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# QUANTITATIVE LABEL-FREE NANOSCALE LC-MS ANALYSIS OF HIV DRUG-TREATED HUMAN T-CELL CULTURES

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## INTRODUCTION

**HIV is one of the main health risks in today's society in many regions of the world. Most current methods of treatment are based on HIV-reverse transcriptase and protease inhibition to reduce the rate of HIV replication. There is however a clear need for more effective treatment methods and drug-candidates to effectively treat the disease. Some of these target the interaction between virus and T-cell, the primary cell type infected by HIV. Whereas classical virology studies show interesting anti-HIV effects, it remains unclear whether or not the drug candidate may have other consequences on the T-cell itself. One modern way of studying the molecular mode of action of a potential next generation class of anti-HIV compounds, as well as to get a first insight into possible side effects (safety issues) is to look at the effect on the proteins expressed by compound treated T-cells versus untreated ones.**

**A promising compound (CADA, Figure1) was selected for a feasibility study and a large batch of CD4+T-cells – a human T-cell line SUPT-1 – were grown[1,2]. Part of the culture was treated with an effective dose of compound and harvested at a relevant time thereafter. Their protein content was comparatively analyzed with that of an equivalent amount of untreated T-cells from the very same culture.**

**The results presented here are from a label-free quantitative LC-MS analysis of Human T-cell line proteins. The effect of the treatment was monitored by utilizing the relative quantification results, which provided invaluable input for the investigation on the mechanism of action of the compound on targeted and non-targeted cellular components.**

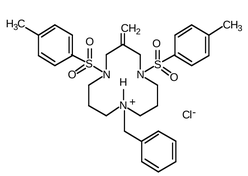


*Figure 1. Chemical structure of cyclotriazadisulfonamide (CADA)*

## METHODS

**Sample preparation**

To study the effect of CADA on surface CD4 receptor expression, human T-cells were incubated with the compound, stained with anti-CD4 monoclonal antibody and analysed by flow cytometry as illustrated in Figure 2. The effect of CADA on T-cell HIV infection is shown in Figure 3.

Membrane and soluble protein fractions have been studied. In this study, the focus is on the soluble proteins.

The soluble protein content of the investigated cell line was extracted and subsequently denatured with RapiGest™ SF surfactant (0.1%) (Waters Corp.), reduced (10 mM DTT), alkylated (10 mM IAA) and enzymatically digested with trypsin, 1:50 (w/w) enzyme:protein ratio.

**LC-MS conditions**

LC-MS quantification experiments were conducted using a 1.5 hr reversed phase gradient from 5 to 40% acetonitrile (0.1% formic acid) at 250 nL/min on a nanoACQUITY UPLC™ System (Waters Corp.). An Atlantis® 3µm C18 75 µm x 15 cm nanoscale LC column (Waters Corp.) was used, with all samples run in triplicate. Typical on-column sample loads were 0.5 µg protein digest—which is the equivalent of less than 50,000 cells.

The Q-Tof Premier™ mass spectrometer (Waters Corp.) was programmed to step between normal (5 eV) and elevated (20-35 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over the m/z range 50-1990. Protein identifications and quantitative information were generated by the use of dedicated algorithms (Waters® Protein Expression Informatics), and searching against a human species-specific database.

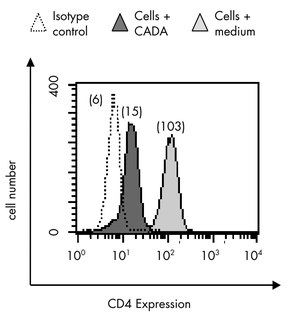


*Figure 2. CD4 down-modulation in human T-cells after incubation with CADA (5 µg/ml). Cell surface CD4 expression of untreated and CADA-treated cells after staining with the specific anti-CD4 mAb shown. Mean fluorescence intensities are indicated between brackets. An isotype control is included to measure the background staining.*

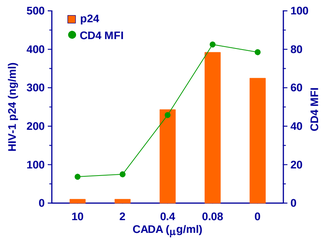


*Figure 3. Correlation between anti-HIV potency and CD4 down-modulating capability of CADA. T-cells were infected with HIV in the presence of different doses of CADA. After 4 days, supernatant was collected and analyzed for its p24 (HIV antigen) content (vertical bars). In parallel — uninfected T-cells were treated with the same doses of CADA — and CD4 expression was analyzed flow cytometrically after 4 days of incubation. The MFI of the Leu3a-FITC staining is depicted for the different doses of CADA (line).*

## RESULTS & DISCUSSION

**LC-MS$^E$ data**

Low and elevated spectra and chromatograms are obtained in a parallel fashion. Typical spectra are shown in Figure 4. The low energy data are used for the quantification of the peptides and subsequently proteins, whereas the high-energy information is utilized for qualitative, identification purposes[3].

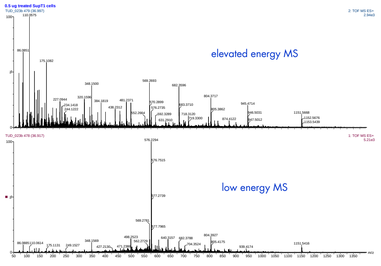


*Figure 4. Example low (bottom pane) and elevated energy mass spectra (top pane) taken at 36.9 min of the treated T-cell sample.*

The quality of the clustered LC-MS data was accessed prior to quantification. Parameters that are typically measured are retention time reproducibility, mass precision and intensity variation of accurate mass/retention time clusters[3,4]. The quality assessment results are illustrated in Figures 5, 6 and 7, respectively.

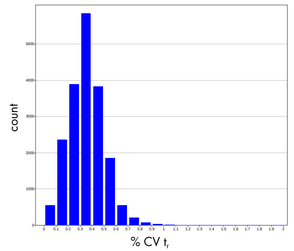


*Figure 5. Retention time reproducibility distribution accurate mass/retention time clusters (replication rate ≥ 3 out of 6).*

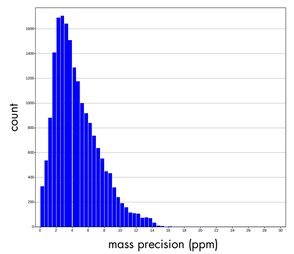


*Figure 6. Mass precision distribution accurate mass/retention time clusters (replication rate ≥ 3 out of 6).*

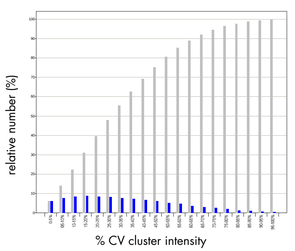


*Figure 7. Relative and cumulative distribution of the variance coefficient of the intensities for the accurate mass/retention time clusters of the complete dataset.*

**Relative Quantification**

The intensity measurements were normalized on the intensity measurement of the internal standard peptides utilizing the three best ionizing peptides identified to a protein[5]. The normalized intensity measurements were subsequently expressed as relative values of which the results are shown in Figures 8 to 10.

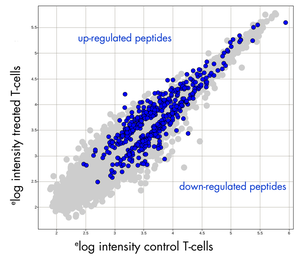


*Figure 8. Accurate mass/retention time clusters (*log intensity control vs. *log intensity treated). The blue annotated clusters are significantly regulated peptides — student T-test.*

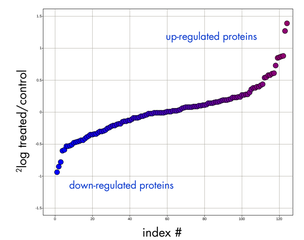


*Figure 9. Relative protein concentration (2log ratio) of both conditions (control and treated) commonly identified proteins (2 ≥ fragment ions/peptide, 2 ≥ peptides identified; replication rate ≥ 2)*

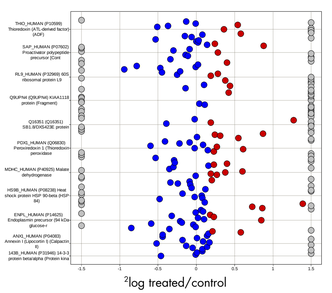


*Figure 10. Common and uniquely identified proteins in both investigated conditions. Blue = up regulated in the treated sample; red = down regulated in the treated sample; grey = condition unique identification*

The results shown in Figures 9 and 10 suggest that the regulation of the commonly identified proteins is very mild and that the number of significantly regulated proteins very low (~ 5 - 10). Furthermore, certain proteins are identified in only one of the investigated conditions— see Figure 11. To date it has not been established if this is a reflection of the biology or the experiment — above/below detection threshold due to sample dynamic range constraints.


*Figure 11. Number of identified proteins across both investigated samples — i.e. control and CADA-treated T-cells*

**Absolute Quantification**

The absolute amounts of all identified proteins — not taking into account post-translational or chemical modifications — were estimated using a recently published absolute concentration formula[5]:

$$\frac{\sum_{i=1}^{n} \text{normalized peptide intensity protein } x}{\sum_{i=1}^{n} \text{normalized peptide intensity internal standard}} \cdot [\text{internal standard}]$$

The total amount of protein that could be quantified for the control sample equaled 0.349 µg/0.5 µg injected with 2 ≥ fragment ions/peptide, 2 ≥ peptides identified, and replication rate ≥ 2. For the treated sample the justifiable amount was 0.311 µg/0.5 µg.

## CONCLUSION

- **The analytical protocols used in this study are capable of reproducibly measuring the intensities of peptides in a very complex protein mixture allowing subsequent quantification**
- **The soluble protein content of the investigated T-cell line is hardly affected by CADA treatment — i.e. no significant peptide and protein regulation has been observed by means of the employed label free LC-MS technique.**
- **The biochemical data are not reflected in these results (CD4 is a membrane protein and therefore not likely to be represented in the currently studied sample)**
- **Absolute LC-MS quantification methods offer means to assess the amount of detected protein**

**References**

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