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Review

Discovery and Development of Safe-in-man Broad-Spectrum Antiviral Agents

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Abstract: Viral diseases are one of the leading causes of morbidity and mortality in the world. Broad-spectrum antiviral agents (BSAAs) are key players in control of human viral diseases. Here, we reviewed the discovery and development process of BSAAs, focusing on compounds with available safety profiles in human. In addition, we summarized the information on approved, investigational and experimental safe-in-man BSAAs in freely accessible database at https://drugvirus.info/. The number of approved BSAAs will be increased as well as their spectrum of indications will be expanded pending the results of further pre-clinical and clinical studies. This will ultimately reinforce the arsenal of available antiviral options and provide better protection of general population from emerging and re-emerging viral diseases.

Keywords: virus; antiviral drug; drug discovery; drug development; broad-spectrum antiviral agents, BSAAs

1. Introduction

Viruses are one of the major causes of morbidity and mortality in the world (1-4). Antiviral drugs and vaccines are used to fight viral infections in human (5, 6). Previously, there has been a focus on "one drug, one virus" dogma, which relied on targeting virus-specific factors. An counterpoint to this is "one drug, multiple viruses" paradigm, which came with the discovery of broad-spectrum antiviral agents (BSAAs), small-molecules that inhibit a wide range of human viruses (7-12). This paradigm was based on the observation that different viruses utilize similar pathways and host factors to replicate inside a cell (13). Although the concept of BSAAs has been around for almost 50 years, the field received a new impetus recently with resent outbreaks of Ebola, Zika, Dengue, influenza and other viral infections, the discovery of novel host-directed agents as well as development of drug repositioning methodology.

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Drug repurposing, also called repositioning, redirecting, reprofiling, is a strategy for generating additional value from an existing drug by targeting disease other than that for which it was originally intended (14, 15). This has significant advantages over new drug discovery since chemical synthesis steps, manufacturing processes, reliable safety, and pharmacokinetic properties in pre-clinical (animal model) and early clinical developmental phases (phase 0, I and IIa) are already available. Therefore, repositioning of launched or even failed drugs from one disease to viral diseases provides unique translational opportunities, including a substantially higher probability of success to market as compared with developing new virus-specific drugs and vaccines, and a significantly reduced cost and timeline to clinical availability (9, 16, 17).

Here, we will describe repositioning of BSAAs, focusing on those antivirals, which have been already tested in human as antivirals, antibacterials, antiprotozoals, anthelmintics, etc. Moreover, we will detail the steps of drug development process, from discovery of novel antiviral activities in cell culture to post-market studies (Fig. 1). Finally, we will discuss future perspectives of safe-in-man BSAAs and their combinations for treatment of emerging and re-emerging viral infections and co-infections.

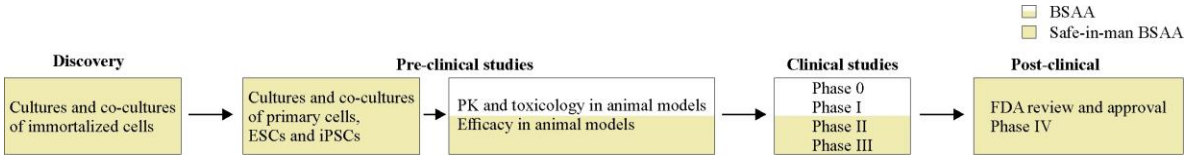

Figure 1. Discovery of novel activities and follow-up development of broad-spectrum antiviral agents (BSAAs). Yellow shading indicates a process of discovery and development of safe-in-man BSAAs, for which pharmacokinetic (PK) properties in pre-clinical (animal model) and early clinical developmental phases (phase 0-IIa trials) are already available. Abbreviations: ESCs, human embryonic stem cells; iPSCs, human induced pluripotent stem cells (iPSCs).

2. Discovery of novel activities of safe-in-man BSAAs in immortalized cell cultures and co-cultures

The discovery of novel activities of BSAAs starts with exposing cells to the candidate antiviral agent at different concentrations and infecting the cells with a virus of interest or mock. Immortalized cancerous cell cultures and co-cultures, which express appropriate viral receptors, are most commonly used in this first step. The half-maximal cytotoxic concentrations (CC_{50}) for a compound is calculated based on their dose-response curves obtained on mock-infected cells. The half-maximal effective concentrations (EC_{50}) are calculated based on the analysis of curves obtained on infected cells. Statistical analyses can help to determine if the differences between CC_{50} and EC_{50} are significant, given the inherent variability of the experiment (18). A relative effectiveness of a drug is defined as selectivity index ($SI = CC_{50}/EC_{50}$).

Cell viability assays and cell death assays are commonly used to assess the cytotoxicity and efficacy of BSAAs (Fig. 2). Cell viability assays include MTT, MTS, resazurin or similar assays, mitochondrial membrane potential-dependent dyes-based assays, esterase cleaved dye-based assays, ATP-ADP assays, and assays that measure glycolytic flux and oxygen consumption. Other cell death assays include LDH enzyme leakage assays, membrane impermeable dye-based assays, and apoptosis assays, such as Annexin V, TUNEL, and caspase assays (www.abcam.com/kits/cell-health-assays-guide) (19). For example, the Cell Titer Glo assay quantifies ATP, an indicator of metabolically active living cells, whereas Cell Tox Green assay uses fluorescent asymmetric cyanine dye that stains the DNA of dead cells (https://no.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/) (13, 20-22).

Viral strains or cell lines expressing reporter proteins are also used to assess the efficacy of BSAAs in infected cells. For example, TZM-bl cells expressing firefly luciferase under control of HIV-1 LTR promoter allowed quantitation of BSAA action on HIV-1 infection (tat-protein expression by integrated HIV-1 provirus) using firefly luciferase assay (23, 24). RFP-expressing RVFV, nanoLuc-expressing CHIKV and RRV, as well as GFP-expressing FLUAV, HCV and HMPV also allowed identification of novel activities of several BSAAs (13, 21, 25-31). In addition, qPCR/RT-qPCR,

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RNA/DNA sequencing, RNA/DNA hybridization, CRISPR-CAS immunofluorescence and plaque assays were used to detect inhibitory effects of BSAAs on viral replications (32-39).

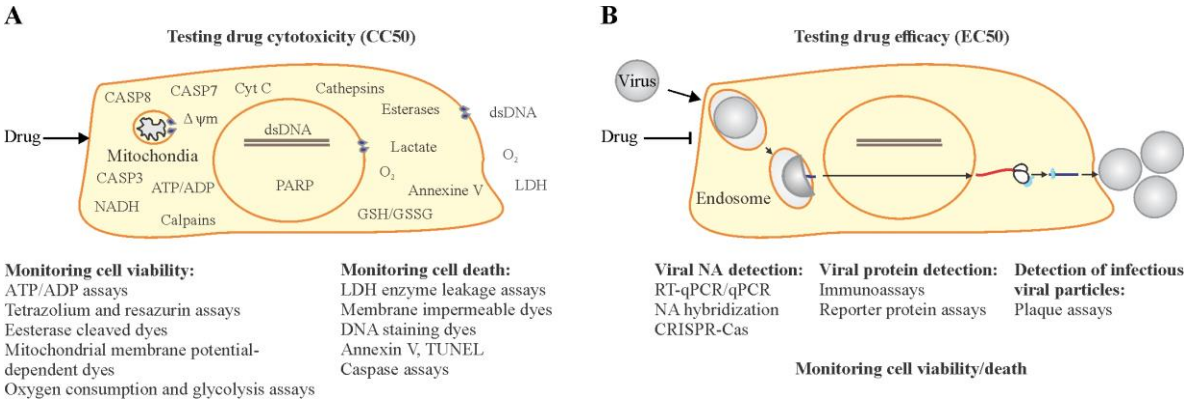

Figure 2. Testing BSAA toxicity (A) and efficacy (B) in immortalized cell cultures and co-cultures.

3. Pre-clinical evaluation of safe-in-man BSAAs

3.1. Evaluation of safe-in-man BSAAs in human primary cell cultures

Immortalized cancerous cell cultures/co-cultures and reporter viral strains represent excellent model systems for the discovery of novel activities of safe-in-man BSAAs. However, these genetically modified systems have certain limitations (attenuated or incomplete virus replication cycle, accumulation of mutations during repeated cell and virus passaging, defective innate immune responses and viral counter-responses, etc.) (40). Thereby, novel antiviral activities of BSAAs should be further validated in primary human cells using different viral strains (including wild-type viruses), different viral loads, different times of compound addition, different endpoint measurements and compound concentration range. Primary cell cultures give more accurate images of drug responses (41-44). They have a low population doubling level and therefore more closely recapitulate the physiological conditions observed in vivo.

Primary cells are cells isolated directly from tissues or blood using enzymatic or mechanical methods. The cells are characterized by their high degrees of specialization, are often fully differentiated and thus require defined culture conditions (serum-free media) in order to preserve their original phenotype. Peripheral blood mononuclear (PBMC), placental, amniotic and fetal primary cultures as well as vaginal/cervical epithelial and male germ cells have been used intensively to validate BSAA activity (42, 45-48). Although primary cell cultures are relevant systems for validation of BSAAs, there are technical difficulties limiting their use, such as ethical issues, purity of population of primary cells, and limited shelf life of the cells. In addition, age, race, sex and other genetic and epigenetic factors of donor cells should be considered. For example, common genetic variants in IRF7 and IFITM3 gene loci which is associated with innate immune responses to FLUAV infection in monocyte-derived dendritic cells, could influence on the results of BSAA efficacy experiments (49, 50).

3.2. Evaluation of safe-in-man BSAAs in human embryonic stem cell and human induced pluripotent stem cell cultures and co-cultures (organoids)

The obstacles associated with use of human primary cell cultures can be bypassed using human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs). ESCs are isolated from surplus human embryos, whereas iPSCs are obtained by reprogramming somatic cells. These cells proliferate extensively and retain multi-lineage activity, which allows to generate virtually any cell type of the body. The ESCs- and iPSC-derived cells have been used successfully to investigate the efficacy of several BSAAs against HBV, ZIKV, CHIKV and HSV-1 infections (Table S1) (51-56).

iPSCs, ESCs and primary tissue cells can be used to generate complex cultures termed organoids. Organoids are miniature and simplified version of organs. Establishing human airway, gut, skin, cerebral, liver, kidney, breast, retina and brain organoids allowed researchers to study toxicity and

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efficacy of several safe-in-man BSAAs against coronaviruses, influenza, enteroviruses, rotaviruses and flaviviruses (51, 57-63) (https://organovir.com/). However, iPSCs, ESCs and iPSCs/ESCs-derived organoids, have the same disadvantages as human primary cells (genetic differences, line-to-line and organoid batch-to-batch variability). On the other hand, these models allow researchers to predict the behavior of viruses in vivo and therefore to reduce animal use and in cases where animal models are unavailable to initiate clinical trials.

3.2. Evaluation of safe-in-man BSAAs in animal models

In vitro and ex vivo models do not fully reflect the complexity and physiology of living organisms. Therefore, several in vivo models have been developed to test novel antiviral activities of BSAAs. These include immunocompetent and genetically or chemically immunocompromised mice, guinea pigs, hamsters, ferrets, pigs, macaques and other animals (Fig. 3) (44, 64-68). PK/PD studies determine drug absorption, dosage and half-life of BSAAs. Toxicological studies determine if the drugs have any adverse effects on the tissues and organs of the animals and defining the dosage of adverse effects (69-71). Studying the efficacy of BSAAs is generally done by treating the animal with the drug or vehicle and infecting it with a virus of interest. Endpoints are usually body weight/ mortality (depending on the virus), histopathology, virus titers in organs, presence of clinical signs and development of immunity (72, 73). Although animal models can give the initial characterization of BSAA, it is important to keep in mind that they differ significantly from humans, with respect to symptoms, disease manifestation, susceptibility, immune responses, pathogenesis, and pharmacokinetics (74, 75).

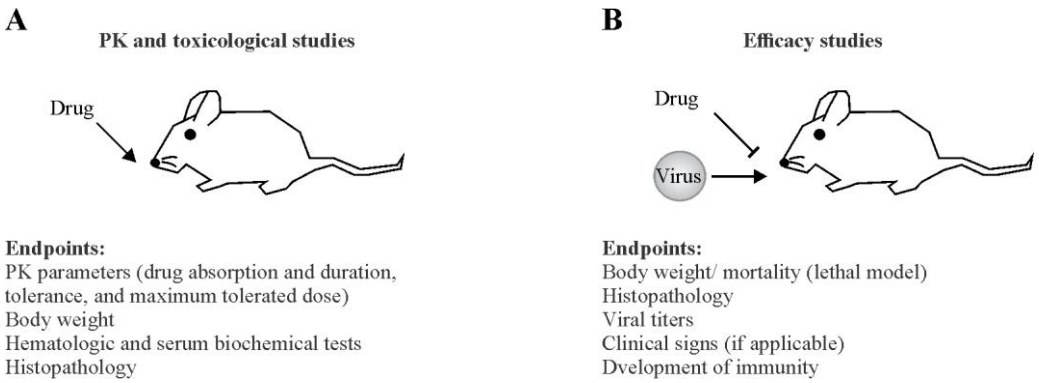

Figure 3. Testing toxicity and efficacy of BSAAs in animal models. (A) PK/PD and toxicity studies. (B) Efficacy studies. If BSAA is repositioned from another disease (i.e. its PK/PD and toxicity profiles are available for the animal model) it could bypass the safety studies.

4. Clinical trials and post-clinical studies of safe-in-man BSAAs

Clinical trials are the most critical and time-consuming step of a drug candidates' journey to being approved (Fig. 4). However, safe-in-man BSAAs make this journey relatively short, because they have been already at phase 0, I and sometime at IIa of clinical trials as antibacterial, antiprotozoal, anticancer, etc. agent; i.e. they have been administered at sub-therapeutic doses to healthy volunteers to ensure the drugs are not harmful to the participants. Thus, safe-in-man BSAAs enter phase II and III, which assess the efficacy, effectiveness, safety and side effects of the drugs in clinic. For this, patients with the viral disease in question are invited to join the study, where they are administered the BSAAs at the ideal therapeutic doses. Phase III is the longest of the phases, and include multiple levels of securities to the studies, such as the use of placebos and double-blinded studies, to ensure the data is as unbiased as possible. Upon completing phase III, depending on its performance and efficacy, BSAAs may end either being approved or dropped. The U.S Food and Drug Administration (FDA) estimates that only 25-30% BSAA candidates which enters phase III are approved for use in the public (76). After approval and marketing of the drug, phase IV may be initiated to follow up on the use of the drug in public, to surveil for rare effects (76, 77).

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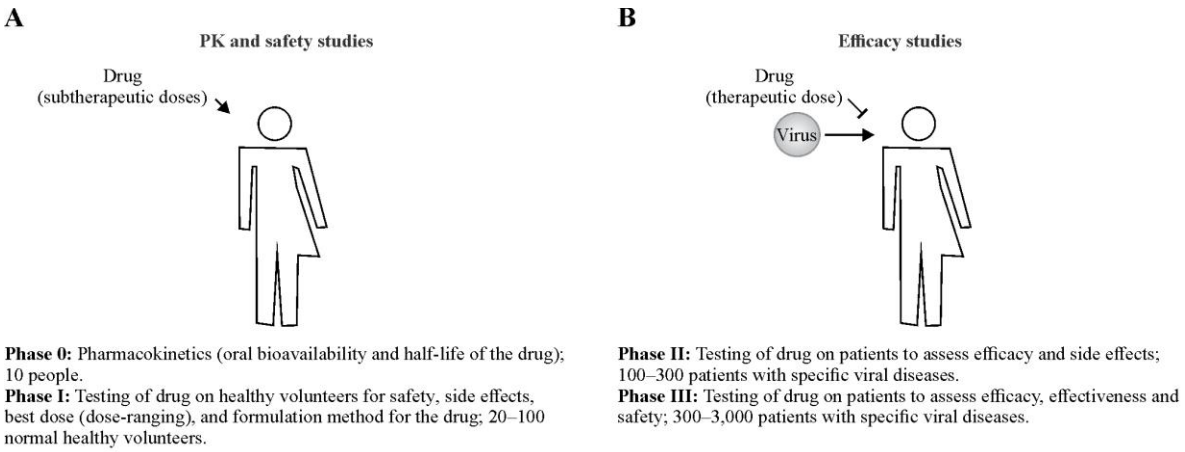

Figure 4. Clinical trials of BSAAs. (A) Pharmacokinetics (PK) and safety studies. (B). Efficacy studies. If BSAA is repositioned from another disease (i.e. its safety profile in man is available) it will bypass the PK and safety studies.

5. Database of safe-in-man BSAAs

We have developed a database for safe-in-man BSAAs, which is available at https://drugvirus.info/. The drug annotations were obtained from PubChem, DrugBank, DrugCentral, PubMed and clinicaltrials.gov databases (Table S1) (78-80). The information on virus families were exported from Virus Pathogen Database and Analysis Resource (Table S2) (81). The database summarizes activities and developmental stages of BSAAs (Fig. 5). The database allows interactive exploration of virus-BSAA interactions. It also includes information on BSA targets. A feedback form is available on the website. The website will be updated upon request or as soon as a new safe-in-man BSAA emerge or novel activity for an existing BSAA is reported.

The database includes 21 BSAAs which were approved by FDA, EMA or other agencies. These BSAAs altogether target 15 viruses. For example, favipiravir, also known as T-705, was approved against FLUAV in Japan; cidofovir is an injectable antiviral medication used as a treatment for CMV retinitis in people with AIDS; ribavirin, also known as tribavirin, is used for treatment of RSV and HCV infections; pleconaril is used against viruses in the picornaviridae family, including enterovirus and rhinovirus; and valacyclovir is used against CMV, EBV, HBV, HSV-1, HSV-2 and VZV infections. Twenty BSAAs are undergoing surveillance studies (phase IV). Azithromycin, chloroquine, cyclosporine, ezetimibe, mycophenolic acid, nitazoxanide and rapamycin progressed to phase IV studies without approvals from national or international authorities (NCT01779570, NCT02058173, NCT02564471, NCT00821587, NCT03360682, NCT02328963, NCT02768545, NCT01624948, NCT01770483, NCT02683291, NCT01624948, NCT01469884, NCT03901001, NCT01412515, NCT02990312).

The database also includes 48 safe-in-man BSAAs, which undergo clinical studies as antivirals. There are currently 21 compounds in phase I, 34 agents in phase II and 11 compounds in phase III clinical trials. For example, nitazoxanide, remdesivir and brincidofovir are under clinical investigations against different viral infections (NCT03336619, NCT00302640, NCT03605862, NCT03719586, NCT01276756, NCT03905655, NCT01529073, NCT03395405, NCT03216967, NCT01431326, NCT02087306, NCT01769170). The rest of safe-in-man BSAAs are still in pre-clinical or discovery stages. Of the drugs in this group, niclosamide is one of the interesting compounds because it showed the broadest spectrum of activities in vitro and in some cases in vivo (31, 61, 82-89). We believe that emetine and gemcitabine could be also pursued as potential BSAA candidates (31, 42, 90). ABT-263, also known as navitoclax, is another interesting BSAA, which is, by contrast to other compounds, facilitates death of infected cells without affecting non-infected cells (20, 91, 92).

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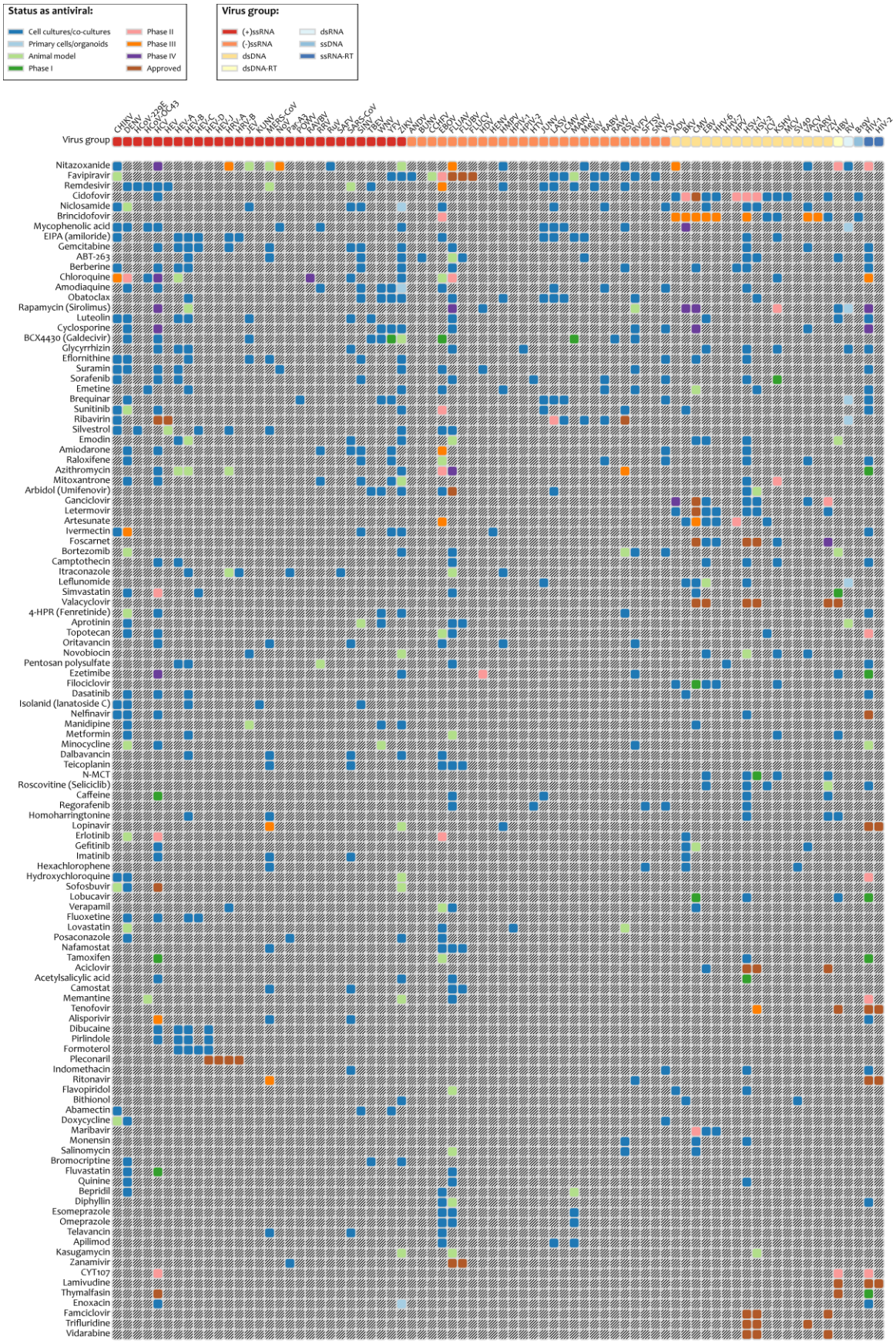

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Figure 5. Hundred and nineteen safe-in-man broad-spectrum antiviral agents (BSAAs) and viruses they inhibit. A snapshot is taken from https://drugvirus.info/ website. Viruses are clustered by virus groups. BSAAs are ranged from the highest to lowest number of targeted viruses. Different shadings indicate different development status of BSAAs. Gray shading indicates that the antiviral activity has not been either studied or reported. Abbreviations: ds, double-stranded; RT, reverse transcriptase; ss, single-stranded.

Altogether, the database contains 119 approved, investigational and experimental safe-in-man BSAAs, which inhibit 83 human viruses, belonging to 25 viral families. The BSAAs inhibit viral, host or both viral and host factors (Table S1). Analysis of BSAA targets and structures (Fig. 6) revealed that the most abundant are nucleotide and nucleoside analogues which inhibit viral RNA and DNA polymerases. Imatinib, erlotinib, gefitinib, and dasatinib that inhibit tyrosine kinases are the most abundant host-directed BSAAs. Most of the host targets (except Bcl-xL protein) are essential for viral replication but redundant for the cell, which is critical for reducing putative toxicities associated with blocking cellular pathways. The limited diversity of the targets and scaffolds could slow down the development of BSAAs.

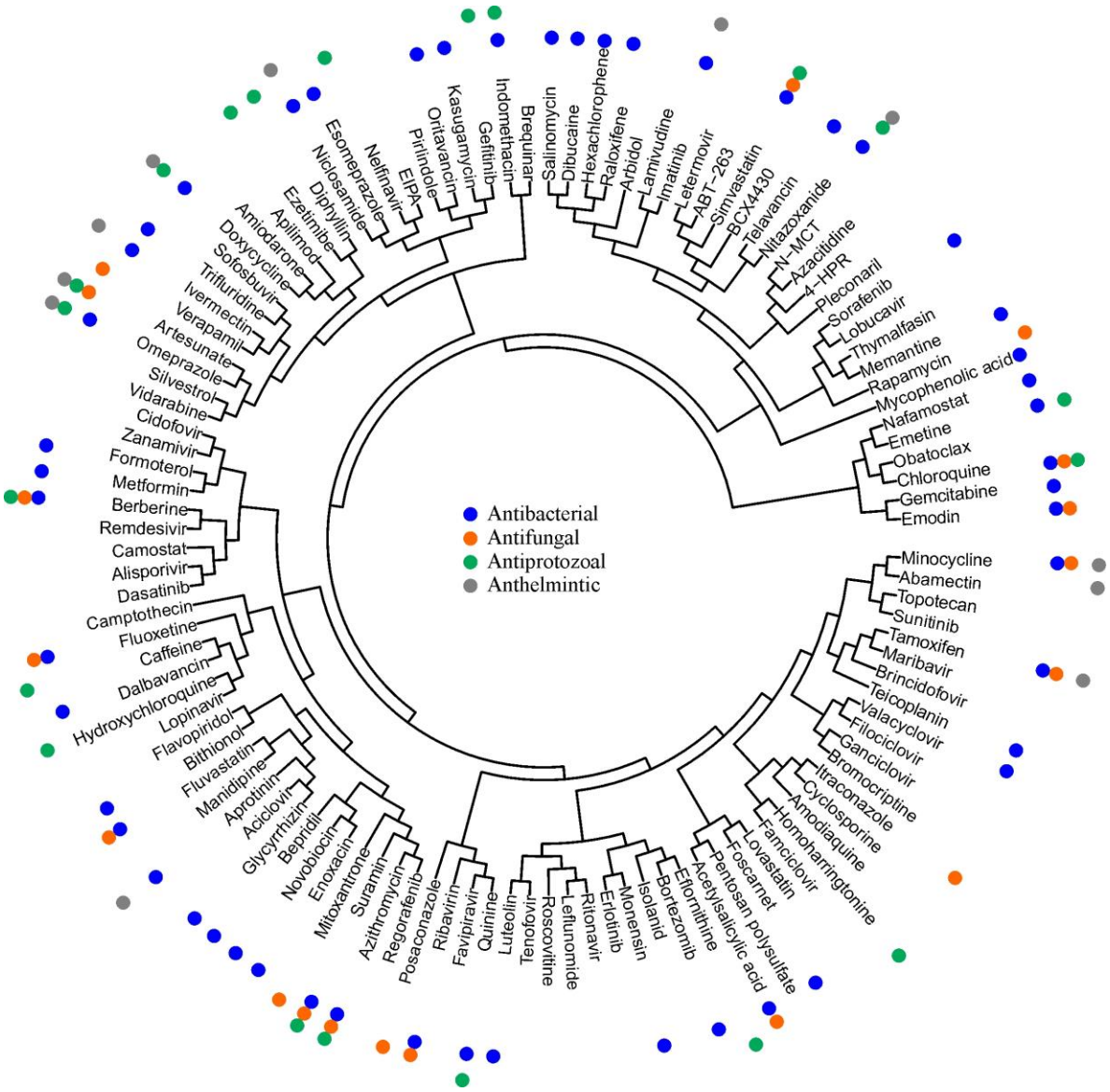

Figure 6. Structure-activity relationship of safe-in-man broad-spectrum antiviral agents.

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6. Conclusions and future perspectives

Here, we reviewed the discovery and development processes of safe-in-man BSAAs. In addition, we developed a database which consists of 119 BSAAs. These BSAAs block viral replication completely, reduce the viral burden to a level at which host immune responses can deal with it or facilitate apoptosis of infected cells. The database will be updated as soon as a new safe-in-man BSAA emerge or novel activity for an existing BSAA is reported.

Emerging BSAAs, such as 5,6-dimethoxyindan-1-one, saliphenylhalamide, and GS-5734 (22, 42, 90, 93-95), whose safety profiles in humans are not yet available, could serve as valuable antivirals in the future, pending the results of further pre-clinical and clinical investigations. The follow-up studies as well as the results of on-going, finalized or terminated clinical trials should be made publicly available to allow posterization and translation of emerging and existing BSAAs into clinical practice.

BSAAs could be combined with other antiviral agents to obtain synergistic or additive effects against certain viruses (17, 96). For example, it was reported that obatoclax and saliphenylhalamide, as well as gemcitabine and pimodivir (JNJ872) possessed synergistic effects against ZIKV and FLUAV infections in vitro, respectively (90, 97). Moreover, many combination therapies, which include BSAAs, became a standard for the treatment of HIV and HCV infections. These include abacavir/dolutegravir/lamivudine (Triumeq), darunavir/cobicistat/emtricitabine/tenofovir (Symtuza), lopinavir/ritonavir (Kaletra), ledipasvir/sofosbuvir and sofosbuvir/velpatasvir (98-100).

By contrast to individual drugs, combinations of 2-3 BSAAs could be used to target even broader range of viruses (17, 101). Such combinations could serve as front line therapeutics against poorly characterized emerging viruses or re-emerging drug-resistant viral strains. For example, a cocktail of nitazoxanide, favipiravir, and niclosamide could be developed for the treatment of infections of viruses belonging to 11 families.

Fifty BSAs possess not only antiviral but also antibacterial activity (Fig. 6; Table S1) (102). Moreover, 13 of the 50 agents are approved as antibiotics (2 withdrawn). These agents with dual activity could be used for treatment of viral and bacterial co-infections or for protection of patients from the secondary infections. For example, azithromycin could be used against FLUAV and *Chlamydophila pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae* or *Streptococcus pneumoniae* infections (NCT01779570) (103).

In addition, BSAAs showed activity against a wide range of other medically important human pathogens, including fungi, protozoa and parasites (Table S1) (104), pointing out that some pathogens utilize common mechanisms to infect hosts. Moreover, structure-activity relationship analysis of BSAAs suggest that some agents, such as doxycycline, artesunate, omeprazole, nitazoxanide, suramin, azithromycin, minocycline and chloroquine, could have novel antibacterial, antiprotozoal, antifungal or anthelmintic activities (Fig. 6). If confirmed, this could lead to development of broad-spectrum anti-infective drugs.

BSAAs could also serve as treatment of other co-morbidities simplifying the therapy and lowering its cost (Table S1). For example, the concomitant actions of ezetimibe and statins could be beneficial for treatment of both hypertension and several viral infections in patients with these co-morbidities (NCT00908011, NCT00099684, NCT00843661, NCT03490097, NCT00994773, NCT00441493).

In conclusion, BSAAs could play a pivotal role in the battle against emerging and re-emerging viral diseases. Discovery of novel BSAAs as well as repositioning existing safe-in-man BSAAs may shorten time and resources, needed for development of virus-specific drugs and vaccines. In the future, BSAAs will have global impact by decreasing morbidity and mortality from viral and other diseases, maximizing the number of healthy life years, improving the quality of life of infected patients and decreasing the costs of patient care.

Supplementary Materials: The following are available online at http://www.mdpi.com/XXXX. Table S1: Safe-in-man broad-spectrum antiviral agents; Table S2: Human viruses and associated diseases.

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Conflicts of Interest: The authors declare no conflict of interest.

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