High Throughput screening for modulators of tissue-kallikrein expression

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ABSTRACT

Human tissue kallikreins (KLKs) comprise a subgroup of 15 homologous secreted serine proteases. Primarily known for their clinical use as cancer biomarkers (e.g. PSA), KLKs are found to be implicated in cancer-related processes including invasion, metastasis and angiogenesis. Therefore, modulators of expression of KLKs might help us understand their regulatory pathways and be of therapeutic value.

A cell-based high throughput screening of 3 compound libraries (~4000 small molecules) was performed under fully automated robotic facilities and sensitive immunoassays (ELISA) were used to measure KLKs in a panel of different cell lines. The 'validated leads' were further assayed in-vitro, to measure their effectiveness (IC50s) at protein (ELISA) and mRNA levels (RT-PCR), cytotoxicity (LDH) and cell viability (MTT assay).

The initial screening resulted in 66 'putative hits' that decreased KLK expression by at least 50% over control. Secondary screening and mini-dose-response assays reduced this number to 21 'leads'. These were classified into 3 different functional groups. IC50 calculations revealed the different potency of the compounds within the same group and the most potent ones were further analyzed in vitro. Novel inhibitors of KLK expression, acting at low concentrations (10-50 nM) were identified. Further experiments to elucidate their exact mode of function are ongoing.

This HTS-technique proved effective in identifying inhibitors of expression of our target protein throughout large chemical libraries. The precise pathways that seem to be blocked by these inhibitors and the therapeutic applications of their use, are currently under investigation.

INTRODUCTION

- The KLK family is a subgroup of secreted serine peptidases, comprised of fifteen members[1].
- Although primarily known for their clinical applicability as cancer biomarkers (eg PSA), accumulating evidence implicates KLKs in many cancer related processes, such as cell-growth regulation, angiogenesis, invasion and metastasis [2]
- Given that KLK5 is shown :
  - to efficiently cleave the extracellular matrix components, collagens type I, II, III, IV, fibronectin and laminin *in-vitro* [3]
  - to be responsible for the degradation of corneodesmosomal cadherin in LEKTI (-/-) knockout-mice [4]
  - to represent important signalling molecule through its ability to regulate PARs in physiological conditions[5],

  → *we assumed that it can potentially represent a novel tharapeutic target*
- Identification of small molecules as modulators of KLK5-expression would help us elucidate their regulatory pathways, understand the mechanisms that lead to their aberrant expression in many different cancers and measure their biological impact in cancer related processes.
- High-throughput screening (HTS) plays a major role in modern drug discovery, since it allows screening of large compound libraries against your putative target [5]

OBJECTIVES

- Development of a cell-based high throughput screening assay, suitable for the screening of large chemical libraries
- Optimization of the following in-vitro validation assays, in order to eliminate the false-positive initial hits and focus on the 'actual hits'
- Measure the potency of these hits, in parallel with their cytototoxic or apoptotic potential
- Elucidate the pathway that regulates KLK5 expression. Check specificity with the other KLKs.
- Measure their therapeutic potential with biological assays.
- Development of 'compound-cocktails' against KLK5, or cocktails of compounds which specifically target different KLKs (tissue specificity)

RESULTS

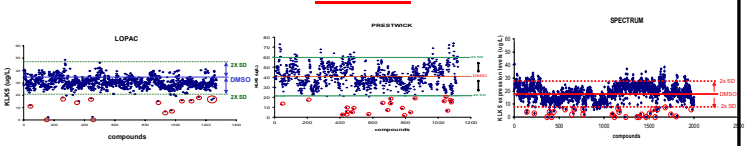


Fig.1. Initial Screening of the 3 libraries (~4000 compounds) identified 66 small molecules as putative hits. *Those were further analysed in the 'secondary-validation' step.*

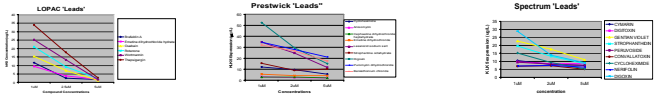


Figure 2. Mini dose-response treatments with the 'putative hits' separated the 'real leads' from the false positives. *A total of 21 lead-compounds were identified and proceeded to the IC50-calculation step.*

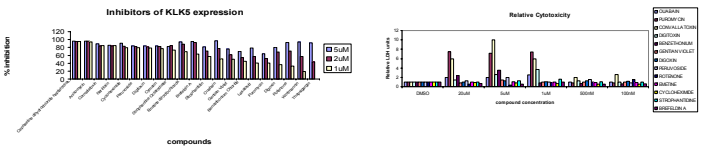


Fig.3. Mini-dose responses with the 21 lead-compounds (A) and their relative cytotoxicity (LDH measurement)

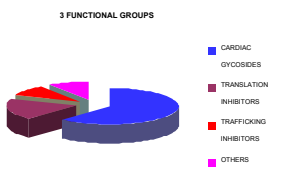


Fig.4. Classification of the 21 compounds. *According to their mode of function these compounds can be grouped to three categories:*

1. *Ion pump inhibitors (cardiac glycosides)*
2. *Translation inhibitors*
3. *Trafficking inhibitors*

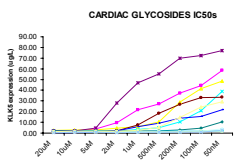


Fig.5. Striking differences in the potency of compounds despite their very high structural similarities (same family).

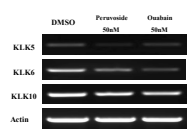
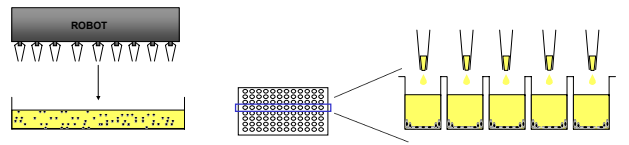


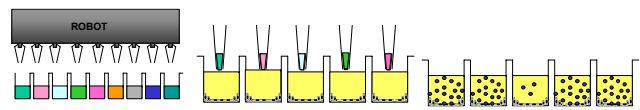
Fig.5. Semi-quantitative RT-PCRs show a similar decrese in mRNA levels (in non-cytotoxic & non-apoptotic concentrations of the compounds)

METHODS

A) Initial screening

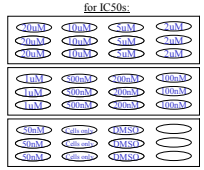


Step 1. The robot collects and seeds a 50000 cells/well in a 96-well plate. The cells stay in serum containing media for 48 h, in order to detach to the wells.



Step 2. The robot washes the wells and changes the medium (serum-free). Then it dispenses the chemical library. We leave 30h for the KLKs to accumulate, and the robot collects the supernatants, which are sent to ELISA.

B) Selected compound concentrations for IC50s:



IC50 calculation:

In 12-well plates seed 500000 cells/well. Following the same protocol (manually), collect the supernatant and send to ELISA (+measure LDH, +collect RNA)

ELISA reader

CONCLUSION

The HTS worked very efficiently for our assay. Out of a total of 4000 compounds, 21 molecules (~0.5%) are validated as inhibitors of KLKs (5,6 and 10), which belong to 3 functional-families of compounds. Furthermore there was a very small overlap among the 3 libraries and these molecules came up as hits from all different libraries, reflecting the reproducibility & sensitivity of the technique.

RT-PCR experiments of treated cells show that these molecules block a certain pathway which regulates the expression of some proteins, including KLKs(data in progress). Western Blot experiments and further quantitative proteomic techniques seem to be the way to further elucidate such pathways.

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