Pyrido[2,3-*b*]pyrazines as anticancer agents and isomerism in their asymmetric condensation reaction

PhD thesis

Dr. László Kékesi

School of PhD Studies of Semmelweis University

Supervisor: Dr. György Kéri, professor, D.Sc.

Opponents: Dr. Zsuzsa Majer, associate professor, Ph.D
Dr. Andrea Czompa, adjunct professor, Ph.D

Head of Examination Committee: Dr. Éva Szökő, professor, D.Sc.

Members of Examination Committee: Dr. József Nyitrai, professor, D.Sc.
Dr. György Dombi, professor, D.Sc.

Budapest
2014

1. Introduction

One of the new trends in the modern cancer research is targeting a small subset of the tumor cell population that responsible for recurrence that occurs after the effective treatment. We aimed to prepare new drug candidate molecules, which can inhibit tumor initiating cells in cancerous diseases. Our strategy was to identify inhibitors of acquired resistant cell line via a pre-selection phenotypic screen. The base of this theory was that the inhibitors of both sensitive- and resistant cell lines had potential inhibition spectrum, which was appropriate to inhibit the increased resistant resistance causing cells.

1.1. Targeted therapies and acquired resistance

In the developed countries one in four deaths is due to cancer nowadays. Cancerous diseases are consequences to genomic failures. Normal cell transformation to a tumor cell is the consequence of several genomical changes and a growth factor activations, which are called surviving factors. Recently the three main cancer therapy methods are used: local surgery, radiation therapy and systemic drug therapy (chemotherapy). In the third case the application of cytostatics is based on the fact that they mainly affect on rapidly dividing cells.

Malfunction of an element of the inter- or intracellular signal transduction system causes a false sign and signal transduction cascade, resulting pathological behavior, for example uncontrolled cell division. The background of a significant portion of cancerous diseases is signaling problems, for example false survival or proliferation signals. Drugs, which are designed against dysfunctional regulatory elements, allow targeted therapies, making the treatment more effective and reducing the risk of possible side effects. There are known small molecule tyrosine kinase inhibitors (TKIs), like gefitinib, erlotinib, imatinib, dasatinib, lapatinib, sunitinib, sorafenib, which targeting the false signaling pathways of survival factors.

Gefitinib was the first selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase. It is indicated in non-small-cell lung carcinoma (NSCLC) in all lines of treatment for patients harbouring EGFR mutations. Gefitinib was followed by EGFR inhibitor erlotinib.

These inhibitors are effective in NSCLC cells harbouring EGFR-sensitizing mutations, but ineffective in harbouring wild type EGFR. EGFR TKIs are widely used in NSCLC. Tumors harbouring EGFR activating mutations respond well to these agents. According to literature and

clinical practice well known problem is the recurrence after effective gefitinib or erlotinib treatment, so-called acquired resistance. It can be driven by various mechanisms. This resistance might attributable to T790M mutation of EGFR (so-called gatekeeper mutation) at 50-60 % of the patients. One is often investigated cell lines, linked to resistance, is human NSCLC PC9 cell line.

The acquired resistance may be the consequence that there is a small proportion of the tumor cells survives the treatment, and causes resistant recurrences or metastases. These cells express survival factors required by more malignant transformations and are more protected than the therapy-sensitive tumor cells, which prevent the successful therapy. Thereby these resistant cells adapt and survive even radiotherapy or chemotherapy, causing recurrence in appropriate circumstances. When the tumor cell population has defined gene amplification or mutation, the most of the first generation of tumor can be removed by the inhibition of the dominant mutation (driving force), but the second generation population is driven by an uninhibited pathway. These signals must be inhibited simultaneously to remove the first treatment resistant cells. Since normal cells do not depend on these false signals they can survive the treatment.

1.2. Resistance causing cells, tumor-initiating cells (cancer stem cells)

In course of chemotherapy the problem is usually not only the absence of primary response for the drugs, but also a significant problem is the tumor relapse or recurrence after effective treatment. In this process the so-called tumor-initiating cells (TIC) are supposed to have a critical role. The literature mentioned them as cancer stem cells (CSCs) or resistance-causing cells also. In the clinical development those drugs are preferred that exhibit maximal reduction of the tumor mass. However, this method ignores that these treatments are most survived by tumor-initiating cells. These tumor cells initiate tumor formation by self-renewal and differentiation, similarly to the normal stem cells. The resulting rapidly dividing, differentiated tumor cells do not possess these properties. They feature is self-renewal, and not the high proliferative capacity.

Unlike to the differentiated tumor cells these cells have high capacity to colonize. Cell suspensions from human tumor tissue injected into immunosuppressed mice these cells are capable of a higher rate of colonization and tumorigenicity.

In the 90s John Dick and colleagues identified a potential cancer stem cell hierarchy, which followed the normal hematopoietic stem cell hierarchy.

Solid tumors models were demonstrated similar the hierarchical organization of leukemia, with TIC on the top of the hierarchy (breast cancer, brain cancer, colon cancer, melanoma, pancreatic-, prostate-, ovarian-, liver-, lung-, gastric cancer, head and neck cancer, mesenchymal cells).

A lot of known markers are used to identify tumor-initiating cells. Such markers are: CD133 glycoprotein, CD44 antigen, aldehyde dehydrogenase-1 (ALDH1) enzyme, ABCB1 and ABCG2 transporters, BMI1 oncogene.

Efficacy of the cytotoxic agents or ionizing radiation often depends on intrinsic or acquired resistance. Signaling pathways regulating the resistance of TICs may serve as therapeutic targets. Self-renewal signaling pathways described in the literature include: Wnt, Hedgehog pathway, Notch pathway, BMI1, PTEN, BMP, TGF-β. Resistance causing cells, which can be selected on the basis of cell surface markers ($CD133^{+}$) show greater sensitivity, reduced migration and invasion as a consequence of the inhibition of Akt kinase and their survival and proliferation are related to Akt kinase activation.

2. Objectives

We aimed to prepare new, patentable drug candidate molecules which can inhibit tumor initiating cells in cancerous diseases. Our approach was a pre-selection method, in which we could identify inhibitors of acquired resistant cell line. Best compounds were tested in clonogenic assay, which is a suitable model system for of resistance-causing cells inhibition.

We synthesized new analogues of pyrido[2,3-*b*]pyrazine hit molecule. In the synthetic procedure two regioisomer of core structures could form. We aimed to prove the structure of the required, biologically effective compound, and the optimization of the selectivity of the reaction.

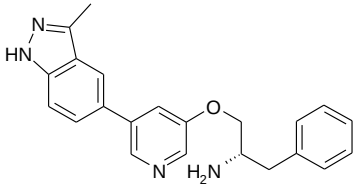
3. Methods

3.1. Biological methods

We identified inhibitors of acquired resistant cell line as a pre-selection method in an *in vitro* model. These compounds are effective on not only erlotinib-resistant, but also on erlotinib-sensitive NSCLC cell line, which contains EGFR mutation. We used PC9 cell line and its erlotinib resistant PC9-ER variant in a phenotypic screen.

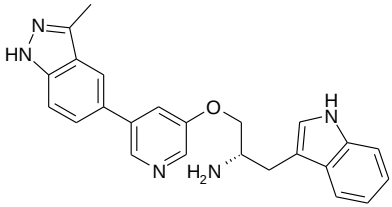
3.2. Synthesis of Akt1 kinase inhibitor reference compounds

I prepared three Akt1 kinase inhibitors known in the literature as reference compounds, which were part of our compound library. They were chosen by their Akt1 inhibitor effect. I synthesised A-674563 (**1**), A-443654 (**2**) and 2,3,7-tri-2-thienylpyrido[2,3-*b*]pyrazine (**3**) compounds (Scheme 1.) according to published methods.



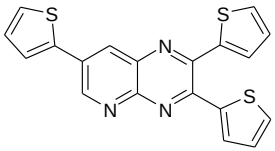
A-674563

1



A-443654

2

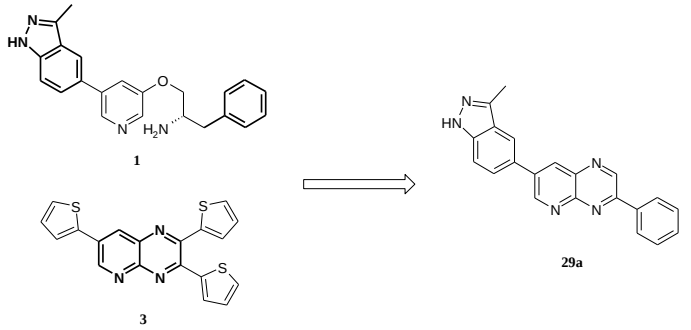


3

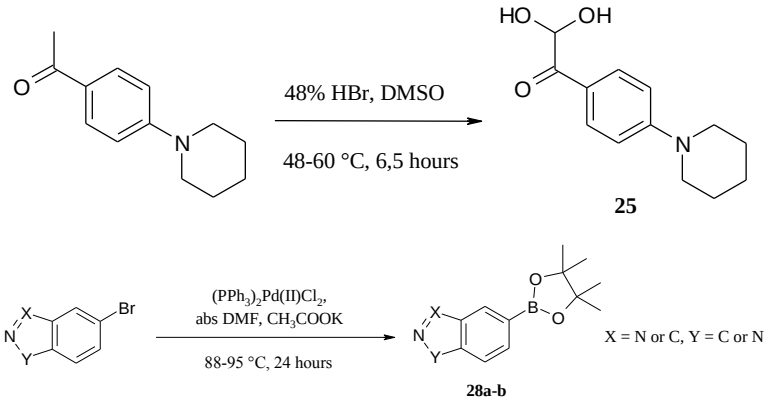
Scheme 1. Akt1 kinase inhibitor reference compounds

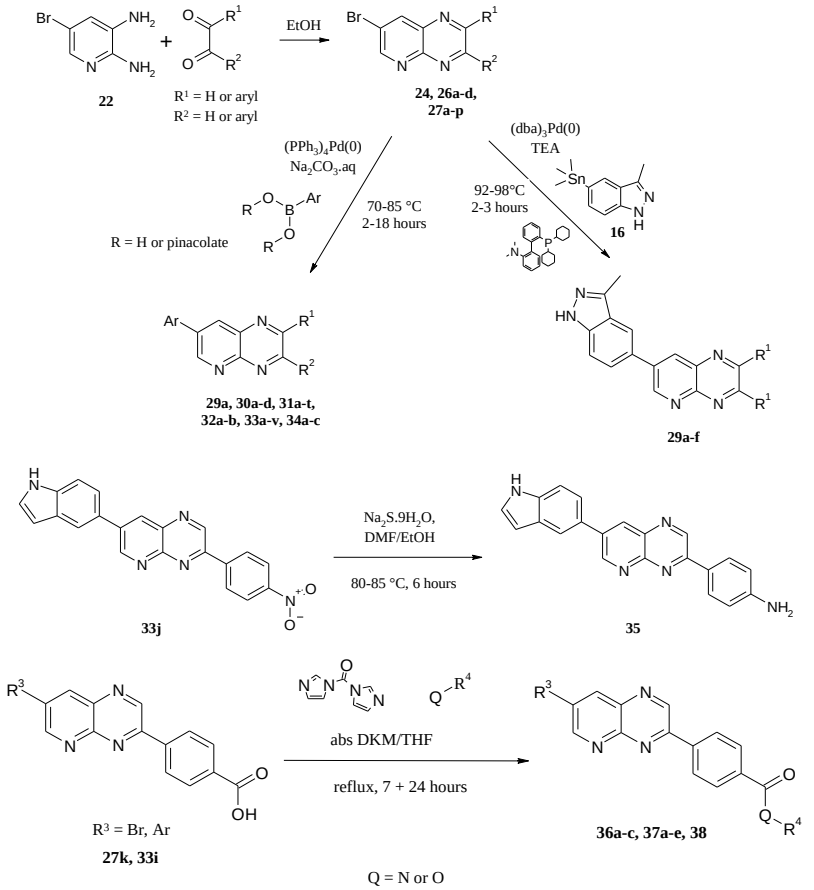
3.3. Combination of the structure of reference compounds, designing new derivatives and focused compound library

I design new structure by combination of compound **1** and **3** (**29a**) (Scheme 2.). I established a focused compound library by changing moieties of this molecule (Scheme 3.).



Scheme 2. New structure (**29a**) was designed from the reference Akt1 kinase inhibitors.





Scheme 3. General synthetic method of patentable compounds.

Moieties of the new structure **29a** were changed by the following steps:

I. substitution of 2-position with aromatic- and heteroaromatic rings;

II. substitution of 3-position with substituted phenyl rings and heteroaromatic rings, furthermore 3-position can be unsubstituted;

III. substitution of methylindazole ring in 7-position with other aromatic- and heteroaromatic rings.

Effect of the set of compounds bearing different moieties on cell growth was investigated. Further optimization was done on the bases of the most effective compounds. Five steps of iteration cycles led to the best molecules.

4. Results

4.1. Inhibition of cell division of PC9, PC9-ER cells

The control compound erlotinib had 0.005 μM EC_{50} value on PC9 and 5.65 μM on PC9-ER cells. We determined the EC_{50} values of the two regioisomers, as well (A-674563: PC9 – 0.91 μM, PC9-ER – 5.65 μM; **3**: PC9- >30 μM, PC9-ER – 15.64 μM).

The most effective compound is unsubstituted in 2-position, it has 2,3-dihydro-1,4-benzodioxin-6-yl group in 3-position, and 1*H*-indol-5-yl ring in 7-position (**33m**, EC_{50} PC9: 0.09 μM; PC9-ER: 0.15 μM).

Structure-activity relationship

The following structural elements are responsible for biological effect:

a) pyrido[2,3-*b*]pyrazine core should be unsubstituted in 2-position;

b) substituent in 7-position should be 4-indolyl, 5-indolyl or 5-indazolyl, which is unsubstituted in 1-position, thus NH in the appropriate position is essential;

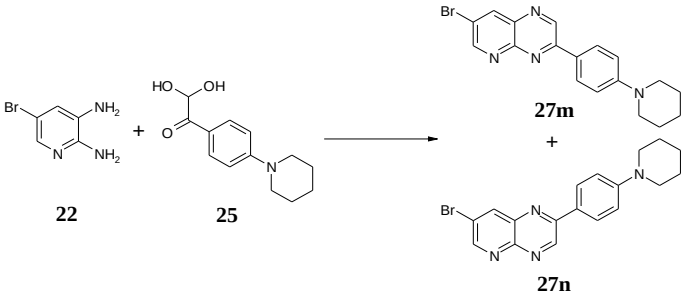
c) the best compound **33m** which contains 2,3-dihydro-1,4-benzodioxin-6-yl moiety in 3-position. Unsubstituted phenyl-ring in 3-position or substituted by aqueous solubility increasing chains decreased the biological effects.

4.2. Regioisomerism of condensation reaction, optimalisation of selectivity and identification of regioisomers

According to the structure-activity relationship the substitutions of 2-, 3- or both position of pyrido[2,3-*b*]pyrazine core have essential role. We optimised the reaction conditions to obtain the required isomer selectively.

The two isomers (7-bromo-2- or 3-(4-piperidin-1-ylphenyl)pyrido[2,3-*b*]pyrazine) formed in the same ratio in the condensation of **22** diamin- and **25** dioxo-compounds without

catalyst, at ambient temperature (Scheme 4.). The effect of the reaction conditions on the selectivity were examined in this reaction. Isomer ratios were determined by LCMS.



Scheme 4. Formation of regioisomers in the condensation reaction.

Thermal effect

We examined the effect of reaction temperature in the range of stanadard reaction temperatures: -25 °C, 0 °C, ambient temperature (22 °C), 70 °C and 120 °C. It was found that lower temperature resulted higher selectivity.

A decomposition product appeared at 70 °C and 120 °C. The analysis proved that it was 4-piperidin-1-ylbenzoic acid (**39**) formed from **25** starting material (2.5 hours, 70 °C).

We could identify on the chromatogram the intermediates of the condensation reaction due to partial water elimination at ambient temperature.

At 0 °C selectivity increased but reaction rate significantly decreased. At lower temperature, at -25 °C the reaction practically stopped.

Effect of acid-base catalysis

Effect of acid and base catalysis on selectivity was examined in DMF solution. 10 % basic catalyst 1,8-diazabicycloundec-7-ene caused partial decomposition of **25** dioxo-compound. Besides the previously mentioned **39** decomposition product oxo(4-piperidin-1-ylphenyl)acetic acid (**40**) was detected, as well.

Five equivalent acetic acid changed the **27m**:**27n** isomer ratio from 58:42 to 70:30. DMF was replaced by acetic acid, then by even more acidic trifluoroacetic acid (TFA), resulting higher selectivity. Not only the selectivity increased in acidic solvent but the reaction rate as well. Diluting TFA with DMF the selectivity decreased.

As lower temperature increases selectivity, I investigated isomer ratio in TFA at 0 °C, and observed that **27m**:**27n** was 98:2.

Identification of regioisomers

Regioisomers were characterized by their HPLC retention time and NMR spectra. There were no significant

difference in mass spectrometry fragments and UV spectra. Structure of **27m** isomer was proved by X-ray crystallography too.

4.3. Clonogenic assay

Seven compound effective on both PC9 and PC9-ER cell lines, were tested in clonogenic assay on EGFR mutation containing HCC827 cell line. Compound **33r** and **33a** were more effective than gefitinib control, by approximately an order of magnitude (4.14 and 3.32 %), for decrease the survival of resistant cells than the clinically used EGFR inhibitor gefitinib (23.08 %).

4.4. Akt1 and EGFR kinase inhibition and kinase inhibition profiling

The examined two tumor cell lines were EGFR mutant ones, and the parent hit compound was a derivative of two Akt1 inhibitors mentioned above. Some of the best compounds were tested on wild type, L858R and L858R/T790M mutant EGFR kinases, on Akt1 kinase and the compound **33a** on a kinase panel assay. It was found that these kinases are not responsible for the mechanism of inhibition. Consequently mechanism of cell growth inhibition needs further investigations.

5. Findings

According to the results of this work we can conclude:

1. I resynthesized three known Akt1 inhibitor compound A-674563, A-443654 and 2,3,7-tri-2-thienylpyrido[2,3-*b*]pyrazine, and by the combination of their structural elements I designed the 7-(3-methyl-1*H*-indazol-5-yl)-3-phenylpyrido[2,3-*b*]pyrazine. The moieties of this compound were changed in 2, 3 and 7 position and sixty-tree new, patentable compounds have been prepared.

2. According to these compounds biological efficacy — inhibition of growth of erlotinib-sensitive and –resistant PC9 cell lines — I determined the structure-activity relationship in five iterative cycle. The following structural elements are responsible for the effect: a) 2-position of pyrido[2,3-*b*]pyrazine core must be unsubstituted; b) the most effective substituent in 3-position was 2,3-dihydro-1,4-benzodioxynyl among the examined ones; c) effective moieties in 7-position were nitrogen-containing [5+6] ring system heterocycles in which the position of carbon-carbon bond between the indol or indazol and the pyrido-pyrazine

ring must be 4’ or 5’ moreover their 1’-position nitrogen must be unsubstituted.

3. In order to increase the aqueous solubility property of the molecules, we inserted basic amine containing chains into the compounds. These compounds had weaker effects than the parent compound. However their aqueous solubility was better than the **31n** parent compound.

4. In the preparative process the condensation reaction resulted two regioisomers. The preferable isomer – according to biological effect – could be prepared in a selective reaction at 0 °C, in TFA solution. Acidic solvent could increase the reaction rate, as well. The structure of regioisomers was proved by NMR and X-ray crystallography.

5. The most effective **33m** compound was roughly as effective on erlotinib-sensitive cell line as the erlotinib itself, but 30-fold more effective on erlotinib-resistant cell line. We can conclude that we could find compounds that were good inhibitors of both erlotinib-sensitive and erlotinib-resistant cell lines.

6. Inhibitors of acquired resistance containing cell line were tested in clonogenic assay in which we examined inhibition ability of EGFR containing HCC827 cells. We found those compounds which were more effective, by approximately an order of magnitude, on clonogenic cells than the clinically used gefitinib.

Well defined structure-activity relationship, biological efficacy, and optimized regioselective synthetic procedure provide that these submicromolar inhibitors might be new antitumor candidates.

6. List of publications

Papers of the thesis work:

Kékesi L, Sipos A, Németh G, Pató J, Kéri Gy, Őrfi L. Pyridopyrazines as anticancer agents. WO2014106763 patent application. (7. January 2014.)

Kékesi L, Sipos A, Németh G, Pató J, Breza N, Baska F, Őrfi L, Kéri Gy. (2013) Synthesis and biological evaluation of novel pyrido[2,3-*b*]pyrazines inhibiting both erlotinib-sensitive and erlotinib-resistant cell lines. Bioorg Med Chem Lett, 23(22): 6152-6155. doi: 10.1016/j.bmcl.2013.09.005.

Kékesi L, Dancsó A, Illyés E, Boros S, Pató J, Greff Z, Németh G, Garamvölgyi R, Baska F, Őrfi L, Kéri Gy. (2014) Preparation of pyrido[2,3-*b*]pyrazine ring system via regioselective condensation reaction. Lett in Org Chem, 11(9): 651-656. doi: 10.2174/15701786116661406062050 28.

Baska F, Szabadkai I, Sipos A, Breza N, Szántai-Kis Cs, Kékesi L, Garamvölgyi R, Nemes Z, Baska F, Neumann L, Torka R, Ullrich A, Kéri Gy, Őrfi L. (2014) Pharmacophpre and binding analysis of known and novel B-RAF kinase inhibitors. Curr Med Chem, 21(17): 1938-1965. doi: 10.2174/092986732166614030415 2606.