The identification and characterization of novel protein kinase D1 inhibitors on inflammation related cell models

PhD thesis

Attila Varga

Semmelweis University
Doctoral School of Pharmaceutical and Pharmacological Sciences

Supervisor: Dr. Tibor Vántus, Ph.D, senior research fellow

Official reviewers: Dr. Katalin Schlett, Ph.D, associate professor
Dr. Beáta Törőcsik, Ph.D, senior lecturer

Theoretical exam committee:

President:	Dr. Tamás Török, DSc, professor
Members:	Dr. András Telekes Ph.D, private professor
	Dr. László Nyitrai, DSc, professor
	Dr. Kornélia Tekes, DSc, professor

Budapest

2015

1. Introduction

Protein kinase D1 (PKD1) is a member of protein kinase D enzyme family with two other kinases, PKD2 and PKD3. According to its structural homology, PKD1 belongs to the group of calcium/calmodulin mediated kinases, and regarding to the function, it is a serine/threonine kinase. PKD1 is implicated in several signaling pathways and cellular functions as an important regulator, for example in gene expression by the inactivating phosphorylation of histone deacetyalses (HDAC), in promoting cell survival in oxidative stress, in the regulation of cell migration, and in Golgi vesicle transport as well. In endothelial cells, PKD1 is the part of the VEGFR2 signaling pathway, which has a dominant role in angiogenesis. In this pathway, PKD1 is involved in the regulation of endothelial cell proliferation, migration and angiogenic gene expression. Furthermore, PKD1 has a proven role in inflammation related immune cell functions, for example in Fcγ-receptor mediated superoxide production in neutrophils, Fcε-receptor initiated degranulation of mast cells, toll-like receptor regulated cytokine production in macrophages, and in the signaling of B-cell receptor in B-lymphocytes. Angiogenesis and inflammation are coupled together in several diseases, for example in rheumatoid arthritis, Crohn-disease, atherosclerosis or in certain tumors. At the chronic inflammatory site, immune cells and hypoxic conditions produced angiogenic factors can promote angiogenesis. The newly formed blood vessels recruit more immune cells to the site of inflammation, promoting more serious tissue injury. Aberrant signaling pathways are present in numerous diseases, such as in pathological angiogenesis and inflammation as well. However, these pathways can be specifically targeted by kinase inhibitors. Kinase inhibitors are in clinical application, mainly in tumor treatment, including tumor angiogenesis, additionally the number of kinase inhibitors as anti-inflammatory agents are increasing. Although, PKD1 is a validated drug target, there is no PKD1 inhibitor in clinical trials, furthermore there are only a few PKD1 inhibitors are available for research use.

2. Aims of the study

According to the literature, PKD1 is a validated drug target in several diseases, for example in pancreatic cancer, pathological angiogenesis and inflammation related diseases. However, there is no PKD1 inhibitor in clinical trials, and there are only a few PKD1 inhibitors are available for research use. Therefore, the aim of my PhD study was to identify novel kinase inhibitors from the kinase inhibitor library of Vichem Chemie Research Ltd., which could effectively inhibit PKD1 enzyme and could be a potential candidate for further drug development.

The main topics of my PhD work were the followings:

- The screening of the kinase inhibitor library of Vichem Ltd. against recombinant PKD1 enzyme, using *in vitro* kinase assay.

- The further characterization of the selected PKD1 inhibitors, regarding their structure-activity relationship and selectivity.

- Determination of certain ADME-Tox parameters of the selected inhibitors.

- To select cellular models, in which PKD1 is a validated drug target.

- The investigation of the selected inhibitor(s) on the above mentioned cellular models, regarding their intracellular effects and their influence on certain cellular functions.

3. Methods

Recombinant kinase assays

IMAP® assay

The inhibitory effect of the compounds was tested using IMAP®-assay, developed by Molecular Devices. The base of the method is that the fluorescently labeled peptide substrate and ATP containing reaction mixture produces low fluorescence polarization signal, when the peptide substrate is in non-phosphorylated state. However, when the kinase phosphorylates the peptide substrate, it bounds to a nanoparticle complex and the fluorescence polarization signal increases. The high signal means that the kinase is active.

Transcreener® assay

The recombinant *in vitro* VEGFR2 inhibitor study was performed applying Transcreener®-method developed by BellBrook Labs. During the assay, fluorescently labeled (Alexa633) ADP and ADP antibody are added to the reaction mixture. When the kinase is active, the labeled ADP bounded to the ADP antibody is replaced with the ADP produced in the kinase reaction. Hence, the fluorescence polarization signal is decreased, which means the kinase is active.

Cell culturing

Our endothelial cell model was the immortalized hybrid EA.hy926 cell line (ATCC®-CRL2922™), which was originally made by the fusion of the primary endotehlail HUVEC and the lung adenocarcinoma A549 cell line.

Cytotoxicity study

The potential cytotoxic effect of the inhibitors on EA.hy926 cells was investigated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) – assay. The base of the assay is that living cells produce purple formazan from the MTT crystals, using the mitochondrial reductase enzymes of the cells. After the solution of the prple formazan crystals, we can measure the optical densitiy and determine the amount of viable cells.

Membrane permeability assay

For the membrane permeability assay, we applied 96-well PAMPA plates (BD Biosciences). These plates contain artificial double layer lipid membrane. Therefore, it is a good model to investigate the passive transport of different compounds *in vitro*. In these experiments, we used 200 μM of the molecules, according to the manufacturer description. Before the experiment absorbance spectrums of the compounds were determined, and according to the absorbance maximum, the penetration of the compounds was calculated.

Cell proliferation assay

The anti-proliferative effect of the inhibitors was determined by cell counting after trypan blue staining. The cell counting was performed using Countess (Invitrogen) cell counter.

SDS-PAGE and Western blot analysis

Prior to the activation with 25 nM PMA or 25 ng/ml VEGF for 20 minutes EA.hy926 cells were pretreated with the inhibitors for 1 hour. Then, cells were lysed in RIPA-buffer on ice. The protein concentrations were determined using Bradford method. The samples were loaded into 8 or 10% SDS-poliacrylamide gels and the proteins were transferred onto PVDF membranes. The investigated proteins were the followings: pSer473-Akt and total Akt, β-actin, pSer498-HDAC5 and total HDAC5, pSer744/748-PKD1, pSer916-PKD1, and total PKD1, pTyr951-VEGFR2, and total VEGFR2. ECL-method was applied to determine the results.

Cell migration assay

For the investigation of cell migration we used wound healing assay. At the beginning of the experiment, the confluent cell layer was scratched by a pipette tip, and the inhibitor containing medium was added. The cells were let to migrate to the scratched area for 18 hours then, photos were taken using inverted phase contrast microscope. During the quantification, the area of the scratched surface was determined at the beginning and at the end of the experiment as well and the wound closure was calculated. The pictures were quantified applying ImageJ software.

In vitro angiogenesis assay

To investigate angiogenesis *in vitro*, we used tube formation assay. The method is based on the phenomenon, that endothelial cells placed on extracellular matrix, form capillary-like tube structures. The inhibitor treated cells were allowed to form capillary-like tube structures for 18 hours, then photos were taken using inverted phase contrast microscope. The photos were quantified using the Angiogenesis Analyzer Plugin of ImageJ software.

Amplex® Red assay

The superoxide production of EA.hy926 cells were measured using Amplex® Red assay. During the experiment the produced superoxide transforms to hydrogen-peroxide, which turns the Amplex® Red reagent to fluorescently detectable rezorufin in 1:1 ratio. Hence, the amount of the produced superoxide could be measure.

The isolation of neutrophil granulocytes

Neutrophil granulocytes were isolated from the venous blood of healthy volunteers. After dextrane sedimentation, ficoll gradient centrifugation was performed, then the remaining red blood cells were lysed using NaCl solution. The separated neutrophils were suspended in Ca^{2+}/Mg^{2+}-free HBSS/Hepes solution and were used for the following experiments.

Neutrophil superoxide production assay

We used 96-well ELISA plates for these experiments. For the immobilized immune-complex activation, human serum albumin and anti-human serum albumin antibody were used. For the adhesion-dependent activation, the wells were pretreated with 150 µg/ml fibrinogen containing PBS and prior to adding the cells 20 ng/ml TNFα was pipetted into the wells. For PMA activation, the wells were pretreated with 10% FCS containing PBS and before the addition of the neutrophils, 100 nM PMA were pipetted into the wells. Prior to the seeding of the cells into the plate, neutrophils were incubated with the inhibitors for 30 minutes at 37^{o}C in 0.5 mM Ca^{2+} containing HBSS/Hepes buffer. Immediately before the addition of the neutrophils into the plate, 1 mM Mg^{2+} and 100 µM ferricytochrome c were also added into the wells. The amount of the produced superoxide could be measured by the reduction of ferricytochrome c.

Neutrophil transmigration assay

Using 24 well transwell migration plates (Corning), the migration of neutrophils was studied. The transwell inserts contain a polycarbonate filter with 3 μm pores. Before the experiment, this filter was coated with fibrinogen. First the neutrophils were incubated with the inhibitors for 30 minutes at 37°C in Ca^{2+} containing HBSS/Hepes buffer and then they were transferred into the inserts, when Mg^{2+} was also added. The cell migration was initiated by 100 nM fMLP as chemoattractant. The amount of the transmigrated cells was determined applying para-nitophenyl-phosphatase enzyme reaction.

Statistical analysis

Every experiment was performed at least three times. The statistical analysis was performed applying Graph Pad Prism 5 software, Microsoft Excel and XLfit. In the *in vitro* recombinant kinase assays, Z' values were calculated, which indicates the reliability of the experiment. The IC_{50} values were determined using non-linear regression. For statistical comparison of the groups one way ANOVA-test, which followed by post hoc Dunett's Multiple Comparison Test was used. p value of < 0.05 was considered statistically significant.

4. Results

In vitro recombinant kinase studies

The screening of the molecular library

First, we have screened the Vichem Chemie Research Ltd's EVL (Extended Validation Library) for recombinant PKD1, using IMAP®-assay. EVL contains known small molecular inhibitor compounds and their structural analogues. After the screening of the 2000 compounds, 72 molecules showed more than 75% inhibition at 10 μM, then they were selected for biochemical IC_{50} value determination and 24 molecules showing less than 1 μM IC_{50} value were further characterize. Investigating the structures of the compounds, we observed that 20 of the 24 molecules had pyrido[2,3-*d*]pyrimidin-7-one moiety. We focused on these compounds in the further experiments.

Structure-activity relationship

Next, we investigated the relationship between the molecular structure and the PKD1 inhibitory effect of the molecules. For this investigation, non-PKD1 inhibitor compounds with pyrido[2,3-*d*]pyrimidin-7-one moiety were also selected. We have observed that the effective PKD1 inhibitors had a basic tertier (or secunder) amine group at the 2^{nd} position of the pyrido[2,3-*d*]pyrimidin-7-one basic structure. Furthermore, these molecules did not contain any function group at the 6^{th} position.

Selectivity study

In the selectivity studies, we examined the inhibitory effect of our best compounds against kinases showing high structural or functional similarities with PKD1. According to the results, the most selective compound was the VCC251801. Hence, this inhibitor was selected for further characterization. Further *in vitro* kinase studies revealed VEGFR isoforms as other important targets of VCC25801. Amongst the VEGFR isoforms, VEGFR2 is the best characterized and the most important drug target. Therefore, beside PKD1 we focused on VEGFR2 as well in our further investigation. Using *in vitro* kinase assay, we observed that VCC251801 inhibited the activity of PKD1 at the same concentration rate like the

kb-NB142-70, which is one of the best available PKD1 inhibitor on the market. Furthermore, VCC251801 also effectively blocked the activity of VEGFR2 at the same concentration rate like the VEGFR inhibitor Axitinib, which compound is in clinical use. Since PKD1 and VEGFR2 have a proven role in pathological angiogenesis and in different inflammatory processes, we selected angiogenesis- and inflammation-related cellular models.

Early ADME-Tox studies

We have investigated the potential cytotoxic effect of VCC251801 and its penetration through artificial lipid membrane.

Cytotoxicity assay

For cytotoxicity study, the widely used MTT-assay was applied. The endothelial cells were treated with the inhibitors in 0.4 – 50 µM concentration range. The results showed that the presence of VCC251801 was well tolerated by the endothelial cells. Moreover, the inhibitor did not caused total cell death after 24 or 48 hours. In the further experiments, the concentration range was selected according to the cytotoxicity studies.

Membrane permeability assay (PAMPA)

In this method, a special, cell membrane modeling plate system was used, in which the transport of the drug candidate molecules could be investigated. The results showed that VCC251801 could penetrate the artificial lipid membrane by passive transport.

Studies on angiogenesis model

Our chosen angiogenesis model was the widely used endothelial derived immortalized EA.hy926 cell line.

Endothelial cell proliferation study

At 0.5 and 1 µM doses VCC251801 did not caused significant decrease in cell proliferation after 24 or 48 hours. However, the 5, 10 and 50 µM concentration treatments strongly reduced endothelial cell proliferation in the same time span.

The intracellular effect of VCC251801

In these experiments, we examined the intracellular inhibitory effect of VCC251801 on PKD1 and VEGFR2 kinases and on their regulated pathways. Hence, we selected two different activation methods, whereby the activity of PKD1 and VEGFR2 could be investigated separately. These agents were the phorbol-ester PMA and the specific activator VEGF.

First, the PMA induced PKD1 activation was examined performing western blot analysis. VCC251801 reduced the autophosphorylation of PKD1 and the phosphorylation of the PKD1 substrate HDAC5 dose dependently. However, the transphosphorylation of PKD1 remained unaffected by the treatment. Therefore, we concluded that, the VCC251801 did not inhibited the PKC isoforms upstream of PKD1.

Next, we focused on the VEGFR2 signaling pathway. Hence, we stimulated the endothelial cell with VEGF. VCC251801 effectively blocked the VEGFR2 signaling pathway activation, which was monitored by its key components: VEGFR2, PKD1, HDAC5 and Akt. The effect was dose dependent and was significant at 5 μM on every investigated enzyme. It was interesting, that the kb-NB142-70 treatment drastically increased the phosphorylation of Akt. There is a negative feedback regulation between PKD1 and Akt, which phenomenon was described previously. Although, the treatment with Axitinib could effectively blocked Akt phosphorylation due to the inhibition of the VEGFR2, it could not inhibit significantly the phosphorylation of HDAC5. Only the VCC251801 treatment could reduce significantly the phosphorylation of both kinases.

We can conclude that, VCC251801 could effectively inhibited the PMA induced PKD1, and the VEGF stimulated VEGFR2 signaling pathway activation in endothelial cells.

Endothelial cell migration assay

In our further experiments, we studied the effects of VCC251801 on certain endothelial cell functions. First, wound healing method was used to investigate cell

migration. The inhibitor was applied at the previously used concentration range, namely 1, 2.5 and 5 μM. The effect of VCC251801 treatment was dose dependent: cell migration was reduced by 35% at 1 μM, and by 55% at 2.5 μM concentration. The reduction of cell migration was also significant at 5 μM concentration, but the cells were beginning to detach probably due to cell death.

In vitro angiogenesis

The widely used tube formation method is a good model to investigate angiogenesis *in vitro*. The method is based on the phenomenon, that endothelial cell placed on extracellular matrix, form capillary-like structures. Regarding to our previous experiments, the endothelial cell form the tubes after 18 hours. Hence, the incubation tame span with the inhibitors was the same. After the experiment three kind of parameters were determined: the number of the tubes, the length of the tubes and the area covered by the tubes. VCC251801 dose dependently reduced all three parameters. The effect was less effective at 1 μM, because only the length of the tubes was decreased significantly, but the number of the tubes and the area covered by the tubes were not. However, the VCC251801 treatment at 2.5 és 5 μM concentration could reduce all of the three analyzed parameters. This reduction was really strong at 5 μM concentration, probably due to the potential cell death. In summary, VCC251801 could effectively reduce angiogenesis *in vitro*.

Superoxide production of the endothelial cells

The superoxide production of endothelial cells in pathological conditions has been described in several diseases. In endothelial cell, the dominant superoxide producing NADPH-oxidase isoform is the NOX4. In these experiments, we investigate the potential role of PKD1 in endothelial superoxide production, because it has not been described yet. The cells were pre-incubated with the inhibitors at 10 μM for 30 minutes, then Amplex Red assay was performed. The results showed that none of the PKD1 inhibitors (VCC251801 and kb-NB142-70) affected the endothelial superoxide production.

Studies on inflammatory cell model

Neutrophil granulocytes were another important inflammation related cellular model in this study. Neutrophil provide the first line of defense against bacterial and fungal pathogens, but also contribute to several different pathological mechanisms as well.

The superoxide production of neutrophil granulocytes

In neutrophils, the superoxide production by NOX2 enzymes is an important response mechanism, which could induce by different stimuli. In our experiments, we have used two specific and one aspecific activation mechanisms. First, we performed the immune-complex induced neutrophil activation, which could be a cellular model of autoimmune inflammation. VCC251801 effectively decreased the superoxide production by 50% at 0.3 and 1 μM, and almost by 100% at 3 and 10 μM concentrations. Since, the role of PKD1 in this mechanism has been described previously, the reference PKD1 kb-NB142-70 could decrease superoxide production as well. The next neutrophil activation mechanism was the adhesion dependent TNFα induced cell response, which could be a cellular model of the inflammatory conditions. Although, the role of PKD1 was unknown in this mechanism, surprisingly VCC251801 effectively reduced the neutrophil superoxide production, which was decreased by 50% at 0.3, by 70% at 1 μM, and almost by 100% at 3 and 10 μM concentrations. The PKD1 reference inhibitor kb-NB142-70 was also effective in these experiments. The third kind of superoxide inducing method was the non-physiological and aspecific activation by PMA. However, this kind of cell response was not affected by the inhibitors.

The transmigration of neutrophil granulocytes

The next investigated cell function was the neutrophil transmigration through an artificial membrane. This method is a good model of the neutrophil extravasation process *in vitro*. In these experiments, 100 nM fMLP was used as a chemoattractant, which is released in inflammation state, for example in bacterial infection. Before the experiment, the artificial membrane was coated with the β2-integrin ligand fibrinogen to induce neutrophil cell adhesion. The addition of fMLP significantly increased the transmigration of the cells in the untreated sample.

However, the fMLP induced transmigration did not change in the VCC251801 treated samples.

5. Conclusions

The summarized conclusions of my PhD study are the followings:

1. We have identified effective PKD1 inhibitors with pyrido[2,3-*d*]pyrimidin-7-one moiety. We observed that the presence of a secunder or tertier amine group at the 2nd position of the pyrido[2,3-d]pyrimidin-7-one is essential for the PKD1 inhibitory effect. Furthermore the presence of an aryl or tienyl group at the 6th position decreased the PKD1 inhibitory effect.

2. We have selected our best PKD1 inhibitor compound, which was selective against kinases showing high structural (CAMK, MLCK) or functional (PKC isoforms) similarities with PKD1. Moreover, VEGFR2 revealed as another important target of VCC251801, which was inhibited by our compound at the same concentration rage like the clinically validated VEGFR inhibitor Axitinib.

3. In early ADME-Tox studies, VCC251801 was well tolerated by endothelial cells. Furthermore, our inhibitor could effectively penetrate the artificial lipid membrane, which could mean that VCC251801 was able to get into the cells by passive transport.

4. In endothelial cells, VCC251801 could effectively block the VEGFR2 signaling pathway by the simultaneous inhibition of PKD1 and VEGFR2. The inhibition of the VEGFR2 signaling pathway by VCC251801 reduced endothelial cell proliferation, migration and *in vitro* angiogenesis.

5. The endothelial superoxide production was not affected by either VCC251801 or the PKD1 reference compound kb-NB142-70. We could conclude that PKD1 was not involved in this mechanism in endothelial cells.

6. VCC251801 could inhibit specifically neutrophil cell responses induced by different kind of physiological stimuli, such as immune-complex or adhesion dependent TNFα induced activation. In contrast with immune-complex activation, the role of PKD1 in adhesion dependent TNFα induced neutrophil cell response has not been described yet. However, our results suggested the potential role of PKD1 in this mechanism.

6. Publications related to the dissertation

[1] **Varga A**, Gyulavári P, Greff Z, Futosi K, Németh T, Simon-Szabó L, Kerekes K, Szántai-Kis C, Brauswetter D, Kokas M, Borbély G, Erdei A, Mócsai A, Kéri G, Vántus T (2015). Targeting vascular endothelial growth factor receptor 2 and protein kinase D1 related pathways by a multiple kinase inhibitor in angiogenesis and inflammation related processes in vitro. *PloS One 10, e0124234.* **IF: 3,53**

[2] Borbély G, Huszár M, **Varga A**, Futosi K, Mócsai A, Őrfi L, Idei M, Mandl J, Kéri G, Vántus T (2012). Optimization of important early ADME(T) parameters of NADPH oxidase-4 inhibitor molecules. *Medicinal Chemistry 8, 174-181.* **IF: 1,37**