Structural perspective on revealing and altering molecular functions of genetic variants linked with diseases

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Abstract

Structural information of biological macromolecules is crucial and necessary to deliver predictions about the effects of mutations – whether polymorphic or deleterious (i.e., disease causing), wherein, thermodynamic parameters, namely, folding and binding free energies potentially serve as effective biomarkers. It may be emphasized that the effect of a mutation depends on various factors, including the type of protein (globular, membrane or intrinsically disordered protein) and the structural context to which it occurs. Such information may positively aid drug-design. Furthermore, due to the intrinsic plasticity of proteins, even mutations involving radical change of the structural and physico-chemical properties of the amino acids (native vs. mutant) can still have minimal effects of protein thermodynamics. However, if a mutation causes significant perturbation of either folding or binding free energies, it is quite likely to be deleterious. Mitigating such effects is a promising alternative to the traditional approaches of designing inhibitors. This can be done by structure-based in silico screening of small molecules for which binding to the dysfunctional protein restores its wild type thermodynamics. In this review we emphasize on the effects of mutations on two important biophysical characteristics, stability and binding affinity and how structures can be used for structure-based drug design to mitigate the effects of disease-causing variants on the above biophysical characteristics.

Key words: mutations, disease-causing mutations, polymorphism, folding free energy change, binding free energy change, drug discovery, in silico screening

1. Effect of mutations on stability and binding

Study of the effect of amino acid mutations within proteins has been a traditional chapter in protein science. Earlier studies were applying site-directed mutagenesis to assess the importance of an amino acid for stability and function of the corresponding protein. Nowadays, the focus has shifted to understanding the effects caused by genetic variants, namely, non-synonymous single nucleoside polymorphisms (nsSNP), with respect to disease predisposition. The phage-T4 lysozyme, for example, has served as one of the most well studied systems with regard to mutations [1]. These studies were facilitated by the availability of X-ray structures (native and mutants) allowing for structural investigations of the effects on protein packing, stability and activity. At the same time, lysozyme mutants in human have also been studied to characterize the molecular mechanism of diseases like hereditary systemic amyloidosis [2]. Similarly, the barnase-barstar protein-inhibitor complex was subjected to extensive mutagenesis to reveal the role of various residues on binding affinity [3–5] . This high-resolution complex has served as a model system to study protein-protein recognition by single and double mutant cycles [4,5] . Such studies have also served to rationalize optimization theories in the binding energetics [5,6] generally applicable to protein-protein recognition. Recent advances in the study of genetic (DNA) variants in the same system have also explored their influence in the manifestation of differential immunogenicity and this very property has then been applied in bio-therapeutics, for example, by constructing heterodimeric barnase-barstar DNA vaccine molecules [7], path-breaking in the development of novel DNA vaccines. Effectively, numerous works in molecular biophysics were and are focusing on understanding the effects of mutations on protein stability and binding. Below we review the relevant concepts and works associated with the two most fundamental biophysical events in protein science, folding and binding.

To begin with effects of amino acid substitutions on protein folding, we emphasize that the same substitution may have different effect occurring in globular, membrane or intrinsically disordered proteins. It is perhaps good to reiterate the fact that globular proteins are characterized by the presence of densely packed interiors (hydrophobic core) with packing densities (0.7 to 0.8) resembling to that of crystalline solids [8] and, that, interior packing is known to be one of the most dominant forces in protein folding [9], also related to the stability, dynamics and the *de novo* design of the foldable globules. The dense interior packing within globular proteins is known to be achieved by a nucleation-condensation of 'packing motifs' [10] , concomitant to the rapid collapse of hydrophobic residues in an aqueous environment. On the other hand, helix packing in integral membrane proteins [11] inserted within the lipid bilayer does not involve the 'hydrophobic effect' and yet scales to an equivalent magnitude of packing to that of the globular proteins [12] . The polar vs. hydrophobic environment presented in the two cases demand differential amino acid compositions in the two types of proteins to achieve an equivalent degree of packing in both. Interestingly, small hydrophobic (Gly, Ala) [11,12] and small hydroxyl-containing (Ser, Thr) [12] amino acids have been found to be contributing the most in tight packing of helices in membrane proteins as opposed to large hydrophobic and aromatic residues [10,13] in globular protein interiors. Apart from the tight packing of helices, membrane proteins are also known to involve distinct pattern of charges [14,15] embedded in their sequence to remain stable and active within the amphiphilic lipid bilayer. In dramatic contrast, in case of intrinsically disordered proteins (IDPs), the interior packing is practically negligible [16], since, (unlike globular proteins) the few hydrophobic residues in them are so placed that forbids the possibility of a hydrophobic collapse to attain a stable fold with a well packed core. This, in fact, enables them to retain their characteristic disorder or dynamic flexibility by means of existing as conformational ensembles rather than a single stably folded global minima structure likewise to either globular [9] or membrane proteins

92 [17]. The major component in retaining this dynamic flexibility in IDPs is electrostatic interactions
93 [18] involving hydrogen bonds, salt-bridges, charge-dipole, and dipole-dipole interactions. Hence,
94 when subjected to mutational studies, the sites to perform the mutations are chosen based on the
95 knowledge-based prediction of the expected differential effect in folding, stability and dynamics for
96 the three major class of proteins. For example, salt-bridge mutations have served to constitute one
97 of the prime chapters in understanding the *modus operanddi* in IDPs [19,20] while the study of
98 hydrophobic core mutations have traditionally served to probe interior packing within globular
99 proteins [21] to be discussed in more details in the next section. Electrostatics also serves as an
100 indispensable component in the folding and stability of globular proteins [22]. For membrane
101 proteins, mutations have been chosen mostly based on structure-function relationships [23] like
102 oligomerization [24], thermostability [25] etc. involving both packing and electrostatics. There have
103 also been instances of strategic point mutations (e.g., involving proline and/or glycine the well-
104 known helix-breakers) introducing kinks **(Fig.1)** and wedging on transmembrane helix-helix
105 interfaces [26]. Apart from the specific emphasis on individual structure-functional attributes of
106 these different classes of proteins, mutational studies have also been attempted as a mean to trace
107 their evolutionary origin (or common ancestor), particularly relevant in the context of the 'globular-
108 disordered interface' [27,28] in proteins.
109

110 **Figure 1. Plausible effect of mutations in membrane proteins:** Helical kink is introduced due to
111 *in-silico* mutations of two successive are residues (100-Ile, 101-Thr) to glycine (helix breaker) in a
112 KcsA potassium channel protein (PDB ID: 1J95).

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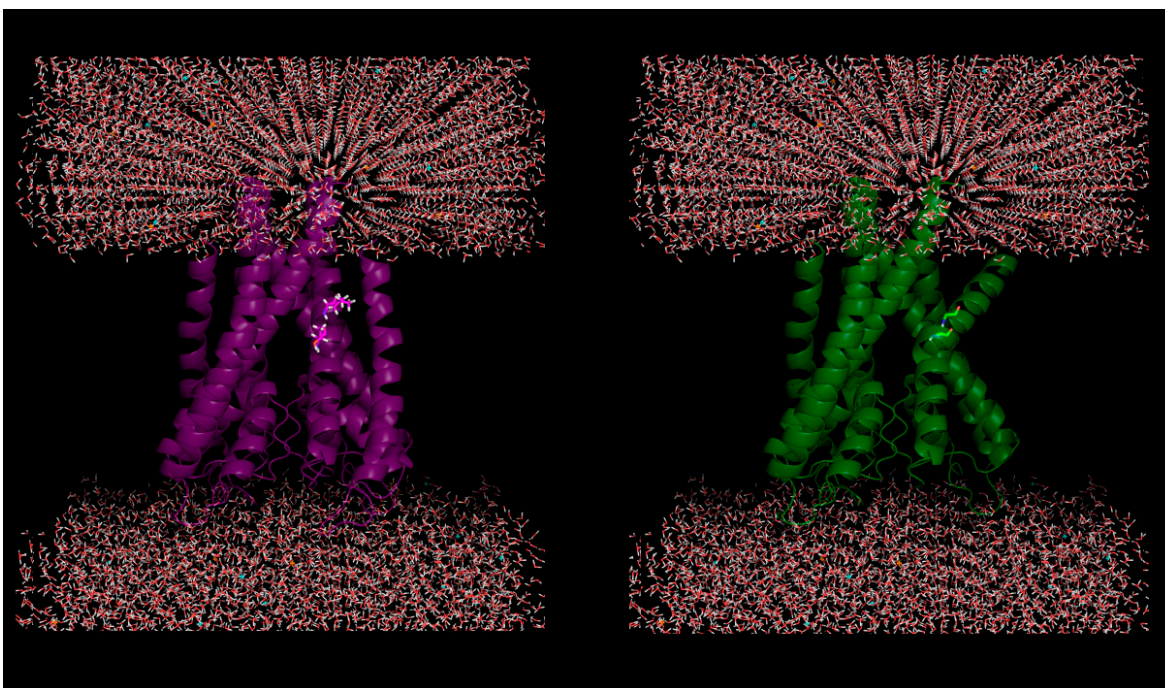
134 **2. Mutation and its Compensation: Structural Plasticity and Conformational Relaxation**
135
136 Plasticity in the context of protein conformations [29–31] describes their adaptability in response to
137 applied external forces (for example, by introducing mutations). This is a key physical property for
138 protein dynamics, wherein, it serves to facilitate protein evolution and other protein functions, such
139 as allostery and self-assembly [32]. Structural studies have further shown how conformational
140 relaxation of both main- and side-chain atoms could compensate the deleterious effects of
141 mutations, thereby preserving the overall fold [33,34]. The random mutation of the 12 out of 13
142 core residues of ribonuclease barnase is an example where 23% of the mutants retained their
143 enzymatic activity *in-vivo* [35]. Other similar studies followed and the idea of introducing strategic
144 multiple mutations was eventually extended into the realm of *de novo* design of proteins [36].
145 Parallel $(\alpha/\beta)_8$ – TIM barrel [37,38] served as an exemplary early model system, wherein, the
146 specificity in side-chain packing as well as the pattern of hydrophobicities, both, were detected to
147 play their part. However, from all such studies, it was unmistakable that conformational plasticity is
148 an inherent feature in proteins, resulting in structural relaxation to reduce the effect of mutations –
149 particularly applicable in the context of multiple core mutations **(Fig.2)** in foldable globules [39],
150 also relevant for IDP's [40,41].
151
152 **Figure 2**. **Effect of mutations in globular proteins:** 7 core (hydrophobic) residues mutated to
153 methionine (left panel: native, right: mutant) in phage T4-lysozyme and yet the fold is preserved
154 without almost any marked distortions. This happens because of 'structural relaxation' in proteins
155 due to their inherent conformational plasticity (adaptability to changes).
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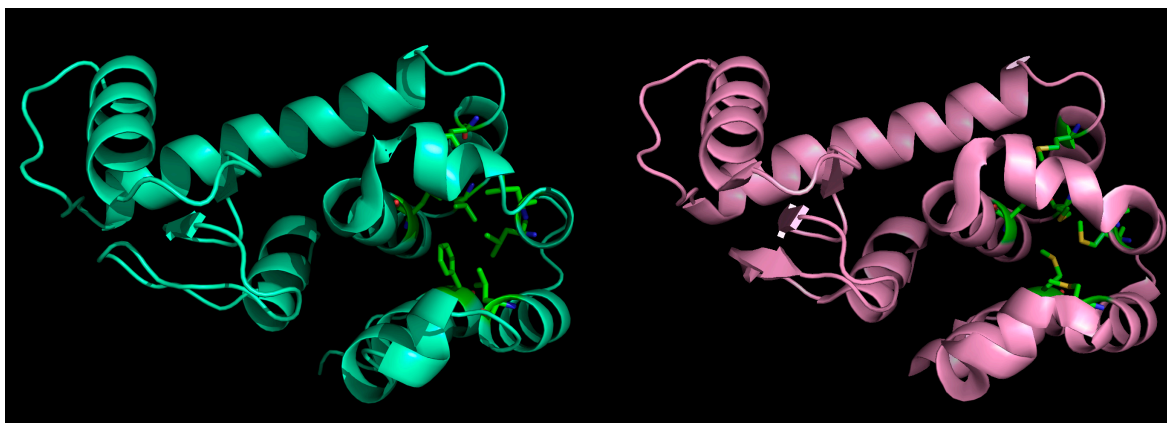
3. Mutations in IDP's as compared to globular and membrane proteins

Although one of the hallmarks of IDP's is to harbor a high degree of structural plasticity, this may not always guarantee compensation of the deleterious effects caused by certain missense mutations. We should recall that many human diseases like cancer, diabetes, neurodegenerative and cardiovascular disorders are associated with IDPs. Interestingly, similar mutational prototypes have generally been found to be more damaging in IDPs than in globular proteins [42–44] – which is somewhat paradoxical, given the fact that IDPs have a substantially greater degree of structural plasticity and therefore is expected to have a corresponding greater potential to compensate for the mutational damage than that of globular proteins. However, the step-wise molecular and cellular consequences of a certain mutational prototype is hierarchical, multi-factored and complex. For example, amyloidosis may be defined as the formation of amyloid fibrils in protein polymers consisting identical monomeric units which is the macromolecular end-effect responsible for many neurodegenerative disorders (e.g., Parkison's, Alzheimer's) which, in-turn, is a consequence of β-aggregation. Again, β-aggregation may be accounted for by hydrophobicity and / or β-sheet propensity of a protein region [43]. Comparative studies in α- and γ-synuclein have revealed increased aggregation in the former with a higher propensity for β-sheets – which further suggest that increased α-helical propensity in the amyloid-forming region may protect against γ-synuclein aggregation [45]. Interestingly, IDPs have been found to be more prone to amyloidosis in spite of having a much lower aggregation propensity (having only 1/3rd of aggregation nucleating regions) compared to globular (and membrane) proteins [43]. This high aggregation propensity also explains the considerable amount of structural frustration in globular proteins [43]. However, it may not be straightforward to draw a correlation between the solution conformations of amyloidogenic proteins and their pathogenicity. Though, lately, biophysical characterisation of misfolded states and their aggregation mechanisms have gained considerable attention [46], aggregation pathways remain complex (whether pathogenic or not), often involving peculiarities of protein misfolding and characterized by remarkable polymorphism, wherein, the final product may consist of soluble oligomers, fibrils as well as amorphous aggregates [47].

As a matter of fact, the 'disease-associated missense mutations' in IDPs are also found in a higher prevalence with greater functional impact [48] than the 'neutral polymorphisms' [49]. More importantly, the IDP-disease-mutations are found to be associated with the 'disorder-to-order transitions' [41] at a far greater frequency than the polymorphic ones [46]. The cancerous mutations in p53 [48,50] in its DNA-binding domain are classic examples of IDP-disease-mutations, wherein, dramatic destabilization of the domain renders it disordered at physiological

192 conditions [51]. Overall, there are many investigations associated with mutational studies on IDPs
193 revealing their molecular evolution   [27]   and pathological features [20]. Traditionally, the
194 mutations can be viewed as mostly 'hereditary' [20], chosen on the basis of geographic and ethnic
195 variations, pedigree of individual families with a history of a certain (say, the Alzheimer's) disease.
196 At the molecular level, one of the major insights revealed by these mutational studies have been the
197 influential role of salt-bridges in mediating the 'mutation-induced rigidity' associated with
198 enhanced aggregation of the candidate IDP – which has also found support by recent molecular
199 dynamic (MD) studies exploring the nitty-gritty and transient nature of salt-bridge dynamics **(Fig.3)**
200 in IDPs [19].

201

202 **Figure 3. Effect of mutations in disordered proteins.** Four transient (flitting) salt bridge forming
203 charged residues (1-Asp, 6-His, 16-Lys, 28-Lys) mutated to alanine in beta amyloid (Aβ42)
204 resulting in the dismantling in salt-bridges globally throughout the structural ensemble (Left Panel:
205 Mutant compared to the Right: Native). These transient salt-bridges continuously keep altering their
206 partners throughout the whole simulation trajectory supporting different conformations at different
207 time points and thereby supporting a conormational ensemble (illustrated in **Fig. 3.** of ref [19]). The
208 yellow dashed lines in the left panel (native) shows the salt-bridges found individually in the five
209 randomly chosen conformers (within 4 Å) while the same connections are shown by thinner cyan
210 dashed lines in the right panel to portray the absence of these ionic interactions (far far greater than
211 4 Å). Molecular Dynamic simulation trajectories collected from ref. [19]. Briefly, explicit-water
212 MD simulation was performed with AMBER 12 at T = 300 K using the ff99SB force field with
213 periodic boundary conditions and TIP3P water model. Figure reconstructed in Pymol.

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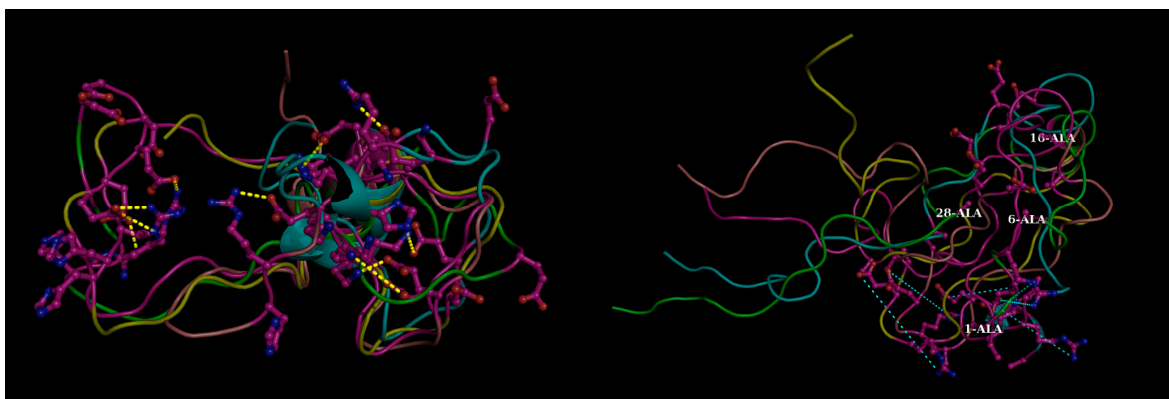
216 Another effective and important way to classify mutations may be based on the actual consequence
217 of a mutation as to whether it purely disrupts the structural integrity of a protein [52] or affect
218 protein functions. For example, proximal residues may co-evolve together in a protein fold to
219 preserve global stability, while, point mutations (including insertion-deletions) may potentially fine-
220 tune protein function, modifying functional sites and protein interactions [53]. Again, functional
221 mutations may be proximal or direct to the catalytic / active site [54,55] as well as distal (allosteric
222 and regulatory). The effect of distal mutations have been found to propagate throughout the whole
223 protein fold affecting both its dynamics and catalysis, wherein, low frequency torsional oscillations
224 [56,57] appear to play a pivotal role. Mutational hot-spots [58] have been identified (e.g., in Human
225 Monoacylglycerol Lipase, Human DNA Polymerase β) based on such long range communication

226 hubs in protein conformational dynamics [59–61]. Such information may also potentially facilitate
227 developing novel ligands with therapeutic value [59].
228

229 **4. Probing the role of mutations in diseases: tracking changes in thermodynamic parameters**
230 Changes in folding and binding free energies (ΔΔG) are the standard thermodynamic measures to
231 probe the effect of mutations on protein stability and binding [50]. It has been demonstrated that for
232 assessing the effect, one needs to take into account the relative change in ΔΔG with respect to the
233 ΔG_{WT} rather than considering ΔΔG alone [63]. Changes in ΔΔG were used to characterize sequence
234 and structural patterns on human disease-causing amino acid variants [64]. Particular attention was
235 paid on mutations involving reversal of biophysical characteristics of the wild type residue(s). For
236 example, salt-bridge mutations have been found to be typically disease-causing as demonstrated in
237 the case of hyper-aldosteronism, wherein, mere removal of the charge (while keeping intact the side
238 chain geometry) on a single strategic amino acid site (Glu → Gln) [65] and thereby effectively
239 dismantling a critical salt-bridge was found to be nitpicking. Salt-bridge mutations in IDPs have
240 also been found to be deleterious with enhanced aggregation of the proteins (e.g., in Alzheimer's
241 and Parkinson's Diseases) [20]. Recent MD simulation studies on IDPs have explored a plausible
242 interpretation of the corresponding molecular events, wherein, considerable reduction in the
243 conformational variation was found in $A\beta_{42}$ upon dismantling both high persistence as well as
244 transient salt-bridges [19].
245

246 Several computational approaches have been developed to predict folding and binding free energy
247 changes (ΔΔG) as a mean to link them with pathogenicity of mutations. These approaches vary
248 from sequence based [66,67], to structure based [68–70], depending on the input [62,64].
249 Methodologies vary from empirical approaches [71,72], first-principle approaches [73],
250 combination of knowledge-based terms and physics [74–76] to machine learning approaches
251 [77,78]. It should be emphasized that for effective drug discovery, one needs to know not only the
252 thermodynamic effects of mutation, but also the 3D structure of the target biomolecule(s). Notably,
253 for amyoidogenic proteins, kinetics may take over thermodynamics as aggregation is often found to
254 be kinetically driven [79]. It is also important to consider that a protein is never isolated in a cellular
255 context, rather, all biochemical cellular processes take place in heterogeneous, highly volume
256 occupied, crowded environments [80,81], wherein, stabilization of a particular protein [82] may
257 occur by complex formation with specific partner molecules. Taking this into account, most
258 biophysical experimental assays as well as computational methods may be seen as reductionist
259 approaches, wherein, free energy calculations may strongly be biased and would therefore require
260 corrections by appropriate normalization factors [83], also taking into account convergence and
261 sampling [84]. It is therefore of utter importance to at least perform cross-validations of the
262 thermodynamic parameters calculated between experimental (calorimetric and/or other indirect
263 spectroscopic techniques) and structure driven computational approaches [85] wherever applicable
264 and possible. Also, to that end, there have been studies vividly addressing the thermodynamic
265 consequences of excluded volume and macromolecular crowding, both, *in vitro* and *in vivo* using
266 labeled tracer macromolecules [80]. Strategies have also been proposed to extend quantitative
267 analyses of crowding from simple model systems to systems with increasing complexity up to the
268 labels of intact cells [80].
269

270 **5. Statistical classification of mutations based on their degree of harmfulness**
271 The effect of some mutations are more pronounced [86] than others. To that end, statistical studies
272 [64,87] have broadly classified the nsSNPs into two major categories: (i) polymorphic (or harmless)
273 and (ii) disease variants. The influential causal factors considered in such statistical studies are

274   genetic variations, frequency of occurrence and statistical measure(s) of the degree of harmfulness
275   [64,88]. The object of the exercise was to find empirical correlations between the variation type and
276   the degree of harmfulness, if any. To that end, the entire combinatorial space of 380 possible amino
277   acid mutations[1] (that can occur from a set of 20 naturally occurring amino acids) were explored and
278   the frequency of each mutation in the corresponding database was recorded. To overcome any
279   possible database-bias, the calculations were repeated as a mean to cross-validate the results on
280   updated database(s). Major observations were that in the HumVar dataset [89], 108 out of 380
281   possible mutations were never found, while, contrastingly, the top 26 most frequent variants made
282   up as much as 46% of the whole dataset. As a matter of fact, only about 1/4th (only 87 out of 380) of
283   the variants were found to belong to the "harmless" category [64] in the same database. As a cross-
284   validation, when the analysis was repeated in an expanded dataset of more than 3-fold increased
285   size, a jump was observed in the 'polymorphic-to-disease variant' ratio from 0.74 to 1.54 in the new
286   compared to the older dataset. Such indifference resulting from database bias inherent to all these
287   statistical / knowledge-based approaches actually speak in favor of using $\Delta\Delta G$ as a more reliable
288   and preferred probe to be applied on a case-to-case basis to make predictions about the effect of a
289   particular mutation in relation to pathogenicity.

6. Mitigating and clustering the effects of disease-causing genetic variants in relation to drug design:

293   With the rapid development of computer techniques, computer-aided approaches have been widely
294   applied in aiding early-stage drug discovery both in industrial as well as in academic projects [90–
295   93]. By discovering the potential compounds that target and affect the function of specific proteins,
296   biological processes can be modulated to mitigate or eliminate the disease-causing effects [90,92].
297   Advances in human genome projects have provided a large plethora of target proteins for drug
298   discovery projects [94,95]. Meanwhile, breakthroughs in structural biology have offered in-depth
299   structural information of more and more targets and elucidated the disease mechanisms at the
300   molecular level [96–99]. Such advances have further stimulated the application of computational
301   approaches to integrate the available structural information, functional mechanism and physico-
302   chemical properties related to drug discovery [91,100]. Drug discovery traditionally is a time- and
303   energy-consuming process and it would be difficult to imagine that the process can be reduced to
304   the time-span of (say) cancer illness of a single patient. Then again, discovery of compounds to
305   mitigate or eliminate the disease-causing effects induced by a specific amino acid mutation is the
306   main goal of Personalized Medicines [101]. In other words, benefiting from individual's genomic
307   information (by means of comparing to the sequence consensus of the standard human genome),
308   followed by the identification of drug-like compounds such as screening of FDA approved drugs
309   over a particular novel mutation may potentially provide precise treatment - which would target
310   specific disease-associated mutations on these individuals. In addition, the individual's genomic
311   information can be of great help to include or exclude patients most appropriate for clinical trails at
312   the final stage of drug development - which would not only increase the safety of the patients but
313   also accelerate the drug testing process.

315   In a drug-design methodology, targeting specific disease-causing mutations and elucidation of the
316   mutational effects together is of great importance, especially for the approaches requiring structural
317   information of the target protein. Free energy calculation methods are used to determine the
318   dominant effects of mutations, whether affecting protein stability, protein binding or both. With the
319   in-depth analysis of the effect of mutations at the molecular-level, the disease-causing mutations in

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[1]   20 amino acids each can be replaced by one out of the other 19 makes it 380

320   the target proteins can further be clustered by their major effects such as destabilizing mutation,
321   catalytic mutations, mutations affecting dimerization or protein conformations [79,85–87]. Such
322   type of classification can help designing drugs for certain groups of mutations with similar effects
323   and is thus applicable to a broader spectrum of diagnosis and therapy.
324
325   **7. Structure-based approach in drug design:**
326   Structure-based drug design (SBDD) is the computational approach that rely on knowledge of 3D
327   structures **(Fig.4)** of the biological targets to identify or design the potential chemical structure
328   suitable for clinical tests [100,105]. With the explosion of genomic, functional and structural
329   information in recent decades, majority of biological targets with 3D structure have been identified
330   and stimulated the applications of structure-based approaches in the current design pipeline. SBDD
331   is popular for virtual screening to filter the drug-like compounds from a large library of small
332   molecules, including widely applied approaches such as docking and structure-based pharcorphore
333   design [75]. While the established high-throughput screening (HTS) allows for automatic testing of
334   a wide range of compounds (up to millions), the low success rate and high cost together limit its
335   applications. Alternatively, one can use computational approaches to reduce the number of
336   compounds subjected to testing [73,89], wherein, docking and structure-based pharmacophore
337   design are the two most popular approaches, targeting delatorious mutations.
338
339   **Figure 4.** Schematic presentation of drug discovery process to mitigate the effects of disease-
340   causing mutations.
341

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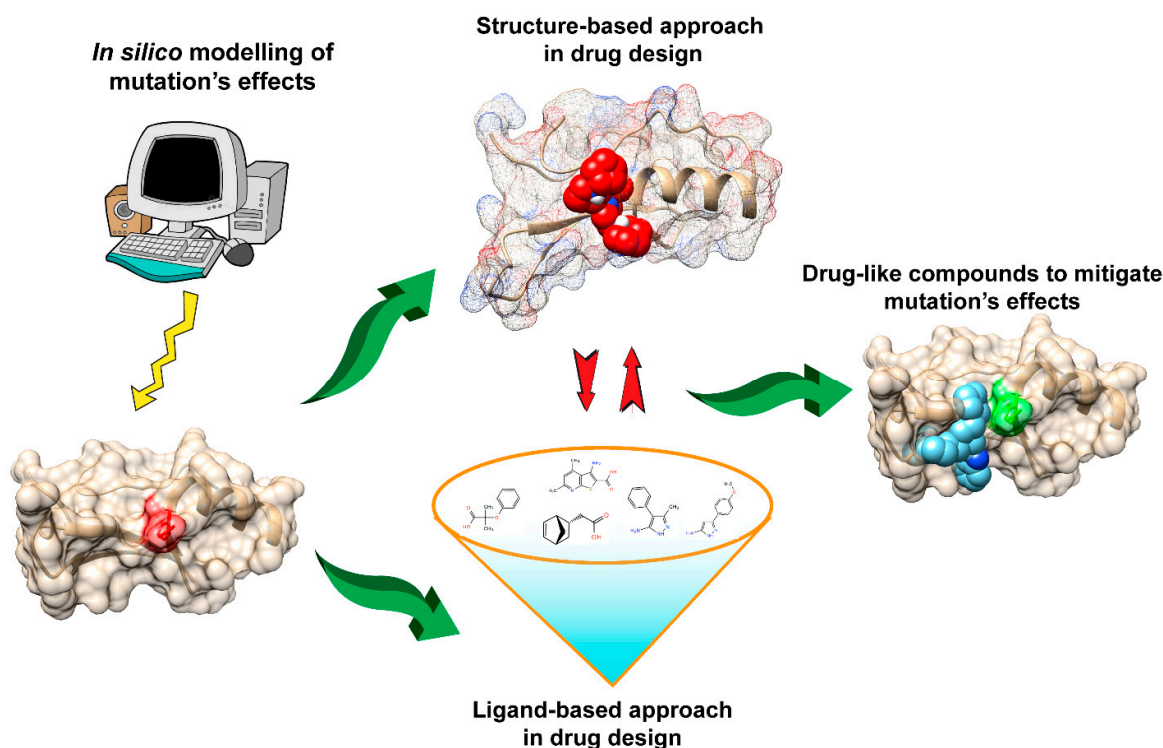
343
344   **7.1. Docking:**

345 Docking is one of the most common approaches for compound screening and the basic idea is to
346 use scoring functions to evaluate the fitness of the target protein in complex with the docked
347 compound [92]. Currently, vast docking programs have been developed to perform fast docking
348 calculations with a wide array of protocols and scoring functions, such as Dock6 [90], Autodock
349 Vina [91], Glide [109], Surflex [110] and many others. Such approaches require the structure of the
350 target protein, either experimentally solved or computationaly modeled. As mentioned above, one
351 should do intensive modeling to generate the best representative structure or set of structures to be
352 subjected to docking [91,111]. In the past, SBDD has been widely applied in mitigating the effects
353 of mutations related to many common diseases. Examples include the p53 protein, which is the so-
354 called "guardian protein" in cancer, functioning as a tumor-suppressor [95]. Again, only, some
355 mutations in p53 result in the malfunctioning of the protein and increases the risk of cancers [108].
356 In cancer patients, mutations destabilizing the DNA binding to p53 are frequently observed and
357 rescuing the native function(s) in the 'mutant p53 protein' is one central objective in current cancer
358 research [114,115]. In the past, it has been shown that binding of small molecules can stabilize the
359 DNA binding domain and rescue mutant functions [110]. Recent work modeled the wild-type and
360 several mutants [116] to elucidate the mechanism of p53 reactivation [116]. A novel transiently
361 open L1/L3 pocket was identified and indicated the exposure of Cys-124 in the formation of such
362 cavity [116]. Such finding is crucial as Cys-124 has been suggested to be the covalent docking site
363 for known alkylating p53 stabilizers [100] while, compounds can be docked onto this pocket to
364 search for other potential stabilizers. As a matter of fact, 1,324 compounds from the NCI/DTP Open
365 Chemical Repository Diversity Set II were docked onto the generated ensemble structures of
366 R273H cancer mutant out of which 45 compounds were selected for biological assay [116]. Finally,
367 one compound, stictic acid (NSC-87511) **(Fig.5)** was experimentally validated to be an efficient
368 reactivation compound for mutant p53 [99].
369
370 **Figure.5.** Rescuing the activity of mutant p53 by binding stictic acid into the open L1/L3 pocket.
371 The representative scheme is generated using the structure of p53 core domain complex with DNA
372 (PDB: 1TSR).
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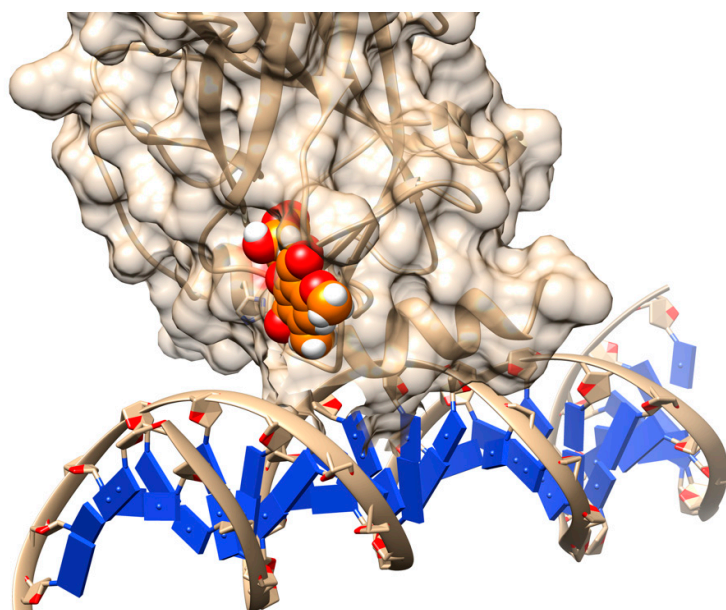
376 Besides cancer research, docking based screening has also been used in rare diseases. Snyder-
377 Robinson Syndrome (SRS) is a rare X-linked mental disease, caused by the malfunctioning of an
378 important human enzyme, the spermine synthase [101]. Spermine synthase functions as homo-
379 dimer and mutations affecting the dimerization such as G56S are shown to abolish the enzyme
380 activity to result in the disease [63,118]. Recent work has targeted identification of dimer stabilizers
381 by binding to the mutant homo-dimer interface [119]. Integrated large commercial compound
382 libraries were used for this docking-based virtual screening with the representative structures of the
383 dimer [119]. The best-ranked 51 compounds were then subjected to experimental screening out of
384 which three top-ranked compounds (also known as 'leads') have been shown to enhance the
385 catalytic activity up to 30% [119,120].
386
387 **7.2. Structure-based Pharmacophore design:**
388 Pharmacophore models can be used to make an ensemble of abstract steric and electronic features
389 representing macromolecular (target protein) interactions with drug-like small molecules
390 [121,122]. In other words, three-dimensional arrangement of these features such as hydrophobic
391 centroids, aromatic rings and hydrogen bonds are representation of the binding mode between the
392 ligand and the target [122,123]. Pharmacophores are generated from common features of active
393 ligands, which are identified by aligning or superimposing the conformers of either ligand-target
394 complexes or known active molecules [123]. Multiple degenerate atomic models can potentially be
395 output from pharmacophore modeling programs requiring further optimization and validation to
396 select the best one. Pharmacophore models are commonly used for virtual screening of active small
397 molecules from large compound databases [121–123]. Such approaches can be more efficient than
398 docking for certain targets, especially when a large number of existing known active compounds are
399 available [124].
400
401 Pharmacophore models have also been used to identify active molecules to mitigate the effects of
402 mutations in many diseases [108–111]. For the cases where a sufficient number of active molecules
403 are previously known for generating high-quality pharmacophore models, pharmacophore proves to
404 be a powerful tool for drug 'lead' identification [105]. Recent work has applied structure-based
405 pharmacophore analysis to identify the novel ROS-1 inhibitors to curb the drug resistance problem
406 caused by mutations [110]. Proto-oncogene receptor tyrosine kinase ROS-1 is ectopicly and
407 oncogenicly expressed in many cancers, mainly in non-small cell lung cancer (NSCLC) [112].
408 ROS-1 is highly homologous with the kinase domain of anaplastic lymphoma kinase (ALK) and
409 FDA approved ALK inhibitors such as Crizotinib are experimentally validated as therapeutics
410 against ROS-1 driven tumors [127]. However, these commercial ROS-1 inhibitors lack a broad
411 spectrum of activity due to the growing resistance from ROS-1 mutations, primarily G2032R [113].
412 Following on, a pharmacophore model was built using the complex structure of both wildtype and
413 mutant ROS-1 with previously known inhibitors to identify more general inhibitors against both
414 WT and mutant [110]. Pharmacophore-based virtual screening was then performed to selected
415 candidates from commercial databases with further filtering and scoring analysis. Five hits were
416 eventually identified with good binding affinities to both WT and mutant [110].
417
418 Thus, pharmacophore essentially defines the interaction framework among the active ligands and
419 their specific targets [105,106] and the corresponding models can also be built with libraries of
420 active ligands alone, in the absence of the 3D structure of the target – an approach known as ligand
421 based pharmacophore. The models, therein, can then be trained for discrimination between active
422 and inactive molecules [105]. In fact, this serves as the prime reason of widespread use of
423 pharmacophore models in virtual screening especially when lacking the target structure. In addition,

424   as the pharmacophore model represent the binding (or interaction map) of 'active compounds-target
425   interaction', it provides a plausible relationship between the structure and the ligand activity and
426   could help to elucidate the underlying biochemical mechanism to further guide the design of the
427   novel active compounds [122]. For example, by exploring the different pharmacological properties,
428   recent studies have seem to improve the potency of existing pharmacophore and designed novel
429   epidermal growth factor receptor (EGFR) inhibitor potentially inhibited by primary mutants
430   (L858R, del9) and drug-resistant mutants, such as, L858R/T790M [111].
431
432   **8. Ligand-based approaches in drug design:**
433   In the lack of structural information of the target protein(s), the aforementioned structure-based
434   approaches may not be suitable for drug design. As an alternative, ligand-based drug design
435   (LBDD) can be applied to aid such cases [114]. Ligand-based methods only focus on the analysis of
436   physico-chemical properties of known ligands that interact with the target of interests. Most popular
437   approaches, however, are the quantitative structure activity relationship (QSAR) models and the
438   ligand-based pharmacophore modeling [114]. In terms of drug design, targeting the mutant proteins,
439   LBDD could be efficient for novel mutations whose effects have not yet been investigated.
440
441   The basic assumption in ligand-based drug design is that small molecules with similar shape and
442   biophysical properties will likewise interact with the same target receptor [123,131]. By identifying
443   the fingerprints of known active ligands and constructing LBDD models, large databases can be
444   screened to retrieve the novel compounds as potential leads for the target of interest [114]. QSAR is
445   a widely applied LBDD approach, which utilizes mathematical models to correlate the
446   physiochemical properties of compounds to their experimentally measured bio-activity. Generally,
447   QSAR methodology identifies the molecular descriptors associated with properties of the ligands
448   and further uses mathematical models to discover correlations between the molecular descriptors
449   and their biological activity. Finally, these QSAR models are tested and validated for the predicted
450   biological activity of the compounds. As it stands, the current *state-of-the-art* is to apply the QSAR
451   models widely in computer-aided drug design, targeting the mutant protein(s). One major success is
452   the discovery of the potential corrector for cystic fibrosis (CF) mutations, namely, F508del in cystic
453   fibrosis transmembrane conductance regulator gene (CFTR) [115]. F508del is the most frequent CF
454   causing mutation, which leads to the improper folding of the protein and its degradation [116].
455   Subsequent to the identification, QSAR analysis has further been applied to guide the synthesis of
456   novel compounds to treat CF by improving the trafficking of the mutant CFTR (the CF corrector)
457   [117]. Recent works have collected all compounds known to improve the F508del trafficking and
458   then applied QSAR analysis to decipher the critical chemical descriptors for the potential F508del
459   correctors [117]. A novel predictive model was then constructed with these descriptors to provide
460   further guideline to the design and optimization of the novel corrector [117]. Again, the
461   combination of ligand and structure-based approaches is expected to add significantly more to the
462   current *'state-of-the-art'* [135,136]. Such combinations can either be sequential, parallel or hybrid,
463   integrated contextually into a drug discovery pipeline, and, have already shown much promise
464   [136].
465
466   A more sophisticated case would be to consider targeting proteins that lack both 3D structures and
467   known active ligands and therefore won't have sufficient information to build robust
468   pharmacophore and QSAR models. On such instances, one may switch on to sequence-based ligand
469   predictor approaches such as meta-structure [137]. The basic idea behind developing meta-structure
470   is the transformation of 3D structural information into the topological space via calculating the
471   residue interaction networks from a database [137,138]. The residues and the corresponding

472 neighborhood relationships are represented by nodes and edges. The predictor is trained against sets
473 of representative protein 3D structure to derive statistical topological information for all possible
474 amino acid pairs and thus can be subsequently used to perform predictions solely based on primary
475 sequences [137]. Based on the sequence analysis, the quantitative information about the local
476 secondary structure and residue compactness for each residue can be acquired to describe the
477 intricate interaction networks in the topological space for the target protein [137]. Such meta-
478 structure features can further be applied in drug development especially for target proteins that lack
479 3D structural information. For example, inspired by the protein-structure similarity clustering
480 (PSSC) approach in structure-based drug development [139], the meta-structure similarities in
481 ligand binding site can be used to cluster proteins with similar ligand-binding properties. Thus, the
482 meta-structure features of any one member of the cluster would serve as a valuable starting point for
483 ligand development of other members in the cluster [137].
484
485 **9. Aiding drug design by the knowledge of mutations on globular, membrane and disordered**
486 **proteins**
487
488 Understanding of molecular mechanism of disease-associated mutations can directly be applied to
489 drug design [91,101]. Structural biology has been instrumental in such understanding, effectively
490 contributing to early drug discovery [140], and, also elucidating the impacts of disease-associated
491 mutations and drug resistance in cancers and infectious diseases. Information regarding the
492 differential effects of mutations on globular, membrane and disordered proteins may serve
493 beneficially to select and apply the most appropriate and effective strategy to design potential drug-
494 like molecules for each individual case. Majority of diseases are directly associated with the
495 alterations of binding stability or folding stability of mutated proteins [63] - probed by binding or
496 folding free energies. Such information also indicates as to what extent the mutations are disrupting
497 the protein interactions or structural integrity providing important guidelines towards the design of
498 stabilizers and/or inhibitors to mitigate or eliminate the deleterious effects of mutations.
499
500 In addition, having 3D structures of target proteins is of great advantage to be used in free energy
501 calculations coupled with MD simulations to extensively investigate the underlying structural
502 mechanism (e.g., disruption of the hydrophobic core or loss of hydrogen bonding) of the mutational
503 effects on binding or folding. As discussed in an earlier section, such information has been
504 successfully used to identify the correct drug-like molecules targeting the mutations related to
505 Snyder-Robinson Syndrome (SRS), a rare X-linked mental disease. SRS is caused by the
506 malfunctioning of the human enzyme, spermine synthase (a globular protein), wherein, the known
507 existing delatorious mutations affect the native protein functionality by a wide range of molecular
508 mechanisms such as dedimerization, destabilization of the monomer and disruption of the catalytic
509 core [96,119,120,141].
510
511 Mutational resistance towards drugs also limits the lifetime of many successful drugs. As an
512 alternative to the design of novel drug-like molecules to overcome such resistance, strategies like
513 ensemble-based protein design [142] have been developed to be administered early in the
514 development process to predict and overcome the effects of possible mutational resistance (e.g., in
515 dihydrofolate reductase of *Staphylococcus aureus)*. Such design protocol has a dual attribute,
516 namely, positive design to maintain catalytic function and negative design to interfere with binding
517 of a lead inhibitor simultaneously.
518

519   Alteration in protein conformation and dynamics are also closely related to tons of human diseases
520   [143–145]. Computational approaches such as MD or mote carlo (MC) simulations are powerful
521   tools to study protein dynamics. Mutations can alter protein dynamics in various ways such as
522   altering local flexibility, transition in conformational states, allosteric regulations etc. Exploring
523   allosteric regulations may serve as potential alternatives for the cases where the native binding
524   pocket deems too difficult to bind with small molecules [91]. Solving experimental structures of
525   destabilizing mutants are often found difficult, particularly for membrane proteins due to their
526   inherent insolubility and instability [146,147] and in such cases molecular modeling of mutant
527   structures can give some guidelines about the mutational effects. Such alternative structures are
528   frequently subjected to docking of compound libraries in virtual screening – a methodology known
529   as "ensemble-based drug design" [148,149]. Especially, for IDPs where there is a definite lack of
530   ordered structures, molecular modeling and MD simulation have together been widely applied to
531   retrieve the representative structural ensemble in structure-based drug design [145,150].
532
533   On the other hand, high-throughput screening and rational drug design has considerably aided
534   drugging membrane protein interactions as they are accessible on the cell surface and can directly
535   alter cellular signaling [151]. This in fact is the key reason why the majority of therapeutics target
536   membrane proteins. Techniques like alanine scanning has also served to identify stabilizing
537   mutations in the computational design of membrane proteins as well as in drug development [152].
538   To that end, frameworks like RosettaMP have been developed to provide a general membrane
539   representation that interfaces with scoring, conformational sampling, and mutation routines offering
540   great ease and flexibility to integrate them into new design protocols [153]. Peptide architectonics
541   [154] has been a relatively new addition in the subject, wherein, the idea is to select for sub-
542   sequences of a native peptide, selectively toxic towards the pathogenic membrane proteins alone.
543   As an alternative to drugging (as there is often a lack of structural information for transmembrane
544   proteins), engineering of protein therapeutics [147] has also been attempted to membrane protein
545   targets, though, its full potential is yet to be explored.
546

10. Conclusion:

548   Macromolecular structural analyses may potentially be used to aid probing of genetic variants
549   linked with disease. Such studies are usually complemented by a wide range of biophysical solution
550   assays and computational modeling. Research along these directions have also opened up avenues
551   towards developing diagnostic tools and plausible therapeutics. In such a context, it is of foremost
552   importance to conceptualize (i) how traditionally mutational effects on protein stability and binding
553   have been probed and (ii) the basis of the differential affects of mutations to different class of
554   proteins (globular, membrane and disordered proteins) based on conformational relaxation,
555   structural plasticity, compensation and other physico-chemical factors. In the second half of the
556   paper we take the opportunity to discuss how this knowledge-base of the effect of mutations in
557   globular, membrane and disordered proteins may potentially aid drug design. As a probing
558   technique, we particularly highlight the importance of tracking changes in thermodynamic
559   parameters (ΔG_{WT}) and also take the opportunity to discuss the limitations of knowledge based
560   approaches such as the statistical classification of mutations based on their degree of harmfulness.
561   The review particularly highlights the emergence of the ever-so-promising recent approach to
562   computationally mitigate the effects of disease-causing genetic variants, alternative to the traditional
563   approaches in designing inhibitors. A wide array of structure-based approaches in drug design
564   including docking, structure-based pharmacophore design and ligand-based approaches have been
565   vividly discussed along with their proper context of applicability, as to whether they are to be aided
566   in presence or absence of the experimental coordinates of the target protein and/or known ligands.

567

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570

571　**References**

572

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