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Combating Mutations In Genetic Disease And Drug Resistance: Understanding Molecular Mechanisms to Guide Drug Design

Abstract

Introduction: Mutations introduce diversity into genomes, leading to selective changes and driving evolution. These changes have contributed to the emergence of many of the current major health concerns of the 21st century, from the development of genetic diseases and cancers to the rise and spread of drug resistance. The experimental systematic testing of all mutations in a system of interest is impractical and not cost-effective, which has created interest in the development of computational tools to understand the molecular consequences of mutations to aid and guide rational experimentation.

Areas covered: Here we discuss the recent development of computational methods to understand the effects of coding mutations to protein function and interactions, in particular in the context of the 3D structure of the protein. Using these methods, novel insights into the mechanistic effects of mutations in disease and drug resistance can be obtained, which can be used to guide treatment options and design better, more efficient and personalised therapeutics.

Expert opinion: While significant progress has been made in terms of innovative tools to understand and quantify the different range of effects in which a mutation or a set of mutations can give rise to a phenotype, a great gap still exists when integrating these predictions and drawing causality conclusions linking variants. This often requires a detailed understanding of the system being perturbed. However, as part of the drug development process it can be used preemptively in a similar fashion to pharmacokinetics predictions, to guide development of therapeutics less prone to the development of resistance, and help guide the design and analysis of clinical trials, patient treatment and public health policy strategies.

Keywords

Mutational Analysis, Genetic Diseases, Drug Resistance, Cancer, Drug Design, Molecular Mechanism, Genotype-Phenotype Association.

Article highlights

- Scalable and reliable structural based computational approaches are providing detailed insight into the molecular consequences of coding mutations.
- These have been used to guide patient treatment strategies for renal cell carcinoma and genetic diseases.
- Using these methods, drug resistance mutations can be identified and predicted.
- Used in a preemptive fashion, these can help guide drug development in the search for new therapeutics less likely to develop resistance.
- Mutations can give rise to a phenotype through different molecular mechanisms which can be assessed via integration of computational methods.

1. Introduction

Changes at the genetic level can result in drastic changes in cellular phenotypes and behaviour. These changes can lead to disease, or provide selective advantages that promote the development of drug resistance. In particular, non-synonymous single nucleotide polymorphisms (nsSNPs) within the protein coding regions of the genome have been strongly associated with occurrence and predisposition of human disease and drug resistance, sparking great interest from the research community.

The rapid developments in high-throughput sequencing, including dramatic drops in the cost, have created vast opportunities to understand the link between our genomes and phenotypes. This has opened up the promises of personalised medicines, targeted therapies and targeted public health policies. In order to fully realise the potential of these developments, however, we still need to improve our understanding of what are the molecular consequences of a given mutation, and how do these lead to a given phenotype.

While considerable resources have been invested in the experimental evaluation of genomic mutations, characterizing mutation effects is a challenging task and impractical to systematically experimentally evaluate all possible mutations for a given protein of interest, even more considering the range of different mechanisms in which mutations can affect protein function and interactions. Traditional experimental approaches are also not efficient enough or don't achieve scalability required to provide real time guidance into patient treatment and public health policy. This has led to significant interest in the development of computational approaches to rapidly and accurately evaluate the effects of mutations. Figure 1 summarises how *in silico* mutation analysis can be helpful in deconvoluting genotype-phenotype associations obtained from the wealth of genomic variation generated from sequencing efforts, including shedding light into disease predisposition and its mechanisms in a molecular level. Such methods can also be used to mutation prioritization for further experimental investigation, identification and anticipation of resistant variants and resistance hot-spots, knowledge that can be applied in the design of drugs less prone to resistance as well as to drive the development of public health policies and aid in establishing more appropriate and personalised treatments.

2. Analysing the effects of mutations

The two most commonly used methods by clinical geneticists to look at the effects of coding nsSNP mutations in the human genome are SIFT [1] and Polyphen [2]. Other approaches include CADD[3] and MutationTaster[4]. These approaches use the protein sequence to evaluate whether a given mutation is likely to be pathogenic or not. However, they have been limited by the lack of mechanistic information they provide and their over-estimation of mutations likely to be pathogenic [5]. Structural approaches can complement these analyses by providing detailed mechanistic information, but historically have involved a trade-off between scalability and molecular level mechanistic information, with molecular dynamics approaches providing greater atomic detail, but proving impractical for comprehensive analysis of a large number of different mutations.

In the 1990's, efforts to utilise the expanding structural information available for many proteins led to the development of SDM [6] the first method for predicting the effects of mutations on protein folding and stability. Subsequent efforts by other groups led to a range of methods to predict the same effects, improving upon the accuracy but not considering the other potential structural effects mutations might lead to.

This was first addressed through the systematic application of cut-off scanning matrices [7, 8] to quantitatively and scalably predict the effects of mutations on the binding affinities to other ligands, including other proteins, nucleic acids, small molecules and metal ions[9-14]. Table 1 presents a summary of the main structure-based methods proposed over the past years to analyse the different effects of mutations on coding regions. While this started to allow the deconvolution of the individual molecular changes that might be occurring, the big question limiting their application, especially in a clinical setting, was how do these individual effects combine to lead to a phenotype? Recent efforts have started to integrate these structural effects in order to better understand phenotypes, and have been used to look at a number of different human health problems driven by mutations in protein coding regions [14-22].

Table 1. Recent structure based computational methods for analysing the effects of coding mutations.

Method	Web server*	Publication year	Reference†
Effects of Mutations on Protein Stability and Folding			
SDM	http://www-cryst.bioc.cam.ac.uk/~sdm/sdm.php http://structure.bioc.cam.ac.uk/sdm2	2011 2017	23 24
PoPMuSiC 2.1	http://babylone.ulb.ac.be/popmusic	2011	25
mCSM-Stability	http://structure.bioc.cam.ac.uk/mcsm/stability	2014	13
DUET	http://structure.bioc.cam.ac.uk/duet	2014	12
ENCoM	http://bcb.med.usherbrooke.ca/encom.php	2015	26
MAESTROweb	https://biwww.che.sbg.ac.at/maestro/web	2016	27
STRUM	http://zhanglab.ccmb.med.umich.edu/STRUM/	2016	28
ELASPIC	http://elaspic.kimlab.org	2016	29
Effects of Mutations on Protein-Protein Binding Affinity			
BeAtMuSiC	http://babylone.ulb.ac.be/beatmusic/	2013	30
mCSM-PPI	http://structure.bioc.cam.ac.uk/mcsm/protein_protein	2014	13
mCSM-AB	http://structure.bioc.cam.ac.uk/mcsm_ab	2016	9
MutaBind	https://www.ncbi.nlm.nih.gov/projects/mutabind	2016	31
Effects of Mutations on Protein-Nucleic Acid Interactions			
mCSM-NA	http://structure.bioc.cam.ac.uk/mcsm/protein_dna http://structure.bioc.cam.ac.uk/mcsm_na	2014 2017	13 11
Effect of Mutations on Protein-Small Molecule Interactions			
mCSM-Lig	http://structure.bioc.cam.ac.uk/mcsm_lig	2016	14
CSM-Lig	http://structure.bioc.cam.ac.uk/csm_lig	2016	10

* The URL links to the webserver to run the method. Links current as of April 2017. † The primary reference describing the method, and which should be cited if used.

3. Using mutation analysis to guide treatment: towards personalised treatments

3.1. Cancers

By analysing the molecular effects of mutations in common renal cell carcinoma genes, including *p15* and *SDHA*, these have been correlated to a patient's risk of developing renal carcinoma. This was best demonstrated by recent studies looking at mutations in the von Hippel-Lindau protein (VHL) associated with the development of clear cell renal cell carcinoma (ccRCC) [15, 16, 32, 33]. By assessing whether a mutation affected the stability of the protein, or disrupted interactions to Elongin or HIF-1α, a patient could be classified into high, medium and low risk groups that could help guide screening strategies and provide more focussed genetic counselling. The available clinical data from over 100 patients was integrated with a saturation mutagenesis analysis of all possible mutations on VHL producing Symphony, a relational database mapping experimental and predicted risks of mutations to its molecular mechanism, aiding the characterization of newly discovered variants.

Understanding cancer genetics has been important for the diagnosis and treatment of a range of other cancers[34, 35], with increasing interest in how the structural impacts of mutations can be used to interpret sequence information. This has led to recent efforts to map the COSMIC database onto protein structures.

3.2. Mendelian Genetic diseases

Alkaptonuria (AKU), also known as ochronosis or black bone disease, is a rare recessive inherited genetic disease and first metabolic disorder firstly described over 100 years ago. AKU is caused by coding mutations that disrupt structure and function of the enzyme homogentisate 1,2-dioxygenase (HGD), related to phenylalanine and tyrosine metabolism. HGD gene product folds to form a homo-hexamer disposed as two stacked trimers, quaternary structure which is necessary for enzyme function.

Two comprehensive analysis on AKU causing mutations were carried out in an attempt to characterize the potential molecular mechanisms on which mutations could disruption enzyme activity [17, 18].

Mutation effects on protein monomer stability as well as protein-protein and protein-ligand affinity were predicted with the DUET, mCSM-PPI and mCSM-Lig web servers respectively. Three mutation clusters emerged from this analysis, regarding the molecular mechanism for structure and function disruption: (a) mutations that greatly affected monomer stability, therefore preventing oligomer formation; (b) mutations greatly reducing protein-protein affinity between the hexamer components, also preventing proper oligomer formation and (c) mutations with mild effects on both monomer stability and protein-protein affinity, which together caused functional impairment. The structural analysis of mutations in other Mendelian diseases, for example Ornithine Transcarbamylase deficiency[36], have identified that disease causing mutations lead to altered protein stability and interactions. Mutations with these molecular consequences occurred in roughly similar proportions to those observed in AKU.

These observations have been validated experimentally and expanded to examine all known disease causing mutations for inclusion in the HGD mutation database[37], which could hopefully guide the development of new, more effective and personalised drugs to treat this condition. For example, subsequent efforts have identified molecular stabilizers that reverse the effects of the destabilising mutations, analogous to the recent successes on p53. They have also been used to classify patients in the SONIA2 clinical trial, as we know that the molecular mechanism of a mutation can alter how patients may respond to therapeutics[38].

Structural mutation analysis techniques have started to play important roles in the diagnosis of rare Mendelian genetic diseases. For example, establishing the genetic basis of epilepsy is a fundamental step for disease prognosis and choice of patient treatments[38]. Recently these methods were used to not only identify the genetic cause of a previously undiagnosed or characterized human cohesinopathy but also characterize the molecular mechanism, subsequently experimentally validated[39]. The potential for the structural characterisation of mutations to impact upon clinical practice will only continue to grow with the increasing availability of structural information, and routine use of exome sequencing in patient care.

3.3. Screening for drug resistance in tuberculosis

The reduction of sequencing costs, and improvements in accuracy and sensitivity, have led to interest in using high-throughput sequencing to diagnose patients, and identify drug resistance mutations. For infectious diseases such as tuberculosis (TB), where the drug susceptibility screening is time consuming and costly, genomic sequencing opens up the possibility of being able to more rapidly identify the correct treatment strategies for a patient, but also to guide public health policy by following the spread of resistance. Experimental innovations have allowed researchers to sequence the TB genome based on a sample of the patient's sputum, and Public Health England is now sequencing all new TB cases in the UK.

Many resistance mutations in TB have been well characterised, but one of the limitations of these approaches is how to interpret novel mutations identified within the genome. Due to the lack of horizontal gene transfer, TB is an ideal pathogen to apply structural based mutational analysis approaches. Looking at mutations in rpoB and katG, which leads to rifampicin and isoniazid resistance respectively, clear structural features were identified that correlated strongly with the resulting effectiveness of the drugs (MIC) [40]. A number of resistance mutations have also been observed across protein-protein interfaces, which raises the interesting hypothesis that similar to Mendelian disease mutations, those at interfaces might be prone to lead to disease and resistance because they have a lower fitness cost associated to them than those in the active site that completely disrupt activity [36, 41, 42].

While previous experimental and clinical knowledge about the effect of a given mutation in a given strain on drug susceptibility will always provide the gold standard for predicting and identifying drug resistance, structural based approaches complement this limited available information by providing the power to look at novel mutations.

4. Targeting resistance mutations: towards resistance-resistant therapies.

4.1. HIV protease 1 Inhibitors

HIV protease catalyses the cleavage of the polypeptide precursors into mature enzymes and structural proteins, an essential step in the HIV-1 replication cycle. Inhibitors targeting the HIV protease have been in clinical use since 1995 and include darunavir, amprenavir, atazanavir, nelfinavir, indinavir, saquinavir and lopinavir [43, 44].

Due to the HIV's error prone replication, resistance mutations against these inhibitors have evolved rapidly and been widely observed clinically, limiting the effectiveness of these therapies. These include mutations in the active site (V32I, L33F, I54M and I84V) that through changes in hydrogen bonding and Van der Waals interactions between the inhibitors and the catalytic site amino acids, can reduce their binding affinities [45, 46].

A better understanding of the effects of mutations on inhibitor binding and their molecular mechanism giving rise to resistance are crucial for designing novel drugs, more effectively and less prone failure. Computational structure-based methods have an important in tackling this challenge. The mCSM suite was successfully used to predict the effect of the aforementioned mutations upon the binding affinities. Molecular dynamics simulations have also been used to elucidate the effects of the protease inhibitor resistance mutations D30N, I50V, I54M, and V82A, providing interesting mechanistic information on how these mutations alter binding affinities, including changes in the binding conformation (I50V), conformational changes (I54M) and large enthalpic changes reducing binding affinity (V82A) [47]. While genomic methods have proven unreliable for phenotypic characterisation of HIV [48], this potentially offers a means to better leverage this information and suggests ways to guide new designs that avoid these common hot-spots.

The last HIV protease inhibitor approved, darunavir, was designed with this in mind and is capable of inhibiting the replication of both wild-type and multidrug-resistant strains of HIV-1. While earlier inhibitors interacted with the side-chains of Asp-28 and Asp-30, darunavir contained a *bis*-tetrahydrofuranylurethane functional group that made close, tight interactions with the main chain of these residues, making only minimal interactions with the side chains [49]. This made darunavir less sensitive to substitutions in either of these positions. Figure 2A depicts an alignment between darunavir and a non-peptidic inhibitor GRL-085 and the interactions made by the inhibitors (Figures 2B and 2C, respectively).

Many resistant strains against darunavir, however, have emerged. These mutations often lead to a change in the conformation of the active site residues, reducing affinity for darunavir, but also leading to a significant fitness cost [50]. In the effort to avoid these resistance mutations, current medicinal chemistry efforts have identified potent inhibitors that differ from the currently approved protease inhibitors by the number and proximity of contacts to the main chains of these catalytic amino acids [49]. These compounds will be hopefully even more effective therapeutics that are significantly less prone to develop resistance.

4.2. Influenza neuraminidase inhibitors

Influenza neuraminidase inhibitors (NAIs) are the major specific anti-influenza drugs used clinically, despite the emergence of resistance [51]. Currently, the NAIs oseltamivir, zanamivir, peramivir and laninamivir (currently approved only in Japan) have been approved to prevent and treat influenza A and B [51-54]. Many governments have stockpiled resources of these drugs in the event of an Influenza outbreak. During the recent H1N1 and H7N9 Influenza outbreaks significant resources were focussed on identifying and monitoring potential resistance mutations, primarily through genetic screening, with sporadic oseltamivir-resistant 2009 H1N1 virus infections identified. Thus, understanding the mechanisms of influenza NA drug resistance is crucial to develop drugs that can get around mutations and be more successful to fight the epidemics and pandemics [51].

A strong correlation has been observed between mutations that affect the slow binding and dissociation of these NAIs, and the association with resistance [55]. Resistance mutations that have been observed to residues E119 and I222 of Influenza A lead to high and slight resistance to oseltamivir and zanamivir respectively [56]. Figures 3A and 3B highlight these resistance hot-spots on the solved complex of the neuraminidase with oseltamivir and the interactions established on the wild-type protein. Mutations on E119, include substitutions to Gly, Asp, Ala, Ile and Val, lead to the loss of a salt bridge to the inhibitors [57], with zanamivir showing less susceptibility due to the presence of the 4-guanidino group that maintains typical interactions [51].

Mutations at I222 alter the hydrophobic drug binding pocket. While I222R leads to a reduction in oseltamivir, peramivir and zanamivir effectiveness [52, 58, 59], the I222L mutation, which is also found in Influenza B, has been reported to not lead to significant drug resistance [51]. The other common mutation in N2 is R292K, which leads to resistance against oseltamivir and peramivir and a slight reduction of zanamivir and laninamivir effectiveness [52].

Following treatment with oseltamivir, the N1 subtype specific substitution H274Y has also been observed, leading to resistance to this drug and also peramivir, but not to zanamivir and laninamivir [60, 61]. The change in volume of the side chains upon this mutation causes the carbonyl group of E276 to be shifted into the binding site of the enzyme, disturbing the hydrophobic pocket that would accommodate the pentyloxy group of oseltamivir [61].

Therefore in efforts to overcome some of these resistance problems, the guanidino group of zanamivir and the hydrophobic pentyloxy group of oseltamivir were merged [60]. The guanidino group was capable of inhibiting the spread of Influenza A with the hydrogen bond interactions between the guanidino group and neuraminidase binding site crucial for the inhibition of the enzyme and virus replication [61, 62]. However, the inhibition profile of MS-257 and zanamivir was comparable against the E119V and I222L mutant strains [51].

The sequence database compiled by the WHO containing lists of amino acid substitutions in the neuraminidase has been widely used to identify key mutations and regions, guiding genomic analysis of resistance and proving invaluable for testing new compounds targeting inhibition of neuraminidase [63, 64]. It has also facilitated the use of next-generation sequencing to detect resistance markers in the NA gene and predict the effect of drug treatment [65], which

have been complemented by the use of structural based approaches to identify likely resistance mutations.

4.3. Kinase drug development

4.3.1 Kinase Inhibition

Abnormal regulation of kinases through occurrence of mutations is responsible for many human diseases, including metabolic disorders and certain types of cancer [66]. The development of small molecule kinase inhibitors has therefore been seen as an attractive treatment option [67]. Unlike conventional chemotherapy (cytotoxic), molecular targeted therapies using kinase inhibitors are designed to act at specific biological points that are essential for development of tumour cells [68].

The design of kinase inhibitors has great impact on their efficacy and sensitivity to resistance. The first kinase inhibitors developed targeted the ATP binding site via competitive binding. As resistance to these inhibitors was identified, other strategies including allosteric and covalently bound inhibitors were used to avoid these common resistance mutations [67].

4.3.2 ATP-competitive inhibitors - First generation

ATP-competitive kinase inhibitors inhibit ATP binding in the catalytic site of the target kinase, or bind at alternative sites to induce conformational molecular changes that inhibit the activity of the enzyme [68]. Imatinib was the first kinase small molecule inhibitor clinically approved by the U.S. Food and Drug Administration (FDA) for treatment of chronic myeloid leukemia [69]. Imatinib binds to the active site of the target enzyme preventing other substrates from phosphorylation and consequently inhibiting kinase activity. Figure 4A shows the Abelson tyrosine-protein kinase 2 (ABL2) in complex with imatinib. The inhibitor only binds to the enzyme when it is in inactive conformation. Another example of an inhibitor with a mechanism similar to imatinib is gefitinib which is used for treatment of non-small-cell lung cancer through inhibition of the epidermal growth factor receptor (EGFR).

Despite the success of imatinib, studies have shown that patients can develop resistance and relapse after initial response to therapy. The effect of mutations linked to imatinib resistance were analysed by mCSM-Lig [14], which could correctly identify resistance mutations located even quite distal from the active site. mCSM-Lig quantitatively predicts the effect of mutations on small molecule affinity. Resistance mutations of competitive inhibitor, however, can exist by shifting the preference of the protein towards the natural ligand (ATP), not necessarily by dramatically reducing the affinity of the protein to the drug. Interestingly, using a fold-ratio between the predicted affinity effect on the natural ligand and the drug, mCSM-Lig was successful in identifying the majority of the imatinib resistance mutations.

Several mechanisms of resistance have been observed, including mutations in the BCR-ABL kinase domain, with the most common resistant observed the gatekeeper mutant T315I [70]. This amino acid substitution eliminates a critical oxygen molecule needed for hydrogen bonding between imatinib and the ABL kinase, and also introduces a steric clash preventing drug binding. The gatekeeper residue determines the relative accessibility of a hydrophobic pocket located adjacent to the ATP binding site, which is important for imatinib binding given that hydrophobic interactions are crucial for inhibitor binding affinity [67, 71, 72]. In fact, mutations

in gatekeeper residues have also been studied for other kinases in different types of cancer, such as the Threonine 790 of EGFR in Lung cancer that mutates to a methionine (T790M) increasing the affinity for ATP and making it difficult for the gefitinib to compete for the binding site [73-75]. Such mechanisms of resistance have contributed to the development of more sophisticated generations of inhibitors with mechanisms to overcome resistances conferred by these gatekeeper mutations.

4.3.3 ATP-competitive inhibitors - Second Generation

The second generation of small molecule kinase inhibitors preferentially bind to regions outside the ATP binding site, for example to the inactive conformation, also known as DFG-out, of the protein kinase. The transition from the active conformation to DFG-out conformation exposes additional hydrophobic pockets adjacent to the ATP site that can be used by the inhibitors to stabilize the kinase in its inactive conformation [76], preventing ATP binding.

Dasatinib is a multitargeted tyrosine kinase inhibitor that targets oncogenic pathways and is a more potent inhibitor than imatinib that binds only when the ABL enzyme is in its inactive conformation. Dasatinib is also effective against several imatinib-resistant ABL mutations that occur in regions that are in contact with imatinib or mutations involved in stabilization of specific inactive imatinib-bound conformation of the enzyme. However, the T315I gatekeeper mutation is also resistant to dasatinib due crucial hydrogen bond with the T315 side chain [77]. Figure 4B shows ABL1 in complex with dasatinib. The main residues involved in the binding of the drug are highlighted, including T315.

4.3.4 Allosteric inhibitors - Third Generation

These inhibitors regulate the kinase activity in an allosteric manner, exhibiting a higher degree of selectivity due the exploitation of binding sites and regulatory mechanisms that are specific to a particular kinase [67]. Figure 4C shows the allosteric inhibitor CI-1040 binding MEK1 immediately adjacent to the ATP binding site.

This class of inhibitors can bind either to the kinase domain (or close to the ATP binding site) or to sites outside the kinase domain. These range of options for inhibiting the catalytic activity of kinases represent clear advantages over the ATP-competitive inhibitors [78, 79]. However, the lack of methods to identify such inactive conformations or binding modes in kinases to drive the development of this type of inhibitor still remains a challenge [80]. Inhibitors that disrupt formation of the higher order oligomers, which play an important role in achieving high signal-to-noise throughout the signal transduction process, have also proven to be effective kinase inhibitors that avoid the common ATP resistance mutations [81-83].

ABL001, also known as Asciminib, is a potent and selective third generation kinase inhibitor with activity against chronic myeloid leukemia and Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia. ABL001 binds to the myristoyl pocket of ABL1 kinase leading to a formation of an inactive kinase conformation[84]. Recent studies have shown that treatment with ABL001 combined with ATP-competitive inhibitors can help prevent resistance in chronic myeloid leukemia[85, 86].

4.3.5 Covalent inhibitors - Fourth Generation

Recent studies [87, 88] described a fourth class of kinase inhibitors that are capable of forming covalent bonds to the kinase active site, most frequently by reacting with a nucleophilic

cysteine residue. Unlike first and second generation inhibitors, the fourth generation blocks the binding of ATP irreversibly preventing the kinase from being activated. Figure 4D shows the fourth generation inhibitor Neratinib (HKI-272) in complex with EGFR kinase T790M mutant, making a covalent bond to Cysteine 797.

4.3.6 Tackling kinase inhibitor resistance

Much of the effort to target and avoid resistance against common kinase inhibitors has focussed on the development of inhibitors with different modes of action. This has in part been driven by the lack of selectivity of the early inhibitors that targeted the ATP binding site- which is highly conserved among many proteins. Structural methods such as mCSM-lig and molecular dynamics approaches have been able to correctly identify and predict likely resistance mutations, which could also potentially facilitate the design of new inhibitors avoiding these resistance hot-spots, similar to the efforts in anti-viral inhibitor design. However, more practically, as sequencing of cancers is becoming more routine, these methods offer the opportunity to help guide the selection of the most effective therapeutics- facilitating the widespread implementation of personalised medicine.

The advent of fast and precise computational methods to predict effect of mutations can be leveraged to assist and guide the development of new drugs. Since resistance can emerge from different molecular mechanisms, current predictors can be integrated in novel drug resistance identification methods that can then be used in large-scale screening to identify better protein targets, identify and avoid potential resistance hot-spots as well as optimize ligand affinity and selectivity, driving the experimental design of better, more potent and efficacious drugs.

5. Expert Opinions

While significant progress has been made in terms of innovative tools to understand and quantify the different range of effects in which a mutation or a set of mutations can give rise to a phenotype, a great gap still exists when integrating these predictions and drawing causality conclusions linking variants, compounded by the need for detailed information regarding the system/protein. The availability of scalable, effective computational methods to assess mutation effects creates new opportunities of development of such integrated approaches and decipher complex genomic background patterns, shedding light into their role in the emergence of a given phenotype and molecular mechanisms of action. This capability can then be used to systematically study, for instance, how drug resistance emerges on specific drug targets, aiding the drug development process. Initial efforts on that matter have focussed on preparing predictors and databases for specific diseases and proteins, however greater effort needs to be invested in making these predictors user friendly, integrated and accessible to geneticists. This is particularly important considering that most structural information is a snapshot of a protein conformation, but how mutations affect the equilibrium between different states can play a very important role in disease and drug resistance [89]. A complementary and important effort refers to the collection and curation of experimental data regarding mutation effects linked to phenotype in comprehensive databases. This information forms the evidence set necessary for the proposal of novel computational methods as well as the improvement of current approaches. Initiatives like the Platinum database [90], the first curated online database

linking effects of mutations on protein-small molecule affinity for complexes with known structures, are fundamental.

Despite this limitation, these methodologies have already provided invaluable insights into many diseases. Current genomic analyses are dependent upon pre-existing information; either extensive genomic or biochemical analyses. This limits the insight and information that can be drawn regarding novel mutations. As these structural methods become more widely used, they will complement traditional analyses methods to provide much greater power from genomic analysis.

In the shorter term, the ability of these methods to predict likely resistance mutations before they arise offers enormous potential throughout the drug development process. Peter Coleman first suggested that the design of inhibitors that resemble transition state analogues should be more resilient to the development of resistance. Out of this Zanamivir was developed, the first successful structure guided drug development, but as we have seen over the intervening years resistance against Relenza has been widely reported, although it has been less prone to resistance than Oseltamivir.

During the development of a recent class of *Mycobacterium tuberculosis* IMPDH inhibitors, structural guided mutational prediction was used to identify likely resistance mutations, defined in this case as point mutations that disrupted inhibitor binding, but did not affect NAD binding, protein solubility or formation of the active tetramer. One mutation in particular, Y487C, was highlighted, and subsequently confirmed to be one of the few mutations to arise during resistance screening [91]. Subsequent drug development attempts avoided this resistance hot-spot and were active against the Y487C mutant [92]. This also enables the analysis of multiple mutations, some of which have been characterised to facilitate the development of resistance. In many cases, these seem to increase protein stability or natural ligand binding, which can be decreased due to the primary resistance mutation.

While current medicinal chemistry efforts are currently normally retroactive - we observe which mutations arise in the lab or clinic and then design new generations of inhibitors to target or avoid them - the power of computational mutational analysis enables us to pre-emptively identify likely resistance hot-spots, and to take this information under consideration when optimising candidate molecules. In a similar fashion to how experimental structures[93-97] and pharmacokinetic predictors are now widely used to guide medicinal chemistry efforts [98], playing a role in dramatically reducing failure rates of clinical trials due to these problems. The use of *in silico* mutational analysis in the development of new therapeutics will hopefully avoid likely resistance mutations. While the evolutionary forces and the constant selective battle makes the development of resistance somewhat inevitable, this will hopefully aid in the development of the next generation of therapeutics that are more resistant to the development of resistance.

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Novel method for calculating and displaying all possible intra and inter molecular interactions.

Figures

Figure 1. The use of in silico mutational analysis to tackle drug resistance and genetic diseases. Sequencing efforts generate a wealth of genomic variation. Computational mutation analysis can help deconvolute genotype-phenotype associations aiding in understanding the molecular mechanism of diseases and disease predisposition as well as in mutation prioritization for experimental validation, identification of resistant variants and resistance hot-spots, which can then fed into drug design pipelines as well drive the development of public health policies and choice of more appropriate and personalised treatments.

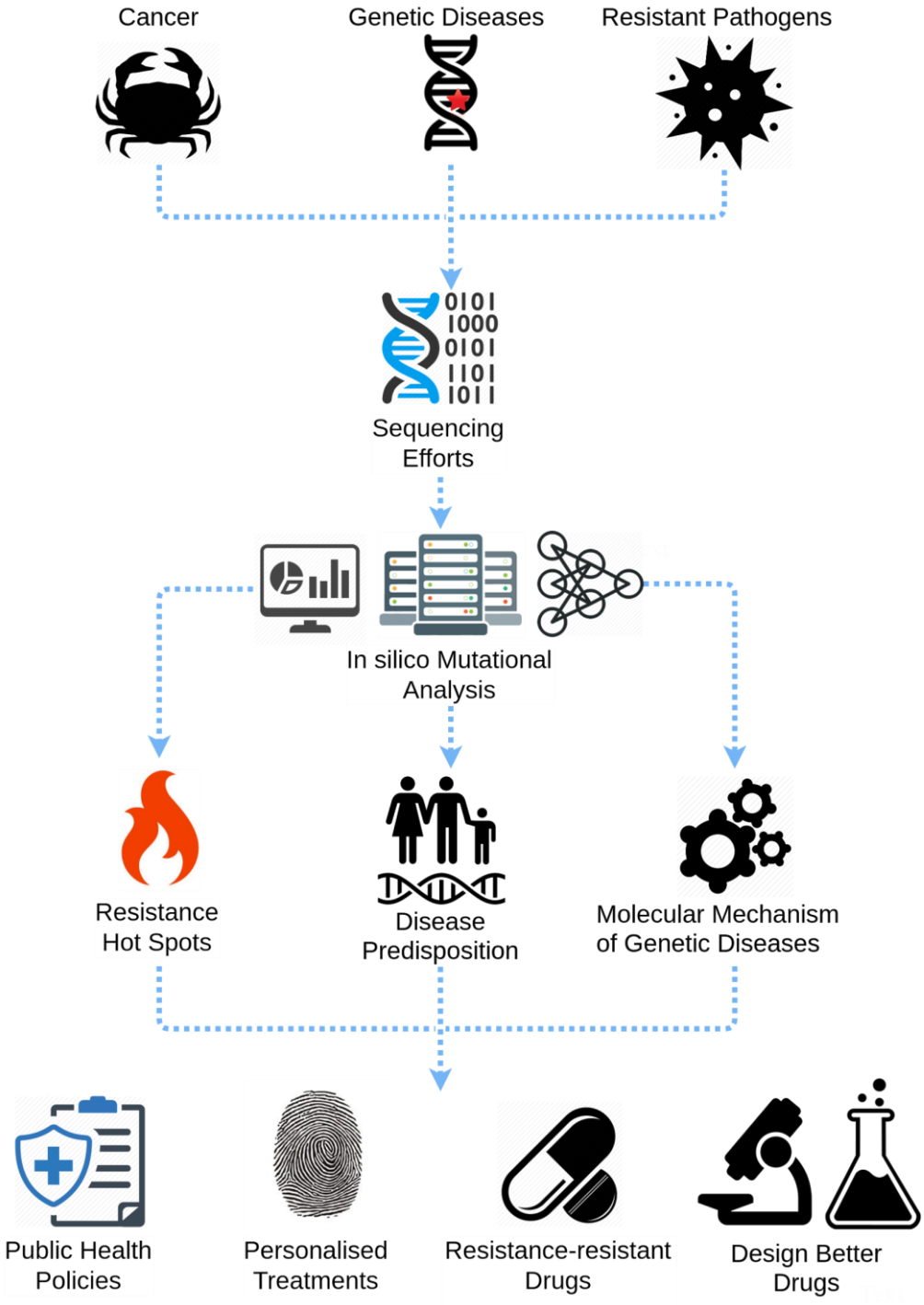

Figure 2. HIV-1 protease in complex with the non-peptidic inhibitor GRL-085 and darunavir (PDB: 5COO and 4HLA, respectively). A shows the two aligned structures of HIV-1 protease in complex with GRL-085 (light gray) and darunavir (dark gray). B depicts the main interactions between the key residues of the binding site of HIV-1 protease and darunavir. C shows the interactions between GRL-085 and the wild-type protease, calculated by Arpeggio [99].

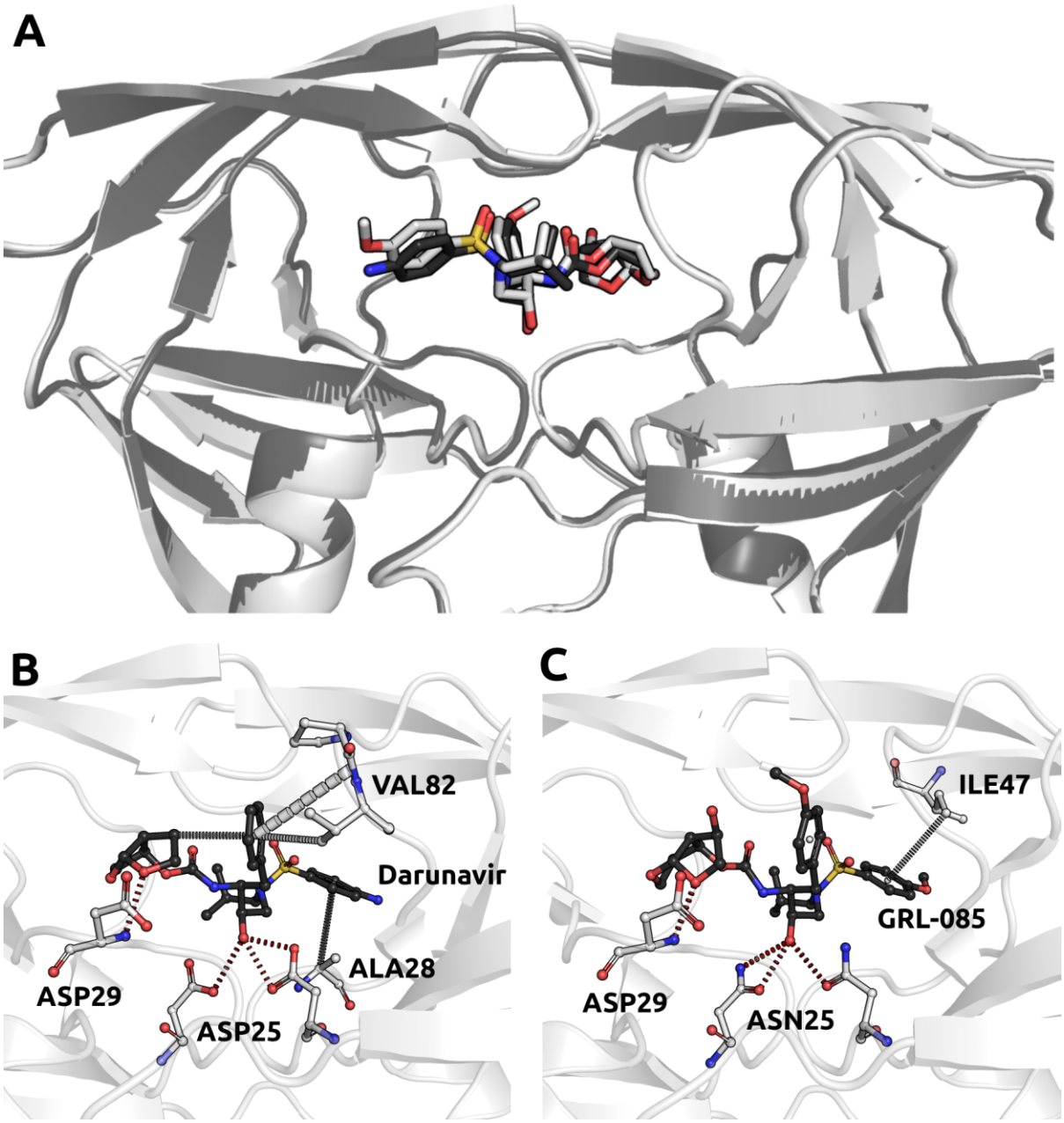

Figure 3. Neuraminidase subtype 2 of Influenza A in complex with Oseltamivir (PDB: 4GZP). A shows the main resistance hot-spot residues Glu119, Asp151 and Ile222 shown as sticks. The two negatively charged residues interact with Oseltamivir via ionic interactions shown as dashes, as calculated by Arpeggio [99]. Arg292, another important binding residue is also shown. B shows the four aforementioned residues and the oseltamivir molecule in a surface perspective.

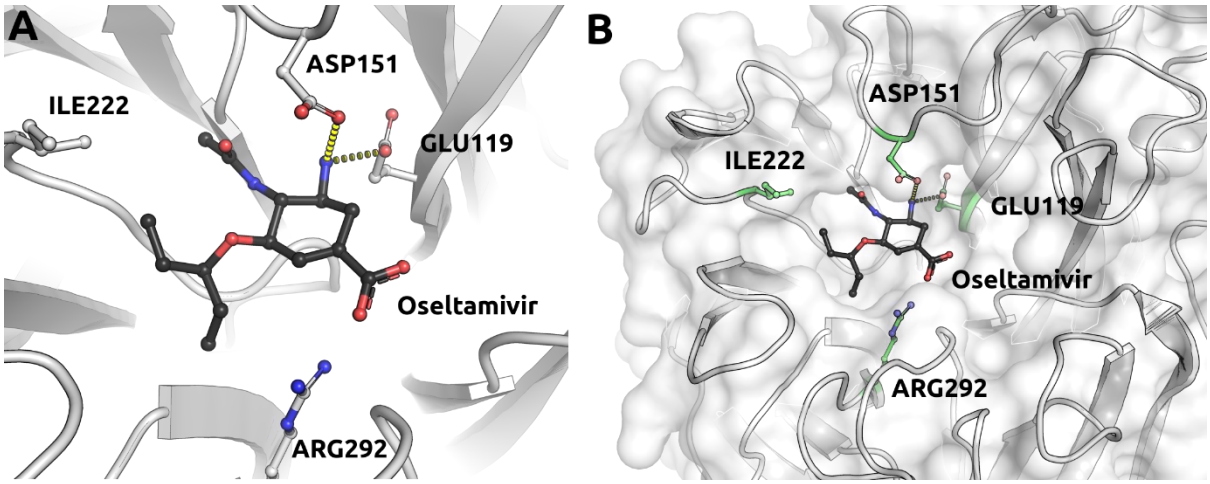

Figure 4. Four generations of kinase inhibitors. A shows ABL2 in complex with first generation kinase inhibitor Imatinib (PDB: 3GVU). Imatinib binds to the active site of the enzyme preventing other substrates from phosphorylation only when the ABL2 is in inactive conformation. B shows ABL1 in complex with second generation inhibitor Dasatinib (PDB: 2GQG). Dasatinib is a multitargeted tyrosine kinase inhibitor more potent than Imatinib due to its capability of binding to the enzyme in inactive imatinib-bound conformation, also effective against several imatinib-resistant mutations, except for T315I gatekeeper mutation as a result of a crucial hydrogen bond with T315 (underlined) for the stabilization of the complex. C shows MEK1 in complex with CI-1040 allosteric kinase inhibitor adjacent to the ATP binding site of the enzyme (PDB: 1S9J). The third generation of kinase inhibitors can bind either to the kinase domain or to other sites giving them clear advantage over ATP-competitive in first and second generation. D shows EGFR mutant T790M/L858R in complex with fourth generation kinase inhibitor Neratinib (PDB: 3W2Q). Unlike first and second generation inhibitors, this fourth generation inhibitor binds covalently to the kinase active site, blocking ATP binding.