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. Virus-specific vaccines and antiviral drugs are the most powerful tools to combat viral diseases. However, broad-spectrum antiviral agents (BSAAs) could provide additional protection of general population from emerging and re-emerging viral diseases reinforcing the arsenal of available antiviral options. Here, we reviewed discovery and development of BSAAs and summarized the information on 119 safe-in-man agents in freely accessible database (https://drugvirus.info/). Future and ongoing pre-clinical and clinical studies will increase the number of BSAAs, expand spectrum of their indications, and identify drug combinations for treatment of emerging and re-emerging viral infections as well as co-infections.

1. Introduction

Viruses are one of the major causes of morbidity and mortality in the world (DALYs and Collaborators, 2018; Disease et al., 2018; Howard and Fletcher, 2012; WHO, 2015). Antiviral drugs and vaccines are used to fight viral infections in human (De Clercq and Li, 2016; Marston et al., 2014). Previously, there has been a focus on "one drug, one virus" dogma, which relied on targeting virus-specific factors. A

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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 (Bekerman and Einav, 2015; de Clercq and Montgomery, 1983; Debing et al., 2015; Ianevski et al., 2019; Rada and Dragun, 1977; Sidwell et al., 1972). This paradigm was based on the observation that different viruses utilize similar pathways and host factors to replicate inside a cell (Bosl et al., 2019). Although the concept of BSAAs has been around for almost 50 years, the field received a new impetus with recent 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.

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 (Nishimura and Hara, 2018; Pushpakom et al., 2019). 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 (Figure 1). Therefore, repositioning of launched or even failed drugs 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 (Ianevski et al., 2019; Pizzorno et al., 2019; Zheng et al., 2018).

Here, we detail the steps of BSAA repurposing, from discovery of novel antiviral activities in cell culture to post-market studies. Moreover, we summarized currently available information on BSAAs in freely available database, focusing on those antivirals, which have been already tested in human as antivirals, antibacterials, antiprotozoals, anthelmintics, etc. Finally, we discuss future perspectives of using safe-in-man BSAAs for treatment of emerging and re-emerging viral infections as well as viral and bacterial co-infections.

2. Discovery and development of safe-in-man BSAAs

2.1. Discovery of novel BSAA activities 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 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

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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 (Meneghini and Hamasaki, 1967). 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 (Figure 2A). 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 (Shen et al., 2019). For example, the Cell Titer Glo (CTG) 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 (Bosl et al., 2019; Bulanova et al., 2017; Ianevski et al., 2018; Muller et al., 2014).

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 (Sarzotti-Kelsoe et al., 2014; Xing et al., 2016). 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 (Andersen et al., 2019b; Bosl et al., 2019; de Graaf et al., 2007; Habjan et al., 2008; Ianevski et al., 2018; Jupille et al., 2011; Kittel et al., 2004; Lee et al., 2017; Utt et al., 2016). In addition, qPCR/RT-qPCR, RNA/DNA sequencing, RNA/DNA hybridization, immuno- and plaque assays as well as CRISPR-CAS9 systems could be used for detection of inhibitory effects of BSAAs (Boonham et al., 2014; Fischer et al., 2017; Konig et al., 2019; Laamiri et al., 2018; Landry, 1990; Perez et al., 2013; Sashital, 2018; Zhou et al., 2018). Interestingly, CRISPR-Cas9, siRNA and shRNA approaches were used for identification of BSAA targets (Deans et al., 2016; Puschnik et al., 2017).

Novel anti-HSV-2 and anti-EV1 activities of emetine were discovered recently using CTG/plaque assays in human non-malignant RPE cells. Moreover, novel antiviral activities of the drug were identified using RFP-expressing RVFV, and GFP-expressing HMPV or FLUAV strains in RPE cells (Andersen et al., 2019a). Given that emetine also inhibits ZIKV, EBOV, RABV, CMV, HCoV-OC43 and HIV-1 infections (Chaves Valadao et al., 2015; MacGibeny et al., 2018; Mukhopadhyay et

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al., 2016; Shen et al., 2019; Yang et al., 2018), and that it is an FDA-approved anti-protozoal drug, it may represent a promising safe-in-man BSAA candidate.

2.2. Evaluation of BSAAs in human primary cell culture and co-cultures

Immortalized 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.) (Carter and Shieh, 2015). 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 (Alves et al., 2018; Denisova et al., 2012; Koban et al., 2018; Postnikova et al., 2018). 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 (Barrows et al., 2016; Denisova et al., 2012; Fink et al., 2018; Rausch et al., 2017; Robinson et al., 2018). 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 to determine common biological effect across a significant number of donors thereby avoiding minor variants (Lee et al., 2014; Zhang et al., 2013).

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

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(Table S1) (Ferreira et al., 2019; Iwasawa et al., 2019; Lanko et al., 2017; Simonin et al., 2019; Xia et al., 2017; Zhou et al., 2017).

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 efficacy of several safe-in-man BSAAs against coronaviruses, influenza, enteroviruses, rotaviruses and flaviviruses (Li et al., 2017; Sacramento et al., 2017; Watanabe et al., 2017; Xu et al., 2016; Yin et al., 2015; Yin et al., 2018; Yin et al., 2016; Zhou et al., 2017). 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.

For example, novel anti-ZIKV activities of enoxacin, amodiaquine and niclosamide were discovered recently using human neural progenitor cells, human pluripotent stem cell-derived cortical neural progenitor cells, and human induced neural stem cells, respectively (Cairns et al., 2018; Xu et al., 2019; Zhou et al., 2017). Enoxacin is an oral broad-spectrum fluoroquinolone antibiotic, which also possesses anti-HCV and anti-HIV-1 activities in immortalized cell cultures (Kashiwase et al., 1999; Young et al., 2010). Amodiaquine is an anti-malaria drug, which also possesses antiviral activities against DENV, HCV, RRV, SINV, WNV, EFV, EBOV, LASV, RABV, VZV, and HSV-1 in immortalized cell cultures (Boonyasuppayakorn et al., 2014; Hulseberg et al., 2019; Mazzon et al., 2019). Niclosamide is an orally bioavailable anthelmintic drug, which inhibits the broadest range of viruses in vitro and, in some cases, *in vivo* (Cairns et al., 2018; Fang et al., 2013; Huang et al., 2017; Hulseberg et al., 2019; Jurgeit et al., 2012; Kao et al., 2018; Mazzon et al., 2019; Stachulski et al., 2011; Wang et al., 2016; Wu et al., 2004). These safe-in-man BSAAs represent promising drug candidates.

2.3. Evaluation of 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 (Figure 2B) (Alves et al., 2018; Haese et al., 2016; Louz et al., 2013; Morrison and Diamond, 2017; Taylor, 2017; Thangavel and Bouvier, 2014). PK/PD studies determine drug absorption, dosage and half-life of

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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 (Alabaster V. and In Vivo Pharmacology Training Group, 2002; Parasuraman, 2011; Rizk et al., 2017). 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 (Oh and Hurt, 2016; Smee and Barnard, 2013). 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 (Barré-Sinoussi and Montagutelli, 2015; Shanks et al., 2009).

For example, aminoglycoside antibiotics, kasugamycin and neomycin were successfully tested against ZIKV, FLUAV, and HSV-2 infections in mice (Gopinath et al., 2018). Polyether antibiotic salinomycin also showed anti-FLUAV effect in mice (Jang et al., 2018). In addition, investigational anticancer agent, flavopiridol, was effective against FLUAV in mice (Soderholm et al., 2016). These findings support further development of these and other BSAA.

2.4. Clinical trials and post-clinical studies of BSAAs

Clinical trials are the most critical and time-consuming step of a drug candidates' journey to being approved (Figure 2C). 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 (U.S Food and Drug Administration, 2018). 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 (U.S Food and Drug Administration, 2018; Umscheid et al., 2011).

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Forty-eight safe-in-man BSAAs 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).

Twenty-one BSAAs 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).

3. BSAA database

We have developed a database for safe-in-man BSAAs, which is available at https://drugvirus.info/ (Figure 3). The drug annotations were obtained from PubChem, DrugBank, DrugCentral, PubMed and clinicaltrials.gov databases (Table S1) (Kim et al., 2019; Ursu et al., 2019; Wishart et al., 2018). The information on virus families were exported from Virus Pathogen Database and Analysis Resource (Table S2) (Pickett et al., 2012). The database summarizes activities and developmental stages of BSAAs. 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 database will be updated upon request or as soon as a new safe-in-man BSAA emerges or novel activity for an existing BSAA is reported.

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 or host factors and block viral replication completely, reduce the viral burden to a level at which host immune responses can

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deal with it or facilitate apoptosis of infected cells (Table S1). Analysis of BSAA targets and structures (Figure 4) 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 BSAA concept.

Emerging BSAAs, such as 5,6-dimethoxyindan-1-one, saliphenylhalamide, and GS-5734 (Denisova et al., 2012; Kuivanen et al., 2017; Muller et al., 2011; Muller et al., 2014; Patil et al., 2017; Sheahan et al., 2017), whose safety profiles in humans are not yet available, are not included in the database. However, they could serve as valuable antivirals in the future, pending the results of further pre-clinical and clinical investigations.

4. BSAA combinations

BSAAs could be combined with other antiviral agents to obtain synergistic or additive effects against certain viruses (Cheng et al., 2019; Zheng et al., 2018). Several combination therapies which include BSAAs (such as abacavir/dolutegravir/lamivudine (Triumeq), darunavir/cobicistat/emtricitabine/tenofovir (Symtuza), lopinavir/ritonavir (Kaletra), ledipasvir/sofosbuvir and sofosbuvir/velpatasvir) became a standard for the treatment of HIV or HCV infections. Several synergistic drug combinations such as, obatoclax/saliphenylhalamide and gemcitabine/pimodivir could enter clinical studies and become effective treatment of ZIKV and FLUAV infections (Fu et al., 2016; Kuivanen et al., 2017).

By contrast to individual drugs, combinations of 2-3 BSAAs could be used to target even broader range of viruses (Foucquier and Guedj, 2015; Zheng et al., 2018). 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 viruses belonging to 11 families.

5. Other activities of BSAAs

Fifty BSAAs possess not only antiviral but also antibacterial activity (Figure 4; Table S1) (Schor and Einav, 2018). 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 the protection of patients from

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the secondary infections. For example, azithromycin could be used against FLUAV and *Chlamydophila pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae* or *Streptococcus pneumoniae* infections (NCT01779570) (Mandell et al., 2007).

In addition, BSAAs showed activity against a wide range of other medically important human pathogens, including fungi, protozoa and parasites (Table S1) (Montoya and Krysan, 2018), 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 (Figure 4). If confirmed, this could lead to development of broad-spectrum anti-infective drugs.

BSAAs could also serve as treatment of other co-morbidities, therefore, 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).

6. Conclusions and future perspectives

Here we reviewed a process of BSAA development and summarized information on 119 safe-in-man agents in freely available database. We hope that further pre-clinical and clinical studies on BSAAs will be harmonized, and data collection will be standardized. Furthermore, the follow-up studies as well as the results of on-going, finalized or terminated clinical trials will be made publicly available to allow prioritization and translation of emerging and existing BSAAs into clinical practice. This would allow BSAAs to play a pivotal role in the battle against emerging and re-emerging viral diseases.

For example, safe-in-man BSAAS could be effective against emerging 2019-nCoV coronaviruses (Hui et al., 2020). In particular, oritavancin and dalbavancin, could be repurposed for treatment of 2019-nCoV infections. Both drugs are approved antibiotics that have been shown to inhibit MERS-CoV and SARS-CoV infections in vitro (Zhou et al., 2016).

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.

Supporting information

The following are available online. 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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Ethical Approval

Approval was not required.

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Figure legends

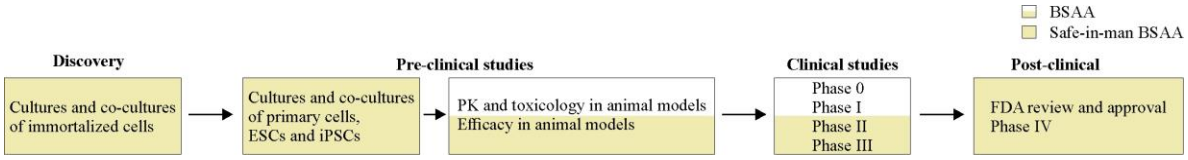
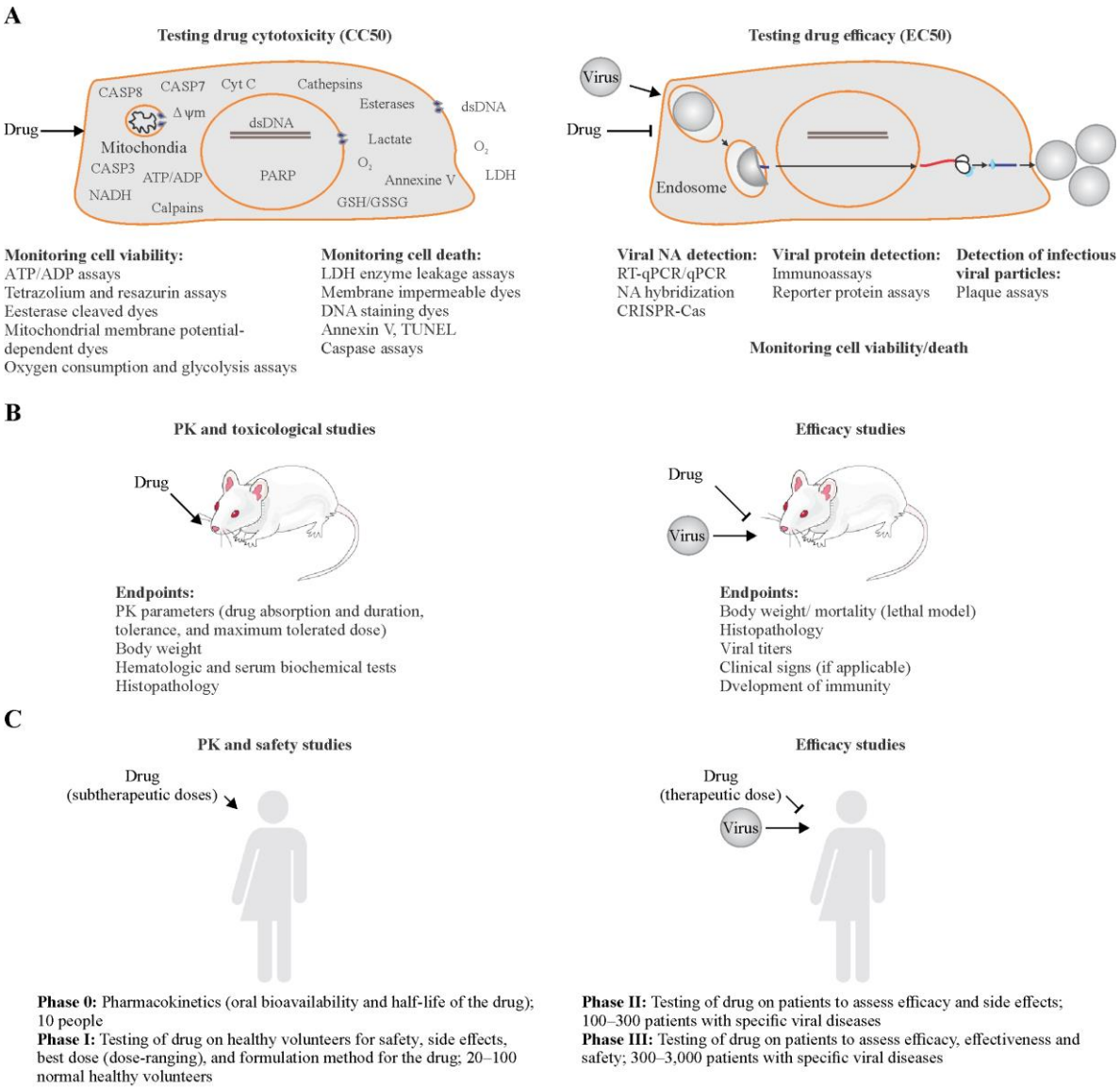


Figure 1. Discovery of novel activities and follow-up development of broad-spectrum antiviral agents (BSAAs). Yellow shading indicates a process of discovery and development of safe-in-man BSAAs, for which pharmacokinetic (PK) properties in pre-clinical (animal model) and early clinical developmental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



Phase 0: Pharmacokinetics (oral bioavailability and half-life of the drug); 10 people
Phase I: Testing of drug on healthy volunteers for safety, side effects, best dose (dose-ranging), and formulation method for the drug; 20–100 normal healthy volunteers

Phase II: Testing of drug on patients to assess efficacy and side effects; 100–300 patients with specific viral diseases
Phase III: Testing of drug on patients to assess efficacy, effectiveness and safety; 300–3,000 patients with specific viral diseases

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Figure 2. ABC of BSAA development process. (A) Testing BSAA toxicity (left panel) and efficacy (right panel) in immortalized cell cultures and co-cultures. (B) Testing BSAA toxicity (left panel) and efficacy (right panel) in animal models. 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. (C) Clinical trials of BSAAs. (Left panel) Pharmacokinetics (PK) and safety studies. (Right panel) Efficacy studies. If the drug is repositioned from another disease (i.e. its safety profile in man is available) it could bypass the PK and safety studies in man.

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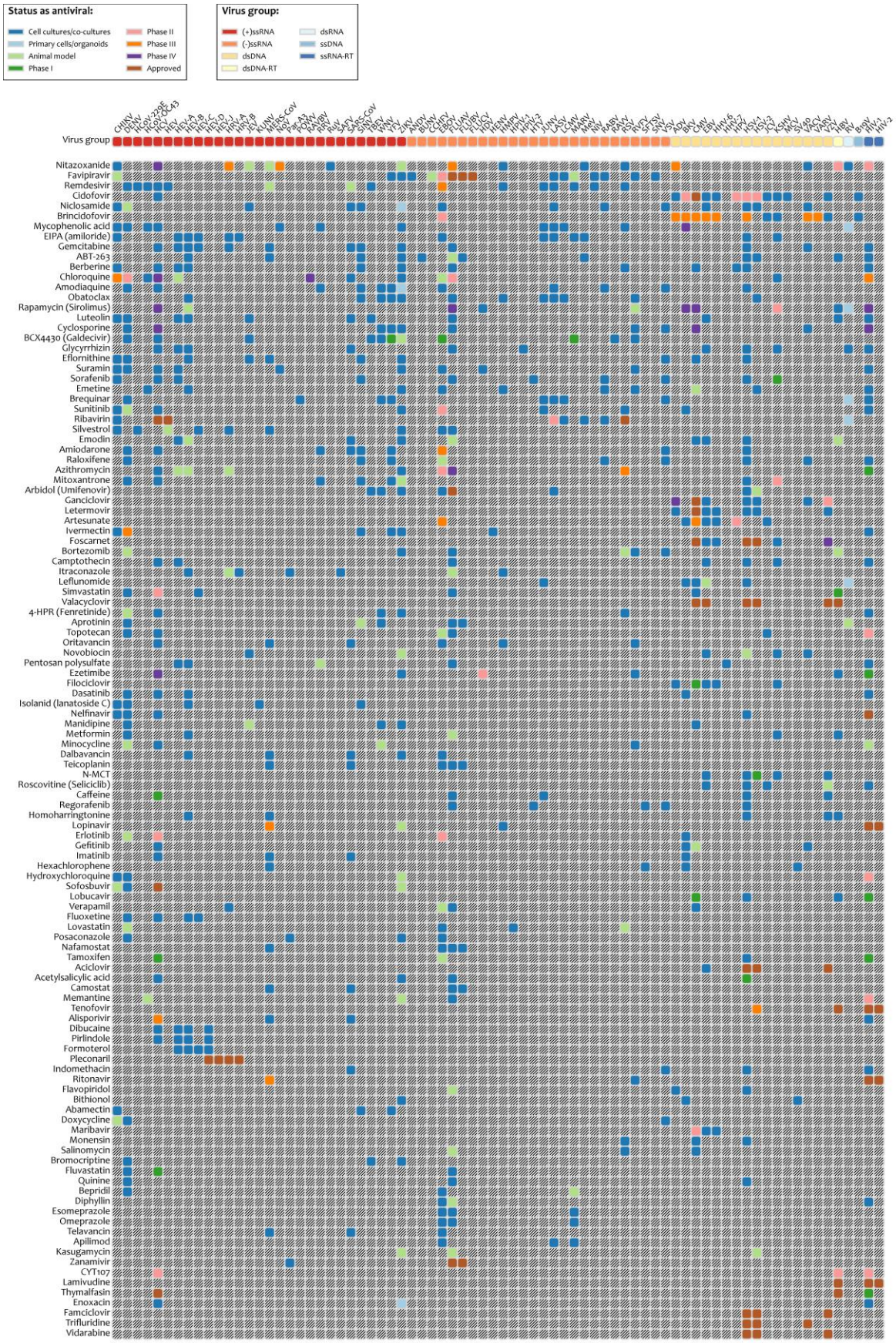

Figure 3. Hundred and nineteen safe-in-man broad-spectrum antiviral agents (BSAAs) and viruses they inhibit. A snapshot is taken from

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.

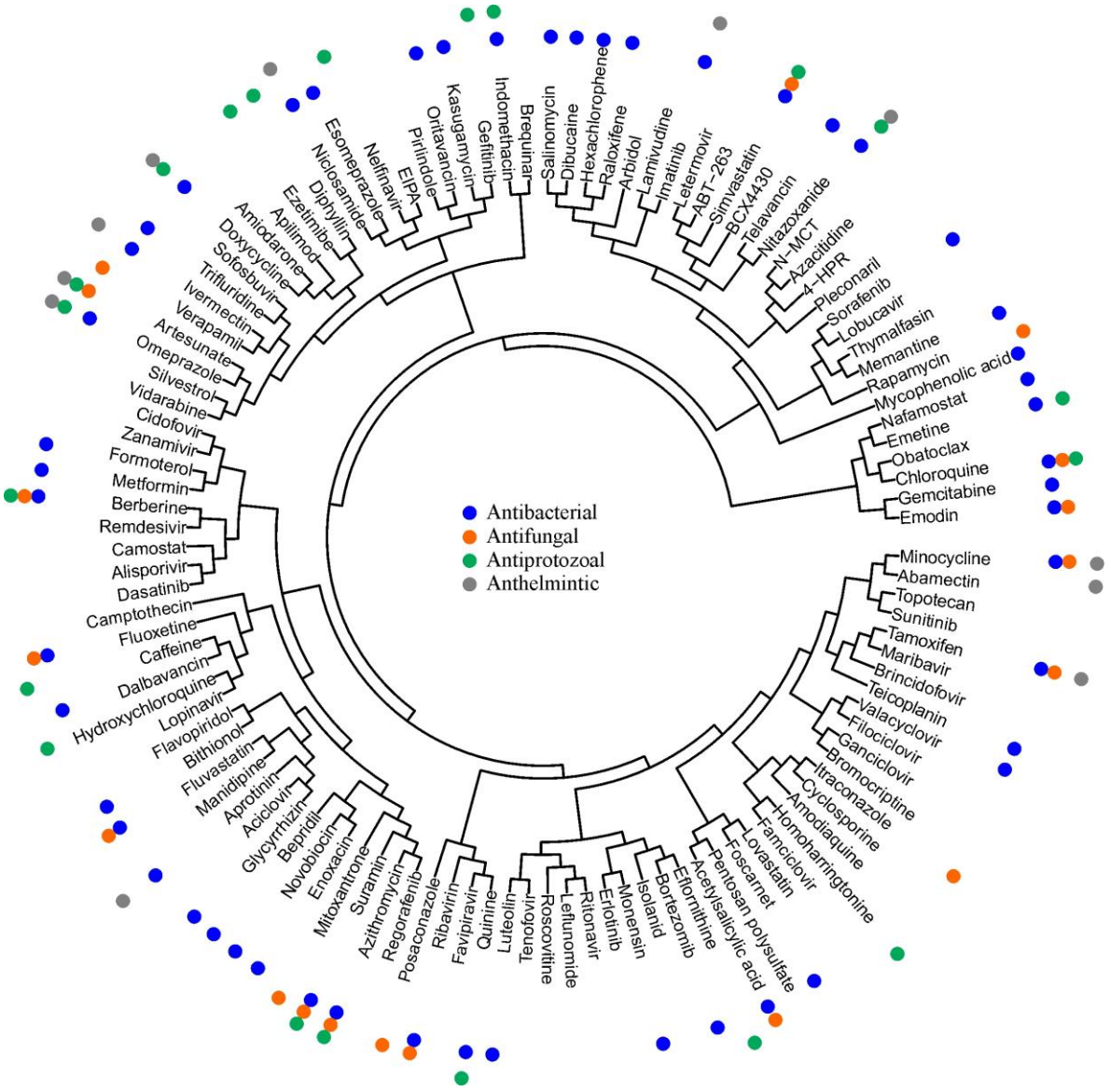

Figure 4. Structure-activity relationship of safe-in-man BSAAs. Web-application serves C-SPADE was used to cluster BSAAs based on their structural similarities and visualize them as a dendrogram of compounds augmented with their functional annotations (https://cspade.fimm.fi/)