Targeting the HIV-1 Nucleocapsid Protein to fight Antiretroviral Drug Resistance

Final Report Summary - THINPAD (Targeting the HIV-1 Nucleocapsid Protein to fight Antiretroviral Drug Resistance)

Executive Summary:
The Joint United Nations Program on HIV-1/AIDS (UNAIDS) estimated that at the end of 2015 there were approximately 36.7 million people infected by HIV and about 40 million people have died of AIDS-related causes since the beginning of the epidemic. During the last 25 years the arsenal of drugs to fight HIV-1 infection has increased continuously. However, most of these drugs experienced clinical failure due to Drug Resistance (DR), i.e. the ability of HIV-1 to mutate and overcome the effects of a given drug to prevent its replication. DR has been the most critical limitation in antiretroviral therapy since its advent, as substantiated by the World Health Organization in establishing the “Global HIV-1 Drug Resistance Network”. Although strategies to fight DR may span from the development of an effective vaccine to target host proteins essential for HIV-1 replication, the most commonly and realistic adopted strategy is still the design of potent and selective inhibitors of target proteins. However, novel compounds developed as inhibitors of well-known HIV-1 target proteins often lack full activity because of DR. Thus, future anti-HIV-1 drug research efforts should focus on new HIV-1 targets. In this context, the objective of the THINPAD project is to discover and develop novel anti-HIV-1 agents targeting the HIV-1 Nucleocapsid protein (NC), which is one of the most conserved sequences within HIV strains and is highly required for HIV-1 replication, being therefore a primary target to overcome antiretroviral DR.

On these bases, the research output of the THINPAD, funded by the European Commission, have been the following:
1) We have designed and developed novel NC Inhibitors (NCIs) which are active against commonly occurring HIV-1 drug resistant strains and may lead to new clinical candidates. These compounds have been optimized in the Pharmacokinetics (PK) and ADMETox (Absorption, Distribution, Metabolism, Excretion and Toxicity) profiles.
2) We have clarified the mechanism of action of candidate NCIs and defined DR pathways induced by NCIs when employing different HIV strains. Conclusions: a) NCIs were able to slow down the cTAR/dTAR annealing reaction, suggesting that the hits compete with cTAR and dTAR for binding to NC. b) Using a combination of assays monitoring the late phase of the HIV-1 cycle, we found that GagNC should be a major target of the tested compounds resulting in inhibition of the steps where Gag is involved. One of the main
consequences of these hits is an inhibition of the production of cell-free viral particles. c) Only one compound I-0155069 showed a concentration-dependent inhibition of Reverse transcriptase (RT) activity indicating that this compound targets both RT and NCp7. d) All tested compounds (2 aminothiazoles + 4 pyrimidines) show moderate inhibition of integrase (IN), with IC50 values in the range of 10 – 250 µM.

3) We have developed novel very promising anti-HIV-1 candidates by following a SME (Small and Medium Sized Enterprises) oriented research plan. One patent application has been filed by the THINPAD partners, demonstrating that the consortium was able to translate research results into industrial application.

Project Context and Objectives:
One of the most widely accepted strategy to fight antiretroviral Drug Resistance (DR) is to target HIV proteins that are highly conserved among phylogenetically distant viral strains and do not evolve in response to pressure by currently used drugs, or to target viral proteins deputed to interact with highly conserved DNA or RNA motifs. In fact, these structures hardly mutate since mutations often result in complete loss of virus infectivity, thus determining a potent antiretroviral effect. Among these proteins, the HIV-1 nucleocapsid protein (NC) is a highly conserved protein in diverse HIV subtypes that plays a central role in virus replication. Of particular interest is the fact that several point mutants of this protein lead to fully non-infectious defective viruses and that viruses resistant to a first generation of anti-HIV molecules targeting NC could not be selected, despite extended efforts to generate them in cell culture. In addition, NC appears to exert multiple roles, chaperoning reverse transcriptase (RT) and integrase (IN) in reverse transcription and integration steps, respectively, further to assembly. This opens the possibility to inhibit multiple steps in HIV life cycle with just one compound, a unique property not shown by any of the other antiretroviral classes. Therefore, NC appears as a highly profitable drug target to overcome antiretroviral DR. Based on these general remarks, THINPAD was aimed at achieving the following general objectives:

a) To develop effective NC inhibitors (NCIs) as clinical candidates for AIDS therapy, particularly in patients harboring drug resistant variants, ready for early clinical trials. All the steps of the pharmaceutical development will be addressed by THINPAD, bringing NCI clinical candidates from the discovery to preclinical trials.

b) To bring together leading scientists with an expertise in NC. THINPAD partners are at the forefront of the NC research, either in characterizing the biochemical role of the NC within the viral replication cycle and its critical involvement in HIV-1 infectivity or in discovering anti-NC lead candidates endowed with drug-like properties. A significant part of the state of the art NC-related literature has been produced by members of the THINPAD consortium.

c) To realize an SME-oriented pharmaceutical pipeline in which SME participating in the consortium are the IRBM Science Park that comprises the core medicinal chemistry team behind the discovery of the first-in-class IN inhibitor (INI) Raltegravir, and ViroStatics that has a a research focus on HIV. Besides being actively involved in the activity of workpackages, SMEs will particularly focus on coordinating the whole consortium research with the aim of translating research results into industrial application with a potential benefit for health, as specified in the guidelines of the call HEALTH.2013.2.3.1-1.

On these bases, the research objectives proposals were the following:

1) To design and develop novel NCIs active against HIV-1 drug resistant strains, leading to new clinical candidates, based on the knowledge previously acquired especially by UNISI and UNISTRA. New hits identified using in silico methods will be evaluated in in vitro tests.

2) To study the possible emergence of DR induced by NCIs in several HIV strains and to monitor the efficacy of NCIs against HIV-1 drug resistant strains retrieved from clinical patients under HAART treatment. Molecules showing anti-NC and anti-HIV activity in cell culture will be processed. Sequence analysis of the NC sequence from subjects both naïve to and pretreated with different antiretroviral classes will be also part of the work.

3) To optimize the PK and ADME profile of NCIs developed by the THINPAD consortium up to the preclinical evaluation of NCIs toxicity and efficacy. These activities will be mostly SME-oriented. The production of optimized NCI leads will be also achieved, to boost the preclinical evaluation of NCIs toxicity and efficacy.

4) To develop novel anti-HIV-1 clinical candidates by following a SME-oriented research plan aiming at translating research results into industrial application. The first outcome of preclinical studies on NCIs, as well as activity profiles in HIV-1 drug resistant strains will be available, allowing for the preparation and establishment of a suitable exploitation plan, which will be finalized at the end of the THINPAD project.

The development of a new drug is a time and resource consuming multidisciplinary process, which requires the tight collaboration between scientists of different areas of expertise both disease and technology related. Academics and industry should work together.

To meet the continuous need for novel anti-HIV agents active against drug-resistant and multidrug-resistant HIV-strains, THINPAD takes advantage of a SME-driven pipeline, blending scientists from both academia and industry, which focus on an innovative anti-HIV target. Innovative approaches as taken in THINPAD intrinsically own greater risks than anti-HIV drug design targeting classical targets. The early drug discovery research therefore lies beyond the immediate research focus of the multinational pharmaceutical industries but nevertheless constitutes a focus of interest for those industries. Multiple private-public partnership initiatives of pharmaceutical
industries illustrate the tendency to outsource target validation and early drug development to academic institutions. Therefore it represents an avenue of opportunities for drug development where academia and SMEs can benefit from mutual interaction and develop a strong competitive advantage.

In details, the THINPAD workplan was the following:

**Hits selection and hits/leads optimization:** This task was accomplished by following two different approaches:

1. Based on the available NCI hits already characterized by some THINPAD partners, focused libraries have been built to explore SAR of these available hits.
2. The virtual screening of large compound libraries was performed to discover novel molecular scaffold for possible NCIs.

Molecules have been delivered and tested. In addition, UNISI performed the rational optimization of the most promising NCIs. Partners UNISI and IRBM synthesized new interesting NCIs. A scale up of the synthetic procedures was done by IRBM, in order to produce the required quantity of NCIs to support all in vitro/in vivo studies performed within THINPAD.

HTS, IC50 evaluation and characterization of the mechanism of action. NCIs coming from WP2 were first screened in a HTS assay developed by UNISTRA. Confirmed NCIs have been further assayed in vitro by IDIBAPS, UNISI and UNISTRA to monitor their antiviral effect in infected cells, to characterize their mechanism of action in HIV-1 replication and to monitor their ability to elicit DR. Preliminary cytotoxicity, apoptosis and mitochondrial toxicity data were collected by Virostatics and were used for the selection of non-toxic, non-proapoptotic NCIs for further development. We have identified interesting NCI hits/leads, which belong to the Dihydroxypyrimidine family.

Monitoring NCIs on HIV drug resistant strains. NCIs discovered and optimized were further tested on HIV-1 strains retrieved from clinical practice. A bioinformatics study coupled with sequencing HIV-1 genome started at the beginning of THINPAD to monitor the mutation rate of NC in patients undergoing antiretroviral therapy and to select HIV-1 strains for further monitoring the efficacy of NCIs against DR. We proved the benefit of a possible combinatorial strategy based on NCIs and classic anti-HIV drugs to overcome HIV-1 DR.

Pharmaceutical optimization. Early feedback from pharmacokinetics (PK) and drug metabolism studies influenced and drove the design strategy, ensuring the advancement of drug-like candidates. IRBM optimized pharmaceutical properties in vitro and in vivo (clearance, oral bioavailability, lack of metabolic liabilities) of novel NCIs developed within THINPAD. In addition, genotoxicity was studied by Virostatics using histidine dependent mutants of Salmonella typhimurium and tryptophan dependent mutants of E. coli. The file patents search covering the new drugs and their application as NCIs has been submitted in August 2016.

Preliminary preclinical toxicity & formulation studies and Preclinical efficacy of NCIs in animal model. The main goal of this activity was to determine the safety profile of a novel NCI clinical candidate in preclinical animal models, to produce an active pharmaceutical ingredient (API) for toxicity studies to monitor the NCIs efficacy in a humanized mouse model of HIV-1 infection. This activity was coordinated by IRBM with expertise in clinical development. Unfortunately, no strong antiviral effect was scored for the experimental drugs tested (see D6.5 for the details). We scored a modest viral load reduction in 3 out of 4 animals. However, such a modest reduction was also observed in two out of the three infected/non-treated animals. It therefore remains questionable whether this modest reduction in viral load of the mice indeed reflects an antiviral effect. The observations that none of the mice treated with the experimental drugs developed resistance mutations, even after a three week treatment period, indicates that the pressure exerted by the drug on the virus is indeed very limited, if present at all.

Exploitation and dissemination strategy. Due to THINPAD strong commitment towards commercialization, WP7 has been explicitly dedicated to dissemination and exploitation of the results. The partners will further apply for funding to proceed towards clinical development or, eventually, by seeking for collaboration with, or out-licensing to, EU pharmaceutical companies with R&D facilities. Dissemination of the results to the public was guaranteed through publications, conference participation and the THINPAD dedicated website. However the consortium will keep on protecting any commercially exploitable innovations developed within the project. A patent application has been filed to protect the class of best compounds as HIV NC inhibitors: European patent application No. 16186511.8 ~ 1452, date of filing Aug 31, 2016. The applicants are IRBM Science Park (ownership 85%), University of Siena (ownership 10%) and University of Strasbourg (ownership 5%).

Coordination and project management. UNISI monitored the progresses of the THINPAD research activity, with a particular focus on milestones and deliverables achievements and timeline. The project management was smooth and very efficient.

**Project Results:**

**WP2: Hits selection and hits/leads optimization**

**PYRIMIDINES**

In silico studies

The optimization in silico of NCI hits and leads was performed by using two different protocols, based on the different chemical and physicochemical nature of the two series of compounds under development, namely pyrimidines and aminothiazole dimers.

Pyrimidines have already emerged as candidate NCIs from the first virtual screening performed at the beginning of WP2 activities (among selected pyrimidines, compounds I-0003389, I-0044811, I-0005276 that proved to inhibit the NC in vitro). Further tests
emphasized the relevant inhibition of the NC by this class of molecules, which were therefore selected as potential NCI leads. It is worth mentioning that pyrimidines share a noticeable pharmacophoric similarity with one of the NCI fragments identified and published by Partner 3 in 2009 (Biochimie 2009, 91, 916–923).

To this end, the computational protocol, previously refined based on biological activity data (see Deliverable 2.1) was used to predict the binding mode of pyrimidines to the NC. Based on the mechanism of NC inhibition suggested by in vitro tests, namely the competition with nucleic acids for the interaction within the hydrophobic pocket of the NC, molecular docking was performed within the hydrophobic pocket. Such as in previous works, the binding site was centered on the side chain of W37 (J Chem Inf Model 2010, 50, 638–650; J Chem Inf Model 2011, 51, 446–454; Vir Res 2012, 169, 377–387 and ACS Chem Biol 2014, 9, 1950–1955).

Docking results unequivocally show that pyrimidines dock in a similar manner to the hydrophobic pocket (See Figure 1.2A and 1.2B of D2.2) independently from the nature of the substituents. The pyrimidine core is stacked on the W37 side chain, while the two OH groups establish H-bond interaction with the backbone of key residues such as K33, G35, W37 and M46. The carbonyl function is projected towards the protein and interacts with the backbone of M46. The pyridyl ring is projected towards the solvent area. Molecular docking highlighted the relevance of 2-pyridyl ring instead of phenyl or 3- or 4-pyridil derivatives, showing that the pyridyl nitrogen atom at position 2 may establish H-bond interaction with the side chain of Q45. The lipophilic substituents at the amide (or ester) moiety occupy a sub-pocket of the NC that is projected towards its N-terminal end, and is occupied also by the BOE-1 inhibitor or oligonucleotides as observed in available NMR structures (J Mol Biol 2008, 383: 1112-1128; J Mol Biol 2013, 425: 1982-1998). This interaction scheme is exemplified in figure 1.2C and 1.2D of D2.2 for the derivative I-0216298, one of the most interesting and promising hit developed by the THINPAD consortium. The binding mode predicted by docking suggests that bulky and preferable hydrophilic substitutions should be applied to the para position of the pyridyl ring, whereas medium sized groups may be connected to the position meta. In contrast, only small substituents seem to be allowed in the ortho position, most likely endowed with hydrophobic character (i.e. methyl group or halogen atoms). The central ring seems to be the core pharmacophore for binding to the NC, and should be preferably kept unmodified, whereas the amide (or ester) group may be substituted with functional groups of increasing length and, generally, of hydrophobic character.

The optimization process derived from the in silico studies has led to the discovery of new inhibitors of the HIV-NC protein that show improved profiles and drug-like properties with respect to the original hit compound I0169073. The conducted SAR studies elucidated the role that critical regions of the molecule play and identified substitution patterns that generate improved activity and drug-like properties. Good levels of activity have been reached both in the biochemical NC-inhibition assay and in the BiCycle antiviral assay. Cytotoxicity has been monitored carefully in different cell lines and human primary cells. Results showed that it is possible to diverge antiviral activity from the intrinsic cytotoxicity of the molecule, and further efforts to increase the selectivity window will be invested. The metabolic stability studies that have been conducted point out a liability in human plasma for a small set of compounds, and this behavior is currently under investigation. However compounds combining good activity and plasma/hepatocytes stability were identified, and several of these have been advanced into in vivo pharmacokinetic studies in mouse to determine their suitability for efficacy studies.

Aminothiazoles
In silico studies

Besides pyrimidines, aminothiazoles proved to be valuable starting points for the design of optimized NCIs. Starting from previously identified active molecule, 2 amino-4-phenylthiazole derivative named AN3, the hypothesis to elongate the aminothiazole scaffold to contact simultaneously the C- and N-terminal ends of the protein was formulated, also taking into consideration the structural features of a reference inhibitor of the NC (namely, BOE-1). Moreover, the evidence that another aminothiazole AN1 was found by NMR to bind within the N-terminal end of the NC further supported this hypothesis, and suggested that dimerization of these molecules (AN3 and AN1) may lead to NCIs with improved activity. Biological assays showed that aminothiazole dimers are highly promising NCIs in cells, even though the NC Inh assay did not provide positive outcomes due to the low water solubility of the molecules. For this reason, the in silico optimization has been performed with the aim to improve the affinity of aminothiazole dimers for the NC and to enhance the water solubility of these molecules. In contrast to what reported above for pyrimidines, molecular docking is not the best tool for studying aminothiazole dimers, because these molecules have a high number of rotatable bonds and are quite large in dimension. To overcome this issue, a molecular dynamics protocol was established. This analysis suggested that the first dimers synthesized and designed (namely, I0155696 and I0155698) are rather too long, even if they are able to contact both terminal ends of the protein by means of hydrophobic and H-bond interactions, respectively. Moreover, to improve the water solubility of these molecules some chemical modifications were suggested.

After the synthesis of the best molecules of this class, their activity was evaluated using NC Inh, EMSA and BiCycle assays and the results suggested different hypothesis:

- aminothiazole family could acts as a pro-drug releasing the active molecule inside the cell
- NC could not be the main target of aminothiazole
Introduction of the 1,2,4-oxadiazole ring decreased the flexibility of the aminothiazole chain leading to a decrease of binding affinity with the target protein. The aminothiazole family was not further studied in the scope of THINPAD.

During the THINPAD project, aimed at the discovery of potential new pharmaceutical agents, different synthetic approaches have been employed depending on the project phase. In an early stage of the THINPAD project, the synthesis goals were the production of small amounts of many molecules for biological evaluation and to facilitate structure activity relationship (SAR) elucidation. At that stage the yields in the final products ranges from very low to medium and the purification was usually performed by automated reverse phase HPLC. The typical amount produced was 15-20 mg, enough to supply material (as powder or DMSO solution) for NC inhibition, EMSA, antiviral, cytotoxicity and in vitro pharmacology assays. The low yields and instrument-intensive purification approaches are acceptable on small scale as a trade off against speed/efficiency of driving the lead optimization effort. However, as compounds are selected for further advances in vitro and in vivo studies, the increased compound requirements (hundred milligram scale) necessitate the optimization of synthetic and purification procedures. Ultimately a robust synthetic process was required to allow the production of 5-10 gr for a limited number of compounds to support advanced stage in vivo studies (efficacy and toxicology) studies as well as formulation activities.

The synthesis of pyrimidine based HIV-NC inhibitors, the main series developed during the THINPAD project, was optimized and scaled up. In addition, IRBM optimized also the synthetic strategies applied during SAR exploration of an alternative less explored series of catechol compounds that are analogs of I-0155069. The detailed synthetic procedure have been reported in a clear and detailed way, together with the characterization of the molecules in D2.4.

WP3 HTS, IC50 evaluation and characterization of the mechanism of action

The objective of this activity is to provide an overview on the screening efforts done in order to select lead compounds from two most promising series identified previously (refer to D5.4 and D3.2): aminothiazoles and pyrimidines. According to the screening funnel described in the Figure 1 of D3.2 in the first step, compounds were tested for their capacity to prevent the binding of NC to its target DNA sequences into two biophysical assays, NC Inh (Partner 3) and EMSA (Partner 2). Compounds found positive at this step were further screened for their antiviral effect into four antiviral assays, providing complementary information. This work was realized by: Partner 1 who is performing TZM-bl cell line based assays using the HIV-1 reference wild type NL4-3 strain (Mono- and BiCycle assays); Partner 2 who is working on TZM-bl cells infection by viruses produced by chronically infected T cells (ACH-2) and induced by Vorinostat (RevCycle assay) and Partner 3 who is checking the effect of the same molecules on HeLa cells infection by pseudotyped non replicative particles (mimicking the early phase of the virus) (PSEUDO assay). All four assays were done in parallel in different laboratories in order to test a large number of molecules and to increase the reliability of the results.

More than 200 compounds belonging to 2 chemical classes, aminothiazoles and pyrimidines, were tested following this screening funnel (results reported in D3.2 and D3.8). In D3.6 we have reported the results about antiviral resistance obtained by the lead candidates and the evaluation of their cellular toxicity. The most interesting hits were evaluated in a panel of cytotoxicity assays by Partner 5 (ViroStatics).

Cytotoxicity determination

Peripheral blood mononuclear cells obtained from healthy donors were stimulated with Phytohemagglutinin and Interleukin-2 and treated with different concentrations of the compounds. After seven days, cytotoxicity was measured through both Trypan blue (exclusion dye) staining and Mitochondria Targeting Sequence assays (MTS). Trypan blue exclusion assay is based on the fact that the cell membrane of live cells is intact and prevents the dye from entering, therefore live cells remain bright. Dye enters dead cells through their compromised cell membranes and colours them blue. Live cells are counted at the microscope using a hemocytometer.

The MTS assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes activity reflects the number of viable cells present in the cell culture. These enzymes are capable of reducing the tetrazolium dye MTS into formazan. The absorbance of this colored solution can be quantified.

In both assays, viability was expressed as percentage of living cells compared to the non-treated control, indicated as 100%. TD50 (Toxic Dose 50%) was calculated as the concentration that kills 50% of cells compared to control.

Mitochondrial toxicity (MT) analysis

BxPC3 cells (a human pancreatic adenocarcinoma cell line) were exposed to different concentrations (50 and 10 μM) of the NCIs.
Analysis of mitochondrial functionality was performed at day 14 with JC-1 staining and flow cytometry analysis. JC-1 is a lipophilic carbocyanine that exists in a monomeric form and can accumulate in mitochondria. In the presence of a high mitochondrial membrane potential, JC-1 can reversibly form aggregates that, after excitation at 488 nm, emit in the orange/red channel (FL2). Monomers emit in the green channel (FL1). The collapse in mitochondrial membrane potential provokes the decrease in the number of JC-1 aggregates and a consequent increase of monomers (decrease in FL2/increase in FL1 channel). 500 nM of Valinomycin was used as a positive control. The ratio between the median fluorescence intensity in FL2 and in FL1 was used to calculate the Mitochondrial Toxicity Index (MTI), which represents the percentage mitochondrial toxicity, assuming the negative control value to be equal to 0, and the maximal toxicity of Valinomycin to be equal to 100%. EC50 and 10xEC50 (as per BiCycle assay results) concentrations were used in mitochondrial toxicity determination. The compound was considered as “toxic” if MTI value was >40%, otherwise the compound was considered as “safe”.

Apoptosis induction
Peripheral blood mononuclear cells obtained from healthy donors were stimulated with Phytohemagglutinin and Interleukin-2 and treated with different concentrations of the compounds. After 7 days, compounds were assessed for apoptotic effect. Cells were stained with Annexin-V and 7-AAD to measure early, late and total apoptotic events induced by pharmacological treatment. 7-AAD is binding to DNA while Annexin-V to phosphatidylserine (PS). In healthy cells, PS is predominantly located along the cytosolic side of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution in the phospholipid bilayer and translocates to the extracellular membrane, which is detectable with fluorescently labeled Annexin V. In early stages of apoptosis, the plasma membrane excludes viability dyes such as 7-AAD, therefore cells which display only Annexin V staining (7-AAD negative) are in early stages of apoptosis. During late-stage apoptosis, loss of cell membrane integrity allows Annexin V binding to cytosolic PS, as well as cell uptake of 7-AAD. Annexin V staining, paired with 7-AAD or PI is widely used to identify apoptotic stages by flow cytometry. EC50 and 10xEC50 (as per BiCycle assay results) concentrations were used in apoptosis determination. Percentage of total apoptosis increase compared to untreated control was calculated. The compound was considered as “toxic” if this value was >40%, otherwise the compound was considered as “safe”.

Among tested aminothiazoles, the most interesting is compound I-0196121: it showed a good activity in NC Inh and EMSA assays, and is the best compound in this series in BiCycle assay. The poor water solubility is the limitation in this series. Among the tested pyrimidines, the most promising are the changes in the left part of I-0195615 either with introduction of new substituents on the pyridine ring or complete change of this part with bicyclic heterocycles. The champions for NC inhibition assays in this series are compounds I-0216298, I-0216395, I-0216333, I-0216336 and I-0216339. The champions in BiCycle assay are compounds I-0216339, I-0216333, I-0217078, I-0198965 and I-0216235. Particularly interesting is compound I-0218640 which gives high toxicity/activity ratio. Compounds I-0216333, I-0216339 and I-0218706 were selected as leads in this series. All the tables with the results of the in vitro inhibition, antiviral and toxicity tests have been reported in D3.8.

In vitro antiviral resistance
The isolation of NCIs resistant mutants was performed through in vitro resistance selection (IVRS) experiments, which allowed the identification of mutations selected by the pressure of one or more drugs in cell culture systems. We applied this method with investigational NCIs showing a low micromolar antiviral activity against the reference HIV-1 strain NL4-3 together with favourable toxicity profiles.

The NCIs considered for IVRS testing were:
- Aminothiazoles I-0168934 and I-0196121
- Triazoles I-0168793 and I-0193813
- Pyrimidines I-0195611, I-0195740, I-0195689, I-0216395, I-0216339, I-0216298

Despite the fact that screening of candidate NCIs led to the identification of several compounds with improved antiviral activity (IC50 < 1 μM), only a limited number of molecules were considered for IVRS experiments since many NCIs induced cellular toxicity at concentrations close to the IC50 values.

As the results of our experiments, no mutation were selected in the NCp7 coding region during IVRS testing performed so far, while two aminoaacidic substitutions selected by NCIs in the GAG p24 coding region showed none or limited impact in the susceptibility to NCIs. Theoretically, these data suggest a high genetic barrier of resistance of NCIs, but the absence of selection of resistance mutations could be explained in different ways:
- The highest concentrations of NCIs used in IVRS experiments (e.g. around 10 times higher than IC50 values for pyrimidines) were not sufficient to induce the selection of resistance mutations.
- The mechanism of action of NCIs is different from expected and the escape from NCIs pressure could not involve GAG proteins.
Further experiments are being carried out to clarify the mechanism of action of the candidate NCIs developed so far and clarify their resistance profile.

NCIs susceptibility of viruses carrying mutations conferring resistance to antivirals commonly used in HIV-1 therapy

Simultaneously with the screening of candidate NCIs in the cell based assay with the NL4-3 wild type reference strain, some of the most promising compounds were also tested with viruses carrying resistance mutations to drugs approved for HIV-1 treatment. This analysis was performed to determine if NCIs could represent a valuable option to overcome drug resistance conferred by licensed anti-HIV antivirals. The resistant viruses were obtained through the NIH AIDS Reagent Program (www.aidsreagent.org) and have been already characterized by the Phenosense Assay (Monogram Biosciences), considered as the reference assay for phenotypic investigation of HIV drug resistance due to its large application in clinical trials.

This analysis showed that all the compounds retain full activity against viruses carrying mutations conferring high level resistance to NRTIs, NNRTIs and INIs. A 10-fold decrease in susceptibility was shown against the highly INI-resistant virus 11842 for some compounds belonging to the pyrimidine series, which deserves further investigation.

In conclusion, the evaluation of antiviral activity with viruses carrying resistance mutations showed that all the compounds inhibited the replication of the resistant strains with IC50 values comparable to the wild-type strain, consistent with previous observations on compound I-0155059 and the reference compound I-0154294 (BOE-1). These data indicate that NCIs could overcome drug-resistant strains selected by treatment with drugs currently approved for clinical practice.

Mechanism of action

A number of different assays was used to evaluate the mechanism of action of the best selected hits. The most important conclusions are the following:

- Tested pyrimidines (I-0195618, I-0216298, I-0216395, I-0216333 and I-0216339) and catechol I-0155069 were found to show affinity to NC protein in the μM range.
- Catechol I-0155069 was able to prevent the binding of NC to its specific binding site on (-)PBS.
- Tested aminothiazole I-0196121, pyrimidines (I-0216333 and I-0216339) and catechol I-0155069 were able to slow down the annealing of cTAR with dTAR, suggesting that these hits compete with cTAR and dTAR for binding to NC.
- Only I-0155069 showed a concentration-dependent inhibition of RT activity indicating that this compound targets both RT and NCp7. None of the other tested compounds was found active against RT.
- Tested compounds (2 aminothiazoles + 4 pyrimidines) show moderate activity on integrase, with IC50 values in the range of 10 – 250 μM.
- GagNC appears as a major target of the tested compounds resulting in an inhibition of the production of cell-free viral particles.

WP4: Monitoring NCIs on HIV resistant strains

For evaluating the sensitivity to NCIs of common PI/NRTI/NNRTI/INI resistant strains, we have calculated the 50% inhibitory concentration (IC50) of candidate NCIs with low micromolar NC binding activity by using an HIV-1 wild type reference strain (NL4-3) in a cell based assay. In addition, we have evaluated the activity of selected compounds against viruses carrying mutations conferring resistance to antivirals commonly used in HIV-1 therapy.

The compounds were proposed for testing with HIV resistant strains after their selection through screening funnel as described in WP3 (Figure 1).

To achieve the objectives of WP4 we performed several experiments by using our phenotypic assays to investigate antiviral activity of candidate NCIs with both HIV-1 reference virus and resistant viral strains. Below the most important conclusions on the mechanism of action of the most interesting and active class of compounds, as derived by experimental work:

1. Among the compounds found to interact with NC as result of biochemical assays, we identified seven compounds as candidate NCIs with IC50 values lower than 10 μM calculated with the wild type reference NL4-3 HIV-1 through the BiCycle Assay.
2. The results of MonoCycle assay suggested that compounds belonging to the triazole and pyrimidine series inhibit viral replication during the late phases of HIV life cycle, while compounds belonging to aminothiazole series could inhibit both early and late steps of viral replication. More specific experiments are required to investigate how these molecules interact with NC at different steps of HIV replication cycle.
3. Preliminary evaluation of antiviral activity with viruses carrying resistant mutations showed that all the compounds inhibited the replication of the resistant strains with IC50 values comparable to the wild-type strain, consistent with the previous observations on compound I-0155059 and the reference compound I-0154294 (BOE-1). These data indicate that NCIs could overcome drug-resistant strains selected by treatment with drugs currently approved for clinical practice.

With the regards to the identification of mutants resistant to NCI leads, the isolation of NCIs resistant mutants has been performed...
through in vitro resistance selection (IVRS) experiments, which allowed the identification of mutations selected by the pressure of one or more drugs in cell culture systems. We applied this method with investigational NCIs showing a low micromolar antiviral activity against the reference HIV-1 strain NL4-3 together with favourable toxicity profiles.

The NCIs considered for IVRS testing were: Aminothiazoles I-0168934 and I-0196121; Triazoles I-0168793 and I-0193813; Pyrimidines I-0195611, I-0195740, I-0195689, I-0216395, I-0216339, I-0216298.

In conclusion: no mutation was selected in the NCp7 coding region during IVRS testing performed so far, while two aminocdic substitutions selected by NCIs in the GAG p24 coding region showed none or limited impact in the susceptibility to NCIs. Theoretically, these data suggest a high genetic barrier of resistance of NCIs, but the absence of selection of resistance mutations could be explained in different ways:
- The highest concentrations of NCIs used in IVRS experiments (e.g. around 10 times higher than IC50 values for pyrimidines) were not sufficient to induce the selection of resistance mutations
- The mechanism of action of NCIs is different from expected and the escape from NCIs pressure could not involve GAG proteins.

Further experiments are being carried out to clarify the mechanism of action of the candidate NCIs developed so far and clarify their resistance profile.

As a part of this WP4 we also analyzed the theoretic positioning of NCIs in the actual combination therapy against HIV. The first antiretroviral agent was introduced in 1986-87. It was zidovudine in monotherapy. It was effective in terms of reducing mortality but resistant viral quasispecies were selected after 3-6 months of treatment. We had to wait more than 10 years (up to 1996-97) to introduce what we now call highly active antiretroviral therapy (HAART) or combined antiretroviral therapy (cART). Used to be a combination of three antiretroviral agents against two different HIV-1 targets (HIV-1 reverse transcriptase and HIV-1 protease). These initial cART regimens proved to be very effective, dramatically reduced mortality and the incidence of opportunistic AIDS associated events (malignancies and/or opportunistic infections). Viral strains with resistance mutations were not selected among adherent and responding patients. Yet, tolerance to medications was poor and consequently compliance with prescribed medication was relatively low, virological failure rate was relatively high and selection of resistance mutations was frequent among patients with virological failure.

Starting from year 2002, better tolerated drugs, compounds active against novel viral targets (entry inhibitors and integrase inhibitors) and drugs with a higher genetic barrier (several mutations need to be selected and accumulated to develop resistance) were introduced. The final scenario is the present situation in year 2016: vast majority (≥ 95%) of our patients receiving cART have a plasma viral load below the limit of quantitation (BLQ) by our routine laboratory tests, medication use to be pretty well tolerated and consisting of a single pill (containing 3 different compounds) per day.

So, a relevant question is whether or not do we need antiretroviral agents directed against new HIV-1 targets. The clear answer is yes for a variety of reasons as long as these new agents fulfill a minimum of requirements event if they are first compound in a new class or family. The main reason is that a novel compounds against a new viral target will be active against viral strains who have accumulated resistance mutations to other drug classes. There is mounting evidence for increasing HIV drug resistance in low-middle income countries progressively accessing cART and the issue of resistance is not yet solved in high income countries either (for example treatment emergent resistance has decreased significantly in latest years but transmitted drug resistance remains stable). An additional reason is that agents targeting other steps of the virus life cycle may potentially overcome some of the limitations of the already existing agents including residual replication, limited penetration in some tissue sanctuaries, drug-drug interactions. Minimum requirements should be excellent intrinsic potency (e.g. short-term monotherapy pilot study showing significant reduction in viral load), once daily dosing and good tolerability. Within this framework the THINPAD compounds are well positioned and are very promising. A new NCI may play an important role not only in salvage therapy (thanks to the lack of cross resistance) but also replacing some older agents against wild type viruses depending on its intrinsic potency, genetic barrier to resistance, tolerability and pharmacokinetics properties. The detailed virological considerations about the strategical interest of NCIs and NCI resistance by mutations within the NC core as a consequence of NC polymorphism are reported in D4.6.

WP5: Pharmaceutical optimization

In vitro/in vivo ADMETox/PK profiling and genotoxicity determination were carried out on selected NCI leads generated within the project. The profiling of these compounds culminated in the selection of the best candidates to be advanced into preliminary in vivo studies that are required prior to conducting efficacy studies in an animal model.

The SAR studies were conducted in the three main series (triazoles, pyrimidines and aminothiazoles) and allowed identification of the weaknesses and of the potential activity of the compounds belonging to each class.

The triazole series offers only a limited opportunity for development due to low solubility that appears to be an inherent problem related to the physicochemical properties of the central core. Efforts to improve solubility have not been successful and this parameter is not
expected to be easily improved. The most notable results in the tricyclic series is the achievement of cell-based antiviral activity, a feature that was lacking in the original lead. However the difficulties in this series, and in the related bicyclic triazole series likely outweigh the benefits, therefore further development of these series has been put on hold.

Exploration of the aminothiazole series led to the identification of optimized compounds that have an interesting antiviral profile. Metabolic issues and solubility liabilities were however identified and need to be addressed in any future work.

The optimization process in the pyrimidine series has led to the discovery of new inhibitors of the HIV-NC protein that show improved profiles and drug-like properties with respect to the original hit compound I-0169073. The SAR studies conducted so far elucidated the role that critical regions of the molecule play and identified substitution patterns that generate improved activity and drug-like properties.

Good levels of activity have been reached both in the biochemical NC-inhibition assay and in the Bicycle antiviral assay. Cytotoxicity has been monitored carefully in different cell lines and primary cells. Results showed that it is possible to diverge antiviral activity from the intrinsic cytotoxicity of the molecule. The metabolic stability studies that have been conducted point out a liability in human plasma of the original lead. However, compounds combining good activity and plasma/hepatocytes stability were identified, and several of these have been advanced into in vivo pharmacokinetic studies in mouse to determine their suitability for efficacy studies.

A series of pyrimidine based inhibitors of HIV-NC protein has been the subject of extensive optimization during the THINPAD project. In the final phases of this work efficacy studies in animal model have been under investigation. This work requires the availability of compounds on significant scale with respect to initial compound screening and moreover further material is needed to perform formulation studies, preliminary in vivo studies and to assess the dosage and treatment length. There is therefore a need for scale-up chemistry for advanced candidate compounds to provide multi-gram quantities of the active ingredients in adequate purity to support these experiments. Chemistry optimization was described in deliverable D2.4 and allowed the preparation of the project most interesting compounds on sufficient scale to cover biological assays and explorative pharmacokinetic studies.

As it was described in deliverable D2.4 the synthesis of the dihydroxypyrimidine core was well depicted in the literature (Angew. Chem. Int. Ed. 2008, 47, 4134–4136). Overall this process is robust and scale-up work has typically translated smoothly with improved yields, mainly reflecting the higher recovery of the final product during its isolation as a precipitate. The starting amide oxime is accessible in typically high yield from the corresponding nitriles that were either commercially available or can be prepared large scale to support the projects chemistry work. The final transamidation step was optimized to facilitate parallel synthesis during the lead optimisation process, and these procedures were adapted for larger scale work by employing thermal heating rather of microwave irradiation to promote the reaction. The purification of the final amides on large scale has proven to be demanding. Reverse phase chromatography however gave good results when performed in the presence of trifluoroacetic acid as additive. The neutral final compound is needed to allow the preparation of different salt forms that are needed for formulation studies and for in-vivo studies. The use of TFA however generates trifluoroacetamide salts as the initial product when a basic amine is present in the final target compounds. In such cases the neutral compound could be obtained by reverse phase chromatography conducted in absence of the additive, and though yields were lower this approach avoided the need for a desalting step (which typically proved low yielding). To ensure a high purity level of the final compounds that were destined for in vivo experiments, additional purification was achieved by precipitation/crystallisation of the neutral final compound prior to any final salt formation step.

WP6 Preliminary preclinical toxicity & formulation studies and Preclinical efficacy of NCIs in animal model

The preparation and formulation of the selected NCI candidates for pre-clinical studies were a task of WP6. In D6.2 are reported details on optimized aminothiazole and pyrimidine analogs, the compound series that were found to be the most promising. Early formulation studies are commonly prepared for drug compounds at both discovery and preclinical stages and are used to select suitable dosing vehicles for administration to animals via various routes such as oral gavage or intravenous dosing. They serve the purpose of evaluating the compounds on a broad range of pharmaceutical interests, notably pharmacology (activity/efficacy), pharmacokinetics (PK), and toxicology. Poor aqueous solubility that can result in limited bioavailability during preclinical screening, and ultimately in drop-out of potential NCIs with promising therapeutic activity, is an issue that can be mitigated through adequate formulation studies. Selection of a suitable formulation based on the physicochemical properties of compounds was therefore essential to avoid solubility-related absorption and bioavailability issues.

Four compounds (I-0216339, I-0216333, I-0216395 and I-0218706) have been selected as potential candidates for efficacy studies in mouse (see report D5.3). As part of the profiling of these compounds it was necessary to assess their tolerability after repeated dosing and to determine the plasma concentrations achieved after this dosing. The vehicle selected for in vivo efficacy studies was MediDrop®, a sucralose based vehicle suitable for administration of compounds as part of their drinking water. Administration of drug candidates in MediDrop® sucralose medium is desirable as it is suitable for suspensions of compounds, allowing for consistent delivery, as it uniformly suspends non-soluble compounds. It also provides an alternative to medicated water or oral gavage to deliver compounds, providing a less stressful method of administration for animals. MediDrop® eliminates the need for daily shaking of water
bottles and also has the advantage of masking unpleasant tastes. In view of these properties MediDrop® was selected by the subcontractor, Professor Berkhout from the Academic Medical Center of the University of Amsterdam, to deliver compounds to humanized mice in efficacy studies.

To ensure the testing of the compounds at the maximum tolerated concentrations a series of preliminary experiments were conducted. Efficacy studies are normally performed at the maximum tolerated concentrations to optimize the antiviral response, but it was found out that tolerability of I-0216339 at 3.8 mg/mL is not adequate to perform efficacy studies. Lower concentrations of I-0216339 are required. In the following tests, results at 1 and 2 mg/mL are described.

The observed mean plasma concentration at Day 7 for I-0216339 and I-0216333 at both tested dosing concentrations was higher than the EC50 concentration in the BiCycle antiviral assay. I-0216339 at 2 g/L showed plasma concentrations at Day 0 and 7 of 0.56 and 1.79 μM, respectively, circa 14 and 44 times higher than the EC50 in the BiCycle assay. I-0216333 at 2 g/L showed plasma concentrations at Day 0 and 7 of 0.26 and 0.80 μM, respectively, circa 2 and 7 times higher than the EC50 in the BiCycle assay. Based on these results both compounds were selected for in vivo efficacy testing.

The plasma concentrations observed for I-0216395 at 0.5 g/L were too low (lower than EC50 concentration in the BiCycle antiviral assay), this compound was not included in the in vivo efficacy assay.

The observed mean plasma concentrations at Day 7 for I-0218706 was higher than the EC50 concentration in the BiCycle antiviral assay. Because of this, I-0218706 was selected for in vivo efficacy testing. The experimental procedures for determining the plasma concentration of I-0216339, I-0216333, I-0216395 and I-0218706 are reported in D6.4.

Describe the results on the in vivo tests: max ONE page

WP7: Exploitation and dissemination strategy

The THINPAD Consortium has been working constantly and hard in order to reach the objectives fixed in the Granted Project. Thanks to the expertise of 5 Partners it has been possible to realize the planned work, to overcome some difficulties met, and to achieve the Milestones of the Project.

Various accomplishments within THINPAD project have been obtained in the course of the three years period, and all of them have been properly disseminated.

Without any doubt, THINPAD significantly moved forward the state of the art of NCp7 inhibition and made a great improvement to the discovery of NCIs allowing the identification of NCIs with a 100 fold increase in terms of potency with respect to the starting point of the Research activities.

It has been demonstrated that THINPAD's NCIs inhibit HIV replication in cells, including drug resistant HIV strains. The Consortium selected the most promising NCIs (4 compounds from 2 different scaffolds) for running the in vivo studies in order to prove their efficacy and to further support the validation of NCp7 as an antiretroviral target.

A patent application has been filed to protect the class of best compounds as HIV NC inhibitors.

In compliance with what stated in the DoW, particular attention has been addressed to dissemination activities: THINPAD counts about 70 presences to National and International conferences, 14 papers (2 open access article) published on International Scientific Journals and a web page as a source of information and contact point for stakeholders.

Furthermore two International Workshops in Drug Synthesis (V EWDSy and VI EWDSY) have been organized with the sponsorship of THINPAD that strongly supported financially the event.

In terms of exploitation, our strategy is to license out the manufacturing, commercialization and marketing rights of the NCI clinical candidate to a Pharmaceutical Company with an HIV franchise. The NCI needed to undergo a series of steps before being handed by the Pharmaceutical Company. Such steps involve the completion of “first-in-man” enabling studies and then a Phase 1 Clinical study.

Some of the participant of the THINPAD project, e.g. Partners 4 and 5, will be able to provide the expertise to support such studies. While performing the preclinical in vivo studies contacts with the Pharmaceutical industry have been initiated. Actually the partners started to do networking since 2015. This work has been reported in D7.6. The business partnering conference BIO-Europe is Europe's largest conference serving the global biotechnology industry and has represented the major contact point with Pharma, in fact the 58% of the companies that it is possible to meet and negotiate with are from the biotech and pharma market segments. As previously said the final goal of the contacts is to establish a negotiation table for out licensing the foreground of the THINPAD project. The process will continue during the 2017, since further studies will be necessary in order to strengthen the foreground and due to the complexity of the operation.

As essential part of the exploitation plan the knowledge generated during the project has been protected by filing a European patent application (Application No. 16186511.8 – 1452, date of filing Aug 31, 2016). The applicants are IRBM Science Park (ownership 85%), University of Siena (ownership 10%) and University of Strasbourg (ownership 5%).

Potential Impact:

The THINPAD project contributed towards the expected impacts of the work programme by developing inhibitors of the highly
The SMEs in THINPAD will benefit from the project results in three ways: to take at least 10-12 years, as there are certain limitations due to the clinical trials timelines. At the end of this period, though, the SMEs such exploitation has the final result of improving the EU position in terms of anti-HIV market. The overall drug development is foreseen companies with R&D facilities, or selling the product to big pharmaceutical multinational companies for final development. Either way, the exploitation plan the knowledge generated during the project has been protected by filing a European patent application (Application No. 1618561.8 – 1452, date of filing Aug 31, 2016). The applicants are IRBM Science Park (ownership 85%), University of Siena (ownership 10%) and University of Strasbourg (ownership 5%).

In the D7.4 an analysis of the most interesting series of compounds identified as part of the THINPAD project with respect to the IP landscape was reported. From these analyses above patentability issues were raised regarding the pyrimidine series, mainly due to the fact that its dihydroxypyrimidine core is claimed by Merck in the patents originating from the international application WO2003/035076 referring to HIV integrase inhibitors for use in the treatment of HIV infection. The THINPAD HIV-1 NC inhibitors fall within the scope of Merck's WO2003/035076 and the different mechanism of action per se cannot be used to support the patentability with respect to the novelty and/or inventive step requirements. However, when the discovered previously unknown property of a compound provides a new technical effect, such property could involve a valuable and inventive contribution to the art thus constituting patentable subject matter (see decision of the Enlarged Board of Appeal G2/88). Data and SAR that were developed by the THINPAD team identified specific structural features that conferred to the compounds some advantageous properties that cannot be foreseen by WO2003/035076 and that therefore constitute a new technical effect. A patent application claiming the dihydroxypirimidine as NC inhibitors for use in the treatment of HIV-1 infection and in particular for use in the treatment of drug resistant HIV-1 infection was prepared and filed on the basis of the above technical features. The inhibition of HIV-1 NC can be regarded as a pharmaceutical functional feature and the effect underlying this feature was not made available to the public by WO2003/035076 or by any of the prior art literature available to the team. In conclusion, three structurally different series were developed by the THINPAD project: 1,2,4-Triazoles, Aminothiazoles and Dihydroxypirimidines. The dihydroxypirimidines gave the most interesting results and, to date, have been selected for further development. For this reason, the consortium decided to protect the series by filing a patent application claiming the compounds as HIV-1 NC inhibitors.

In terms of exploitation, the strategy is to licence out the manufacturing, commercialization and marketing rights of the NCI clinical candidate to a Pharmaceutical Company with an HIV franchise. After the completion of THINPAD, the NCI will need to undergo a series of additional steps before being handed by the Pharmaceutical Company. Such steps involve the completion of "first-in-man" enabling studies and then a Phase 1 Clinical study to be carried out immediately after the end of the THINPAD project and requiring around 1-2 years. Some of the participants of the THINPAD project, e.g. IRBM and Virostatics, will be able to provide the expertise to support such studies. After the completion of these steps, the SMEs will develop a strategy to proceed for further development in collaboration with EU Pharma companies. The choice of licensing out the rights to the NCI at the end of preclinical development is consistent with a consolidated trend: at least 50% of recent deals between SMEs and large Pharma companies have closed at the preclinical or Phase 1 development stage [MedTrak survey]. The nature of the deal could be by either out-licensing the intellectual properties to EU Pharma companies with R&D facilities, or selling the product to big pharmaceutical multinational companies for final development. Either way, such exploitation has the final result of improving the EU position in terms of anti-HIV market. The overall drug development is foreseen to take at least 10-12 years, as there are certain limitations due to the clinical trials timelines. At the end of this period, though, the SMEs in the consortium will be prime movers in the NCIs field, ensuring a good market positioning and potential blockbuster products. The SMEs in THINPAD will benefit from the project results in three ways:
a) by increasing their patent portfolio;  
b) by progressing in the development of a drug at the most critical stage, between discovery and clinics, where financial support is largely unavailable, as the stage is too advanced to attract limited academic and public resources and too early to attract significant investment and Pharmaceutical Industry support;  
c) by being able to seek out partnerships with the large Pharmaceutical companies for further clinical development (funding or buying out).

Regarding the concrete contacts for the exploitation of the THINPAD results so far, even if initial contacts with industries started during 2014 with the European Workshop in Drug Synthesis, a different approach within the negotiation tables started with the Bio-Europe conference experiences since 2015. The final goal was to establish contacts for out licensing the foreground of the THINPAD project. However, during the project, it was not possible to duly protect the foreground (as the research activities were still ongoing) and, as a consequence, to reveal confidential information. For this reason, the consortium decided to move in parallel toward the protection of the knowledge by filing a patent application.

The current global HIV drug market is worth approximately $24.23 billion (Figure 2), representing 43% of sales of antiviral agents and 20% of the total anti-infectives drug market. The HIV drug market has grown with a compound annual growth rate (CAGR) of 8.6% in the past 5 years. Gilead's Truvada dominated the NRTI class, with estimated sales of $4.15 billion in 2015, accounting for 17.1% of the market. In the same period, Atripla (a STR combining Sustiva with Truvada) generated revenue of $3.7 billion and is the NNRTI class market leader. Stridil, which accounted for sales of $2.34 billion, is the InSTI market leader, and Prezista, with sales of $1.89 billion, is the PI market leader. The United States remains the dominant national market for HIV therapies, accounting for 66% of the total sales. Over the next several years, NRTI and PI class sales are expected to decline because of impending generic competition, as are sales of branded NNRTIs because of the move towards the InSTI class. Launches of new branded agents such as elvitegravir, raltegravir, DCF/TAF and doravirine, as well as continued growth of the InSTI class, are expected to offset these declines. The HIV drug market is projected to reach $25 billion by 2019.

Since the nearly thirty single and multiple antiretroviral drug products currently on the market all share a single common strategy to reduce viral replication (directly targeting HIV and its viral enzymes and thus making them prone to DR), NCIs identified by THINPAD and developed by the SMEs will be able to address an important issue in HIV therapy. In relation to the state of the art, the THINPAD consortium proposes an innovative strategy that targets a highly conserved protein sequence such as NC, to avoid the emergence of DR and to develop new candidate drugs that could be used alone or in combination with first-line antiretroviral drugs in the clinics. At the end of the clinical development process, the aim is to provide drugs with the concrete potential to form the basis of first-line therapy, which represents the most lucrative line of therapy in the treatment of HIV disease. THINPAD products will be able to be used in conjunction with other antiretrovirals and as part of multidrug cocktails, increasing their marketability.

Development Timeline and License Revenue
The Objective is to secure THINPAD NCIs a co-development/license deal by 2017. Table 3.3.3.2 illustrates the development perspectives of the project.
Assumptions:  
- Because of their low likelihood of inducing resistance, THINPAD NCIs are best positioned for early and long-term use with potential blockbuster status  
- Market HIV 2017: $17.3 billion [GlobalData]  
- Market Share: 10%; assume no other NCIs on the market  
- Royalties: 8% of net sales  
- Total Sales: 2026: €150M; 2030 €1,500M (25% marketing and manufacturing costs)  
- Total value of the deal: €350M, including upfront and milestones revenues

List of Websites:  
www.thinpad.unisi.it/