	2.53	0.9	12.79	15.98
4.8	-1.27	-1.23	-0.72	28.77	9.3

438

439 **Mutated antibody interaction with simulated epac2** The mutated antibody was
440 again subjected to docking with epac2. The results as expected showed increase in
441 interactions with higher number of hydrogen bonding. There was a total of 8 hydrogen
442 bonding interactions and 1 salt bridge (Fig 5d). We also found 2 clashes in the

interaction but each followed by a hydrogen bonding each would negate the effects of
stearic clashes. The antibody would be bound with increased efficiency. (Supp table 3)

Antibody interaction with native epac2 A native epac2 is the structural conformation
of epac2 before the BrefA1 induced simulation. The results suggest (Supp Table 4) the
interaction efficiency to be lower and the docking will not be stable due to presence of
clashes. From the top 10 interactions viewed, the best interaction has total of 59
clashes. The interactions also showed formation of 2 salt bridges between Asp 61-Lys
706 and Arg 99-Glu 585. The stability of salt bridges is not efficient enough over the
clashes occurring in the interaction. The lack of hydrogen bonding decreases the
stability of interactions too. This study provides enough evidence with lack of antibody
not interacting with sufficient stable interactions with epac2(Supp table 4).

This study proves that the designed antibody shows preferential binding ability with
simulated epac2 over native one. This antibody can be used for identification and
quantification of epac2 after the conformational changes with BrefA1.

Conclusion

The reprofiling of known inhibitor via combinatorial strategy is an efficient protocol to
understand the impact of side chain fragments on protein targets. The need to discovery
of new compounds can be avoided if the known compounds can be utilized more
efficiently via minor substitutions. In the current study, new variants of Brefeldin A
were docked against EPAC2 and best docked variant had better binding efficiency than
cAMP.

This has a direct impact on the protein conformation, due to the hinge mechanism of EPAC2. The change in conformation of EPAC2 makes its N-terminal domain inaccessible to the RAP1B protein. Herein Brefeldin A, a natural inhibitor due to minor substitution behaves as an antagonist to EPAC2. The known compounds/inhibitors will provide an advantage in its ADME properties over the design of newer compounds. The simulation studies and setting up a minimum time of 200ns will enable better understanding of structural changes on the target due to ligand interactions. The longer interaction period will provide insights into target structure stability which cannot be achieved over smaller simulation runs. This simulation results can avoid minimizing the time and resources needed for *In-vivo* studies by selecting only those interactions which have stable structures by end of simulation course.

In-silico design of antibodies prove to be an efficient method in understanding the interaction behaviour. The single domain antibody (sdAb) is specifically designed to bind with EPAC2 protein after binding with BrefA1 and change in conformation. The sdAb has relatively greater binding efficiency over native EPAC2 structure. The study includes CDR grafting and mutagenesis provide more confidence in the results. Single domain antibodies Selectively bind to antigens, less molecular weight, higher heat resistance and stable towards detergents. Diagnostic applications include Fusion with fluorescent protein called chromobody.

Studies including a robust workflow like these can be fruitful by saving time and resource by speeding up the process in wet lab validation.

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Declaration

Author Contribution

Methodology, analysis and drafting of manuscript by AUC. VN was involved in refining the methods, conclusions and review of final manuscript. All authors have reviewed the manuscript and agreed for submission.

Competing interests

The authors claim NO competing interests with any research lab.

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Supplementary Information

S1 Supplementary Figure 1: Similarity and sequence coverage of 4F7Z over other EPAC2 crystal structures available in PDB

The selection of 4F7Z as receptor for docking studies has been justified by providing a comprehensive view of domain and sequence coverage of the selected structure over others. The PDB ids are highlighted in yellow.

S2 Supplementary table 1: docking score and prime energy of lead like molecules from MM-GBSA output.

561 **S3 Supplementary Fig 2: Benchmarking results for Protein-Protein interactions**

562 The evaluation of protein-protein docking was benchmarked by comparison with
563 known interaction of epac2 with rap1b. The interaction residues are highlighted in red

564 **S4 Supplementary table 2: Antibody interaction with simulated epac2**

565 **S5 Supplementary table 3: Mutated antibody interaction with simulated epac2**

566 **S6 Supplementary table 4: Antibody interaction with native epac2**

a)

d)

b)

c)

e)

a)

b)

c)

d)

panel a

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panel b

Panel (e)