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Drug Repurposing Techniques in Viral Diseases

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Abstract

Since the advent of the twentieth century, several severe virus outbreaks have occurred—H1N1 (1918), H2N2 (1957), H3N2 (1968), H1N1 (2009) and recently COVID-19 (2019)—all of which have posed serious challenges to public health. Therefore, rapid identification of efficacious antiviral medications is of ongoing paramount importance in combating such outbreaks. Due to the long cycle of drug development, not only in the development of a “safe” medication but also in mandated and extensive (pre)clinical trials before a drug can be safely licensed for use, it is difficult to access effective and safe novel antivirals. This is of particular importance in addressing infectious disease in appropriately short period of time to limit stress to ever more interlinked societal infrastructures; including interruptions to economic activity, supply routes as well as the immediate impact on health care. Screening approved drugs or drug candidates for antiviral activity to address emergent diseases (i.e. repurposing) provides an elegant and effective strategy to circumvent this problem. As such treatments (in the main) have already received approval for their use in humans, many of their limitations and contraindications are well known, although efficacy against new diseases must be shown in appropriate laboratory trials and clinical studies. A clear in this approach in the case of antivirals is the “relative” simplicity and a high degree of conservation of the molecular mechanisms that support viral replication—which improves the chances for a functional antiviral to inhibit replication in a related viral species. However, recent experiences have shown that while repurposing has the potential to identify such cases, great care must be taken to ensure a rigorous scientific underpinning for repurposing proposals. Here, we present a brief explanation of drug repurposing and its approaches, followed by an overview of recent viral outbreaks and associated drug development. We show how drug repurposing and combination approaches have been used in viral infectious diseases, highlighting successful cases. Special emphasis has been placed on the recent COVID-19 outbreak, and its molecular mechanisms and the role repurposing can/has play(ed) in the discovery of a treatment.

Keywords: viral infectious disease, COVID-19, drug development, drug repurposing, drug repurposing strategies, applications

1. Introduction

The development of a new drug is an extensive, intricate, highly risky and expensive process. According to the study of 12,728 transitions over the last decade

(2011–2020), the success rates of clinical drug development were 52.0% (Phase I), 28.9% (Phase II) and 57.8% (Phase III) separately [1]. Most of the candidates failed in the early drug development process for reasons of efficacy and safety concerns [2]. This causes a huge cost in drug development, ranging from tens of millions to billions. In addition, it typically takes at least 10 years from initial laboratory evaluation for a new drug to be approved. All these factors lead to low output disproportionately to the high input and make it challenging for the pharmaceutical industry to respond to emergent infectious diseases within a time frame that is able to impact the course of an outbreak. As a result, the development of vaccine candidates will likely remain to be the optimal mechanism to address outbreaks in the immediate future. Notwithstanding the extraordinary developments in vaccine technology—exemplified by recent events [3, 4]—there still remains a need for therapeutics as a sufficiently successful virus will rapidly become pan/endemic, creating a constant need for treatment of those unfortunate enough to not have access to a vaccine due to socioeconomic, age or immunostatus issues. Indeed, in the case of a pandemic, there is likely to be constant strain on health systems to treat patients, which will significantly enhanced in the absence of an effective therapy as it will instead rely heavily on patient support technologies such as ventilation or oxygen therapy.

A potential solution to this conundrum is the examination of already proposed (ideally approved) medications—repurposing—to assess their potential in the treatment of an emergent diseases. Drug repurposing as a strategy to identify potential new indication areas of approved/old drugs has many advantages. Firstly, low risk. Most repurposed drugs have at least been tested in early clinical trials. Hence, the failure rate of repurposing candidates caused by safety is very low. Secondly, low cost. Most old or approved drugs have clear safety, pharmacokinetics and pharmacodynamics data, which reduces the studies that need to be performed before the drugs extension to a novel indication. Thirdly, a higher success rate. The drugs used for repurposing are enriched from previous studies. This means less promising compounds are filtered out, allowing for a higher success rate [5]. Finally, while the molecular complexity of the protein targets of diseases is extremely broad, areas of essential molecular function can be identified as conserved in many diseases. For example, tyrosine kinase activity is a frequent target for the development of cancers from highly diverse tissues. A common feature of all kinases is the presence of an ATP-binding site, which has resulted in a large number of targeted cancer therapies (tyrosine kinase inhibitors, TKIs) which have a strong resemblance to ATP on the molecular level. This has further resulted in the clinical testing of TKIs for cancers distinct to those for which they were developed, as the evolutionary pressure to retain a function ATP-binding site provides a precondition for potential TKI cross-reactivity [6].

In the past decades, the successful application of drug repurposing shows a promising direction for drug development. For example, Thalidomide was firstly synthesized by Ciba in 1953 and came on to the market to relieve morning sickness in 1957. In 1961, Thalidomide was taken off the market due to the severe teratogenic effect on the developing fetus. In the following years, an Israeli researcher found that thalidomide could be used as a treatment against autoimmune diseases. In 1998 it was approved by FDA for the repurposed use in the treatment of ENL [7–9]. In this chapter, we present an overview of the benefits and drawbacks of drug repurposing in viral disease, including approaches, applications and outlook.

2. Drug repurposing

Drug repurposing is the process of identifying new indications and uses for approved/existing drugs [10]. It mostly involves approved drugs or compounds

under study, which have clear pharmacokinetics and pharmacodynamics that provide data on metabolic stability, tissue distribution and clearance rates. In the past decades, a large quantity of new molecular entity drugs was approved or studied, meaning that not only the ~2000 FDA approved compounds can be screened [11, 12], but also a much larger potential library of compounds that have been developed to have appropriate physico-chemical properties for their use as drugs, but that may have failed clinical trials due to lack of action in their original disease class. This, perhaps, is the key benefit of repurposing—as all potential repurposing candidates possess “drug-like” properties. However, these properties should be borne in mind by the researcher as they strongly impact the potential of repurposed compounds in clinical use.

The classic description of “drug-like” properties has grown significantly since the original introduction of the “Rule of 5” by Lipinski (Ro5) [13]. This initial classification arose from the observation that successful drugs shared common properties: low molecular weight (Mw), a relative scarcity of potential electrostatic interactions (H-bond donors and acceptors) and a partition coefficient (logP) that indicated the molecules would be able to passively diffuse across cell membranes. While, the most important descriptors remain unchanged a summary of Lipinski’s and others rules is provided in **Table 1** [13–17]. As repurposing candidates will be drawn from compounds that are likely to be enriched for these properties and therefore the potential route of administration for the repurposed disease should be compatible with these. For instance, compounds that are a good fit to Ro5 would be relatively poorly applied to diseases for which administration would be via inhalation [18]. Thus, the availability of this pharmacological information and likely route of administration is of key importance in deciding on which compounds should be assessed for repurposing.

In summary, these studies provide a wealth of information about the clinical application and mechanism of action, aiding the rapid development of the drug repurposing. When compared with *de novo* drug discovery, drug repurposing accelerates the development process and significantly reduces development risk [19].

2.1 Drug repurposing approaches

At its broadest level drug repurposing approaches can be divided into two general types: computer-based and experimental techniques [20, 21]. Within both these approaches there are three main angles of attack, drug-centric, target-centric and disease-centric methods [22]. As indicated by its name, drug-centric approaches start from the point of view of a drug, with the aim to find efficacy against diseases other than the initial indication. In the case of a disease-centric approach the disease is the focus, with the purpose being to identify and repurpose a drug

RO5	RO3	Ghose rules	Veber’s Rules	MDDR-like rules
MW ≤ 500	MW < 300 Da	160 ≤ MW ≤ 480	NRTB < 10	RNG ≥ 3
HBD ≤ 5	HBD ≤ 3	−0.4 ≤ logP ≤ 5.6	PSA < 140 Å	RGB ≥ 18
HBA ≤ 10	HBA ≤ 3	30 ≤ AMR ≤ 130		NRTB ≥ 6
LogP ≤ 5	cLogP ≤ 3	20 ≤ NA ≤ 70		
	NRTB ≤ 3			

Abbreviations: MW, molecular weight; HBD, H-bond donor; HBA, H-bond acceptor; logP, octanol-water partition coefficient; clogP, calculated octanol-water partition coefficient; AMR, molar refractivity; PSA, total polar surface area; RGB, the number of rigid bond; HB, hydrogen bond; NAT, the number of atoms; NRTB, number of rotatable bonds; RNG, number of rings.

Table 1.
Summary of ‘druglikeness’ rules applied in drug development.

specifically against that disease. Target-centric methods utilize drugs that bind to well-characterized targets that are known to be, or at least suspected to be, involved in other diseases besides the drugs' original indication. What they have in common is that at the core these strategies often employ similarity assessment to identify drugs that can potentially be repurposed.

2.1.1 Computer-based approaches

Traditional drug repurposing often relies on the *in vitro/in vivo* identification of active drugs or alternative targets. Whilst this can provide promising compounds with reliable, desired activity, it can be expensive, involves physical access to the drug libraries and requires setup and optimization of the assays [23]. With the rapid development of bioinformatics and the accumulation of vast amounts of experimental data, the development of drug repurposing, especially the initial stage, has moved from traditional biological experiments towards an increasing diversity of computational screening approaches, partially due to the lower cost and lower barrier to entry [24].

2.1.1.1 Virtual screening

Virtual screening is an essential computational approach in drug discovery, and particularly in drug repurposing. It involves the use of computer programs to evaluate compound libraries on a specified criterion, usually similarity or calculated binding energy. Virtual screening can be classified into two categories: ligand-based and structure-based virtual screening [25, 26].

Ligand-based screening focuses on analyzing the structure-activity information of known active ligands against a certain indication to identify other potentially effective drugs. This analysis relies on similarity in the form of pharmacophores and geometric shape which can be informed by structural knowledge of the ligand-target complex to identify key pharmacophores or without structural information, relying on the structure-activity relationship information from experimental approaches to identify the pharmacophores [27]. Pharmacophores are the chemical moieties of drugs that play essential roles in the interaction with their targets. Pharmacophore features—including features such as hydrogen bond donors, hydrogen bonds acceptors, charge groups, aromatic rings and hydrophobic centroids are then identified together with their spatial characteristics and mapped into a string [28]. This string can serve as a fingerprint, which can be used for easy similarity matching between different drugs, potentially identifying drugs that are also active against the disease.

This approach can be successful for small molecules as they are relatively simple molecules from a chemical perspective. Their pharmacophoric features are limited and usually rely on a few strong, deeply buried interactions with the target, making it easy to map and identify drugs with similar characteristics [29]. In contrast, biologics, such as peptides and antibodies, are far less suitable to these techniques as their method of actions typically dependent on mimicking protein-protein interactions, which are characterized by large, flat interaction surfaces [30, 31]. This makes them highly specific for their target, allowing for targeted therapies with typically less side-effects, but that specificity also prevents them from being repurposed for a different target.

In contrast, structure-based virtual screening uses the three-dimensional structure of a target of therapeutic interest [32, 33], which is screened against a virtual library of approved drugs in order to identify those that show interactions with this novel target. Drugs are docked against the target and interaction analysis

is performed based on binding energy and binding geometry. Many different docking software packages have been developed with the key differences being in the docking methodologies and the scoring functions used to rank the drugs [34]. Classical scoring functions usually rely on experimental data or prior information to rank the drugs. However, these have been consistently getting outperformed by machine learning based scoring functions, especially when specific target data is available to train on [35, 36].

Developments in computational power have made virtual screening approaches highly accessible to labs all over the world as they do not require nearly the amount of financial resources compared to wet-lab experiments. In addition, due to the speed at which the screenings can be performed nowadays, huge libraries containing 100's of millions of compounds can be screened against a target rapidly massively increasing the chemical space explored [37]. Though these advances are very useful in early drug discovery, where it can be used to screen fragment and compound libraries that cover a diversity in chemical space, they are less impactful when it comes to drug repurposing since the amount of approved drugs is limited and does not comprise wide chemical space. A downside of structure-based screening is the need for the actual structural information, which can be difficult to obtain for novel targets. Fortunately the number of entries available in the PDB has been growing at an exceptional rate, more than doubling in the last decade [38], meaning more and more targets have structural information available. In addition, the recent achievements of AlphaFold [39], including the prediction of the 3-dimensional structures of the entire human proteome, might alleviate this issue [40]. Overall this still has a positive impact on drug repurposing strategies as the more structural information is available the better scoring functions can become, aiding both ligand-based and structure-based methods in identifying drugs that can be repurposed.

2.1.1.2 Machine learning approaches

Machine learning is an overarching term used to describe diverse algorithms that use data sets to perform intelligent predictions [41]. The algorithms can be trained on large datasets to identify patterns and interactions. The trained algorithm can then be applied to novel data to identify or predict outcomes or interactions.

Computer based drug repurposing techniques utilizing machine learning have been gaining a lot of traction due to a large increase in available omics data in a variety of databases and the development of sophisticated algorithms that can utilize this data [42–44]. It is carried out using computational biology, bioinformatics and database tools, which allows for economical and high efficiency drug discovery [45]. Machine learning techniques used for drug repurposing include: k-nearest neighbor algorithms, decision tree, random forest, artificial neural networks, k-means clustering and principal component analysis [20, 46, 47].

In recent years researchers have not been able to keep up with the amount of information being generated by omics experiments, creating a need for different data analysis methods. Where previously they would manually comb through the data looking for patterns and connections, there has been a shift towards big data analysis utilizing machine learning approaches, which have shown several specific applications in drug repurposing [48].

Signature matching is an approach where complex patterns and profiles—signatures—are generated for diseases and drugs by machine learning algorithms from large omics datasets. By looking for negative correlations between differential

signatures resulting from diseases and from drug treatments, drugs can be identified that can serve as treatments for those diseases outside of their original indication [5, 20]. Simultaneously, drug signatures can also be compared with the signatures of structurally dissimilar drugs, with the idea being that if drugs show a similar signature they can share a therapeutic application irrespective of chemical similarity. For both these applications there is an alternative signature that can be compared, the clinical phenotype signature. Even though some diseases or drugs might show little to no similarities in direct transcriptomic, metabolomics or proteomic patterns, they could still have similar clinical phenotypic outcomes, which can also allow for the identification of repurposing uses of drugs [49].

Another use of signature matching is in finding similar chemical features of drugs and mapping a network based on shared features. This allows for the identification of drugs that may potentially be repurposed—as similarity in pharmacophores tends to correlate with a similarity in biological activity.

Related to signature-based methods, application of genome-wide association studies (GWAS) have also shown to be valuable within the field of drug repurposing [50]. GWAS data can be analyzed using machine learning approaches to identify interaction and association patterns of genes linked to diseases [51]. Genes identified by GWAS to associate with a disease tend to be enriched with druggable targets. By cross-referencing the disease enriched genes with databases containing drug-target information drugs can be found that inhibit specific genes that are involved in other indications but also seemingly play a role in the GWAS investigated disease, potentially being able to reuse that drug. In addition if a gene is shown to be associated with a disease it could become a novel drug target, which can be screened against using approved drug libraries.

Even though GWAS identified genes can be associated with a disease that does not mean that the target is druggable. Pathway mapping could be a potential tool to leverage the information gained with GWAS and expand upon it [52]. By analyzing the pathways or protein interaction networks up and/or downstream of the GWAS identified genes, other, previously elusive, proteins can be identified that could play a role in disease progression. This can either yield new drug targets or repurposing opportunities of drugs that already inhibit the elucidated target. For example, pathway analysis was performed on data sets containing gene expression data from human hosts infected with many different respiratory viruses. This identified 67 conserved biological pathways that could play an important role in respiratory viral infections. Comparing these pathways to a drug-target database resulted in drugs like pranlukast and amrinone, drugs with a different indication, that could potentially be used in treating viral infections [53].

2.1.2 Experiment-based approaches

Empirical evidence is still highest order of evidence and remains the golden standard for drug screening, including drug repurposing. Since experimental assays provide the most immediate evidence of drug activity [51] they are not only used to discover potential repurposing candidates from libraries but they are also essential in validating hits from computational approaches.

Inhibition assays can serve to identify target-specific drug efficacy, including inhibition constants. Binding assays are very powerful as they can also provide binding constant information [54]. Immediate use can be made of the identified binding drug that might not be highly specific or effective but it could serve as a temporary stop gap in emergency situations (like pandemics). Whilst the repurposed drug is being used as a sort of band aid, drug development can be undertaken in parallel, using the drug as the starting point. Rapid SAR approaches can then be utilized to improve the drug binding

and efficacy [55]. The fact that the resulting drug would ideally be quite similar to the approved drug could lead to accelerated approval processes.

2.1.2.1 Binding assays

Binding assays aim to detect the interaction(s) between two (bio)molecules, such as protein-protein, peptide-protein, nucleic acid-protein, small molecule-protein, or small molecule-nucleic acid and ideally also evaluate the degree of the interaction [56]. These assays can be used in two ways, in screening approaches to qualitatively identify hits that interact with the target and in a quantitative way to characterize the binding affinity.

There are many examples of different types of qualitative assays that have been used in drug repurposing approaches. Among the most common are immobilization or affinity chromatography, where either the target or the drug are immobilized on a matrix or column followed by exposure to a drug library or potential binding targets [57]. The complexes that have formed can then be eluted and identified using analytic methods. DNA-encoded libraries encompassing wide chemical space have been used in such approaches. After eluting complexes binding compounds are identified by sequencing the DNA-barcode attached to the binding compound. This technique can also be applied to approved drug libraries [58].

The aforementioned assays are aimed at screening large libraries for hits. However, obtaining detailed binding information such as dissociation constants (K_D), is crucial in the identification and development of potent drugs. Several biophysical techniques are available to quantify these interactions. Microscale Thermophoresis (MST) can be used to measure binding affinity by detecting changes in molecular motion in a temperature gradient in the presence and absence of different compound concentrations [59]. Differential scanning fluorimetry (DSF) can be used to measure protein unfolding temperature by monitoring in fluorescence of a probe that binds to hydrophobic moieties in a denaturing temperature gradient. Upon binding of drugs to the protein it can stabilize the complex, leading to a shift in unfolding temperature. By using a range of drug concentrations and measuring the effect on the thermal shift the K_D can be calculated [60]. Surface plasmon resonance (SPR) is a technique in which the target or drug of interest is immobilized on a thin metal film. A light source is aimed at the other side of the film and the surface Plasmon resonance angle is detected. When a drug or target binds to the immobilized partner the local mass at the sensor surface changes, causing a shift in the angle of reflection proportional to the mass. By measuring these changes in the presence and absence of drug or target, association and dissociation constants can be determined [61]. Isothermal titration calorimetry (ITC), one of the golden standards in K_D determination, can directly measure all binding parameters by measuring heat transfer. When binding of drug to target occurs, enthalpy changes (heat absorbed or released) of the system can be measured by a highly sensitive calorimeter. By titrating ligand against the target of interest the K_D , stoichiometry, enthalpy and entropy can be directly measured in the native states of the binding partners since no modifications are required [62].

2.1.2.2 Phenotypic screening

Where binding assays are typically focused on identifying target-drug interactions, phenotypic screening takes a more disease-centric approach. Phenotypic assays aim to identify compounds that show effects on disease-relevant outcomes [63]. These are usually performed on cell lines or organelles engineered to function as disease models. Since the assay is target agnostic less, or no, information about specific targets is obtained. However, the fact that it is agnostic also means that there are more potential

targets available within this complex environment, which could lead to the discovery of new targets that would otherwise be left unexplored [63]. There are also additional benefits to this approach in the context of drug repurposing. Since the assays are disease based and compounds are approved drugs or clinical candidates it means that if positive outcomes are obtained the drug already has positive properties and shown efficacy in more complex systems, which is beneficial to real world applications [64].

2.1.3 Side effect based or “serendipitous” drug repurposing

One of the most frequent reasons for drugs failing in (pre)clinical trials is the determination of a side effect that cannot be ignored. Most commonly this is determined to be a dangerous side effect that argues against further clinical investigation of the compound. However, one man's meat is another man's poison. These drugs with unwanted side effects can be given new indications through a drug repurposing strategy. Side effects-based drug repurposing links indications with clinical effect and is one of the common strategies employed for drug repurposing [65, 66]. A key example in this area is Sildenafil, which was originally entered into clinical trials as a drug to treat hypertension and angina [65, 67–70]. Unfortunately, Phase I clinical trials suggested that it had little effect on angina. However, use of Sildenafil causes a significant side effect: marked penile erections. This led to the discovery that Sildenafil could be used as a treatment for erectile dysfunction (ED) [71]. In 1988, Sildenafil was approved by the FDA for the treatment of ED. Such repurposing approaches could be termed serendipitous repurposing, as the new indication area is revealed during clinical trials. As a result, such repurposing is relatively rare.

3. Drug repurposing in viral diseases

Over the last decades the world has seen multiple severe viral outbreaks resulting in millions of deaths. Among the deadliest were the Influenza pandemics such as H1N1 (1918), H2N2 (1957), H3N2 (1968) and H1N1 (2009). The HIV/AIDS epidemic that was first recognized in the 1980s and went global has also caused up to an estimated amount of 36 million deaths and is still ongoing. Besides the large, deadly pandemics there have been smaller but very impactful localized epidemics such as Dengue virus (DENV), Zika virus (ZIKV), Ebola virus (EBOV) and Middle East respiratory-syndrome corona virus (MERS-CoV) which pose serious challenges to public health. Most recently in 2019 there was an outbreak of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which caused the COVID-19 pandemic, affecting nearly every country in the world with over 226 million reported cases to date [72]. Despite the advances in controlling viral pathogens that come with the widespread mass vaccination, there are no approved specific (effective) therapies for the treatment of most viral infections.

By exploring new targets and mechanisms, drug repurposing provides new indications for old drugs. The major time advantage of repurposing is that this approach allows repurposed drugs to quickly enter clinical trials, which is of significant importance in reacting to disease outbreaks, especially in the case of worldwide pandemics.

3.1 Drug repurposing for COVID-19

The outbreak of COVID-19, caused by SARS-CoV-2, has spread across the world. There is, as yet, no specific treatment for COVID-19 approved. Drug repurposing provides a fast and economical option for the identification of medications targeting SARS-CoV-2.

SARS-CoV-2 is a member of the betacoronaviruses family. It is a single-stranded RNA virus, characterized by large crown-like spikes protruding on the viral surface and an unusually large RNA genome which encodes four main structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) [62, 73, 74]. Like other coronaviruses, SARS-CoV-2 cell entry is mediated by the spike glycoprotein. The spike glycoprotein is composed of two subunits, S1 and S2, which mediate viral-host attachment and viral-host membrane fusion cascade, respectively [75]. SARS-CoV-2 spike recognizes and binds to the human ACE2 (hACE2) receptor through its receptor-binding domain (RBD) and is primed and activated by proteolytic cleavage by enzymes such as Furin and transmembrane serine protease 2 (TMPRSS2) [76, 77]. Spike, ACE2, Furin and TMPRSS2 have been shown to play a key role in mediating viral-host fusion attachment and fusion, and the Furin cleavage site on the Spike protein has been indicated as one of major reasons SARS-CoV-2 is so infectious [78, 79]. This makes these potentially promising drug targets for COVID-19 treatment [80, 81].

3.1.1 Drug repurposing targeting viral fusion

Inhibition of the Spike-Ace2 interaction is a primary target for drug repurposing as it is crucial to viral entry. *In silico* approaches have been performed and identified Simeprevir, an HCV NSP3A/4 protease inhibitor, as a potential blocker of Spike-Ace2 interaction by binding the RBD [82]. However, other *in vitro* studies showed that Simeprevir is not necessarily active against the RBD but is targeting the viral replication [83].

Another promising target is TMPRSS2, a serine protease. TMPRSS2 is associated to the host endothelial cell surface and cleaves the viral spike glycoprotein after binding to ACE2, activating it. The activation of spike protein then facilitates viral entry [84]. Camostat mesilate (a serine protease inhibitor) has been approved for the treatment of chronic pancreatitis, postoperative reflux esophagitis and kidney or liver disease fibrosis [81, 84, 85]. Since it is an established serine protease inhibitor it is a prime candidate to inhibit the TMPRSS2. An *in vitro* study indeed showed that camostat mesilate can suppress viral replication by halting the fusion of virus-cell membranes through the inhibition of TMPRSS2 [84]. Clinical trials using this drug are currently ongoing [86]. However, recently the results of a small double-blind randomized clinical trial using camostat mesilate performed in patients hospitalized with COVID-19. The trial determined no adverse effects of treatment with camostat but also no increase in positive clinical outcomes [87].

3.1.2 Drug repurposing targeting endocytosis of SARS-CoV-2

Besides direct membrane fusion, SARS-CoV-2 can also invade cells via endocytosis [88, 89]. This route involves several proteins that play an important role in endosome formation, such as two-pore channel 2 (TPC2), Cathepsin L (CTSL) and Vacuolar-type ATPase (V-ATPase) [90]. These proteins are indicated to be potentially interesting therapeutic targets for COVID-19 treatment.

Tetrandrine is a bisbenzylisoquinoline and calcium channel blocker, known for its anti-inflammatory, immunosuppressive, oncological, and cardiovascular bioactivity [48–49]. The compound has been shown to be effective in the treatment of silicosis [90–92]. According to an *in vitro* study, tetrandrine is a low micromolar inhibitor of viral replication that functions by blocking the two-pore channel 2 (TPC2), which impedes Ca^{2+} release which in turn prevents acidification of the endosome [92].

The now infamous anti-malarial drugs chloroquine (CQ) and hydroxychloroquine (HCQ) were also posited to inhibit endocytosis of SARS-CoV-2 [93, 94]. CQ and HCQ are potentially involved in blocking cleavage of spike by raising the pH of the endosomes, preventing cathepsin L-mediated proteolysis, which is a key element in membrane fusion after binding ACE2. Whilst many potential mechanisms of action have been suggested, none have been rigorously demonstrated. In addition, a recent large meta-data analysis has shown that there is no evidence that treatment with CQ or HCQ reduces COVID-19 mortality in patients [95]. To the contrary, evidence is available that shows HCQ is responsible for a small increase in mortality outcomes. These compounds garnered lots of attention when the presidents of prominent countries started promoting CQ and HCQ as wonder drugs that could combat COVID-19 [96]. However, as mentioned before most evidence points towards the contrary and the WHO recommends against the treatment with these drugs [97]. These cases have shown an important risk in the use of drug repurposing: in the age of hyper connectivity and social media echo chambers dangerous, unfounded ideas can avoid scrutiny and rigorous investigation, leading to large groups of people self-medicating with alternative treatments that have no scientific basis. This poses a problem in general drug development but even more so in drug repurposing cases where these compounds tend to be far more easily obtainable by the general public as they are approved and often available for purchase in pharmacies.

3.1.3 Drug repurposing targeting viral replication

After invading host cells, the coronavirus comes into the next stage of its life cycle: translation, replication, transcription and Assembly. This process mainly involves five different proteins: Mpro, RdRp, nsp14, MTHFD1 and Plpro, which have different functions [74, 98]. Mpro is also known as 3C-likeprotease (3CLpro) and proteolytically processes the majority of the polyprotein into functional polypeptides [99]. Similar to 3CLpro, Plpro is a viral protease that is responsible for cleaving polyproteins to generate a function replicase complex [100]. RNA synthesis, critical for viral replication, is performed by RdRP and its cofactors nsp7 and nsp8. Nsp14 has an exonuclease activity that supports RNA synthesis with an unusual RNA proofreading function. A study performed by Tsinghua University showed that knockdown of MTHFD1, a key enzyme in cellular production of purine, dTMP and methyl groups, significantly inhibits viral replication [101]. As a result of their key functions in the viral life cycle these proteins are promising potential targets for antiviral drugs development. Clofazimine is an anti multi-bacillary leprosy drug which was approved for medical use in 1986. A recent study performed by The University of Hong Kong showed that clofazimine inhibits both viral spike glycoprotein mediated cell fusion and replication of SARS-CoV-2 *in vitro* [102].

Genome analysis has demonstrated that SARS-CoV-2 and SARS-CoV genes globally share >80% nucleotide identity and >89% similarity [73, 103, 104]. As a result, the key steps in the CoV family viral life cycle within the host cell are likely to be highly conserved. A key feature of this process is the expression of non-structural proteins (nsps). Subsequent to cell entry, two extended polypeptides (pp1a and pp1ab) from the CoV viral genome are generated by the host cell translation machinery [105, 106]. These two polypeptides then self-cleave into 37 distinct non-structural (nsp) proteins [107] and analysis has demonstrated that the CoV family possesses several proteases involved in this essential self-cleavage process: the papain-like protease (PLpro), and the 3C-like proteinases (3CLpro or Mpro) [108]. CoV generally encode two PLpros within nsp3, with the exception of gamma-CoV,

SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 [100]. Thus, 3d unlike structural/accessory protein-encoding genes, which can show significant sequence variation in order to select between different potential host cell receptors, CLpro plays a central and critical role in CoV replication in host cells and the similarity in essential function leads to a high sequence similarity between the proteases of the CoV family, in particular beta-CoV [105, 106]. This increased similarity in the structure of the 3CLpro has the consequence of high structural between 3CLpro of different CoV family members—and the concomitant increased likelihood of cross-species function of CoV 3CLpro inhibitors and the potential to repurpose these inhibitors. The high sequence homology within Cov 3CLpros also provided high quality model templates—subsequently supported by the availability of high-resolution diffracting crystals—to perform both computational docking experiments, as well as molecular validation by X-ray crystallography [109]. There also exists the potential for the discovery and the development of a pan-anti-CoV inhibitor [110, 111].

This potential has been partially realized in not only the discovery of entirely novel SARS-CoV2 3CLpro inhibitors [99, 112, 113] (refs), but also in a number of reports describing successful identification of potential repurposing candidates [109].

For example, we and others have previously reported the results of a molecular docking experiment that indicated a class of well tolerated compounds (gliptins) as potential SARS-CoV2 inhibitors. For example, Anagliptin, a DPP4 inhibitor, is a well-established treatment for diabetes that is used by millions of patients. It has an excellent safety profile [114]. Computational docking demonstrated an efficient binding, with predicted H-bonds made to the backbone atoms of Gly163, Gly271, and Tyr268 and the side chain of Tyr273. Docking experiments also proposed that α -ketoamide inhibitors of hepatitis C virus (HCV) protease would be potential inhibitors of SARS-CoV-2 3CLpro. Efforts were concentrated on brocprevir and telaprevir as the docking poses were supported by experimental structure analyses (**Figure 1**). Subsequent biochemical assays demonstrated that brocprevir indeed displays strong binding to isolated 3CLpro of SARS-CoV2 and inhibits viral replication in cellular assays.

Similarly to CQ and HCQ, ivermectin, originally an anthelmintic, also gained widespread attention as a potential treatment for COVID-19 and some *in vitro* evidence of SARS-CoV-2 replication inhibition in cell cultures has been provided [115]. The suggested mechanism of action is the inhibition of importin alpha/beta-1 nuclear transport proteins which the virus uses to enter the nucleus and is an important part of the replication cycle as it suppresses host-immune response [116]. Despite the *in vitro* effect no clinical data has supported the therapeutic use of ivermectin at concentrations approved for use in humans. However, it gained attention in the media and people started buying ivermectin meant for use in large animals to self-medicate against the virus [117]. Even though ivermectin is approved for human use and is generally safe, the doses used in the treatment of animals are several times larger than recommended for humans and can cause side-effects ranging from mild diarrhea to seizures and coma. This once again shows the care that needs to be taken when repurposing drugs in how scientific results are communicated to the general public, as it can lead to potential dangerous situations [118].

3.1.4 Drug repurposing targeting immune response modulators to treat SARS-CoV-2

Clinical symptoms resulting from SARS-CoV-2 infection are heterogeneous. Recent reports have shown that the cytokine storm effect may play a significant

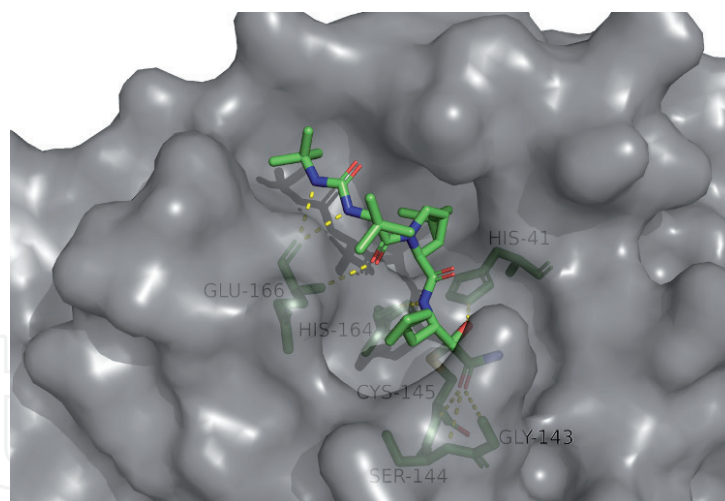


Figure 1.

Boceprevir (green), a HCV NS3/4A protease inhibitor, bound to SARS-CoV-2 3C-like protease (gray surface representation) with several key drug-protein interactions shown. Hydrogen bonds are shown as yellow dotted lines. PDB accession code: 6zru.

role in disease progression, potentially leading to multiple organ failure and death [119]. Immune response-related proteins have been proposed as potential targets for treatment options [120, 121]. Even though effectively suppressing cytokine storm does not directly combat the viral infection itself, it can be a crucial treatment option against COVID-19. It has previously been demonstrated that melatonin has beneficial effects on infection induced models of respiratory disease and associated complications [122]. Recently evidence also surfaced that it can inhibit COVID-19 induced cytokine storm [123]. Tocilizumab, an interleukin antagonist used for rheumatoid arthritis, is an immunosuppressor and was thus posited to be effective at reducing inflammation caused by SARS-CoV-2 [124]. It has recently become one of the first drugs to be recommended by the WHO as an effective treatment against COVID-19 [125]. Even though this is very promising there is an issue with the availability and affordability of this drug [126].

Researchers at Johns Hopkins found that the drug prazosin, an alpha-1 blocker used to treat high blood pressure, can prevent cytokine storms and that it significantly strengthened survival following inflammatory stimuli in preclinical models. This study is now in clinical trials [127].

3.1.5 Drug repurposing targeting pyroptosis

Sepsis is a systemic inflammatory response syndrome resulting from dysregulation of host immunity. It is one of the deadliest clinical symptoms of severe SARS-CoV-2-infected patients [128]. Sepsis treatment normally mainly relies on the administration of intravenous antibiotics. However, since SARS-CoV-2 viral sepsis is not bacterial in nature the efficacy is low. Treatment is difficult, typically consisting of supplying oxygen and assisted breathing using a ventilator. Cocktails of antivirals and immune suppressors are also given but usually only have limited effect [129]. Approximately one-third of the discharged patients will die and one-sixth will suffer severe persistent impairments in the following year [130]. These facts taken together demonstrate the urgency and significance to find new treatments.

Sepsis is associated with pyroptosis (inflammatory programmed cell death) that is triggered by proinflammatory signals [131]. When viruses invade the host cell, inflammasomes are activated which in turn triggers an inflammatory response [132]. Pore-forming protein gasdermin D (GSDMD) is cleaved by activated Caspase-1, releasing its N-terminal domain [133]. The GSDMD N-terminal domain

induces the formation of a large plasma membrane pore, resulting in pyroptosis [134]. Under normal circumstances pyroptosis can be good response, being able to trigger cell death of infected cells, releasing the pathogens and stimulating subsequent phagocytosis, protecting against infections [135]. However, excessive activation of pyroptosis will exacerbate sepsis or excessive cell death, causing immunity dysregulation [136, 137].

Disulfiram is approved for the treatment of chronic alcoholism. In a study conducted by Boston Children's Hospital, researchers found that disulfiram possesses inhibiting potential towards GSDMD both in *in vitro* cell assays and in *in vivo* mouse experiments. The experiment results showed that disulfiram inhibit the formation of the GSDMD pore by covalently modifying Cys191 of human GSDMD [138]. These results indicate that disulfiram could potentially be used to combat the pyroptotic effects induced by sars-cov-2 infection, hopefully reducing the negative clinical outcomes.

3.2 Drug repurposing for other viral diseases

Not only large global pandemic diseases are worth investigating for drug repurposing opportunities. Smaller, localized viral epidemics still plague many countries to this date. These diseases tend to fly under the radar since they typically occur in poorer regions of the world, meaning less research money is being spent on novel drug development. Drug repurposing could be the solution for these diseases due to the far faster and cheaper development pipeline.

3.2.1 Dengue virus

Dengue virus (DENV) is a single-stranded RNA virus, enveloped by a bilayer lipid membrane. The premembrane (prM) protein and envelope glycoprotein adhere to the membrane. Dengue virus can infect humans through mosquito bites. Symptoms, that include high fever, severe headache, muscle and joint pain, nausea, vomiting, swollen lymph nodes and rash, usually appear 3–14 days post-infection [139]. Most patients will recover in 2–7 days, while a small number of patients' conditions may worsen accompanied by bleeding, thrombocytopenia and plasma protein effusion. Up to 22,000 people die from Dengue annually and currently there are no therapies to treat this infection [140].

Ulipristal, a FDA approved small molecule, is an elective progesterone receptor modulator (SPRM), that has been demonstrated to be a potent inhibitor of DENV, most likely by blocking viral entry [141]. The antiviral activity was evaluated by *in vitro* DENV infection assay using Vero E6 cells. The results show that ulipristal has an antiviral effect against DENV in Vero E6 cells with an EC₅₀ of $8.3 \pm 0.1 \mu\text{M}$. The anti-DENV effect of ulipristal was further confirmed using a murine infection model. The ulipristal-treated group presented less weight loss and disease symptoms compared the control group. A significant drop was also detected in the degree of viremia in the blood of the ulipristal-treated group. This study showed that ulipristal has desirable anti-DENV effects *in vitro* and *in vivo* [141].

3.2.2 Zika virus

Zika virus (ZIKV) is another virus that is propagated by mosquitoes and belongs to the genus of flaviviruses. ZIKV infection generally causes only mild symptoms, including fever, rash, conjunctivitis, muscle and joint pain, and headache. However, it has shown severe tetatogenic impacts, being able to cause a range of neurological complications, such as Guillain-Barre syndrome and microcephaly, in the fetuses of infected pregnant women [142].

There are no currently approved specific therapies for ZIKV infection [143]. However, a screening study utilizing 774 approved drugs has shown promising results. *In vitro* studies showed that ivermectin (anthelmintic), mycophenolic acid (an immunosuppressant), and daptomycin (a lipopeptide antibiotic) can inhibit ZIKV, resulting in reduced infection rates [144].

3.2.3 Ebola virus

Ebola virus is one of numerous hemorrhagic fever viruses, which was first discovered in 1976. It can cause severe viral haemorrhagic fever with case fatality rates vary from 25 to 90% [145]. It is characterized as a non-specific febrile illness (symptoms may include anorexia, arthralgia, headache, malaise, myalgia and rash) in the early infection and progresses to severe gastrointestinal symptoms (nausea, vomiting and high-volume diarrhea) in the first week [146]. To date, a monoclonal antibody (mAb114) and a cocktail of three antibodies (REGN-EB3) have been approved for the treatment of Ebola [147, 148]. Besides these biologics there has also been attempts at drug repurposing for this disease. Several drugs such as Amiodarone (anti-arrhythmia), bepridil (anti-angina pectoris), teicoplanin (antibiotic), amiodarone (ventricular fibrillation/tachycardia) and favipiravir (RNA polymerase inhibitor) have shown therapeutic potential for Ebola, but their efficacy requires further confirmation [149–151].

3.2.4 MERS-CoV

A warning of the potential for a coronavirus pandemic was provided by the Middle East respiratory syndrome coronavirus (MERS-CoV). While the impact of this outbreak was significantly less than that of the current SARS-CoV2 outbreak the urgent need for MERS-CoV treatments was recognized, also including a focus on repurposing approaches and a call for the development of pan-corona virus inhibitors [152]. Suggested repurposing agents included GS-5734, which has previously demonstrated antiviral against multiple viral families, including *Coronaviridae*. GS-5734 activity *in vitro* was supported by reduced disease effects in mouse models and, while resistance mechanisms emerged, they were associated with a loss in viral fitness *in vitro* and *in vivo*—supporting the further analysis of GS-5734 as a pan-corona inhibitor [153, 154].

Similarly, lopinavir-ritonavir (a molecule designed as an inhibitor of the HIV-1 protease inhibitor) was proposed as a repurposing target of the 3CLpro of both SARS-CoV and MERS-CoV during their respective outbreaks [155, 156]. Combination therapy approaches in both cases resulted in improved patient outcomes, thereby offsetting the lacking of designed affinity that is a hallmark of repurposed compounds. In the example of SARS-CoV, a study on a combined therapy with ribarivin (a guanosine analog with activity against multiple viral families that inhibits viral RNA synthesis by RdRp) demonstrated both reduced viral load and improved clinical outcomes [157]. Whereas, a clinical trial of lopinavir-rotonavir in combination with IFN- β 1b targeted therapies was proposed for MERS-CoV patients in Saudi Arabia [158]. Ribarivin itself was also a focus for repurposing during the SARS-CoV and MERS-CoV outbreaks. However, while efficacy of ribarivin alone could be demonstrated *in vitro* the doses required for a clinical response could not be supported by patients [159, 160].

Screening of an FDA-approved compound subset against viral replication in culture identified lopinavir and an additional 3 compounds with IC₅₀ values in the low micromolar range (chloroquine, chlorpromazine, and loperamide) [156]. This again demonstrates not only the potential for experimentally based repurposing screens

to identify potential agents, but also suggests that the relatively limited potency of the agents identified may require the assessment of combination therapies to provoke a clinical response. This additional limitation of identifying appropriate combination therapies may well represent a common theme as a complicating factor in repurposing strategies.

4. Conclusions

In summary, the relatively conserved elements of the viral life cycle offer many opportunities to reexamine compounds developed to address previous outbreaks for efficacy against novel outbreaks. Clear examples are shown in the results against non-structural proteins above, which often maintain significantly higher sequence homology across species due to a conserved mechanism than structural proteins. However, while this sequence conservation indeed leads to a degree of “cross-talk” between nsp inhibitors, the required exquisite and intricate nature of the interaction between a successful drug and its target will almost inevitably reduce the efficacy of a monotherapy. As a result, it is likely that while repurposing can identify promising candidates, care must be taken not to hope for a single effective solution in existing drugs (e.g. Ivermectin, hydroxychloroquine, etc.). Rather, functional (clinical) solutions are much more likely be found in careful clinical trials of combination therapies of drugs identified through repurposing screens.

The current combination of virtual, *in vitro* and *in vivo* screening is well positioned to perform rapid repurposing experiments on the relatively small number of clinically approved candidate molecules. However, significant research effort should be expended globally to continue to identify potential viral inhibitors and further populate the potential repurposing list.

Response speed is a key factor facing outbreaks. Drug repurposing is a practical solution that provides multiple benefits beyond classical drug discovery. Perhaps the greatest advancement in this area has been the improvements in computational techniques, that has developed in parallel with advances in structural biology—both of which continue to improve. These structural views of the proteins driving disease expand the number of experiments that can be performed *in silico*, providing both an increase in speed of hypothesis generation, as well as an important pre-filter stage to select out candidate molecules for screening. In our opinion a key aspect that should not be overlooked is the *in vitro* validation of a molecular effect on a proposed target. Certain recent experiences have shown that attempts to bypass this stage and short-cut the process by directly jumping into clinical trials can produce conflicting results, leading to confusion and loss of confidence of the public. However, despite successful application of drug repurposing, no single golden standard as yet exists to give relatively predictable results.

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Conflict of interest

The authors declare no conflicts of interest in the contents of this manuscript.

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