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The human immunodeficiency virus (HIV) causes an acquired immunodeficiency syndrome (AIDS) due to exhaustion of the immune system characterized by CD4+ lymphocyte depletion. Current medication can inhibit infection of cells almost completely, prolonging life expectancy of people living with AIDS considerably. However, normal life expectancy is not reached yet. Besides side-effects of the medication, residual expression and replication of the virus might contribute to this observation. The Nef protein expressed by lentiviruses such as HIV is an important pathogenic factor in humans and other simians. Several of its functions believed to contribute to this property would therefore be a relevant target for therapy. In this project, partners worked together to discover small molecule inhibitors of Nef functions. The functions assayed were activation of Hck; CD4 and MHC-I trafficking; NFAT hyperinduction; virological synapse formation and infectivity enhancement. Besides small molecules, the existence of putative partner proteins Nef needs to perform its functions were searched for.

The project took advantage from small molecule compound libraries available to the partners, as well as from new molecules synthesized by one of the partners (P#5 EcoSynth). A function of Nef shown to be important for pathogenicity in vivo is the association with SH3 domains, mediated by the PXXP domain and typically measured by Hck association. Our consortium discovered several molecules that could block activation of Hck by Nef in living cells. By chemical derivation, the most potent were identified. These compounds also inhibited SH3 domain-mediated interaction between the NS1 protein influenza A virus and the SH3 of CrkL, and were non-toxic. Further optimisation will be needed to enhance the specificity for Nef-SH3 binding.

We discovered molecules that inhibited the hyperinduction of the transcription factor NFAT by Nef in activated cells. By inducing NFAT hyperinduction in infected cells, Nef could contribute to viral replication and immune exhaustion. Therefore, inhibiting this function might inhibit progression towards AIDS in HIV infected patients. Optimal inhibitors were synthesized within the consortium, further development to delineate the mechanism and specificity for Nef is warranted.

Other molecules identified by our consortium were shown by NMR spectra shifts to bind to the Nef protein molecules. Detailed analysis revealed the cargo binding pocket (“sorting motif recognition site”), just adjacent to the binding site of the SH3 RT-loop, to be bound by some compounds. This cargo binding pocket appears to be well conserved over Nef proteins, pointing to its importance for Nef functions as well as its attractiveness as a drug target. At present, affinity of binding is too low to detect functional consequences, and further lead development should be undertaken. During this work, the crystal structure of protein derived from a HIV-1 Nef SF2 allele (45-210) was solved.

We have identified several molecules that affect HIV infection and replication. These were different from the ones mentioned above, some belonged to a class IV phosphodiesterase inhibitors. Our project allowed to characterize the effect on HIV in detail of members of this family of inhibitors, reported previously to inhibit HIV infection.

Finally, by performing a shRNA screen, we identified transcripts likely to be involved in CD4 downregulation by Nef, and in this way identified targets that can be used to block this function of Nef.

In sum, during this project several new compounds and targets were discovered that can inhibit Nef functions and HIV infection.
1.2 Summary description of project context and objectives.

Since the introduction in the human population, human immunodeficiency virus (HIV) infection that causes an acquired immunodeficiency syndrome (AIDS) has taken the lives of at least 50 million people, and more than 33 million are living with HIV/AIDS today, according to the latest UNAIDS figures. Fortunately, the world-wide availability of current therapy of HIV infection (targeting viral enzymes i.e. protease, reverse transcriptase and integrase; and the gp41 transmembrane protein) has slowed down the death rate in HIV infected people due to efficient block of HIV infection. However, the virus is not removed from the body and can still express even up to low but detectable viral levels in the blood. This implies a risk for the emergence of drug-resistant HIV strains and may further maintain immune pathology. One of the proteins that can be expressed and was shown before to be important for immune pathology and progression to AIDS is Nef. The Nef protein is one of the accessory proteins of HIV. In some long-term non-progressors (LTNP), exclusively virus strains carrying Nef deletions were found (Kirchhoff et al., 1995; Deacon et al., 1995). Moreover, experimental SIV infection of rhesus macaques monkeys demonstrated the crucial role of Nef for in vivo viral replication and development of simian AIDS (Kestler et al., 1991). Therefore, it would constitute an interesting new target to be developed, the focus of the call HEALTH-2007-2.3.2.1 launched by the European Commission in 7th Framework Programme (FP7).

Nef is thought to be exclusively involved in virus-host interactions. It affects T cell activation, T cell development, trafficking of several cellular proteins like CD4, CD8, CD28, CXCR4, CCR5, MHC class I and II. Many of these functions were discovered by partners in this consortium, such as PAK2 and NFAT activation, NKp44L downregulation, MHC-I, DC-SIGN and CD8 trafficking. We also described immunopathological features of Nef, such as TCR-CD3 modulation by primate lentiviral Nef, the deregulation of T cell development by Nef, and the phenotype of Nef transgenic mice. Nef has no catalytical nor structural function, but serves as an adaptor protein. Well-known cellular partners for HIV proteins are the primary CD4 receptor, the chemokine co-receptors (CXCR4 and CCR5) serving as entry cofactors and the cellular protease required to process the viral gp160 envelope glycoprotein. Surfaces of the viral proteins interacting with host factors are potential targets for therapy. Inhibition of these specific virus-host interactions could in theory be achieved without side-effects and should be less prone to resistance. In addition, activity of specific cellular proteins (e.g. a kinase) might be redundant for cellular metabolism but essential for viral replication and/or pathogenicity. Thus, inhibition of interaction with these cellular co-factors by specific drugs may hamper HIV-1 replication and immune evasion, without cellular toxicity, as illustrated by HIV co-receptor inhibitors. Unlike other viral proteins targeted in HAART, anti-Nef therapy will not only directly affect viral replication, but will also dampen the deleterious impact of the virus on host defence. In this way, such therapy might lead to control of the virus by host defence mechanisms.

The goal of our iNEF project was to target the HIV-1 Nef protein, known to be a key factor in immune evasion by the virus and in the pathogenesis of AIDS. Our consortium consisted of academic partners with long-standing research interest in HIV and Nef; and both a university department and a company with expertise in medicinal chemistry.

Our specific objectives could be summarized as follows:

1. Optimization of existing and creation of new assays to identify and develop small molecule inhibitors of Nef function.
Identification of compounds that inhibit the Nef functions and further development of selected hits to highly active derivatives.

3. Assessment of the inhibitors’ effect on viral replication.

4. Evaluation of known and newly identified Nef interacting cellular partners for antiretroviral therapy.

To reach these objectives, the consortium organized for efficient collaboration and communication (WP1). Sharing of reagents and assays was planned, and management structures designed. The consortium engaged to follow two strategies that were complementary.

- First, small molecules were screened in search for molecules that inhibit Nef functions. To this end, screening assays available needed to be improved, or new assays had to be developed. For instance, partner Kalle Saksela had experience in the development of small molecule protein-protein interaction inhibitors for blocking HIV-1 Nef function (Saksela, 2004). They have a long-standing track record on the role of cellular SH3 domain-containing proteins as mediators of the AIDS-promoting effects of Nef. They developed and validated an “ELISA-like” biochemical assay for high-throughput screening (HTS) of compounds that could block SH3 domain binding by Nef. This assay was used to screen a large lead molecule library purchased from ChemBridge Inc. This compound library consisted of 65,000 drug-like small molecules rationally selected based on 3D pharmacophore analysis to cover the broadest part of biologically relevant diversity space, and has previously been highly recognized and proven as a primary screening tool for a wide range biological targets. This screening resulted in three small heterocyclic compounds that consistently inhibited SH3 binding by Nef. For further screening, improvement of the screening assay and development of an in cellulo assay was put forward. Screening of libraries available within the consortium was planned, as was chemical derivation to optimize hits from the different assays. These leads were distributed over the partners to enter testing in functional Nef assays. Important Nef functions tested were Hck-SH3 binding (WP3), T cell hyperactivation (WP4), CD4 and MHC-I down-regulation (WP5), immune synapse formation, and T cell reactivity (WP6). Hits were assayed for their effect on HIV infection (WP7). Promising compounds were planned to be tested in HIV- and Nef-transgenic mice to look for in vivo efficacy, and on NKp44L expression at the CD4+ T cell surface, and subsequent sensitivity of these cells to NK lysis (WP10). Moreover, molecular modelling and measurement of the Nef protein interaction with small molecules (WP11) was performed.

- Second, a whole genome RNA interference approach was planned to discover new cellular partners of Nef (WP8), that might lead us to new potential therapeutic targets. Throughout the project, care was taken to transfer results to the public domain, without jeopardizing the potential valorisation (WP9).

Performance and research indicators for measurement of progress were defined as follows:

i) Development of new tools, chemical libraries and shRNA library that allow identification of Nef inhibitors and Nef co-factors
ii) Identification of new lead compounds that inhibit Nef/SH3 interactions

iii) Identification of new lead compounds that inhibit Nef mediated T cell hyperactivation

iv) Identification of new lead compounds that inhibit Nef mediated changes in immune synapse formation and T cell reactivity.

References


1.3 Description of the main scientific and technological results

1.3.1 Work package 1: Project coordination and management

Objectives To establish a consortium that collaborates efficiently towards the objectives, while protecting intellectual property

The initial partners (#1 to #6, see Table W1) worked together to agree on a consortium agreement drafted from a DESCA template provided by P#1. The General Assembly met, either in person or by teleconference, on June 19th, 2008, December 13th 2008, August 24th 2009, October 17th 2009, December 9th 2009, July 12th 2010, October 23rd 2010, February 2nd 2011; external scientific expert Dr Lishan Su, Professor of Microbiology & Immunology, School of Medicine, The University of North Carolina, Chapel Hill, NC was present as a witness on the General Assembly, December 13th 2008. Partner #7 to #8 joined the consortium after the first reporting period (July 1st 2009), non-funded partner #9 signed the grant agreement but did not sign the consortium agreement and was not involved in the experimental work.

Table W1

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<td>France</td>
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Deliverables were all met
1.1 Consortium agreement (month 6)
1.2 Establishment of management bodies (General Assembly, external advisory group, WP leaders; month 1)
1.3 Reports on progress issued by General Assembly (on the average every 6 months)
1.4 Advices of external advisory group (after 18 months, Nikos Dedes, chair Policy WG, European AIDS Treatment Group reviewed the project)
1.3.2 Work package 2: Standardization and delivery of tools for the identification, validation and analysis of Nef/co-factor interaction and Nef function

Objectives To obtain robust assays for measuring Nef function

1.3.2.1 Reporter viruses
Several hundred HIV-1- and a variety of SIV-based reporter viruses have been generated.

1.3.2.2 Chemical libraries
In a 65000 compounds library from ChemBridge, #P3 previously discovered 3 compounds affecting Nef-SH3 binding. Leads from these compounds were developed by P#5. In addition, over 40 years several generations of medicinal chemists from #P8 laboratory have prepared small molecules as part of their research programs in chemical biology or medicinal chemistry. An open access high throughput screening facility in Strasbourg was established to accelerate the discovery of novel biologically active molecules that might represent interesting research pharmacological probes or precursors of clinical candidates. This initiative was extended to the level of France and #P8 currently participates in the EU-OPENSCREEN ESFRI infrastructure preparatory phase (Coordinator: Ronald FRANK). For our project, 4800 structurally diverse and very ‘drug-like’ molecules were selected, shown in previous screens to yield interesting, validated hits for about 70% of targets. The collection was sent to #P2 and #P3. The structures of all compounds were also made available to the Consortium. Some hit resynthesis and analoging within the limits of P#8 back-up collection was needed. Some screenings generated more than 200 validated hits. To select the most relevant structure following strategy was used:

- Frequent hitters, fluorescent or coloured compounds, non drug-like compounds such as Michael acceptors, chemically instable compounds, exotic and nitro compounds were identified and discarded.
- Derivatives belonging to a same chemical family (common scaffold or a well-defined generic formula) or structurally-related compounds sharing a common pharmacophore were clustered.
- Sometimes one representative molecule for each series was selected.

1.3.2.3 RNA interference platform
A commercially available library targeting around 38.500 genes (FIV based, GeneNet 50K, System Biosciences, Mountain View, CA) was purchased and used in WP8.

1.3.2.4 Reporter cell lines
Several reporter cell lines are in use by the partners. They are available to the consortium.

1.3.2.5 Generation of a new and optimized screening assay
We aimed to develop an improved, cell-based HTS assay for the Nef/SF3 interaction. In addition to taking place in the intracellular milieu, ideally such an assay would have fewer steps to perform, show less sample-to-sample and assay-to-assay variation, and would preferably be a cell-based assay.
To this end, we focused on developing an assay based on a principle known as bimolecular fluorescence complementation (BiFC), which enables direct visualization of diverse protein interactions in living cells (Kerppola T, Annu Rev Biophys, 2008, 37:465-87).
Deliverables were all met
2.1 Bicistronic HIV reporter constructs panel extended and available to all partners (month 6)
2.2 Chemical library panel extended and available to all partners (month 11)
2.3 Reporter cell line panel extended and available to all partners (month 6)
2.4 Single polypeptide screening tool for inhibitors of Nef/Hck-SH3 binding available (month 36, replaced by the BiFC assay mentioned above)

1.3.3 Work package 3: Evaluation of potential inhibitors of Nef-SH3 protein-protein interactions

Objectives To discover new inhibitors of Nef/Hck-SH3 binding

1.3.3.1 Screen for novel inhibitors of Nef/Hck-SH3 binding

P#3 previously developed and validated an “ELISA-like” biochemical assay for high-throughput screening (HTS) of compounds that inhibit SH3 binding of Nef. In this primary screen, 65,000 ChemBridge compounds were screened at 10 µM concentration, followed by a secondary screen. In these screens 2 small molecules (L20-K03 and L170-G08) inhibited Nef-SH3 interaction dose-dependently. The same assay was used on the library provided by P#8 (see WP2). Although small in size (4,800 compounds), this compound collection should be more divergent than the Chembridge collection.

The primary screen gave a relatively high rate of false positives, from 1000 possible hits only 3 consistently inhibited SH3 binding by Nef. Therefore P#3 further optimized this “ELISA-like” assay to be more suitable for HTS and gain much lower intra- and inter-plate variation. With the optimized assay, P#3 could confirm 4.5% of the hits from the primary screen of the P#8 library, when in the previous screen only 0.3% of the potential hits were confirmed.

With the optimized “ELISA-like” assay, compounds were tested for their ability to inhibit Nef/S3H interaction at 50 µM concentration. From this library, 4 small molecule compounds that consistently inhibit SH3 binding by Nef dose-dependently (Figure W3.1) were identified. These chemicals were further characterized for their ability to inhibit Nef-unrelated protein-SH3 interactions as well as other protein-protein interactions.

The chemical structures of these 4 compounds were used to models to generate additional molecules by P#5 to find an optimal Nef-inhibitor (see later).
Figure W3.1. Dose-response analysis of selected anti-Nef lead compounds in Nef/SH3 ELISA assay. All tested compounds were selected from the initial screen as “hits”, and the inhibitory potential of compounds LPS 02-30-L-F07, LPS 01-16-L-B05, LPS 01-05-L-A07 and LPS 05-02-L-H03 was confirmed in this secondary Nef/SH3 ELISA assay. A validated hit chemical L20-K03 was used as a positive control.

These molecules were re-synthesized in large amounts and lead candidates LPS 02-30-L-F07 (estimated IC50 value of 35 µM), LPS 01-16-L-B05, LPS 01-05-L-A07 (estimated IC50 value of 150 µM) were confirmed, the first being the most potent.

1.3.3.2 Identification of new lead compounds that inhibit Nef/SH3 interaction

Based on our own findings and the structure of a potential Nef-inhibitor reported by Collette et al., (Collette Y, PNAS, 2007. 104(49):19256-61) a structure of a putative lead molecule (“Structure 1”) was planned to be used for designing novel anti-Nef compounds. Structure 1 was used as a model for identification of potential compounds from existing chemical libraries as well for chemical synthesis of derivatives as candidates for new Nef-inhibitors. Selection of compounds was based on their potential ability to bind in the hydrophobic pocket of Nef where the RT-loop of SH3 interacts. More specifically, the idea of Structure 1 was to have on one side an apolar group that would fit in the “hydrophobic pocket” of Nef and on the other side a polar group to interact with Lys82 or Arg 77. The linker between the hydrophobic group and hydrophilic group will be crucial for a good orientation of both groups.

A selection of 349 interesting Structure 1-like molecules from the Chembridge compound collection was tested in our “ELISA-like” Nef-Hck protein-interaction assay for their ability to inhibit Nef. However, in our screens none of these selected chemical library compounds showed inhibitory potential. In addition, a set of compounds based on Structure 1 was synthesized by P#5 and tested by P#3 for Nef/SH3 inhibition (41 compounds altogether; named ES000167 - ES000230). However, similar to the Structure 1-related compounds
ordered from Chembridge none of the compounds ES000167 - ES000230 showed inhibitory potential in our assay when tested with five different concentrations. However, since ES000219 showed activity in a different type of anti-Nef screening assay performed by P#2, further derivates synthesized by P#5 based on ES000219 (ES000668-ES000675) were also included in analysis by P#3. Similar to their parental compound ES000219, none of these compounds were able to inhibit Nef/SH3 association. Also compounds derived from hit lead compound LPS 02-30-L-F07, LPS 01-05-L-A07, LPS 01-16-L-B05, LPS 01-11-L-A02 and LPS 02-14-L-H02 (altogether 34 compounds) as well as compounds derived Chembridge library compounds failed to inhibit in the Nef/SH3 assay. Additional derivatives (altogether 33 compounds) of the hit compound LPS 01-16-L-B05 with four different concentrations (100 µM to 12.5 µM) were made by P#5 and tested. As shown below (Figure W3.2), several compound derivatives had lost their capacity to inhibit Nef/SH3 along the modification of chemical structure. By titrating the activity of hit lead derivatives (ES507-ES543) we were able to identify a specific molecular group whose presence clearly correlated with compound activity.

![Figure W3.2](image)

*Figure W3.2.* A dose-response analysis for compound derivatives of hit lead LPS 01-16-L-B05. The inhibitory potential of compounds ES000507-ES000543 was tested in the Nef/SH3 ELISA assay, two different concentrations (50 µM and 12.5 µM) are shown. The unmodified hit lead LPS 01-16-L-B05 was used as a positive control.
Testing the inhibitory capacity of anti-Nef lead compounds in living cells

One of the key aspects in drug development is to identify lead candidates that can penetrate through the cell membrane if their target is located inside the cell as is the case of Nef. Therefore P#3 set up a cellular test to analyse how well these lead candidates could inhibit Nef function in cells.

Nef induces phosphorylation of Hck, a Src tyrosine kinase that mediates Nef effects in hematopoietic cells, a function that can be measured with a specific antibody recognizing phosphorylated Hck. In this assay, cells are transfected with Nef and Hck cDNAs resulting in formation of Nef/Hck protein complexes in cells. Cells are lysed and Nef/Hck protein complexes are immunoprecipitated from lysates and incubated with test chemicals. After SDS-PAGE and Western blotting, the amount of phosphorylated Hck present in Nef-Hck complexes is measured with an anti-phospho Hck antibody (Acris Antibodies GmbH, Hiddenhausen, Germany). A group of compounds to be tested in cells was selected based on their activity in Nef/SH3 ELISA assay. In addition, compounds that showed no inhibitory potential in Nef/SH3 assay were included as negative controls along with DMSO control. Below (Figure W3.3) is an example how phosphorylation status of Hck was detected. Based on these results most potent Nef-inhibitors having effect on Nef in cells would be compounds LPS 01-16-L-B05, and the 2 original hits from the ChemBridge library. Since only the compounds that seemed most promising based on our biochemical Nef/SH3 interaction data were yet tested here due to practical limitations (such as requirement of high amounts of tested compound and low-throughput nature of the assay), we cannot rule out that some of the inhibitors with modest potency in the ELISA assay might nevertheless show more promising results in this cell-based assay.

Studies on the effects of our lead compounds on Nef-Hck interaction based on co-immunoprecipitation assays gave inconsistent results (data not shown), and this experimental approach is not well suited for such analysis. Thus, although pHck assay seems to be a sensitive cellular assay to examine the effect our lead compounds on the Nef-SH3 interaction in living cells, a practical cell-based assay that would directly measure the physical association of Nef with Hck-SH3 would be informative. For this purpose P#3 developed a high-throughput cellular Nef/SH3 interaction assay based on bimolecular fluorescence complementation.
Figure W3.3. An example analysis of compound effect on Nef-induced Hck activation. Upper panel shows the level of Hck phosphorylation after SDS-PAGE and Western blotting measured with an anti-phospho Hck antibody. Lower panel shows total Hck (phosphorylated and unphosphorylated) measured with an Hck antibody. pHck and Hck signals were measured and quantified with Odyssey Infrared Imaging system (LI-COR Biosciences).

1.3.3.4 Analysing cytotoxicity of selected anti-Nef lead compounds

The effect of hit compounds and their derivatives on cellular toxicity was evaluated in an assay that measures the amount of ATP present, which signals the presence of metabolically active cells (Promega, Cell TiterGlo): none of the hit chemicals e.g. LPS 01-16-L-B05 induced cytotoxicity when compared to negative control (DMSO) in HeLa cells with 100 µM or 50 µM concentration.

1.3.3.5 Testing the specificity of anti-Nef lead compounds

As a first approximation of the specificity of hit compounds P#3 used the same ELISA-like assay to test their ability to inhibit related and unrelated protein-protein interactions. Lead compounds showed no inhibitory activity towards binding of an anti-Myc epitope antibody towards its Myc peptide target when tested at four different compound concentrations up to 200 µM. By contrast, in SH3 domain-mediated interaction between the NS1 protein influenza A virus and the SH3 of CrkL, compounds LPS 02-30-L-F07, LPS 01-05-L-A07, LPS 01-16-L-B05 all showed inhibitory potential that was almost as strong as that observed towards the HIV-1 Nef - Hck-SH3 interaction. Similar results were derived with Chembridge lead compounds (data not shown).

Thus, it is clear that these lead compounds are not specific Nef inhibitors, but can inhibit at least another proline-directed protein-protein interaction. However, their ability to block Nef-induced Hck activation in cell without apparent toxicity, and failure to inhibit an unrelated (antibody/antigen) interaction suggest that at least some specificity exists. Further analysis of additional derivatives of LPS 02-30-L-F07, LPS 01-05-L-A07, LPS 01-16-L-B05, L20-K03 and L170-G08 including a broader selection of protein interaction pairs are clearly indicated to address this issue.

Deliverables were all met

3.1 Optimized derivatives from lead compounds identified by P#3, inhibiting Nef/Hck-SH3 binding (month 18)

3.2 Novel, optimized inhibitors of Nef/Hck-SH3 binding (month 42)

1.3.4 Work package 4: Inhibition of HIV-1 Nef-mediated hyperactivation of T cells

Objectives To discover inhibitors of NFAT activation by Nef

NFAT-driven luciferase activity in HIV-1-infected and stimulated Jurkat T cells was measured in a NFAT screening assay. The assays system was optimized to detect inhibitors of NFAT hyperinduction by Nef. Briefly, 15 µl of the diluted compounds from the library provided by P#8 were added to 85 µl Jurkat.NFAT cells and infected with 50 µl VSV-G pseudotyped HIV-1. One day later the cells were treated with PHA (1 µg/ml) and after overnight incubation luciferase activities were measured in the cell lysates. Cytotoxicity
assays were always performed in parallel. These analysis showed that about 15% (~500) were cytotoxic and about 6% (~200) reduced the NFAT-dependant luciferase activities without displaying cytotoxic effects. Repetitive testing yielded some hits that were further developed by P#5. From this first generation (e.g. ES000219 and ES000377) some second generation derivatives were most active in the NFAT assay. Figure W4.1 shows an example obtained with several of these compounds

![Figure W4.1](image)

**Figure W4.1** Suppression of NF-AT induction by the indicated compounds. Jurkat cells stably transfected with an NFAT-dependent reporter gene (Fortin et al., 2004) were seeded in 96-well plates (100,000 cells in 100µl RPMI). One hour prior to infection with a VSV-G pseudotyped HIV-1 construct the cells were incubated with diluted compounds (final concentration 10 µg/ml; ± 25 µM). Two days later the cells were stimulated overnight (PHA) and luciferase activities were measured after overnight incubation. The indicated levels of NFAT-dependent luciferase reporter activity are the average (±SD) of quadruplicate infections. Mock specifies uninfected control cells.

**Deliverable was met**

4.1 Novel, optimized inhibitors of NFAT activation by Nef (month 42)

### 1.3.5 Work package 5 Inhibition of HIV-1 Nef induced CD4 and MHC-I down-regulation

**Objectives** To discover inhibitors of Nef-mediated down-regulation of CD4 and MHC-I

P#4 designed cell-based assays, and performed screening activities, in order to identify potential compounds inhibiting the down-regulating activity of Nef on cell surface expression of CD4 and MHC-I molecules. P#4 used different experimental systems, to test a few hundred selected compounds, from various libraries.
1.3.5.1 Design of cell-based systems to study HIV-1 Nef induced CD4 and MHC-I down-regulation.

P#4 developed and validated various experimental systems, based on the transient or stable expression of Nef in T cell lines, that may allow the screening of compounds inhibiting Nef function. First, individual cell clones (derived from CEM cells) stably expressing Nef, that were established in P#4 laboratory (Schwartz et al. J. Virol. 69:528-533 1995) were used. However, a western blot analysis indicated that CD4 expression was very low in these cells, suggesting that some unexpected mechanisms may compensate in these cells for the presence of Nef (not shown). Therefore a Jurkat T cell line, in which Nef is transiently expressed by transduction with a lentiviral vector (Sol-Foulon et al., J Biol Chem 279:31398-408 2004) was used. In these cells, CD4 and MHC-I molecules are down-regulated from the cell surface (Figure W5.1) and accumulate as expected in intracellular compartments.

![Figure W5.1](image1.png)

**Figure W5.1.** CD4 and MHC-I down-regulation in Nef-expressing Jurkat cells.

Compounds ES000199 through ES000230 were tested, by incubating the drugs at various concentrations with T cells, expressing or not expressing Nef. Cell viability was then measured at different periods of time (24 and 48h), using a MTT colorimetric assay. A subset of the compounds (ES000220 through ES000226) displays an important cytotoxicity at the highest concentration (100 µM). None of the compounds tested increased CD4 or MHC-I surface levels in Jurkat-Nef expressing cells, nor in control Jurkat cells (not shown).

Next 01-06-L-A03, 03-05-L-B03, 01-18-L-B06, 02-31-L-D09, 02-13-L-E04, 02-30-L-F07, 02-29-L-G07, 02-30-L-G10 were tested. These compounds were selected from a panel of drugs previously tested in the lab in a preliminary experiment, and displaying minor effects on CD4 or MHC-I surface levels, with or without Nef. In addition, P#4 tested a new set of compounds: ES000470 through ES000494 and ES000507 through ES000543.

The compounds were first tested in the Nef-inducible Jurkat TetOn system described by Witte et al Mol Cell 13:179-90, 2004 (Figure W5.2). This Jurkat cell clone expresses a Nef-estrogen receptor (Nef-ER) fusion protein that becomes active after incubation with tamoxifen. In addition, Nef-ER is under the control of a doxycycline inducible promoter. Nef functional expression is thus induced by the addition of doxycycline and tamoxifen drugs. After 18h of incubation, CD4 and MHC class I surface levels are down-modulated by Nef. To summarize, the compounds to be tested are incubated with Jurkat TetOn-Nef cells at
different concentrations (2.5 to 50 μM) for 24 hours. Then doxycycline and tamoxifen are added and incubated for 18 hours to allow Nef expression: none of the compounds had any significant effect on Nef-induced CD4 and MHC-I down-regulation.

**Figure W5.2** The Nef-inducible Jurkat TetOn system system used to assay effect of compounds Jurkat TetON Nef-inducible cells were pre-treated with compounds for 24h. Then, addition of Doxycyclin and Tamoxifen for 18h induced the expression of Nef. Staining with Class-I and CD4 antibodies was performed afterwards and analyzed by flow cytometry using a FACS CANTO II (BD Bioscience)

**1.3.5.2 Study of HIV induced CD4 and MHC-I down-regulation.**

P#4 tested some of the compounds on HIV-infected Jurkat cells. The effect of the compounds was assayed 48h post-infection. Jurkat E6.1 were infected with NL4-3 viruses for 2h, split into 96 well plates and then incubated with the compounds. Cells were double stained with intracellular Gag and either CD4 or MHC-I antibodies and analyzed by flow cytometry.

**Figure W5.3** Assay to test selected compounds in HIV infected cells

Jurkat E6.1 were infected with NL4-3 viruses pseudotyped with VSV (2 doses; 10 and 50 ng p24/mL) for 2h, split into 96 well plates and incubated with the compounds. Two days later, cells were double stained with intracellular Gag and either CD4 or MHC-I antibodies and analyzed by flow cytometry using a FACS CANTO II (BD Bioscience)

The levels of CD4 and MHC-I were determined in productively infected (Gag+) and bystander (Gag-) cells.
The effect on CD4 and MHC-I down-modulation was tested in Gag negative and Gag positive populations of Jurkat cells. None of the tested compounds affected CD4 and MHC-I down-regulation.
A few compounds induced a moderate effect in the absence of Nef (02-30-L-G10, ES000485, ES000488, ES000514, ES000518, ES000522, ES000526). None of the compounds tested displayed any inhibitory effect on Nef-induced CD4 and MHC-I down-regulation.
This screening did not lead to the identification of hits that will be worth studying further.

**Deliverables**
5.1 Novel, optimized inhibitors of CD4 and MHC-I down-regulation by Nef (not met)

**1.3.6 Work package 6 Assay of selected compounds or siRNAs on immune synapse formation and T cell reactivity**

**Objectives** To evaluate the effect of identified Nef inhibitors on immune synapse formation. To determine role of cellular Nef partners on immune cell function.

HIV efficiently propagates through cell-to-cell contacts, which include virological synapses, filopodia, and nanotubes. P#4 quantified and characterized further these diverse modes of contact in lymphocytes. Viral transmission mainly occurs across the viral synapses and through "polysynapses," a rosette-like structure formed between one infected cell and multiple adjacent recipients (Figure W6.1). Polysynapses are characterized by simultaneous HIV clustering and transfer at multiple membrane regions. HIV Gag proteins often adopt a ring-like supramolecular organization at sites of intercellular contacts and colocalize with CD63 tetraspanin and raft components GM1, Thy-1, and CD59. In donor cells engaged in polysynapses, there is no preferential accumulation of Gag proteins at contact sites facing the microtubule organizing centre. The LFA-1 adhesion molecule, known to facilitate viral replication, enhances formation of polysynapses. P#4 results reveal an underestimated mode of viral transfer through polysynapses. In HIV-infected individuals, these structures, by promoting concomitant infection of multiple targets in the vicinity of infected cells, may facilitate exponential viral growth and escape from immune responses.

![Figure W6.1 A Polysynapse formed between one infected T cell (HIV is in green) and 4 target T cells (in blue).](image)

No molecules were tested yet to assay the effect on immune synapse formation.
**Deliverables**

6.1 Characterisation of Nef inhibitors in their effect on immune synapse formation (not met)
6.2 List of cellular Nef partners as relevant therapeutic partners given their role in immune cell function (not met)

**1.3.7 Work package 7 Effect of Nef inhibitors on Nef enhanced HIV replication**

**Objectives** To evaluate the effect of identified Nef inhibitors on HIV replication and Nef function in vivo. To determine the role of cellular Nef partners on HIV replication. To monitor viral resistance development against Nef inhibitors in vitro.

The available library of 4800 molecules from P#8 was screened by P#2 for inhibition of HIV infection using TZM-bl indicator cells, incubated for 30 min and subsequently infected the culture with HIV-1 R5 (~ 1 ng p24) in duplicate. Two days later the cells were examined for cytotoxic effects and the infection rates were determined using the β-Gal Screen assay. Of the ± 5000 compounds tested about 15% (~750) were cytotoxic and 25 inhibited HIV infection >50% and ten >80% without significant cytotoxic effects. Hits were verified in addition to most compounds produced by P#5, both by P#1 and P#2 using TZM-bl cells, peripheral blood mononuclear cells or CD4+ peripheral blood lymphocytes. Cytotoxicity tests using Celltiter Glo assay (P#1) or MTT assay (P#2) were performed to exclude aspecific inhibition of infection. P#1 also performed one week of culture of HIV infected CD4+ PBL to monitor the effect of the compounds on replication.

Some compounds were detected by both P#1 and P#2 to affect HIV infection without toxicity. These are LPS 02-15-L-D07 and LPS 02-11-L-E02. Both X4 and R5 strains were inhibited by these compounds. As shown in Figure W7.1, upon titration LPS 02-15-L-D07 was the most potent (IC50 estimated 2.5 µM) compared to structurally related compounds (cm51-027, cm51-034, cm51-037 or LPS 02-11-L-E02) to inhibit HIV replication CD4+ peripheral blood lymphocytes, 1 week after infection.
Nef induces actin cytoskeleton changes and impairs cell migration toward chemokines. P#4 further characterized the morphology, cytoskeleton dynamics, and motility of HIV-1-infected lymphocytes. HIV-1 induces a characteristic remodelling of the actin cytoskeleton. In infected lymphocytes, ruffle formation is inhibited, whereas long, thin filopodium-like protrusions are induced (Fig. W7.2). Cells infected with HIV with nef deleted display a normal phenotype, and Nef expression alone, in the absence of other viral proteins, induces morphological changes. P#4 confirmed the induction of long filopodium-like structures in unfixed Nef-expressing lymphocytes. The cytoskeleton reorganization induced by Nef is associated with an important impairment of cell movements. The adhesion and spreading of infected cells to fibronectin, their spontaneous motility, and their migration toward chemokines (CXCL12, CCL3, and CCL19) were all significantly decreased. Therefore, Nef induces complex effects on the lymphocyte actin cytoskeleton and cellular morphology, which likely impacts the capacity of infected cells to circulate and to encounter and communicate with bystander cells.

Figure W7.2. HIV-1 alters the shape of T lymphocytes through Nef expression. Representative images of non infected (NI) or HIV WT or -nef-deleted infected Jurkat cells. **A.** SEM images. HIV virions were stained with anti-Env MAb coupled to gold particles (appearing as white dots). **B.** 3-D reconstruction of confocal images. Cells were stained with phalloidin rhodamine (red) and anti-Gag Mab (green).
Deliverables
7.1 Characterisation of Nef inhibitors in their effect on HIV replication and function of Nef in vivo (met in part at month 42)
7.2 List of cellular Nef partners as relevant therapeutic partners given their role in HIV replication (not met)
7.3 Assessment of viral resistance acquisition to Nef inhibitors affecting HIV replication (not met)

1.3.8 Work package 8 Genome-wide RNA interference screen for novel co-factors of Nef: in search for new therapeutic targets

Objectives To discover novel cellular partners of Nef to identify potential therapeutic targets

As downregulation of CD4 by Nef is a conserved property among most primary isolates, and several other observations also suggest this function to be important for the viral life cycle, P#1 focused on this function to discover new partner proteins of Nef. A HeLa derived cell line was used, stably expressing a high level of CD4 receptor. Cells transduced with a shRNA targeting AP2-µ2, an essential partner of Nef for CD4 downregulation, were used as positive controls.

Two different screening approaches were developed. The first strategy was to transduce Hela-CD4 cells with a retrovirus expressing Nef and GFP reporter protein. Cells positive for GFP (30 to 45%) were sorted two to three times to obtain a cell population homogenously positive for GFP, thus Nef expression. Subsequently, cells were transduced with a lentiviral library targeting around 38.500 genes (FIV based, GeneNet 50K, System Biosciences, Mountain View, CA) at MOI of 0.5 for 16h and submitted to puromycin selection. After one week of selection, sorting was done to isolate single cells showing inhibition of CD4 downregulation. These cells were deposited in multi-well culture plates and allowed to expand as individual clones. The phenotype was verified after clonal expansion (expected to be GFP+CD4+), and genomic DNA was isolated. On this DNA that should contain the proviral insert of the virus expressing the shRNA, PCR was performed to sequence the encoded shRNA present. Using these sequences, the targeted genes were identified by performing blast search analysis. The targets identified are involved in several cellular pathways, such as ubiquitin-dependent protein degradation, proteolysis, transport, etc.

However, this screening strategy was not optimal because only a few hundreds of cells could be sorted per experiment. Also, only 13% of sorted cells grew after FACS single cell deposition in culture plates. It is known that long term expression of active Nef protein in cell lines can lead to toxic effects that could affect the final results, and contribute to poor survival of single sorted cells. Furthermore, only one third of the surviving clones could be sequenced unambiguously.

In the second screening approach long term HIV-Nef expression was avoided (Figure W8.1).

To this end P#1 first transduced Hela-CD4 line with the shRNA lentiviral library at MOI 0.5 or with a control shRNA targeting luciferase. After selection with puromycine for 7 days, the surviving cells were then transduced with the retroviral vector expressing Nef and GFP. After a few days, the Nef transduced cells that had maintained high to medium (upper 5%) of surface CD4 receptor expression were bulk sorted by FACS and grown in culture medium. On cDNA, a two rounds nested PCR generated biotin labeled shRNA sequences.
As the System Biosciences library is constructed using the Affymetrix probes, amplification products can be detected by hybridisation to the Affymetrix human U133 Plus 2 microarray.

Figure W8.1 Bulk sorting strategy to identify overrepresented targeted genes in cells CD4⁺ (Y-axis flow cytometry plot) despite Nef expression (GFP, X-axis flow cytometry plot).

After data summarization using MAS5 algorithm implemented in Expression Console analysis software from Affymetrix, the quality of the hybridization process was very satisfactory. Targeted genes overrepresented in the CD4 high sorted fraction compared to the total non-sorted cell samples were identified and a ratio was calculated between signal intensity for every detected probe. Genes with a ratio higher than two-fold were further analyzed for their relative enrichment in certain pathways using gene ontology tree machine web site tool (http://bioinfo.vanderbilt.edu/gotm/). Certain hits identified were components of vesicle trafficking and endocytic pathways, such as a protein that plays an important role in cargo sorting from trans-golgi network to endosomes and lysosomes for degradation, or proteins implicated in critical step of numerous cellular pathways including signal transduction and membrane trafficking. Several of these proteins were shown before to interact with each other. In addition, several proteins involved in RNA processing were found. This screening strategy therefore yielded very intriguing results. However, as the targets of HIV infection are primary cells such as T cells and macrophages, the role of the identified proteins should preferably be validated and confirmed in these cell types, before further development of the target is justified.
Deliverables

8.1 List of novel cellular partners of Nef in trafficking, NFAT activation and T cell development, in vivo validation for some (met in part)

1.3.9 Work package 9 Dissemination and valorisation

Objectives  To disseminate results generated to the public domain, while protecting potential valorisation of EU investment

All partners delivered content for the website www.iNEF.UGent.be established by P#1. The site informs visitors on the mission of the iNEF consortium and its partners with links to their institutions. The symposium “Recent advances in HIV Biology”, September 23rd 2011 is announced on that site, that will mark the end of the iNEF project and will display the findings in the context of the state of the art in HIV research (http://www.inef.ugent.be/Symposium.htm). At this occasion, members of the consortium will speak on a public forum on their research. This symposium is open to all scientists and the public. The program is as follows:

- Introduction to molecular determinants of HIV infectivity and pathogenicity. Bruno Verhasselt, Ghent University
- The virology and immunology of HIV cell-to-cell transfer. Olivier Schwartz, Institut Pasteur, Paris
- Variation of the HIV-1 envelope protein and its impact on cell entry and pathogenesis. Chris Verhofstede, Ghent University
- Tetherin antagonism by primate lentiviral Nef proteins. Frank Kirchhoff, University of Ulm
- Structure-function relationships in HIV-1 Nef: Where to inhibit? Matthias Geyer, Max Planck Institute Dortmund
- The relation between HIV-1 integration and latency. Linos Vandekerckhove, Ghent University

Most results of these project are considered for exploitation and cannot be disclosed in publications at present. However, several partners published more fundamental results or overviews of research in peer reviewed journals that provide open access or that will allow open access after an embargo period.

A list of dissemination activities and publications can be found on the website www.iNEF.UGent.be

A special issue (to be published early 2012) of the journal Current HIV Research will be devoted to Nef research, with contributions of several partners of the consortium. The support of the European Community FP7 Health funding is acknowledged in these papers. The public report will be posted on the project website after consent of the Commission.

No patent applications were filed at the end of the project (month 42)

Deliverables were met

9.1 Website with public and secure pages (month 6)
9.2 Scientific publications and patent applications (month 42)
1.3.10 Work package 10 Inhibition of HIV-1 Nef induced NKp44L down-regulation

**Objectives** To discover inhibitors of HIV-1 Nef induced NKp44L down-regulation

P#9 did not access to the consortium agreement, and therefore was not involved in research relating to the project iNEF. No experiments were performed within this work package.

**Deliverables**
10.1 Novel, optimized inhibitors of NKp44L down-regulation by Nef (not met)

1.3.11 Work package 11 Structural biology of Nef interactions

**Objectives** To model and analyse the molecular interaction of Nef with partner proteins and small molecule inhibitors

P#7 performed NMR spectroscopy mapping experiments with compounds synthesized by P#5 that (ES000219 (both DMSO and water soluble variant) and 8 derivative compounds (ES000669 through ES000675). When using only 2-fold excess of ES000219 over Nef (NefSF2 (45-210) protein; 0.4 mM) a number of shifts were detected (residues G99 and G100 part of the second hydrophobic groove, that is not the binding side for the RT-loop of the SH3 domain. In consequence, W61 is displaced from this site, which results in a small shift of the side chain (W61-epsilon), and a much larger shift of the backbones (W61 and L62), as shown in Figure W11.1. The control measurement showed no perturbation of the NMR resonance signals of 0.4 mM NefSF2 (45-210) protein upon addition of 1.6% DMSO. All compounds were dissolved in DMSO besides the salt variant of ES000219.
Figure W11.1 NMR spectra reference measurement of the initial hit ES000219 that was used as a lead structure for further optimization trials.
Figure W11.2 NMR spectra of a titration series with molar ratios from 1:1 to 1:16 Nef to ES000219, that show perturbation of several residues in Nef. These shift cluster into the hydrophobic "sorting motif binding site" around residues G99 and G100. Movement of residues W61 and L62 indicates the displacement of these residues from this site by the compound.

The mapping results from the titration experiments point very much to the cargo binding pocket, or "sorting motif recognition site", as the candidate binding surface on Nef for the small molecular anti-Nef compound ES000219 and its various derivatives. From an analysis of Nef sequence conservation it turns out that this binding site is in fact much more conserved than the RT-loop binding site, required for the specific interaction of Nef with SH3 domains. In Figure W11.3, on the left side sequence conservation is displayed on the surface of Nef ranging from 'variable' to 'conserved'. On the right side, polar and hydrophobic residues on the surface of Nef are displayed. It shows that both the RT-loop binding site but also the sorting motif binding site contain several hydrophobic residues.
Figure W11.3 Surface models of NefSF2 showing on the left side sequence conservation (ranging from blue 'variable' to red 'conserved'). On the right side, polar (green) and hydrophobic (yellow) residues are displayed.

Crystallization experiments were performed with different constructs of NefSF2 and compound ES000219. Both co-crystallization and soaking strategies were applied. These crystallization trials were performed with different amounts of the compounds, due to the rather low binding affinity of the small molecule inhibitor. Crystals grew under various conditions at 4°C. However, none of the tested crystals showed occupancy of the small molecule compounds after structure determination.

Figure W11.4 Nef crystals growing at 4°C, under conditions: 1 M Tris/HCl, pH 8.1, 0.2 M lithium sulphate and 25% PEG 4000.
The structure was solved by molecular replacement showing four Nef molecules in the asymmetric unit. The crystal turned out to be very stable, which is likely due to the tight intermolecular interaction shown in Figure W11.5 and Fig W11.6.

**Figure W11.5** Crystal structure of Nef\textsubscript{SF2}. The four Nef proteins are intertwined by mutual N-terminus-to-core domain interactions. The electron density map is shown in the right panel.

Unfortunately, by these assemblies the hydrophobic sorting motif recognition sides in Nef are occupied, such that soaking of the small molecular inhibitors in not feasible.
Figure W11.6 Intermolecular interaction between the four Nef proteins in the asymmetric unit of the crystal structure solved. Upper panels ribbon representation, lower panels surface representation. As can be seen from the surface depiction on the lower right panel, both hydrophobic pockets of Nef molecule C are occupied by the proline-rich N-termini of neighbouring molecules.

 Deliverable was met
11.1 Model of Nef/partner protein or small molecule interaction (month 42)

1.4 The potential impact and the main dissemination activities and exploitation of results.

1.4.1 Potential impact

1.4.1.1 Social impact

The number of people living with HIV/AIDS keeps rising, in Europe and world-wide. An estimated 0.8 million of people were living with HIV/AIDS in Western and Central Europe by the end of 2009, a 30% increase compared to 2001 (UNAIDS data). In Eastern Europe the increase was even more dramatic, totalling in more than 2 million infected people in the whole of Europe. Over the same time period, the global incidence rate increased by 16%, indicating that the prevention measures need to be reinforced in the whole of Europe. The social impact of this epidemic is enormous in some regions. The most active generation and their children have become infected at considerable degree. The inherent morbidity and mortality leads to disruption of social and family ties and structures. In addition, stigmatisation occurs in both treated and non-treated patients. Fortunately, highly active anti-retroviral therapy (HAART), a cocktail of reverse transcriptase inhibitors and protease inhibitor that can be modified or supplemented with integrase inhibitors or entry inhibitors, has become available worldwide. At present, this therapy can suppress viremia in almost all patients, reducing morbidity and transmission. However, occasional ‘blips’ of low level viral replication occur in a considerable fraction of patients. Moreover, a continued evolution of
the immune pathology has been observed in these and other treated HIV infected patients. This urges us to continue the search for medication that targets Nef, an important mediator of HIV immune pathology.

1.4.1.2. Economical impact

With more than 33 million people living with HIV/AIDS today, the potential market for antiretroviral therapy is still growing. Not only the number of people, but also the demographic profile (i.e. children and younger adults) jeopardise the economical future of the affected region, on top of the human suffering and costs caused by morbidity and mortality. Enzyme inhibitors (reverse transcriptase, protease and integrase inhibitors) keep to be the most important segment of the anti-viral medication market. To keep pace with the development of resistance by the virus, HIV fusion inhibitors, HIV attachment inhibitors, HIV maturation inhibitors, HIV replication inhibitors, and HIV entry blockers (e.g. CCR5 antagonist maraviroc) are being developed or have hit the market recently. Several European major pharmaceutical companies are active in the antiviral medication (Boehringer Ingelheim, Sanofi Pasteur, Pfizer, GlaxoSmithKline, Novartis, Hoffmann-La Roche,...). 

Nef is a novel target: not essential for viral infection and replication, but important for the induction of immune pathology ultimately resulting in AIDS. In this project, several candidate molecules were identified. For some, a direct interaction with Nef was shown, others inhibited Nef function or HIV infectivity although more research is needed to demonstrate the specificity for Nef rather than a broader inhibitory spectrum. Such broad inhibitors of SH3 interactions (inhibiting e.g. as well Nef-Hck association as NS1-CrkL association) can have applications in research or other fields of medicine. The consortium decided not to file IP rights now, but however to continue research in this leads to strengthen claims on exploitation.