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Exploring antiviral strategies:

From innate immunity modulation to viral proteins targeting.

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Ph.D. Student: Annalaura Paulis

Supervisor: Enzo Tramontano

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*Dedicated to the strong women
of my life, who have spent their
life in serving others.*

*Your perseverance, sacrifice,
and unselfishness were never
praised enough.*

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To plant a garden, is to believe in the future.

There is no better metaphor that could represent this incredible journey in becoming a researcher: a little plant nourished with passion, curiosity, and sake for science has grown into a blooming tree.

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Abstract

The search for new targets and antiviral agents is continuously growing, especially for agents with broad-spectrum activity. In fact, even though vaccines are the elective way to limit diseases and possibly eradicate pathogens, they are not always effective, particularly in people with compromised immune defenses, and they can also lose activity against rapidly evolving pathogens. Hence the identification of novel antiviral agents is a priority for the health systems, and the number of approved antiviral drugs is increasing yearly: until today over one hundred antivirals have been approved. The most common strategies to develop antiviral drugs are based on the identification of molecules targeting viral proteins and blocking viral replication as direct acting agents. Although successful, this strategy must consider that most viruses are easily capable to select drug resistant strains. A novel approach for the identification of potential broad-spectrum antivirals is to target cellular proteins inducing innate immune response, so avoiding the high mutagenesis rate occurring for viral proteins. An important help in fighting viral spread can arrive from the Traditional Chinese Medicine (TCM), widely used in China to treat infectious diseases both alone and in cooperation with western medicines.

This thesis will investigate three possible paths for the identification of potential antivirals i) the identification of cellular targets to develop broad-spectrum antivirals, ii) target viral proteins in order to impair viral replication selectively, iii) study the effect of immunomodulators with unknown targets in order to identify potential broad-spectrum antiviral.

Among the cellular proteins possibly used as drug target, a recently discovered one is STING: a downstream actor in the detection of non-self cytosolic nucleic acids related to viral infections and tumor conditions. The Stimulator of Interferon Genes (STING) plays a pivotal role in counteracting viral infections, independently from whether the viral genome is DNA or RNA, mounting a strong innate immune response driven

principally by type I Interferon (IFN-I). On the one side, when cytosolic DNA is detected cGAS produces cyclic GMP-AMP (2'3'cGAMP) that directly binds STING determining TBK1 phosphorylation (pTBK1) and hence transcription of IFN-I. On the other side, when the RIG-I pathway is activated in response to viral RNA detection, STING interacts with activated Mitochondrial Antiviral Signaling protein (MAVS) determining pTBK1 mediated IFN-I production.

The SARS-CoV-2 pandemic have highlighted the need of specific as well as potent antivirals; among SARS-CoV-2 proteins the Papain Like protease (PL-pro) is an important protein involved in viral life cycle and in the innate immune evasion, hence it will be investigated as potential viral target for the development of selective antiviral drugs.

Finally, we will investigate the mechanism of action of a series of compounds derived from one molecule isolated from herbal extracts used as immune stimulator in the TCM, in order to verify their potency in inducing the innate immune response.

List of Publications

- ◇ Annalaura Paulis, Angela Corona, Enzo Tramontano. “Chapter Five - Inhibitors of Ebolavirus targeting innate immune evasion”. Annual Reports in Medicinal Chemistry, Academic Press, Volume 57, 2021, Pages 153-174. <https://doi.org/10.1016/bs.armc.2021.09>.
- ◇ Annalaura Paulis, Enzo Tramontano. “Unlocking STING as therapeutic antiviral strategy”. International Journal of Molecular Sciences. (Accepted for publication)

As part of my work during the PhD, I’ve been involved in the following publications:

- ◇ Angela Corona; David Strayer; Simona Distinto; Gian Luca Daino; Annalaura Paulis; Enzo Tramontano; William M. Mitchell. “Ebola virus disease: In vivo protection provided by the PAMP restricted TLR3 agonist rintatolimod and its mechanism of action”. Annual Reports in Medicinal Chemistry, Volume 212, 2023, DOI: <https://doi.org/10.1016/j.antiviral.2023.105554>.
- ◇ I. Stefanelli, A. Corona, C. Cerchia, E. Cassese, S. Improta, E. Costanzi, S. Pelliccia, S. Morasso, F. Esposito, A. Paulis, S. Scognamiglio, F.S. Di Leva, P. Storici, M. Brindisi, E. Tramontano, R. Cannalire, V. Summa, Broad-spectrum coronavirus 3C-like protease peptidomimetic inhibitors effectively block SARS-CoV-2 replication in cells: Design, synthesis, biological evaluation, and X-ray structure determination, European Journal of Medicinal Chemistry (2023), doi: <https://doi.org/10.1016/j.ejmech.2023.115311>.

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Chapter I

Introduction

1.1 The innate immune response

Organisms have learnt how to coexist with invading pathogens by developing barriers that could counteract pathogen's invasion and spread. The first step for an invading pathogen is to overcome chemical and physical barriers that limit pathogen invasion such as skin, mucous membranes, antimicrobial enzymes, and peptides, acidic or basic environments [1]. However, in some cases these barriers are not enough to counteract the invasion and pathogens can attack the organism getting access to the main organism's delivery system, the blood flux, through which they reach all the body districts. At the same time however, pathogens spread also put them into contact with the intrinsic cellular defense system, allowing the start of the organisms' countermeasures [1]. The pathogens' detection occurs through specific proteins called Pattern Recognition Receptors (PRRs) which recognize and bind pathogens structures such as lipopolysaccharide (LPS), nucleic acids, and pathogens proteins; more in general the so-called Pathogen Associated Molecular Patterns (PAMPs) [2], [3]. Different PRRs are involved in the pathogen recognition depending on the PAMP detected, among them are the Toll Like Receptors (TLRs), the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) as well as the NOD-like receptors (NLRs) and the cyclic GMP-AMP synthase (cGAS) [4]–[7] (Fig. 1). TLRs are transmembrane receptors located on the cellular surface or on the membrane of intracellular vesicles such as endosomes and lysosomes that detect a wide variety of PAMPs such as lipoproteins, LPS, flagellin, C—phosphate—G (CpG)-DNA, viral ssRNA, dsRNA. Differently, RLRs, NLRs and cGAS are all located in the cytoplasm of the cells. RLRs detect long and short dsRNA and 5'triphosphate RNA inducing pTBK1 dependent

IFN-I transcription; NLRs detect peptidoglycan component (iE-DAP) and intracellular muramyl dipeptide (MDP); cGAS recognizes viral dsDNA and tumor derived DNA producing cyclic dinucleotides that bind the Stimulator of Interferon Genes (STING) inducing IFN-I transcription [4]–[10] (Fig. 1).

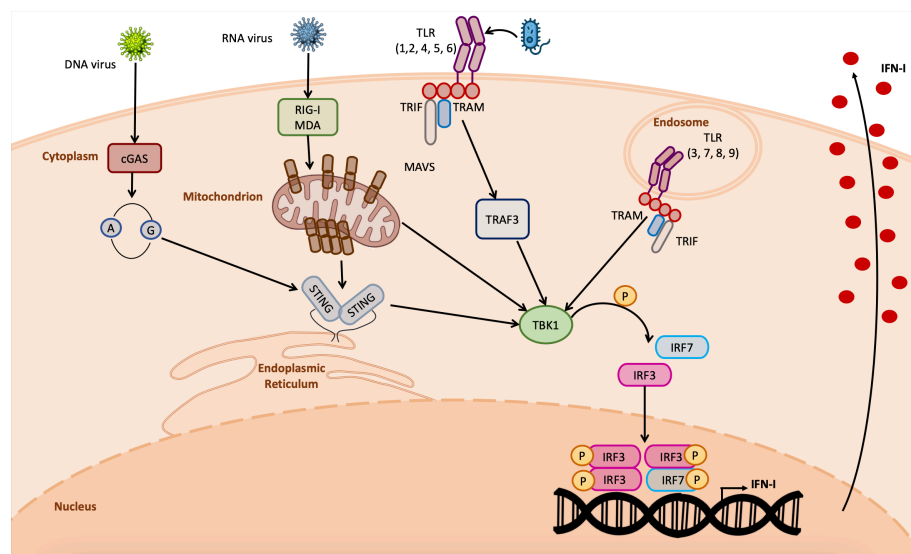


Figure 1. Overview on IFN-I induction by viral infection [10].

PAMP detection is the starting point of the host organism innate immune response that involves the activation of phosphorylating cascades and leads to the production of cytokines and chemokines. Cytokines are low molecular weight (15-20 kDa) proteins or glycoproteins [8]. Among the cytokines, there are specific ones principally involved in response to viral infections called Interferons (IFNs) for their ability to interfere with viral replication [9]. IFNs can be divided into type I type II and the more recently identified type III, each of them triggers different response interacting with specific receptors [9], [11]. IFN-I includes 13 IFN- α isoforms and only one IFN- β as well as IFN- ϵ , κ , δ , τ , ω , ζ . [11]. IFN-II has only one member (gamma) [11], while IFN-III includes 11, 2, 3, 4 [12], [13]. The IFN-I, once released by the cells, binds to

IFN- α/β receptors (IFNAR1/2) on the surface of neighbouring cells, these receptors are permanently associated with kinases belonging to the family Janus (JAK) (Fig. 2). The JAK/STAT pathway includes four members, JAK 1, 2, 3 and tyrosine kinase (Tyk) and seven Signal Transducer and Activation of Transcription (STAT 1–4, 5a, 5b and 6) [14]. IFNARs activation by IFN-I determines the phosphorylation of tyrosine residues on the receptor tail [15], and this causes the recruitment of STAT1 and STAT2 through phosphorylated Tyk2 and JAK1 [16]. Upon binding with JAK1, STATs are phosphorylated by JAKs and are released as heterodimer of phosphorylated STAT1 and STAT2 [17]. The STAT1-STAT2 heterodimer interacts in the cytoplasm with the Interferon Regulatory Factor 9 (IRF9) protein forming a complex called IFN-stimulated gene factor 3 (ISGFR3) STAT1-STAT2 that is translocated into the nucleus due to the activity of some of the karyopherin alpha (KPNA) proteins, namely, the NPI-1 KPNA (KPNA1, KPNA5 and KPNA6). The complex binds a specific sequence in the DNA called IFN Stimulated Responsive Element (ISRE) leading to transcription of Interferon Stimulated Genes ISGs [18], [19]. Such ISGs can be divided into two categories due to their role, either antiviral “effectors” response or “regulators” of the response. The first ISGs improve the viral recognition and target the viral life cycle to stop viral infection and recruit immune cells, while second ISGs are essential in returning to normal balance cellular homeostasis post-infection, modulating the anti-viral response that could potentially damage the host [17].

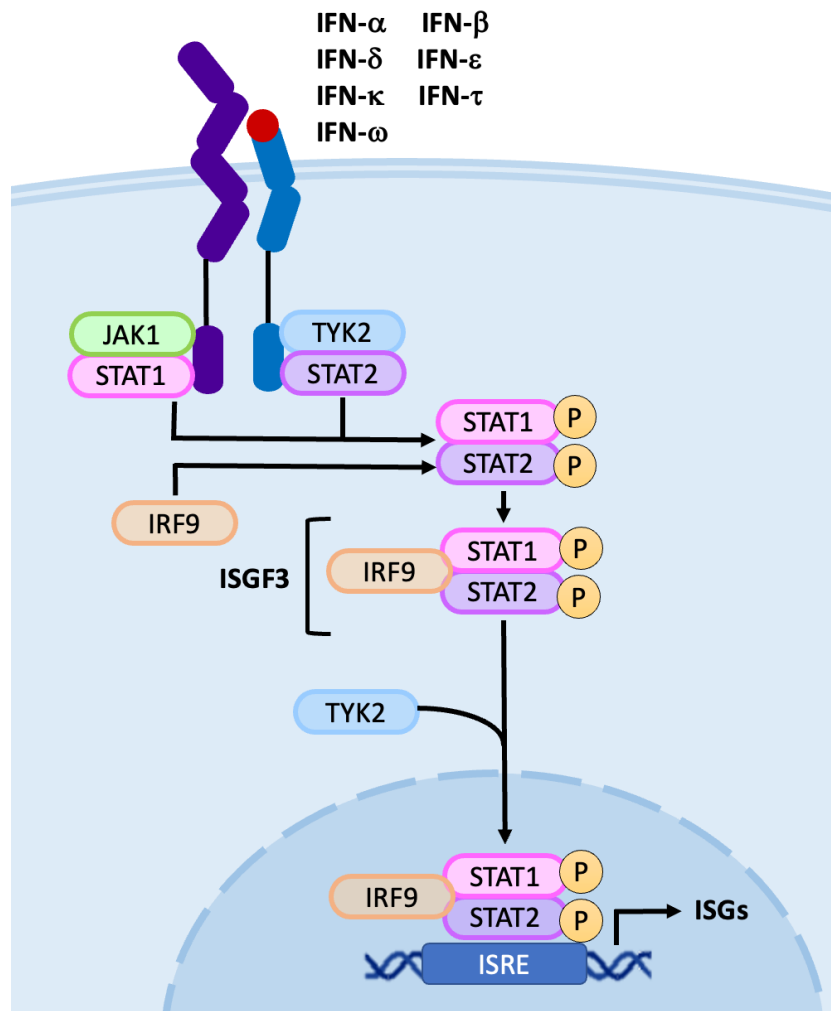


Figure 2. IFN-I signaling and activation of ISGs production. (Created with PowerPoint)

The IRF is a major family of 9 transcription factor (IRF1-9) [20].-The first IRF was discovered in 1988 and named IRF1; it was found in cells infected with Newcastle Disease Virus (NDV) following DNA affinity chromatography [20]. IRF1 expression is driven by the binding to the IFN- γ activated sequence (GAS) of unphosphorylated STAT1/STAT1, phospho- STAT1/STAT1 and phospho-STAT1/STAT2 respectively named U-STAT1, IFN- γ (gamma) activating factor (GAF) and GAF-like (Fig. 3) [21]. Interestingly, two mechanisms leading to IRF1 production can be

identified, in the first, IFN-independent, the U-STAT1 activates the GAS promoter and is hence responsible for the basal cellular IRF1 levels required for the physiologic state of alert against invading pathogens; in the second one, IFN-dependent, IFN-I and II activate the cellular receptors GAF as well as GAF-like bind GAS promoter [21]. However, the IRF1 mRNA and protein itself have both a short half-life suggesting a high IFR1 protein turnover [20]. In addition to its IFN-I signaling through the previously described activation mechanisms, IRF1 is also activated following the stimulation of Tumor Necrosis Factor Receptors (TNFRs), RLRs and TLRs that leads to the production of pro-inflammatory nuclear factor kappa-B (NF- κ B), thus it can fit perfectly in the ISGs category [22]–[24]. Once activated the IRF1 binds the IRF binding element (IRF-E) inducing ISGs transcription [25]. Many studies have highlighted the importance of IRF1 basal activity in counteracting viral replication, in IRF1 knockout hepatocytes the constitutive expression of diverse classes of antiviral effectors acting on lipid metabolism, protein degradation pathways, antiviral ISGs and Oligo-A synthase (OAS) was downregulated indicating a pivotal role for IRF1 in counteracting pathogens' infection but also to maintain a constitutive basal defense [20].

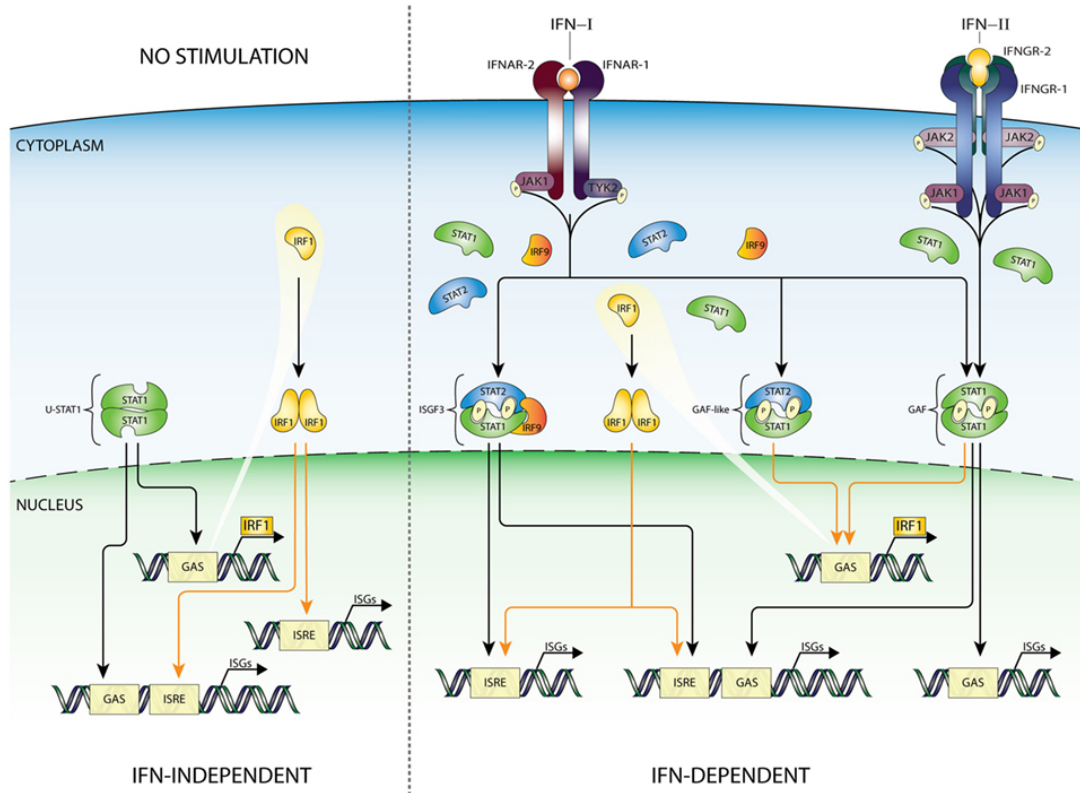


Figure 3. IRF1 IFN-independent and IFN-dependent production [21].

1.2 STING as potential cellular target for the development of broad-spectrum antivirals.

1.2.1 STING functional Role

STING protein is the product of Tmem173 gene, it is a transmembrane protein identified in the first decade of the new century by different research groups simultaneously, starting from a previously uncharacterized molecule supposed to be an inducer of the INF-I response [26]–[28]. STING itself cannot be considered a PRR,

in fact its activation is an event downstream of dsDNA detection in the cytosol by cGAS that determines the production of 2'3' cGAMP; hence cGAS is the actual PRR in the cascade. The cGAS produced cyclic dinucleotide (CDN) in turn directly binds STING leading to its dimerization and phosphorylation (Fig. 5). Once activated, STING determines TBK-1 mediated IRF3 phosphorylation; pIRF3 hence dimerizes and translocates in the nucleus mounting the antiviral state [10], [29].

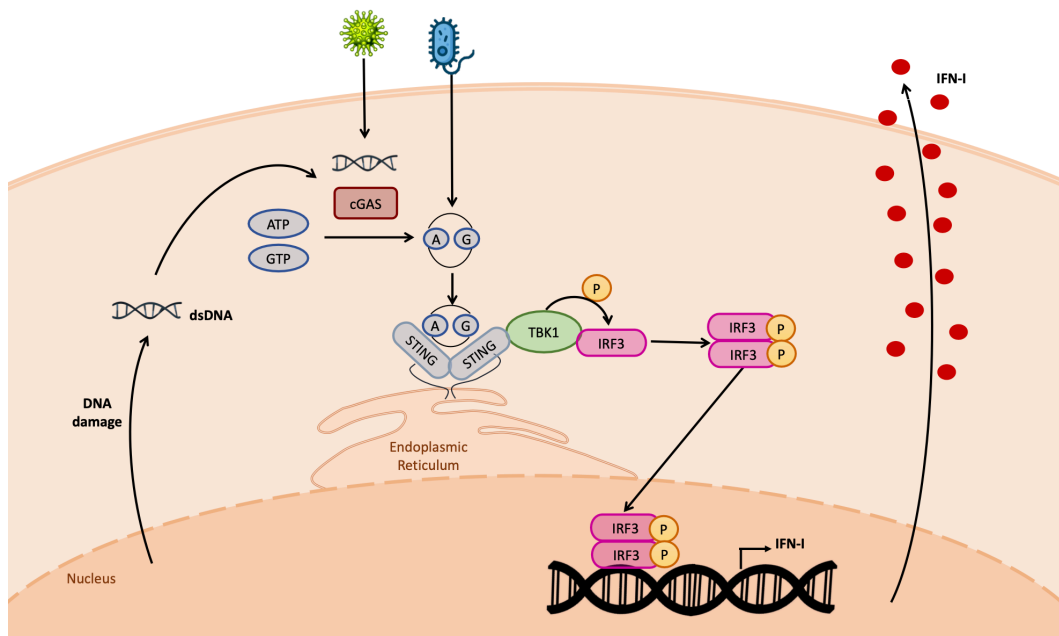


Figure 4. cGAS-STING pathway [10].

Comparative analysis between wild type cells expressing STING and STING^{-/-} cells, demonstrated that knocking out of STING reduced IFN- β as well as pro-inflammatory cytokines production, resulting in higher susceptibility to viral and bacterial infections [30]–[32]. In addition to cGAS activation, the STING pathway was also found to be triggered during *L. Monocytogenes* infections, an intracellular pathogen that expresses di-adenylate cyclase (DAC) which produces as CDN a 3'3' cGAMP, that can also be

detected by STING leading to IFN- β production. Interestingly the DAC enzyme was further identified through bioinformatic analysis in bacteria and archaea including *Staphylococci*, *Streptococci*, *Mycobacteria*, *Chlamydia* and *Mycoplasma spp.*, possibly suggesting that all these pathogens might induce a STING mediated immune response [33].

The cGAS/CDN mediated pathway is not the only possible STING activation pathway. In fact, several studies reported that when viral RNA in the cytosol is detected by RIG-I or MDA5 (depending on whether the RNA is short or long, respectively [4], STING may contribute to the RIG-I mediated innate immune response through direct interaction with RIG-I, but not MDA5, since STING is not able interact with MDA5 [34]. Of note, as confirmation of the importance of STING in RNA viruses infection, RNA viruses have evolved strategies to target STING protein in order to overcome the innate immune response [10], [28], [35]–[37].

In addition, it was also demonstrated that long term exposure to the genotoxin etoposide induces the activation of DNA damage inside the nucleus leading to a non-canonical STING activation without cGAS activation nor cGAMP production. In this case, the protein ATM (ataxia telangiectasia mutated) and PARP1 (poli-ADP-ribose polymerase 1) are responsible for p53 phosphorylation that leads to the interaction with IFN-Inducible factor 16 (IFI16) in the nucleus leading to the interaction with the cytosolic E3 ubiquitin ligase tumor necrosis factor receptor associated factor 6 (TRAF6). This complex, assembled straddling the nuclear membrane, interacts with STING located in the ER surface, leading to a STING TRAF6-mediated K-63 linked poli-ubiquitination [38], [39]. The non-canonically activated STING preferably activates NF- κ B rather than IRF3 inducing a different gene profile transcription with respect to the canonical STING activation [38].

1.2.2 STING structure and interaction with ligands

STING is composed of 379 amino acids (aa) and can be divided into nine domains (Fig. 5A). Starting from the amino-terminal portion, STING presents four transmembrane domains (TMD) responsible of the localization in the organelles' membrane, endoplasmic reticulum, and mitochondrion. The main domain, including aa 139-379, the cytosolic domain (CTD), is located in the carboxyl-terminal region in which resides the ligand binding domain (LBD) and the highly conserved dimerization motif GXXXS required for STING dimerization; the remaining domains are considered linker domains [40]. STING exerts its function through homodimerization by adopting the $\alpha+\beta$ fold involving five-stranded sheets in the centre and four helices in periphery [40] (Fig. 5B). The dimerization motif localized in the transmembrane region adjacent to the LBDa1, essential for STING activity as demonstrated through mutagenesis studies [41]. The resulting homodimer is the functional element able to bind the signalling nucleotide 2'3'cGAMP (Fig. 5B-C). Its binding site is located in the interface between the two chains where the Tyr167 in the LBDa1 is responsible for the binding with 2'3'cGAMP (Fig. 5A).

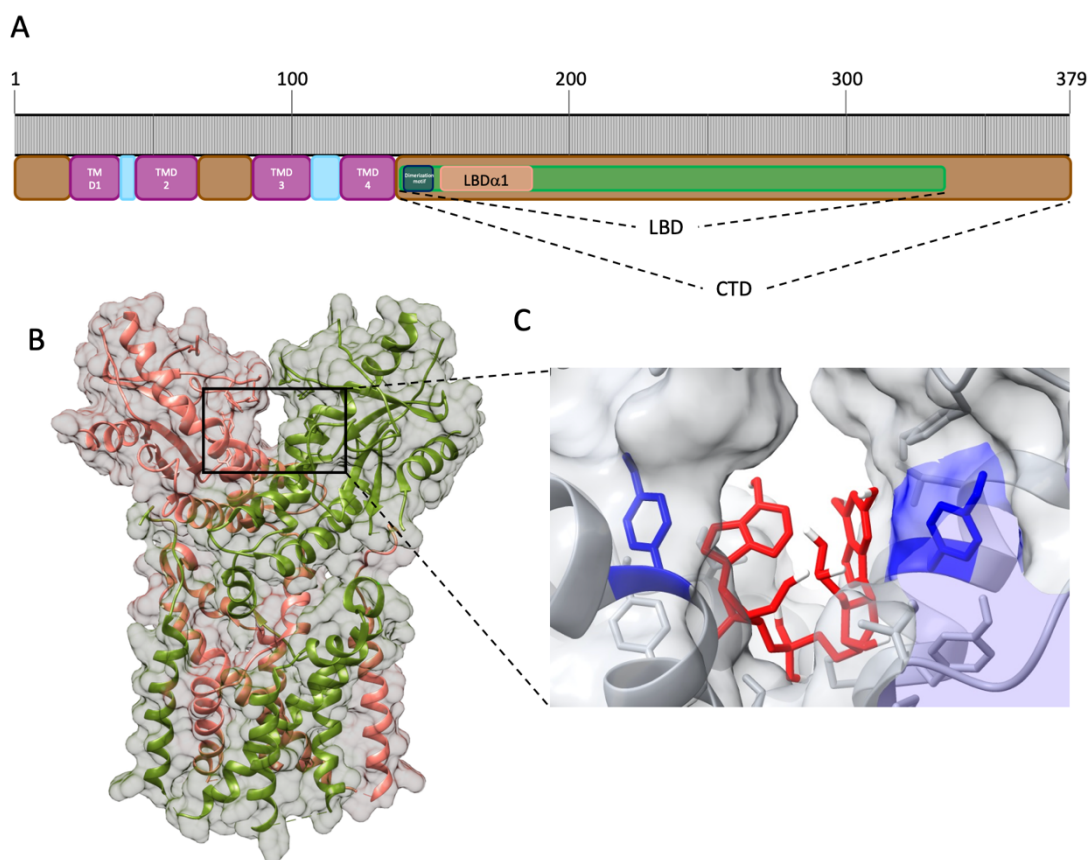


Figure 5. Human STING (*hSTING*) full-length structure. A. Domain subdivision: TMD stands for transmembrane domain, LBD stands for ligand binding domain and CTD stands for C-terminal domain; the dimerization domain is indicated in blue. B. *hSTING* homodimer tridimensional structure. C. Focus on *hSTING* binding pocket bond to 2'3' cGAMP with highlighted Tyr167 residues [10].

The binding requires charge-charge interaction between the phosphates in the 2'3'cGAMP and the protein residues, and determines the conformational change from an open to a closed STING active form [41]–[45]. Of note, bacterial CDN, 3'3' cGAMP, detected by STING, can only form a weak interaction with STING in the binding site, suggesting lower effectiveness in the activation of STING with respect to the interaction with the 2'3'cGAMP, indicating STING while potentially involved in mounting innate immune response also against bacteria cannot be the major

detector of bacterial infection [16], [33], [46]. STING interacts in a constitutive manner with TBK1 through its cytosolic domain, more in detail with the LBD. The binding to 2'3' cGAMP induces STING multimerization leading to trans-autophosphorylation of TBK1 [47]. In turn, pTBK1 attracts the IRF3 forming the STING-TBK1-IRF3 complex. Although the complex formation dynamics are still unclear, the interaction leads to IRF3 dimerization and phosphorylation mediated by pTBK1 [40]. Then the active IRF3 dimer translocate in the nucleus where it binds the IFN-I promoters, leading to IFN-I production [27], [48], [49].

1.2.3 STING connection with Mitochondria

Mitochondria are cellular organelles with a potent role in cellular metabolism as they are the centre of ATP production and serve as metabolic hubs. They are factories for the biosynthesis of macromolecules and lipids, proteins and nucleotides [50] and they also play a pivotal role in redox homeostasis responding to cell's stressors, both cellular and environmental, as well as in programmed cell death, responding to pathogenic conditions leading to instauration of a pro-apoptotic state or triggering the activation of the innate immune response [50]–[52].

One of the most important mitochondrial proteins involved in the innate immune response is Mitochondrial Antiviral Signaling protein (MAVS), a transmembrane protein located in the outer membrane (OM) of the mitochondria. MAVS is an effector of the RIG-I pathway, involved when RNA viruses are detected. MAVS aggregates and interacts with STING to trigger TBK1 phosphorylation and then IFN-I transcription [50] (Fig. 6A). MAVS is also modulated by bioproducts of the metabolism. In fact, in the OM it is associated with the glycolytic enzyme hexokinase 2 (HK2), that prevents MAVS multimerization in normal conditions when RLRs are not activated [47], [50].

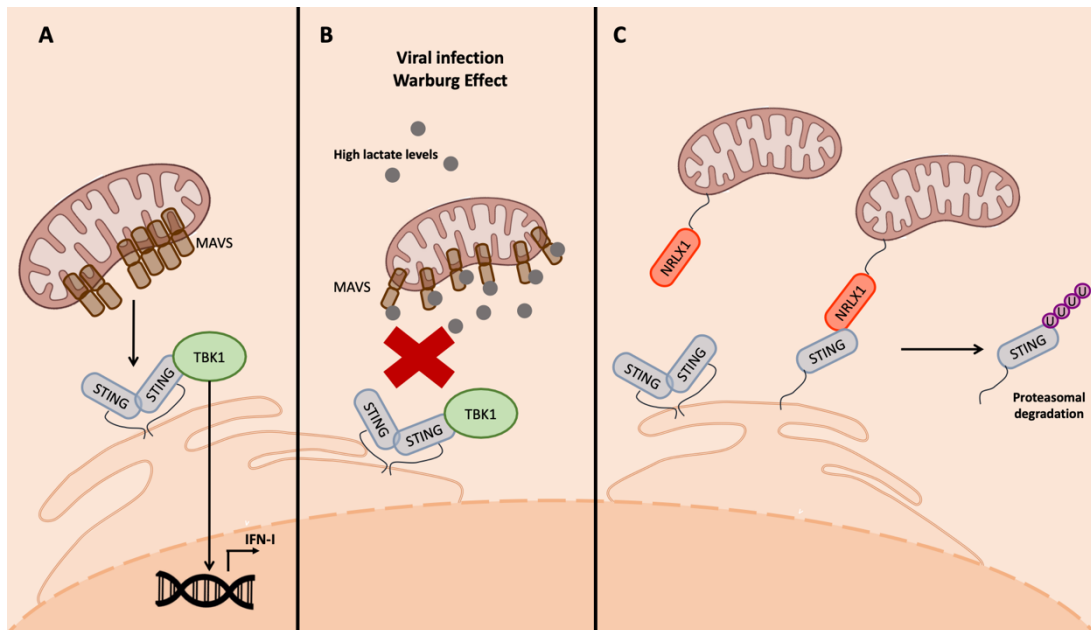


Figure 6. Modulation of STING interaction with MAVS. A. STING interact with MAVS inducing IFN-I. B. High lactate levels caused by viral infection or Warburg effect block MAVS aggregation and interaction with STING inhibiting IFN-I production. C. NLRX1 mediates STING ubiquitination leading to its proteasomal degradation [10].

Among the viral strategies developed to inhibit innate activation are the ones to manipulate mitochondria metabolism to inhibit IFN production, and an interesting example is the small molecule lactate [50] a bioproduct of the lactate dehydrogenase complex (LDH) that generates it starting from acetyl-CoA in absence of oxygen. This production can actually occur even in presence of oxygen due to the aerobic glycolysis, a condition called Warburg effect frequently promoted during viral infections and in cancer cells [48], [53]–[55]. Lactate then binds MAVS, preventing its aggregation and the interaction with the downstream effectors of the cascade involved in the IFN-I production that are TBK1 and STING [50] (Fig. 6B).

Another important mitochondrial protein that interacts with the STING signaling pathway is the nucleotide binding oligomerization domain (NOD)-like receptor 1 (NLRX1). It belongs to the NLR family, it is ubiquitously expressed and possesses

the NACHT domain, responsible for hetero-dimerization, the leucine-rich repeats domain (LRR) in the C-terminus and is the only NLR localized on the OM due to presence of a mitochondrial targeting sequence in the N-terminus [4], [56]. This localization was reported to be important for the interaction with proteins involved in innate immunity such as MAVS and STING, as well as cytoplasmic proteins such as TRAF6 and IKK complex [4], [57]. The interaction of NLRX1 with the effectors of the innate immune response occurring through the NACHT domain promotes their K-48 ubiquitination and consequently proteasomal degradation, resulting in the inhibition of NF- κ B and IRF signaling following viral infection (Fig. 6C) [57].

In fact, in cells infected with Sendai virus, Influenza A virus and Hepatitis C Virus (HCV) the RIG-I/MAVS signaling pathway was negatively affected by NLRX1 [56], [57]. This inhibition is related to glucose levels: high glucose levels activate NLRX1 that binds poly(rC) binding protein 2 (PCBP2) which drives MAVS, unable to aggregate due to the presence of lactate covalently bond as just described, to ubiquitination and degradation [56], [57]. A very similar mechanism was reported for STING in cells infected Human immunodeficiency virus type-1 (HIV-1), in which reverse transcribed viral DNA induces IFN-I transcription through cGAS-STING and in Human Papilloma Virus 16 (HPV16) infected cells in which the viral protein E7 also promotes NLRX1-mediated STING degradation [58], [59].

1.2.4 STING triggering through mtDNA release

The mitochondrial genetic system, constituted by a double stranded circular DNA, was identified studying the various encoded proteins mostly involved in the oxidative phosphorylation [60]. It is organized in protein-rich structures highly controlled and

regulated, it is present in at least one copy per mitochondrion, but it can arrive also to hundreds copies per cell. The loss of this highly controlled structure is called “mtDNA stress” and leads to the release of mtDNA in the cytosol [61]. The mtDNA in the cytosol is detected as foreign DNA, triggering cGAS-STING-IRF3 dependent IFN-I transcription and consequently ISGs transcription (Fig. 7)[61].

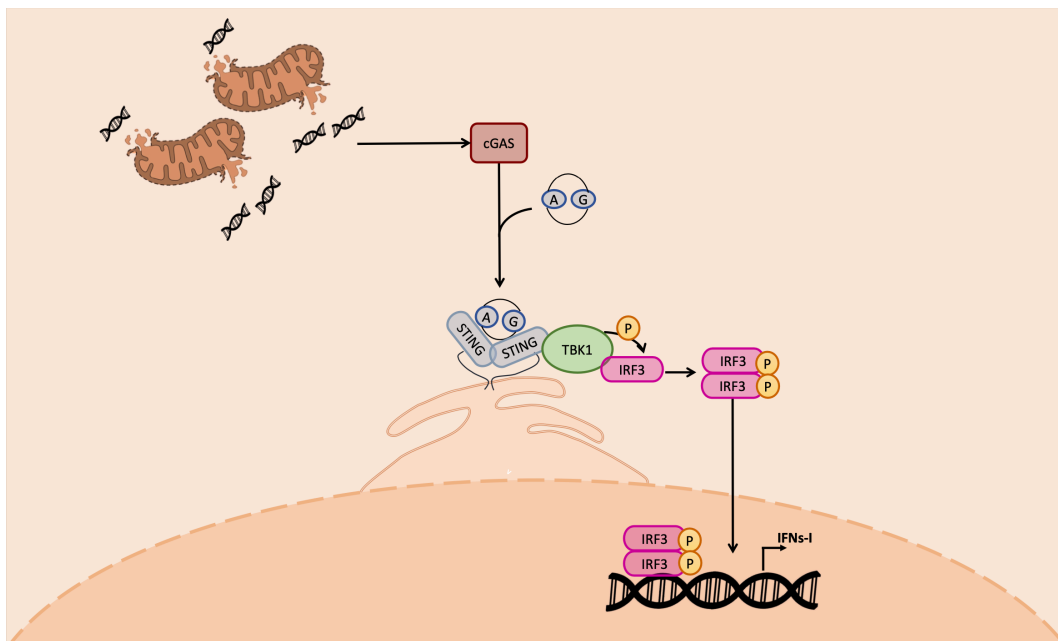


Figure 7. mtDNA stress activates cGAS-STING pathway [10].

Viruses interact with mitochondria and mtDNA in different ways. Herpes viruses have been reported to induce mtDNA stress and consequently its release in the cytosol, activating the STING pathway. In particular, the alpha herpesvirus protein UL12.5 localizes in the mitochondria where contributes to the mtDNA depletion, often coupled with its release. Indeed, the absence of this viral protein was found to reduce mtDNA depletion leading to mount a less potent TBK1 STING-dependent phosphorylation with respect to the wild type, confirming a mtDNA dependent innate immune response activation [61]. Dengue virus (DENV) infection also determines cellular damage causing mtDNA release and inducing IFN-I transcription in a cGAS-

STING dependent way. As countermeasure, DENV produces the NS2B protease which cleaves cGAS neutralizing its activity [62].

Mitochondria also play a key role in nucleotide biosynthesis as central hub for dNTP salvage synthesis pathway [50]. Briefly a multifunctional protein complex including i) the carbamoyl-phosphate synthetase (CPSase), ii) the aspartate transcarbamylase (ATCase), and iii) the dihydroorotase (DHOase), termed hence CAD, the dihydroorotate dehydrogenase (DHODH) located in the inner mitochondrial membrane, and uridine monophosphate synthetase (UMPS) are responsible for de-novo pyrimidine biosynthesis starting from glutamine, aspartate, and bicarbonate. At the same time the salvage pathway processes uridine and cytidine in order to produce intermediates embedded in the de-novo nucleotide synthesis cascade [50], [63]. Some enzymes involved in pyrimidine biosynthesis such as DHODH and CAD were found to be targeted by antiviral compounds leading to pyrimidine deprivation impairing viral genome production and as consequence, viral replication [63]–[70]. Moreover, pyrimidine deprivation is interestingly related to mtDNA release and, as consequence, to the activation of a cGAS-STING mediated IFN response [64], [71]. However, it is still unclear whether this mechanism takes place in infected cells as antiviral defense, inducing spontaneous mtDNA release to trigger innate immunity.

1.2.5 Ubiquitin role in the STING mediated innate immune response.

The ubiquitin proteasome system includes Ubiquitin (Ub) a highly conserved small protein of 76 amino acids and three enzymes: the E1 Ub-activating enzyme, the E2 Ub-conjugating enzyme and the E3 ligase [72], [73]. The best-known function of Ub conjugation is to target proteins to modulate their half-life, driving target proteins to

proteasomal degradation. However, a second role is to regulate protein activity [72]–[74]. The Ub chain is bound to the target protein starting from its activation: E1 adenylates the Ub C-terminal group, then the E2 transfers the activated Ub to the active site, finally E3 catalyses the formation of a covalent bond between a lysine, belonging to the target protein, and the Ub C-terminal [72].

Ubiquitination can occur as mono- or polyubiquitination in selected Ub lysine residues: K6, K11, K27, K29, K33, K48, K63 [75]. Only two of them, K29 and K48, are related to proteasomal degradation; all the others modulate protein activity during inflammation, innate immune response, endocytic trafficking, transcriptional regulation and DNA repair [76]–[78]. These Ub chains can be removed from target cells through the activity of enzymes called Deubiquitinases (DUB or USP) [79]. Ubiquitination and deubiquitination are tightly regulated since the absence of regulation in processes as important as the innate immune response could lead to pathogenic conditions like immune diseases [80].

STING activity is controlled and regulated through Ub conjugation and deconjugation, in fact, while binding to CDNs is sufficient to induce the conformational changes required for STING multimerization and IFN-I induction, ubiquitination can enhance its activity or regulate protein's half-life [81].

Enhancing ubiquitination requires the activity of two main E3 ligases, tripartite motif (TRIM) protein 56 and 32, that have been found to link K-63 ubiquitin to STING. TRIM56 ubiquitinates K150 residue, while TRIM32 ubiquitinates multiple lysine residues (20/150/224/236) [82] (Fig. 8A). In addition, the E3 ligase ring finger protein 26 (RNF26) mediates K11 Ubiquitination that protects STING from K48 Ub conjugation and prevents its degradation [83] (Fig. 8A).

The proteasomal degradation, on the other hand, is induced through K-29 and K-48 ubiquitination: RNF5, TRIM29 and the heat shock protein 27 (HSP27) mediate K-

48/K-29 Ub conjugation leading to STING degradation [84]–[86] (Fig. 8B). In addition, cellular USPs modulate the STING ubiquitination by removing Ub chains hence preventing STING degradation: USP18, USP20 and USP44 target STING promoting K-48 deubiquitination (Fig. 8B), while USP49 was found deconjugating K-63-linked ubiquitination of STING inhibiting its activity [82], [87]–[89].

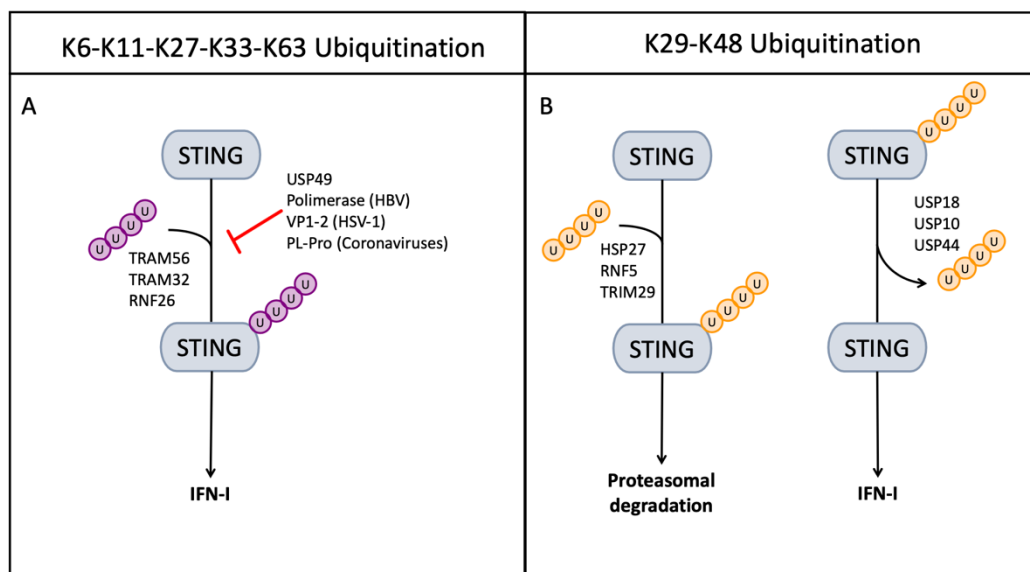


Figure 8. STING ubiquitination. A. Positive STING ubiquitination by cellular proteins, the red arrow indicates the inhibition of activating ubiquitination by both cellular and viral proteins. B. Negative STING ubiquitination by cellular and viral proteins, Ub-conjugation induces proteasomal degradation, deconjugation prevents proteasomal degradation [10].

Small Ub-like Modifier conjugation (SUMOylation) is comparable to ubiquitination as it modulates protein activity, function, stability, subcellular localization, and interaction with other proteins [90]. Four different SUMOs are known: SUMO1-2-3-4; SUMO 2 and 3 are isoforms, SUMO1 shares 50% homology with the SUMO2/3, while SUMO4 is the least studied. The conjugation mechanism for Ub requires the maturation of the SUMO, the E1 mediated SUMO activation, the E2 mediated conjugation and the E3 mediated ligation [91]. TRIM38 is a E3 ligase found to ligate SUMO to both cGAS and STING in uninfected cells and in the early phases of

infection, regulating their activity. However, both cGAS and STING were de-SUMOylated by sentrin specific peptidase 2 (Senp2) leading to their degradation [74].

Viruses have evolved proteases and mechanisms to mediate either Ub conjugation or deubiquitination in order to turn intrinsic cell processes to their advantage. As an example, Hepatitis B virus (HBV) possesses two relevant proteins: the viral polymerase deubiquitinates K-63 STING downregulating cytoplasmic dsDNA sensing, while the HBx protein downregulates the accumulation of cGAS expression by promoting its ubiquitination and autophagy [92], [93]. In Herpes simplex virus 1 (HSV-1) infected cells, cellular USP49 and protein 1-2 (VP1-2) have deubiquitinating activity of STING targeting K-63 linked Ub and inhibiting its activity [94]. In cells infected with HSV-1, the cellular protein RNF5 plays a major role in inhibiting innate immune response mediated by STING by K-48 ubiquitination (Fig. 8B)[86]. In cells infected by Pseudorabies virus (PRV), a herpesvirus causing domestic and wild animals' diseases, the HSP27 protein promotes PRV proliferation targeting the cGAS-STING pathway, inhibiting IFN-I transcription [95]. In Epstein-Barr virus infected cells, TRIM29 ubiquitinates STING inducing its proteasomal degradation to inhibit the innate immune response [85]. All coronaviruses, (+) ssRNA viruses, encode for at least one Papain Like Protease (PLPs) that act as DUB removing Ub chains from target proteins, moreover the intrinsic characteristic of these proteases make them able to cleave ISG15, an ISG with similar activity as Ub [96]–[98].

1.2.6 Direct viral STING inhibition

STING is also directly targeted by viral proteins determining its degradation or inactivation. HSV-1 encodes three different proteins that inhibit STING signaling:

ICP27 interacts with the activated STING-pTBK1 complex preventing phosphorylation and activation of the transcription factor IRF3 [99]; UL46, a tegument protein, binds both STING and TBK1 with different domains preventing STING and TBK1 interaction [100]; γ 134.5 prevents STING phosphorylation also by direct interaction [101]. STING plays a major role in Kaposi sarcoma virus (KSHV) detection during primary infection and, in the reactivation from latency, the viral protein vIRF3 interacts with STING preventing its binding to TBK1 [102]. The Human Cytomegalovirus (HCMV) tegument protein UL82, was found to inhibit STING trafficking towards the Golgi blocking IFN-I activation [103]. More recently, it was described that the HCMV protein UL42 inhibits cGAS-STING pathway directly interacting with cGAS and STING [104]. Moreover, HCMV IE86 protein promotes RNF5 activity determining STING proteasomal degradation [105]. In HCV infection, the viral NS4B protein disrupts STING interaction with TBK1 and MAVS to prevent the innate immune response activation [35], [106], [107]. The same mechanism was reported for DENV NS4B protein. Moreover, the DENV protease NS2B3 has been reported to cleave both cGAS and STING to block IFN-I production [36], [37], [62].

1.2.7 STING agonists as broad-spectrum antivirals

The involvement of STING in activating the INF production as well as all the efforts that viruses put in shutting down STING activity clearly indicate that STING is a potential target for small molecules that acting as STING agonists may be broad-spectrum antiviral agents. Indeed, computational studies have confirmed that STING is a druggable target showing the possibility to accommodate small molecules acting

as STING agonists [108]–[111]. Until now a few studies have been already published on this topic.

1.3 Pathogenic human Coronaviruses and SARS CoV2 PL-pro

1.3.1 Epidemiology

Coronaviruses (CoVs) belong to the Nidovirale order, are RNA viruses with spherical enveloped particle of around 120-160 nm and are divided into four genera: Alpha-, Beta-, Gamma- and Deltacoronaviridae, identified according to the results of viral genome comparison [112]. The identified hosts are both mammals and birds, among mammals: pig, cat, dog, bat, mouse, rat, cow, horse and human, among birds: chicken, pheasant, goose, pigeon, mallard [112]–[118]. CoVs infection causes principally respiratory syndromes or enteric diseases and more rarely neurologic illnesses or hepatitis. [112]. CoVs are considered rapidly evolving viruses due to their high genomic nucleotides substitution rates, this characteristic, together with the increasing urbanization and poultry farming, have permitted the mixing of species facilitating the zoonosis onset [119], [120].

Seven Human CoVs (HCoVs) have been identified so far: HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 (Fig. 9). [121], [122]. HCoVs cause respiratory diseases with different levels of severity such as common cold, bronchitis and pneumonia [123]. HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 are globally diffused and associated with over one third of the common colds, although in elderly patients, immunocompromised people and children can evolve in severe pneumonia and bronchitis [121], [123]–[127].

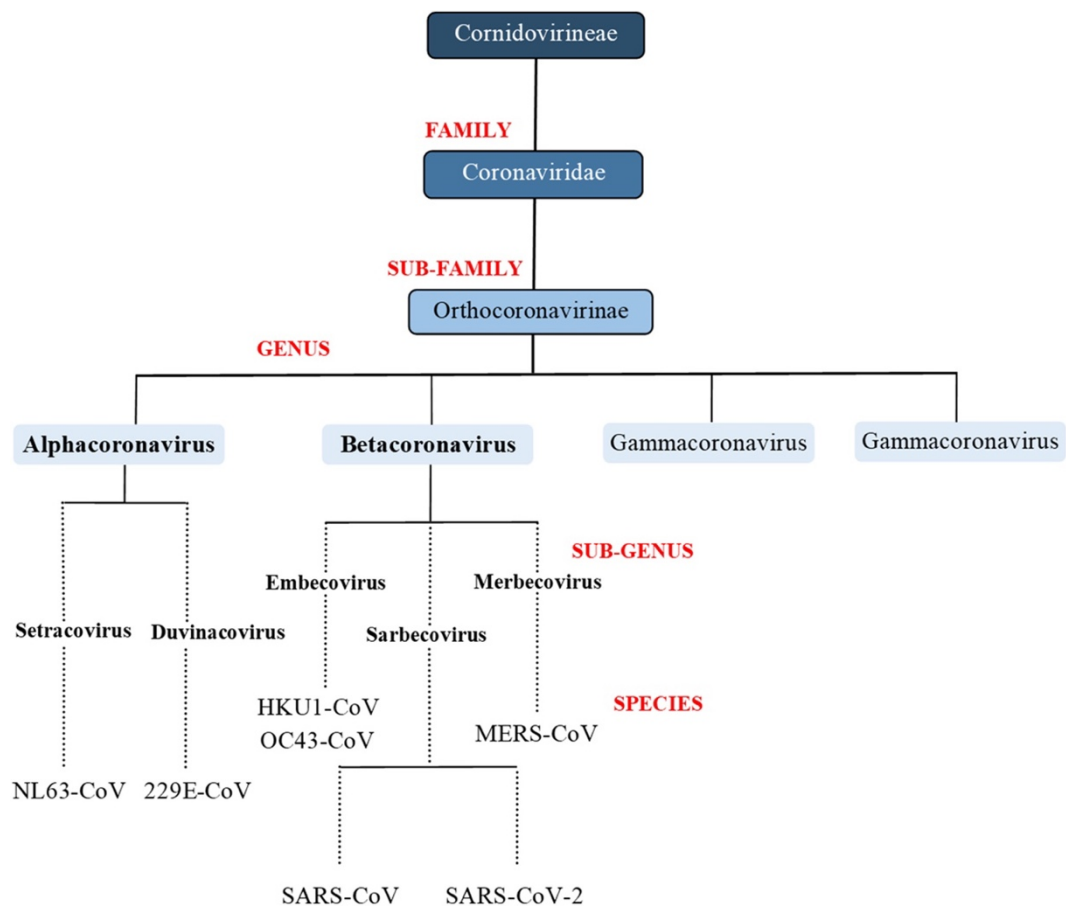


Figure 9. The classification of human coronaviruses by the International Committee on Taxonomy of Viruses 1 [127].

SARS-CoV was first detected in 2002-2003 in Guangdong, China, during the spread of an atypical pneumonia with fever, cough declining to respiratory failure and acute respiratory distress syndrome [128]. The SARS-CoV spread was attributed to a zoonosis, in fact palm civets were first indicated as natural reservoir, despite following studies indicated the link between the SARS-like virus and SARS-CoV through phylogenetic analysis of the spike protein sequence. [129], [130]. Considering the severe symptoms and the high fatality rate (3%), SARS-CoV diffusion was promptly limited by halting all human-to-human transmission, isolating infected patients and quarantine contacts [131].

MERS-CoV spread in 2012 in Saudi Arabia causing similar symptomatology as SARS-CoV but with super-spreaders events, limiting MERS-CoV diffusion geographically. Although, to date, have been reported cases of people, mostly travellers, infected with MERS-CoV in the Middle East and few outbreaks outside this region indicating that the infection is currently ongoing [128]. MERS-CoV is highly pathogenic with a case fatality rate (CRF) of 32.7% [132].

SARS-CoV-2 is the etiologic agent of the Coronavirus related disease 19 (COVID 19), it was first detected in Wuhan in late December 2019 and, despite SARS-CoV and MERS-CoV, rapidly spread globally leading to the declaration of a pandemic in March 2020 [133]. To date the COVID 19 has caused around 7 million deaths and 800 million cases worldwide [122], [134].

The respiratory virus SARS-CoV-2 is transmitted through droplets and aerosol and the median incubation period is 2-14 days before symptom onset; it generally causes flu-like state including fever, cough, headache, myalgia, lethargy, and diarrhoea [135]. Critical complications generally occur concurrently with increased age, obesity, cardiac arrhythmia, diabetes kidney failure, hypertension, and chronic pulmonary disease [136]. Severe COVID-19 regards 20% of the totality of infected patients and is characterized by pneumonia with dyspnoea, tightness-chest pain and fatigue, these symptoms correspond to the acute respiratory disorder syndrome (ARDS), a form of lung injury characterized by inflammation pulmonary vascular leakage and loss of aerated lung tissue [135], [137]. ARDS is also related to the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8 and tumour necrosis factor (TNF), ferritin and C-reactive protein (CRP)[137]. Hospitalized patients with COVID-19, are not likely to survive notwithstanding treatment with antivirals, since the virus has already spread into the lungs and triggered immunopathology [137]

As explained earlier, HCoV-NL63, HKU1, OC43 and 229E are most likely to cause mild diseases although immunocompromised patient can develop severe disease. In

mild diseases no particular treatments are needed, and no specific treatment has been developed for HCoV-NL63, HKU1, 229E and OC43.

Differently after the insurgence of the recent pandemic many efforts have been made in the development of vaccines and medicines to inhibit SARS-CoV-2, while much less efforts were applied to cure for previous epidemics of SARS-CoV and MERS-CoV.

In fact, during SARS-CoV epidemic, patients were only treated with convalescent plasma (CP) showing lower mortality rate and recovered sooner than patients not receiving CP [127], [138].

In addition, to date, no Food and Drug Administration (FDA)-approved vaccine or medicines are available for MERS-CoV and treatments are mainly supportive. However, neutralizing antibodies could be used also in this case [139].

Since SARS-CoV-2 represented a main issue for public health worldwide, a wide array of studies has been out in place to identify vaccines and drugs. The main goal in the treatment and prevention of COVID-19 diffusion has been the development of effective vaccines: FDA approved vaccines that target the S protein are the messenger RNA (mRNA; Pfizer-BioNTech Comirnaty - BNT162b2 & Moderna - mRNA-1273), protein-based (Novavax – NVX-CoV2373) and viral vector-based (Johnson & Johnson Janssen - Ad26.COV2.S, Oxford-AstraZeneca - AZD1222/ChAdOx1, Sputnik V - Gam-COVID-Vac-rAd26/rAd5) vaccines, while traditional inactivated vaccines (Sinopharm - BBIBP-CorV, Sinovac - CoronaVac, Covaxin – BBV152) target the entire virus [140]. The development and administration of effective vaccine demonstrated to induce protection from the insurgence of severe symptoms and reduced the diffusion of SARS-CoV-2, although it could not stop completely viral spread leading to the insurgence of variant of concerns (VOCs): Alpha, Beta, Gamma, Delta, and Omicron the most frequently mutated among the VOCs [141], [142].

The rapid onset of VOCs made evident that, notwithstanding the protection against COVID19 severe symptoms development and the viral spread limitation obtained worldwide with the vaccine administration, vaccines cannot be considered sufficient countermeasures in the fight against COVID19. Moreover, vaccine distribution and storage resulted challenging in remote and hard-to-reach locations, such as for example central Africa and India. For this reason, vaccines need to be supported by effective and specific medicines. The scientific community made a huge effort during the COVID19 pandemic to develop and repropose effective antivirals. To date the approved medicines include small molecules targeting viral proteins, monoclonal antibodies targeting both viral and cellular proteins and immunosuppressors: the combination of Nirmatrelvir/Ritonavir inhibits the M-pro and is commercially available as Paxlovid [143]; Molnupiravir, a nucleoside analogue incorporated in viral RNA thus blocking viral replication, is at the moment under evaluation by the European Medicines Agency (EMA) for marketing approval [143] Remdesivir, commercially available as Veklury, selectively blocks the RdRp [144]; Tocilizumab, Sotrovimab, Regdanvimab, are monoclonal antibodies that bind the IL-6 controlling the cytokine storm; Casirivimab/Imdevimab and Tixagevimab/Cilgavimab selectively bind the S protein [145]–[148]; meanwhile Anakinra is an immunosuppressive drug [149]. The use of monoclonal antibodies and immunosuppressors is limited due to the possible side effects and, although viral proteases are highly conserved among the viral strains, the insurgence of variants can reduce the drug efficacy leading to resistance, hence, new targets and drugs are needed.

1.3.2 HCoV genome and proteins

HCoVs virions are characterized by a spherical morphology; the external surface is covered by a lipidic bilayer envelope that presents viral proteins: the Spike (S) protein responsible of the binding with cellular receptors, the Membrane (M) protein and the Envelope (E) protein [112]. Inside the virion it is located the nucleocapsid with helical symmetry formed by the Nucleocapsidic (N) protein and the positive single strand RNA molecule of around 30 kilobases (Fig. 10) [112], [150].

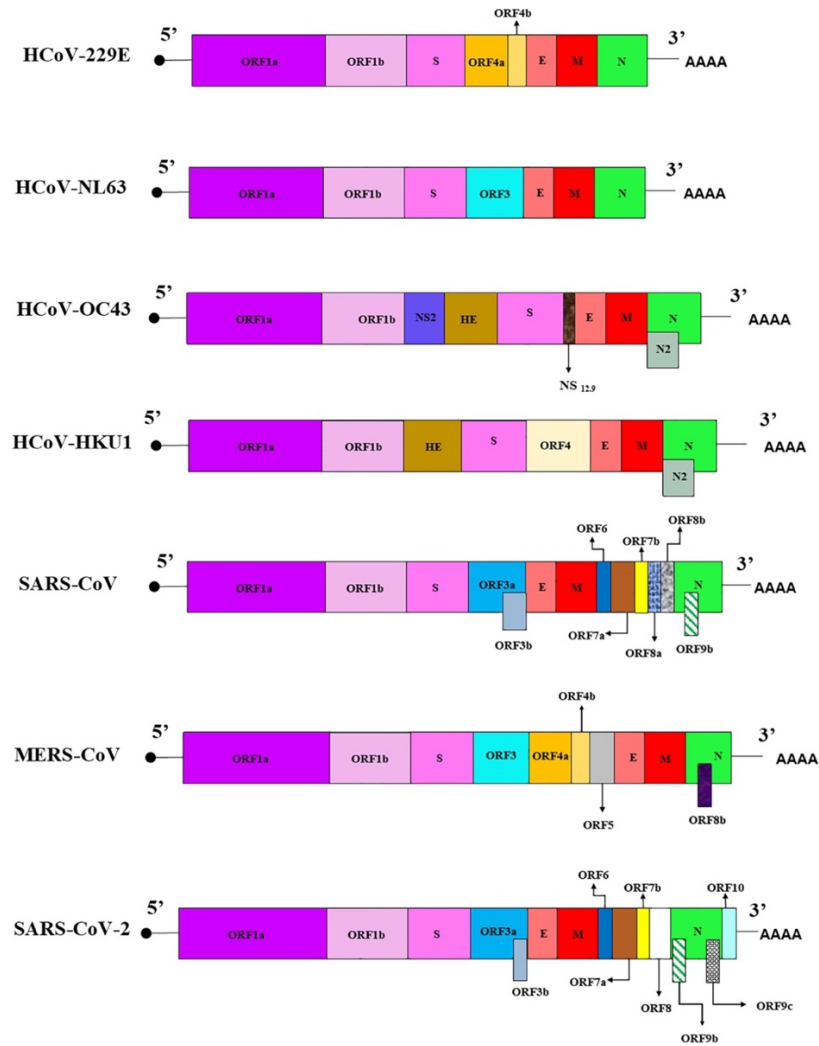


Figure 10. Genomic organization of HCoVs [127].

Viral RNA genome presents a 5'-cap and 3'-poly-A tail, and it acts as mRNA during the beginning of viral infection [112]. It is divided into one main open reading frame (ORF) called ORF1ab including two thirds of the viral genome that is translated into a main polyprotein, while the remaining part is divided into an average of 9 ORFs also coding for structural proteins (S-E-M-N) [112], [127].

ORF1ab polyprotein undergoes viral proteinases activity releasing 16 non-structural proteins (nsps). HCoV encode for at least two proteases: 3 cathepsin like protein (3CL-pro) also called Main protease (M-pro) and, depending on the HCoV, one or two papain like proteases (Pl-pro) [112], [151], [152].

The 16 nsps present specific roles in HCoV replication, the most important are nsp3 the Pl-pro, nsp5 the M-pro, nsp12 the RNA dependent RNA polymerase (RdRp), nsp13 the helicase while the nsp15 is the endoribonuclease; nsp10-14 cooperate with the nsp16, the 2-O' metil transferase, nsp7 and nsp8 interact to promote RdRp activity. The S protein is responsible for the binding to cellular receptors required for viral internalization; the cellular receptors are specific for every HCoV: HCoV-NL63, SARS-CoV related viruses and, as consequence, SARS-CoV-2 with which shares 80% sequence identity with SARS-CoV, interact with the angiotensin converting enzyme 2 (ACE2), HCoV-OC43 and HCoV-HKU1 bind 9-O-acetylated sialic acids (9-O-Ac-Sias), meanwhile HCoV-229E recognizes the human aminopeptidase (hAPN) and MERS binds dipeptidyl peptidase 4 (DPP4) (Table 1) [150], [153]–[155].

Table 1. HCoV's cellular receptors.

<u>HCoV</u>	<u>Cellular Receptor</u>
HCoV 229E	Human Aminopeptidase (hAPN)
HCoV-NL63	Angiotensin converting Enzyme 2 (ACE2)
HCoV-OC43	9-O-acetylated sialic acids (9-O-Ac-Sias)
HCoV-HKU1	9-O-acetylated sialic acids (9-O-Ac-Sias)
SARS-CoV	Angiotensin converting Enzyme 2 (ACE2)
MERS CoV	Dipeptidyl Peptidase 4 (DPP4)
SARS-CoV-2	Angiotensin converting Enzyme 2 (ACE2)

1.3.3 HCoVs life cycle

HCoVs infection starts with the interaction with cellular receptors, the bond induces conformational changes in the S protein and the viral envelope's fusion with the host's membrane. Viral genome is then released in the cytosol where is detected as RNA template for the production of viral proteins (Fig.11) [112], [127], [156]. The replicase complex include the first translated nsps: soon after production the replicase starts the synthesis of negative strand RNA copies, both subgenomic mRNAs and genome-length that are in turn used for the synthesis of positive strand RNA in convoluted membranes [156]. The translation follows with the production of S, E and M membrane-associated proteins, that are inserted in the ER and then translocated in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) where the virion assembly occurs: the progeny genome assembles with the N protein in the ERGIC forming the nucleocapsid, the virions are released through budding into the ERGIC [112], [127], [156]. The newly produced virions are then transported to the cellular membrane in vesicles or Golgi sacs and then released outside the cells. In some cases,

the S protein creates syncytia in the cell membrane in order to allow viral infection of adjacent cells avoiding the extracellular immune detection [112], [127], [156].

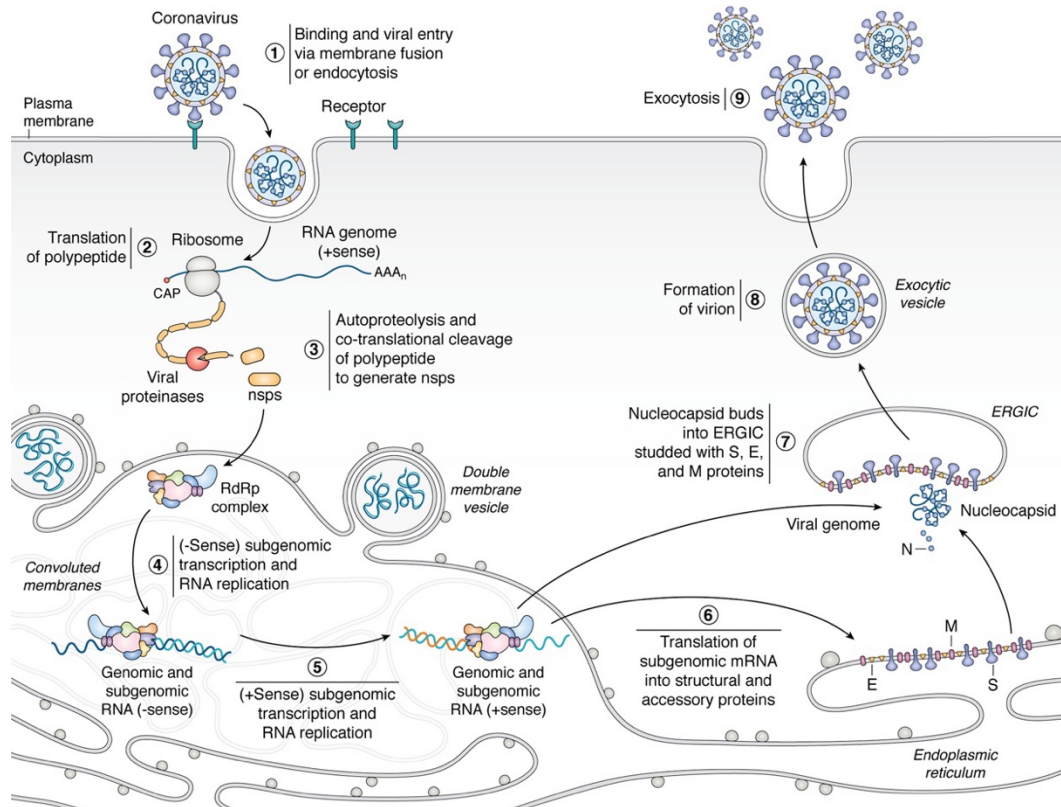


Figure 11. HCoV life cycle [156].

1.3.4 SARS-CoV 2 Papain-Like protease

The SARS-CoV-2 PL-pro presents 83% homology with the SARS-CoV PL-pro meaning that is a highly conserved protein [157]; nsp3 is the largest multifunctional protein encoded by SARS-CoV-2 with an average mass of 200 kDa, it is subdivided in various domains that can differ among the HCoVs, however eight of them are conserved among all the HCoVs: the Ub-like domain 1 (Ub11), NAB (nucleic acid

binding domain), ADRP (ADP-ribose phosphatase, SUD (SARS-unique domain) the ubiquitin-like domain 2 (Ubl2), the papain-like protease (PL-pro), the TM (Transmembrane domain), and finally the Nsp3 ectodomain (also called “zinc-finger domain” ZnF) (Fig. 12).

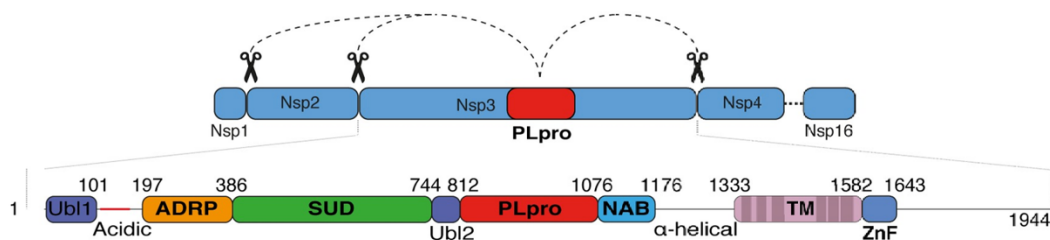


Figure 12. SARS-CoV-2 nsp3 cleavage scheme and polyprotein subdivision [158].

PL-pro is responsible for cleaving in correspondence of the LXGG sequence present in nsp1, nsp2 and nsp3; moreover, this sequence is also present in cellular Ub and ISG15 [159]. The enzyme, in fact, possesses an N-terminal ubiquitin-like (Ubl) domain and a catalytic core domain that features an open-right-hand fold, with thumb, palm and fingers subdomains (Fig. 13) [160]. The thumb domain includes four α -helices, the palm on the other hand is formed by six β -sheets. The protein acts through the catalytic triad (C112, H273, D287) located between the palm and the thumb domain [159]–[161].

The nsp3 is an essential component for the viral cycle and plays an important role antagonizing the host innate immune response acting on post-translational modification of host proteins through de-MARylation, de-PARylation (possibly), deubiquitination or deISGylation [162].

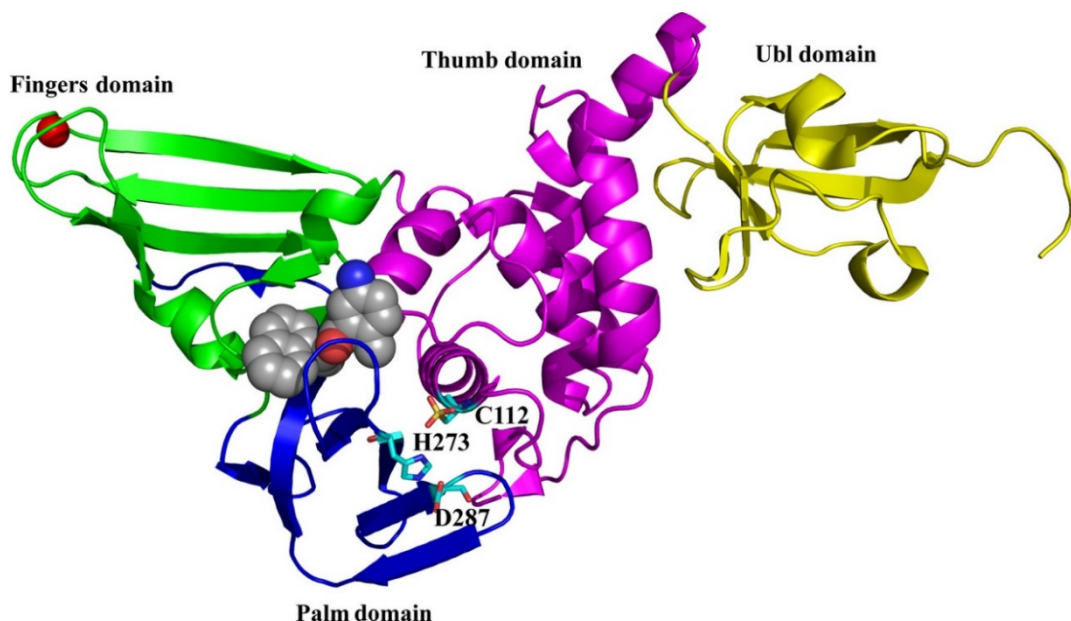


Figure 13. PL-pro tridimensional structure [160].

1.3.5 SARS-CoV-2 innate immune evasion.

SARS-CoV-2 as most RNA viruses is detected by the RIG-I/MDA pathway inducing the transcription of IFN-I. Comparative analysis showed that in SARS-CoV-2 infected cells IFN-I levels were lower with respect to other HCoV infected cells, suggesting an improved innate immune evasion [163], [164]. Indeed, SARS-CoV-2 possesses numerous proteins able to block the innate immune response at different levels (Fig. 14)[164].

Upon infection, the RNA detection is inhibited by nsp8 and orf9b at MDA5 level, meanwhile RIG-I detection is blocked by orf9b; MAV signalling is impaired by the activity of orf10, orf8 and the M protein. The nsp8, nsp13, nsp6, orf9 and M inhibit phosphorylation at different levels: IKK ϵ in complex with TBK1, NF- κ B and the IRF3 dimer. IRF3 nuclear translocation is inhibited by the activity of nsp12, nsp14, nsp15, orf6 and orf9b.

SARS-CoV-2 also inhibit IFN-I signalling preventing STAT1 as well as STAT2 phosphorylation, viral proteins involved are nsp1, nsp6, nsp13, orf3a and orf7b. Finally, STAT1/STAT2 phosphorylated dimer nuclear translocation is blocked by nsp1 and orf6.

Although RNA viruses are mostly detected by the RLRs, it was also reported the activation of cGAS-STING signalling in both infected cell cultures and COVID-19 patient samples. More in detail, it was reported a specific activation of NF- κ B mediated by cGAS-STING recruitment. Moreover, cGAS-STING activity was detected in lung samples of COVID-19 associated with type I IFN responses [165].

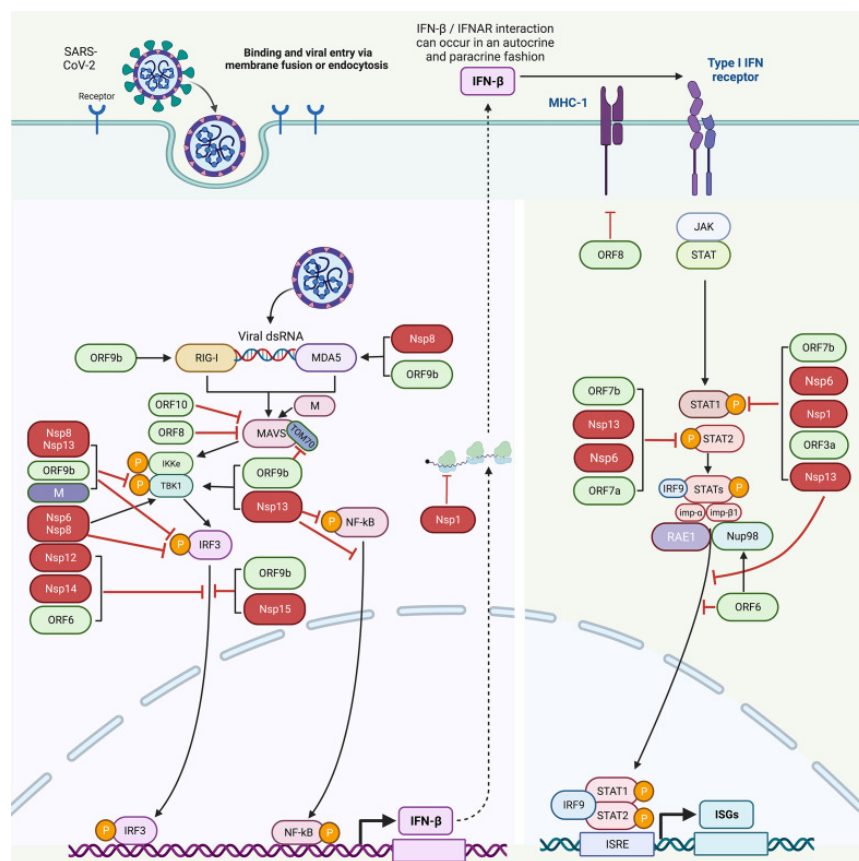


Figure 14. SARS-CoV-2 innate immune evasion [164].

1.3.6 PL-pro inhibits innate immune evasion through deubiquitination and deisgylation.

The innate immune response is tightly modulated through posttranslational modifications as ubiquitination and ISGylation.

ISG15 is a small protein (15 kDa) produced following viral infections and conjugated to target proteins in order to modulate their activity in the antiviral response[166]–[168]. The ISGylation has both activating and inhibitory functions on target proteins, it was reported to modulate cellular proteins in order to promote the antiviral response as well as target viral proteins inhibiting their functions [168]–[170]. This small protein is structurally linked to Ub, it presents tandem Ub-like domains connected with a flexible linker and hence is considered an Ub-like protein [171]. ISG15 chains are linked to target proteins in a similar way as compared to ubiquitination: the E1 recognizes the C-terminus domain but both C-terminus and N-terminus domains are required for the chain formation [166], [167], [172]. ISGylation is a reversible process since ISG15 deconjugation is mediated by the USP18 (exclusive for ISG15) which acts on one ISG15 domain, leaving the second available for a second protease [173]–[175]. Free ISG15 can have a secondary effect: once deconjugated, the ISG15 can be either secreted, acting as a cytokine in the neighbor cells, or it can bind USP18 downregulating deconjugation [173]. Given the important role of ISG15 production and conjugation, different viruses try to evade this system with different approaches: some viruses alter the ISG15 conjugation by removing the ISG15 from target proteins, others act through regulating free ISG15 levels hence inhibiting the migration to neighbor cells or the binding to USP18 [173].

CoVs encode a USP that also acts as deISGylase in order to directly interfere with host cell ISGylation. In fact, it was demonstrated that SARS-CoV-2 PL-pro preferentially cleaves ISG15 and Ub (Fig. 15)[176].

SARS-CoV-2 PL-pro inhibits innate immune response at different levels by removing Ub chains or ISG15 from target proteins. The IFN-I production is blocked by PL-pro through deubiquitination of RIG-I/MAVS following by RNA detection [177], it also blocks DNA mediate IFN-I production through targeting Ub-STING [96]; moreover, it impedes the NF- κ B dimer translocation in the nucleus through k-48 deubiquitination inhibiting I κ B degradation. TRAF6 and TRAF3 are also deubiquitinated, hence they are not able to start the phosphorylating cascade leading to IFN-I production; IFN - signaling is inhibited through ISG15 deconjugation [177]. The innate immune response is, hence, overall suppressed by PL-pro activity.

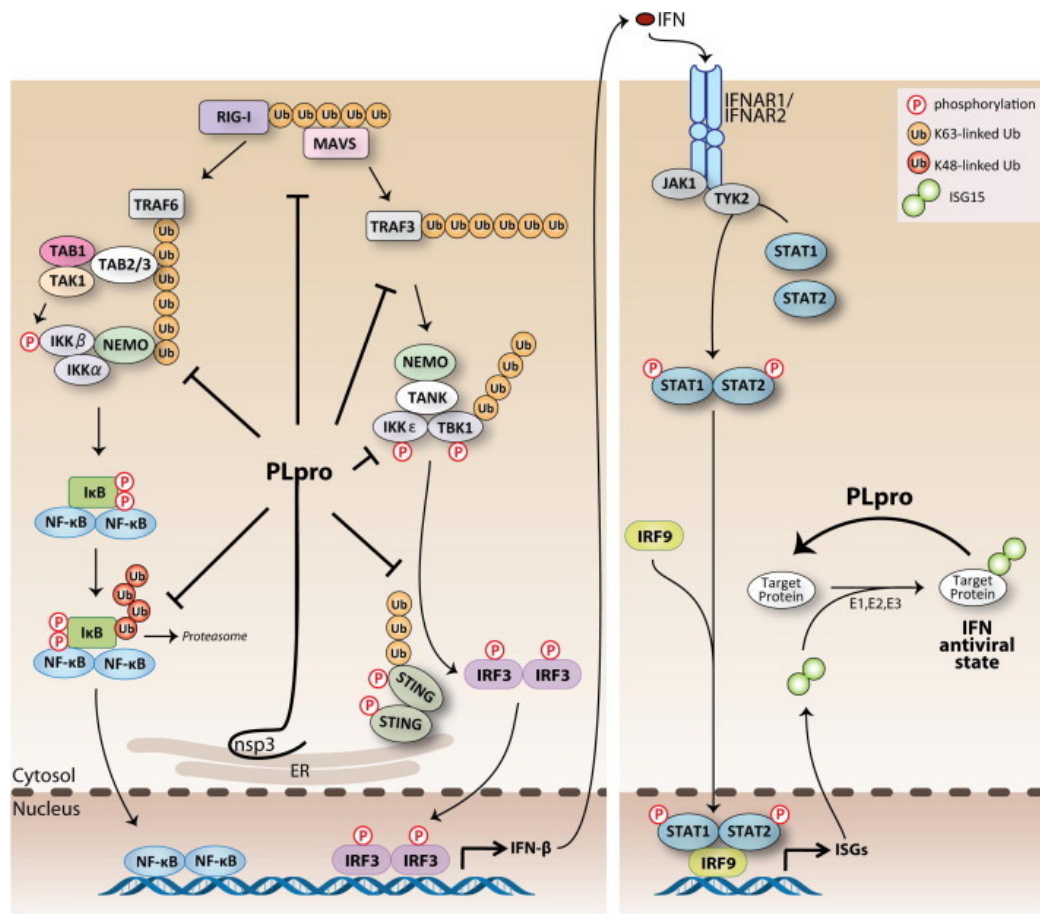


Figure 15. Inhibition of Innate Immune response mediated by SARS-CoV-2 PL-pro [177].

1.3.7 SARS-CoV-2 PL-pro inhibitors

Overall, the SARS-CoV-2 PL-pro roles on the innate immune response evasion and the pivotal role in viral replication make of it an excellent anti-viral drug target [178], [179]. During the SARS-CoV-2 pandemic, the main issue was the identification of effective drugs that could inhibit viral replication and spread, the starting point was the drug-reproposing strategy [178], [180].

A few molecules have been identified that resulted able to inhibit PL-pro activity in infected cells restoring the innate immune response. GRL0617 was reported to inhibit SARS-CoV PL-pro, hence it was shown to be able not only to restore IFN response by inhibiting the PL-pro interaction with ISG15 C-ter but also to inhibit viral replication in cell-based assays [181]. 6-Thioguanine (6-TG), an FDA-approved drug for the treatment of childhood leukemias, was found able to inhibit SARS-CoV and MERS-CoV PL-pro catalytic domain in vitro. 6-TG can inhibit SARS-CoV-2 PL-pro-catalyzed viral polyprotein cleavage and deISGylation in cell-based assays, moreover it inhibits SARS-CoV-2 replication in Vero E6 and Calu3 cells [182], [183]

1.4 Inhibitors of Ebolavirus targeting innate immune evasion

1.4.1 Ebola virus

Ebola virus (EBOV) belongs the order *Mononegavirales*, *Filoviridae* family, genus Ebolavirus, which includes six species of enveloped, filamentous, linear non-segmented negative-sense RNA viruses (NNSVs): (EBOV), Sudan, Reston, Tai Forest, Bundibugyo, and Bombali viruses [184]–[186]. EBOV possesses a ssRNA- of approximately 19 kb that encodes for three structural proteins and seven non-structural proteins [187], [188]. The viral glycoprotein (GP) mediates virion

attachment and entry into the cell, the nucleoprotein forms the nucleocapsid with three other viral proteins (VP) VP30, VP35 and the large (L) RNA dependent RNA polymerase (RdRp), while VP24 and VP40 form the viral matrix. Other non-structural proteins are the secreted GP (sGP), the super small sGP (ssGP) and the D-peptide. These three proteins are encoded by the virus but not present in the virion: sGP and ssGP are involved in the viral entry, while D-peptide is involved in the proteolysis process. Although their functions seem to be identified, their mechanism of action has yet to be fully explained [188]–[191].

EBOV is the etiological agent of Ebola Virus Disease (EVD), a severe, most often fatal haemorrhagic fever that affects humans (HP) and non-human primates (NHP). During the last decade, a major outbreak in West Africa spread between 2014-2016, that caused more than eleven thousand deaths, was followed by recurring minor outbreaks, especially in Democratic Republic of the Congo (DRC). The last EVD epidemic event started in 2018, it is still ongoing and up to date has caused more than three thousand deaths only in DRC, and since 2021 it is also present in Guinea [192]. Due to the results of animal surveillance, both snakes and fruit bats are supposed to be natural reservoirs of this virus [193]. EBOV transmission follows a specific chain of events: first it is probably common an animal-to-animal infection, then the transmission occurs from animal-to-human and lastly from human-to-human. Several mechanisms are involved in this: consumption of potentially contaminated food, direct contact with body fluids (blood, saliva, sweat), vertical transmission (in utero and during delivery), contact with contaminated surfaces and materials [194], [195].

Once into the organism, EBOV preferentially infects macrophages and dendritic cells (DCs) following their differentiation from monocytes, causing high levels of cytokines and chemokines such as IL-1 β , IL-6, IL-8, IL-10, MCP-1, MIP-1 α , MIP-1 β and TNF α that increase the overall endothelial permeability [190]. On the other hand, coagulation abnormalities are initiated early during EBOV infection followed by

over-activation of the extrinsic pathway of coagulation that may promote disseminated intravascular coagulation [196].

During the major outbreak in 2014-2016 the vaccine Ervebo, a recombinant vesicular-stomatitis virus-based vaccine, conferred immunogenicity against EBOV. Due to this result the European Medicine Agency (EMA) allowed its use during the 2018 outbreak in DRC as a possible transmission prevention tool available and for investigation. Ervebo was approved by FDA in 2020 [197] together with Inmazeb, a monoclonal antibody cocktail that targets the EBOV glycoprotein (FDA, 2020). However, the road to the global protection against EVD is still long and would need a more systematic chronic treatment, economically sustainable, due to the persistence of residual virus in survivors that leads to long-term sequelae [198].

The EBOV lethality is related to its ability to evade host innate immune response, especially during the early stages of infection [199]. It has been demonstrated that immune evasion functions of the EBOV VP35 protein are critical for virulence in NHPs [200], [201]. In fact, a VP35 mutant EBOV does not cause lethal disease and instead elicits adaptive immune responses that can protect animals from wild-type EBOV challenge [200]. So as VP35, VP24 plays an important role on the evasion of innate immune response [202]. In fact, infection with a VP24 mutant EBOV results in marginal effects on the innate immune response pathways [202]. Moreover, pharmacological amplification of the innate immune response has been shown to overcome EBOV replication [203]. This proves that the IFN- β pathway restoration by the block of viral components involved in immune evasion could lead to an effective antiviral approach.

1.4.2 VP35

VP35 is a 340 amino acid protein that contains three main functional domains: i) the N-terminus, that possesses an NP-chaperoning domain [204] (PDB 4YPI), ii) a homo-multimerization domain (PDB 6GBP), able to form various multimers, tetramers and trimers in solution [205]–[207] and iii) the C-terminus domain presenting the IFN Inhibitory domain that binds dsRNA (RBD/IID) and is required to inhibit the RLRs signalling (PDB 3L25) [208]–[211] (Fig.16). Similarly, to its orthologous counterpart in the *Mononegavirales* order, VP35 is a key component of the viral RdRp complex [212]. VP35, bridging NP and RdRP, interacts with the NP-chaperoning domain, to prevent NP non-specific associations with RNA, keeping it in the monomeric form and mediates the delivery of the NP-encapsidated template to viral polymerase complex for viral RNA synthesis. In addition, VP35 has been demonstrated to interact with the N-terminal region of the viral L protein but this molecular function remains to be characterized [213]. In this context, VP35 is also involved in viral genome packaging and nucleocapsid formation [214]. The recently discovered VP35-associated NTPase and helicase-like activities suggest its potential involvement also in viral RNA remodeling [215]. Of note, its homo-multimerization domain is responsible for binding the viral polymerase L [216], [217] and it is also required for full IFN antagonism.

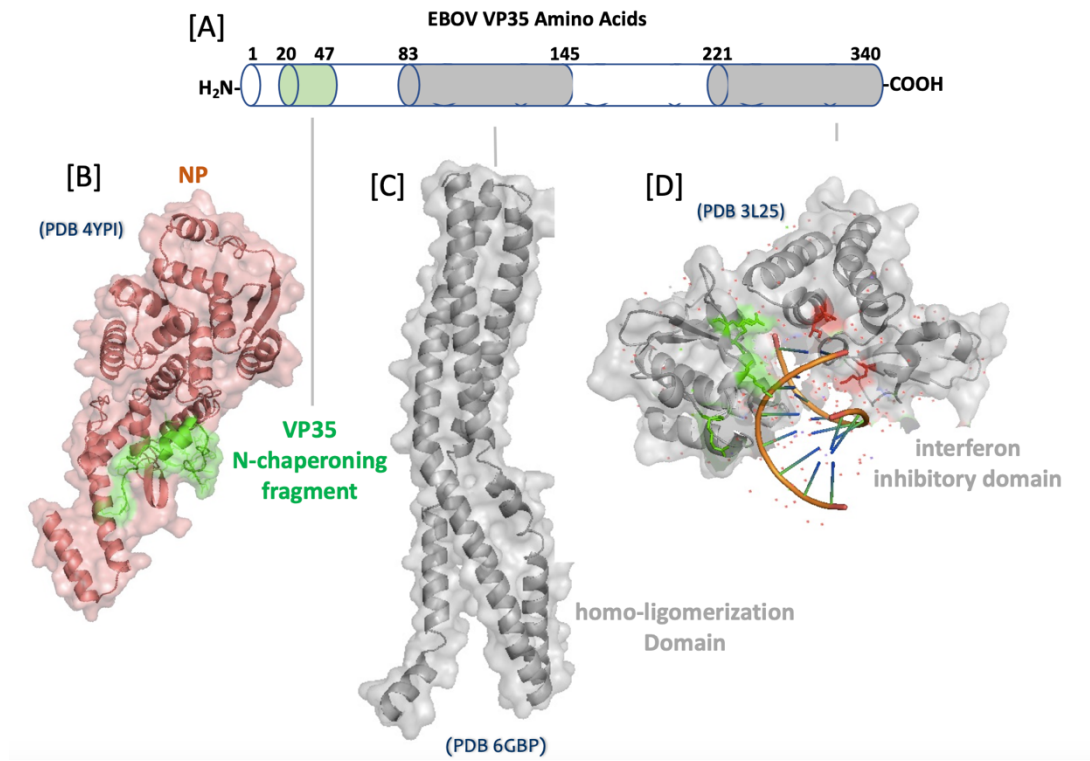


Figure 16. VP35 protein structure. A. VP35 subdomains; B. Structure of Ebola virus nucleoprotein N-terminal fragment (in orange) bound VP35 N-chaperoning fragment; [C] oligomerization domain of VP35; [D] VP35 interferon inhibitory domain with “backbone capping residues” (green) and “end capping residues” (red), in complex with dsRNA [218].

In the VP35 C-terminus domain the preservation of the IID structure is critical for EBOV. Crystallographic analyses that solved the structures of EBOV IID in complex with dsRNA [219] and Reston IID dsRNA [220] revealed that VP35 uses a bimodal strategy to bind the dsRNA. The protein acts as a functional dimer where one monomer binds the terminal nucleotides and the proximal phosphate backbone (“backbone-capping”) while a second monomer binds the sugar-phosphate backbone of both dsRNA strands (“end-capping”) (Fig. 16 D). IID mutations have been shown to suppress the ability of VP35 to bind to dsRNA and further resulted in loss of its IFN antagonistic function [209]. In the “backbone-capping” monomer, R305, K309, R312 and K339 amino acid residues directly interact with the dsRNA phosphate

backbone, while in the “end-capping”, amino acid residues K339, Q274 and I278 bind dsRNA terminal nucleotides [220], [221]. Mutations in residues R305, K309, R312, K319, R322, F239 and K339 strongly affect suppression of IFN- β [221]–[228]. Interestingly, VP35 double mutant R319A/K322A (KRA) EBOV has been shown to be completely avirulent in guinea pigs [222], and the simultaneous presence of F239A, K319A, and R322A mutations (VP35m), in EBOV decreased the viral growth rate and attenuated the virulence in animal models [229]. Moreover, the interaction with the host protein 8 kilodalton dynein light chain (DLC8 or LC8) enhances VP35 stability during viral replication, while the VP35 IID also binds the dsRNA-binding protein, 76 KD (DRBP76; also referred to as NFIL3, NF90 and other names) [230].

The VP35 suppression of the IFN- β production is exerted at several levels of the RLR cascade (Fig. 17), by preventing the RLR activation by sequestering viral dsRNA [209], formed as viral replication intermediate, and also inhibiting the RIG-I cascade at several levels [231], [232] interacting i) with RNA-activated protein kinase (PKR) activator (PACT) to disable its interaction with RIG-I [233], [234]; ii) with TBK-1 and IKK ϵ [235] to block their interactions with their substrate IRF-3; iii) with both IRF-3 and IRF-7 to promote their SUMOylation [236], hence blocking type I IFN production mediated by tripartite motif-containing protein 6 (TRIM6), [237] [238]–[240], and iv) repressing the stress granule assembly [241], [242].

it undergoes nucleocytoplasmic trafficking regulating its activity. In fact, VP24 contains a nuclear export sequences (NES) signal, required for the export of the protein from the nucleus to the cytoplasm where is required for the viral particles' formation [245]. The secondary, but relevant, activity of VP24 is the inhibition of the cellular IFN signalling cascade through the interaction with the NPI-1 subfamily of karyopherin- α proteins (KPN α 1, KPN α 5, and KPN α 6) and by blocking the translocation of PySTAT-1 to the nucleus (Fig.18) [18], [246], [247]. The interaction with KPN α has been characterized by crystal structure analysis [248] and involves a hydrophobic portion including amino acid residues L115, L121, D124, W125, T128, T129, N130, T131, N135, R137, T138, R140, Q184, N185, H186, L201, E203, P204, D205 and S207 (Fig. 18) [248]. However, also amino acid residues 142–146 were reported to be important for the ISG54 inhibition, since mutations at residues 142–146 were able to increase the expression of ISG54 reporter up to 90%, thus drastically reducing VP24 anti-IFN activity [247].

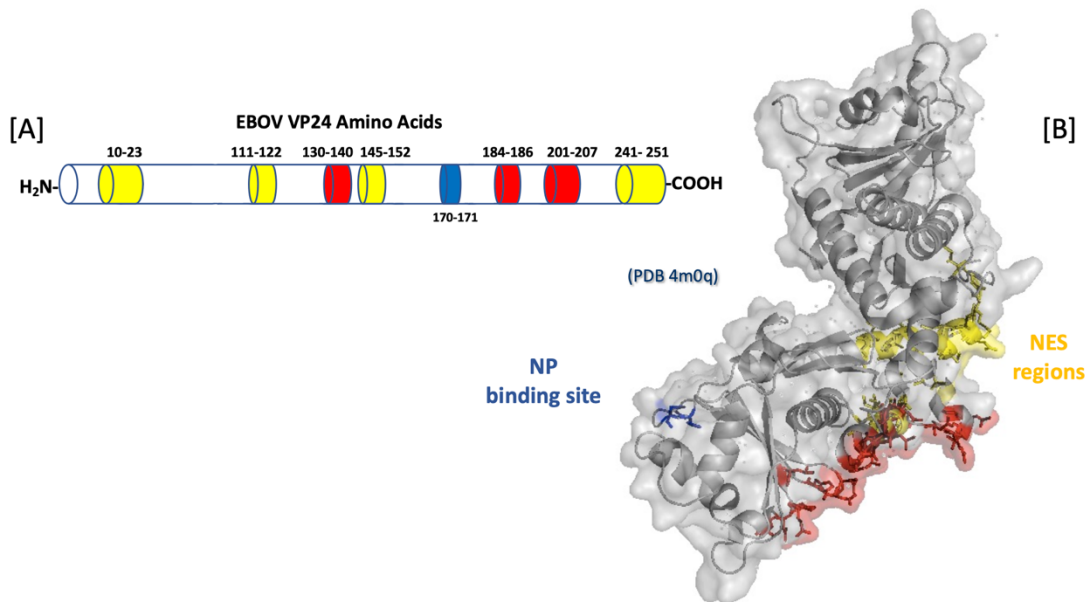


Figure 18. VP24 Structure (grey) with residues critical for karyopherin- α interaction (red), NES signals (yellow), NP binding site (blue) [218].

As demonstrated by Vidal et al, VP24 undergoes SUMOylation [249]. This modification is essential for the stability of the protein, and in fact VP24 mutated in the putative SUMOylation site has a significantly reduced ability to interact with KPNA5 and to block the IFN activity, it exhibits decreased stability, and shows a reduced capacity to inhibit IFN-induced nuclear translocation of phosphorylated STAT1 [249]. In a recently published work, VP24 has been shown to interact with STAT3, inhibiting its activity, in infected cells; the two proteins co-localize in cytoplasmatic viral replication/inclusion bodies, suggesting that STAT3 is included into these virions. The interaction was also confirmed in a cell line defective for IFN, Vero cells, excluding possible impact by virus-induced IFN-I. The interaction does not affect STAT3 ability to bind DNA and is moreover STAT1-independent. Further studies are required to determine how this interaction can affect innate immune response in viral infected cells (Fig. 19) [250].

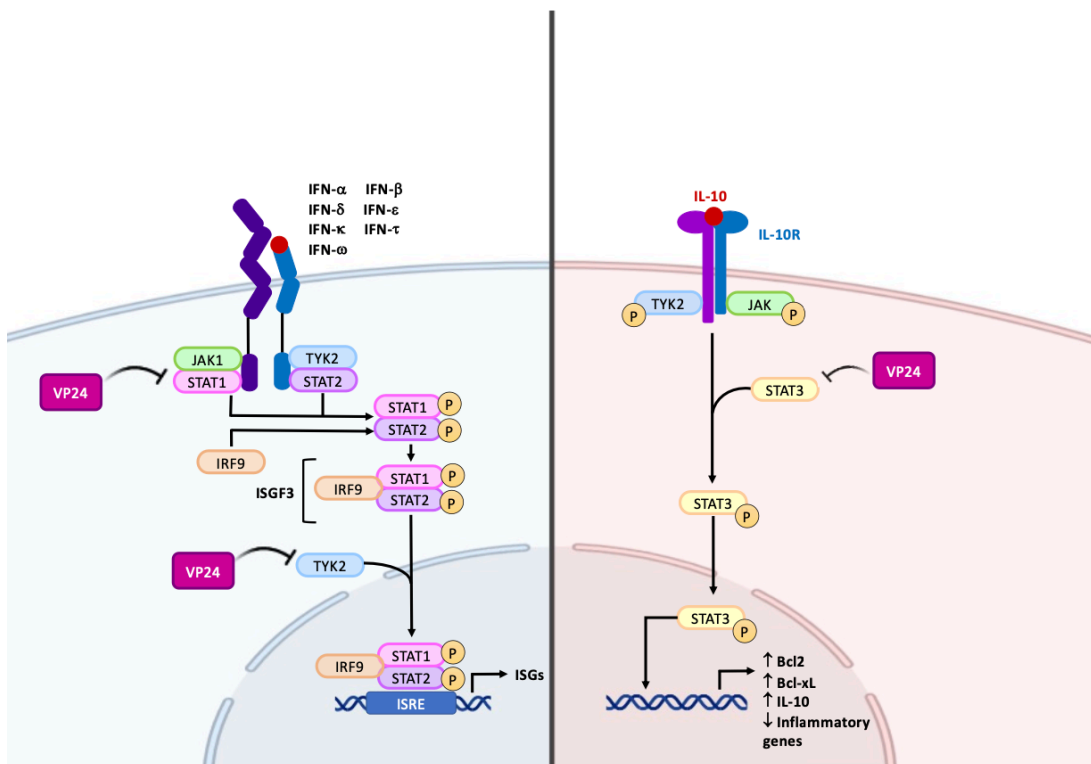


Figure 19. Scheme of EBOV VP24 inhibition of IFN- α/β signalling [218].

1.4.4 EBOV inhibitors targeting innate immune evasion

The suppression of innate immune response is critical during EBOV infections. As mentioned above, EBOV has evolved two principal strategies to avoid the interruption of viral replication cycle: VP35 and VP24 viral proteins block the IFN production and the IFN signalling, respectively, and are key elements for EBOV virulence. Several mutational studies at the level of binding sites for both VP35 and VP24 have demonstrated how their contribution in evading host innate immunity is important to allow the viral replication. As reported by Ilinykh et al. disabling VP35 and VP24 innate response antagonizing domains (IRAD) result in the modification of the expression of multiple genes involved in maturation of DC and the induction of the innate and adaptive immune responses in a time dependent manner and with different consequences for the two proteins [202]. On the one hand, the abolition of VP35 activity increases the expression of multiple genes involved in DC maturation [251], on the other hand disabling the VP24 IRAD resulted in the decrease of expression of many genes whose level was upregulated in response to EBOV infection. The combined effect of the two mutated IRAD increases the levels of transcripts of almost all subtypes of IFNs in DC compared to the wt EBOV infection [202]. The characterization of the differences of binding affinity of the complex VP24- KPNA among EBOV species demonstrated that a higher affinity corresponds to higher IFN inhibitory activity [252]. All of these considerations lead to the conclusion that these two proteins are validated drug targets [253]–[256]. In fact, VP35 and VP24 are currently investigated as promising drug targets since a therapy targeting their IFN inhibitory functions and the consequent restoration of the IFN response will have a positive impact in the treatment of early stages of EVD. In the last decade a number of *in silico*, *in vitro* and *in vivo* studies have been performed, identifying potential active compounds among synthetic and naturally derived molecules.

1.4.5 VP35 inhibitors

Since the determination of the first VP35 IID crystal structure [257], the purification of the first soluble His-tagged full length rVP35 [258] and the availability of effective pull-down assays [225], [259], *in silico* and *in vitro* studies have been carried out to identify ligands possibly able to inhibit VP35 RdRp cofactor activity and revert its IFN inhibitory effect.

At first, a screening of FDA approved drugs docked on the VP35 structure (PDB ID: 4IBI) [260], lead out to a high LibDock score for amodiaquine and chloroquine (Fig. 20). Interestingly those molecules previously demonstrated anti-EBOV activity [261]. Later, the compassionate treatment of EVD patients with a combination of amodiaquine-artesunate resulted in an increased survival [262], while no increase of the survival benefit was registered for treatment of animals or EVD patients with chloroquine [263]–[265].

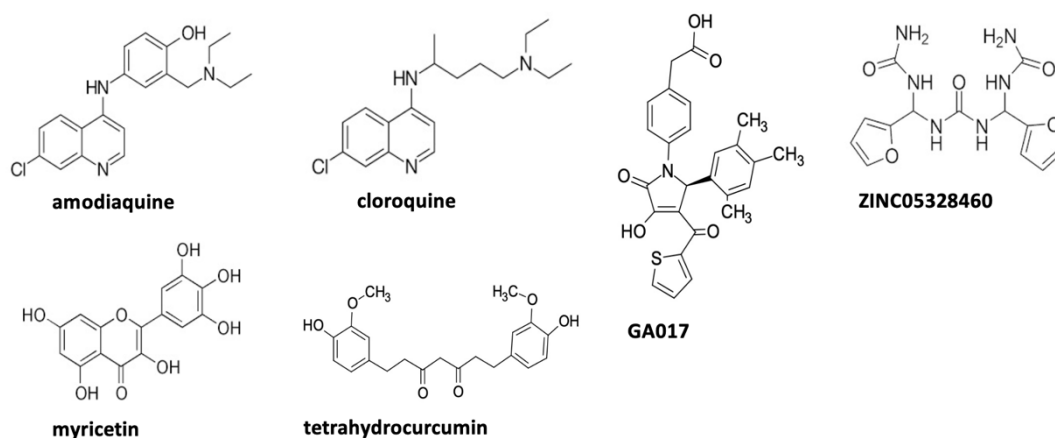


Figure 20. Chemical structure of VP35 inhibitors [218].

Later, Brown and colleagues docked a total of 5.4 million drug-like small molecules from the ZINC library to the VP35 IID (PDB ID: 3FKE), and identified and co-

crystallized N-aryl pyrrole-containing compounds bound to the VP35 IID, showing that they bind near a pocket formed by residues from the alpha-helical and beta-sheet subdomains, including amino acid residues A221, R225, Q241, L242, K248, K251, P293, I295, I297, D302, and F328, and proved they are able to disrupt the interaction between VP35 and NP, so affecting VP35 polymerase cofactor function [266] and that compound GA017 (Fig. 20) is able to block over 90% of viral replication at 125 μ M concentration. Unfortunately, the impact of such compound on VP35 IFN antagonist function was not investigated. Later, a sensitive in vitro high-throughput screen was developed to identify inhibitors targeting the VP35–NP interface [267].

Differently, Glanzer and colleagues, applied an ensemble-flexible strategy reducing the search space for docking to CBP of VP35 IID (PDB ID: 3FKE) and in particular the molecular space around the amino acid residues R305, K309, R312, K319, R322 and K339, known to be involved in the dsRNA binding, among which residues R305, K309, R322 and K339 were designated as flexible. Among the 80,000 compounds, ZINC05328460 (Fig. 20) showed the highest predicted affinity showed the greatest inhibition of VP35-dsRNA binding ($IC_{50}=4 \mu$ M) [268].

Also, VP35 IID (PDB ID: 3FKE) was used for a pharmacophore based virtual screening predicting a potential high binding affinity for FDA-approved deslanoside and digoxin as well as the deslanoside analogue ZINC77291634, with predicted binding energies of -12.9 kcal/mol, -12.2 kcal/mol, and -10.0 kcal/mol, respectively, [269]. A further pharmacophore model (HypoA of 4-acetyl-3-hydroxy-1-phenyl-1H-pyrrol-2(5H)-one derivatives) was used to identify seven VP35 potential inhibitors (cpd 1 – 7) with 3D QSAR prediction and molecular docking method [270].

Later, Daino and colleagues that utilizing a fluorescent dsRNA oligomer ligand, found that epigallocatechin 3-O gallate (EGCG) and the flavonol myricetin (Fig. 20) are able to suppress the VP35-dsRNA binding with an IC_{50} values of 43.5 μ M and 2.7 μ M, respectively [271]. Of note, among natural products, also the curcuminoid metabolite

tetrahydrocurcumin (Fig. 20) was predicted by molecular dynamics simulations to potentially bind EBOV VP35 (PDB ID: 4IBB) [272].

The effect of the VP35 inhibition of the RIG-I pathway was investigated by development of gene-reporter assays [273], used to screen natural extracts identifying some activity from the extract from the leaves of *Asphodelus microcarpus* Salzm. et Viv. against the viral protein. The extract was able to counteract the IFN VP35 inhibitory effect, partially restoring IFN production [274], [275].

The possibility to Inhibit VP35 was investigated also by cell-penetrating peptides (CPPs). Serving as “intracellular blockers”. These molecules were developed to the aim of facilitating the entry of active compounds into cells [276], [277]. Differently from conventional antibodies that are unable to target viral proteins localized within the cytoplasm, these molecules take advantage of electrostatic interactions between the negatively charged membrane components and their positive charged portions, such as CPP the nona-arginine (R9), displaying a lack of cytotoxicity that would make transbodies-based therapy a promising approach compared to the current antibodies’ treatments [278], [279]. Seesuw and colleagues used R9 to mediate the transport of human single chain variable antibodies fragments (HuscFvs) or transbodies directed against VP35 that specifically recognize the IID function [278].

HepG2 cells transiently expressing EBOV VP35 were treated with R9-HuscFv3 and R9- HuscFv8 and gene expression of IFN- β promoter and EIF2AK2, encoding for PKR, were evaluated, showing a restoration of IFN- β promoter and EIF2AK2 expression compared to the expression of IFN- β and EIF2AK2 in not treated VP35-transduced cells, leading to the host innate immunity rescue [278].

R9-HuscFv3 was predicted to interact with IID residues of the CBP (R305, K309, R312, R322, and K339), the end-cap motif (F235, F239, and I340), other residues known to interact with the dsRNA (P233, T237, Q274, C275, I278, A306, and S310), and also with D271 [278]. R9-HuscFv8 was shown to interact with the CBP residues R322 and K339, the end-cap residue I340 and with amino acid residues S272, C275,

I278, and Q279, important for the VP35-dsRNA binding, as well as other residues (E269, Q270, and D271) [278]. In addition, the lack of cytotoxicity renders transbodies-based therapy a promising approach compared to the current antibody's treatments [278], [279]. Similarly, Flego and colleagues targeted VP35 in the cytoplasm by as single chain fragment variable (scFv) identified by Phage display technology, all scFvs were expressed in cell cytoplasm as intrabodies; a luciferase reporter gene inhibition assay performed in A549 cells showed that two of the scFvs can significantly hamper the inhibition of the IFN- β -induced RIG-I signaling cascade mediated by EBOV VP35 [280]. All the selected scFvs were able to interact with VP35 in ELISA assays, one of the scFvs being also able to react in Western Blot assay (WB).

1.4.6 VP24 Inhibitors

With the aim of developing VP24 inhibitors, several studies have been performed leading to the identification of a number of promising small molecules (Fig. 21).

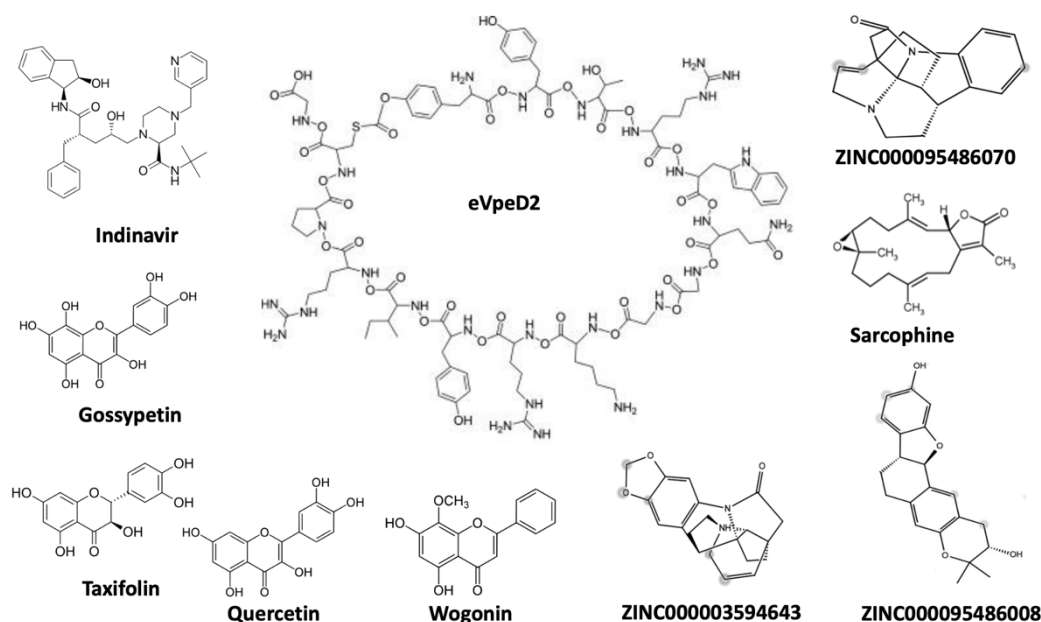


Figure 21. Chemical structure of VP24 inhibitors [218].

The investigation of an FDA-approved database of antivirals, identified the HIV protease inhibitor, indinavir (Fig. 21), as potential VP24 interactor due to the putative three hydrogen-bonding interactions between: i) the O2 atom of Indinavir and the nitrogen atom from the sidechain of residue Gln94 in VP24, ii) the atom N4 of Indinavir and the oxygen atom in the sidechain of VP24's Gln94, and iii) the O4 atom of Indinavir and the oxygen atom from the main chain of Gln94 of VP24 [281].

An in-silico study has designed antisense phosphorodiamidate morpholino oligomers (PMOs) to target Ebola EBOV virus genes [282]. AVI-7537 is a PMO_{plus} that binds directly to the viral VP24 transcript RNA blocking the protein production and showing 61% of protection from lethal challenges. Of note, meanwhile 100% of protection was reached with AVI-6003, a PMO combination of AVI-7287 (targeted to VP24) and AVI-7288 (targeted to NP) [282].

Some macrocyclic peptides, generated with the by RaPID (Random non-standard Peptides Integrated Discovery) system have been shown to be able to disrupt the

interaction between eVP24 and KPNA5, demonstrating that this interaction is druggable and could lead to the production of unique anti-viral drugs [283]. The most promising one peptide, eVpeD2 (Fig. 21), was able to inhibit this interaction in a dose-dependent manner, with an IC₅₀ value of approximately 9 μ M [283].

On the basis of an *in-silico* study that identified as potential EBOV inhibitors the natural compounds Gossypetin (Fig. 21), isolated from the flowers and the calyx of *Hibiscus sabdariffa*, and Taxifolin, present in *Taxus chinensis*, *Larix sibirica*, *Pinus roxburghii* and *Cedrus deodara* [284], a library of flavonoids have been screened to identify molecules that shared a similar structure with the previously identified molecules. Quercetin and Wogonin were selected to be tested together with Gossypetin, Taxifolin (Fig. 21) and Tricetin in a luciferase assay to evaluate the inhibition of VP24 activity. The most interesting molecule was Quercetin, that showed a significant efficacy in restoring IFN stimulation in a dose-dependent manner. To further characterize its mechanism of action, Quercetin ability to block the VP24 inhibition of P-STAT1 translocation into the nucleus was evaluated performing an immunofluorescent assay, observing that it is able to block VP24 function and induce the recovery of P-STAT1 translocation. Since VP24 is known to interact with KPNA5, to determine whether Quercetin was able to inhibit this interaction, a co-Immunoprecipitation was performed confirming a reduction in VP24/KPNA5 co-immunoprecipitation and indicating that Quercetin interferes with this interaction. Quercetin was then finally tested on EBOV replication in an IFN-susceptible cell-line model [285]. Of note, when with the derivative form Quercetin 3- β -O-D-glucoside (Q3G) derivative was tested on EBOV replication it was less efficient than the non-glycosylated form suggesting that Quercetin indeed mainly targets the VP24 anti-IFN function while Q3C probably mainly targets EBOV entry [285].

Kwofie et al. with the purpose of identify putative lead compounds from the African flora with the potential to inhibiting the activity VP24 functions screened an integrated library of > 7600 African natural products obtained from the AfroDB and NANPDB

databases, using pharmaco-informatics and molecular docking methodologies. For 19 of these compounds, it was characterized the physicochemical and pharmacological profiles, using ADMET Predictor™, SwissADME and DataWarrior, and the VP24 putative binding mechanism using LigPlot+, predicting the activity spectra to determine their anti-EBOV activity. Four natural products, namely ZINC000095486070, ZINC000003594643, ZINC000095486008 and sarcophine (Fig. 21), were found to be potential VP24 inhibitors and will deserve further biological testing to verify their ability as potential EBOV biotherapeutic agents [286].

1.4.7 Immunomodulators from Traditional Chinese Medicine

The Traditional Chinese Medicine (TCM) originated around 2500 years ago. TCM was reported in the first Chinese medical book “Yellow Emperor’s Canon of Medicine” in which it was described the physique difference existing among the individuals from their birth through their eldest age by sorting physique people’s characteristics as stature, pathological features but also emotional and psychological characteristics as temper and emotion-thought, yin-yang and the five elements. This first analysis of human nature related physique conditions to the onset and the development of diseases, suggesting that treatment methods should have been modified according to the patients’ physique difference even for same diseases [287].

The most recognized definition of TCM proposed by prof. Wang “is s defined as an integrated, relatively stable and natural specialty of individual in morphosis, physiological functions as well as psychological conditions formed on the basis of innate and acquired endowments in his/her life process and determines the susceptibility to some pathogenic factors as well as tendency towards pathogenic modes” that can be summarized in the familiar Chinese expression: “An excellent

doctor treats diseases before it arises but not diagnosed diseases”. In TCM, hence, the patient is treated before symptoms onset before the insurgence of an energy imbalance [288].

The basis for TCM treatments include: acupuncture, a technique in which practitioners stimulate specific points on the body, usually by inserting thin needles through the skin, Tai-chi, that combines certain postures, gentle movements, mental focus, breathing, and relaxation improving balance and stability, and Chinese Herbal Products used to treat many medical problems, including stroke, heart disease, mental disorders, respiratory diseases and infectious diseases [289].

The Chinese Herbal Products have been widely used for the treatment of infectious diseases as SARS-CoV and MERS-CoV in 2003 and 2012 respectively, reducing hospitalization and side effects from steroids treatment [290], [291], as well as in the treatment of Influenza A (H1N1) viral infection in the 2009 reducing the mean time of defervescence and the duration of viral shedding [292]. Of note, one particular TCM product was used for the treatment of EBOV infection, in fact tetrandrine, which was isolated from the TCM medicinal herb *Stephaniae Tetrandrae Radix*, was reported to inhibit EBOV infection both in vitro and in vivo [293].

To date hundreds of natural TCM ingredients as well as dozens of Chinese herbs have reported to possess antiviral activities [294]. TCM drugs are usually composed of herbal components with known and unknown active ingredients that can target various pathways [289].

It is common, among the TCM treatments to have immunomodulatory activity especially in infectious diseases as influenza and other viral infections [295], hence we decided to investigate the activity of a molecule isolated from Chinese herbs that have strong immunomodulatory effect.

Objectives

IFNs, due to their potency in inhibiting viral replication, have been studied and used as potential medicines to counteract viral infections such as Hepatitis C virus (HCV) and Hepatitis B virus (HBV) infection [296]. However, treatment with IFNs is not complication-free since in INF- α treated patients were found to develop autoimmune disorders such as psoriasis, vitiligo, rheumatoid arthritis, autoimmune hepatitis have been reported, indicating that direct exposure to IFNs can be comparable to an uncontrolled cytokine expression incompatible with physiologic conditions where the innate immune response is tightly regulated [297].

In the fight for spreading, viruses have evolved strategies to evade the innate immune response at different levels. A number of virus-specific mechanisms through which viruses mask to prevent PRR detection have been identified [298]. Viral evolution has indeed occurred by selecting strains possessing proteins capable to interact with cellular proteins preventing the transcription factors activation or their translocation in the nucleus impeding the IFNs or ISGs transcription [252], [298], [299]. Moreover, viral enzymes were shown to be able to degrade cellular proteins by direct cleavage or by regulating the cellular proteasomal mediated degradation [300]–[302].

The aim of this project focuses on i) the exploitation of STING as cellular targets for the development of compounds with broad spectrum antiviral activity inducing the innate immune response activation, ii) the development of compounds selectively active against SARS-CoV-2 PL-pro and iii) the characterization of compounds able to revert the inhibitory effect of EBOV proteins on the innate immune response.

Potential immunomodulator compounds have been tested in biological assays to investigate their potency in inducing the IFN response STING-dependent, inhibit viral replication, and characterize their mechanism of action. Meanwhile the potential PL-pro inhibitors identified through *in-silico* screening and confirmed through

biochemical assays, were tested in a biological system in order to evaluate the ability to subvert PL-pro mediated IFN- β inhibition. Finally, a selection of molecules deriving from TCM products have been tested for their ability to revert EBOV modulation of the innate immune response, the best hit compounds were then tested in biological system in absence of viral proteins to study the cellular factors involved in their mechanism of action.

Chapter II

Methods

2.1 Cells and reagents

HEK293-ISRE reporter and HUH7-ISRE reported, kindly provided by Pierre-Olivier Vidalain, and HEK293T (ATCC® CRL-3216™) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco), 10% v/v fetal beef serum (FBS; Gibco) and 1x Pen-strep (Euroclone); BEAS-2B cells, kindly provided by Pierre-Olivier Vidalain, were maintained in DMEM/F-12 (Gibco), 5% v/v FBS HI (Gibco), 1% Kanamycin (Thermo-Fisher Scientific). Vero E6 GFP, kindly provided by Janssen Pharmaceutical, were maintained in DMEM (Gibco) supplemented with 10% v/v FBS (Gibco), 0.075% Na bicarbonate (7.5% solution, Gibco), and 1 x Pen-strep (Euroclone). Calu 3 were maintained in DMEM, 10% FBS, 1% Pen-strep, 1mM Na Pyruvate (Euroclone), 1mM Essential Amino Acids (Euroclone). MRC-5 cells (ATCC: CCL-171™) were maintained in MEM (Gibco), 10% v/v FBS (FBS HI; Gibco), 1 mM Na Pyruvate (Euroclone), 1 mM Non-Essential Amino Acids (Euroclone) and 1x Pen-strep (Euroclone). All cells were maintained under 5% CO₂ on 37°C.

HCoV229E (ATCC® VR-740™) was propagated in MRC5 maintained as previously described; the SARS-CoV-2 strain is the BetaCov/Belgium/GHB-03021/2020 was provided by KU Leuven. All SARS-CoV-2-related work was carried out in certified, high-containment biosafety level-3 facilities at the University of Cagliari.

Plasmid pGL-IFN- β -luc was kindly provided by Prof Stephan Ludwig from the Institute of Molecular Virology, (University of Münster, Germany). pRL-TK was

purchased from Promega (Promega Italia S.r.l. Milan, Italy). pUNO1-hSTING-HA3x was purchased from Invivogen. pDEST-HA was kindly provided by Pierre-Olivier Vidalain.

2.2 Plasmid mutagenesis

The plasmid pUNO1-hSTING-HA3x was mutated with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's indications. Primers used were forward CCG TGC GGA GAG GGA GTT GCT TTT CCA TTC CAC T reverse: AGT GGA ATG GAA AAG CAA CTC CCT CTC CGC ACG G, mutagenesis was confirmed through sequencing.

2.3 Gene reporter assay for the evaluation of IFN β induction STING dependent.

HEK293T were seeded 2×10^4 cells/well in a white 96 well plate and incubated overnight in order to reach 90% confluency. 24 hours later cells were transfected with 40 ng pGL-IFN- β -luc, 10 ng pRL-TK, plasmid concentrations for the reporter luciferases had already been determined [303], 10 ng pUNO1-hSTING-HA3x or pDEST-HA or pUNO1-hSTING-HA3xP371Q, using JetPrime transfection reagent (Polyplus) according to manufacturer's indications. 24 hours post transfection, cells were treated with indicated concentration of compound diluted in complete culture medium. The day after, luciferase activity was detected using homemade solutions: Lysis buffer (50 mM Na-MES [pH 7.8], 50 mM Tris-HCl [pH 7.8], 1 mM dithiothreitol, and 0.2% Triton X-100). Luciferase assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM magnesium acetate, and 2.5 mg/mL ATP). Luciferin solution 1mM D-Luciferin in 5mM KH₂PO₄. Coelenterazine assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM magnesium

acetate, 5mM KH₂PO₄, 10 μ M coelenterazine). Luminescence was detected with Victor Nivo5 PerkinElmer both after adding Luciferin solution and Coelenterazine assay buffer. The relative light units (RLU) were normalized against renilla luciferase luminescence as the fold induction over unstimulated controls.

2.4 Gene reporter assay for the evaluation of IFN- β induction in presence of PL-pro or VP35.

HEK293T were seeded 2*10⁴ cells/well in a white 96 well plate and incubated overnight in order to reach 90% confluency. 24 hours later cells were transfected with 60 ng pGL-IFN- β -luc, 10 ng pRL-TK, for the PL-pro assay: 30 ng of pcDNA3.1 (empty vector, EV) or pcDNA3.1-PL-pro Flag, for the VP35 assay :10 ng of pcDNA3 (EV) or pcDNA3-VP35 [303], 24 hours post transfection, cells were stimulated with 200 ng of Influenza-A Virus (IAV) RNA transfected with T-PRO NTR III according to manufacturer indications in presence and absence of indicated compounds. Luciferase activity was detected 24 hours post treatment using homemade solutions: Lysis buffer (50 mM Na-MES [pH 7.8], 50 mM Tris-HCl [pH 7.8], 1 mM dithiothreitol, and 0.2% Triton X-100). Luciferase assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM magnesium acetate, and 2.5 mg/mL ATP). Luciferin solution 1mM D-Luciferin in 5mM KH₂PO₄. Coelenterazine assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM magnesium acetate, 5mM KH₂PO₄, 10 μ M coelenterazine). Luminescence was detected with Victor Nivo5 PerkinElmer both after adding Luciferin solution and Coelenterazine assay buffer. The relative light units (RLU) were normalized against renilla luciferase luminescence as the fold induction over unstimulated controls.

2.5 Gene reporter assay for the evaluation of ISRE induction in presence of VP24.

HEK293T were seeded 2×10^4 cells/well in a white 96 well plate and incubated overnight in order to reach 90% confluency. 24 hours later cells were transfected with 60 ng pGL-IFN- β -luc, 10 ng pRL-TK, 30 ng of pcDNA3.1 (empty vector, EV) or pcDNA3.1-VP24 [304], 24 hours post transfection, cells were stimulated with compounds at indicated concentrations in presence of 10 ng/ml of human- α -IFN (IFN- α). 8 hours after treatment luciferase activity was detected using homemade solutions: Lysis buffer (50 mM Na-MES [pH 7.8], 50 mM Tris-HCl [pH 7.8], 1 mM dithiothreitol, and 0.2% Triton X-100). Luciferase assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM magnesium acetate, and 2.5 mg/mL ATP). Luciferin solution 1mM D-Luciferin in 5mM KH₂PO₄. Coelenterazine assay buffer (125 mM Na-MES [pH 7.8], 125 mM Tris-HCl [pH 7.8], 25 mM magnesium acetate, 5mM KH₂PO₄, 10 μ M coelenterazine). Luminescence was detected with Victor Nivo5 PerkinElmer both after adding Luciferin solution and Coelenterazine assay buffer. The relative light units (RLU) were normalized against renilla luciferase luminescence as the fold induction over unstimulated controls.

2.6 Gene reporter assay with ISRE reporter cell lines.

HEK293 and HUH7-ISRE reporter cells are stably transfect with a plasmid encoding for a luciferase under the control of ISRE promoter [305]; cells were seeded 2×10^4 cells/well in a white 96 well plate and incubated overnight in order to reach 90% confluency, 24 hours later cells were treated with compounds at indicated concentrations. Luminescence was detected using the Bright-Glo™ Luciferase Assay

System (Promega). The relative light units (RLU) were used to calculate the fold induction over unstimulated controls.

2.7 RNA silencing on ISRE reporter cell lines.

HEK293 and HUH7-ISRE reporter cells were seeded 2×10^4 cells/well in a white 96 well plate and incubated overnight in order to reach 90% confluency; on day two the cells were transfected with indicated siRNA using DharmaFECT 1 (Horizon – Perkin Elmer) according to customer's indications. 24 hours after transfection, cells were treated with compounds at indicated concentrations. Luminescence was detected using the Bright-Glo™ Luciferase Assay System (Promega). The relative light units (RLU) were used to calculate the fold induction over unstimulated controls.

2.8 Western Blot

HEK293 were seeded in 12 well plate, 10^5 cells per well; 24 hours after seeding cells were treated with indicated compound diluted in culture medium at 10 mM, Actinomycin D was used as control of genotoxic effect at 10 nM. Cells were incubated O/N in incubator at 37°C, 5% CO₂. After 24 hours, the compounds' dilution was removed, cells were washed with cold Phosphate Buffer Saline (PBS) and proteins were extracted with 200 µl RIPA buffer (0.05M Tris-HCl, pH 7.4, 0.15M NaCl, 0.25% deoxycholic acid, 1% NP-40, 10mMEDTA) supplemented with protease and phosphatase inhibitor (PhosphoSTOP™ - Roche). Cells were lysed with RIPA buffer for 20' in orbital shaker at 250 rpm. Whole Cell lysates were centrifuged 20' at 12000 g in order to remove cell debris and membranes. Protein concentration was quantified with DC™ Protein Assay Kit II (BioRad) and 20 ng of proteins were processed with 4X Loading Buffer and boiled 3', then loaded in SDS-Page (NuPage 4-12%) for

protein separation. Proteins were blotted with Trans-Blot® Turbo™ Transfer System on polyvinylidene difluoride (PVDF) membrane. Membrane was blocked with 5% BSA in Tris Buffer Saline – 0,1 % Tween, 1 hour at room temperature in orbital shaker 50 rpm. The antibodies used were diluted in blocking solution: Anti-p53 (#9282 CST Rabbit 1:1000), Anti-Rabbit (#7074 CST HRP-linked 1:3000).

2.9 HCoV-229E CPE assay

2×10^4 cells per well were seeded in transparent 96 well plate and incubated overnight in order to reach 90% confluency. 24 hour later cells were infected with hCoV-229E m.o.i. 0.06 in DMEM/F-12 in presence of compound or 0,1% DMSO (untreated controls). The cells were incubated for 2 hours at 35°C with 5% CO₂, then the virus was removed, replaced with complete medium with or without compound, and incubated at 35°C with 5% CO₂. After 72 hours, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma-Aldrich), dissolved in PBS at 7,5 mg/ml, were added to each well and incubated at 37°C with 5% CO₂ for 1 hour. Then the supernatant was removed, and cells were lysed with 100 µl/well of: 10% 2-Propanol, 0,004% Triton-X-100 (Sigma-Aldrich), 0,0004% HCl, then the absorbance was read at 570 nm with a plate reader Victor Nivo5 PerkinElmer.

2.10 SARS-CoV-2 Viral replication assay in BEAS2B cells

Cells were seeded 3×10^5 per well in transparent 12 well and incubated overnight in order to reach 90% confluency. 24 hours later, cells were infected a viral dilution 1:50 and treated with indicated concentrations of compound for 1 hour at 37°C with 5% CO₂. One hour later, the inoculum was removed and substituted with compounds diluted in complete medium. After 48 hours of infection, RNA was extracted with

TRIzol™ Reagent (Invitrogen). Extracted RNA was then reverse transcribed and amplified using a Luna universal one-step quantitative real-time PCR (RT-qPCR) kit (New England BioLabs), mRNA expression levels were normalized to the level of GAPDH. Results are expressed as percentage of cytopathic effect calculated with respect to the infected control. GC376 compound [306] was used as positive control of viral inhibition.

2.11 SARS-CoV-2 Viral replication assay in VERO-E6 GFP

Cells were seeded at 10^4 cells/well in 96-well black cell-treated plates (PerkinElmer). The following day, cells were incubated with the control compounds at different concentrations and the virus at MOI 0.01. GC376 compound [306] was used as positive control, in presence of 2 μ M p-gp inhibitor CP-100356 [307]. 72 hours post infection the media was removed and total well GFP fluorescence was measured with a Victor 3 with 485/535 nm excitation wavelength. The inhibition of viral replication was calculated as percentage of viral induced cytopathic effect on infected untreated controls, minus blanks (empty wells). EC₅₀ value was calculated with Prism 9. Version 9.1.2 via non-linear regression. The experiments represent average and standard deviation of at least two independent experiments in triplicate.

2.12 Real Time PCR.

HUH7 ISRE reporter cells were seeded in 24 well plate, 5×10^3 cells per well, 24 hours after were stimulated with compounds at 30 μ M in presence and absence of 3 IU/ml of IFN- α . 24h after treatment the mRNA was extracted with Direct-zol RNA Miniprep Kits (ZymoResearch) and subjected to reverse transcription with the High-Capacity RNA-to-cDNA kit (Applied Biosystems). The cDNA was used to perform q-PCR

using LightCycler® FastStart DNA Master SYBR Green I (Roche), primer used are listed in Table 2. Results were normalized to housekeeping gene, S9.

Table 2. Primer Sequences

Gene	Primer Sequence
qIRF1-F	CACCTCTCACCAAGAACCAG
qIRF1-R	TGTAGCCTGGAAGTGTGTAGC
qIRF9-F	CCACCGAAGTTCCAGGTAACAC
qIRF9-R	AGTCTGCTCCAGCAAGTATCGG
qRIGI-F	GACCCTGGACCCTACCTACA
qRIGI-R	TCCAAAAAGCCACGGAACCA
qISG15-F	ATGGGCTGGGACCTGACG
qISG15-R	GCCAATCTTCTGGGTGATCTG
qIL6-F	ACTCACCTCTTCAGAACGAATTG
qIL6-R	CCATCTTTGGAAGGTTCAAGTTG
qMxA -F	ACAGGACCATCGGAATCTTG
qMxA -R	CCCTTCTTCAGGTGGAACAC
qS9-F	GTCTCGACCAAGAGCTGAAGCT
qS9-R	GGTCCTTCTCATCAAGCGTCAG

Chapter III

Benzofuran derivatives inhibit HCoV viral replication through STING dependent Innate Immune activation.

3.1 Exploitation of STING as antiviral target for drug development

As described in chapter I, a possible strategy for the development of antivirals with broad spectrum activity is to target cellular proteins involved in the innate immune response in order to mount an interferon response strong enough to subvert viral replication but with more a limited side effect than IFN- α treatment. Among the cellular targets that could be explored is STING that, as described, is activated in response to viral and bacterial infections [308]–[310].

Since the discovery and characterization of STING activity, several studies have been performed in order to identify potential agonists with antiviral activity.

The second messengers 2'3' cGAMP (Fig. 22) has been studied as STING natural ligand in the treatment of viral infection due to its potential antiviral activity. Both in *in vitro* cell-based infection and in mice infected with HSV-2, local and systemic delivery of 2'3' cGAMP induced a strong IFN-I and ISG production. Of note, 2'3' cGAMP showed a stronger antiviral effect with respect to TLR agonists [310]–[312]. However, despite their strong IFN induction, CDNs do not result suitable for drug therapy because of their poor membrane permeability and metabolic instability [313], [314].

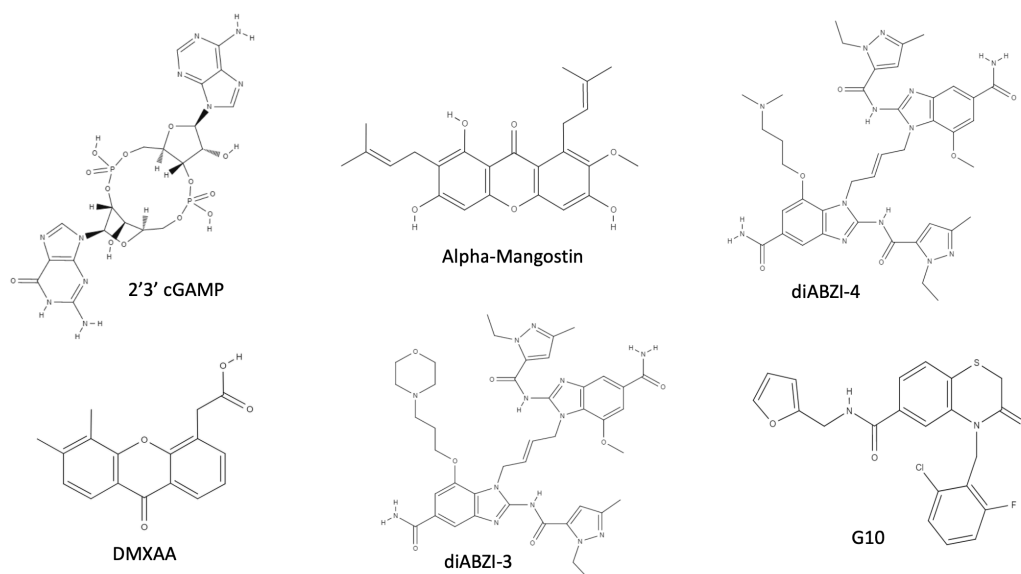


Figure 22. Chemical structure of STING agonists [10].

DMXAA (Fig. 22) is a well-known antitumor agent used to induce the disruption of the tumor vasculature and the release of chemokines by the activation of tumor-associated macrophages [315]. Although, once moved to human clinical trials, it failed in showing any effect against human tumors. The compound was also demonstrated to be a potent antiviral in murine and mouse models but showed no effect in human cells, further studies demonstrated that DMXAA selectively binds murine and mouse STING but not human STING, explaining the inefficacy of the compound in human models [316], but demonstrating that STING agonists may be effective as antiviral agents.

Alpha-Mangostin (Fig. 22) is a xanthone with antimicrobial properties that was reported to induce IFN-I production in a STING dependent manner, binding to the STING CTD. Alpha-Mangostin has been shown to have antiviral properties activity against DENV and HBV replication in cell-based assays, while *in vivo* studies have not been yet reported [317]–[321].

The dimeric amidobenzimidazoles (diABZIs) are STING agonists identified through *in silico* studies that showed the ability to induce IFN- β , α -chemokine CXCL1 and IL-6 transcription and have an anti-tumor activity [322]. Two of them, have been tested for their ability to inhibit viral replication: di-ABZI-3 (Fig. 22) was tested in primary human bronchial epithelial cells infected with parainfluenza virus 3 and rhinovirus subverting viral infection in STING dependent manner [323]. More recently it resulted active in inhibiting the HCoV-OC43 and SARS-CoV-2 replication [324], [325]. The di-ABZI-4 (Fig. 22), that differs from di-ABZI-3 for its more favorable solubility profile, was also tested in vitro in hACE2-A549 cells and in animal model for its ability to inhibit SARS-CoV-2 replication showing good results in vitro, although in vivo studies were limited by the high SARS-CoV-2 morbidity [326].

The result of an high throughput in vitro screen identified the 4-(2-chloro-6-fluorobenzyl)-N-(furan-2-ylmethyl)-3-oxo-3,4-dihydro-2H-benzo[b][1,4]thiazine-6-carboxamide, also called G10 (Fig. 22), that was found to inhibit the replication of the alphaviruses Chikungunya virus (CHIKV), Venezuelan equine encephalitis virus (VEEV), and Sindbis Virus (SINV) in human cells [327]. Due to the proven STING druggability in the development of antivirals, based on the published molecules, we decided to investigate the effect of compounds for their ability to inhibit viral replication through STING-dependent IFN induction.

In the present chapter we show the results obtained by testing potential STING agonists identified through *in-silico* screening on the IFN-I induction, in cells with wt STING or a mutated inactive form of STING that lacks the ability to bind TBK1, hence it is not able to induce IFN-I [47]. Then, DNA damage pathway have been investigated in order to clarify other possible mechanisms of action of the compounds and the compounds were tested for their ability to inhibit CoVs replication in two different cell lines.

3.2 *In-silico* screening and synthesis of STING agonists.

In collaboration with Prof. Simona Distinto, from the University of Cagliari, an *in-silico* screening for the identification of STING agonists was performed.

A ligand-based virtual high throughput screening (vHTS) was carried out utilizing STING agonists as references (queries) to find new potential STING agonists (Fig. 23). Six compounds were considered: 2',3' cGAMP, diABZI-3, diABZI-4, Mangostin, MSA2, G10 (Fig. 23).

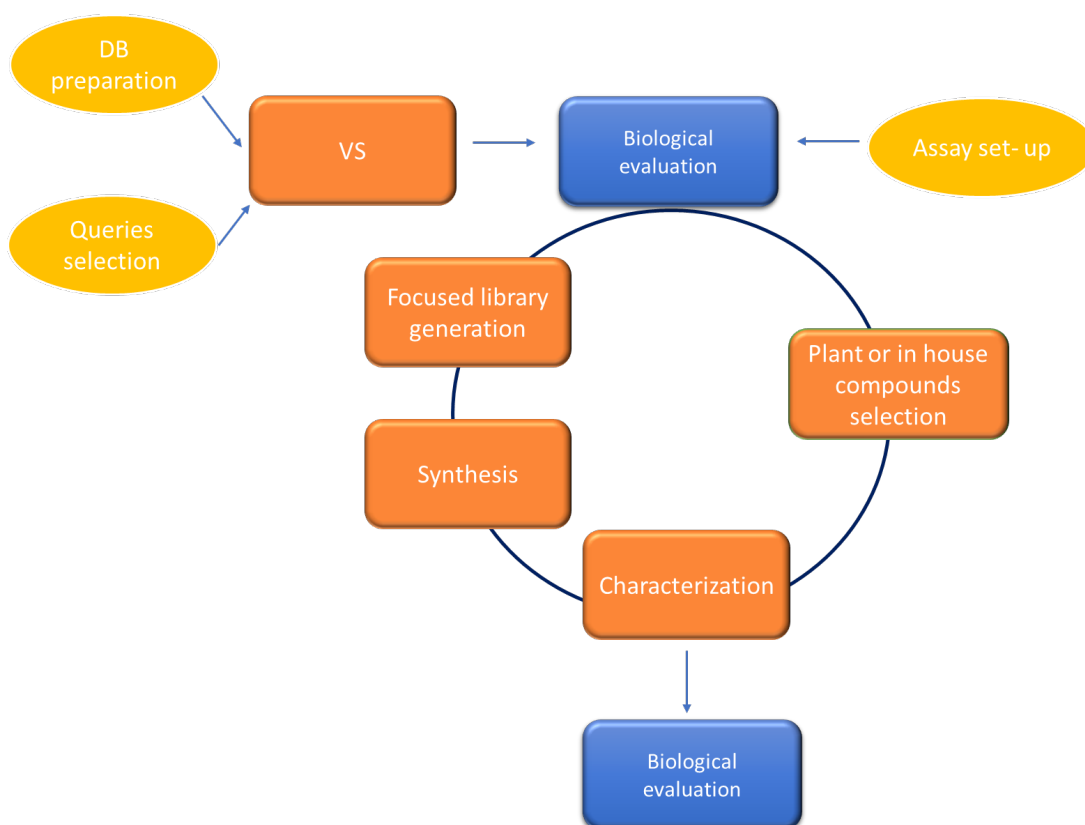


Figure 23. vHTS workflow.

The databases (DB) screened include an "in-house" DB containing synthetic compounds (cp) (3370 cp), a in house and Sardinian natural compounds DB (754 cp) [328], natural compounds and metabolites (UNDP DB, 200400 cp) [329]. In order to perform the screening, the necessary Pipeline Pilot protocols have been created for the DBs generation, preparation, while other protocols were set up for similarity fingerprints screening, employing ECFP4, and subsequent merge and analysis of the results.

Twenty-four promising and diverse molecules were chosen. Among them, eleven benzofuran derivatives (BZFs), provided by Prof. Giovanna Delogu from the University of Cagliari, have been chosen from the synthetic DB and subjected to biological assay (Fig. 24).

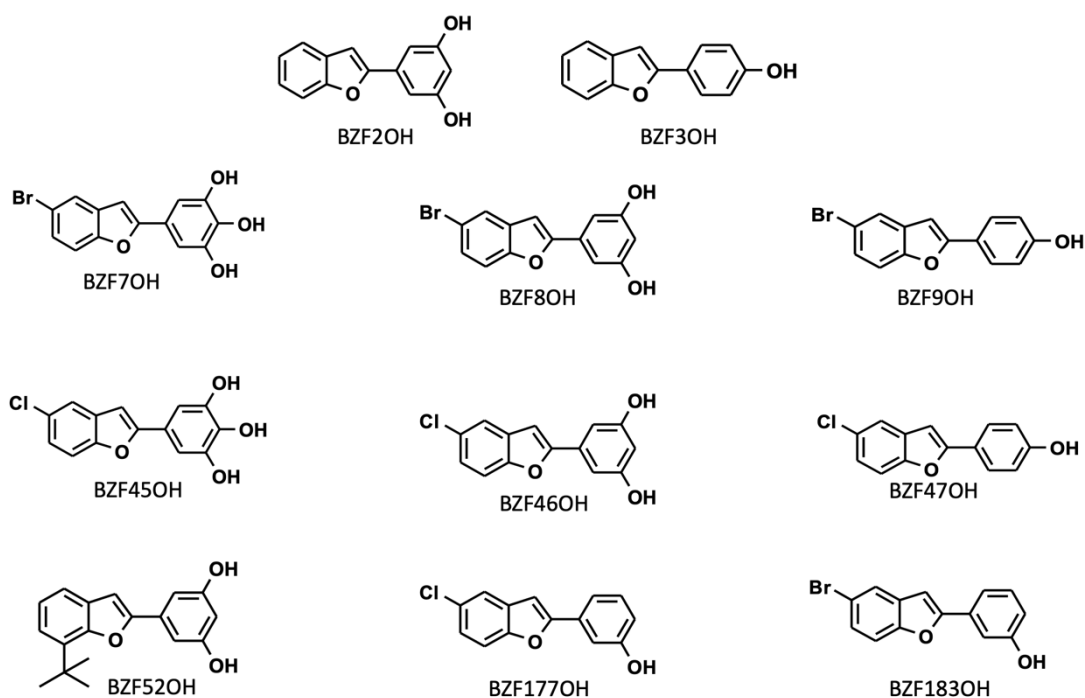


Figure 24. BZFs structures.

It is worth to note, that benzofuran is a common moiety found in many biologically active natural and therapeutic products, and thus represent a very important pharmacophore. They are present in many medicinally important compounds that show biological activity, including anticancer and antiviral properties [330]. Some benzofuran derivatives are also known as 5-lipoxygenase inhibitors, antagonists of the angiotensin II receptor, blood coagulation factor Xa inhibitors, ligands of adenosine A1 receptor [330], [331] and more recently as MAO inhibitors [332]–[335] and selective inhibitory against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme [336]–[338]. Many benzofuran derivatives, of natural or synthetic origin, have been approved clinically, and some of which are fused with other heterocyclic moieties [339]. These numerous properties could represent a considerable added value to the molecules identified in the VS.

3.3 Establishment of gene reporter assay

In the first place, the BZFs have been tested in biological assays for the evaluation of IFN-I induction. It must be considered that STING is expressed in most immortalized cell lines and hence their use for testing STING agonists could be correctly performed, while in transformed cell lines often the cGAS-STING pathway, as well as the DNA damage response, could be somehow altered [340]–[342]. For the aim of this study, it was important to evaluate the IFN- β induction in a system defective for STING in order to confirm the selectivity of the compounds and the IFN- β induction is evaluated in presence of exogenous wild type (wt) STING and in presence of a mutated STING. For this purpose, it was selected a cell line defective for STING, the Human Embryonic Kidney 293T (HEK293T). As reported in literature, the parental cell line HEK293 was stably transfected with the Simian Virus (SV40) Large Antigen T to obtain a cell line highly responsive to DNA transfection; during the HEK293T clone characterization it was determined the absence of STING [343]. In order to evaluate

the ability of BZFs to induce IFN-I as STING agonists, a gene reporter assay was used. Briefly, HEK293T were transfected with a luciferase under the control of IFN- β promoter, the plasmid encoding for wt STING or an empty vector (EV) or a mutated inactive form of STING (STING P371Q) and a plasmid encoding for renilla luciferase under the control of a constitutive promoter that is used to normalize the luminescence signals. The cells were then stimulated with the indicated compounds and, 24 hours later, the luminescence was read. As positive control for induction, a small molecule named MSA-2 reported as STING agonist with antitumor activity was used [344]. In fact, although the STING natural ligand is the 2'3'cGAMP, this was considered not suitable for such assay since the CDN's delivery requires the use of transfection reagents or digitonin solution due to its poor membrane permeability [345], while MSA-2 and BZFs did not require permeability solutions for cell delivery. Hence, MSA-2 possesses chemical characteristics more similar to the BZFs to be tested and it was selected as reference molecule for the IFN-I induction.

Since it is reported that STING overexpression is sufficient to induce IFN- β production [21], in order to determine the ideal plasmid concentration to be transfected to obtain a good IFN- β induction with the lowest background, we transfected the cells with 50 ,10 and 1 ng of plasmid expressing wt STING, 50 ng of plasmid encoding for mutated STING, and 50 ng of Empty Vector; the cells were stimulated 24 hours after transfection with MSA-2 10 μ M.

Results indicate that the STING protein levels when 50 ng of plasmid expressing wt STING are transfected, are high enough to induce IFN- β production even in absence of stimulation and the MSA-2 stimulation boosts STING effect (Fig. 25)

Hence, we selected 10 ng of pUNO-STING to perform the experiment.

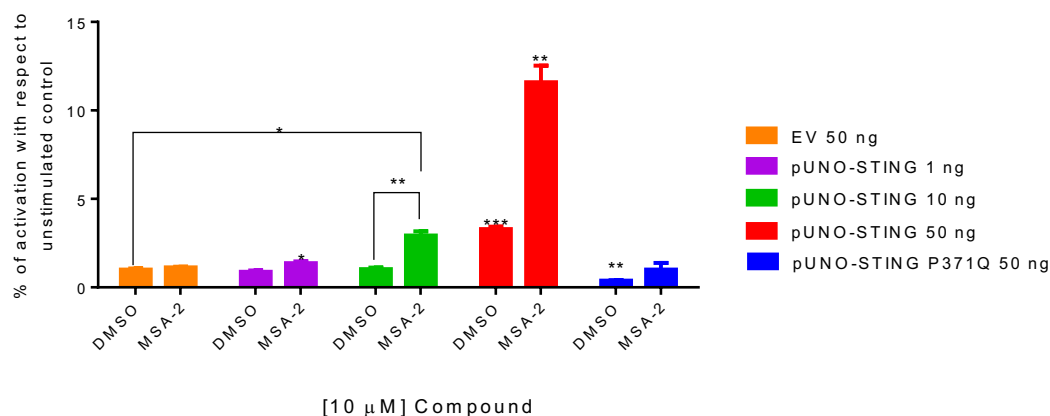


Figure 25. Establishment of pUNO-STING concentration for the IFN-I induction gene-reporter assay. HEK293T transfected with pGL-IFN- β -luc (60 ng/well) and 50, 10 or 1 ng of pUNO-STING or Empty Vector (EV), as well as in presence of 50 ng of pUNO-STING P371Q. 24 hours after transfection cells were stimulated with indicated compounds at 10 mM; on day four the cells were harvested, and luciferase activity was measured. Results are shown as pGL-IFN- β -luc folds of induction over not stimulated control in presence of EV. Error bars mean $\pm$ SEM. Asterisks indicate a significant difference (two-tailed unpaired Student's *t*-test, $n \geq 3$) * $P < 0,05$, ** $P < 0,01$, *** $P < 0,006$.

3.4 Benzofurans induce IFN-I in STING dependent way.

The 11 BZF derivatives were hence tested in the above-described cell-based assay. Results showed that in presence of wt STING BZF are able to induce strong IFN- β transcription (Fig. 26). BZF2OH, BZF3OH, BZF8OH, BZF9OH, BZF46OH were shown to significantly induce the IFN-I gene reporter system (Fig. 24). Similarly, the MSA-2 was able to induce significantly the IFN-I gene reporter activation. In order to confirm that these 5 BFZ derivatives indeed induced the IFN-I gene reporter system acting as STING-agonists, we also tested the activity on a STING protein mutated in the amino acid residue Pro371 substituted with Gln that is not able to properly bind TBK-1 and hence induce INF-I production.

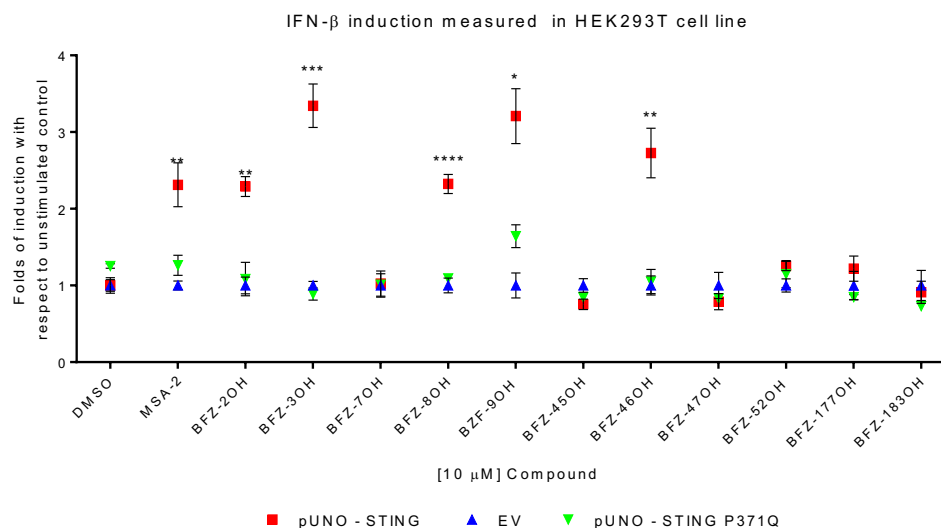


Figure 26. Gene reporter assay in 96-well plates. HEK293T transfected with pGL-IFN- β -luc (40 ng/well) and pUNO-STING or Empty Vector (EV) or pUNO-STING P371Q (10 ng/well), 24 hours after transfection cells were stimulated with indicated compounds at 10 mM; on day four the cells were harvested, and luciferase activity was measured. Results are shown as pGL-IFN- β -luc folds of induction over not stimulated control in presence of EV. Error bars mean $\pm$ SEM. Asterisks indicate a significant difference (two-tailed unpaired Student's *t*-test, $n \geq 3$) * $P < 0,05$, ** $P < 0,01$, *** $P < 0,006$.

Results showed that the 5 BFZ derivatives did not induce the IFN-I gene reporter system using mutant P371Q STING, confirming that they act as STING agonist.

3.5 BZFs do not induce DNA damage.

One of the possible PAMP that can trigger the cGAS-STING pathway is the DNA released during damage repair, in which the DNA damage response is related to the increase of p53 levels [346], and the DHODH inhibition can lead to the production of IFNs. Hence in order to confirm that BZF compounds acted as STING agonists, we wanted to exclude that they could induce any genotoxic effects by evaluating both p53 induction and DHODH inhibition.

For this aim we selected the HEK293 ISRE reporter cells, stably transfected with a luciferase under the control of ISRE promoter, that are the HEK293T parental cell line, these cells express basal levels of wt STING, hence they do not require plasmid transfection in order to evaluate STING activation.

Two BZFs, BZF46OH and BZF 177OH, were selected on the bases of their higher IFN-I induction, HEK293-ISRE reporter cells were then treated for 24 hours, cell lysates were subjected to western blot analysis to evaluating p53 induction. Actinomycin D was used as positive control of genotoxic effect (Fig. 27).

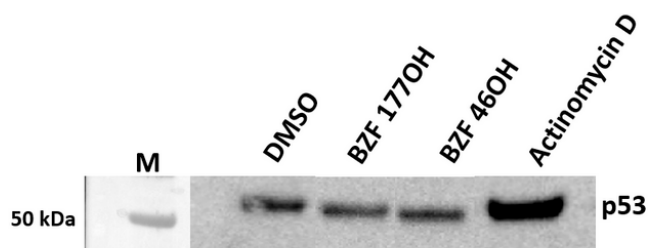


Figure 27. p53 expression. HEK293 ISRE reporter cells were treated with BZFs 10 mM or Actinomycin D 10 nM for 24 hours; then 20 μ g of cell lysates were subjected to western blot. The experiment was repeated three times independently with similar results.

Results showed that p53 levels in presence of compounds were comparable to the ones detected in the untreated control, while they were highly increased in presence of Actinomycin D.

Moreover, DHODH is a key enzyme in the production of pyrimidines: DHODH inhibition reduces the pyrimidine availability leading to the instauration of DNA damage response and the ISGs production (Fig. 28). Uridine is a pyrimidine precursor, hence, when DHODH activity is abolished the treatment with uridine, restores the pyrimidine production pathway leading to the DNA damage response shutdown.

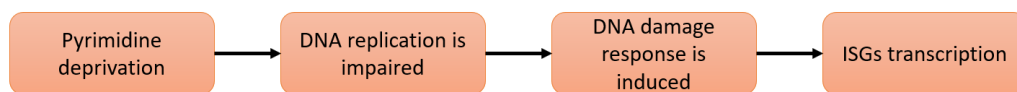


Figure 28. Pyrimidine deprivation induces ISGs transcription.

Hence, in order to exclude the inhibition of DHODH, ISRE expression was measured in HEK293 ISRE reporter cells treated with two BZF compounds selected on the bases of their ability to induce IFN-I (BZF 90H and BZF 177OH) and one BZF derivative lacking this ability (BZF 52OH). The cells were treated with IFN- α in order to induce ISRE promoter transcription, then compounds were added in presence and absence of uridine.

Results showed that the compounds were able to boost the effect of IFN- α , due to their effect on IFN-I production, and levels of ISRE induction measured in presence and absence of uridine were unaltered indicating that the effect on ISRE promoter was not depending on the inhibition of DHODH activity, but on the IFN-I production that boosted the effect of IFN- α (Fig. 29).

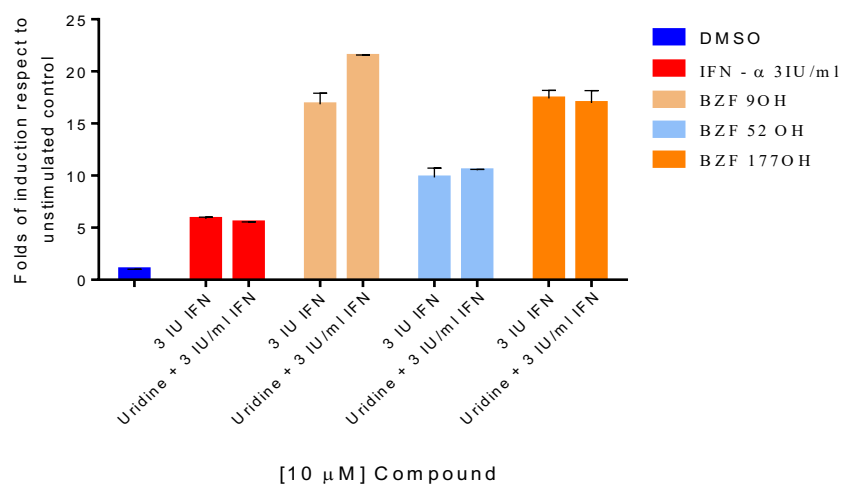


Figure 29. ISRE induction in reporter cell lines. HEK293-ISRE reporter cells were treated 24 hours after seeding with indicated compounds in presence of IFN- α and in presence and absence of Uridine; 24 hours later, cells were harvested, and luminescence was read. Results are shown as folds of ISRE induction calculated respect to untreated control. Error bars mean $\pm$ SEM.

3.6 BZF2OH inhibits HCoV-229E replication.

Once observed the IFN- β induction STING dependent of the BZF derivatives, we wanted to evaluate their potency of inhibition of CoV replication. To this aim, we used the BEAS-2B cell line since they are immortalized but not transformed cells and possess wt STING and can sustain HCoV-229E replication [347]. As described in chapter II, BEAS-2B cells were infected with HCoV-229E for 72 hours, in the presence or in the absence of the compounds, and then cells were treated with MTT for viability measurement. MS2-A and the known CoV protease inhibitor GC376 were used as positive controls.

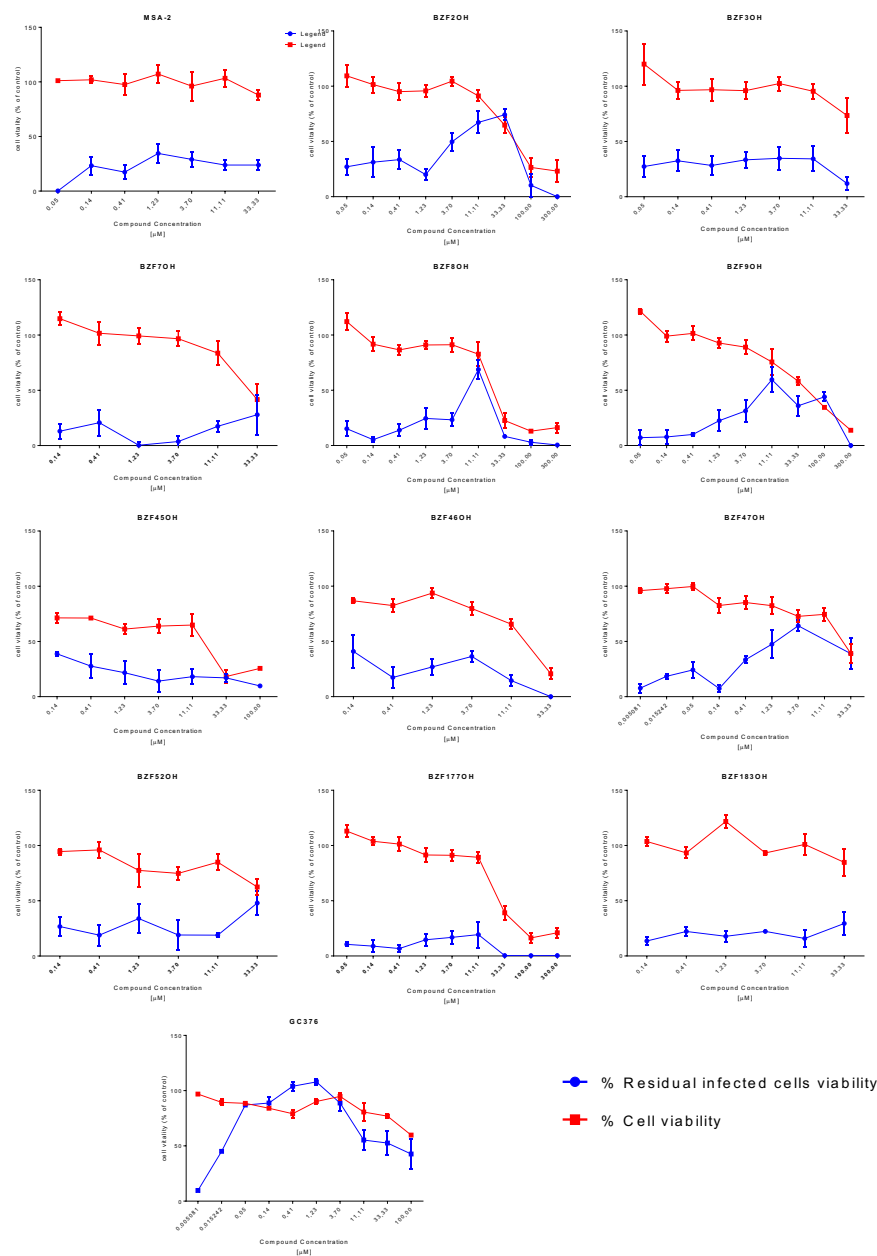


Figure 30. Evaluation of the effect of BZFs on HCoV-229 replication. BEAS-2B cells have been infected with HCoV-229E and incubated at 35°C, in parallel was evaluated the cytotoxic effect of the compounds; 72 hours after infection, cells were treated with MTT and incubated 1h at 37°C. Then cells were harvested, and absorbance was read. Results are shown as CPE (blue) calculated respect to the untreated control and cytotoxic effect (green) calculated with respect to untreated control; values represent the mean $\pm$ SEM of three independent experiments based at least on 6 compounds' concentrations in triplicate.

Results showed that of the 11 tested compounds, and of the 5 compounds able to induce IFN-I production, only one compound, BZF2OH, was able to inhibit HCoV-229E replication (Fig. 30). In the Table 2 are reported calculated CC_{50} and EC_{50} for every compound; for this assay it was used as reference compound the GC376 [307].

Of note, while IFN-I stimulation inhibited viral HCoV-229E replication, MSA-2 chosen as STING the reference compound was not able to inhibit viral replication. Indeed, at the best of our knowledge no report has been published showing an MSA-2 antiviral effect.

Among the 5 compounds that were observed to induce IFN-I production in HET293T cells, only BZF-2OH was able to inhibit HCoV-229E replication in BEAS-2B cells. Compounds BFZ-8OH, BFZ-9OH and BFZ-46OH showed a BEAS-2B cytotoxicity that could be the reason of their lack of antiviral effect. Differently, it is possibly dependent on the cell line specificity the lack of antiviral effect of MSA2 and BFZ-3OH.

Table 3. Activity of MSA-2 and BZF derivatives as HcoV-229E inhibitors

Compound Name	^a Anti-229E Beas2B EC ₅₀ (μM)	^b BEAS2B CC ₅₀ (μM)	^c SI
MSA2	>30	>30	-
BFZ-2OH	14,7 ± 3,0	70,1 ± 7,0	4,8
BFZ-3OH	>30	>30	-
BFZ-7OH	>17,3	17,3 ± 4,4	-
BFZ-8OH	>8,7	8,7 ± 5,5	-
BZF-9OH	>30	30,6 ± 10,2	-
BFZ-45OH	>8,2	8,2 ± 1,4	-
BFZ-46OH	>13,4	13,4 ± 0,7	-
BFZ-47OH	>18,9	18,9 ± 3	-
BFZ-52OH	>30	>30	-
BFZ-177OH	>25,22	25,22 ± 10,7	-
BFZ-183OH	>30	>30	-
GC376	0.12 ± 0.03	>100	>828

Table 3. ^aEC₅₀ = compounds concentration able to rescue 50% of the 229E cytopathic effect in BEAS2B cells, as compared to the untreated control, as measured with colorimetric formazan based MTT assay; values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate. ^bCC₅₀ = compounds concentration able to reduce BEAS2B cells viability by 50%, as measured with colorimetric formazan based MTT assay. Values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate. ^cSI = selectivity index calculated as the ratio between CC₅₀ and EC₅₀ values.

3.7 BZF2OH inhibits SARS-CoV-2 viral replication in BEAS2B cells but not in Vero E6.

Aiming to investigate the broad-spectrum antiviral potency of the BZF2OH, we tested the effect in BEAS-2B cells on the replication of another CoV, this time a Betacoronavirus, SARS-CoV-2. The total RNA of BEAS-2B cells infected with

SARS-CoV 2 and treated with BZF2OH was subjected to qPCR and results were reported as percentage of inhibition of cytopathic effect.

Results showed that BZF2OH was able to inhibit SARS-CoV-2 replication in BEAS-2B cells with an $EC_{50} = 23,55 \mu M \pm 2,26$ (Fig. 31).

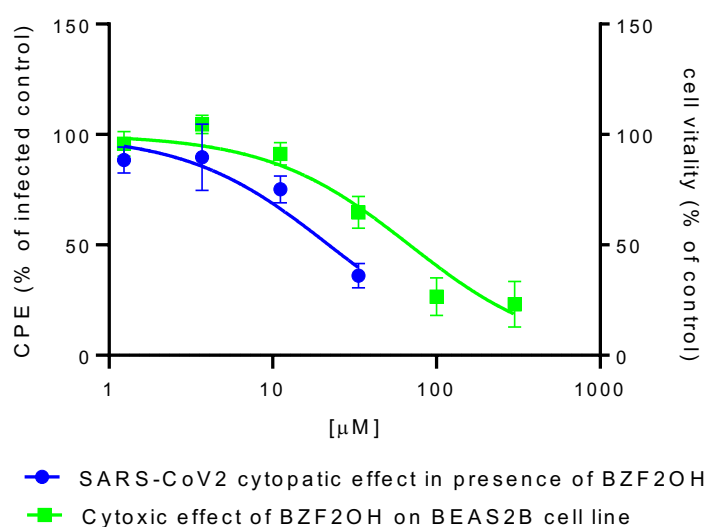


Figure 31. Evaluation of the effect of BZF2OH on SARS-CoV-2 replication in BEAS-2B cells. BEAS-2B cells have been infected with SARS-CoV-2 and incubated at 37°C, in parallel was evaluated the cytotoxic effect of the compound; 48 hours after infection, total RNA was extracted, and RT-qPCR was performed. Results are shown as CPE (blue) calculated respect to the untreated control; values represent the mean $\pm$ SEM of three independent experiments based at least on 4 compounds concentrations in duplicate. The cytotoxic effect (green) was evaluated with MTT based assay and calculated with respect to untreated control; values represent the mean $\pm$ SEM of three independent experiments based at least on 6 compounds concentrations in triplicate.

In order to confirm that BZF2OH inhibitory effect on CoVs replication was dependent on IFN-I induction, we decided to test the inhibitory effect on BZF2OH on SARS-CoV-2 using a different cell line, Vero E6. Vero E6 cells are commonly used as model for SARS-CoV-2 infection, moreover these cells lack the ability to produce IFN-I

[348]. As expected, BZF2OH could not inhibit viral replication, moreover it resulted highly toxic in the cell line ($CC_{50} = 9,56 \mu\text{M} \pm 2,27$) (Fig. 32).

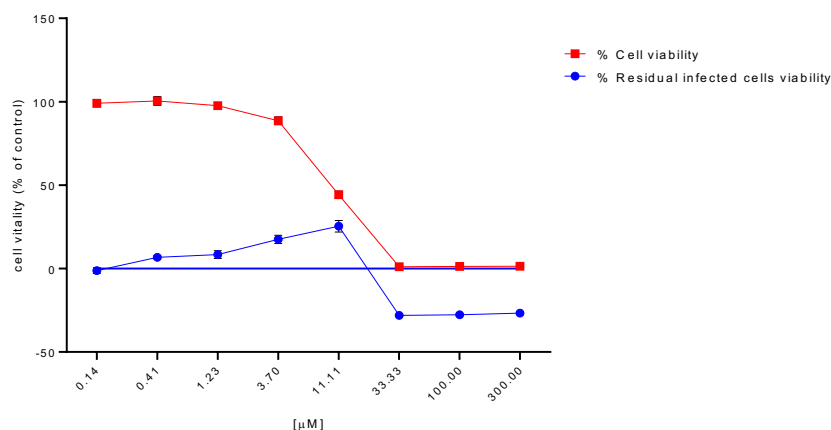


Figure 32. Evaluation of the effect of BZF2OH on SARS-CoV-2 replication in Vero E6 GFP cells. Vero E6 GFP reporter cells were infected with SARS-CoV-2 at m.o.i: 0,01 in presence of indicated compound. in parallel was evaluated the cytotoxic effect of the compounds; 72h after infection, fluorescence was read. Results are shown as residual infected cell viability (blue) calculated respect to the untreated control and cytotoxic effect (red) calculated with respect to untreated control; values represent the mean $\pm$ SEM of two independent experiments based at least on 6 compounds concentrations in triplicate.

3.8 Discussion

The continuous viral evolution and the insurgence of novel viral strains represent a major issue worldwide; hence, innovative medicines are required in order to counteract and control viral spread.

During the last decades, a new protein named STING, involved in the innate immune response, was discovered, and characterized [32], [37], [46]. STING plays a central role in the innate immune response, mediating the IFN production in response to cytosolic DNA (viral, tumor derived and mitochondrial DNA) and acting as a linker among the different responses to RNA viruses and bacteria [28], [36], [50], [154].

In the search of novel antivirals, one strategy is the development of molecules with broad spectrum antiviral activity targeting cellular proteins, hence we wanted to exploit the cellular protein STING as target for the development of immune modulators with broad spectrum antiviral activity.

To date, there are no STING agonists in clinical trial, in fact many obstacles have been encountered in the development of STING agonists as antivirals. Among them, their deliverability, in fact CDNs, the firsts to be studied for their potential antiviral effect, showed poor membrane permeability and, even when efficiently delivered in the cells, their half-life was short due to the presence of endonucleases, indicating that it was necessary to improve the compatibility to the cellular membrane as well as the permanence in the cells without inducing side effects as uncontrolled STING induction or cytotoxicity.

To this aim, we firstly developed an IFN- β gene-reporter assay in order to evaluate the potency of compounds to induce IFN-I production in presence and absence of the active wt STING as well as in presence of a mutated inactive form of STING lacking the ability to interact with TBK1. The resulting gene reporter assay was then used to test 11 molecules identified through in silico screening and 5 of them resulted able to

significantly induce IFN- β production in presence of wt STING. Of note, the IFN- β induction in presence of the mutated STING was comparable to the one obtained in presence of the empty vector confirming compound selectivity for the wt STING.

To further characterize the mechanism of action and confirm STING selectivity, we explored two pathways involved in DNA damage response, p53 induction and DHODH inhibition, that are reported to induce the ISGs production and to induce an antiviral response [68], [346], such experiments allowed us to exclude these two mechanisms reinforcing the observation of IFN-I induction by STING agonists.

We next moved to the antiviral assay testing the compounds against two HCoVs, HCoV-229E and SARS-CoV-2, in such assays one compound, the BZF2OH resulted able to inhibit both viruses with an EC₅₀ in the μ M range. As confirmation of the viral inhibition dependent on the activation of innate immune response, we tested the compound against SARS-CoV-2 in Vero E6 cells that lack the ability to produce IFN-I, having no effect on viral replication and confirming the hypothesis that the BZF2OH was inhibiting HCoVs replication inducing IFN-I production.

The reason behind the lack of activity of some compounds in the antiviral assay performed in BEAS-2B cells, as compared to the IFN- β induction performed in HEK293T cells, could reside in a selective cytotoxicity of BZFs on the BEAS-2B cells with respect to the HEK293T cells that might have reduced compounds efficacy. However, questions about the role of hydroxyl and halogen groups on the selectivity towards STING are still answered. This selective BEAS-2B cytotoxicity also raised a question that will need further investigation using different cell lines in order to select the less cytotoxic BZFs.

Overall, present results indicate that the identified BZFs are suitable for the development of antivirals with a broad-spectrum activity exploiting STING as cellular selective target. Improvements are required in order to increase the binding specificity. Since it was demonstrated that dimeric structures bind more stably STING

as reported for MSA-2 and diABZI, one possible path to be followed is the synthesis of dimeric BZFs starting from the ones here described. However, dimer synthesis is complicated by the presence of hydroxyl substituents. We are currently optimizing the synthetic procedure of several dimers and will soon be able to test them to confirm their activity. We can conclude that vHTS produced relevant results and enabled the discovery of a new scaffold for the development of STING agonists.

Chapter IV

As described in the introduction, drugs that inhibit SARS-CoV-2 are highly demanded, and PL-pro is a validate drug target. Hence, we decided to focus on the identification of potential PL-pro inhibitors that counteract the virus by inhibiting the PL-pro activity in viral replication and by restoring the innate immune response (see chapter I).

4.1 Identification of PL-pro selective inhibitors.

The UNICA molecular virology research team has been involved in an EU H2020 project entitled Excalate4CoV aimed to develop high throughput screening (HTS) assays for the identification of novel antivirals, to select the best hit compounds and to characterize their mode of action. As part of the Excalate4Cov project, a study was performed in order to identify molecules that could inhibit PL-pro activity. Within the HTS campaign against SARS-CoV-2, a library of around 6000 molecules that could potentially inhibit PL-pro was tested (Fig.33). The primary screening was performed with a biochemical assay evaluating the protease activity of PL-pro using an ISG15 labeled with a fluorophore measuring the effect of compounds at one concentration. Such screening identified 54 compounds that inhibit over 50% PL-pro activity, 49 of them were further confirmed and subjected to dose response curve. This second round of assays reduced the selection to 23 compounds with an IC_{50} value $< 5 \mu M$. The compounds were then tested in cell-based assay for SARS-CoV-2 inhibition using Calu-3 cells, and 4 of them resulted active in SARS-CoV-2 antiviral assay (Table 4) (Kurzikov et al., in preparation). Next, we wanted to verify whether these compounds could have any effects on the innate immune response restoring IFN-I production.

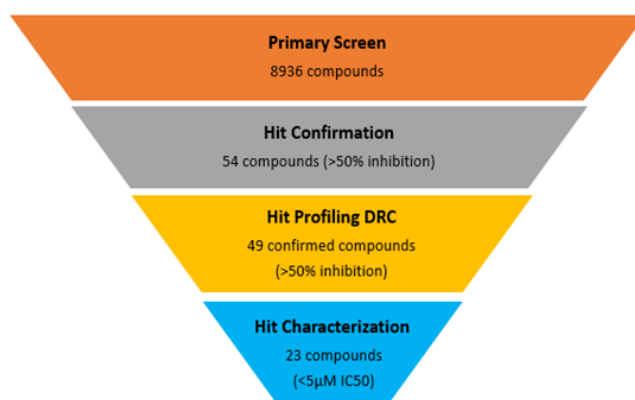


Figure 33. Screening process block diagram.

Table 4. Activity of compounds on PL-pro enzymatic activity and SARS-CoV-2 replication

Compound	^a IC ₅₀ (µM)	^b Anti-SARS-CoV-2 Calu 3 EC ₅₀ (µM)	^c Calu 3 CC ₅₀ (µM)	^d Anti-SARS-CoV-2 Vero E6 EC ₅₀ (µM)	^e Vero E6 CC ₅₀ (µM)
Walricin B	0,04 ± 0,01	4,56 ± 0,87	11,61 ± 1,24	>10	>10
NSC-663284	0,22 ± 0,06	7,42 ± 1,85	11,47 ± 2,31	>10	>10
α-Lapachone	4,43 ± 0,80	nd	nd	>10	>10
BVT-948	0,50 ± 0,11	10,50 ± 2,71	74,8 ± 7,4	nd	nd
Propidium-Iodide	0,30 ± 0,07	nd	nd	nd	nd
Ryuvidine	0,26 ± 0,03	6,78 ± 0,75	28,27 ± 1,65	nd	nd

^aIC₅₀ = compounds concentration able to rescue 50% of the PL-pro enzymatic activity, as measured with fluorescence -AMC based assay; values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate. ^bEC₅₀ = compounds concentration able to rescue 50% of the SARS-CoV-2 replication in Calu3 cells, as compared to uninfected control, as measured with qPCR values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate. ^cCC₅₀ = compounds concentration able to reduce Calu3 cells viability by 50%, as measured with colorimetric formazan based MTT assay. Values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate. ^dEC₅₀ = compounds concentration able to rescue 50% of the SARS-CoV-2 replication in VeroE6-GFP cells, as compared to uninfected control, values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate. ^eCC₅₀ = compounds concentration able to reduce VeroE6-GFP cells viability by 50%, values represent the mean ± SDs of three independent experiments based at least on 6 compounds concentrations in triplicate.

4.2 Establishment of assay conditions in IFN- β reporter assay.

Firstly, we established a cell-based assay to measure the SARS-CoV-2 PL-pro inhibition IFN- β production using a gene reporter assay methodology. Briefly, HEK293T cells were transfected with a plasmid encoding for a luciferase under the control of IFN- β promoter, a plasmid encoding for SARS-CoV-2 PL-pro or an empty vector and a plasmid encoding for renilla luciferase under the control of a constitutive promoter that is used to normalize the luminescence signals. The cells transfected were then stimulated with vRNA for 24 hours and treated with compounds. The RFU obtained were normalized in order to calculate the induction in presence an absence of PL-pro.

Prior to perform the screening, a dose response curve of the effect of the pcDNA3.1-PL-pro (coding the PL-pro) plasmid concentration in HEK293T transfected with pGL-IFN- β -luc was performed, the resulting RFU were normalized to empty pcDNA3.1 (Fig. 34). From the dose-response curve, the concentration of 30 ng of plasmid per well (blue square), giving around 50% of inhibition of IFN- β transcription, was selected for further testing.

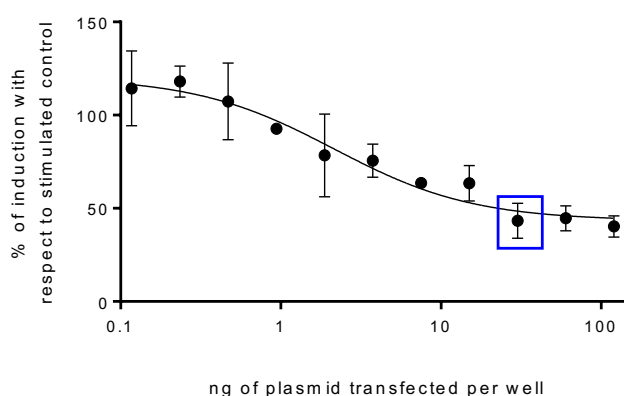


Figure 34. PL-pro plasmid concentration curve. HEK293T transfected with pGL-IFN- β -luc (60 ng/well) and indicated concentration of pcDNA3.1 PL-pro, 24 hours after transfection cells were stimulated with RNA; on day four the cells were harvested, and luciferase activity

was measured. The blue square indicates the plasmid concentration chosen for the screening. Results are shown as pGL-IFN- β -luc percentage of induction over not stimulated control in presence of EV. Error bars mean $\pm$ SEM of three independent experiments.

4.2 Compounds revert PL-pro inhibition of IFN- β transcription.

Among the 12 of derivatives that were able to inhibit SARS-CoV-2 replication in cell-based assays, 8 were available for further studying. Hence, we decided to test them at the concentration of 1 μ M due to the IC₅₀ obtained in the biochemical assay, to evaluate their ability to subvert SARS-CoV-2 PL-pro inhibition of the INF- β production in the gene-reporter assay previously described (Fig. 35). Out of the eight tested compounds, three of them (α -Lapachone, Walrycin B and NSC-663284) were able to restore the IFN- β transcription inhibited by PL-pro indicating that the compounds are able to inhibit also the deubiquitinating activity of PL-pro.

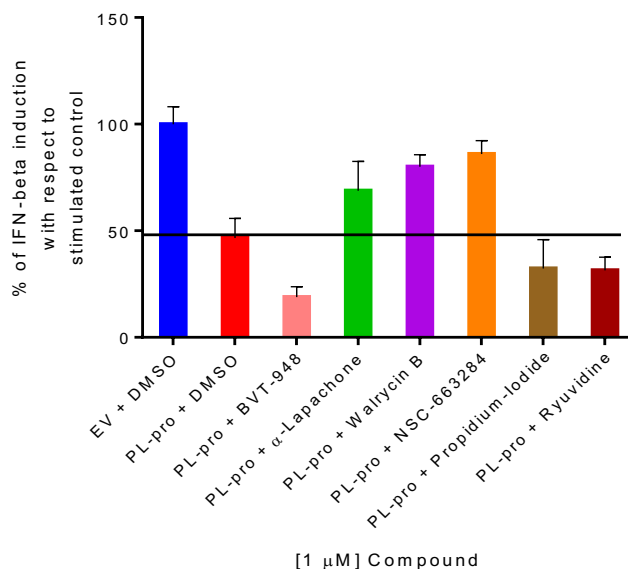


Figure 35. Effect of compounds on PL-pro inhibition of IFN- β promoter transcription. HEK293T were transfected with pGL-IFN- β -luc (60 ng/well) and Empty Vector (EV) or

pcDNA3.1 PL-pro (30 ng/well), 24 hours after transfection cells were stimulated with RNA and treated with 1 μ M of indicated compounds; on day four the cells were harvested, and luciferase activity was measured. Results are shown as pGL-IFN- β -luc percentages of induction over not stimulated control in presence of EV. Error bars mean $\pm$ SEM of three independent experiments.

4.3 Dose response curve.

Next, we wanted to determine the EC₅₀ value for PL-pro inhibition of the three active compounds in the above-described assay, by performing a dose-response inhibition curve (Fig. 36). Results showed that Walrycin B and NSC-663284 were able to inhibit in a dose response manner PL-pro activity, and it was possible to calculate the IC₅₀ that are respectively 0,14 μ M $\pm$ 1,3 and 0,4 μ M $\pm$ 1,26; although it was not possible to determine an IC₅₀ value for α -Lapachone.

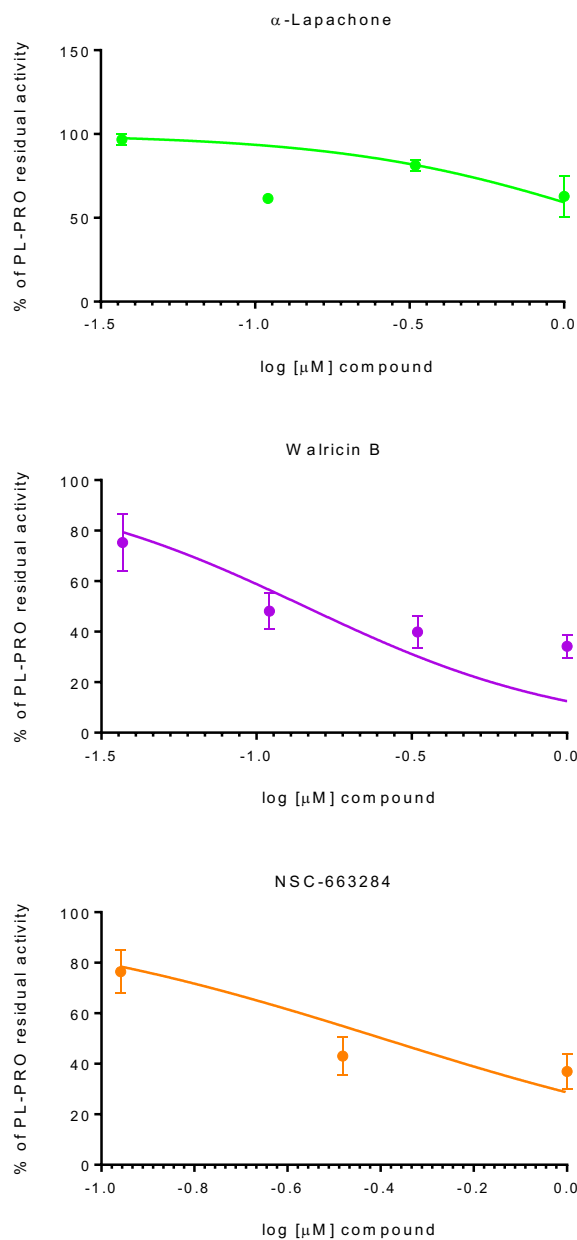


Figure 36. Dose response curve of inhibitory compounds on PL-pro activity. HEK293T were transfected with pGL-IFN- β -luc (60 ng/well), pRL-TK (10 ng/well) and Empty Vector (EV) or pcDNA3.1 PL-pro (30 ng/well), 24 hours after transfection cells were stimulated with RNA and treated with decreasing concentrations of indicated compounds; on day four the cells were harvested, and luciferase activity was measured. Results are shown as percentage of pGL-IFN- β -luc expression respect to PL-pro control. Error bars mean $\pm$ SEM.

4.4 Discussion

The SARS-CoV-2 PL-pro is involved in poly-protein maturation and represents the viral weapon against the cellular innate immune response implied in silencing the antiviral defense mounted by the host.

This double role, one on viral replication and the other on innate immune evasion, made of it a valuable target to be evaluated in order to develop selective SARS-CoV-2 antivirals.

As part of the Excalate4CoV European project, we were involved in the identification and characterization of SARS-CoV-2 inhibitors using multiple targets, and one of them was the viral protein PL-pro. Through virtual screening around 9000 compounds that were tested in biochemical assays and 23 compounds were identified to have an IC₅₀ value on PL-pro lower than 5 μ M.

The 23 compounds were tested in Calu-3 cells to evaluate their inhibitory effect on SARS-CoV-2 replication. Results confirmed that 4 compounds, Walrycin B, NSC-663284, BVT-948 and Ryuvidine were able to inhibit viral replication with an EC₅₀ value in the μ M range (Kurzikov et al, in preparation).

Then, aiming to demonstrate that the viral replication inhibition was due to PL-pro inhibition leading to the restoration of the innate immune response, Walrycin B, NSC-663284, and α -Lapachone were tested in the Vero-E6 GFP cells, widely used for the evaluation of SARS-CoV-2 replication and defective for IFN-I production system. Results indicate that the compounds were not able to inhibit viral replication in this cell line, corroborating the above suggested theory [306].

Hence, the four compounds active in the Calu-3 cells were tested in a gene reporter assay to evaluate the effect on the PL-pro inhibition of IFN-I production. In addition, two more compounds, Propidium Iodide and α -Lapachone, were tested in the gene

reporter assay to broaden the molecular structure spectrum of possible PL-pro inhibitors, moreover α -Lapachone resulted active in the biochemical assay but inactive on SARS-CoV-2 replication in the Vero-E6 GFP. The cells transfected with the IFN- β reporter plasmid and the PL-pro plasmid, were treated with the previously described compounds. Results indicate that three of them, Walrycin B, NSC-663284 and α -Lapachone were able to restore the IFN- β production, indicating the inhibitory effect on PL-pro. It was also possible to determine an IC₅₀ value for two of them, Walrycin B and NSC-663284, but not for α -Lapachone.

Walrycin B and NSC-663284 were identified in the previously described virtual screening as repropounded drugs. In fact, NSC-663284 also known as DA3003-1, is a quinone reported as antitumor agent targeting Cdc25 family of phosphatases [349]; meanwhile Walrycin B is reported as antibacterial agent active on *Staphylococcus aureus* methicillin resistant strains [350], moreover through high throughput screening Walrycin B was already found to be active in inhibiting SARS-CoV-2 M-pro in biochemical assay and active in vitro with an EC₅₀ value in the μ M range [351].

The challenges behind the development of SARS-CoV-2 PL-pro inhibitors reside in the identification of compounds that selectively bind PL-pro, without targeting the cellular DUBs or generally target cysteine proteases reducing the off-target effect [352]. Moreover, it is still unclear whether PL-pro inhibition is sufficient to inhibit viral replication, in fact no PL-pro inhibitor was found to be able to inhibit SARS-CoV-2 replication in animal models [352].

Our results indicate that targeting PL-pro is a suitable strategy in order to develop selective SARS-CoV-2 antivirals, moreover the restoration of IFN- β production is sufficient to inhibit viral replication in vitro. Further tests on PL-pro knock-out viral strains could be of help in confirming this mechanism.

Chapter V

The TCM uses Chinese herbal products to prevent and eventually treat illnesses. Not much is known about the mechanism of action of these products, however their efficacy and safety were proven during thousands of years of administration. In the last decades, however, the focus was also put on their molecular composition, their characteristics and biological target/s.

Considering the interest in the characterization of the unknown active substances that constitute these products, a selection of them including pure molecule, water extract and methanol extract was provided by Prof. Liang Lin from the Taipei Medical University (Taiwan).

These substances were first tested in two screening assays previously developed by the Molecular Virology team to investigate the ability of compounds to revert the effect of EBOV viral proteins VP35 and VP24 on the innate immune response. These two viral proteins, in fact, block the IFN-I induction (VP35) and the IFN-I signalling (VP24).

Among the tested compounds, one resulted able to inhibit the innate immune modulation of two EBOV proteins. Moreover, it also induced a strong IFN-I production as well as strong IFN-signaling. The encouraging results brought us to start investigating their mechanism of action evaluating the ISRE induction in the HUH7 reporter cells, the induction of ISGs through quantitative PCR.

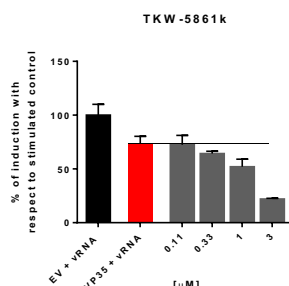
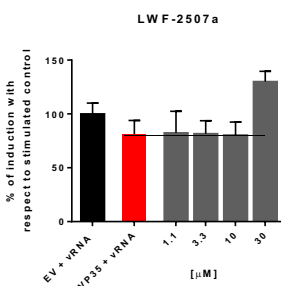
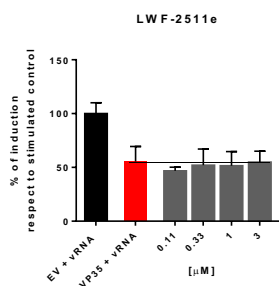
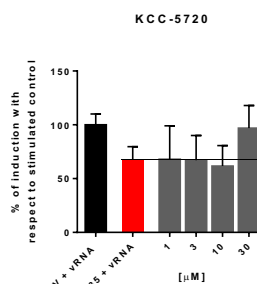
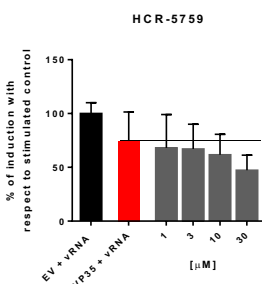
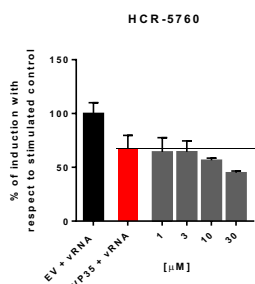
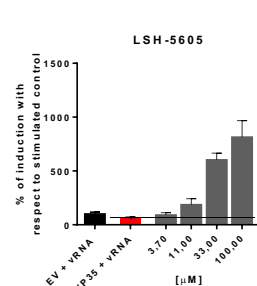
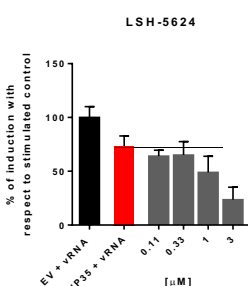
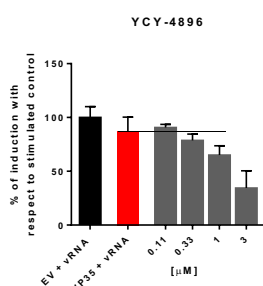
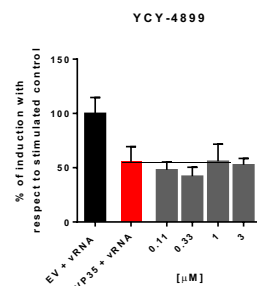
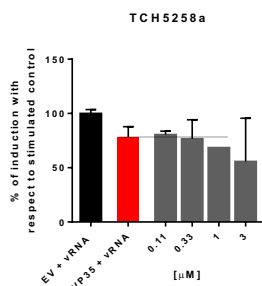
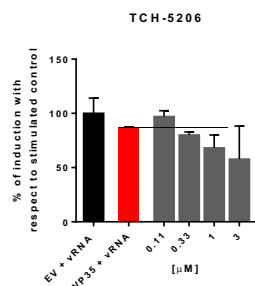
Results

5.1 Evaluation of TCM compounds on VP35 and VP24 inhibitory effect on the innate immune response.

The selection of 22 compounds including 3 water extract and 3 methanol extracts and 16 pure compounds, were tested in two gene reporter assays evaluating the modulation of innate immune response by VP24 and VP35.

The first gene reporter assay was used to investigate the IFN-I production inhibited by EBOV VP35. Cells were transfected with a plasmid encoding for a luciferase under the control of IFN- β promoter, a plasmid encoding for the VP35 or an empty vector, and a plasmid encoding for renilla luciferase under the control of a constitutively expressed promoter used to normalize the signal. The transfected cells were then stimulated with vRNA in presence and absence of compounds.

Results indicate that among the tested compounds, some were active in subverting VP35 effect on IFN-I production (Fig. 37). TCH-5206, KCC-5720, LWF-2507a, TKW-5864n, WE11 and ME02 were able to restore IFN- β production in presence of VP24. The most interesting compound, however, was the LSH-5605, in fact, as shown in the graph, it was able to completely restore IFN- β production and to potentiate the signal.



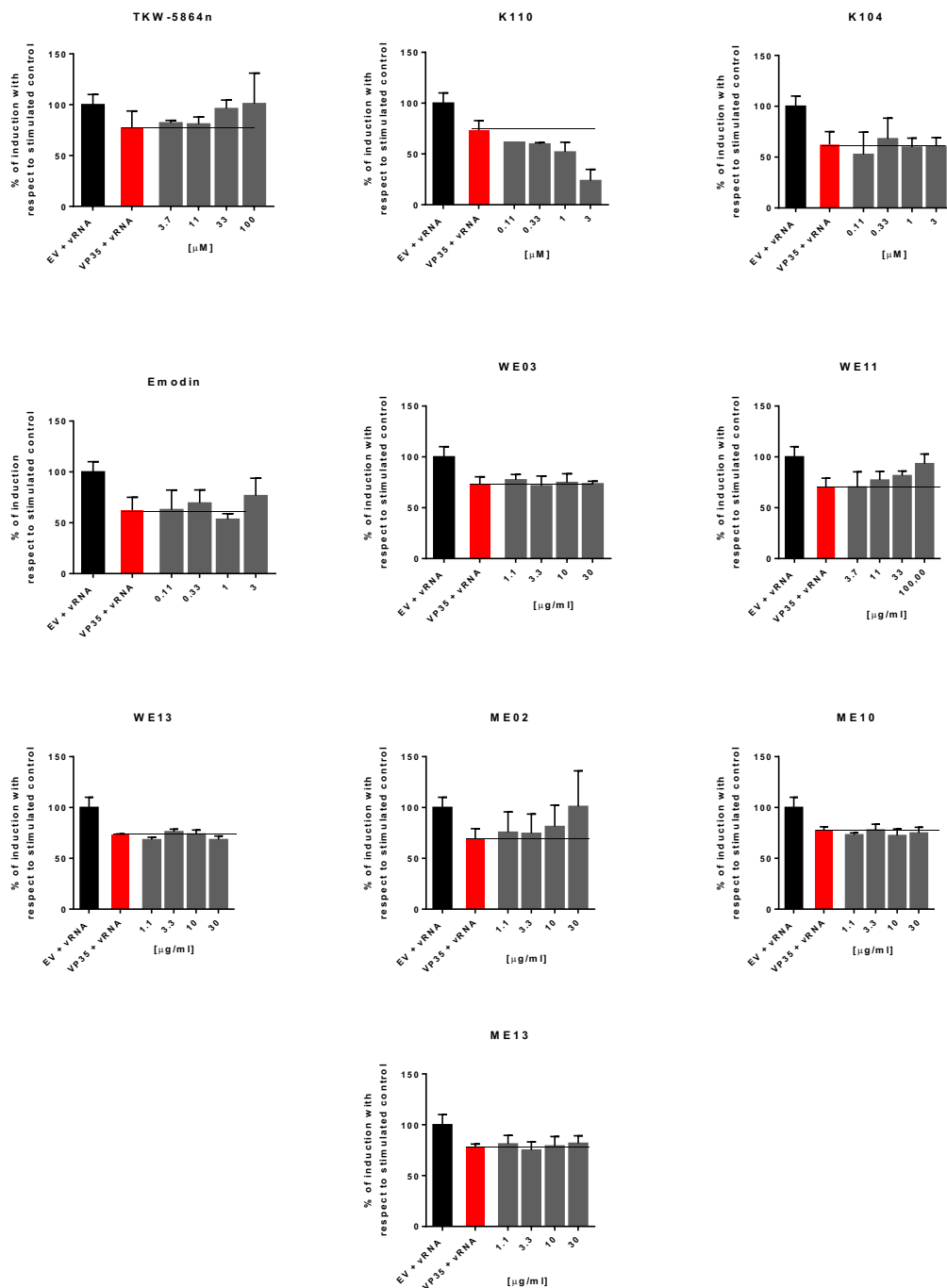
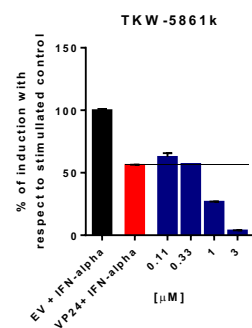
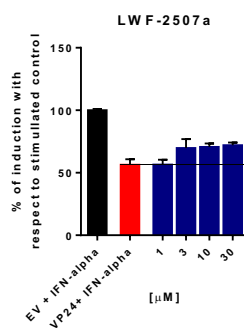
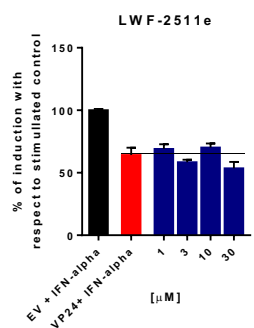
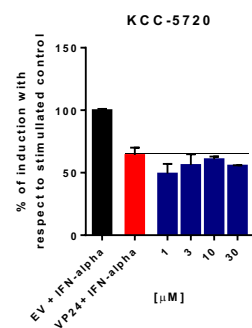
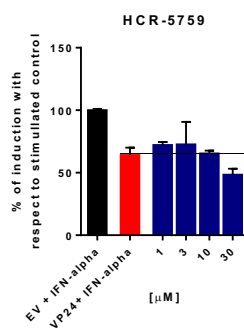
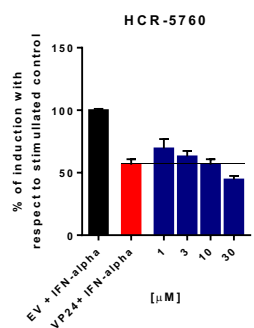
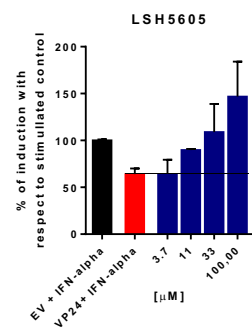
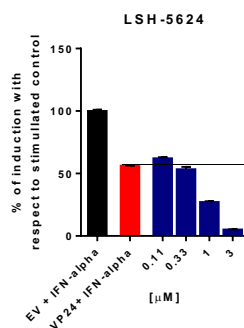
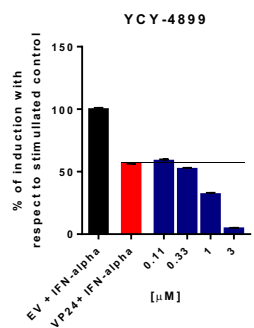
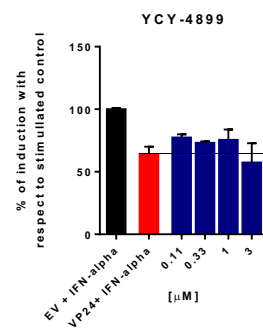
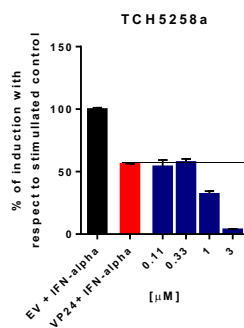
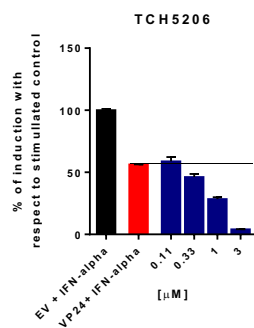


Figure 37. Effect of TCM compounds on EBOV VP35 inhibition of IFN-I production. VP35 inhibits IFN- β production by inhibiting RIG-I activity, LSH5605 reverted the effect of VP35 promoting IFN- β production.

In parallel it was performed a gene reporter assay used to investigate the effect of the EBOV VP24 on the IFN-I cascade using a luciferase under the control of ISRE promoter and VP24. The trasfected cells were then stimulated with copounds or IFN- α in order to see the effect on the IFN-I signalling inhibition mediated by VP24. Results show that the only one copound that was able to revert VP24 effect on ISRE production was the LSH5605 (Fig. 38).



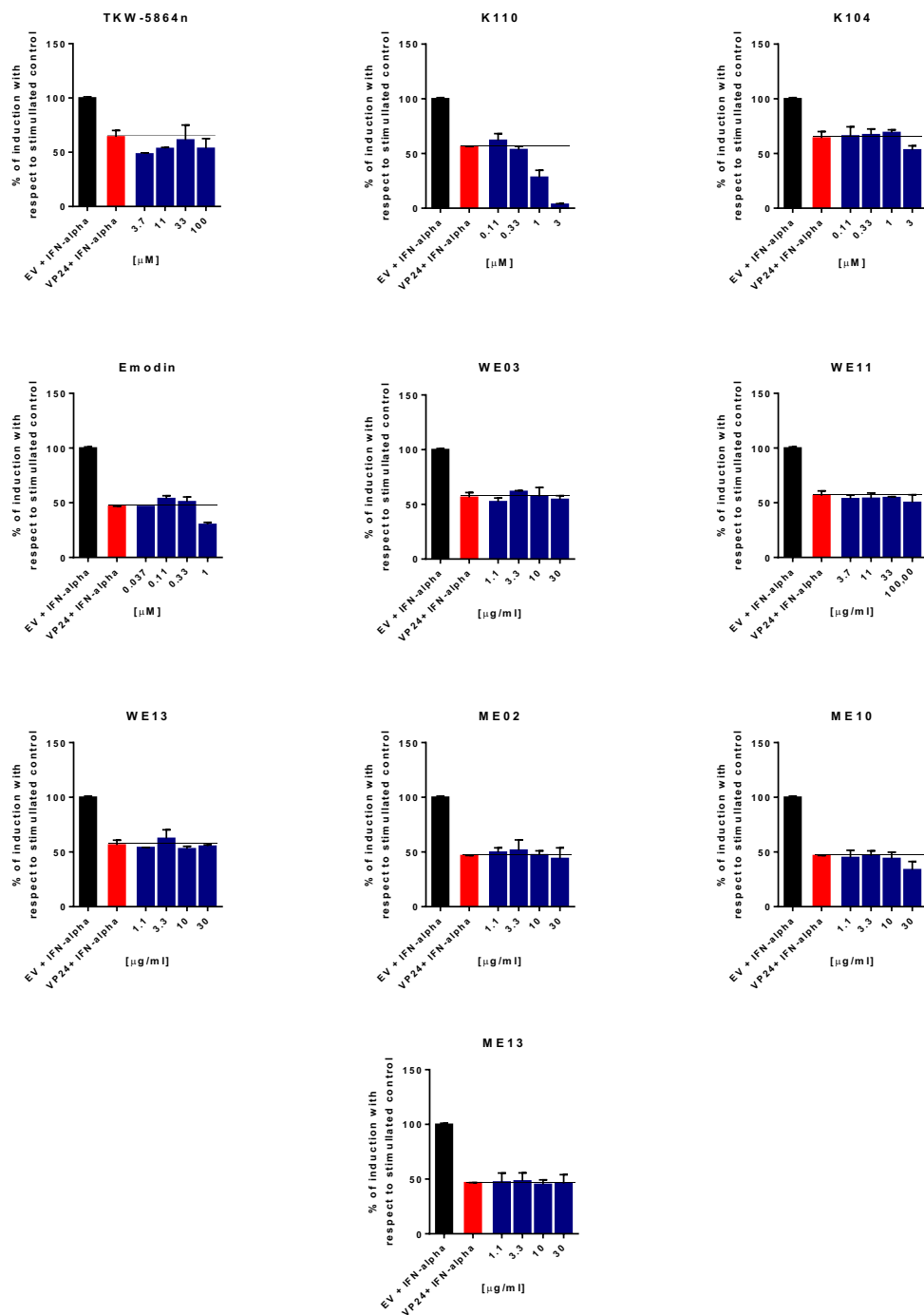


Figure 38. Effect of TCM compounds on EBOV VP24 inhibition of IFN-I signaling. A. VP35 inhibits IFN- β . V24 blocks STAT1/STAT2 translocation in the nucleus inhibiting ISRE transcription, LSH5605 reverted its effect promoting ISRE transcription.

5.2 Evaluation of LSH5605 innate immune response.

Since the compounds were identified as immunomodulators, the best hit compound, LSH5605, was tested in the previously described gene reporter assay in absence of viral proteins.

Results indicates that the compound was able to induce INF-I production and signalling in a dose response manner, boosting the effect of both RNA and IFN- α (Fig. 39).

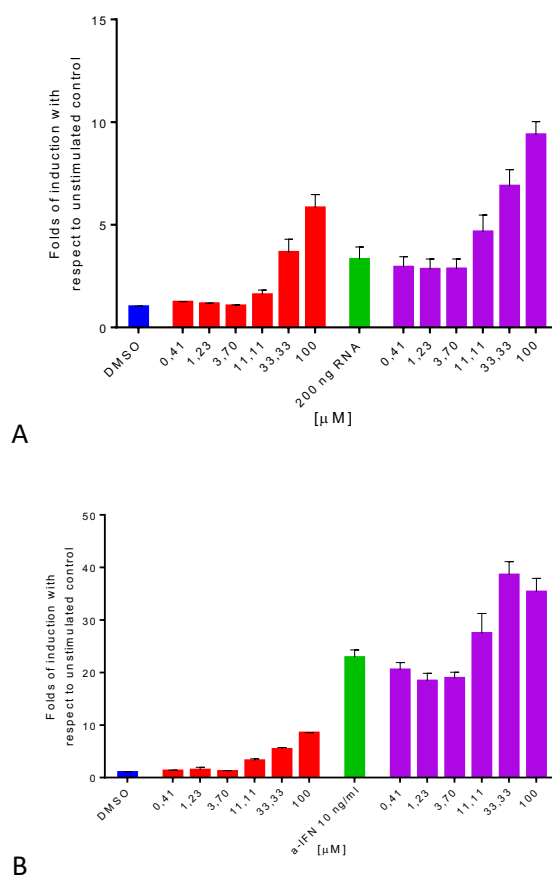


Figure 39. Dose response curve of LSH5605. A. HEK293T transfected with a luciferase under the control of IFN- β promoter were stimulated with compound at indicated concentration in absence (red) and presence (violet) of vRNA. B. HEK293T transfected with a luciferase under the control of ISRE promoter were stimulated with indicated concentration of compound in

absence (red) and presence (violet) of 10 ng/ml of IFN- α . Results are shown as folds of ISRE induction calculated respect to untreated control. Error bars mean $\pm$ SEM.

To further explore the effect on the IFN-I signalling LSH5605 was tested in two reporter systems. The HEK293 ISRE reporter cells are HEK293T parental cell line, while the HUH7 ISRE reporter cells are tumor cells stably transfected with a luciferase under the control of ISRE promoter.

The ISRE induction was evaluated in both cases in presence and absence of IFN- α . Results confirm the agonist effect of the compound on the IFN-I signalling previously described, indicating that the effect is not cell line selective (Fig. 40).

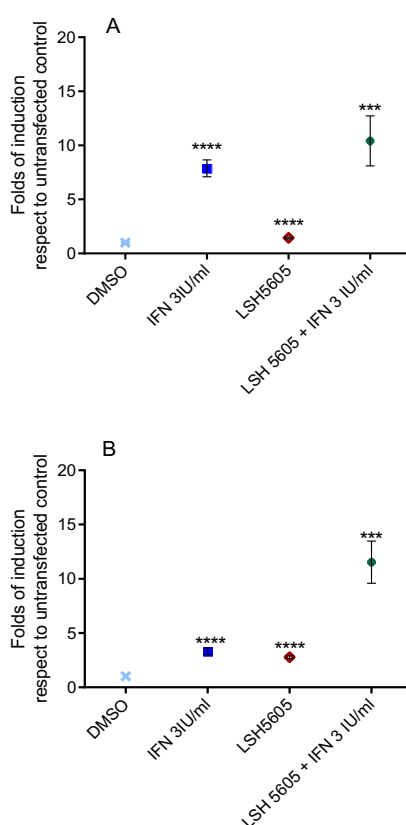


Figure 40. ISRE induction in reporter cell lines. (A) HEK293-ISRE reporter cells and (B) HUH7-ISRE reporter cells were treated 24 hours after seeding with LSH5605 at 30 μ M in

presence and absence of IFN- α ; 24 hours later, cells were harvested, and luminescence was read. Results are shown as folds of ISRE induction calculated respect to untreated control. Error bars mean $\pm$ SEM. Asterisks indicate a significant difference (two-tailed unpaired Student's t-test, $n \geq 3$) * $P < 0,05$, ** $P < 0,01$, **** $P < 0,0001$.

5.3 ISGs modulation evaluated in HUH7-ISRE reporter cell line.

Considering the ISRE induction in the reporter cell lines, it was evaluated the modulation of selected ISGs in presence of compound with and without IFN- α : IRF-1, IRF-9, MxA, ISG15, RIG-I, IL-6.

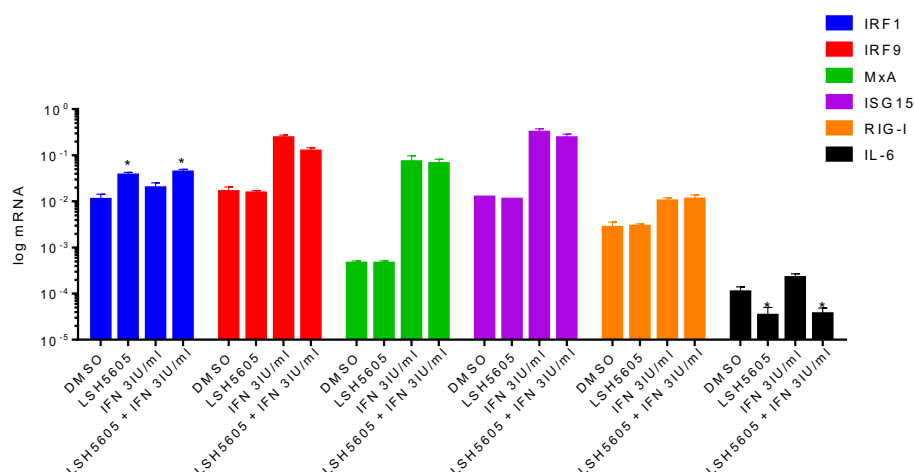
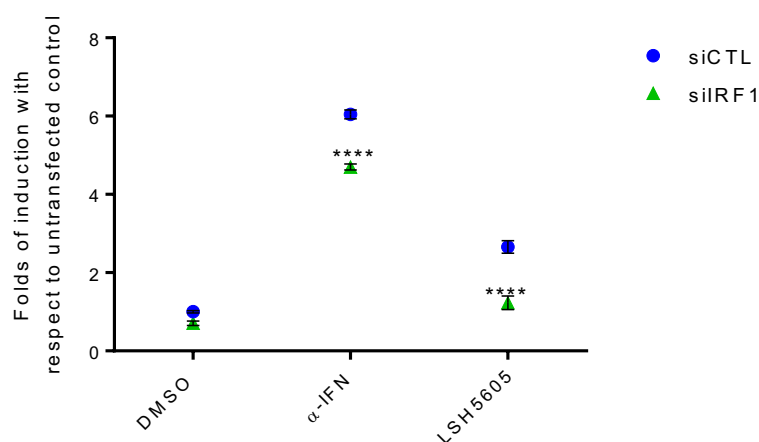


Figure 41. ISGs modulation in HUH7-ISRE reporter cell line. The total mRNA was retrotranscribed and cDNA was used to perform qPCR, results were obtained through normalization vs the housekeeping gene S9. Error bars mean $\pm$ SEM Asterisks indicate a significant difference (two-tailed unpaired Student's t-test, $n \geq 2$) * $P < 0,05$.

Interestingly, IRF9, MxA, ISG15 and RIG-I expression was unchanged (Fig. 41). IRF-1 was significantly modulated by the compound in presence and absence of IFN- α increasing its expression, as well as IL-6, although in this case the IL-6, cytokine involved in inflammation, was downregulated.

5.4 siIRF1 reduces ISRE induction in HUH7 ISRE- reporter cell line.

The up-regulation of IRF-1 was further analyzed through silencing RNA. HUH7-ISRE reporter cell were transfected with siIRF1 and treated with compound in presence of compound.

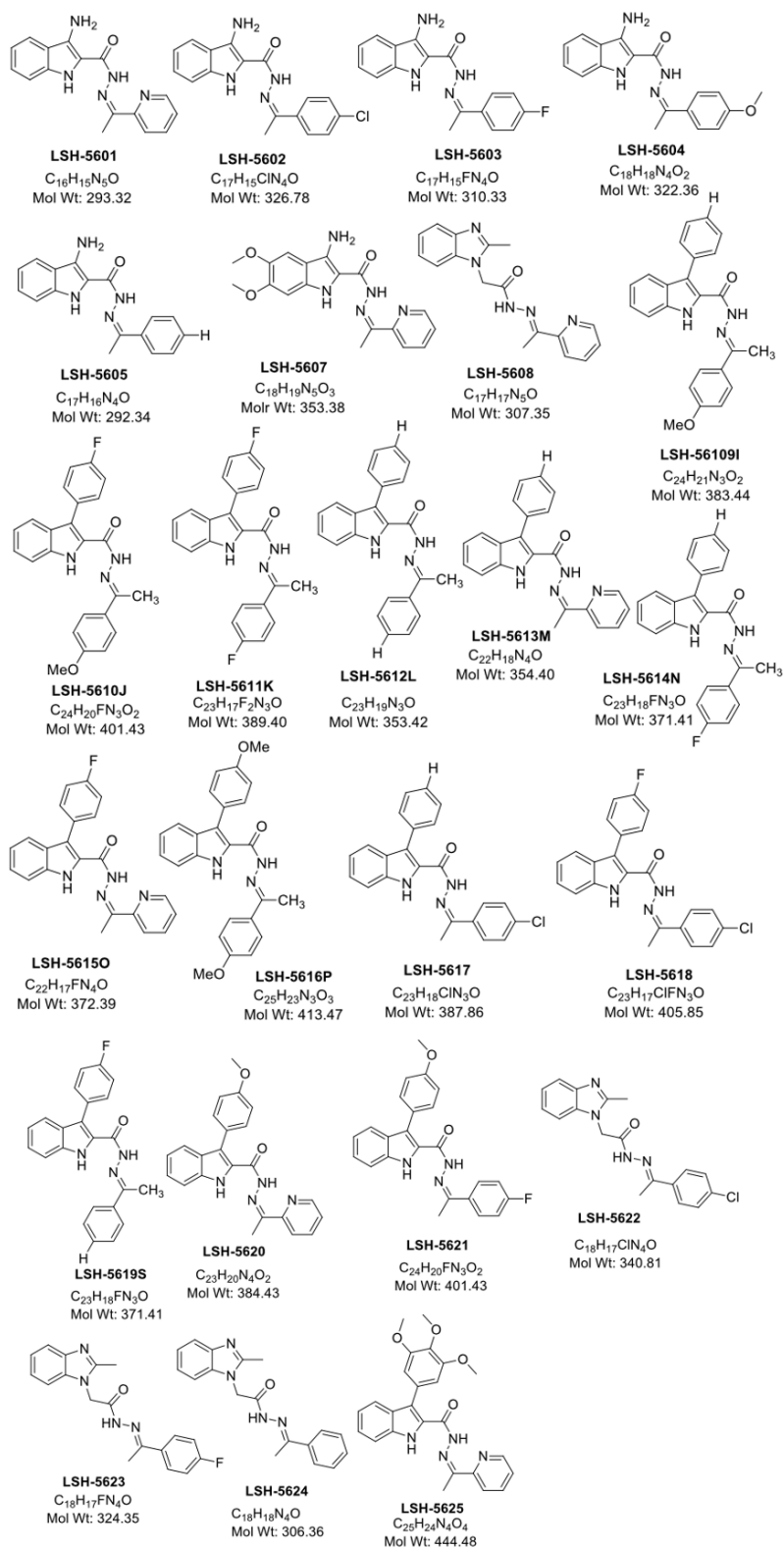


*Figure 42. IRF1 silencing in HUH7-ISRE reporter cell line. HUH7-ISRE reporter cells were transfected with indicated siRNA and treated 24h after transfection with indicated compound at 30 μ M or IFN- α 3 IU/ml; 24 hours later, cells were harvested, and luminescence was read. Results are shown as folds of ISRE induction calculated respect to untreated control. Error bars mean $\pm$ SEM Asterisks indicate a significant difference (two-tailed unpaired Student's *t*-test, *n*=3) *****P*<0,0001.*

As expected, results show that the ISRE induction was significantly impaired by IRF1 silencing indicating that IRF1 plays a major role in LSH5605 mechanism of action (Fig. 42).

5.5 Development of LSH56- derivatives

The LSH5605, was used as scaffold for the development of LSH56- derivatives. 35 derivatives were synthesized and analyzed in the HEK293-ISRE reporter cells (Fig. 43).



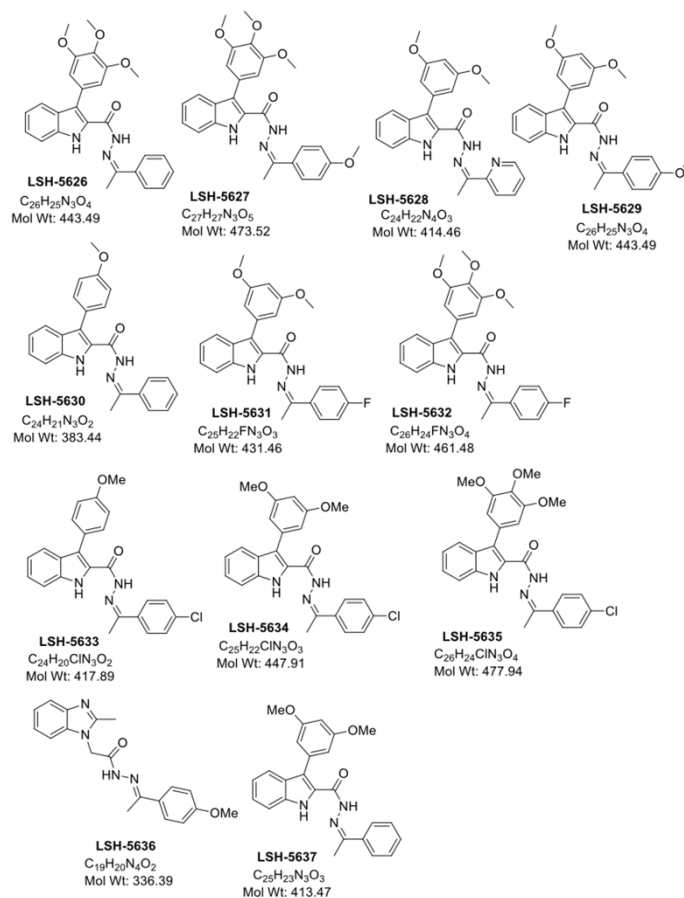


Figure 43. LSH56- series compounds' structures.

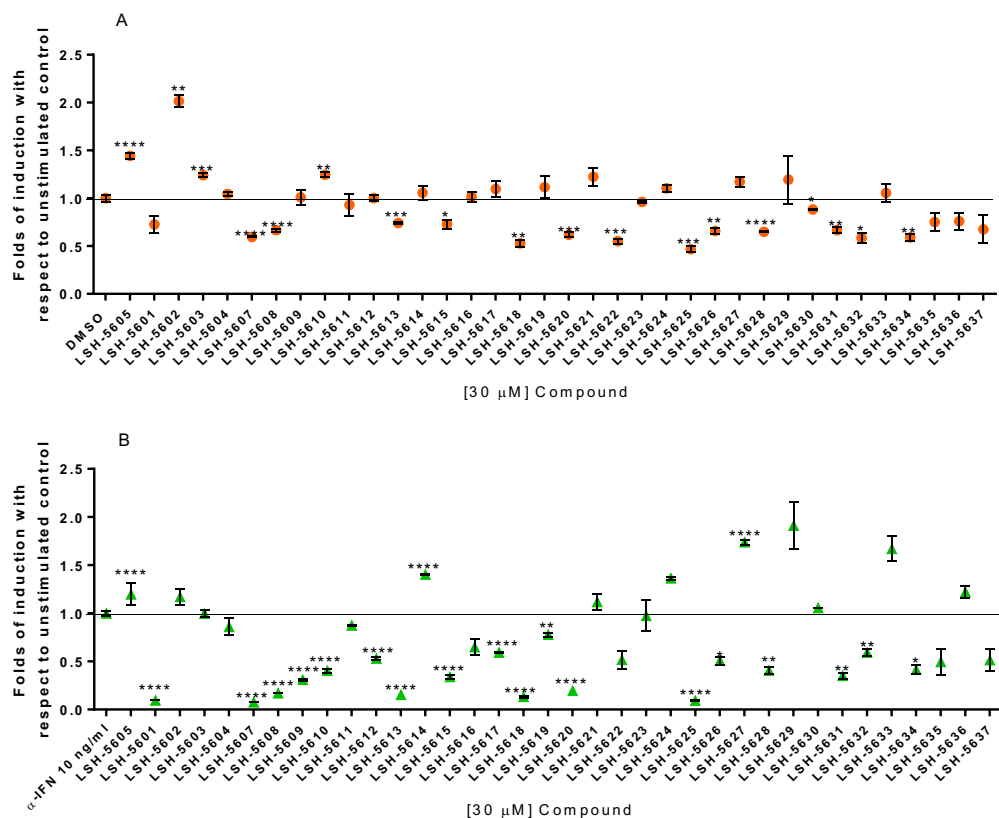


Figure 44. ISRE induction in reporter cell lines. HEK293-ISRE reporter cells were treated 24h after seeding with indicated 30 μ M compound in absence (A) and presence (B) of 1 ng/ml of IFN- α ; 24 hours later, cells were harvested, and luminescence was read. Results are shown as folds of ISRE induction calculated respect to untreated control. Error bars mean $\pm$ SEM Asterisks indicate a significant difference (two-tailed unpaired Student's *t*-test, $n \geq 3$) * $P < 0,05$, ** $P < 0,01$, *** $P < 0,007$, **** $P < 0,0001$.

Results indicate that the compounds were significantly modulating the ISRE expression in absence of IFN- α in a negative way (Fig. 44A), only the LSH5602 was significantly inducing ISRE. In presence of IFN- α the majority of the compounds negatively modulated the ISRE expression, although LSH5614, LSH5624, LSH5627, LSH5629 e LSH5633 were able to boost the effect of IFN- α (Fig. 44B).

5.6 Discussion

The TCM products have demonstrated their efficacy during the past centuries, being administered to treat a wide variety of pathologies.

Our work has investigated the effect of a list of substances derived from TCM products through gene reporter assays for the study of EBOV VP35 and VP24 on the innate immune response. These proteins represent the major effectors for EBOV in counteracting cellular defenses, in fact it was demonstrated that direct inhibition of VP24 and VP35 resulted in inhibition of viral replication [304].

Among these substances tested in the gene reporter assays for the inhibition of VP35 and VP24 only one has shown ability to revert viral proteins' effect of innate immunity in a dose response manner and showing induction of both IFN-I production and signaling.

The compound LSH5605 was selected to further investigate its mechanism, to this aim, it was evaluated its activity in the IFN-I production and signaling in absence of viral proteins. In both cases the compound showed a strong inducing effect on both cascades suggesting that the inhibitory effect on the VP24 and VP35 activity was not due to direct binding could be due at least in part to innate immune response strengthening.

Through testing the compound on the HUH7-ISRE reporter cell line, we also demonstrated that the effect on ISRE production was not cell dependent.

We then moved to the characterization of the compound effect on the ISGs modulation through qPCR. Results showed that the compound was significantly modulating the transcription of IRF1 (up-regulation) and IL-6 (down-regulation).

As described in chapter I, IRF1 is constitutively expressed aiming to maintain the alert state in the cell, it's also considered an ISG due to the production following IFNs

production. IRF1 upregulation reinforce the obtained results indicating that the compound acts upstream IFN-I production.

IL-6 is an inflammatory cytokine having a promoting effect on differentiation or proliferation of several nonimmune cells, stimulation of antibody production and of effector T-cell development [353]; moreover, it was reported to be highly expressed in COVID19 patients suggesting they may contribute to the cytokine storm of severe COVID-19 [354].

Hence, we could hypothesis that the combination of the two effects obtained with LSH5605 treatment, IRF1 upregulation and IL-6 downregulation, could lead to strong IFNs production and reduced inflammation.

In order to understand more deeply the role of IRF1 on LSH5605 ISRE promoter induction, we decided to silence IRF1 in the HUH7-ISRE reporter cell line. As expected, the ISRE induction was significantly reduced ($p < 0,001$) in presence of siIRF1 confirming the important role of IRF1 in LSH5605 activity.

Results obtained in the characterization of LSH5605 brought us to investigate the possibility to further improve the effect of LSH5605 by synthesizing a series of LSH56- derivatives and testing them in the HEK293-ISRE reporter cells as screening model for the evaluation of ISRE induction, comparing the effect of novel compounds with the previously characterized LSH5605.

Results indicate that LSH5605, LSH5614 and LSH5627 were able to significantly boost the effect of IFN- α ; although the majority of the compounds were inhibiting the ISRE induction, or they had no effect.

Since it has been reported that blocking VP24 and VP35 effects on innate immunity led to inhibit EBOV replication, the compound LSH5605 and the most promising derivatives will be tested in vitro on EBOV replication to confirm that the restoration of IFN production and signaling in presence of EBOV can inhibit viral replication.

Chapter VI

Conclusions

Infectious diseases represent a major issue worldwide especially in low-income countries where the medical treatments are inadequate and insufficient to counteract diseases' infection and spread. The limited treatment availability leads to the insurgence of medical emergencies as epidemics and pandemics, having devastating consequences on health and economy worldwide. The recently COVID19 pandemic has highlighted the necessity to have medicines that could counteract viral infection limiting its spread. In fact, the use of a broad-spectrum antiviral could have limited the number of death as well as the economic consequences of the pandemic. SARS-CoV-2 is the clearest example of the reason why, now more than ever, broad-acting agents are required to treat and prevent the insurgence of novel viral strains.

IFNs represent a very strong wake-up call for the human organism, so that the detection of a danger occurring in the cells evokes the activation of innate immune defense mechanisms. It is well known that dysregulation in the IFNs production and its downstream activity can involve the development of autoimmune diseases.

The aim of this PhD thesis was the exploitation of the virus-host interaction in order to develop antivirals. For this purpose, we explored three different strategies willing to i) target the cellular protein STING as pharmaceutical target, ii) target the SARS-CoV-2 PL-pro to develop antivirals, iii) identify natural compounds from TCM with immunomodulatory properties.

In particular, in the first approach we investigated the protein STING due to its role in mounting the innate immune response against both DNA and RNA viruses, representing a potential target for the development of broad-spectrum antivirals.

Hence, pathways leading to the production of IFNs must be tightly regulated [155]. STING is an example of such fine-tuned regulation mechanism. In fact, its activity is modulated by cellular proteins through Ub conjugation and deconjugation in order to control its activation and proteasomal degradation, as well as trafficking between ER and mitochondria where it interacts with several mitochondrial proteins [97], [108], [112], [113], [156]. Since the mitochondria is an important hub for cellular metabolism, the interaction with mitochondrial proteins, influenced by metabolism alterations, suggests that STING regulation is also related to metabolism perturbation [77], [157]–[159].

Viruses are well known for their ability in mutating to adapt to and overcome the cellular defense weapons. Viral proteins target cellular proteins to cleave them entirely, or partially by removing post-translational modification required to modulate protein's activity, as for example through deubiquitination [18]–[20], [110], [113], [160]–[165]. STING is targeted by both DNA and RNA viruses since it is recognized as driver for the instauration of the antiviral response, moreover modulation of cell metabolism alters the ability of STING to interact with upstream and downstream effectors involved in IFNs production pathways.

The request for novel antivirals is continuously increasing, especially due to the rapid onset of novel viral strains leading to the identification of targets among the cellular proteins involved in cellular defense, for the development of broad-spectrum antivirals [21], [166]. STING's central role in innate immunity and the viral mediated inhibition brought to focus attention on this protein as potential cellular target for the development of broad-spectrum antivirals. A few molecules identified by computational approaches have been studied in vitro showing the ability to induce a strong IFN response and, in some cases, to inhibit viral replication in a STING dependent way [167]–[178]. Notwithstanding the promising results obtained in vitro, none of the identified compounds active on the human STING have been tested in animal models.

Our study has investigated the potential agonistic effect of putative STING agonists through cell-based assays. By performing an *in-silico* screening using a ligand-based approach 24 molecules were identified and among them, 11 benzofuran derivatives.

The BZFs were tested for their ability to induce IFN- β promoter using a cell line defective for STING and then transfecting the cells with two different forms of STING, the wt form and a mutated inactive form that lacks the ability to bind TBK1. The results obtained indicated that 5 out of 11 compounds significantly induce the IFN-I gene reporter system, BZF2OH, BZF3OH, BZF8OH, BZF9OH, BZF46OH, while the remaining six compounds were not able to induce IFN- β promoter transcription. The BZFs' selectivity for STING was then confirmed with the luciferase assay performed in presence of the mutated inactive STING; in fact, the IFN- β levels detected in cells expressing the mutated STING and treated with the 5 agonists were comparable to the levels detected in absence of STING.

Considering the involvement of STING in the DNA damage response, we wanted to evaluate the effect of the compounds on this pathway measuring p53 levels and DHODH activity in cells treated with compounds. We were able to exclude that the compounds are inducing DNA damage by increasing p53 or inhibiting DHODH activity.

The 11 BZFs were then tested to evaluate their potential antiviral effect on HCoV-229E replication in BEAS-2B cells, expressing wtSTING. Results showed that the BZF2OH was the only one able to inhibit viral replication while for the other compounds it was detected a cytotoxic effect in BEAS-2B cells. The BZF2OH was then tested for its ability to inhibit SARS-CoV-2 replication in both BEAS-2B and Vero E6, cells defective for IFN-I production. In this case, results indicate that BZF2OH was able to inhibit SARS-CoV-2 replication in BEAS-2B but not Vero E6 confirming that the inhibitory effect involves IFN-I production.

Overall, this study represents a proof of concept of the potential antiviral activity of identified BZFs. More studies are required to further improve the BZFs' activity by developing either dimers or other derivatives, to increase the potency of antiviral effect on viral replication.

The second approach aimed to identify antivirals targeting SARS-CoV-2 PL-pro. As part of the Excalate4CoV European project, we have studied possible SARS-CoV-2 PL-pro inhibitors. The PL-pro plays an important role in viral protein maturation cleaving the nsp3, and it is also an important weapon in the inhibition of innate immune response by cleaving Ub-chains and ISG15 conjugated from target proteins.

During the Excalate4CoV studies, have been identified around 9000 molecules potential PL-pro inhibitors that were screened in diverse consecutive biochemical assays up to the identification of twenty-three molecules with an IC₅₀ lower than 5 μM. The best hit compounds were then tested for their potency in the inhibition of SARS-CoV-2 replication in Calu-3 cells, identifying four compounds able to inhibit viral replication in Calu-3 (Kuzikov et al., in preparation); among them two compounds, Warlycin B, NSC-663284, as well as one more compound identified with the first biochemical screening, α-Lapachone, resulted inactive in Vero E6, suggesting a possible involvement of the IFN-I possibly restored by the inhibition of PL-pro [306]. Hence, Walrycin B, NSC-664382, α-Lapachone, Ryuvidine, Propidium iodide and BVT-948 were chosen among the 23 identified compounds for further testing firstly for their inhibitory effect of PL-pro, secondly for their activity on viral replication when present and finally to have a wide molecular structure spectrum of PL-pro inhibitors.

We then established a gene reporter assay to evaluate the inhibitory effect of PL-pro on IFN-β production, the cells transfected with the luciferase under the control of IFN-β promoter and expressing PL-pro were treated with the previously identified compounds. Results showed that the compounds restored the IFN-β levels, hence we

performed a dose-response curve to determine the IC₅₀, but only for two of them was possible to obtain a dose-response curve.

To date, no PL-pro inhibitor has been developed as SARS-CoV-2 antiviral, in fact, studies are not conclusive about the efficacy of targeting PL-pro in order to inhibit SARS-CoV-2 although the double role of the viral protein in viral maturation and innate immune evasion. The compounds identified through the *in-silico* screening, able to inhibit SARS-CoV-2 replication in Calu3 cells, were not able to inhibit SARS-CoV-2 replication in Vero E6 cells. With our assay we could show that the compounds are possibly inhibiting the DUB activity of PL-pro and hence, restoring the IFN- β production.

For the third approach, we investigated the activity of molecules belonging to TCM. The TCM products have been commonly administered alone as well as in combination with western medicine since centuries treat infectious and chronic diseases. Recently, a strong interest in the understanding of the molecules that compose these substances have started a deeper study on these products' mechanism of action [287], [290], [292].

With this aim in collaboration with the Taipei Medical University (Taiwan), we tested a selection of around 20 substances including pure molecules, water extracts and methanol extracts obtained from herbs used in TCM products. These substances have been studied in two gene reporter assays developed to evaluate the activity of two EBOV proteins, VP35 and VP24, effective on the IFN- β production and ISRE induction, respectively [303], [355]. Both proteins play a major role in inhibiting the innate immune response, hence represent an important target for the development of antivirals.

Among the compounds tested on the two assays, one compound LSH5605 showed a strong effect in both assays, completely reverting the effect of VP35 and VP24 on the innate immune response. Hence, we decided to evaluate the effect of the compound

on IFN-I production and signaling, in absence of viral proteins. Results showed a very strong inducing effect of both cascades suggesting that the induction previously detected in presence of the two viral protein was strong enough to revert their effect.

Hence, to further characterize the mechanism of action, the compound was tested in two reporter cell lines, HEK293 and HUH7, stably transfected with a luciferase under the control of ISRE promoter. In both cell lines, the compound was able to induce the ISRE promoter transcription and boost the effect of IFN- α confirming that the ISRE induction was not cell line dependent.

The next step was the evaluation of ISGs modulation through qPCR which showed that the compound was modulating significantly IRF1 in a positive way and IL-6 in a negative way, the first implied in the early innate immune defense and the second implied in the inflammatory response. These results indicate a double role of the compound that induces the antiviral response but downregulates the inflammation.

In order to improve the LSH5605 activity, 35 derivatives were developed by our collaborators in Taipei. The compounds were tested in the HEK293-ISRE reporter cell line in absence and presence of IFN in order to evaluate their stimulating activity in ISRE. Overall, among the compounds tested, LSH5605, LSH5614 and LSH5627 were significantly modulating the ISRE induction in a positive way in presence of IFN- α while the rest of the selection was decreasing the ISRE production.

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