REPORT ON NKFI-118176 PROJECT

entitled: ERA-NET HIVERA: DEVELOPMENT OF A NOVEL FAMILY OF NATURAL AND NATURAL PRODUCT BASED SYNTHETIC HIV INTEGRASE INHIBITORS

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immune deficiency syndrome (AIDS). There are approximately 37 million people currently infected with HIV worldwide. In the last decades, a combination antiretroviral therapy (cART) was introduced in treatment of HIV, which use different classes of drugs, such as protease inhibitors, nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTIs/NNRTIs), entry inhibitors (CCR5 coreceptor antagonists, fusion inhibitors, and postattachment inhibitors), and integrase inhibitors (INIs). HIV integrase inhibitors target HIV IN, the enzyme that inserts the viral genome into the chromosomes of the host cells. However, resemblance of IN with RAG1 (recombination-activating gene 1) protein the essential enzyme assembling the functional genes encoding for the variable region of antibodies and T cell receptors, makes this enzyme an also vulnerable target for these compounds, therefore INi treatment can induce immunodeficiency by which makes them unavailable for treatment in newborns and young infants. Our project aimed to solve this major drawback of IN inhibitors, and a novel strategy was applied for design and screen new INi drugs, first for HIV-IN inhibition at various steps (DNA binding, nicking and strand transfer reactions) and second to subject the best candidates of the first screen to RAG interference scrutiny.

This NKFI research was performed as a work package of HIVERA proposal “INinRAGI”.

2. AIM OF THE STUDY

The main objective of the present project was to identify new scaffolds inspired by compounds with natural herbal origin shown as specific inhibitors of HIV-integrase. Our aim was to screen a natural product library using two complementary in vitro fluorescence assays of HIV IN: LTR vDNA binding and 3’ single strand processing catalytic assays. The most active compounds of these investigations were selected for future cell based cytotoxicity and antiviral tests on MT4 and MT2 cells, reverse transcriptase and RAG recombinase assays.

3. RESULTS

3.1. Providing compound library

Altogether 270 natural products, representing a wide chemical diversity, were selected for the first *in vitro* screening. These compounds originated from a large number of plant species and were obtained at >95% purity during our previous phytochemical studies. The compounds cover all major chemical classes of secondary plant metabolites including flavonoids (all sub-classes represented) and other phenolic compounds (phenanthrene and anthracene derivatives, coumarins, lignans, tannins, naphthalenes, phenanthrene, anthracene derivatives, phenylpropane analogs, etc.), mono-, sesqui-, di- and triterpene derivatives, ecdysteroids, pregnanes, cardiac glycosides, saponines, nucleosides, different types of alkaloids. In addition, some semisynthetic ecdysteroids and protoapigenone derivatives were also assayed.

3.2. Preparation of plant extracts

Altogether 368 extracts of plant or mushroom origin were completed for screening investigations. Extracts were prepared with solvents of different polarity, including *n*-hexane, chloroform, methanol, aqueous methanol and water, and, in certain cases where complexity of the extracts justified it, solvent-solvent extraction steps were also performed in the initial step. Further purifications were performed by different kind of chromatographies, including CC, RPC, VLC, preparative TLC, and HPLC. At various levels of complexity and polarity range, the prepared 368 samples cover the secondary metabolite profile of altogether 84 species:

3.3. Screening of extracts and compound library for HIV integrase inhibitory activity in vitro

The retroviral integrase acts on the linear double-stranded viral DNA product of reverse transcription. Integrase binds to sequences flanking the viral DNA called LTR (Long Terminal Repeats) and cleaves specific phosphodiester bonds at 3’ ends (the 3’ processing reaction). In our study the HIV IN activities of natural compounds and extracts were investigated using LTR vDNA binding and 3’ single strand processing catalytic assays. Altogether 638 natural products were studied in the screening.

Methods: Fluorescence assays were used for testing HIV-IN LTR vDNA binding and 3' single strand processing catalytic inhibition using purified recombinant HIV-1 integrase and a 27 bp synthetic double stranded DNA with the viral 5' LTR sequence. The compounds were tested at 100 μM concentration in comparative tests with the use of positive controls unlabeled 5' LTR viral DNA for IN DNA binding, and L708-906 for catalytic 3' processing inhibition assay. For binding assay purified recombinant integrase was adsorbed on 96 well plates at 40 °C overnight. The next day, short double stranded DNA (LTR sequence) labelled with Alexa488 was added and incubated with integrase for 1 hour. The plates were further washed four times and after each washing step the residual fluorescence was recorded (which corresponded to the amount of DNA bound by integrase). Data was acquired from 6 independent experiments and percent binding was calculated based on the maximum value of fluorescence recorded in each plate.

Results: The IN-LTR viral DNA binding inhibition of **plant extracts** was measured. Among the tested extracts, the following were identified as promising hits (% inhibition): *Heterotheca inuloides* (flower, aqueous; 45 +/-17%), *Juglans regia* (leaf, chloroform; 27 +/-25%), *Juglans regia* (leaf, aqueous; 27 +/-20%), *Arnica montana* (flower, aqueous; 26 +/-15%), *Heterotheca inuloides* (flower, chloroform; 22 +/-24%), *Arnica montana* (flower, n-hexane; 20 +/-21%), aqueous methanol; 16 +/-29%), *Arnica montana* (flower, aqueous methanol; 15 +/-22%), *Juglans regia* (leaf, n-hexane; 15 +/-21%), *H. inuloides* (flower, aqueous methanol; 14 +/-13%), *A. montana* (flower, chloroform; 13 +/-21%).

LTR viral DNA binding inhibitory test of the **pure compounds** (Table 1) revealed >30% activity of some flavonols (physetin, quercetin, rhamnetin, hyperoside, quercetin-3-arabinoside, myricitrin, gossypitrin, herbacitrin), flavons (5,7,4',5'-tetrahydroxy-6,3'-dimethoxyflavon, 6-methoxytricin, isoorientin), the anthraquinone frangulin A, the alkaloid liriodenin, the naphthalene musizin, the sesquiterpene matricin, the phenyl carboxylic acids (ellagic acid, rosmarinic acid, caffeic acid methyl ester), the semisynthetic protoflavonoid (protoapigenone 1'-propargyl ether), and the ecdysteroid (5αH-2-deoxyponasterone A) (positive control LTR 83.6%).

Table 1. LTR viral DNA binding inhibitory activity of the most potent compounds

compound	LTR viral DNA binding inhibition (%)*	IC_{50} (μM)
physetin	42.0	-
quercetin	61.1	34.16
rhamnetin	47.7	-
hyperoside	32.0	-
quercetin-3-arabinoside	32.2	-
myricitrin	38.6	-
gossypitrin	56.8	55.41
herbacitrin	44.3	-
5,7,4',5'-tetrahydroxy-6,3'-dimethoxyflavon	38.9	-
6-methoxytricin	44.1	-
isoorientin	35.1	-
frangulin A	51.9	42.98
liriodenin	50.2	-
musizin	48.8	-
rotenone	28.0	-
matricin	34.5	-
ellagic acid	74.6	54.38
rosmarinic acid	68.4	55.05
caffeic acid methyl ester	57.0	64.54
protoapigenone 1'-propargyl ether	62.2	60.58
5αH-2-deoxyponasterone A (	47.1	-
positive control LTR	83.6	-

*at 100 μM

In the 3' processing catalytic inhibitory assay the highest activity was exerted by glycyrrhetinic acid. Ellagic acid, 2-methoxystipandron, β-amyrin, gossypitrin and herbacitrin, rotenone, ursolic acid, diosgenin, and the semisynthetic compounds 20-hydroxyecdyson-2,3,22,25-tetraacetate, and 2-deoxy-20-hydroxyecdyson-20,22-acetonide showed remarkable inhibitory effects (Table 2).

After the first screening of the compound library, the dose response curves were determined for the most potent compounds, and IC_{50} values were calculated. These experiments revealed that the most effective 3' processing inhibitor is glycyrrhetinic acid, followed by 2-methoxystipandrone, rotenone, and ellagic acid. On the other hand, the most active compounds in the LTR vDNA binding assay were quercetin, frangulin A, ellagic acid, rosmarinic acid, gossypitrin, protoapigenone 1'-propargyl ether, and caffeic acid methyl ether.

Table 2. 3' processing catalytic inhibitory activity of the most potent compounds

compound	3' processing catalytic inhibitory activity	
	inhibition (%)*	IC_{50} (μM)
glycyrrhetinic acid	78.0	16.24
ellagic acid	52.1	118.6
2-methoxystipandron	58.9	32.08
β-amyrin	38.3	-
gossypitrin	44.0	-
herbacitrin	37.6	-
rotenone	63.1	67.2
ursolic acid	32.1	-
diosgenin	39.0	-
20-hydroxyecdyson-2,3,22,25-tetraacetate	42.3	-
2-deoxy-20-hydroxyecdyson-20,22-acetonide	39.0	-
protoapigenone 1'-propargyl ether	28.0	-
control L708-906	95.9	

*at 100 μM

With regard to the in vitro screening data, the following samples were selected for future cytotoxicity and antiviral tests: musizin, quercetin, gossypitrin, herbacitrin, ellagic acid, glycyrrhetinic acid, frangulin A, liriodenin, and musizin. A next compound, (*R*)-6,9-dihydroxy-1-oxo-14-noreudesm-5,7,9-triene, which was isolated from the fruit of *Elaeagnus rhamnoides*, was also involved in the antiretroviral investigations considering its structural similarity to musizin. Semisynthetic derivative of glycyrrhetinic acid was prepared, because it was supposed that its methyl ester could have better cell-membrane penetration and physicochemical properties as glycyrrhetinic acid. Structural analogs of protoapigenone 1'-propargyl ether were prepared, and the cytotoxic symmetric dienone pharmacophore was eliminated from these compounds, in order to obtain non-cytotoxic compounds with a higher chance of measurable antiviral activity. Further semisynthetic compounds were prepared through continuous-flow hydrogenation. Reduced curcuminoid metabolites were obtained, such as the octahydro- and tetrahydro-analogs of curcumin and demethoxycurcumin. These compounds are known to be formed during the metabolism of ingested curcuminoids, and they were of interest considering the published anti-HIV activity of their parent compound curcumin.

3.4. Evaluation of the cytotoxicity of compounds selected for HIV-1 antiviral investigations

Method: To determine the in vitro cytotoxic effect of the compounds, the viability of the treated and untreated MT-4 cells was measured by the MTT colorimetric assay as described by Mosmann, 1983. The absorbance was measured at 550 nm on a microplate-reader. The CC_{50} (50% cytotoxic concentration) values were determined.

Results: Herbacitrin, gossypitrin, quercetin, ellagic acid, glycyrrhetinic acid, its methyl ester, frangulin A, liriodenin, musizin, octahydro-demethoxycurcumin, tetrahydrocurcumin, tetrahydro-demethoxycurcumin, and (*R*)-6,9-dihydroxy-1-oxo-14-noreudesm-5,7,9-triene decreased the cell viability in a dose-dependent manner, and their 50% cytotoxic concentrations (CC50) are shown in Table 3. Liriodenin was tolerated only in low concentrations (CC_{50} 5.31 μM) by MT-4 cells, therefore it was ruled out from further antiviral screening. During the cytotoxicity assay insolubility of some compounds (musizin, glycyrrhetinic acid and its methyl ester) were observed, which did not allow the determination of CC_{50} values and further antiretroviral tests.

Table 3. Cytotoxicity of compounds (CC_{50} in μM)

compound	MT-4	MT-2
herbacitrin	27.8	63.64
gossypitrin	101.0	112.56
quercetin	107.5	157.38

ellagic acid	
glycyrrhetinic acid	
glycyrrhetinic acid methyl ester	
frangulin A	
liriodenin	5.31
musizin	
(*R*)-6,9-dihydroxy-1-oxo-14-noreudesm-5,7,9-triene HYR	
octahydrocurcumin	>80
octahydro-demethoxycurcumin	59.4
tetrahydrocurcumin	55.9
tetrahydro-demethoxycurcumin	55.9

3.5. Determination of HIV-1 antiviral activity of selected compounds

Methods: **Cells and virus**: The permanent human T-cell lines MT-4 and MT-2 were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). HIV-1 (HTLV-IIIB) was obtained from the culture supernatant of MT-4/HTLV-IIIB cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) on MT-4 cells was determined by virus yield assay. The titer of the virus stock was $2.32*10^5$ $TCID_{50}$/ml. **HIV-1 antiviral assay**: MT-4 and MT-2 cells at a density of 15,000 cells/well were incubated in 96-well plates in the presence of compounds at 37°C in 5% CO_2 for 5 days. Simultaneously, cells were exposed to HIV-1 ($2{,}32*10^2$ $TCID_{50}$/ml). Untreated and infected or AZT (3'-azido-3'-deoxythymidine)-treated cells were used as controls. After the incubation period, diluted culture supernatants were analyzed for HIV production by determining the amount of viral core protein using a p24 enzyme-linked immunosorbent assay (ELISA) kit (Fujirebio) according to manufacturer's instructions. The results were expressed relative to the control of untreated HIV-1 infected cells. The experiment was performed in four biological replicates. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test.

Results: Determination of the antiviral activity of compounds in non-toxic concentrations was carried out by analyzing HIV-1 p24 core protein levels in the supernatants of HIV-1 infected MT-4 and MT-2 cell cultures after 5 days of incubation. It was found that herbacitrin and quercetin reduced HIV-1 replication (p24 level on MT-4: 49.8 and 45.1%, respectively; p24 level on MT-2: 23.0 and 62.3%, respectively), whereas other compounds were ineffective.

In parallel to this work, non-cytotoxic protoflavone analogs were tested by a new collaboration partner, Dr. Carole Seguin-Devaux (Luxembourg Institute of Health, Esch-sur-Alzette, Luxemburg) for their anti-HIV activity. U373-CD4-CCR5 cells were infected by pseudotyped virus pNL4.3ΔenvLuc-ADA 8 using spinoculation at 1 200g during 2 hours in the presence of compounds and cultured for two consecutive days in the presence of the compounds. After 48h, luciferase activity expressed as Relative Light Units was measured.

Results: In the pseudotype virus assay, tetrahydroprotoapigenone was found to inhibit viral infection by ca. 50% at the non-cytotoxic concentration of 100 µM. While this represents a rather weak activity, it may still be of interest since this compound was not cytotoxic on the host cells at as high as 500 µM concentration.

3.6. HIV RT and IN inhibition assays of selected compounds

Method: Inhibitory effects of compounds on the HIV-1 reverse transcriptase and integrase activity were measured by a colorimetric RT kit (Roche Diagnostics) and IN assay kit (Express Biotech International) according to the instructions of the manufacturer. Reverse transcriptase assay measures the amount of labeled nucleotides incorporated during the transcription process of RNA. Nevirapine, a nonnucleoside RT inhibitor, was used as a positive control in the RT reaction. HIV-1 integrase assay measures the integrase activity after 3'-end processing of the HIV-1 LTR donor substrate DNA and catalyzing the strand-transfer recombination reaction to integrate the donor substrate DNA into the target substrate DNA. Sodium azide was applied as a positive control compound in the experiments measuring the integrase activity. The RT and IN inhibition assays were performed in two biological replicates. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test, and a planned comparison by unpaired T-test was also performed

Results: To determine the potential target of herbacitrin within the HIV-1 replication cycle, we tested its effect on the activity of recombinant HIV-1 reverse transcriptase (RT) and integrase (IN). We observed that herbacitrin, applied at a relatively high, 21.5 μM concentration, significantly inhibited the HIV-1 reverse

transcriptase (Figure 1(a)). In contrast, the activity of integrase was inhibited already at a lower, 2.15 μM concentration of herbacitrin (Figure 1(b)). These results suggest that herbacitrin may interfere with the replication cycle of HIV at multiple stages.

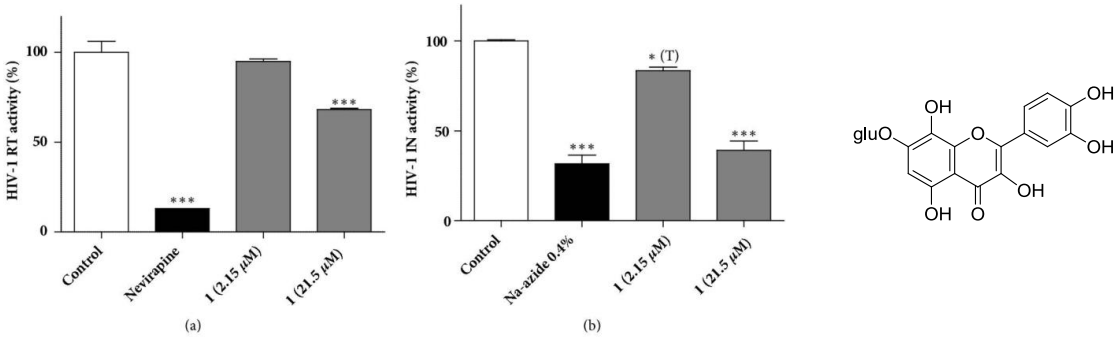

Figure 1. Effect of non-cytotoxic concentrations of herbacitrin (**1**) on HIV-1 reverse transcriptase (a) or integrase (b) activity.

3.7. In vitro testing of the influence on RAG recombinase

Method: To test the influence on RAG recombinase our selected compounds were titrated in catalytic DNA double cleavage reactions performed by RAG. The purified murine subunits of the RAG enzyme were expressed in eukaryotic system (293T human fibroblasts transiently co-transfected RAG1 (384-1040) and RAG2 (1-387)), both fused with MBP (maltose binding protein) and purified by affinity chromatography on amylose columns.

A) IC_{50} 3'P = 118.06 $\pm$ 6.67 µM B) IC_{50} Binding = 54.38 $\pm$ 21.18 µM

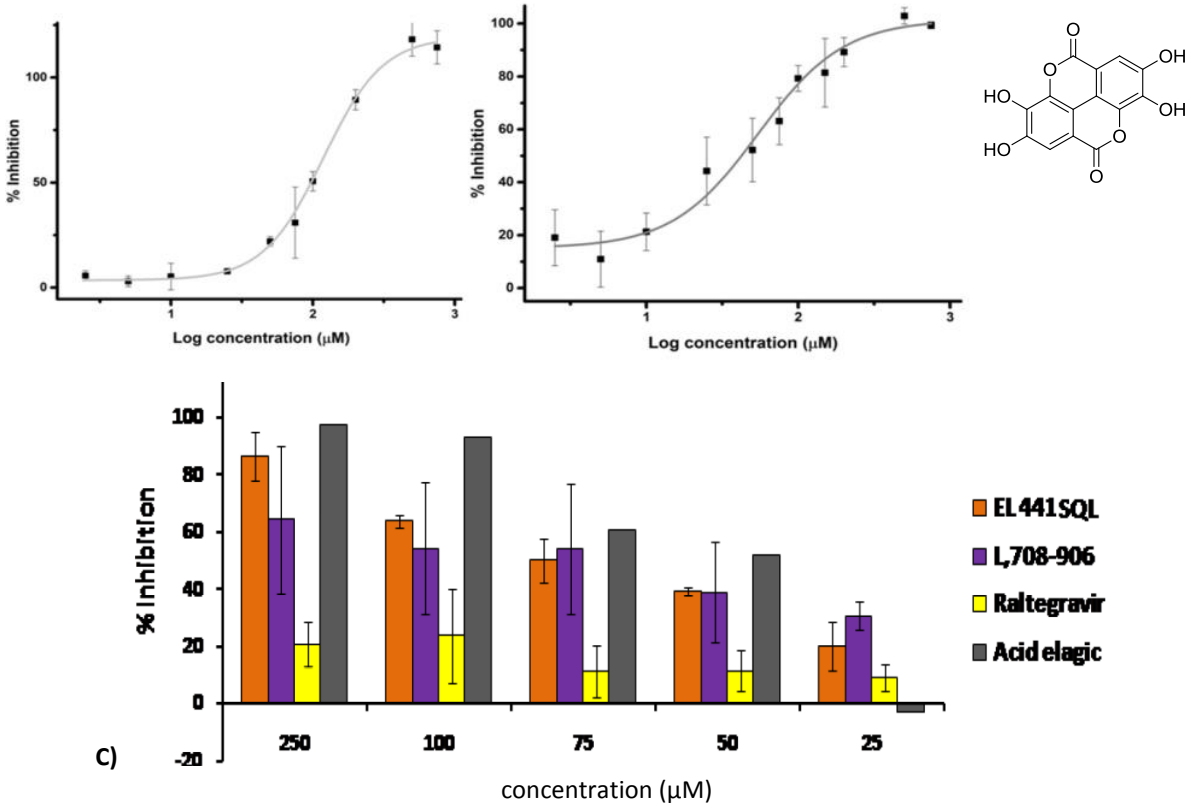

Figure 2. IN (A, B) and RAG (C) inhibitory activities of ellagic acid

Results: Ellagic acid which shows substantial activities on both 3' processing catalytic inhibitory and LTR vDNA binding assays (Figure 2A, 2B) was subjected to RAG recombinase inhibitory test. The results of the analysis are presented as histograms in Figure 2C. The effect of ellagic acid was compared with those of drugs clinically used in the treatment of HIV infected patients (EL441SQL, L708-906, and Raltegravir). It can be stated that ellagic acid similarly as EL441SQL and L708-906, concentration dependently inhibit RAG1, especially at concentrations > 50 µM.

4. SUMMARY

In frame of the present project a plant extract library and a natural compound library was created and transferred for bioactivity screening in two complementary in vitro fluorescence assays of HIV integrase.

The LTR viral DNA binding inhibitory test enabled the identification of a series of compounds with >30% inhibitory activity. The active compounds represent mainly the phenolic type of secondary plant metabolites. In the 3' processing catalytic inhibitory assay the highest activity was exerted by triterpenes and phenolic compounds. On the basis of the in vitro screening data, the samples were selected for further cytotoxicity test and cell based antiviral investigations. The antiviral activities were evaluated by HIV-1 p24, RT and IN tests. We also established a new collaboration that allowed us to test a selected set of compounds on a pseudotype virus assay.

As a result of our studies, multi-level anti-HIV activity of herbacitrin was discovered for the first time. The other promising hits are ellagic and glycyrrhetinic acids with high HIV-INi potency. Further, tetrahydro-protoapigenone was identified as a promising new natural scaffold that, due to its extreme low toxicity, is worthy for chemical structure optimisation aiming to perform an in-depth structure-activity relationship study. In summary, our results serve as good starting point for further studies for identification new scaffolds inspired by natural compounds as specific inhibitors of HIV-integrase.