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Predicting broad-spectrum antiviral drugs against RNA viruses using transcriptional responses to exogenous RNA

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Abstract

All RNA viruses deliver their genomes into target host cells through processes distinct from normal trafficking of cellular RNA transcripts. The delivery of viral RNA into most cells hence triggers innate antiviral defenses that recognize viral RNA as foreign. In turn, viruses have evolved mechanisms to subvert these defenses, allowing them to thrive in target cells. Therefore, drugs activating defense to exogenous RNA could serve as broad-spectrum antiviral drugs. Here we show that transcriptional signatures associated with cellular responses to the delivery of a non-viral exogenous RNA sequence into human cells predict small molecules with broad-spectrum antiviral activity. In particular,

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transcriptional responses to the delivery of Cas9 mRNA into human hematopoietic stem and progenitor cells (HSPCs) highly matches those triggered by small molecules with broad-spectrum antiviral activity such as emetine, homoharringtonine, pyrvinium pamoate and anisomycin, indicating that these drugs are potentially active against other RNA viruses. Furthermore, these drugs have been approved for other indications and could thereby be repurposed to novel viruses. We propose that the antiviral activity of these drugs to SARS-CoV-2 should therefore be determined as they have been shown as active against other coronaviruses including SARS-CoV-1 and MERS-CoV. Indeed, two of these drugs- emetine and homoharringtonine- were independently shown to inhibit SARS-CoV-2 as this article was in preparation. These drugs could also be explored as potential adjuvants to COVID-19 vaccines in development due to their potential effect on the innate antiviral defenses that could bolster adaptive immunity when delivered alongside vaccine antigens.

Introduction

Invasion by viruses presents one of the biggest challenges faced by both prokaryotic and eukaryotic cells. As a consequence, a wide range of mechanisms to counter viral infections have evolved across all life forms. In higher vertebrates including mammals, both innate and acquired immunity are employed as a defense against viral infections. While acquired immunity is driven by highly specialized cell types, for example B and T-cells, innate immunity against viral infections is possessed by nearly all cell types in mammals. Unlike acquired immunity which involves antigen-specific receptors generated through gene rearrangements, innate defense against a broad range of viruses relies on

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germline encoded pattern recognition receptors (PRRs). Antiviral defenses by PRRs recognize specific pathogen associated molecular patterns (PAMPs) that are crucial for viral replication and transmission, for example viral nucleic acid (*1*). However, many viruses circumvent this challenge by targeting PRRs. Thus, activating the intrinsic antiviral defenses in individual cells could provide a means for developing broad-spectrum antiviral drugs.

Here, we hypothesized that the delivery of an exogenous RNA sequence into human cells elicits antiviral defense characterized by a specific transcriptional response, and that small molecules that enhance this response have potential for broad-spectrum antiviral activity. In contrast to viruses which subvert innate cellular defenses to foreign RNA, non-viral exogenous RNA should evoke antiviral responses but fail to subvert these defenses. Thus, studying cellular responses to non-viral exogenous RNA could provide an unbiased view of exogenous RNA defenses. As a proxy to cellular response to the delivery of exogenous RNA, we considered recently published transcriptional responses of a human cell type to delivery of non-viral RNA sequences encoding Cas9 protein and CRISPR gRNA to hemoglobin B locus (HBB), hereafter referred to as 'cas9 mRNA' (*2*). Specifically, the delivery of Cas9 mRNA into hematopoietic stem and progenitor cells (HSPCs) was found to induce a strong antiviral transcriptional response (*2*). Several genes involved in innate antiviral response such as interferon stimulated genes (ISGs), interferon regulatory factors (IRF1, IRF7, IRF9) and the cytosolic foreign RNA sensor RIG-1 were upregulated by Cas9 mRNA delivery (*2*). The activation of innate antiviral defenses upon delivery of exogenous RNA into cells has also been reported for in vitro

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transcribed CRISPR guide RNAs (*3*). As little as 1nm of gRNA triggered a 30 to 50 fold increase in interferon-beta1 (IFNB1) in HEK293T cells and 50nm gRNA led to a 1000-fold induction of IFNB1 (*3*), an effect that is equal to that induced by RNA from Sendai virus or hepatitis C virus PAMP (*4*, *5*). Using the Connectivity Map database of thousands of genetic and chemical perturbations of human cell lines (*6*), we show that several small molecules with broad-spectrum antiviral activity elicit transcriptional responses that are highly similar to those of human cells perturbed by the delivery of an exogenous, synthetic RNA sequence.

Results

Relationship between transcriptional responses to exogenous RNA (Cas9 RNA) and viral infection

To explore the potential regulatory mechanisms underlying transcriptional response to an exogenous RNA, specifically Cas9 mRNA, and their association with key antiviral response regulators, we investigated the similarity of the transcriptional responses to the delivery of Cas9 mRNA to those obtained under overexpression of thousands of genes across different cell lines in the Connectivity Map (CMap) database, a compendium of more than 1 million transcriptional profiles of human cell lines under thousands of chemical and genetic perturbations (*6*) (Fig. 1 and Methods). Interestingly, we found that the overexpression of IFNB1 across several cell lines in the CMap database has a transcriptional signature that is highly similar to the delivery of Cas9 mRNA (Fig. 1A), with a median similarity score (tau, τ) of 99.98 to the Cas9 mRNA signature. This indicates that among a reference set of queries in CMap, only 0.02% have a level of similarity to

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IFNB1 that is higher than that between cas9 mRNA and IFNB1. This high similarity is consistent with previous reports that CRISPR gRNAs induce IFNB1 (*3*, *7*) and shows that the similarity between Cas9 mRNA and IFNB1 signatures is also stronger than that observed between IFNB1and other biological signatures in CMap. In addition, several of the top ranked signatures are of genes involved in innate immunity to viruses such as B-cell lymphoma-2-like protein (BCL2L2), TNFRFS1A, KLF6 and TIRAP. For example, B-cell lymphoma-2-like protein (BCL2L2) inhibition by the anticancer compound ABT-263 accelerates apoptosis of influenza A virus (IAV) infected cells and lowers survival of infected mice (*8*); Kruppel-like factor 6 (KLF6) is a transcription factor that induces the production of the antiviral compound nitric oxide during infection by negative-sense, single stranded RNA viruses such as IAV (*9*) or respiratory syncytial virus (RSV) (*10*); BCL10 is required for antiviral response mediated by the retinoic acid inducible gene 1 (RIG-1) which senses foreign RNA in the cytosol (*11*, *12*), while the toll-interleukin-1 domain adaptor receptor containing protein (TIRAP) is required for innate immune signaling to ligands such as viral nucleic acids in multiple subcellular compartments (*13*).

To further determine whether the observed transcriptional similarity between Cas9 mRNA and overexpression of several antiviral genes (Fig. 1A) mimics an IFN response, we repeated our analysis with an independent list of core interferon stimulated genes (core ISGs) based on a published dataset of ISGs identified as upregulated by IFN treatment of cells across several vertebrate species using a single experimental platform (*14*). Using the core ISGs gene set as a query on the CMap database, we found that genes whose overexpression show high similarity to those elicited by Cas9 mRNA also exhibit

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high similarity to core ISGs (Fig. 1B). For example, out of the top 10 genes with high similarity to Cas9 mRNA signature, 7 are also identified as the top genes with high similarity to the core ISGs gene set. In addition, using the Cas9 mRNA signature is complementary to using the core ISGs gene set. For example, the Cas9 mRNA signature had significant similarity (τ = 94.99, Fig. 1A) to other antiviral response genes such as DLX3 (*15*) which in contrast, had no significant similarity to the core ISGs (Fig. 1B and Supplementary Figure 1). Similarly, using the core ISGs also identifies other antiviral response genes that are missed by the Cas9 mRNA signature query. For example, IRF5 was highly similar to the core ISGs signature (Fig. 1B, τ = 98.78) but not to Cas9 mRNA signature (Fig. 1A, τ < 90). These results show that the transcriptional response to Cas9 mRNA in HSPCs identifies key antiviral response genes across different cell types.

Similarity between transcriptional signatures to exogenous RNA and small molecules

Next, we investigated the similarity between transcriptional response to Cas9 mRNA delivery and small molecules in the CMap database (Fig. 2 A). We have previously shown that the transcriptional signatures of several small molecules in various cell lines are highly similar to those associated with the delivery of CRISPR/Cas9 components, including the delivery of Cas9 mRNA sequence (*16*). Given that transcriptional response to Cas9 mRNA is associated with antiviral transcriptional signatures, we reasoned that some of the small molecules with transcriptional signatures similar to that of Cas9 mRNA could bear broad-spectrum antiviral activity. Since the delivery of Cas9 mRNA may also lead to transcriptional responses unrelated to foreign RNA sensing, we filtered out small

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molecules whose transcriptional signatures are highly similar to that of the delivery of Cas9 protein pre-complexed with gRNA to form a ribonucleoprotein, RNP (Fig. 2B). This process removed small molecules such as etoposide, teniposide and idarubicin which may have similar transcriptional effects to Cas9 mRNA due to downstream effects of Cas9 protein expression in inducing DNA cleavage. In addition, we also compared the similarity between Cas9 mRNA signature and the core ISGs gene set (Fig. 2C). Idarubicin and etoposide were both significantly similar to core ISGs (Fig. 2C, $\tau > 90$), consistent with previous reports that DNA topoisomerase inhibitors trigger IFN response (*17*, *18*). However, in our analysis we cannot rule out a non-specific association between the topoisomerase inhibitor signatures and Cas9 mRNA or IFN because they also showed similarity to RNP. Furthermore, given the genotoxic nature of these inhibitors they would be highly undesirable clinically.

Small molecules with signatures highly similar to core ISGs could similarly indicate potential broad-spectrum antiviral agents (Fig. 2C) as the use of Cas9 mRNA signatures alone may miss some of these molecules. 7 small molecules with high similarity to Cas9 mRNA also had significant similarity to the core ISGs and were within the top 30 molecules in both lists ($\tau > 90$, Fig. 2D). Even though some of the small molecules were in the top 30 list for Cas9 mRNA but not in the top 30 for core ISGs, their similarity to core ISGs was significant ($\tau > 90$, Supplementary Figure 3 for full list). Specifically, homoharringtonine and cephaeline had significant similarity to both Cas9 mRNA and core ISGs at a threshold of ($\tau > 90$). On the other hand, doxorubicin and topotecan were in the top 30 small molecules for core ISGs and although not in the top 30 for Cas9 mRNA, their

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similarity to Cas9 mRNA was significant (Supplementary Figure 2). However, there were also compounds that were unique to Cas9 mRNA and not found to share any significant similarities with core ISGs and vice versa. For example, aminopurvanolol-a, puromycin and cycloheximide were unique to Cas9 mRNA while 3-matida, BRDA69470004 and benzathrone were unique to core ISGs. Given that some of the compounds unique to Cas9 mRNA or core ISGs have antiviral activity against different viruses, using both lists to predict potential broad-spectrum antivirals is complementary. For example, ivermectin which has demonstrated broad-antiviral activity was significantly similar to Cas9 mRNA signature (τ = 91.64, Supplementary Figure 2) but not to the core ISGs. Some of the small molecules identified as unique to Cas9 mRNA or core ISGs may nevertheless be false positives. We provide a full list of small molecules with significant similarity to Cas9 mRNA or core ISGs in Supplementary Material Figure 2 and 3.

Discussion

Implications for broad-spectrum antiviral agents

The innate immune response to viruses protects animals from a wide range of viral infections. While many viruses have evolved mechanisms to evade specific aspects of the innate immune response, small molecules that trigger antiviral responses offer a means to overcome this. In this study, we demonstrate that small molecules whose transcriptional signatures match the signatures of exogenous RNA sequences can be leveraged to predict potential broad-spectrum antiviral agents that act through distinct modes of action. For example, among the top small molecules identified in this study, puromycin, homoharringtonine, emetine (and its structural analog cephaeline),

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anisomycin and cycloheximide are protein synthesis inhibitors (Fig. 2). Except for puromycin, these protein synthesis inhibitors have been shown to have selective activity towards several viruses. Furthermore, at very low concentrations where they do not significantly affect host protein synthesis and cell viability, cycloheximide and anisomycin mimic interferon treatment by inhibiting the synthesis of specific proteins in encephalomyocarditis virus, murine leukemia virus and vesicular stomatitis virus (*19*, *20*). Homoharringtonine is a broad-spectrum antiviral molecule that enhances the degradation of phosphorylated eukaryotic initiation factor 4E (eIF4E) which is required for the synthesis of viral proteins (*21*) while emetine shows broad-spectrum antiviral activity to DNA and RNA viruses such as ebola virus, Zika virus, bovine herpesvirus-1 and Newcastle disease virus (*22*, *23*). Translation initiation and elongation are obligate steps in the replication cycle of many fast replicating RNA viruses, thus these small molecules could inhibit several RNA viruses. Interestingly, eIF4E enhances the anti-proliferative effects of IFN by promoting the translation of interferon stimulated genes (ISGs) demonstrating that translational control is an important part of the innate antiviral defense (*24*). In addition to the protein synthesis inhibitors, we found that thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca2+ ATPase (SERCA), has transcriptional signatures that highly match those of Cas9 mRNA delivery (Fig. 2A). The inhibition of SERCA by thapsigargin results into endoplasmic reticulum (ER) stress leading to the activation of interferon regulatory factor 3 (IRF3), a transcription factor that regulates multiple IFN-inducing pathways triggered by DNA and RNA viruses (*25*). Thapsigargin has antiviral activity at low concentrations without cellular toxicity to several viruses including Newcastle disease virus, peste des petits ruminant virus, murine norovirus and

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flaviviruses (*26–28*). Narciclasine, another top small molecule (Fig. 2A) has been shown to be active against the RNA flaviviruses (Japanese encephalitis, yellow fever and dengue viruses) as well as to the bunyaviruses Punta Toro and Rift Valley Fever virus (*29*).

Implications for broad-spectrum antivirals to emerging viruses, SARS-CoV-2

There is an urgent need to identify broad-spectrum antiviral drugs to emerging viral infections. In particular, the recent emergence of SARS-CoV-2 and its rapid spread through all parts of the world demands fast development of antiviral drugs and vaccines to counter the ensuing COVID-19 pandemic (*30*, *31*). To explore the potential for the predicted small molecules to inhibit SARS-CoV-2 in addition to their known broad-spectrum antiviral effects, we searched published literature on their potential activity on other coronaviruses including SARS-CoV-1, MERS-CoV, HCoV-NL63, HCoV-OC43 and HCoV-229E, mined a public database of broad-spectrum antiviral agents (*32*) and considered whether these molecules have been approved for other diseases. In a recent high-throughput screen of a library of 2,000 approved and pharmacologically active compounds, emetine, pyrvinium pamoate and cycloheximide were found to have broad-spectrum activity against the coronaviruses MERS-CoV, HCoV-OC43, HCoV-NL63 and MHV-A59 at low micromolar concentrations (*33*). Furthermore, in a separate screen of a library of 290 compounds with FDA approval or advanced clinical development, emetine, anisomycin, cycloheximide and homoharringtonine had high activity to both MERS-CoV and SARS-CoV-1 (*34*). More recently, emetine and homoharringtonine were demonstrated to have high activity against SARS-CoV-2 with an EC_{50} of 0.46 and 2.55 μM, respectively (*35*) (Fig. 3A) compared to EC_{50} of 23.15 and 26.63 μM for remdesivir

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and lopinavir (*36*). Besides its antiviral activity, emetine is also an expectorant in cough syrups and has also shown promise for the treatment of pulmonary arterial hypertension (*37*). Emetine is approved in many countries for the treatment of amoebic dysentery, homoharringtonine has been used for 30 years in China for the treatment of several tumors and a semisynthetic derivative of the drug was approved by the US FDA in 2012 for the treatment of chronic myelogenous leukemia (CML) while pyrvinium pamoate is approved by the FDA for the treatment of helminth infections. Notably, out of the compounds screened for activity against MERS-CoV, anisomycin and emetine had the lowest EC_{50} while for SARS-CoV-1, cycloheximide and emetine had the lowest EC_{50} in a previous study (*34*). Cycloheximide was also recently shown to inhibit SARS-CoV-2 replication in vitro with an IC50 of 0.17 μM which had no significant cytotoxicity to human Caco-2 cells (*38*). In addition, ivermectin- an antihelminth drug with activity against SARS-CoV-2 in vitro (*39*) currently under clinical trials for COVID-19- also showed significant similarity (τ = 91.64, Supplementary Figure 2) to the Cas9 mRNA signature. Thus, the predicted molecules appear to have high activity in a range of viruses (Fig. 3B).

Unlike remdesivir and chloroquine which are undergoing a clinical trial for SARS-CoV-2, the drugs we identified in this study have a number of advantages: i) they target host antiviral defenses instead of viral proteins, and may therefore avoid evolution of drug resistance which is rampant in RNA viruses, ii) they are likely to have activity across viruses that have little genetic similarity and infect different cell types, and iii) compared to other drugs that are only active in cells they diffuse in, small molecules that activate

antiviral defenses could result into secretion of antiviral effector proteins, cytokines and chemokines that diffuse to other cells and attract/ activate specialized immune cells.

Several mRNA-based vaccines are under development for COVID-19 including by Moderna Therapeutics, BioNTech/ Pfizer and CureVac (*40*, *41*). These vaccines aim to elicit adaptive immunity which is commonly viewed as independent from the innate antiviral defenses considered in this study. Yet, it is becoming increasingly clear that long-lasting adaptive humoral and cellular immunity involves an interplay with innate immunity (*42–44*). While innate antiviral responses to foreign RNA can negatively impact expression of the encoded antigens in mRNA vaccines, we propose that some of these small molecules could potentially mimic adjuvants. Since PRRs including those that detect foreign RNA are targets of many vaccine adjuvants (*42*, *43*), these small molecules may increase the quantity and quality of immune responses to mRNA vaccines when co-delivered. Hence, we propose that the small molecules identified in this study should be explored both for inhibitory activity against SARS-CoV-2/ COVID-19 as well as for potential enhancement of immunological responses to mRNA vaccines including for COVID-19.

Study Limitations

It is important to point out a number of limitations of this study. First, the transcriptional response to exogenous RNA analyzed in this study is based on data from a single cell type (CD34+ HSPCs) while transcriptional responses to perturbations varies across cell types. Nevertheless, by comparing the transcriptional responses of HSPCs to those of

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cancer cell lines exposed to small molecules in the CMap database, it is evident some of the genes involved in innate antiviral defenses are highly co-regulated across cell types (Fig. 2). Secondly, CD34+ HSPCs have a strong restriction to lentiviruses such as HIV-1 (*45*, *46*), making it possible that the transcriptional responses to exogenous RNA in these cells could be stronger than that in other cells. While this may be the case, there appears to be a considerable extent of co-regulation of the genes involved across cell types. Third, CRISPR/Cas systems evolved as antiviral defenses in bacteria. Therefore, the Cas9 mRNA signature may not entirely mimic a typical viral infection of human cells. Nevertheless, using the Cas9 mRNA signature captures core components of the foreign RNA response mediated by IFN that are not virus specific and, thus help to predict broad-spectrum antiviral agents. In addition, using the Cas9 mRNA signature is an important contribution as CRISPR/Cas systems are being explored as antiviral agents to human viruses including SARS-CoV-2 (*47*) and HIV-1(*48*) through direct cleavage of the viral genomes. In this respect, our work implies that the intrinsic ability of Cas9 mRNA to elicit innate immune responses may in part account for their antiviral activity in human cells. Small molecules that trigger the foreign RNA response such as the ones identified in this study may further boost the antiviral activity of CRISPR/Cas systems when used in combination. Finally, whether any of the identified drugs can be repurposed to novel viruses like SARS-CoV-2 requires a deeper understanding of the drug bioavailability in relevant tissues, the relative tolerability given the disease severity, the cost and scalability in manufacturing which are not addressed in this study.

Figures

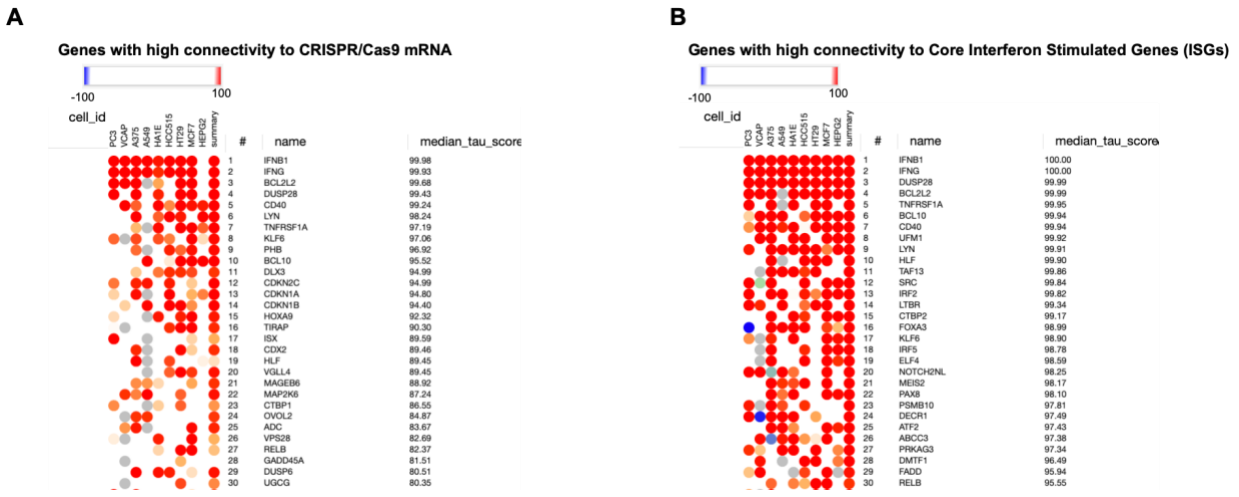

Fig 1: Top 30 genes whose over-expression in different cell lines in CMap are highly similar to transcriptional signatures of Cas9 mRNA (A) or core ISGs (B). Genes are ranked by the median tau score (τ) which measures the statistical significance of the similarity between the query signature (in this case Cas9 mRNA signature or core ISGs) and the signatures in the CMap database. The Cas9 mRNA signature consisted of a set of upregulated and downregulated genes following Cas9 mRNA and CRISPR sgRNA delivery into HSPCs using previously published data (*2*). A high positive similarity implies that the transcriptional signatures are highly similar, i.e. upregulated genes in the query also tend to be upregulated in the matching CMap database experiment and downregulated genes in the query tend to be downregulated in the CMap experiment. In the heatmaps, the 'Summary' column shows the overall similarity of the query signature to the experiments across various human cell lines and is a maximum quantile statistic of the normalized connectivity scores across cell lines between a query and reference signature (see Methods). $\tau > 90$ is considered as significant similarity where upregulated genes in the query are also upregulated in the given perturbation and downregulated

genes in the query are also downregulated in the perturbation. In contrast, $\tau < -90$ shows that the query and perturbation signatures are highly dissimilar, i.e. genes upregulated in one are downregulated in the other and vice versa. The heatmaps are color coded with the value of τ ranging from -100 (highly dissimilar to Cas9 mRNA signature) to +100 (highly similar to Cas9 mRNA signature). White regions of the heatmaps show perturbations where the query signature has no significant correlation to the target perturbation. Grey areas show perturbations for which data was missing. A full list of genes at this threshold for Cas9 mRNA is shown in A while for core ISGs a full list is provided in Supplementary Figure 1.

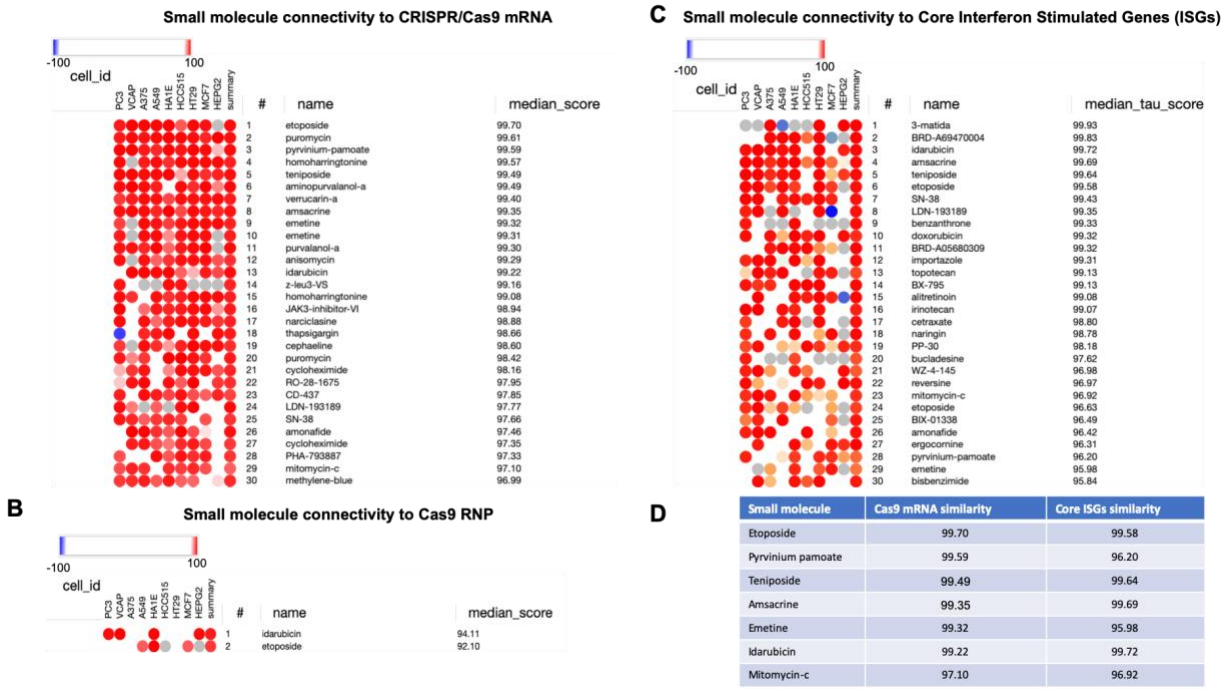

Fig. 2: Top 30 small molecules whose transcriptional signatures in various cell lines are highly similar to those of Cas9 mRNA delivery (A) or core ISGs (B) or Cas9 RNP (C).

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Genes are ranked by the median tau score (τ) which measures the statistical significance of the similarity between the query signature (Cas9 mRNA or core ISGs or Cas9 RNP) and the signatures in the CMap database (details in Methods). Only the top 30 compounds are shown with a full list of compounds (τ > 90) provided in Supplementary Figure 2 and 3. For Cas9 RNP, only 2 compounds shown in C met this criterion. The complete list of top 30 compounds for RNP are provided in Supplementary Figure 4. (D) Small molecules in (A) and (B) with transcriptional signatures highly similar to both Cas9 mRNA signature and core ISGs gene set. The 'Summary' column shows the overall similarity of the query signature to the experiments across various human cell lines. The heatmaps are color coded with the value of τ which ranges from -100 (highly dissimilar to Cas9 mRNA signature) to +100 (highly similar to Cas9 mRNA signature). White regions of the heatmap indicate no significant correlation between the query and perturbation while grey areas show missing data.

A

Drug	SARS-CoV-2 EC_{50} (Reference) 1μM	SARS-CoV-1 EC_{50} (Reference) μM	MERS-CoV EC_{50} (Reference) μM
Pyrvinium-pamoate	Unknown	Unknown	1.84 (Shen et al)
Homoharringtonine	2.55 (Choy et al)	Unknown	0.0718 (Dyall et al)
Emetine	0.46 (Choy et al)	0.051 (Dyall et al)	0.014 (Dyall et al)
Anisomycin	Unknown	0.191 (Dyall et al)	0.003 (Dyall et al)
Cycloheximide	0.17* (IC50 Bojkova et al)	0.043 (Dyall et al)	0.189 (Dyall et al)

B

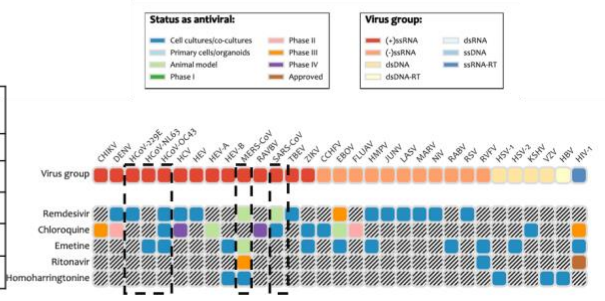

Fig. 3: Evidence of broad-spectrum antiviral activity of some of the identified small molecules based on published studies. (A) Summary of potential anti-viral activity of the candidate small molecules on human coronaviruses based on literature. * For

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cycloheximide effect on SARS-CoV-2,only IC50 values were available from literature (*38*). (B) Comparison of broad-spectrum antiviral activity of remdesivir, chloroquine and ritonavir which are currently undergoing clinical trials for the treatment of SARS-CoV-2 viral infection (COVID-19 disease) vs. emetine and homoharringtonine. Data was obtained from the broad-spectrum antiviral agents database, drugvirus.info (*32*). Highlighted by dashed boxes are the activity of these drugs on different coronaviruses. Boxes with black diagonals highlight cases where information on the activity of a given drug against a specific virus is unavailable based on previous publications in the broad-spectrum anti-viral drugs database (*32*).

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Author contributions

GHS conceived and designed the study. Both GHS and AVN performed analysis. GHS wrote the manuscript. Both authors reviewed the manuscript.

Conflict of interest: The authors declare no competing financial interests in the work presented.

Methods

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Matching Cas9 mRNA transcriptional responses to genes and small molecules in CMap

We obtained differentially expressed genes in CD34+ hematopoietic stem cells (HSPCs) exposed to Cas9 mRNA and sgRNA targeting the hemoglobin B locus (HBB) from Cromer et al (*2*). Briefly, Cromer *et al* reported transcriptional effects following electroporation of Cas9 mRNA containing 5-methylcytidine and pseudouridine modifications to limit innate immune responses and sgRNA containing 2'-o-methyl-3'-phosphothioate modification to enhance tolerance and activity. They performed differential gene expression analysis by computing log2 fold changes between Cas9 mRNA treated samples and those exposed to mock electroporation at 24-hour timepoint to identify genes differentially expressed following Cas9 mRNA delivery. Using these differentially expressed genes from Cromer et al, we constructed a transcriptional signature for Cas9 mRNA response by selecting the top 150 upregulated genes and the 150 most downregulated genes based on differential expression and using Bonferonni-corrected significance threshold of MLogP > 7, i.e. -log10(P value) > 7 (Links to data provided in Supplementary File 1). We restricted the number of up- and downregulated genes in the Cas9 mRNA signature to 150 based on the optimal recommended signature size in the L1000 based CMap (*6*). The Cas9 mRNA transcriptional signature was then used to query the CMap database which contains about 1.3 million transcriptional responses of human cell lines to chemical and genetic perturbation (*6*). To confine the analysis to well annotated genetic and small molecule perturbations in a core panel of cell lines, we queried the Touchstone dataset of CMap which contains about 238,125 expression signatures, approximately half of the total signatures in CMap. The core panel of cell lines in CMap are A375 (human malignant

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melanoma), A549 (human non-small cell lung carcinoma), HCC515 (human non-small cell lung adenocarcinoma), HEP2 (human hepatocellular carcinoma), MCF7 (human breast adenocarcinoma), PC3 (human prostate adenocarcinoma), VACP (human metastatic prostate cancer) and HT29 (human colorectal carcinoma). We performed two independent queries on CMap: i) the first query was to assess the similarity between the Cas9 mRNA signature and overexpression of different genes in human cells lines, ii) the second query was to assess the similarity between the Cas9 mRNA signature and several small molecules. We performed queries using the Clue.io API data version 1.1.1.2 and software version 1.1.1.43.

Matching core ISGs to genes and small molecules in CMap

We obtained a list of genes previously identified as core Interferon Stimulated Genes (core ISGs) based on their upregulation in response to IFN across 10 animal species- human, rat, cow, sheep, pig, horse, dog, microbat, fruit bat and chicken - as previously published (*14*). Briefly, Shaw et al stimulated fibroblasts from each of these species with IFN and then performed differential gene expression analysis on samples obtained when the cells were in an anti-viral state vs. controls (*14*). They determined that cells were in an anti-viral state when IFN stimulation led to at least 75% inhibition of VSV-ΔG-GFP infectivity. We used the 62 genes identified by Shaw et al. to be significantly upregulated (FDR < 0.05) by IFN stimulation across all 10 species in their study (*14*) as core ISGs (a link to this dataset is provided in Supplementary File 1). This core ISG gene set was then used to perform two independent queries on CMap: i) the first query was to assess the similarity between the ISGs gene set and overexpression of different genes in human

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cells lines, ii) the second query was to assess the similarity between the ISGs gene set and several small molecules. We performed queries on the CMap Touchstone database using the Clue.io API data version 1.1.1.2 and software version 1.1.1.43.

Computation of similarity measure (median tau score, τ) between signatures

For each query on CMap, a non-parametric similarity measure between the query and all signatures in the Touchstone database was computed as previously described (*6*). This similarity measure is referred to as the connectivity score (tau, τ). τ is a standardized measure that ranges from -100 to +100 and represents the percentage of reference queries that have a lower normalized connectivity score than that between the query signature (for example the Cas9 mRNA signature) and a given signature. To obtain, τ, a bidirectional version of the Kolmogorov-Smirnov enrichment statistic (ES) previously described (*49*) is used to first compute the Weighted Connectivity Score (WTCS). This score is then normalized to account for global variations in connectivity that might occur across the different cell types and perturbations resulting into a normalized connectivity score. The normalized score between the query and a given signature is then compared to the distribution of normalized scores between reference signatures to determine τ. A 'Summary' score is also obtained to summarize the connectivity across the cell lines using a maximum quantum statistic as described previously (*6*). Because the maximum quantile statistic is very sensitive to signal in a subset of cell lines, we used a median of this score, i.e. median tau (τ) to rank the results for each query. Computation of τ, the summary score and median τ in this study was performed using the CLUE online tool (https://clue.io/). Full details of the computations have been previously described (*6*).

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443

444 **Filtering of predicted small molecules for downstream mRNA delivery effects**

445 To filter out small molecules whose transcriptional signatures may have matched that of
446 Cas9 mRNA delivery due to downstream effects (e.g. translation of the mRNA into Cas9
447 protein that mediates double strand breaks), we also queried CMap using transcriptional
448 signatures constructed from genes differentially expressed after delivery of Cas9 protein
449 combined with sgRNA targeting HBB (ribonucleoprotein, RNP) at 24-hour timepoint from
450 the study by Cromer et al. (*2*). Differential expression after RNP delivery was determined
451 using the same approach described above for Cas9 mRNA differential expression
452 analysis. We then constructed an RNP transcriptional signature by selecting the top 150
453 upregulated genes and the 150 most downregulated genes based on differential
454 expression Bonferonni-corrected significance threshold of MLogP > 7, i.e. -log10(P value)
455 > 7 as reported in Cromer et al (*2*). The RNP signature was then used as a query to the
456 CMap Touchstone dataset using CLUE online tool. A link to the data used to construct
457 the RNP signature is provided in Supplementary File 1. We then excluded small
458 molecules whose signatures were highly similar (τ > 90) to both Cas9 mRNA and RNP
459 signatures.

460

461 **Supplementary Information**

462 **Supplementary File 1:** Contains links to data sources and Supplementary Figures 1 to
463 4.

464 **Supplementary File 2:** Contains an analysis file that can be used to explore the Cas9
465 mRNA signature similarity to Gene Over-Expression signatures in detail.

Supplementary File 3: Contains an analysis file that can be used to explore the core ISGs gene set similarity to Gene Over-Expression signatures in detail.

Supplementary File 4: Contains an analysis file that can be used to explore the Cas9 mRNA signature similarity to small molecule signatures in detail.

Supplementary File 5: Contains an analysis file that can be used to explore the RNP signature similarity to small molecule signatures in detail.

Supplementary File 6: Contains an analysis file that can be used to explore the core ISGs gene set similarity to small molecule signatures in detail.

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