Broad-spectrum host-acting antivirals: identification and characterization of anti-HIV drugs

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SUMMARY

Viruses rely on host factors to complete their life cycles. Indeed, hundreds of host factors related to viral infections like HIV, hepatitis C (HCV), dengue (DENV) or west Nile (WNV) have been identified. As many of these host factors are shared by different viruses, chemical blockade of key virus-associated cellular components may effectively act as broad-spectrum antiviral treatment. Persistent infections like HIV have no effective vaccine and the available antiviral treatments cannot clear the infection. Broad-spectrum host-acting antivirals (HAAs) may reduce treatment complexity and costs, increase adherence to the therapy and may pose a higher barrier to develop resistance.

In this thesis a high-throughput anti-HIV assay was used to screen for virus inhibitory effects of a library of secondary metabolites derived from myxobacteria. Compounds with high anti-HIV activity and low toxicity were classified as hits and two of them (ratjadone A and soraphen A) were selected for further analysis. Ratjadone A inhibits HIV by blocking the CRM1-mediated nuclear export of viral RNA, although significant toxic effects were also detected. However, other studies indicate that Ratjadone A may have a tumour cell selective toxicity, suggesting that toxicity in other cell types, including primary cells, may differ. The *de novo* fatty acid synthesis inhibitor soraphen A blocks HIV by altering the composition of viruses produced in the presence of the drug. Interestingly, blocking fatty acid synthesis has been related to the inhibition of other 14 viruses including HCV, WNV, DENV, influenza, human cytomegalovirus (HCMV) and rotavirus (RV). Its broad-spectrum activity, together with its low toxicity, makes soraphen A a promising candidate for development of a broad-spectrum antiviral. The results presented in this thesis show that broad-spectrum HAAs are a feasible option for antiviral treatment and that the compounds identified can be further studied for hit-to-lead compound development.

RESUMEN

Los virus necesitan factores del huésped para completar sus ciclos vitales. Cientos de factores del huésped relacionados con infecciones por el virus de la inmunodeficiencia humana (VIH), virus de la hepatitis C (VHC), virus del dengue (DEN) o virus del Nilo occidental (WNV) han sido identificados. Como muchos de esos factores del huésped son compartidos por diferentes virus, el bloqueo químico de un componente celular clave asociado al virus podría actuar de forma efectiva como un tratamiento antiviral de amplio espectro. Infecciones persistentes como VIH no tienen una vacuna efectiva y los tratamientos antivirales disponibles son incapaces de eliminar el virus. Antivirales de amplio espectro contra factores del huésped podrían reducir la complejidad y el coste del tratamiento antiviral, incrementar el cumplimiento de la terapia y pueden suponer una barrera mayor al desarrollo de resistencia.

En esta tesis un cribado de alta capacidad de fármacos anti-VIH fue aplicado a una librería de metabolitos secundarios de myxobacteria. Compuestos con alta actividad anti-VIH y baja toxicidad fueron clasificados como hits y dos de ellos (ratjadone A y soraphen A) fueron seleccionados para posteriores estudios. Ratjadone A inhibe VIH bloqueando la exportación de RNA viral desde el núcleo al citoplasma mediada por CRM1, pero también fueron detectados efectos tóxicos significativos. Sin embargo, otros estudios muestran una citotoxicidad selectiva para células tumorales. Esto sugiere que la toxicidad en otros tipos celulares, incluyendo células primarias, podría ser diferente. El inhibidor de la biosíntesis de ácidos grasos soraphen A bloquea VIH alterando la composición de los virus producidos en presencia del fármaco. El bloqueo de la síntesis de ácidos grasos ha sido relacionado con la inhibición de otros 14 virus incluyendo VHC, WNV, DEN, virus de la gripe, citomegalovirus (CMV) y rotavirus (RV). Su actividad de amplio espectro junto con

su baja toxicidad, hacen de soraphen A un candidato prometedor para el desarrollo de un antiviral de amplio espectro. Los resultados presentados en esta tesis muestran que usar antivirales de amplio espectro contra factores del huésped es una opción viable para tratamientos antivirales y que los compuestos identificados pueden ser estudiados para el desarrollo de fármacos.

PROLOGUE

Viral infections affect millions of people every year and are a global public health threat. Infections with viruses like HIV or HCV can become persistent and may produce severe diseases as AIDS or hepatocellular carcinoma, respectively. (Re-)emerging viruses like WNV, DENV or CHIKV are spreading without proper control mechanisms. Thus, there are no effective vaccines and the treatment options against most virus threads are still limited. In this context, the development of new antivirals remains an important issue.

Viruses need cellular host factors to complete their life cycles. Hundreds of these host-factors have been identified. Interestingly, several viruses use the same host factors so that chemical inhibition of these may lead to broad-spectrum viral inhibition. Broad-spectrum host-acting antivirals (HAAs) may increase the barrier to resistance development and reduce the complexity of co-infection treatments.

In this thesis a high-throughput anti-HIV screening was applied to a myxobacteria metabolites library. Compounds with high anti-HIV activity and low toxicity were identified as hits and 2 of them (ratjadone A and soraphen A) were selected for further research. Ratjadone A inhibits the CRM1-mediated nuclear export and soraphen A inhibits the *de novo* fatty acid synthesis. Both drugs inhibit HIV and we described the mechanisms of inhibition of both compounds. The data presented in this thesis suggest that the use of host-acting antivirals as a broad-spectrum therapy is a feasible option.

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INTRODUCTION

1.- Viruses

Viruses are intracellular parasites that depend on cells to replicate their genomes and generate progeny. As of 2014, 105 families with 3186 species of viruses have been described according to the International Committee on Taxonomy of Viruses (ICTV: http://www.ictvonline.org/). There are 7 groups of viruses based in how they replicate their genomes. Groups I and II are DNA double- or single-stranded viruses,

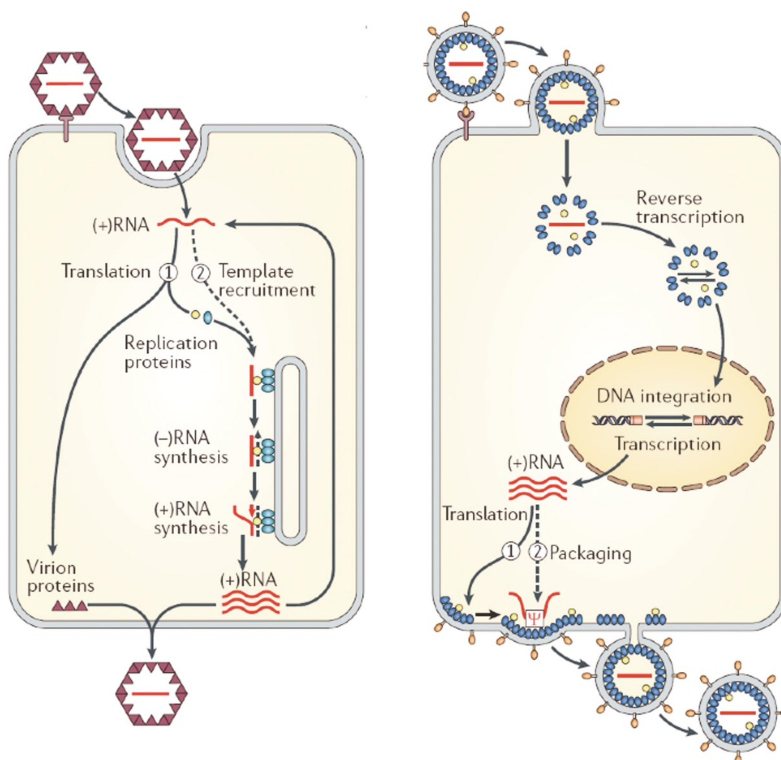

Figure 1. Life cycle of (+)ssRNA viruses and retroviruses. These viruses infect cells through a interaction with cell membrane receptors. Once internalized, (+)RNA viruses use their genome directly to produce viral proteins and templates for new viruses, while retroviruses retrotranscribe their RNA into DNA that goes to the nucleus and integrates in the cell genome. Once there, viral genes are transcribed and translated. Viral proteins and genomic templates are assembled in the cell membrane and progeny virions are produced by budding. Modified from Ahlquist, Nature Reviews Microbiology 2006.

respectively, groups III, IV and V are RNA viruses with double-stranded, positive and negative single-stranded genomes, respectively. Finally the reverse transcribing viruses with RNA and DNA genomes are the groups VI and VII, respectively. Clinically relevant DNA viruses are herperviruses (i.e. HSV, VZV, HCMV and EBV) and poxviruses (i.e. smallpox and VV). The dsRNA viruses most known are rotaviruses that are the principal cause of severe diarrhoea in children around the world (http://www.who.int/topics/rotavirus_infections/en/). (+)ssRNA viruses are the most abundant viruses, representing about 30% of all genera. Some relevant viruses of this group are flaviviruses (i.e. WNV, DENV, YFV or HCV), picornaviruses (i.e. enterovirus and poliovirus) and coronaviruses (i.e. SARS-CoA and MERS-CoV). Two of the most pathogenic viruses with (-)ssRNA are influenza and Ebola virus. Finally, the reverse transcribing viruses with ssRNA or dsDNA comprise HIV and HBV as the best studied representatives, respectively. Examples of viral life cycles of (+)ssRNA viruses like HCV and retroviruses like HIV are shown in figure 1 .

2.- Viruses depend on cells

All viruses have in common that they need the cell machinery to complete their life cycles. There are host factors intervening in virtually every step of viral infections. The most obvious host factors used by viruses are (i) cell surface receptors utilized for viral entry, (ii) transcription factors and (iii) factors of the cellular translation machinery. Cell lipids and membrane proteins are also important for assembly and release of newly-generated viruses. Genetic and proteomic high-throughput screens have revealed a huge amount of host factors potentially interacting with different viruses. siRNA-based screens have revealed, for example, more than 1000 host factors potentially supporting or restricting HIV replication. Around one third of them have a direct

physical interaction with at least one of the HIV proteins (1, 2). Indeed, Jager et al performed a proteomic assay consisting of an affinity purification of HIV-1 proteins and a scoring of the mass spectrometry data to detect physical interactions of host factors and HIV proteins (3). With this approach 497 interactions between HIV and human proteins were detected. In the case of flaviviruses and influenza virus, siRNA screens have detected 262 host factors related to HCV (4), 305 related to WNV (5), 116 related to DENV (6) and from 28 to 616 (depending on the screen) related to Influenza (reviewed in 7). All these host factors used by the viruses constitute a large amount of potential targets for antiviral therapy.

2.1.- Shared use of host factors

Interestingly, the shared use of host factors by different viruses has been reported (8-10). DDX3 and Cyclophilin A are remarkable examples of shared host factors. DDX3 is an RNA helicase detected as a host factor interacting with HIV (see below) but also in HCV, WNV, DENV and influenza (8, 11-13). While cyclophilin A inhibition has been shown to inhibit HCV, HIV, influenza, CoV, HCMV, HBV, HSV, VSV, VV and HPV (14-22). The development of small chemical inhibitors targeting host factors shared by different viruses has been proposed as a strategy to obtain broad-spectrum antivirals (22, 23). This concept is further explained below.

3.- HIV life cycle and host factors

Researchers have been able to map the involvement of host factors throughout the HIV life cycle. Only some of the most known host factors are presented below, more detailed information is reviewed in (24) and in figure 2. The first step in HIV infection is viral entry. The HIV-Env

protein binds to the cellular CD4 receptor and either the co-receptors CCR5 or CXCR4. Binding to these cellular receptors induce conformational changes in Env that bring the viral and cell membranes closer to each other, subsequently leading to their fusion. Once the membrane fusion is complete the capsid is released into the cell cytoplasm and is uncoated. The host factors Cyclophilin A and Pin1 assist in the disassembly of the capsid (composed of HIV-p24 proteins) that releases the viral genome and viral accessory proteins (25, 26). Viral RNA is reverse transcribed into cDNA by the viral reverse transcriptase and the pre-integration complex (PIC) is formed. This complex is transported by cytoplasmic dynein motors along microtubules to the nucleus (27). Nuclear entry of the viral DNA that is bound to viral proteins is facilitated by importins and karyopherins that bind to viral integrase and matrix proteins, respectively, through their nuclear localization signals (NLSs)(28,

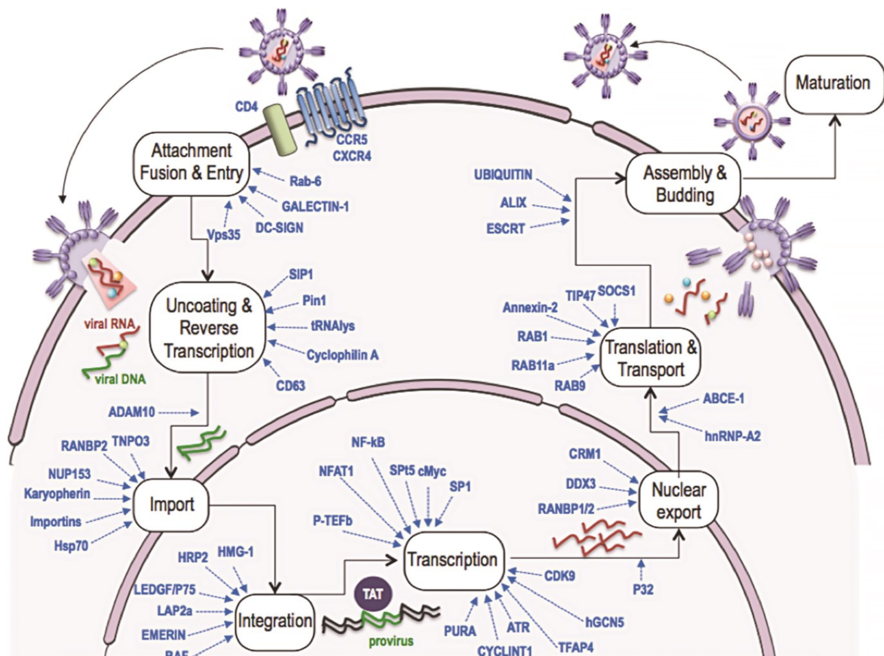

Figure 2. Host factors involved in the HIV life cycle. Main steps in the HIV life cycle are circled. Host factors used by HIV are in blue with arrows that indicate in which step they participate. Modified from Friedrich et al, Virus Research 2011.

29). Viral DNA integration is mediated by the HIV-integrase and several host factors like LEDGF/p75, HMG-I/Y and HRP2 (30-32). HIV-Tat and cellular transcription factors including Sp1, AP1, P-TEFb and NF-κB among others are recruited for transcription of the provirus into genomic and messenger RNAs (reviewed in 33). Viral RNAs are exported to the cytoplasm (see below) where they are translated by ribosomes. Once the encoded HIV polyproteins Gag, GagPol and Env are produced, they traffic to the cell membrane together with unspliced viral RNA molecules. The unspliced viral RNA will be packaged into new virions. Some host factors intervening in the trafficking process to the assembly spots in the cell membrane are Rab GTP proteases, SOCS1, ABCE-1 and ubiquitin (34-37). Assembly is accomplished in specific subdomains of the plasma membrane. These subdomains are composed of lipid rafts and tretraspanin-enriched microdomains (TEMs), and are the platforms for viral assembly and release as explained in section 3.2 (38). Assembly is directed mainly by the viral structural protein Gag that contains 4 proteins, the matrix (MA), the capsid (CA, p24), the nucleocapsid (NC) and p6. MA is responsible for the anchorage to the cell membrane via its myristoylated N-terminal domain and its highly basic region (HBR). It also participates in Env incorporation into the virus. CA facilitates the lateral Gag-Gag and Gag-GagPol interactions that drive the multimerization of these proteins. NC interacts with and incorporates two copies of viral RNA into the nascent virion. In turn, viral RNA has been seen to play an important role in targeting Gag specifically to the plasma membrane and in Gag multimerization (39, 40). Finally, p6 is important in the recruitment of the host endosomal-sorting complex (ESCRT family) machinery that together with the accessory protein ALIX terminates budding by closing the Gag sphere and releasing the virion (41). When the virus is released, it contains around 2500-5000 Gag molecules and 7-14 trimers of Env (42-44). It is not completely understood when the viral proteases are activated but they should be at some point between late budding and early release (45).

Once viral proteases are activated, they cleave Gag, Pol and Env polyproteins followed by a reorganization of the viral components. NC covers the viral RNA, CA proteins generate a conical capsid, MA together with the lipid bilayer taken from the cell membrane keeps the spherical shape of the virion and Env trimers cluster to increase the efficiency of viral entry into new target cells (24, 46). Thus, a large number and varied types of host factors are participating in the HIV life cycle representing a huge potential source of targets for developing antivirals. In this thesis I focused on two host processes associated with HIV replication that might be a target for novel antiviral strategies: CRM1-mediated nuclear export and lipid metabolism.

3.1.- Viral RNA nuclear export

Fully spliced viral RNA is exported from the nucleus to the cytoplasm using the usual pathway for cellular mRNAs mediated by the TAP/p15 complex (47, 48). In contrast, partially spliced and non-spliced HIV RNAs required the early expressed HIV-Rev protein and the host factor CRM1 to be exported to the cytoplasm (figure 3) (49). Rev can shuttle in and out of the nucleus because it contains a nuclear localization signal (NLS) and a nuclear export signal (NES) (50). Once Rev is translated in the cytoplasm, it binds to importin-β through its NLS and is transported to the nucleus. In the nucleus, Rev binds to the Rev-responsive element (RRE) of unspliced or partially spliced viral RNA hiding its NLS and exposing its NES (51). Cell host factor CRM1 recognizes the NES and forms a complex CRM1-Rev-mRNA that is stabilized by the phosphorylated form of Ran (RanGTP). The RNA helicase DDX3 binds to CRM1 and helps the complex to cross the nuclear pore possibly by straightening the viral RNA (52). Once in the cytoplasm, Ran is dephosphorylated (RanGDP) and the complex is disassembled (53, 54). Viral RNA goes into translation or encapsidation, and Rev starts the nuclear export cycle again binding to importin-β and moving into the nucleus.

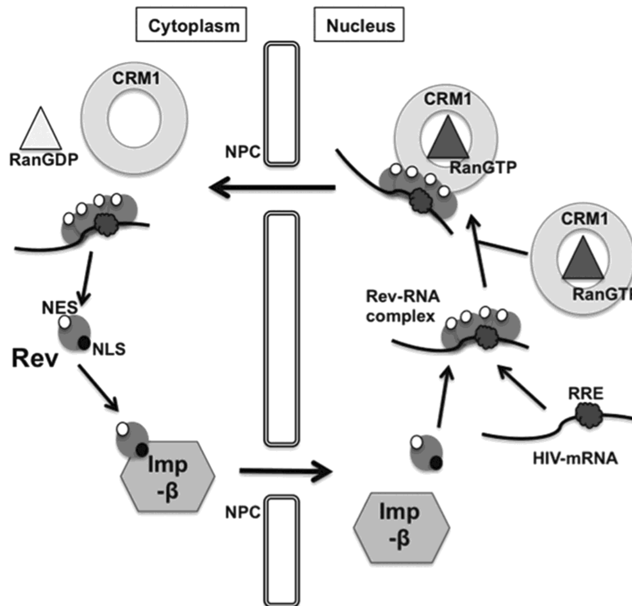

Figure 3. CRM1/Rev-mediated nuclear export of unspliced and partially spliced viral RNAs. A complex with Rev, RRE-containing viral RNA, CRM1 and RanGTP is formed to exit the nucleus. This complex is disassembled in the cytoplasm. Rev starts the cycle again by binding to Imp-β that goes to the nucleus. Figure from own published results: Fleta-Soriano et al, Microbial Cell Factories 2014.

3.2.- Lipid participation in the HIV life cycle

Host lipids, like many other cell molecules, are required in various steps of the HIV life cycle (55, 56). Lipids are a structural part of HIV since this virus is an enveloped virus. The most external viral structure (matrix) is covered with a lipidic bilayer taken from the virus-producer cell membrane. Besides the structural participation, lipids are also required in viral entry, assembly and release.

3.2.1.- HIV entry into target cells

Lipids have an important role in viral entry, the first step of HIV infection. It has been shown that cholesterol is required for entry and together

with the sphingolipid sphingomyelin promotes fusion of viral and cell membranes by increasing aggregation of gp41 which contains the fusion peptide (57, 58). In addition, blocking of sphingolipid biosynthesis not only inhibits entry but also inhibits HIV replication and infectivity (59). Downregulation of CD4 on target cells and alteration of lipid rafts may explain the inhibitory effect (60). Finally, the presence of phosphatidyl-serine (PS) in viral membranes is required for HIV infectivity (61).

3.2.2.- HIV assembly and release

HIV assembly and release require the participation of lipids. The fatty acids myristic acid and palmitic acid, sphingolipids, cholesterol and the acidic lipids PS and, especially, PI(4,5)P_2, are fundamental players in the complex process of HIV assembly (62). Once HIV-Gag is translated, it is post-translationally modified by acylation of its N-terminal glycine residue with myristate, a 14-carbon saturated fatty acid. This covalent fatty acid addition is required for Gag binding to the cell membrane and to initiate the assembly process (63-65). However, Gag myristoylation alone is not enough for a stable interaction with the cell membrane. That is why Gag contains a highly basic region (HBR) in its N-terminal domain that interacts with acidic lipids (negatively charged) as PS and PI (4,5)P_2. These lipids are abundant in the inner leaflet of the plasma membrane and not in other internal cell membranes. PI(4,5)P_2 is critical for the insertion of Gag-myristate into the membrane and makes Gag to target specifically the plasma membrane. Indeed, this lipid is required for an efficient virus release in Hela cells and in CD4+ T-cells (66, 67).

Lipid rafts are highly dynamic cell membrane subdomains with a characteristic lipid composition enriched in cholesterol, glycosphingolipids and other saturated fatty acids. Interestingly, it is known that if cholesterol is chemically depleted, rafts are disrupted and HIV infection is inhibited

(78, 79). Other membrane microdomains related with HIV assembly are TEMs, protein-based microdomains with multiple cell functions like migration or signalling (reviewed in 68). Lipid rafts and TEMs are used by HIV as platforms for assembly and release (69-71). However, it is not clear if these platforms are pre-existent or if the virus initiates their formation. It has been proposed that Gag multimerization triggers the formation of acidic lipid enriched domains (ALEMs) (figure 4) at the inner leaflet of the cell membrane (72, 73). Cellular proteins inducing lipid microdomains have been described before (74) and dynamic modelling of HIV-MA anchoring in a lipidic membrane supports the hypothesis of a Gag-induced formation of lipid microdomains (75). Assembly progresses by coalescence of Gag-enriched ALEMs and merging of ALEMs induces coalescence of lipid rafts and TEMs (76, 77) that will form the platforms from where HIV will be released.

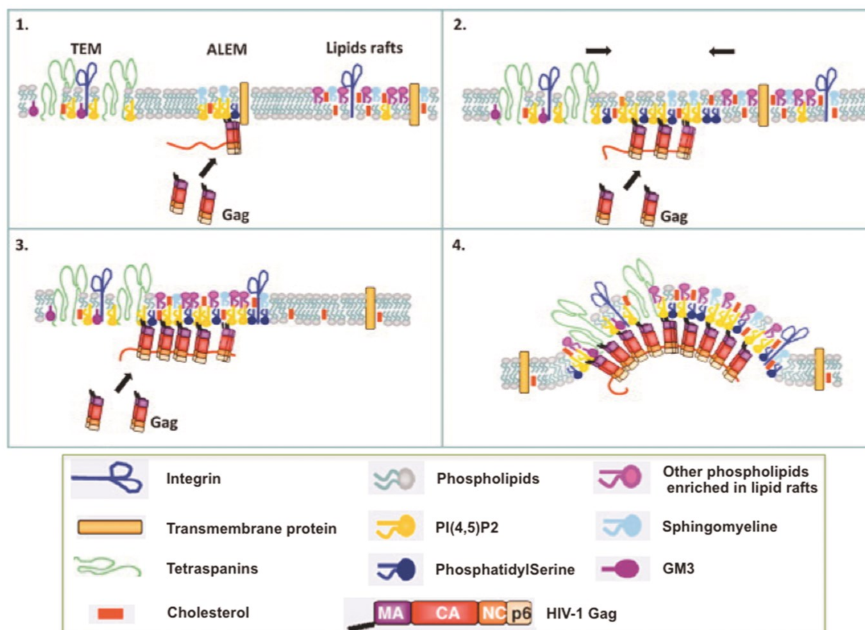

Figure 4. Proposed assembly platform formation. Gag initiates the formation of ALEMs in the inner leaflet of the cell membrane. Assembly of Gag induces coalescence of ALEMs and attracts tetraspanin enriched domains and rafts enriched in cholesterol and other phospholipids. All these components stabilize the assembly sites. Modified from Kerviel et al, Virus Research 2013.

HIV-Env gp160 is a polyprotein precursor that contains gp120 and gp41. These proteins initiate the infection process, gp120 contacts with CD4 and the co-receptors CXCR4 and/or CCR5, and together with gp41 mediate fusion of viral and cell membranes. Env has to target the assembly sites in the cell membrane to be packaged in the nascent virions. It is believed that Env targets the membrane due to interactions of the cytoplasmic C-terminal tail of gp41 with Gag and direct interactions with the cell membrane. The cytoplasmic tail of gp41 contains three lentiviral lytic peptides (LLPs) that are highly conserved in lentiviruses. LLPs are amphipathic α-helices with positive charges on one side that will interact with negatively charged surfaces of the cell membrane increasing incorporation, stability and cell-surface expression of Env (80, 81). In addition, it has been shown that the cytoplasmic tail of gp41 can be palmitoylated in two cysteine aminoacids (C764 and C837) (82) which is known to promote membrane binding. Another notable example of palmitoylation is the HIV co-receptor CCR5 that requires that modification to target the cell membrane (83, 84).

4.- Persistent infections and emerging viruses

Viruses can infect different hosts including plants, fungi, bacteria, animals or humans. Though, every virus has its particular life cycle depending on the viral genome and proteins, and the host. Viruses like VV or RV produce an acute infection that can be cleared by the immune system and usually don't kill their hosts. In contrast, HIV is not eliminated by the immune system and infections become chronic (figure 5).

Viral infections that can become persistent such as HIV and HCV might produce serious diseases like AIDS, cirrhosis and hepatocarcinoma that may lead to death. HIV and HCV have infected 35 and 150 million people worldwide, respectively. In addition, HIV-HCV co-infections have a

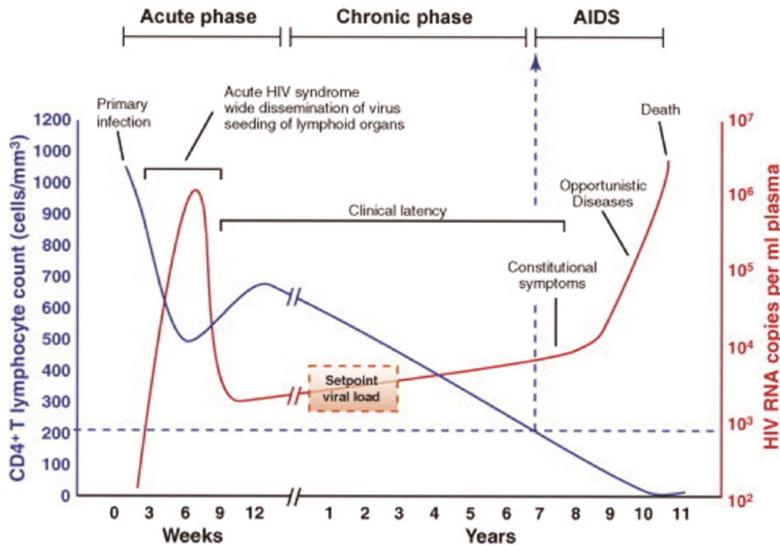

Figure 5. HIV infection course. In the acute phase of HIV infection the amount of viral RNA/ml plasma increases while the number of CD4 T cells decreases. The immune system responds and partially controls the infection. This initiates the chronic phase of the infection in which the HIV RNA level is kept low but the CD4 T cell count is continuously decreased. The last phase of the infection is the development of AIDS. In this phase the HIV RNA increases and CD4 T cells decline leading to the development of opportunistic diseases and death. Modified from An and Winkler, Trends in Genetic 2010.

high prevalence affecting to around 5 million people worldwide. This co -infection worsens the outcome of both diseases with increased mortality and faster liver disease progression (85). Furthermore, the treatment of co-infected individuals is complex since HIV treatment (HAART) comprises a minimum of 3 drugs of different types and HCV treatment comprises at least 2 or 3 drugs (https://aidsinfo.nih.gov/guidelines/html/1/adult-and-adolescent-arv-guidelines/26/hiv-hcv). There are many drug-drug interactions with an increase in liver toxicities and other secondary symptoms. In addition, combination therapy has elevated costs. Finally, the development of viral resistance to existent antiviral drugs is a continuous threat that necessitates constant development of new antivirals (86). Broad-spectrum drugs targeting both viruses could reduce some of those problems.

Another growing concern in public health are infections with (re-)emerging viruses. According to the WHO definition a viral disease is considered emerging when it appears for the first time in a population or when it is rapidly increasing in incidence or geographic range. The climate change, human travel activities and the global commerce have increased the spread of viral infections such as WNV, DENV, Chikungunya virus (CHIKV) or more recently (February 2015) Zika virus (ZIKV) in Latin America (87). These mentioned viruses are Arboviruses transmitted by mosquito vectors. They produce infections in new zones of the planet principally because their mosquito vectors are expanding. DENV is the most relevant mosquito-borne virus worldwide with around 50-200 million estimated new infections per year, although a recent estimation by Bhatt and colleagues suggested around 400 million infections/year (88, 89). Interestingly, sexual transmission of ZIKV has been reported and could be the first case of a sexually transmitted Arbovirus (90, 91). Hence, persistent and emerging infections are major concerns in global human health. Specific treatments are often inexistent or have unsolved issues as mentioned in the previous paragraph. Therefore, there is a clear need for vaccines and antivirals against these viruses.

5.- Therapies

There are two options to fight viral infections: vaccines and antiviral drugs. Vaccines are preventive so they have to be administered before infection and promote the antiviral response of the immune system of the subject. Antiviral drugs fight infections directly by blocking or interfering with viral replication cycles. Drugs are the only option for people already infected.

5.1.- Vaccines

5.1.1.- HIV

There is no effective vaccine available for HIV today. The HIV vaccine clinical trial named RV 144 (thai trial) was a combination of 2 vaccines (ALVAC and AIDSVAX B/E) that previously failed in individual clinical trials. It is the only vaccine candidate in decades of research that showed a modest protection of around 30% (92). The unprecedented hyper-variability of HIV-Env is the main issue to be solved in the development of an effective vaccine (93). To accomplish this task a rational design of vaccines based on targeting the most vulnerable sites of HIV-Env has been proposed (94).

5.1.2.- HCV

There is no vaccine available for HCV. There are several candidates in clinical trials most of them in initial stages. However, a major obstacle in generating an efficient vaccine is the high variability between the HCV genotypes (around 30%) (reviewed in 95).

5.1.3.- Emerging viruses

There are no vaccines available against emerging diseases caused by viruses such as DENV, WNV and CHIKV in humans. However, for all these viruses there are vaccines in different phases of clinical trials. For DENV there are currently 5 vaccines in clinical trials. Two are in phase II and three are in phase I. Only 1 of them, named CYD, is in phase III. This latter is a live attenuated vaccine with the backbone of the yellow fever vaccine (96). There are 3 vaccines against WNV approved for horses but not for humans. However, four vaccines are in initial steps of clinical development (one in phase II and three in phase I) (97). For

CHIKV there is one attempt based on a live attenuated CHIKV181/25 clone that is in clinical phase II and another vaccine based on virus like particles that is in clinical phase I. In addition, at least a dozen different vaccine approaches are in pre-clinical studies against CHIKV (98).

5.2.- Antiviral drugs

Antivirals are small chemical inhibitors that can be classified as direct-acting antivirals (DAAs) or as host-acting antivirals (HAAs). DAAs target viral components or processes while HAAs target host factors or processes of the cell that are required within a viral life cycle. Examples for targets of HAAs are cell receptors used for viral entry or the translation machinery used for viral protein production.

5.2.1.- DAAs

The main advantage of this kind of drugs is that, in principle, they are very specific for the viruses so they should have low toxicity. On the contrary, the main disadvantage is the fast development of resistance especially in viruses such as HIV with a high mutation rate and wide heterogeneous populations (quasispecies) in patients (99, 100). To avoid resistance development, DAAs are administered in combination. This leads to complex therapy regimens that may reduce the adherence to the treatment and increase side effects (101).

In the case of HIV there are currently 24 individual DAAs and 1 HAA approved by the FDA (http://www.fda.gov/ForPatients/Illness/HIVAIDS/Treatment/ucm118915.htm). Drugs targeting fusion, reverse transcription (2 types), integration and maturation are available (figure 6). T20 (Enfuvirtide) is a peptide that binds to HIV-gp41 and blocks the conformational changes required for fusion of viral and cell membranes.

There are two classes of reverse transcription inhibitors. The nucleoside reverse transcriptase inhibitors (NRTIs; 7 drugs available) are analogs of normal nucleosides or nucleotides needed to synthesize the viral DNA. When these compounds are incorporated into the nascent viral DNA, the synthesis stops. In contrast, non-nucleoside reverse transcriptase inhibitors (NNRTIs; 5 drugs available) bind directly to and block the viral reverse transcriptase. Integration of viral DNA in the host genome is blocked with 3 different drugs that inhibit the HIV-integrase by direct binding. Finally, the viral protease is inhibited by 8 approved drugs (PIs) that impede the maturation of HIV virions. Currently, the highly active antiretroviral therapy (HAART) is used to treat HIV. This therapy is a combination of at least 3 drugs of different classes and represents a high barrier for the development of resistance.

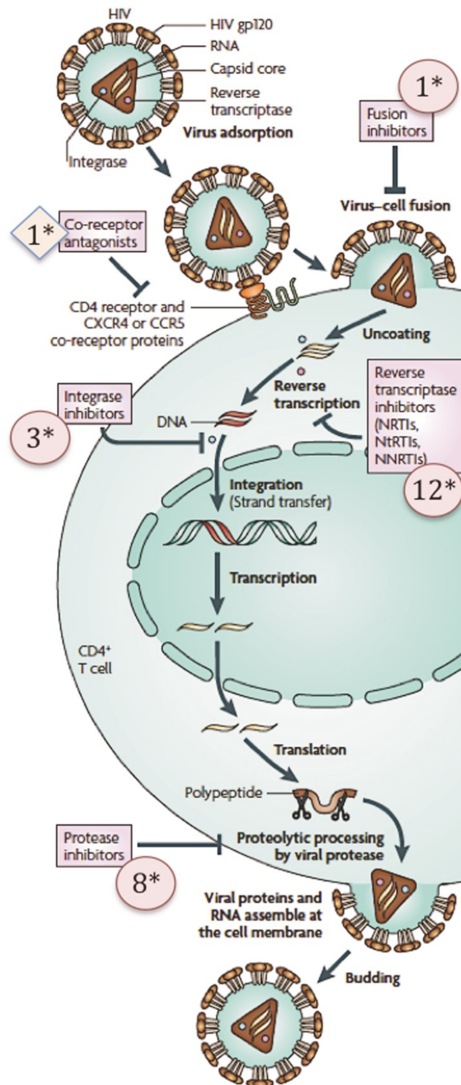

Figure 6. HIV inhibitors approved by the FDA. *Number of FDA-approved drugs in every step of the HIV life cycle. DAAs are shown in circles and the HAA is shown in a diamond. Adapted from: De Clerq, Nature Reviews Drug Discovery 2007.

For HCV there are 9 very recently (2011-2015) approved DAAs, the protease NS3/4A inhibitors boceprevir, telaprevir, simeprevir and

paritaprevir, the RNA polymerase NS5B inhibitors sofosbuvir and dasabuvir and the NS5A inhibitors daclatasvir, ledipasvir and ombitasvir (102). The standard treatment before DAAs development was a combination of ribavirin and peginterferon. Protease inhibitors telaprevir and boceprevir were included in the treatment after their approval in 2011 (103). However, the treatment is rapidly evolving due to the approval of new very potent drugs which make interferon-free treatments possible and highly effective (102). In the case of emerging viruses as DENV, WNV, CHIKV and ZIKV there are no drugs approved for their treatment.

5.2.2.- HAAs

The main advantage of this kind of drugs is the possibility of developing broad-spectrum antivirals targeting central host factors or pathways shared for several viruses (as explained above). Using broad-spectrum drugs may simplify the current antiviral treatments. A simplified treatment can be very important in HIV-HCV co-infected patients because it will reduce undesired drug-drug interactions, improve adherence to the therapy (101) and also can contribute to reduce the high treatment costs. Another interesting advantage of this type of drugs is the higher barrier to develop resistance (104, 105). In addition, these kind of drugs may be very useful in the control of novel pathogens or when quick diagnosis is not possible (22). However, toxicity is a main potential problem since a drug that targets a cellular component may block important cellular functions. Nonetheless, this risk may be reduced or eliminated in different ways. First, there are frequently redundant pathways for specific cellular functions. The inhibition of a host factor that is essential for the virus, may be compensated by other cellular pathways or proteins redundant for that specific cellular function so that its targeting is non-toxic (104). Second, the up-regulation of different pathways under viral infections shows that viruses and cells may have different requirements for a given

cellular component or function (106). It therefore may be possible to adjust the drug dose to block viral replication but keep the cell alive. Finally, it is known that there are non-essential cellular genes whose inhibition may be tolerated and the toxicity in this case will depend on the drug concentration used as well as the treatment duration (104). Indeed, some HAAs are approved by the FDA or are in clinical phase III showing acceptable toxicities. The best example is maraviroc, a CCR5 inhibitor that blocks CCR5-tropic HIV infection without major toxicity and that was approved by the FDA in 2007 (107). However, this antiviral has several limitations. The most important is that CXCR4-tropic HIV is not inhibited by maraviroc and a tropism test is required before prescription. In addition, CCR5 deficiency make individuals more susceptible to some other infectious diseases like for example symptomatic WNV infections. This is due to a defective leukocyte trafficking into the infected brain (108, 109). Finally, Maraviroc is a HAA but not a broad-spectrum drug, since there is no evidence of a shared use of CCR5 by other viruses. A novel example of a broad spectrum HAA is Alisporivir. This drug is in clinical phase III and inhibits HCV by blocking the host factor cyclophilin A with low toxicity (18). Remarkably, cyclophilin A inhibition blocks many other viruses as is shown above (see section 2.1). This example shows that targeting host factors for a broad-spectrum antiviral therapy is a viable option. However, targeting host factors as antiviral therapy have been barely followed until recent years and there are no further FDA-approved HAAs. Thus, more research efforts should be put into this therapy option.

6.- Strategies to find broad-spectrum antiviral drugs

There are two approaches to develop broad-spectrum drugs targeting host factors shared by several viruses (figure 7). One is to identify common host factors or cellular pathways shared by several viruses and then

search for drugs targeting those host factors (host factor → drug). The other approach is to perform overlapping screens of drug libraries against different viruses and pick the shared hits (drug → host factor).

	1st step	2nd step	3rd step
Host factor ↓ Drug	Find shared host factors	Identify or develop drugs against shared host factors	Test antiviral activity and analyse inhibition mechanism
Drug ↓ Host factor	Antiviral screening of drug libraries	Pick hits that inhibit several viruses	Analyse inhibition mechanism

Figure 7. Strategies to discover broad-spectrum antiviral drug candidates. The *Drug* ⟶ *Host factor* strategy is followed in this thesis.

6.1.- Host factor → drug strategy

The first step in this strategy is to identify shared host factors or cellular pathways using the available published information on host-viral interactomes (figure 8). siRNA-based screens have revealed hundreds of host factors implicated in viral infections as mentioned above in section 2. The next step is to find existing drugs targeting shared host factors. The final step is to test those drugs against different viruses. If antiviral efficiency can be proven, the extended approval of the drug for antiviral applications should be rather fast and with relatively little development costs.

6.2.- Drug → host factor strategy

In this approach the first step is to screen drug libraries against different viruses. The use of natural product libraries has the advantage over other clinical libraries that the structural diversity and complexity is very large (22). The next step of this strategy is to identify common hits against

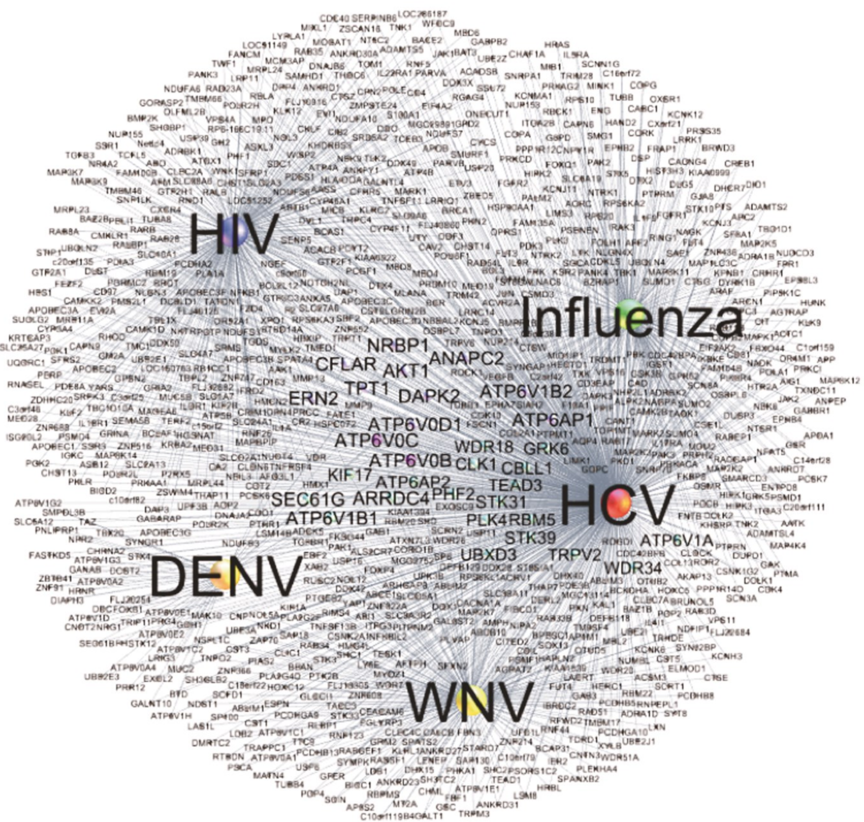

Figure 8. Interactome of HIV, HCV, WNV, DENV and influenza. Viruses are depicted as colored circles bound to host factors by lines. From center to outside: factors shared by all five viruses to only two viruses. The interactome was constructed in Gephi 0.8.2 with data available from genome-wide siRNA screens for HIV, HCV, Influenza, Dengue Virus and West Nile Virus.

several viruses and then characterize further interesting drug candidates for specific applications.

A source of natural products with interesting properties is the library of around 150 different myxobacteria metabolites belonging to the Helmholtz Centre for Infection Research (HZI) in Braunschweig, Germany. Some of the compounds are known for their antifungal or antibiotic activities. For example Corallopyronin A from the myxobacteria *Corallococcus coralloides* has antibiotic properties (110), while

Epothilones from *Sorangium cellulosum* have antifungal activity (111). Additional information on, for example, their cellular targets is available for many of the compounds. This makes them a precious starting point for antiviral screening.

In this thesis, the myxobacteria drug library of the HZI was used to validate an anti-HIV screening method on a robotic platform. Two interesting hits were used for further characterization of their anti-HIV properties.

OBJECTIVES

Persistent infections like those of HIV and HCV, and (re-)emerging viruses like DENV, WNV or CHIKV are a major threat for human health worldwide and there is a continuous need for new antivirals. Broad-spectrum antivirals targeting host factors have not been explored in depth. Objective of this thesis was to follow the drug → host factor strategy and develop a rapid and simple screening procedure for anti-HIV hits. Interesting antiviral hits with broad-spectrum activities were then to be selected and further characterized with respect to their mechanism of action. The specific objectives of this thesis evolved with the experimental progress of the work and were as follows:

Objective 1: To develop an anti-HIV drug screening assay and to identify antiviral hit compounds from a myxobacteria compound library.

Objective 2: To validate a hypothetical mechanism of HIV inhibition by the nuclear export inhibitor ratjadone A, a hit with anti-HIV activity detected under objective 1.

Objective 3: To characterize the mechanism of HIV inhibition by the fatty acid synthesis inhibitor soraphen A, a hit with anti-HIV activity detected under objective 1.

RESULTS

Chapter 1

Identification of myxobacteria-derived HIV inhibitors by a high-throughput two-step infectivity assay

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Chapter 2

The myxobacterial metabolite ratjadone A inhibits HIV infection by blocking the Rev/CRM1-mediated nuclear export pathway

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RESULTS

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Chapter 3

The myxobacterial metabolite soraphen A inhibits HIV infection by altering virion composition*

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THE MYXOBACTERIAL METABOLITE SORAPHEN A INHIBITS HIV INFECTION BY ALTERING VIRION COMPOSITION

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Abbreviations:

ACC: acetyl-CoA carboxylase; CC_{50}: cytotoxic concentration 50; CVB3: coxsackie virus; DENV: dengue virus; EBV: Epstein-barr virus; EC_{50}: effective concentration 50; HCMV: human cytomegalovirus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; LA: lauric acid; OA: oleic acid; PA: palmitic acid; PS: phosphatidylserine; RV: rotavirus; SI: selectivity index; SINV: Sindbis virus; SM: sphingomyelin; USUV: usutu virus; VSV: vesicular stomatitis virus; VV: Vaccinia virus; VZV: varicella-zoster virus; WNV: west nile virus; YFV: yellow fever virus.

ABSTRACT

Human Immunodeficiency Virus (HIV) infections are still a major threat for human health worldwide. The infection is treated with a combination of antivirals. However, even efficient antiretroviral therapy does not eliminate HIV from an infected individual. Co-infections with the hepatitis C virus (HCV) increase the complexity of the treatments leading to multiple drug-drug interactions. Targeting host factors used by both viruses may reduce complexity of the co-infection treatments. Soraphen A (SorA) is a myxobacterial metabolite that inhibits the acetyl-CoA carboxylase (ACC) of the host, a key enzyme in the *de novo* fatty acid synthesis. The anti-HIV activity of SorA was discovered in a high-throughput screen of myxobacterial metabolites. Recently, the SorA-mediated inhibition of HCV has been described. The aim of this work is to describe the mechanism of HIV inhibition by SorA. This compound inhibits mainly late steps of HIV-1 with an EC_{50} of 0.78 ±0.34µM and 0.21±0.08µM in TZM-bl cells and PBMCs, respectively. Gag assembly and HIV maturation are not inhibited by SorA as shown by confocal imaging of gag assembly spots and by transmission electron microscopy, respectively. HIV-Env protein amount is reduced with respect to the p24 content in viruses produced under SorA treatment. This correlates with a reduction of viral entry measured with a CD4 binding assay and a fusion assay. Infectivity and gp120 levels of viruses with Env proteins that can be palmitoylated is recovered when palmitic acid is added to SorA-treated HIV-producer cells. Thus, SorA inhibits HIV by altering the composition of viruses produced in the presence of the drug. Given that it also inhibits HCV and shows low cytotoxicity, SorA is an interesting drug candidate that may be further developed for the treatment of HIV-HCV co-infections.

INTRODUCTION

Broad-spectrum antivirals that target central host factors, pathways or common structural elements shared by several pathogens are a promising option to treat viral infections (1-3). Potential advantages of broad-spectrum host-acting antivirals (HAA) are the higher barrier to develop resistances (4, 5) and the reduction of co-infection treatment complexity. Surprisingly, this strategy to obtain broad-spectrum antivirals has been barely followed until recent years. A novel example of HAA is Alisporivir. This inhibitor of Cyclophilin A has been shown to block HCV replication with low toxicity and is currently in development (phase III trial) (6). Interestingly, inhibition of cyclophilin A blocks as well other viruses including HIV, influenza, Coronaviruses (CoVs) and HCMV among others (7-10). This indicates that targeting shared host factors is a viable option to develop broad-spectrum antiviral drugs.

Cellular lipids have an important role in viral infections (11). In the case of HIV, cellular lipids are crucial in entry, assembly and budding steps (12). For example, cholesterol depletion leads to HIV inhibition due to "lipid raft" disruption (13, 14). "Lipid rafts" are cell membrane subdomains enriched in cholesterol and sphingolipids. Although it is not clear if these subdomains are pre-existent or induced by the virus (15), they are essential for entry and/or budding of HIV. It is also known that inhibition of the glycosphingolipid metabolism impairs HIV infection through blocking Env-mediated fusion (16). Another example of lipid involvement in the HIV life cycle is the covalent addition of a 14-carbon fatty acid (myristic acid) to the N-terminal domain of viral Gag protein. This is required for Gag to target the cell membrane where viral assembly takes place. Indeed, myristoylation has been explored as a target for anti-HIV therapy (17, 18). Thus, the cellular lipid metabolism may be an interesting target for development of new HAAs.

A key pathway in lipid metabolism is the *de novo* fatty acid synthesis. This pathway is mediated by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Fig. 1). ACC mediates the conversion of acetyl-CoA into malonyl-CoA. The first step in this pathway is the addition of a malonyl-CoA molecule to an acetyl-CoA. There are six elongation cycles adding new malonyl-CoA molecules to the nascent fatty acid. The reaction ends when the 16-carbon saturated fatty acid palmitate is produced. Small molecules that inhibit ACC and FAS are known to eliminate the *de novo* fatty acid synthesis. Remarkably, drug-mediated blocking of that pathway has been related with inhibition of HCV, WNV, DENV, YFV, RV, HCMV, VSV or influenza among other viruses (19-24). Soraphen A (SorA) is a myxobacterial secondary metabolite that inhibits ACC. It was identified as an HIV inhibitor in our previous anti-HIV screening (25) and it has been already proven to inhibit HCV very efficiently with a large therapeutic window (selectivity index, SI>100000) (4). Therefore, SorA is a promising candidate for a broad spectrum antiviral drug.

In this article we studied the mechanism of HIV inhibition by SorA. SorA-mediated ACC blocking leads to HIV inhibition both in cell lines and primary cells. We show that processes as HIV-Gag assembly or maturation are not affected by SorA. On the contrary, the viral composition is altered with a decrease of the gp120/p24 ratio that leads to a reduced entry capacity of the virus. In contrast, the total amount of p24 and gp120 in the cell lysate of SorA treated cells is only modestly reduced, suggesting an interference with a step posterior to viral proteins translation. Finally, viruses produced under SorA treatment and pseudotyped with Env variants that cannot be palmitoylated have lower recovery of gp120 levels and infectivity, when treated with palmitic acid than those pseudotyped with Env that can be palmitoylated. Altogether, these results suggest that SorA inhibits HIV by reducing Env amount in newly produced virions.

RESULTS

RESULTS

Soraphen A inhibits late steps of the HIV life cycle

SorA was identified in our previous screening (25) as an inhibitor of HIV acting in a step of the HIV life cycle between viral gene translation and virus maturation. We analysed the potency of SorA in a TZM-bl cell-based assay and in primary cells (Fig. 2). TZM-bl cells produce luciferase upon Tat trans-activation. Production of luciferase is proportional to the level of HIV infection. Small molecules with antiviral activity will reduce the luciferase signal (26). TZM-bl cells or PBMCs were pre-treated with 10-fold serial dilutions of SorA from 1nM to 10µM, infected with HIV_{LAI} at MOI=0.5 and incubated for 48h. The supernatant of these cells was used to reinfect fresh TZM-bl cells. HIV-dependent luciferase activity was measured 48h after reinfection. SorA inhibited late steps of HIV in a dose-dependent manner. The calculated EC_{50} in TZM-bl cells and PBMCs was 0.78 ±0.34µM and 0.21 ±0.08µM, respectively. No toxicity was detected at the concentrations tested. Both titrations indicate that SorA can inhibit HIV at low micromolar concentrations and that the inhibition of HIV in primary cells required lower SorA concentrations.

To rule out a direct effect of SorA on luciferase expression, we performed an immunostaining of p24 in infected MT2 cells treated with SorA (Fig. 3). Cells were treated with the drug, infected and incubated for 48h. Supernatants of these cultures were added to fresh MT2 cells and incubated for 48h. SorA reduced p24 production compared with the DMSO control but did not completely abolish it (Fig 3D left). Nevertheless, when fresh cells were incubated with supernatant from SorA-treated infected cells, no p24 was detected (Fig. 3D right). These data suggest that SorA cannot block completely HIV p24 production but inhibits the generation of infectious virus.

Gag assembly is not impaired in SorA-treated samples

N-terminal addition of myristic acid to the N-terminal domain of HIV Gag is required for viral assembly (17). Since SorA inhibits fatty acid synthesis, we analysed if Gag assembly is inhibited upon SorA treatment. TZM-bl cells pre-treated with SorA or DMSO (vehicle) for 1h were transfected with a plasmid expressing a EGFP-Gag fusion protein. Confocal images were taken at several timepoints and assembly spots were quantified (Fig. 4). At 12 and 24h after transfection it seemed that the number of Gag assembly spots was slightly higher in SorA-treated samples compared with the vehicle control samples. However, at 48h there was no difference in the number of Gag assembly spots between SorA-treated and DMSO-treated cells. These data indicate that a fail in assembly can be discarded as the mechanism of SorA inhibition of HIV.

HIV maturation is independent of SorA treatment

Drug-induced changes in the lipid content of cell membranes can lead to differences in membrane fluidity as shown for cholesterol-depleted cells by Bosch and colleagues (27). HIV lipid bilayer is less fluid than the plasma membranes of their host cell (28). Therefore, alterations in cell membrane fluidity may interfere with the reorganization of HIV structural proteins (29) during viral maturation. To test whether maturation of HIV particles is inhibited by SorA, virions produced in the presence of SorA were analyzed by a transmission electron microscopy (TEM). For this, the HIV-latently infected ACH2 cells were treated with SorA and viral production was activated with Vorinostat. 48h after activation, cells were fixed with glutaraldehyde and stained with osmium tetroxide (Fig. 5). More than 500 viral particles per condition were counted and classified as mature, immature or undetermined. The HIV protease inhibitor Lopinavir (LPN) was used as a positive control. Around 90% of viruses were mature in SorA-treated samples, a number similar to the DMSO control. In contrast, around 90% viral particles were immature in the LPN control. In addition, an assay to determine changes in membrane

fluidity and cholesterol content during SorA treatment was performed (Supp. Fig. 1). No changes in membrane fluidity of Jurkat cells and PBMCs or cholesterol content of Jurkat cells were detected. These data indicate that SorA does not affect cell membrane fluidity and has no effect on HIV virion maturation.

SorA reduces the gp120/p24 ratio and the infectivity of HIVpp and of virions produced from ACH2 and PBMCs

Our data (Fig. 3 and 5) suggest that HIV virions can be produced under SorA treatment but they have a reduced infectivity. To investigate if the virus integrity is compromised, we quantified the viral RNA, p24 and gp120 content present in the pelleted supernatants of transfected 293T cells producing HIVpp, in activated ACH2 cells and in HIV_{LAI}-infected PBMCs (Fig. 6). Virus-producing cells were treated with SorA or DMSO and incubated 48 or 72h. The viral-containing supernatants were clarified to remove cellular debris and the virus was pelleted. The infectivity of SorA-treated samples was reduced around 50 to 70% compared with the control (Fig. 6, upper panels). The reduced infectivity of virions produced from the latently HIV-infected ACH2 cells (Fig. 6A, upper panel) indicated that SorA inhibits a step in the HIV life cycle between translation and maturation. The amount of viral RNA and p24 of treated samples was reduced with respect to the DMSO control. These data suggest a global reduction in viral production. In addition, the gp120/p24 ratio (Fig. 6, lower panels) observed in SorA-treated cells was lower than in the DMSO control suggesting that the amount of gp120 per viral particle was reduced due to the activity of SorA. The reduction of gp120 may lead to a decrease of the fusion capacity of the virus and therefore, to the inhibition of HIV infection.

Viral entry is reduced in viruses produced in the presence of SorA

Every HIV virion has around 7 to 14 trimers of Env exposed on its surface (30, 31). It has been shown that a lower number of Env limits HIV

entry efficiency, slow down viral entry kinetics and impairs the stimulation of proviral expression (32, 33). To study entry of virions produced under SorA treatment into target cells, we performed two assays that allow to dissect the entry process in two steps, the initial CD4 binding and the final fusion of viral and cellular membranes. To test CD4 binding, TZM-bl cells were spinoculated with supernatant (10ng of p24 per condition) from activated and SorA-treated ACH2 cells. After spinoculation cells were lysed and viral p24 quantified. Samples were kept at 4°C during all the assay, consequently the virus could attach to CD4 but could not continue the entry process (34) (Fig. 7B). We show that the CD4 binding capacity was reduced to about 50% with respect to the control. In the second assay the viral fusion capacity was tested. First, viruses containing Vpr-BlaM activity were produced in the presence of SorA, LPN or DMSO as mock-treated sample. Then, Jurkat cells were infected with a normalized p24 amount of produced viruses. The fusion inhibitor T20 was used as a negative control. Infected cells were analysed using flow cytometry to detect β–lactamase activity, i.e. fusion capacity, of the different samples (Fig 7C and supp. fig. 2). We detect a reduction >60% in the fusion of SorA treated cells compared with DMSO treated cells. Intriguingly, samples treated with LPN (a protease inhibitor) with a dose that inhibits completely HIV maturation, still showed 10% of fusion. On the contrary, there was complete fusion inhibition in samples treated with T20. Finally, it was observed that viral binding to the CXCR4 co-receptor is also about 50% inhibited for virus produced under SorA treatment (data not showed). These data together suggest that near all the inhibitory effect in viral entry is due to CD4 binding inhibition and not the inhibition of posterior steps in the entry process. This fits with a model where the HIV virions produced under SorA treatment have less Env molecules on its surface and consequently binding to CD4 receptors is impaired.

Palmitic acid recovers the gp120/p24 ratio and virus infectivity

It is known that HIV-Env proteins can be palmitoylated. Since SorA inhibits fatty acid synthesis and thus palmitic acid (PA) production, it is likely that SorA affects HIV-Env palmitoylation and subsequently incorporation of Env into the lipid membrane of the virion. If true, this would suggest that an exogenous addition of palmitic acid should rescue the gp120/p24 ratio and infectivity of virions produced under SorA treatment. To test the rescue of infectivity, a panel of lipids was added to SorA-treated cells (Supp. Fig. 3). Phospholipase A2 and ceramidase inhibitors were added to phosphatidylserine and sphingomyelin treated samples, respectively, to confirm that the observed effects in infectivity recovery were specific of these lipids and not due to the fatty acids released in their degradation. While oleic acid and sphingomyelin ± Ceranib-2 (ceramidase inhibitor) had no effect in infectivity, the 12-carbon lauric acid produced a decrease in infectivity. The inhibitory effect of lauric acid has been seen before in other enveloped viruses (35). Only PA and phosphatidylserine ± anthranilic acid (phospholipase A2 inhibitor) could revert significantly the SorA-mediated HIV inhibition. When HIV-Gag reach the cell membrane interacts mainly with the acidic lipid phosphatidylserine (15), an increase of this lipid may explain the recovery in infectivity observed. As expected, PA produced the most potent reversion of SorA inhibitory activity. To confirm that PA can counteract SorA activity, it was added to infected SorA-treated PBMCs (Fig. 8A). A partial recovery in the gp120/p24 ratio and in infectivity was detected. As PA is an allosteric inhibitor of ACC and also blocks gp120-CD4 attachment, recovery of gp120/p24 ratio and infectivity cannot be complete (36, 37).

Palmitic acid recovers gp120 and p24 levels in HIVpp but not in cell lysates

To test if the SorA-mediated inhibition of HIV is due to a decrease in the production of the viral p24 and/or gp120 proteins or due to an alteration

of the composition of the virions produced, the amount of p24 and gp120 was measured in pelleted HIVpp and in cell lysates from transfected 293T cells under SorA treatment ± PA addition (Fig. 8B and C). p24 and gp120 levels in pelleted HIVpp were clearly reduced with respect to the control (around 50-70%), while in cell lysates the amount of those viral proteins was modestly reduced (around 10-20%). This suggests that the viral proteins are normally produced but are retained within the cells. When PA was added, a complete recovery of p24 and gp120 levels in pelleted HIVpp was detected but the levels measured in the cell lysate decreased. These data suggest that exogenous PA induces HIVpp formation reducing the amount of viral proteins "trapped" in the cell and increasing their levels in supernatant.

Recovery of gp120 levels and infectivity upon palmitic acid addition depends on the palmitoylation capacity of the HIV envelope variants

HIV-Env consists of gp120 and gp41. gp41 can be palmitoylated in two cysteine aminoacids (C764 and C837) in its cytoplasmic C-terminal tail (38). However, these cysteines are not completely conserved among all isolates (Supp. Table 1), C764 is in 87.4% of the isolates of group M while C837 is only present in 40% of the isolates. The mutations in 764 and/or 837 positions may lead to different responses to SorA treatment and PA addition. As we show above (Fig. 8 and Supp. Fig. 3) exogenous PA counteracts SorA activity in HIV_{LAI} (containing C764 and C837) infections. To evaluate the susceptibility to SorA of viruses with different palmitoylation capacity (i.e. with two, one or none cysteine at positions 764 and 837), we tested a panel of HIVpp pseudo-typed with envelopes with the four possible combinations for 764 and 837 aminoacids (C764/C837, C764/no-C837, no-C764/C837 and no-C764/no-C837) (Fig. 9). Surprisingly, in all the cases SorA inhibited HIVpp infectivity and reduced p24 and gp120 levels. However, when PA was added to the cells, we observed that the recovery in infectivity and gp120 level (Fig. 9A and B right panels, respectively) was proportional to its palmitoylation

capacity, being higher in double cysteine-containing envelopes than in no cysteine-containing envelopes. On the contrary, recovery of p24 levels seems independent of palmitoylation capacity (Fig. 9C right panel). All these data together suggest that SorA produces overlapping inhibitory effects in HIV infection, being the lack of palmitoylation of HIV-Env part of the mechanism of HIV inhibition by SorA.

DISCUSSION

The data presented here indicate that SorA generates overlapping HIV inhibition mechanisms. A general decrease in virus production and a specific infectivity reduction of virions produced in the presence of SorA can be distinguish. Specific infectivity is reduced due to the composition alteration of the produced virions, being the decrease of gp120/p24 ratio the main alteration. This decrease of gp120 with respect to p24 levels impairs viral entry. The low toxicity of SorA and the shared use of the fatty acid synthesis pathway by other viruses suggest that SorA is a good candidate for a broad-spectrum host-acting antiviral (HAA).

The SorA-mediated reduction of gp120/p24 ratio in newly-produced viruses seems the major cause of HIV inhibition. However, we also observed a general reduction of p24 and viral RNA suggesting that fatty acid synthesis inhibition produce a general decrease in virus production. Different functions of the *de novo* fatty acid synthesis in other viral infections have been described. For example, the main function of this pathway in the replication of vaccinia virus is to produce energy (39) and for HCV is to form the membranous web required for its replication (4). The inhibition of HIV and other viruses due to blocking of fatty acid synthesis is most likely a combination of those functions in different proportions depending on the virus and/or the host cells.

We found that HIVpp produced in the presence of SorA and pseudotyped with envelopes that can be palmitoylated recover their gp120 levels and infectivity upon exogenous palmitic acid addition more than those that cannot be palmitoylated. This suggest that palmitoylation of Env plays a role in the SorA-mediated HIV inhibition mechanism. The role of palmitoylation in HIV infectivity is controversial. While Rousso et al. defined env palmitoylation as critical for HIV infectivity (40) and Bhattacharya et al. showed a 60-90% reduction in infectivity with

mutants that cannot be palmitoylated (without cysteines in the C-tail of gp41) (41), Chan et al. were unable to detect a reduction in infectivity in viruses with mutant envelopes without cysteins in the C-tail of gp41 (42). A possible explanation for this discrepancy may be the transfection system used in their assays. The promotors and backbound plasmids used to pseudo-type the viruses were different in these studies. This could translate to differences in the outcomes of their assays. One possibility is that Env expression controlled by a potent promotor could over-express that viral protein. A higher amount of Env protein may increase the passive diffusion to the viral assembly sites (43, 44). In addition, an excess of Env has been shown to increase proviral gene expression that may increase viral production (32). These effects could mask a potential reduction of infectivity due to lack of Env-palmitoylation. Our data suggest that Env palmitoylation plays a role in specific infectivity reduction but the general decrease in virus production is independent of Env palmitoylation.

HIV protease inhibitors and HIV infection leads to lipodystrophy and dyslipidemia that can be the cause of cardiovascular disease and metabolic syndrome. These lipid metabolism disorders produce an increase in plasma total cholesterol, free fatty acids and triglycerides (45-48). Inclusion of fatty acid-lowering agents like SorA in the HIV treatment may reduce the accumulation of fatty acids and triglycerides and thus, the cardiovascular risks of infected patients. The advantage of using SorA with respect to other lipid-lowering drugs is the dual effect of SorA because it inhibits HIV infection and the secondary effects of the infection and treatment at the same time. Further studies should investigate whether SorA holds its promises also in vivo.

HIV and HCV infect around 35 and 150 million people worldwide, respectively. It is noteworthy that 5 million people are HIV/HCV co-infected. This co-infection worsen the outcome of the infections, with

higher mortality of patients and faster liver disease progression. The treatment of this co-infection is very complex because comprises a combination of at least 3 anti-HIV drugs and 2 or 3 anti-HCV drugs. There are multiple drug-drug interactions that complicate the selection of the drugs and increase the liver toxicities. In addition, the viruses may develop resistance to existent drugs, therefore it has been suggested that there will be a continuous need for new antivirals (49). All these problems may be reduced with the development of broad-spectrum inhibitors like SorA. An increase in fatty acid synthase levels in serum of HIV and HCV infected patients has been described. Moreover this increase is exacerbated in co-infected patients (50). This suggest that the *de novo* fatty acid synthesis pathway is used by both infections and thus, may be a promising target for broad-spectrum antiviral drug. SorA is a fatty acid synthesis inhibitor that inhibits HIV and has been already established as a very potent *in vitro* HCV inhibitor acting at low nanomolar concentrations (EC_{50}=0.7nM; SI>100000) (4). TOFA that also blocks ACC, inhibits HCV much less efficiently and with more toxicity. Other fatty acid synthesis inhibitors anti-HCV like Orlistat, Cerulenin or C75 do not completely inhibit the virus even at 10µM concentrations (22). Taken together these data indicate that HIV-HCV co-infection treatment with SorA is an interesting option that should be evaluated further.

The *de novo* fatty acid synthesis pathway is important for the replication of at least 15 different viruses including HIV, HCV, WNV, DENV, influenza, HCMV, RV, VV, EBV, VZV, VSV, SINV, CVB3, USUV and YFV (4, 19-21, 23, 24, 39, 50-52). These viruses are from 9 different families and include DNA and RNA viruses, and enveloped and non-enveloped viruses, thus, the *de novo* fatty acid synthesis is a central pathway used by very different viruses. The implication of the fatty acid metabolism in all that viral infections make SorA an attractive candidate for exploring its development as a broad spectrum antiviral drug. However, it is important to study well the secondary effects of SorA because of the

wide number of cellular processes in which fatty acid metabolism are involved (as structural elements, energy source or intracellular signalling (reviewed in 53). Another concern is the possible modification of the antiviral activity of the drugs due to diets. As we show in our assays, an exogenous addition of palmitic acid can counteract the activity of SorA. Therefore, it is important to study the effects of diets in a treatment based on fatty acid synthesis inhibitors.

SorA has been investigated in other fields as anti-cancer chemotherapy and autoimmune diseases. First, it has been seen that SorA induces cytotoxicity selectively in cancer cells *in vitro* and impairs self-renewal of cancer stem cells (54, 55). Second, SorA treatment reduces the number of Th17 cells that are implicated in several autoimmune diseases as multiple sclerosis and rheumatoid arthritis (56). Indeed, SorA treatment attenuates autoimmune diseases in vivo (57).

In conclusion, SorA has at least two inhibitory effects on the HIV life cycle. It decreases viral particle production and reduces the specific infectivity of the virions produced. The latter seems mediated by gp41 lack of palmitoylation that reduces the gp120/p24 ratio of produced virions and, in turn, the viral entry. The *de novo* fatty acid synthesis pathway is an interesting target for the development of broad-spectrum antiviral drugs. Thus, the research on SorA should continue with other viruses beyond HIV and HCV and especially in animal models. Recent publications in the fields of virology, anti-cancer chemotherapy and autoimmune diseases about SorA and the *de novo* fatty acid synthesis pathway inhibition confirm that there is a growing interest on these topics and more effort should be made for the hit-to-lead SorA development.

MATERIALS AND METHODS

Cell culture

TZM-bl cells (NIH AIDS Reagent Program, catalogue number: 8129) were maintained with DMEM medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), HEPES 25mM and 0.5% Gentamycin. HEK293T cells (ATCC, CRL-11268) were maintained in DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (P/S). MT-2, Jurkat, PM1, latently HIV-infected ACH2 cell lines (NIH AIDS Reagent Program, catalogue numbers: 237, 177, 3038 and 349, respectively) and isolated human peripheral blood mononuclear cells (PBMCs) from several donors were cultured with RPMI medium (Gibco) supplemented with 10% heat-inactivated FCS and 1% of P/S. PBMCs were activated with 5 µg/ml of PHA during 3 days prior to infection. All cell types were incubated at 37ºC and 5% CO_2.

Plasmids

pGag-EGFP (NIH AIDS Reagent Program, catalogue number: 11468) expresses HIV-1 Gag fused to EGFP. pNL-ΔEnv (a kind gift from Yasuko Tsunetsugu-Yokota, Tokyo University of Technology, National Institute of Infectious Diseases, Tokyo, Japan) expresses HIV-1 with a mutation in Env. It was used to pseudo-type HIV-1 with several envelopes. pHXB2 (provided by Prof. Dr. Christian Jassoy, Institute for Virology, University of Leipzig, Lepizig, Germany) expresses HIV-1 Lai envelope. pW61D_TCLA.71, pWITO4160, pSS1196.1, pBal.26 and pMN.3 (a kind gift from Kelli Green, Duke University Medical Center, Durham, USA) express different HIV-1 envelopes. pMM310 (a kind gift from Yasuko Tsunetsugu-Yokota) expresses E. coli β-lactamase fused to the amino terminus of HIV-Vpr. Transfections in HEK 293T and TZM-bl cells were performed with Lipofectamine 2000 (Invitrogen, Paisley, UK) or JetPEI, respectively, according to the manufacturer's manual.

Drugs and lipids

Soraphen A (SorA) is part of the myxobacterial secondary metabolites library of the Helmholtz Centre for Infection Research (HZI) in Braunschweig, Germany. The ACC inhibitor TOFA (Cayman Chemical company, Michigan, USA) was used as a control drug. The HIV protease inhibitor Lopinavir (LPN, Sigma, Missouri, USA), the non-nucleoside reverse transcriptase inhibitor Nevirapine (Sigma) and the entry inhibitor enfuvirtide (Fuzeon, Roche, Basel, Switzerland) were used as controls in several assays. The histone deacetylase inhibitor Vorinostat (Sigma) was used to activate HIV production of ACH2 cells. The fatty acids palmitic acid, lauric acid and oleic acid and the complex lipids sphingomyelin and phosphatydilserine (Sigma) were used in the lipid recovery assay. The phospholipase A2 inhibitor anthranilic acid (Sigma) was used to prevent degradation of phosphatidylserine and the ceramidase inhibitor Ceranib-2 (Sigma) was added at 1µM to prevent degradation of sphingo-myelin.

Virus stocks

HIV-1 viral stocks were produced using pNL4-3 (NIH AIDS Reagent Program, catalogue number: 114) that expresses complete infective HIV-1 or via propagation in PM1 cells or PBMCs. The viruses were ti-trated in TZM-bl cells and stored at -80°C. HIVpp with several enve-lopes were produced by transfection of HEK293T cells (see above).

Dose-response assays

TZM-bl cells were sedeed (10^4 cells/well) in 96-well flat-bottom plates and PBMCs were seeded (50000 cells/well) in 96-well V-bottom plates. They were incubated for 1h with the drug or the vehicle control (DMSO). Five 10-fold dilutions of the drug were tested in triplicate. After incubation, the plates were washed with medium and infected with HIV_{LAI} at MOI=0.5. Fresh drug was added to maintain concentration constant during the experiment. After incubation of 48h (TZM-bl) or 72h

(PBMCs) at 37°C 5%CO_2 the supernatant was used to re-infect fresh TZM-bl cells. 48h after re-infection the luciferase activity was measured using Britelite Plus^{TM} (PerkinElmer, Waltham, USA). In parallel, the citotoxicity of the compound was assessed using the commercial ATP-based system CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA). Mean luciferase values were normalized to untreated controls and effective concentration 50 (EC_{50}) and cytotoxic concentration 50 (CC_{50}) were calculated in GraphPad Prism (GraphPad Software, San Diego, CA, USA) by analyzing the log_{dose} vs. normalized response.

Lipid recovery assay

TZM-bl cells were plated (10^4 cells/well) in 96-well flat-bottom plates and incubated for 1h with 10μM of SorA or DMSO. After incubation, the plates were washed with medium and infected with HIV_{LAI} at MOI=0.5. Palmitic acid, oleic acid, lauric acid, sphingomyelin and phosphatidylserine were added at 50μM and fresh drug was added to maintain concentration constant during the experiment. Ceranib-2 and anthranilic acid were added to PS and SM treated cells at 1 and 10μM, respectively. All conditions were tested in triplicate. Luciferase activity and toxicity of the compounds and lipids were measured as explained for the dose-response assays. Results showed were normalized to the non-lipid control.

Immunofluorescence

MT-2 cells were treated with SorA 10μM, TOFA 10μM or vehicle control (DMSO). After 1h incubation, cells were spinoculated 75′ 1200g 4° C with HIV_{LAI} at MOI=05. 48h after spinoculation cells were incubated 1h on poly-L-lysine-coated microscopy glasses (Thermoscientific) and the supernatant was added to fresh MT-2 cells and incubated 48h more. Samples were fixed (30 minutes in PBS containing 4% paraformaldehyde (PFA)), permeabilized (20 minutes in 0.2% Triton X-100; Sigma)

and blocked (30 minutes with FCS 10%). Samples were stained for 1h with anti-HIV p24 antibody provided by the Centre for AIDS Reagents, NIBSC (UK), and a secondary anti-mouse IgG antibody labelled with Alexa Fluor 647 (Invitrogen) for 45 minutes in the dark, followed by a 15 minutes nuclear staining with 4,6-diamidino-2-phenylindole (DAPI). Glasses were placed in microscopy slides with Mowiol (Sigma) and images were acquired on the Leica TCS SP5 at 63x.

Assembly assay

TZM-bl cells were transfected as explained in the manual with 0.5 µg of EGFP-Gag expressing plasmid and treated with 10µM SorA. After 12, 24 and 48h incubation cells were washed and fixed (4% PFA 10min). Nucleus were stained with 1µg/ml Hoechst (Thermofisher). Z-stacks of around 30 cells at 1.22 µm axial step size were acquired with a Leica TCS SP5 confocal microscope using a 63× 1.4-NA PL APO objective (Leica Microsystems GmbH). Image processing and analysis to obtain the number of gag assembly spots was performed with Fiji software (58). Maximum-intensity projections of the stacks were obtained and they were subsequently processed by using a difference of Gaussians filter to enhance structures within the range of the two Gaussians (sigma 0.5 and 4). The resulting images were thresholded for gag assembly spots segmentation and counting. Only particles within a specified range in size (3–200 pixels) and circularity (0.4–1.00) were counted.

Transmision electron microscopy

ACH2 cells were treated with 10µM SorA, 10µM Lopinavir and DMSO (vehicle). After 1h incubation, the HIV production was activated with 10µM Vorinostat. 48h after activation cells were fix with 2.5% glutaraldehyde in 0.1M phosphate buffer incubating 2x 30 minutes at RT. After washing samples were incubated with 1.1% osmium tetroxide + 0.8%

potassium ferricyanide in phosphate buffer 1-2h at 4°C. Samples were dehydrated and infiltrated in the resine Eponate 12 as previously described (59). Pictures were taken with a transmission electron microscope (TEM) JEM 1010 100 kv (JEOL, Tokyo, Japan) with CCD Megaview 1kx1k at 80 kv and analysed with Imagej software (National Institutes of Health, NIH).

TZM-bl infectivity assay, Immunoassay and viral RNA quantification

Infectivity of supernatants from PBMCs, ACH2 cells, transfected 293T cells and cell lysates was determined with the TZM-bl assay. Cell culture supernatants or cell lysates were added to fresh TZM-bl cells. 48h after supernatant addition luciferase activity was measured as explained above. HIV p24 and gp120 proteins were detected using the ELISA kits INNOTEST® HIV Antigen mAb to detect p24 (Fujirebio, Gent, Belgium) and the HIV-1 gp120 antigen capture assay (ABL, Rockville, USA). Viral RNA was quantified using a semi-nested RT-qPCR assay. Viral RNA was extracted from cell culture supernatants using the QIAamp® viral RNA mini kit (Quiagen) according to the manufacturer´s instructions. RNA was treated with the DNaseI TURBO kit (Ambion) and quantified using the Thermo Scientific NanoDrop 2000 spectrophotometer. Between 20 to 50 ng of RNA was used for first-strand cDNA synthesis performed with the SuperScript® IV Reverse Transcriptase (Invitrogen™) and an HIV-specific RT primer containing an unique tag sequence at the 5´ end (underlined): 5'-CTGATCTAGAGG-TACCGGATCCAAAGCTCGATGTCAGCAGTCTT-3'. The cDNA was treated with RNAse H (New England Biolabs) to hydrolyze RNA:DNA bonds. Quantitative PCR was performed in the QuantStudio® PCR system (Applied Biosystems®) with SYBR® Green technology (Sigma). Primers used for the qPCR were: 5'-GCCGCCTAGCATTTCATCAC-3' (HIV-specific) and 5'-CTGAT-CTAGAGGTACCGGATCC-3' (Tag). Unless indicated, standard curves

were generated with 5-fold serial dilutions of a standard pGEM-T plasmid carrying the specific HIV amplicon. All samples were run in triplicates. To obtain cell lysates, treated (±10µM SorA and ±50µM palmitic acid) and transfected 293T cells producing HIVpp were washed with PBS and lysed with passive lysis buffer 10min at 4°C. Lysates were centrifuged (maximum speed 5′ 4°C) to remove cell debris.

CD4 binding and fusion assay

TZM-bl cells were spinoculated (2095g 4°C 30′) with 10ng p24-containing supernatant from SorA or vehicle (DMSO) treated ACH2 cells that were previously activated with Vorinostat. Cells were washed with PBS and lysed with M-PER 10′ at 4°C. p24 was detected with the ELISA kit INNOTEST® HIV Antigen mAb. Cells were maintained at 4°C all the assay to prevent virus-cell fusion. Fusion assay was performed as in (60). Briefly, HIVpp containing β-lactamase activity were produced by transfection in the presence of SorA, Lopinavir or DMSO. Jurkat cells ($2.5x10^5$ cells/condition) were spinoculated (1200g, 25°C, 2h) with 50 or 200ng p24 of HIVpp. Cells were incubated 2h at 37°C 5% CO_2. After washing, cells were loaded with the substrate for β-lactamase (CCF2-AM) at 1µM. After 1h at room temperature in dark, cells were washed, resuspended in CO_2 independent medium (Gibco) containing 5% heat-inactivated FCS and incubated overnight at room temperature in dark. Cells were washed, stained with propidium ioide (0.5µg/ml) and analyzed with a LSR Fortessa cytometer (BD bioscience). Data were analyzed with Flow Jo software (Tree Star).

Membrane fluidity assay and cholesterol amount determination

Membrane fluidity was determined as explained in (27). Briefly, Jurkat cells and previously 3-days activated PBMCs ($1.5x10^5$ cells/condition) were incubated 48h with SorA at 0.1, 1 and 5µM or DMSO (mock). Cells were stained with 1µg/ml of di-4-ANEPPDHQ (30min, 4°C) that changes its fluorescence emission depending on the fluidity of the mem-

brane. Cells were excited at 488nm and emissions at 530/30nm and 670LPnm were simultaneously recorded with a cytometer. Intensities were converted into a general polarization index (GP) using the equation $GP = (I530/30 - I670LP) / (I530/30 + I670LP)$. GP values range from +1 (more condensed) to -1 (more fluid). To detect changes in cholesterol content, Jurkat cells were similarly incubated with SorA and stained with 0.05 mg/ml of filipin (30′ at RT). The intensity of the staining is proportional to the amount of cholesterol in the cell membrane and was determined using a cytometer. Cyclodextrine (1 and 5mM, 1h 37°C before staining) was used in both assays as a positive control because depletes cholesterol that results in an increased membrane fluidity.

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FIGURES

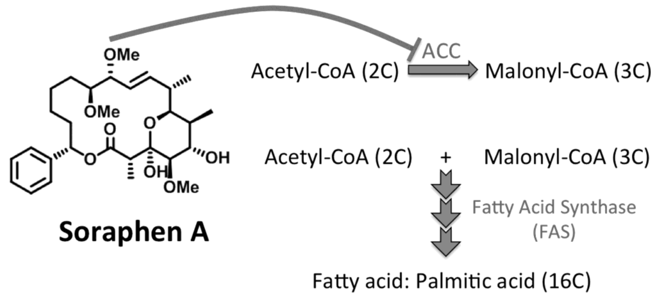


Figure 1. Soraphen A inhibits ACC and blocks the *de novo* fatty acid synthesis. SorA chemical structure and the de novo fatty acid synthesis pathway are shown.

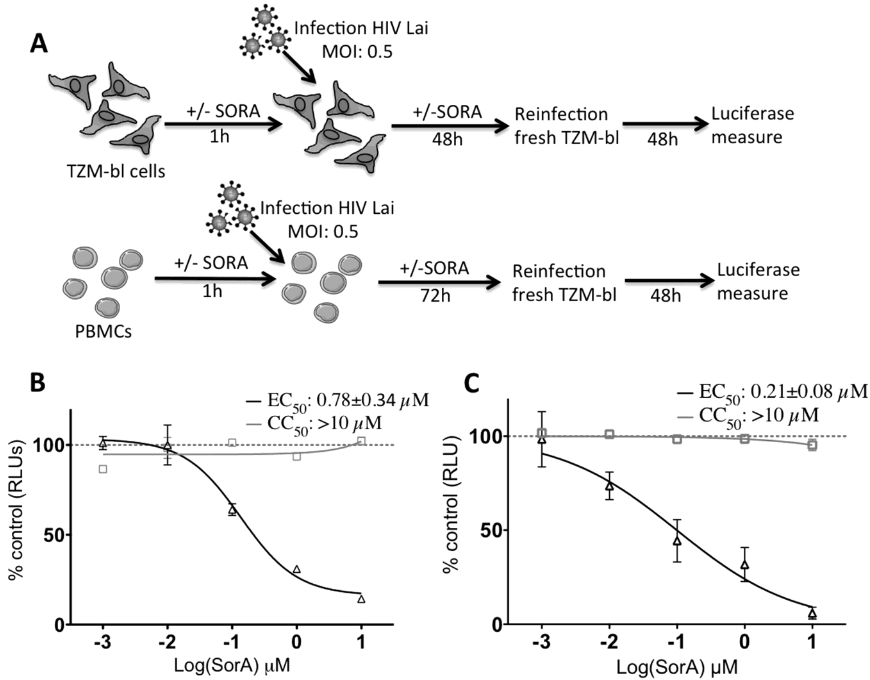


Figure 2. Soraphen A inhibits late steps of the HIV life cycle. (A) TZM-bl cells or PBMCs were seeded in 96-well plates in triplicate and treated with increasing concentrations of soraphen A. TZM-bl cells (B; n=5) or PBMCs (C; n=2) were infected with HIV-1_{LAI} at a multiplicity of infection (MOI) of 0.5. 48h after infection the supernatant was used to re-infect fresh TZM-bl cells and 48h after reinfection cells were assayed for luciferase activity. The mean relative light units (RLU) are plotted as % relative to DMSO (vehicle) for late steps infectivity (triangles) and cell viability (squares). Effective concentration 50 (EC_{50}) and cytotoxic concentration 50 (CC_{50}) were estimated by non-linear regression of log inhibitor vs. normalized response (see Materials and Methods). A representative curve is shown in every case. Error bars: standard error of the mean (SEM).

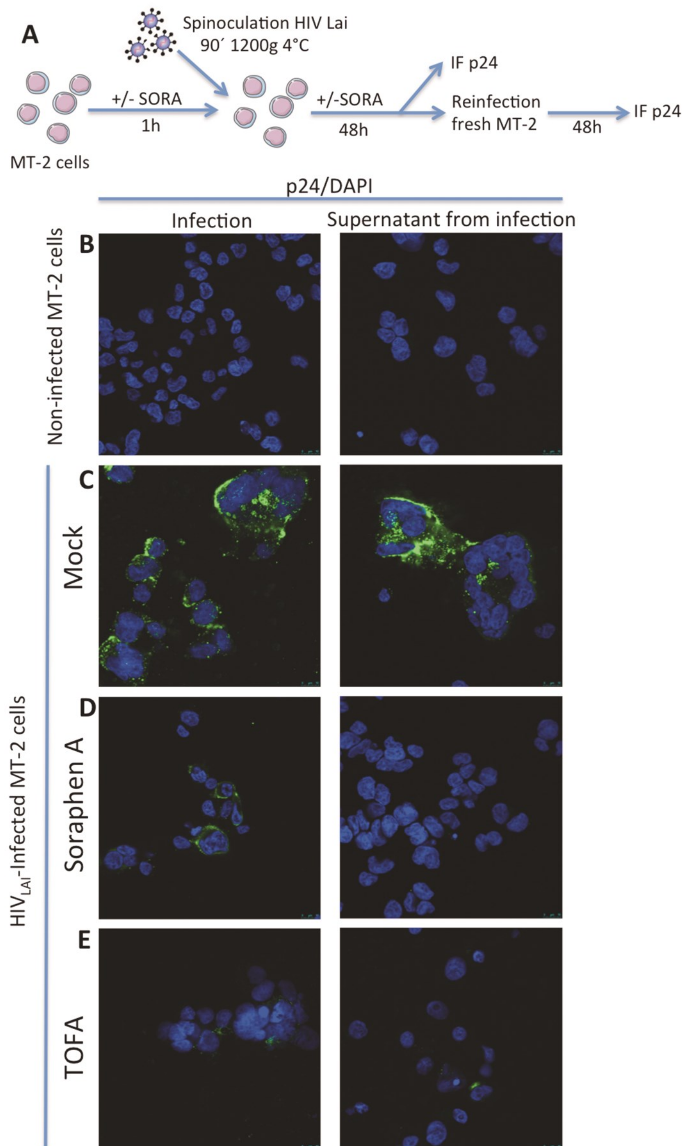


Figure 3. Soraphen A inhibits production of infectious HIV in MT-2 cells. (A) MT-2 cells were spinoculated with HIV_{LAI} and treated with soraphen A or TOFA. 48h after infection cells were fixed and stained for HIV-p24 protein (green signals) and with DAPI (blue signals) (left panels). The supernatant was collected and added to MT-2 fresh cells. 48h after supernatant addition cells were fixed and stained for HIV-p24 and with DAPI (right panels). (B) Not infected cells and (C) infected but untreated cells were used as negative and positive controls respectively. p24 distribution in infected cells treated with (D) soraphen A and with (E) TOFA is shown.

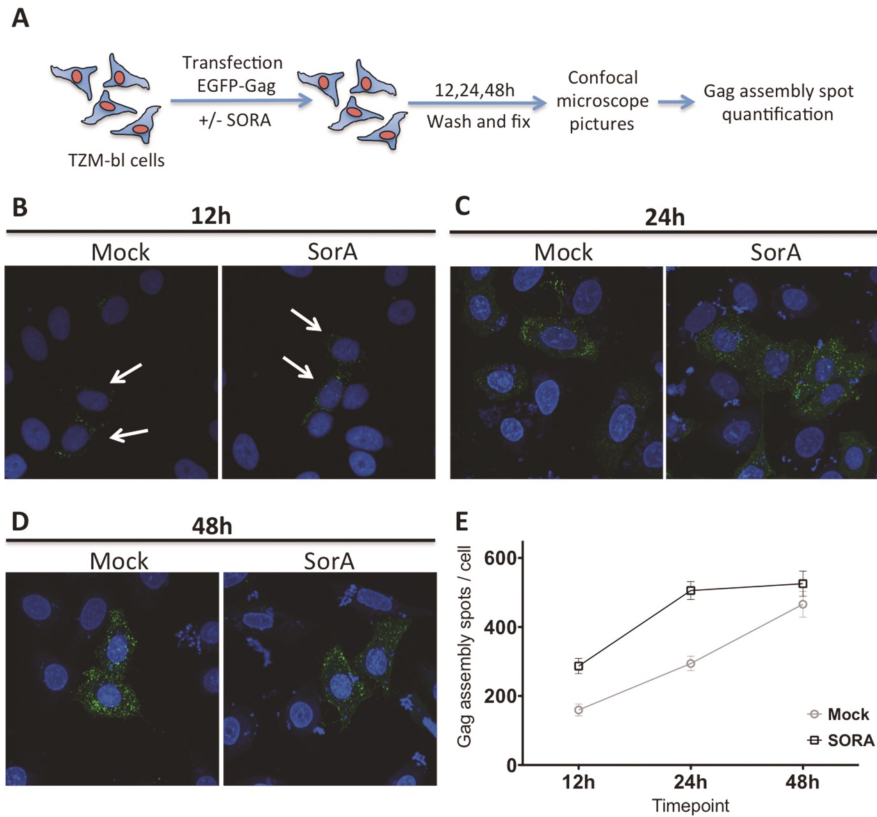


Figure 4. Gag assembly is not impaired in SorA treated samples. (A)TZM-bl cells were pretreated 1h with SorA or DMSO (vehicle) and transfected with the EGFP-Gag expressing plasmid. Cells were fixed and confocal images were taken at 12, 24 and 48h after transfection. Representative pictures of SorA and DMSO (mock) treated cells at 12, 24 and 48h (B, C and D, respectively) are shown. (E) Number of gag-assembly spots per cell from around 30 cells per condition was quantified with ImageJ 1.49g software. Arrows indicate cells with gag assembly spots. Error bars: standard error of the mean (SEM).

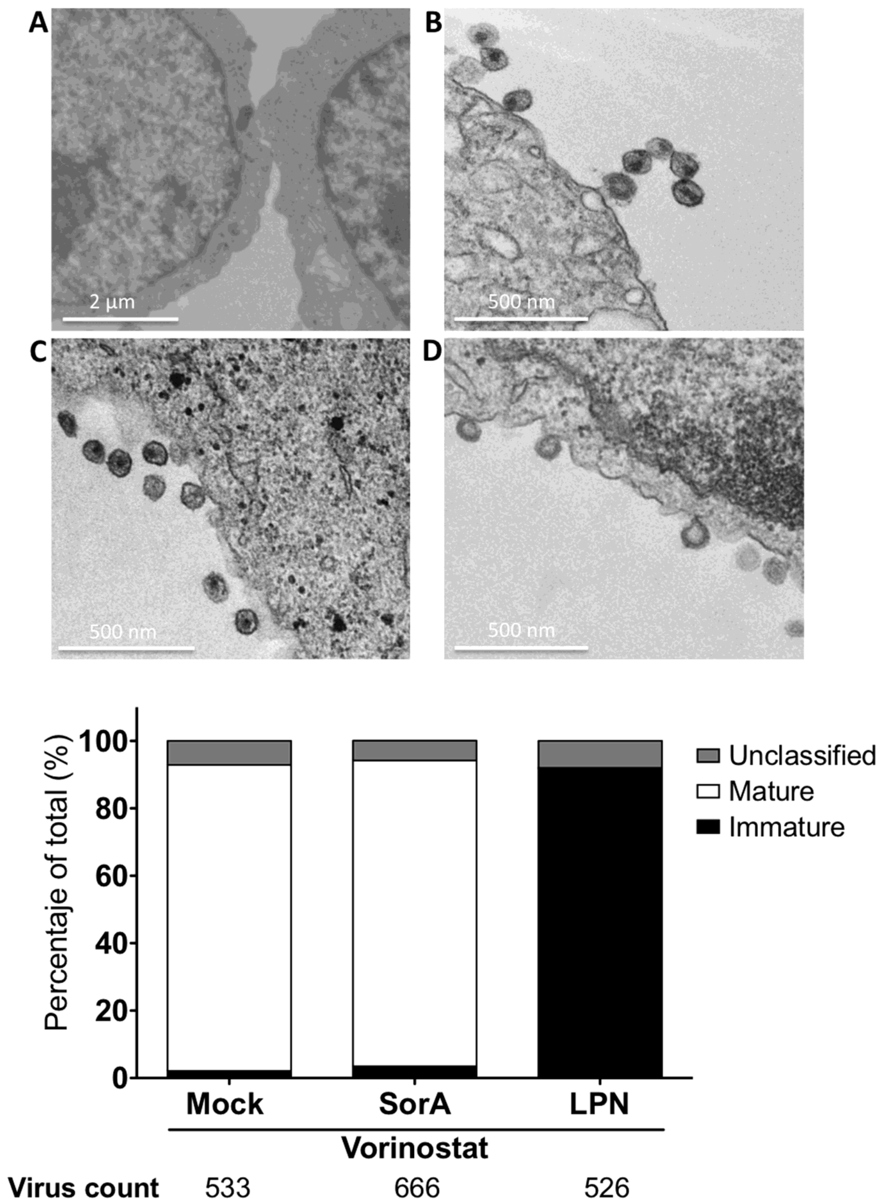


Figure 5. HIV maturation is independent of SorA treatment. ACH2 cells were activated with Vorinostat and treated with SorA (10µM), Lopinavir (10µM) or DMSO (mock). 48h after infection cells were fixed and processed for transmission electron microscopy. TEM pictures were taken of (A) Uninfected cells, (B) DMSO, (C) SorA and (D) Lopinavir treated cells. (E) Number of mature (white), immature (black) and unclassified (grey) viral particles is presented. More than 500 viral particles were counted for each condition.

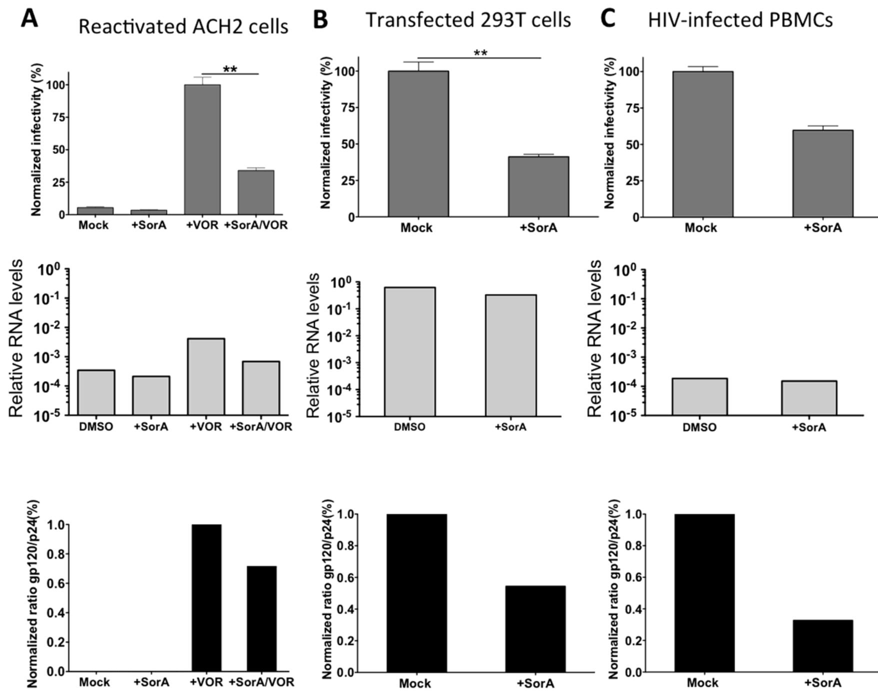


Figure 6. SorA reduces the gp120/p24 ratio in HIVpp and in virions produced from ACH2 cells and primary cells. (A) Latently HIV-infected ACH2 cells were incubated with SorA (10μM) or DMSO (mock), activated with Vorinostat and incubated 72h. After that, supernatants were pelleted (2x16000g 2h 4ºC). (B) 293T cells were incubated 1h with SorA (10 μM) or DMSO (mock) and transfected with pNLE-ΔEnv and pHXB2-env. 48h after transfection supernatant was pelleted. (C) PBMCs from two healthy donors were treated with SorA (10μM) or DMSO (mock) and infected with HIV_{LAI} (MOI: 0.5). 48h after infection supernatant was pelleted. Pelleted samples from (A), (B) and (C) were subjected to a TZM-bl assay, a qRT-PCR, and an ELISA for p24 and gp120. Infectivity (higher panels), viral RNA content (middle panels) and ratio gp120/p24 (lower panels) for the three cell systems are shown. Error bars represent the standard error of the mean. (**, p<0.01).

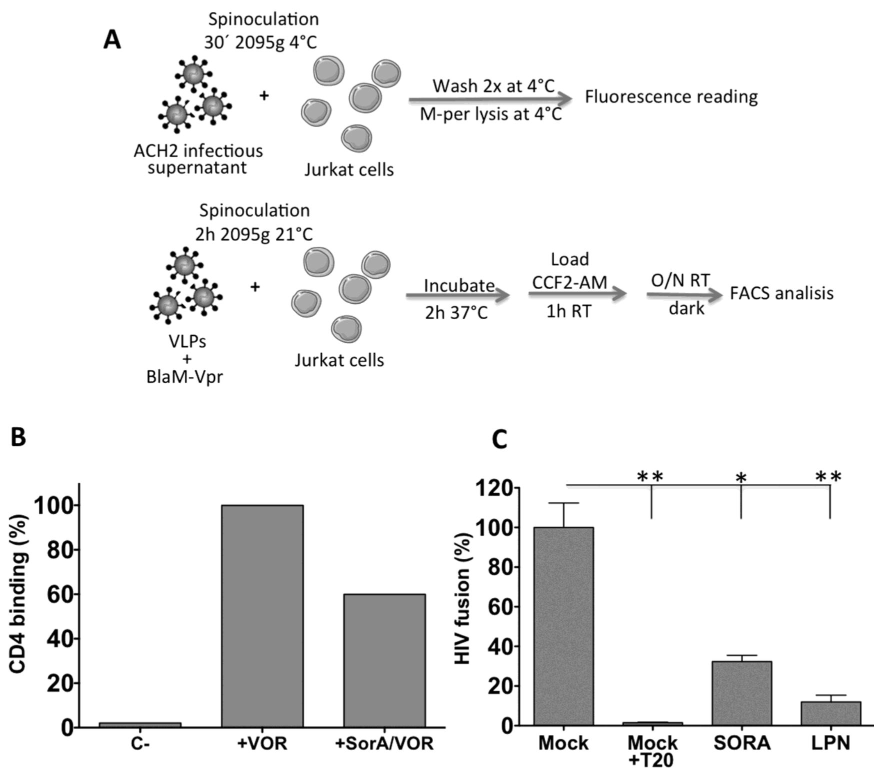


Figure 7. Viral entry is reduced in viruses produced in the presence of SorA. (A) Protocols followed for the CD4-binding and fusion assays are shown. (B) TZM-bl cells were infected by spinoculation with supernatant from ACH2 cells previously activated with Vorinostat and treated or not with SorA. The amount of p24 in the supernatant was normalized to 10ng for every condition (except C-). After washing, cells were lysed with M-Per and analysed for p24 content with an ELISA. Cells were maintained at 4ºC during the entire assay. Normalized CD4 binding is represented (n=2). (C) HIVpp were produced in the presence of SorA (10µM), Lopinavir (10µM) or DMSO (mock) with pNLE-ΔEnv, pHXB2-env and pMM310 (a plasmid that contains Vpr-BlaM). Jurkat cells were infected by spinoculation, incubated at 37ºC to allow viral fusion and loaded with CCF2-AM, the substrate for β-lactamase. Samples were incubated overnight and analysed with a cytometer (60). Normalized fusion is shown (n=5)(*, $p<0.05$; **, $p<0.01$). T20 is an HIV entry inhibitor.

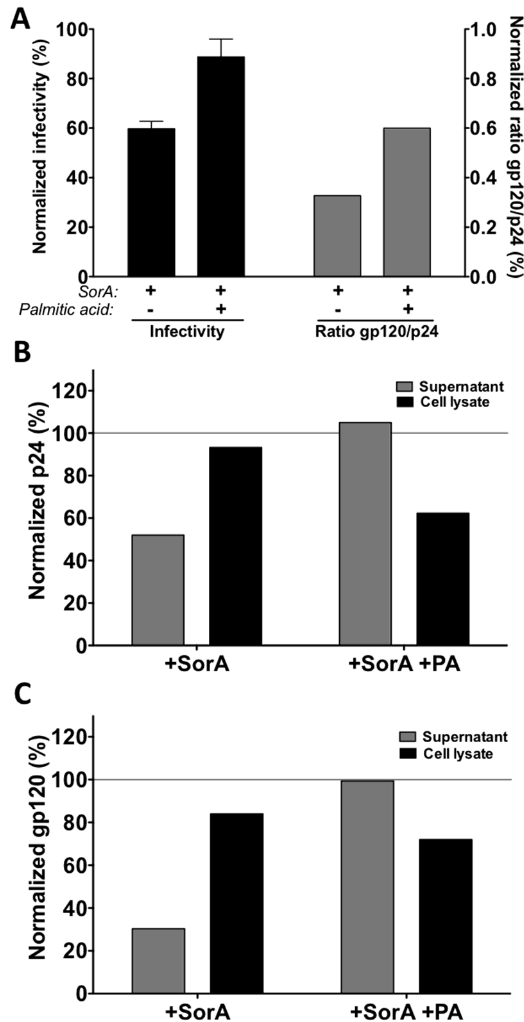


Figure 8. **Palmitic acid addition recovers infectivity in PBMCs and gp120/ p24 levels in PBMCs and in HIVpp produced under SorA treatment.** (A) PBMCs from different healthy donors were treated with SorA (10µM) or DMSO (vehicle) and infected with HIV_{LAI} (MOI: 0.5). Palmitic acid (50µM, PA) or EtOH (vehicle) were added as indicated in the figure. 48h after infection supernatant was pelleted. Pelleted samples were subjected to a TZM-bl assay (black bars) and an ELISA for p24 and gp120 to calculate their ratio (grey bars) (n=2). (B) and (C) 293T cells were incubated 1h with SorA (10 µM) or DMSO (vehicle), transfected with pNLE-ΔEnv and pHXB2 and incubated again with the drug and with or without PA. 48h after transfection supernatant was pelleted and the cells were lysed with passive lysis buffer. Both types of samples were analysed with a p24 (B) and a gp120 (C) ELISA. Pelleted supernatant data is shown in grey bars and cell lysate data is shown in black bars (n=2). In all the cases the data was normalized to DMSO+EtOH or DMSO+PA controls.

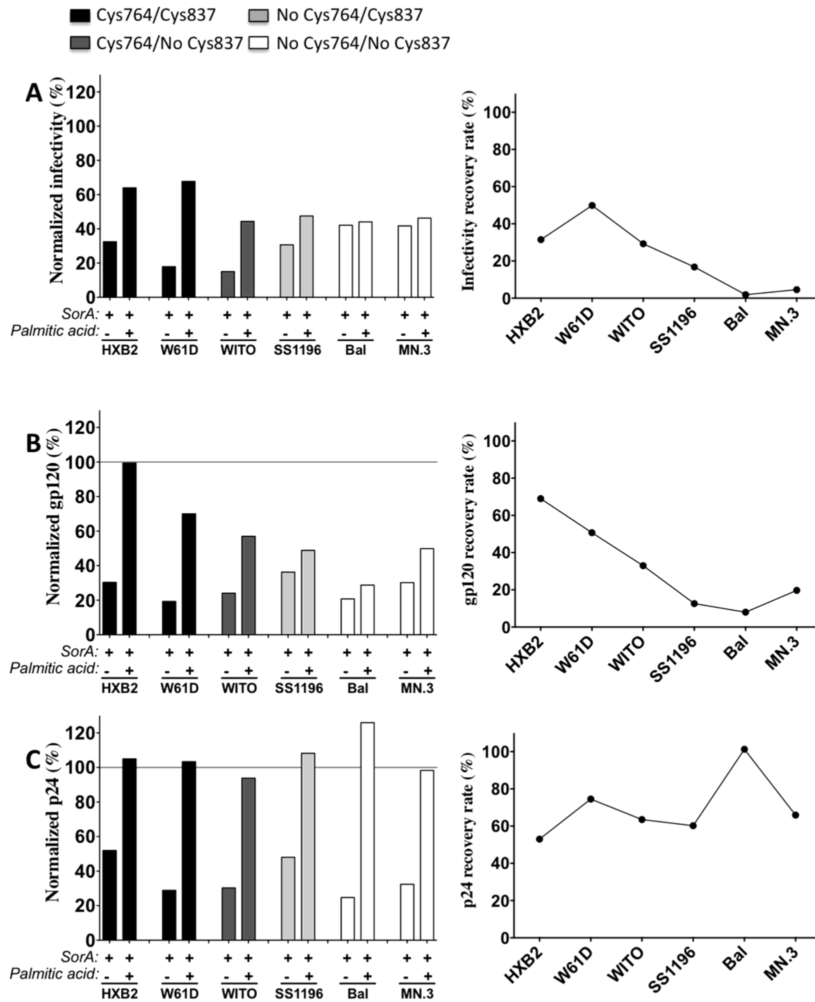
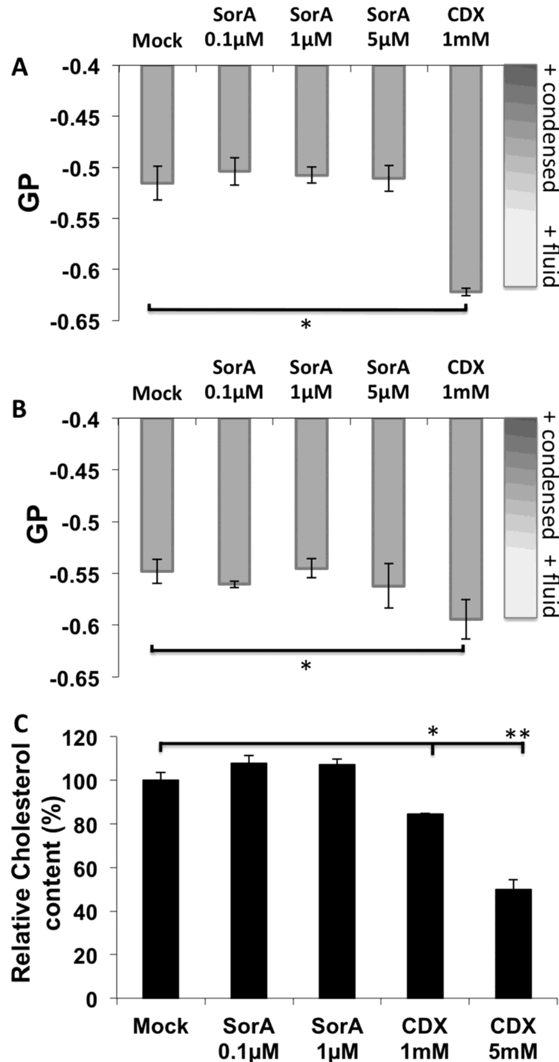
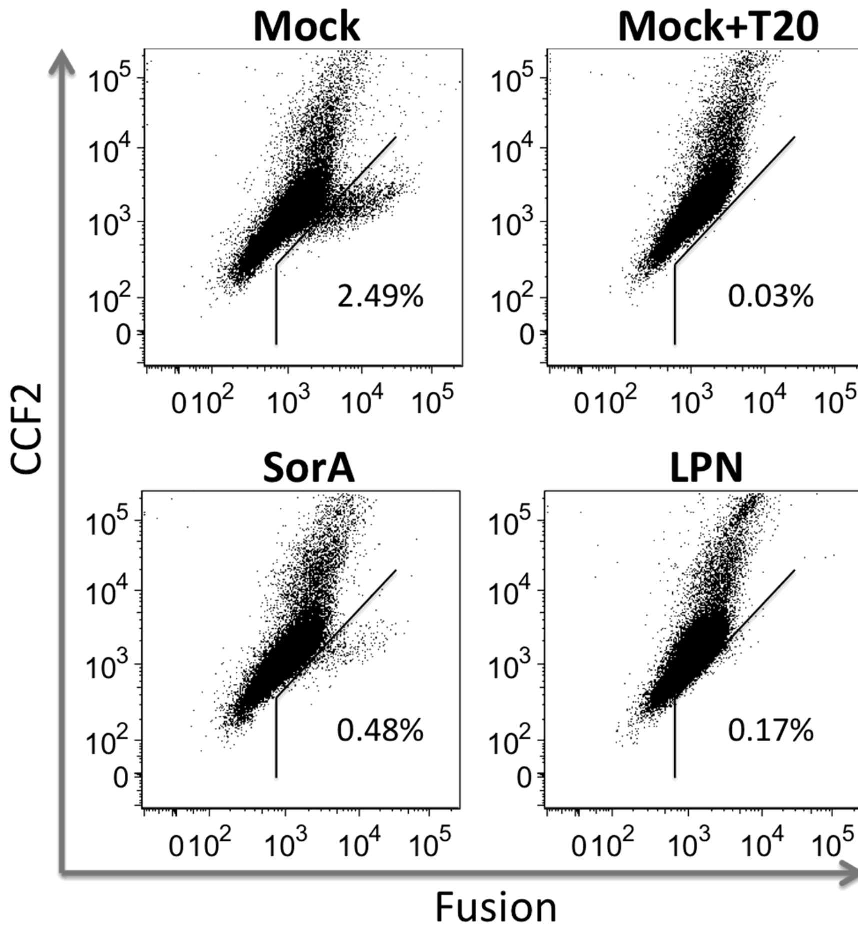


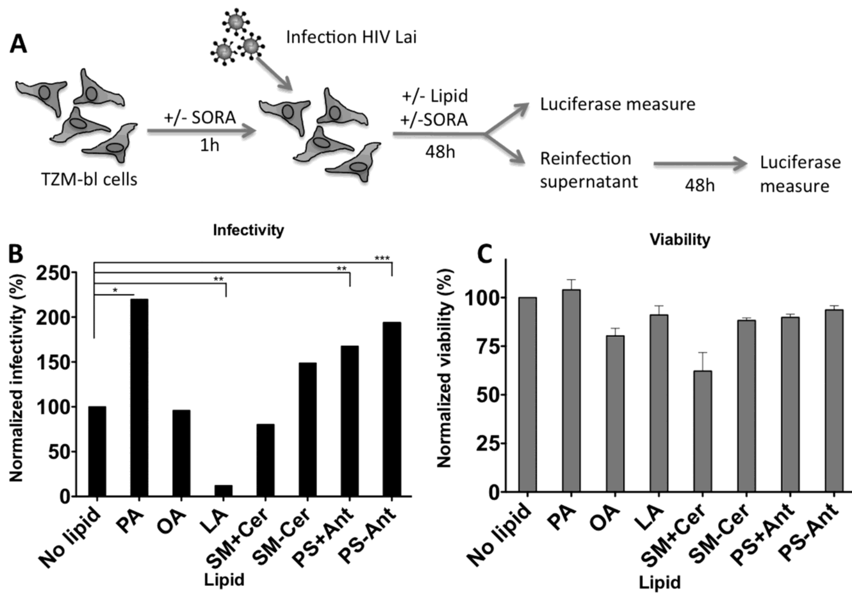
Figure 9. Recovery of infectivity and gp120 levels upon palmitic acid addition depends on the palmitoylation capacity of the envelopes. (A) HIVpp were produced by transfection of pNLE-ΔEnv and 6 different pEnvs in 293T cells in the presence of SorA(10μM) or DMSO (vehicle). Envelopes containing Cys764/Cys837 (Black bars, HXB2 and W61D_TCLA.71), Cys764/No Cys837 (Dark grey bars, WITO4160), No Cys764/Cys837 (light grey bars, SS1196.1) and No Cys764/No Cys837 (white bars, Bal.26 and MN.3) were used. Palmitic acid (PA) or EtOH (vehicle) was added after transfection. 48h after transfection supernatant was pelleted and subjected to a TZM-bl assay and an ELISA for p24 and gp120. Normalized infectivity (A, left panel), p24 (B, left panel) and gp120 (C, left panel) levels for SorA and SorA+PA treated samples are shown for every pseudo-typed HIV particle. In all the cases the data was normalized to DMSO+EtOH or DMSO+PA controls. Recovery rate of infectivity (A, right panel), p24 (B, right panel) and gp120 (C, right panel) is shown. The recovery rate was calculated subtracting infectivity, p24 or gp120 values of samples not treated with PA to the values of the samples treated with PA for each virus.



Supplementary figure 1. SorA treatment does not change membrane fluidity or cholesterol content in Jurkat cells or PBMCs. Jurkat cells and PBMCs were incubated 48h with different concentrations of SorA. (A) Jurkat cells and (B) PBMCs were stained with di-4-ANEPPDHQ to detect changes in membrane fluidity. (C) Jurkat cells were stained with filipin to detect changes in cholesterol content. Cells were analyzed with a cytometer. Cyclodextrin (CDX) was used as a positive control in both assays. Generalized polarization index (GP) values for membrane fluidity and relative cholesterol content are shown. Error bars: standard deviation (SD). (*, $p<0.05$; **, $p<0.01$).



Supplementary figure 2. SorA inhibits HIV fusion. Representative flow cytometry plots from analysis of data obtained for the fusion assay (figure 7C) are shown. Proportion of fusion respect to CCF2 loaded cells in SorA, LPN, mock and mock+T20 treated samples is shown.



Supplementary figure 3. Palmitic acid and phosphatydilserine recover HIV infectivity in SorA treated cells. (A) TZM-bl cells were seeded in triplicates, treated with SorA or DMSO (vehicule) and infected with HIV_{LAI} at MOI=0.5. Palmitic acid (PA), oleic, acid (OA), lauric acid (LA), sphingomyelin (SM) and phosphatidylserine (PS) were added at 50μM. Ceramidase (Cer) and anthranilic acid (Ant) were added to inhibit degradation of SM and PS, respectively. 48h after lipid addition the supernatant was used to re-infect fresh TZM-bl cells. 48h after re-infection luciferase activity was measured. Cell toxicity was measured in parallel. Normalized (B) infectivity and (C) viability respect to non-lipid treated cells is shown. Error bars: standard deviation (SD). (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

DISCUSSION

Screening

Myxobacteria are bacteria from soil that produce a wide range of secondary metabolites (22). These metabolites have uncommon structural elements (112) with around a 40% of myxobacteria compounds described having a novel chemical structure (113). In addition, myxobacteria metabolites are known to have a wide range of biological activities including anti-cancer, antifungal and antibacterial activities (22). Thus, the anti-HIV hits detected in the screening presented in this thesis have additional biological activities. For example, epothilones are approved for cancer treatment and also have anti-fungal activity (111, 114). Others as apicularen and archazolid inhibit the V-ATPase that result in inhibition of HIV, HCV and HPV (this thesis and 115, 116). The unique and varied chemical structures of myxobacteria secondary metabolites make the myxobacteria library a good source of potential candidates for broad-spectrum, small chemical antivirals (117).

High-throughput screening assays as the one presented in this thesis are very powerful tools to find new antivirals (118). However, the number of hits confirmed in follow up assays may be low due to false-positive and false-negatives obtained in this kind of screens (119). Therefore, how to obtain reliable hits is a matter of discussion and different approaches have been proposed to improve the quality of the data obtained in high-throughput screenings (120, 121). For example, Smith et al found differences depending on the drug-delivery methods in the plates where the screening is performed (122), while Malo and colleagues use a statistical approach to analize the positional effects of wells within the drug-testing plates and recommended to alternate positive and negative controls (119). Although our screening was not free of these difficulties, the antiviral activity of 5 of the compounds was confirmed in follow-up assays. SI values in 4 of the confirmed compounds (sulfangolid C, soraphen F, epothilon D and spirangien B) is >15, while the SI of

kulkenon is around 5. These SI values are comparatively low with respect to FDA-approved drugs like Nevirapine (SI>1000) but similar to SI values observed in other natural compounds of different origins (123-125). Interestingly, it is generally observed that when the SI value of a compound is higher than 10, the biological effect is not due to in vitro toxicity (126). Besides this, the natural products tested in the here described screening may be optimized by chemical modifications to increase their antiviral effect and/or decrease their toxicity. Furthermore, discovery of the pharmacophore of every molecule could lead to the design of less complex derivatives with better pharmacokinetic properties, more potent HIV inhibition and a facilitated large-scale production of the drug.

Ratjadone A

Ratjadone A (RatA) inhibits HIV at the nanomolar range with an EC_{50} of 1.7 ±0.2nM. Interestingly, the intrinsic potency of RatA is higher than that of leptomycin B (EC_{50}= 6.8 ±0.8nM), another CRM1 inhibitor, even when the mechanism of action is similar (127, 128). Attempts to inhibit the Rev-RRE interaction with DAAs like neomycin B (129) and diphenylfuran derivatives (130) that bind to the RRE structure of viral RNAs have shown low antiviral activity with an EC_{50} in the millimolar range while the EC_{50} of FDA-approved drugs like Nevirapine is in the nanomolar range. In addition, the development of resistance will be probably fast as in other DAAs. In contrast, targeting some of the numerous host factors related with HIV-Rev activity such as DDX3 or other members of the helicase family (131) could reduce the risk of resistance development and broaden the spectrum of antivirals against viruses like HTLV that also need the CRM1-mediated nuclear export pathway.

RatA inhibits HIV by blocking the CRM1-mediated nuclear export of unspliced and partially spliced viral RNA as is shown in this thesis. Interestingly, this pathway is shared by another retrovirus namely HTLV (132-134). It has also been implicated in viral replication of (+)ssRNA flaviviruses such as DENV (135) and HCV (136), of the dsDNA HPV (137-139) and of the (-)ssRNA influenza virus (140). Thus, the wide range of viruses that use the CRM1-mediated nuclear export pathway makes it a good target for a broad-spectrum antiviral drug. However, the toxicity of RatA detected in our assays in cell lines (CC_{50}=4.6 ±0.7nM; SI=2.7) is high compared to FDA-approved drugs or other hits detected in the screening presented in chapter 1 of this thesis like sulfangolid C, soraphen F, epothilon D and spirangien B (SI>15). Interestingly, a tumour cell selective cytotoxicity has been reported when CRM1 is inhibited (141, 142). Since only cancer-derived cell lines (TZM-bl from cervical cancer) were used in my experiments to measure toxicity of RatA, the possibility of a lower toxicity in primary cells or *in vivo* cannot be excluded. This possibility should be tested in further studies because the anti-HIV activity of RatA is directed against a novel cellular target that may also be used for cancer therapies.

CRM1 is overexpressed in several types of cancer (including cervical cancer) (143) and selective cytotoxicity induced by blocking of the CRM1-mediated nuclear export has been shown for ovarian tumour cells and chronic lymphocytic leukemia (142, 144). In addition, *in vivo* assays with T-cell leukaemia models (145), and breast and ovarian cancer mouse models (146) have shown an effective anti-tumour activity of the CRM1 inhibitors KPT-185 and KPT-330. Finally, it is known that topoisomerase II exportation from the nucleus to the cytoplasm by CRM1 prevents death of multiple myeloma cancer cells when treated with topoisomerase II inhibitors like doxorubicin and/or etoposide (143). Multiple myeloma cancer cells can be sensitized to topoisomerase II inhibitors if they are treated with CRM1 inhibitors such as ratjadone. Thus,

the combination of topoisomerase II and CRM1 inhibitors improves the anti-tumour treatment efficacy (147, 148). Together these data indicate that ratjadone A may be an interesting candidate for the development of a broad-spectrum antiviral and a chemotherapeutic agent against tumours.

Soraphen A

The data shown in chapter 3 of this thesis suggest that the SorA-mediated mechanism of HIV inhibition is to impair the packaging of Env into newly produced virions. The block of viral entry (specifically CD4 binding) of HIVpp produced in the presence of SorA agrees with such a mechanism. Nevertheless, a general reduction in p24 and viral RNA levels is also detected. This suggests that other mechanisms different than the lack of Env may also participate in HIV inhibition by SorA. Indeed, alternative mechanisms of HIV inhibition by blocking fatty acid synthesis have been described for other viruses. For example, the main function of fatty acid synthesis in vaccinia virus infection is to store energy in form of fatty acids that will be used for viral infection (149). In addition, the SorA-mediated mechanism of HCV inhibition is the disruption of the membranous web in which the virus replicates (105). This demonstrates that lipid-containing intracellular structures can be affected by SorA treatment. Thus, three general functions for fatty acid synthesis in viral infections show up. They are used as an (i) energy source, (ii) structural elements and (iii) for acylation of proteins (105, 149, 150). Indeed, inhibition of fatty acid synthesis produces a complex scenario with multiple players and interactions that makes the description of viral inhibitory mechanisms a difficult issue. Most likely viral inhibition is a complex combination of the effects of blocking different functions of fatty acid synthesis in viral life cycle.

HIV-Env can be palmitoylated at two positions in its intra-cytoplasmic

C-terminal tail (cysteines 764 and 837). However, the presence of cysteine at those positions is not completely conserved. An 87.4% of 3433 different HIV isolates of the M group (non-recombinants) carry C764, with just 40% of isolates having C837. The cysteine conservation level varies among clades of M group viruses ranging from 100% C764 of clades F, J and K to 78.2% of clade B. It seems that hydrophobic aminoacids (such as phenylalanine) could mimic the effect of cysteine palmitoylation (151). Indeed, the percentage of phenylalanine substitutions at the 764 position seems to support this notion. The number of isolates of clade B with either cysteine or phenylalanine at 764 position increases to 95%. In the case of all group M viruses, the percentage rises from 87.4% to 96.3%. Thus, cysteine aminoacids or palmitoylation-mimic residues are very conserved among HIV isolates suggesting an important function for Env-palmitoylation in the HIV life cycle.

Incorporation of Env into assembly platforms at the cell membrane and into nascent virions is in part mediated by the interaction between the N-terminal domain of Gag and the C- terminal tail of Env (39). In addition, palmitoylation of the C-tail of Env has been suggested to be required for Env incorporation into virions (150). The data presented in this thesis suggest that lack of Env incorporation into virions produced in the presence of SorA may be related to the palmitoylation capacity of the viral Env protein. After palmitic acid addition the recovery of gp120 levels and HIV infectivity of viruses with full palmitoylation capacity (i.e. C764/C837 containing Env-pseudotyped viruses, Env from W61D and HXB2 isolates) is higher than that of viruses with less palmitoylation capacity, i.e. only with 1 cysteine (Env of WITO4160 and SS1196 isolates) or without cysteine in positions 764 and 837 (Env from Bal and MN.3 isolates). On the contrary, recovery of p24 levels seems to be independent of the palmitoylation capacity of the viruses. Remarkably, the role of palmitoylation in the HIV life cycle is still controversial. Rousso and colleagues found that Env palmitoylation is

essential for the protein to target the cell membrane assembly platforms and therefore to produce infectious HIV viruses (150). Similarly, Bhattacharya et al showed that palmitoylation of Env is required for targeting the assembly platforms and found a reduction of 60 to 90% in infectivity of Env mutants without cysteines in the C-terminal tail (151). On the contrary, Chan et al. showed that palmitoylation of Env is neither needed for Env targeting to assembly platforms nor for production of HIV infectious viruses (152). The differing results presented in those publications could be due to the system used to obtain the data. In the three cited cases, Env-pseudotyped viruses (with or without cysteines at positions 764 and 837) were produced by transfection but the promoters and backbound plasmids used were different. This may translate into different outcomes in their experiments. A more potent promoter could over-express Env compared with transfections with a less potent promoter (153). Hence, a higher amount of Env in the cell may increase the passive diffusion of that protein to assembly platforms independently of palmitoylation as has been proposed (154, 155). In addition, it has been seen that an increase of Env expression leads to an increase in proviral gene expression due to the activation of the transcription factor NF-κB. Consequently, the viral production may have been increased (156). This effect may mask a potential decrease in infectivity due to lack of palmitoylation. In the studies presented here palmitoylation seems to play a role in the infectivity of viruses produced under SorA treatment but some other factors appear to participate since viruses that can not be palmitoylated are also inhibited. Thus, the inhibitory effects of SorA cannot be completely attributed to lack of Env palmitoylation.

HIV-HCV co-infections are a major health problem as explained in the introduction (see section 4). The worsened outcome of the diseases, the complexity of the treatment, the high cost of the co-infection therapy and the development of resistance (85) may be reduced if broad-spectrum antiviral drugs such as SorA are developed further and reach the market.

Interestingly, it has been seen that fatty acid synthesis is increased in HIV or HCV infection and this increase is exacerbated in co-infected patients (157). This suggests a participation of the fatty acid synthesis pathway in both viral life cycles and opens the door to target that pathway to develop a broad-spectrum antiviral drug against HIV and HCV. In this work, we show that SorA inhibits HIV and its potency against HCV (EC_{50}=0.7nM; SI>100000) was recently described (105). Other ACC inhibitors as TOFA were much less efficient and more toxic. Orlistat, Cerulenin and C75 are inhibitors of the fatty acid synthase that also block the fatty acid synthesis but they cannot inhibit HCV completely even at 10µM concentration (158). Our data together with the HCV inhibitory data suggest that SorA is a good candidate for inhibition of HIV-HCV co-infections and should be evaluated further with respect to pharmacological properties in vivo.

The blocking of fatty acid synthesis by SorA inhibits HIV and HCV. Interestingly, it is known that at least 15 viruses from 9 different families need fatty acid synthesis for their replication cycles including WNV, DENV, influenza, HCMV or RV among others (106, 159-161). These viruses include DNA and RNA viruses and enveloped and non-enveloped viruses. Thus, it is clear that the fatty acid synthesis pathway is widely used in viral infections and targeting this pathway to obtain broad-spectrum drugs seems suitable. SorA may be a good candidate to manipulate the fatty acid metabolism and to inhibit these viruses. However, the potential secondary effects of SorA or other fatty acid synthesis inhibitors have to be studied very carefully because fatty acids have very important cellular functions. They are an energy source, structural elements and are involved in intracellular signalling (162). Another potential problem is that a diet rich in fatty acids (especially palmitic acid) might counteract SorA activity. Indeed, we observed an increase in infectivity in SorA-treated samples after palmitic acid addition (especially in Env-pseudotyped viruses with full palmitoylation

capacity). This effect should be taken into account and a diet control of patients should be addressed if needed. In conclusion, targeting the *de novo* fatty acid synthesis may be a good option for a broad-spectrum drug inhibiting HIV, HCV and many other viruses. Consequently, studies in viruses different than HIV and HCV to expand the range of antiviral activity of SorA and studies in animal models should be performed to continue the development of SorA.

HIV and protease inhibitors included in HAART are known to produce lipodystrophy and dyslipidemia with an increase in plasma total cholesterol, free fatty acids and triglycerides (163, 164). These lipid metabolic disorders together with i.e. HCV infection as in HIV-HCV co-infected patients may produce metabolic alterations including insulin resistance, type 2 diabetes and liver steatosis and fibrosis. These alterations increase the risk of cardiovascular diseases (for review see 165). Lipid-lowering agents such as statins that inhibit cholesterol synthesis have been explored in their combination with anti-HIV and anti-HCV antivirals. It was observed that for example simvastatin and lovastatin cannot be administered to patients treated with PIs or NNRTIs due to the increase of toxicity (166, 167). Indeed, serum levels of simvastatin were increased up to 30-fold if administered with saquinavir/ritonavir (168). The inclusion of SorA in the treatment of HIV-HCV co-infected patients may reduce the levels of free fatty acids and triglycerides and, consequently the cardiovascular risk of the patients. The advantage of using SorA with respect to other lipid-lowering drugs is that SorA inhibits HIV, HCV and fatty acid synthesis at the same time. The use of SorA to treat patients with lipid disorders is another interesting path to follow.

SorA has been studied as inhibitor of tumours and autoimmune diseases. Cancer cells are highly active metabolically and proliferate very quickly. Thus, they need more energy for the metabolic processes and more

structural elements than slow proliferating normal cells. Therefore, inhibition of fatty acid synthesis should affect tumour growth. Indeed, it has been seen that ACC activity is required for growth of cancer stem cells and cancer prostate cells. The ACC inhibitor SorA induces cytotoxicity selectively in prostate cancer cells and impairs self-renewal of cancer stem cells (169, 170). Finally, it has been seen that the *de novo* fatty acid synthesis controls the fate between regulatory T cells and Th17 cells. Th17 cells are implicated in several inflammatory and autoimmune diseases as multiple sclerosis and rheumatoid arthritis (171). Remarkably, ACC inhibition by SorA reduces the number of Th17 cells and attenuates autoimmune diseases *in vivo* (172). Viral inhibitory data together with the data in these other fields confirm that manipulation of the *de novo* fatty acid synthesis, particularly with SorA, is still a hot topic that needs further attention.

Broad-spectrum HAAs development

Some important points should be addressed when searching for new broad-spectrum HAAs. First, the detection of host factors involved in viral infections and particularly the description of their relative importance in viral life cycles should be improved. For example a ranking of required host factors for HIV-rev function has been performed and may be expanded to complete viral life cycle (131). This would priories targets worth further investigation. Second, drug screens against different viruses should be performed trying to keep the amount of false positives and negatives low (119, 121). As the analysis of hits is costly in time and money, false positives produce a high waste of resources while by false negatives, potential antivirals might be lost. Finally, effective collaborations should be started to test rapidly confirmed hits for toxicity in vivo and thus, discard candidates that have a low potential to reach clinical trials. The coming toxicity screening tools in zebrafish seem very interesting in this context (173, 174).

To identify broad-spectrum antiviral compounds with the drug → host factor strategy, the screening results for different viruses have to be compared. In our case, anti-HIV data was compared with results from an anti -HCV screening of the same myxobacteria metabolite library (116). 14 compounds of 28 hits detected in our anti-HIV screening have overlapping antiviral activity with HCV. Importantly, Gentzsch and colleagues only tested 114 compounds whereas 154 compounds were tested by us. Therefore, more compounds with overlapping activities are to be expected.

Toxicity is a major concern in HAAs development but it might be handled. Good examples of HAAs that have "acceptable" toxicities are Maraviroc (already approved by the FDA for HIV treatment) and Alisporivir (phase III clinical trials against HCV). The latter is not the only HAA against HCV that is being tested. There are 3 compounds in phase II, 2 in phase I and 4 in mouse models targeting host factors involved in entry, replication and assembly of HCV (18). As far as we know, there are no further HAAs against HIV in clinical trials. This lack of commercial interest maybe related to the biology of HIV persistence. Until today, HIV cannot be completely eliminated from its infected host and thus HIV-infected individuals require life-long treatments. Therefore, anti-HIV drugs should have the lowest possible toxicity. Other viruses like HCV with RNA genomes can be completely eliminated from an infected host and thus may need less strict requirements. As antiviral drug toxicities also depend on the duration of the treatment, side effects have to be carefully considered in relation to the pathogenicity of the virus, its potential for persistence and its elimination kinetics within infected hosts. Acceptance of drug toxicities may then be ranked, with acutely lethal viruses like Ebola at the top and long-term persistent viruses like HIV at the bottom. This should give a sufficient space to develop HAAs with broad-spectrum activities for several applications.

In summary, this thesis describes a high-throughput anti-HIV assay and applies it to screen a small library of myxobacteria. Two compounds named ratjadone A and soraphen A that had the potential of being active against a range of viruses were selected and further analyzed with respect to their mechanism of action. Follow-up studies should now (i) evaluate the therapeutic ranges of RatA against HIV in primary target cells and cancer cells, and (ii) test the antiviral activity of SorA in relevant animal models. Results from these experiments will be decisive for subsequent drug development efforts.

CONCLUSIONS

From the results presented in this thesis, the following conclusions can be drawn:

1. Myxobacteria metabolites with anti-HIV activity and low toxicity were identified as hits using the high-throughput anti-HIV screening developed during this thesis.

2. Ratjadone A inhibits HIV at a nanomolar range by blocking the CRM1-mediated nuclear export.

3. Toxicity of ratjadone A in TZM-bl cells discourages to continue research with this drug. However, toxicity in primary cells should be tested.

4. Soraphen A inhibits HIV at a submicromolar range mainly by altering the composition of viruses produced under the drug treatment.

5. Soraphen A is a promising candidate for a broad-spectrum antiviral because it inhibits the *de novo* fatty acid synthesis pathway that is used by 15 different viruses and shows low cytotoxicity.

6. Development of broad-spectrum drugs targeting host factors is an achievable objective and the hits obtained in our screening are interesting starting points for further evaluation.

APPENDICES

1.- Abbreviations

ACC	Acetyl coenzyme A carboxylase
AIDS	Acquired immune deficiency syndrome
ALEM	Acidic lipid-enriched microdomains
CHIKV	Chikungunya virus
DAA	Direct-acting antiviral
DENV	Dengue virus
EBV	Epstein-Barr virus
HAA	Host-acting antiviral
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
HTLV	Human T-lymphotropic virus
MERS-CoV	Middle east respiratory syndrome-coronavirus
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PS	Phosphatidylserine
RatA	Ratjadone A
RV	Rotavirus
SARS-CoV	Severe acute respiratory syndrome-coronavirus
SorA	Soraphen A
TEM	Tetraspanin-enriched domain
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
VZV	Varicella-zoster virus
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

2.– Patent

I am part of an invention disclosure of an antiviral in preparation for patent application filing.

"ANTIVIRAL AGENTS COMPRISING OLIGONUCLEOTIDES CONTAINING G-QUADRUPLEXES"

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3.- Other publications during this thesis

Low seroprevalence of West Nile virus in blood donors from Catalonia (Spain)*

M. Piron, A. Plasencia, E. Fleta-Soriano, A. Martinez, J.P. Martinez, N. Torner, S. Sauleda, A. Meyerhans, J. Escalé, A. Trilla, T. Pumarola, and MJ. Martinez

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APPENDICES

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