Identification and biological validation of new and selective antitumor CDK9- and FLT3- inhibitors

Ph.D Thesis

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Budapest
2020

1 INTRODUCTION

Cancer is the second leading cause of death in the world causing 9.6 million deaths in 2018 in both developing and developed countries. Mutations in kinases lead to complete or partial loss of function, which can lead to a number of diseases (diabetes, various gamma globulinemia involving the cardiovascular, nervous or immune systems) and cancers (leukemia, neuroblastoma and glioblastoma). The breakthrough clinical success of the anti-tumor drug Bcr-Abl tyrosine kinase, imatinib, in the early 2000s ushered in a new era in targeted therapy for mutant kinases. As a result, kinases have become drug targets over the past few decades. CDK9 is one of the cyclin-dependent kinases (CDKs), a serine / threonine kinase. A cyclin subunit is also required for its activation. CDK9 promotes the synthesis of RNAs required for genetic programs of cell growth, cell differentiation, and viral pathogenesis. The CDK9-cyclinT1 complex plays an important role in the phosphorylation of RNAP-II, inhibiting RNA synthesis, thereby reducing the expression of certain anti-apoptotic proteins. Furthermore, inhibition of the complex has also been shown to initiate p53-independent apoptosis via the caspase cascade. To date, more than 20 CDK inhibitors have been used in various tumor patients with phase I and II. studies. Resistance to conventional chemotherapy remaind the major obstacle in treating many cancers. The intrinsic via p53 mediated apoptosis pathway often disabled. At the beginning it was believed that targeting cancer cells via extrinsic cell

death machinery is the solution in case of resistant tumors. TRAIL can induce p53 independent apoptosis selectively in tumor cells, thus becomes important target in research. The FLT3L the ligand of the FLT3 is a cytokine, plays a pivotal role in the proliferation, survival and differentiation of the hematopoietikus progenitor cells. FLT3 is one of the most mutated genes, observed approximately 30% of AML patients. The most frequent FLT3 mutation is the internal tandem duplication alteration (ITD), being present 25% in AML, associated with poor prognosis. Point mutations within the activation loop of the tyrosine kinase domain (TKD) have also been detected in 7% of AML and 3% of ALL patients. The D835Y TKD mutation, may lead to the aberrant activation of the receptor. Nowdays testing for FLT3 status is mandatory for all AML patients. Given the prognostic impact and the high rate of FLT3-mutations, inhibition of FLT3 has long been recognized as a potential therapeutic target in AML. Toxicity, suboptimal pharmacokinetic results, and off target effects resulting the use of initial first-generation FLT3 inhibitors have yielded exciting results in clinical trials.

2 AIMS

In case of 4-amino-6-phenylpyrimidine derivatives.

1. Testing of a large number of derivatives (focused compound library) in targeted protein-based biochemical systems to identify the most efficient and selective molecules, setting up high and medium throughput (HTS / MTS) biochemical assays for targeted protein-based testing: optimization and validation of CDK9, CDK4, CDK2 kinase activity assays.
2. Investigation of the efficacy of hit molecules identified in protein-based biochemical tests in cellular systems.
3. Determination of the selectivity of the most effective lead compound **20** identified in biochemical kinase and cellular tests.
4. Investigation of the mechanism of action of the compound **20**.
5. Sensitization of the MCF7 tumor cells to TRAIL-induced apoptosis via CDK9-inhibition by **compound 20**.

In case of phenylethenylquinazoline derivatives.

1. Optimization and validation of bichemical assays for target based drug discovery – optimization and validation of kinase activity tests for WT and for ITD- and D835Y-mutationscarrying FLT3-kinases.
2. Validation of the inhibitors is cellular sytems.

3. Identification of the selectivity profile of the lead compound **III**.
4. Investigation of the mechanism of the action on MV4-11 FLT3 ITD-mutant cell line.
5. Determination of ADME properties of the most effective lead compounds.
6. Investigation of the pharmakokinetic profile and *in vivo* effect in mouse xenograft model of the lead compound **III**.

3 METHODS

1. *In vitro* kinase activity assays IMAP (Immobilized Metal Assay for Phosphochemicals)
2. High-throughput kinase selectivity profiling (KINOMEscan™)
3. Luminescent cell viability sssay
4. *In vitro* clonogenic assay
5. Flow cytometry analysis of apoptotic DNA fragmentation
6. Flow cytometry analysis of apoptosis by annexin V-FITC and PI double-labeled cells
7. Investigation of cell cycle distribution of BrdU and PI double-labeled cells
8. Measurement of caspase-3/7 activity with luminescent plate reader
9. Measurement of caspase-3/9 activity with fluorescent plate reader
10. Western-blot analysis
11. Mouse xenograft model
12. Pharmakokinetic Study
13. Investigation of kinetic solubility
14. Permeability assay (PAMPA)
15. Molecular docking

4 RESULTS

In case of 4-amino-6-phenylpyrimidine derivatives.

Compounds were tested with fluorescence polarization-based IMAP™ (Immobilized Metal Assay for Phosphochemicals). The best molecules are low-nanomolar inhibitors of the CDK9 kinase, moreover they showed selectivity over CDK2 and CDK4.

Based on biochemical kinase assay results, proliferation assays were performed with four most potent derivatives **17, 20, 21** and **22**. We asked whether biochemical results would translate to cellular activity in a panel of 21 cancer cell lines. **Compound 20** was found to be the most potent from the series. It dramatically reduced the cell viability of all cell lines, most of the cases at submicromolar IC_{50} values.

The most effective **compound 20** was selected for an experiment with a broad range of kinase enzymes. KINOME*scan*™ binding assay (DiscoveRx Corporation) was performed with 451 different types of kinases. **Compound 20** (1 μM) binds only 1.5 % of tested kinases and the highest binding affinity was found for CDK9.

As **compound 20** showed highest selectivity toward CDK9, we decided to demonstrate its inhibition in MCF7 cells. A significant dose-dependent reduction was observed in the phosphorylation of RNA polymerase II. Blocking the RNA polymerase II by **20** and subsequent inhibition of transcription decreases the expression of antiapoptotic protein Mcl-1 was seen. Decreased level of another short-lived protein ubiquitin ligase

Mdm-2 correlated with observed accumulation of tumor suppresor p53. We also observed decrease in overall CDK9 protein level. **Compound 20** inhibited DNA replication (S-phase), at higher doses cells accumulated in G2/M phases.

U266 cells were treated with **compound 20** and analysed for inhibition of CDK9 and expression of apoptosis-related proteins. Phosphorylation status of RNA polymerase II was consistent with previous experiments with MCF7 cells showing significant decrease of both phosphosites from 1 μM concentration. The treatment also lead to downregulation of proteins Mcl-1 and XIAP, activation of caspases-3, -7, -9 and cleavage of PARP. Activation of caspases-3, -9 was confirmed by fluorimetric enzyme-activity assay which has revealed a dose-dependent effect of **compound 20** on U266 cells. Our experiments on tumor cell lines have demonstrated that combination therapy with lead **compound 20** and TRAIL may provide an opportunity for effective tumor therapy. The results of our research have been the subject of an international patent application. **Compound 20** is currently under clinical development.

In case of phenylethenylquinazolin derivatives

In the first instance all compounds were tested with fluorescence polarization based IMAP™ kinase assay. Based on the results of the biochemical assays we concluded that the best molecules are low-nanomolar inhibitors of the FLT3-ITD and FLT3-D835Y kinases, moreover they showed selectivity over the wild-type FLT3.

After the selective and potent inhibitory effect of the compounds was demonstrated in different biochemical assays (FLT3-WT, FLT3-ITD and FLT3-D835Y), the cell proliferation inhibitory activity was tested on a panel of leukemic and non-leukemic human cells. The compounds (**I-XIII**.) inhibited the cellular proliferation of FLT3-ITD bearing leukemic (MV4-11 FLT3-dependent) cell line. FLT3 signal independent leukemic cell lines (U937 and K562) and HS-5 non-leukemic cell line were either weakly or not inhibited at all by the compounds.

The solubility of the compounds was measured at two pH values using HPLC. Lead **compound III** showed acceptable solubility at physiological pH. Most of the tested compounds demonstrated good penetration values, thus we conclude that some of these derivatives can cross the cell membranes by passive diffusion special, compound formulation is not required for early preclinical development. The **compound III** demonstrated improved solubility and a good membrane penetration value therefore, this molecule was chosen for the selectivity validation by KINOME*scan*™ binding assay.

With the reproducible anti-proliferative effect observed for **compound III**, **VII** and **IX** against the MV4-11 cell line, their mechanism of action was investigated more in details. Apoptosis was demonstrated by staining the cells with Annexin V-FITC and propidium iodide. The cells were treated with increasing concentrations of **compound III**, **VII** and **IX** and the dose-dependent effect on the induced apoptosis was demonstrated. Caspase-3 is a key downstream effector in the apoptosis pathway

therefore Caspase-Glo® 3/7 assay was used for the measurement of caspase 3/7 activity. This effect was as pronounced as the effect of sunitinibe and verified apoptosis induced by the compounds in the MV4-11 cell line in a dose-dependent manner. Together, the data indicate that **compound III**, **VII** and **IX** effectively induce apoptosis.

Compound III has good pharmacokinetic profile. SCID mice were subcutaneously inoculated with MV4-11 cells. 19 days after the inoculation, **compound III** was administered in mice intraperitoneally at a dose of 17 mg/kg in every other day for 16 days. Compared to the vehicle control, the treatment with **compound III** reduced the tumor volume and weight significantly, by 48% and 49% in average, respectively. These results demonstrated that **compound III** effectively reduced the tumor size and was well-tolerated at the dose we employed.

5 CONCLUSIONS

In case of 4, 6-disubstituted pyrimidine derivatives.

1. Promising lead compounds was indentified and characterized among newly synthesized, 4, 6-disubstituted pyrimidine derivatives, that effectively and selectively inhibited CDK9 enzyme.
2. During the optimization process **compound 20** was identified as the most selective CDK9 inhibitor in the series.
3. Results of molecular modeling confirmed the interaction of **compound 20** with CDK9 enzyme.
4. **Compound 20** showed prominent antitumor activity in a cell panel.
5. Our results demonstrated the lead **compound 20** induced apoptosis via inhibition of expression of Mcl-1.
6. **Compound 20** sensitizes TRAIL-resistant MCF7 cells to TRAIL-induced apoptosis *in vitro* via CDK9-inhibition.

In case of phenylethenylquinazolin derivatives.

1. A novel series of phenylethenylquinazoline derivatives synthesized resulted in the discovery of a new, highly selective FLT3 inhibitor (compound **III**). To our current knowledge

compound III unlike the other known FLT3-inhibitors showed an impressive *in vitro* efficacy not only on kinase activating ITD, but on resistance conferring D835Y single mutant enzymes.

2. **Compound III** selectively inhibited the proliferation of FLT3-dependent MV4-11 ITD mutant AML cell line.
3. Lead molecules showed acceptable solubility at physiological pH and could cross the artificial cell membrane. According to the results we can conclude that some of these phenylethenylquinazoline derivatives can cross the cells by passive diffusion.
4. Based on the results of pharmakokinetic profiling and mouse xenograft model, we conclude that **compound III** showed effective *in vivo* antitumor activity, by significantly inhibiting MV4-11 tumor cells.

6 PUBLICATIONS

Publications in the topic of the thesis.

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