CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Drug Control

Prodrug structures to improve the drug delivery of anti-cancer agents

DIPLOMA THESIS

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Specialized supervisor: Kristiina Huttunen, Ph.D.

2016   Vendula Králová

DECLARATION

Hereby I declare that this thesis is my own work. All literature and sources of information I used are listed in the list of used literature and they are properly cited. This work has not been used to gain equal or different degree.

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ABSTRAKT

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Katedra farmaceutické chemie a kontroly léčiv

Student: Vendula Králová

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Název diplomové práce: <u>Syntéza proléčiv vedoucí ke zlepšení dostupnosti protinádorových látek</u>

Rakovina patří v dnešní době mezi jednu z nejrozšířenějších nemocí, a můžeme očekávat, že tomu tak bude i v budoucnu. Proto mnoho vědeckých týmu po celém světě vyvíjí a zkoumá nové léky, které by mohly tento vzrůstající problém zastavit. V dnešní době se farmakologický výzkum zaměřuje především na vývoj cílené léčby onkologických onemocnění, která je zaměřená jen na určité struktury na povrchu nebo uvnitř nádorových buněk, je vymezená jen pro přesně vymezenou skupinu nádorů a zároveň zpravidla neničí zdravé buňky. Na základě klinických studií byla do praxe zavedena řada nových léků, které vykazují vysokou efektivitu při léčbě nádorových onemocnění. Avšak jejich prospěšnost je limitována nástupem získané rezistence, která je v dnešní době velkou překážkou v této léčbě. Proto intenzivně probíhá výzkum, jak tuto rezistenci překonat.

Cílem této práce byla syntéza nových protinádorových léčiv a jejich proléčiv. Tato proléčiva byla připravena tak aby zvyšovala buněčné vychytávání těchto protinádorových léčiv prostřednictvím transportérů, které jsou všudy přítomny v nádorových buňkách.

Jednou z myšlenek bylo připravit analog verapamilu, kde byl použit jako linker thieno[2,3-b]pyridine a methoxyfenylová skupina jako nezbytná část pro potenciální proti rezistenční účinek, který již byl testován a vykazuje slibné výsledky pro další výzkum. Mým cílem bylo tedy připravit proléčivo z této sloučeniny, které by bylo následně transportováno selektivně prostřednictvím transportérů do nádorových buněk.

Druhá část práce byla zaměřena na nukleární faktor-Kβ, který je spojován, mimo jiné, také s rozvojem rakovinného bujení a autoimunitních nemocí. Mým plánem bylo připravit nový inhibitor IKKβ (podjednotka NF-Kβ), a z něj následně vytvořit cíleně transportováno proléčivo, které by mohlo nabídnout nové terapeutické možnosti v léčbě rakoviny. Avšak z nedostatku času se mi nezdařilo připravit zamýšlené proléčivo, syntéza však dále pokračuje na „University of Eastern Finland" v Kuopiu.

Třetí část práce je zaměřena na ganciklovir, jako potenciální léčivo genové terapie maligního gliomu. Mým plánem bylo provést syntézu nových proléčiv gancikloviru, které by díky jejich vyšší lipofilitě mohly proniknout do nádorových buněk ve větším množství než ganciklovir a dosáhnout zde tedy vyšší koncentrace a lepšího účinku.

Hlavním přínosem prezentované práce je experimentální část, nicméně téma syntézy nových protinádorových léčiv s lepšími farmakologickými vlastnostmi je velmi široké a tudíž otevřené k dalšímu vývoji.

ABSTRACT

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Title of diploma thesis: <u>Prodrug structures to improve the drug delivery of anti-cancer agents</u>

Cancer belongs to the most widespread diseases in the world, and we can expect that it will remain among the deadliest illnesses in the future. For this reason, scientists have put enormous efforts into researching new drugs, which would stop this growing trend. Nowadays, pharmaceutical research is focused mainly on the development of targeted treatment of oncological diseases, which aims at specific structures on the surface or in the core of the tumorous cells. This treatment is limited only to a precisely targeted group of tumors, and it usually leaves healthy cells unharmed. Through clinical studies, many new medications have been developed, and they show high efficiency in the treatment of various types of cancer. However, their success is constrained by drug resistance, which has become a significant obstacle to their use.

The topic of this thesis is the synthesis of new anticancer drugs and their prodrugs. These prodrugs were prepared in such a way that they would increase the cellular uptake of the anticancer drugs, via transporters that are over-expressed in cancer cells.

One of the presented ideas is the preparation of an analogue of verapamil, where I used thieno[2,3-b]pyridine as the linker and the methoxyphenyl group as the necessary part of the potential anti-resistance effect. Its impact, which had already been tested, shows promising results for further drug development. My goal was to prepare a prodrug from this compound, which would be transported selectively via transporter into the cancer cells.

The second part of this thesis examines the nuclear factor-Kβ, which is associated with the development of cancerous growth and autoimmune disease. My plan was to prepare a new inhibitor of IKKβ (subunit of NF-Kβ), and its transporter-targeted prodrug, which would introduce new therapeutic possibilities in the treatment of cancer. For the lack of time, I was not able to finish the preparation of this prodrug. However, the synthesis still continues at the University of Eastern Finland, Kuopio.

The third part of the thesis is focused on Ganciclovir - a potential medication for the gene therapy of the malignant glioma. My plan was to perform the synthesis of new prodrugs for ganciclovir, which could, due to their higher lipophilicity, penetrate to the tumorous cells in higher quantity than the ganciclovir, and thus reach higher concentration and better effect.

The main contribution of this thesis is in the experimental part. However, the field of the synthesis of new anticancer drugs with better pharmacological properties is very extensive, and thus still open to more research.

1. <u>Content</u>

2. List of abbreviations

Table 1: Abbreviations

ATP	Adenosin triphosphate
ABC	ATP Binding Cassette
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
DNA	Deoxyribonucleic acid
ECF	extracellular fluid
GCV	Ganciclovir
GDEPT	Gene directed enzyme prodrug therapy
HSV-tk	Herpes simplex virus thymidine kinase
IL-1	Interleukine 1
IKβ	Inhibitor Kβ
LAT1	L-type amino acid transporter 1
MDR	Multidrug resistance
NF-Kβ	Nuclear factor-Kβ
NEMO	NF- Kβ essential modulator (inhibitor of nuclear factor Kβ kinase)
P-gp	P-glycoprotein
TNF-α	Tumor necrosis factor α

*Abbreviations of chemical compounds used in the experimental part for synthesis are listed in the *Table 2.* List of chemical reagents

3. Introduction

Cancer is nowadays considered one of the most common diseases in the world. It is expected that malign tumors will increasingly threaten the human population in the third millennium. In the following years, we can expect another dramatic increase in the number of new cases of cancer. It is estimated that by 2030, the total number of cancer patients will triple and the number of deaths caused by cancer will exceed the number of deaths caused by cardiovascular disease, the current leading cause of death globally. (1)

The increase in the life expectancy is the most important factor contributing to the rising incidence of cancer. Advances in the treatment of non-malignant disease have led to longer life expectancy, thereby paradoxically contributing to the growing number of tumors among the older, more vulnerable population. With rising median age, the increase in the incidence of cancer among older population, especially in the developed countries, is more and more evident. At present, approximately 50% of all cases of cancer occur in people aged 65 and above. As they get older, men are more likely to be affected by the rising incidence of cancer than women. In most European countries, the most frequent type of cancer above the age of 70 is lung cancer or prostate cancer for men, and breast cancer or colorectal cancer for women. The close relationship between age and cancer is due to a longer period that carcinogenic substances can affect the body for and the increased susceptibility of aging tissues to carcinogens from the environment and from the physiological changes associated with the process of aging. Long-term cumulative exposure to carcinogens and the cumulative effect of endogenous processes that cause DNA damage are the main causes of increased risk of cancer in later life. Cancer is considered a disease of civilization, so changing lifestyle is another important factor explaining the increase in new cases of cancer. Smoking, poor diet, lack of physical activity, obesity, increased alcohol consumption, and stress are some of the factors associated with higher risk of developing cancer. Excessive sun exposure and tanning beds are risk factors for developing skin cancer. Hereditary cancer (7-10%) is another considerable group of tumors that may be related to adverse developments in the genepool.

Despite increasing incidence of cancer, the mortality rate has stabilised over the long-term. This is mostly due to new treatment methods and detection of cancer at earlier stages. However, there are still many patients who are not diagnosed until advanced stages of cancer.

For this reason, many research groups worldwide are working on the development of new drugs that would reduce or even stop the disease. (2)

The main goal of this thesis was to synthesize novel anti-cancer agents and their prodrugs. The prodrugs were designed to increase the cellular uptake of these anti-cancer drugs and be selectively transported into cancer cells. Syntheses were quite challenging, however we managed to produce two novel prodrugs and two novel anti-cancer agents, which were highly pure (>95%) and which we could later use for our *in vivo/in vitro* studies.

The first part of the thesis presents a general introduction of cancer and possibility of cancer therapy as well as problems that arise from the current chemotherapy. The second part is mainly focused on chemical synthesis of desired compounds.

4. Theoretical part

4.1 What is cancer

Cancer is a group of diseases involving abnormal uncontrollable cell growth and spreading into surrounding tissues. All cancers begin in cells. Our bodies are composed of more than a hundred million cells. Cancer can emerge in almost any part of the human body, which is made up of trillions of cells. Normally, human cells grow and divide to form new cells as the body needs them. When cells grow old or get damaged, they die, and replaced by new cells. After a cancer develops, this systematic process breaks down. As cells become more and more abnormal, old or damaged cells survive in situations when they should die, and new cells form even when they are not needed. These extra cells can divide further without any restriction and eventually they can form growths which are called tumors. The place where cancer starts is called the primary tumor. We distinguish two main kinds of tumors – malignant and benign. Malignant tumors can spread into, or invade, nearby tissues. In addition, as these tumors grow, some cancer cells can break off and travel to distant places in the body through the blood or the lymph system, and form new tumors far from the original tumor. Sometimes, benign tumors can be quite large, but when removed, they usually do not grow back. Whereas malignant tumors occasionally do. (3) (4)

4.2 Genes and cell division

All types of cells in the body have specific function, but they are in essence very similar. They include a nucleus which is the control center of the cell. Inside the nucleus, there are 23 pairs of chromosomes made up of long strings of DNA (deoxyribonucleic acid) which contain thousands of genes, containing messages that tell the cell how to behave. Each gene is an instruction that tells the cell to create something, decide what sort of cell it should be, what it does, when it divides, and when it dies. (5) (Figure 1.)
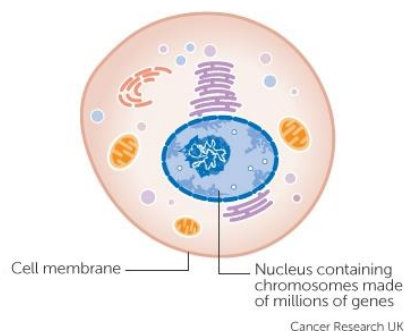

Cell membrane —— Nucleus containing
chromosomes made
of millions of genes

Cancer Research UK

Figure 1. *Structure of the cell* (5)

4.3 Differences between Cancer cells and normal cells

Cancer cells differ from normal cells in many ways that allow them to grow out of control and to become invasive. Unlike genes in normal cells, genes in cancer cells fail to ensure controlled and systematic growth. This failure is often caused by gene mutation during cell division. (4) (3) Mutations can occur randomly while the cell is being divided. Cancer cells can grow out of control and become invasive. One of the main differences from normal cells is that cancer cells are less specialized than normal cells. For this reason that cancer cells can continue to divide without any restriction, they can ignore signals that normally instruct cells to stop dividing, or to initiate the programmed cell death, or to get rid of unneeded cells. It can take many years for a damaged cell to divide and grow and form a tumor big enough to cause symptoms or show up on a scan. (3) (Figure 2.)
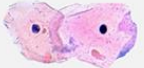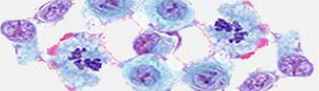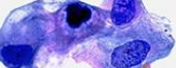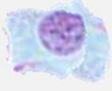

Figure 2. *Difference between cancer cells and normal cells* (6)

4.4 How cancer arises

A mutation can occur by chance when a cell is dividing. It can also be caused by external factors, such as chemicals in tobacco smoke, or by the processes inside cells. Some people can inherit defects in particular genes which might make them more susceptible to developing a cancer. (4)(Figure 3.)

Cancer Development
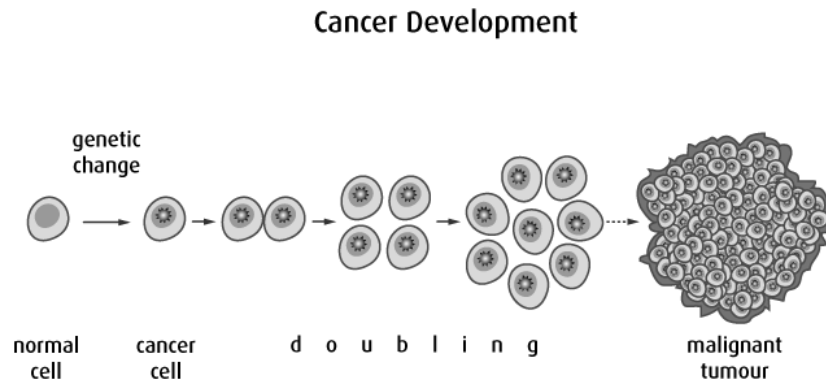
Figure 3. *Cancer development* (7)

4.5 Cancer therapy

Historically, the first treatment of cancer was surgery. Even after development of radiation therapy and chemotherapy, the surgery stay as the first choice of treatment. The types of treatment depend on the type of cancer and on the state of its advancement. Some patients with cancer undergo only one type of treatment, but most people undergo a combination of treatments, such as surgery with chemotherapy and/or radiation therapy. The ultimate objective is to destroy all cancer cells with minimal damage to normal cells. (8) This can be achieved in a number of ways:

Radiotherapy uses high doses of radiation to kill cancer cells and shrink tumors. It is usually used in conjunction with surgical procedures.

Chemotherapy uses a variety of drugs which work preferably, but not exclusively, by stopping or slowing the growth of cancer cells.

Endocrine therapy slows or stops the growth of cancer that uses hormones to grow. For example, it can be used for breast cancer therapy.

Antibodies against specific proteins that are overexpressed in cancer cells may be used to preferentially target those cancer cells.

Targeted therapy that targets those changes in cancer cells which help them grow, divide and spread. They have been developed to exploit the overexpression of targeted proteins in many cancer cells.

Biological response modifiers are a class of compounds, which may be modulated to elicit an immune response against cancer cells. (9) (2)

4.6 Chemotherapy

Chemotherapy is one of the most frequently used forms of cancer therapy. It has been used in the treatment of almost every type of cancer for its ability to slow, or even stop, the growth of cancer cells. (4) We use it to make a tumor smaller before surgery or radiation therapy, to destroy cancer cells that may remain after treatment with surgery or radiation therapy, to help other treatments work better or to kill cancer cells that have returned or spread to other parts of a body. Cytotoxic drugs are normally used only for short periods of time due to their side effects and distress they might cause to patients. (10) This side effects include mouth sores, nausea, fatigue, blood clotting problems depressed immune system, and hair loss. Most of them subside after treatment, but sometimes they can cause permanent damage to the heart, lungs, kidneys or reproductive system. (4) (9) (10)

4.6.1   Failure of chemotherapy

One of the major problems in cancer chemotherapy is the development of resistance. Failure of cancer to respond to a specific therapy can result from one of two general causes:

- Host factors and specific genetic alterations in the cancer cells include poor absorption or rapid metabolism or excretion of a drug, resulting in low serum levels (poor tolerance to effects of a drug resulting in a need to reduce doses below optimal level).
- Inability to deliver a drug to the site of a tumor, as could occur with bulky tumors or with biological agents of high molecular weight and low tissue penetration such as monoclonal antibodies and immunotoxins. (4) (3)

4.6.2   Potential mechanism of resistance

Previous research has identified various mechanisms by which cancer cells, grown in tissue culture, become resistant to anticancer drugs. (Figure 4.) Effective chemotherapy usually consists of a combination of multiple drugs with different mechanisms of entry and different cellular targets. However, cells frequently show resistance to many drugs that are not structurally or functionally related. (8) This phenomenon, known as multidrug resistance (MDR) (11), can result from changes that limit accumulation of drugs within cells by limiting uptake, enhancing efflux, or affecting membrane lipids such as ceramide. (12) These changes block the programmed cell death (apoptosis) that is activated by most anticancer drugs (13), activation of general response mechanisms that detoxify drugs and repair damage to DNA, and alterations in the cell cycle and checkpoints that provide cells relatively resistant to the cytotoxic effects of drugs on cancer cells. (13) (14)
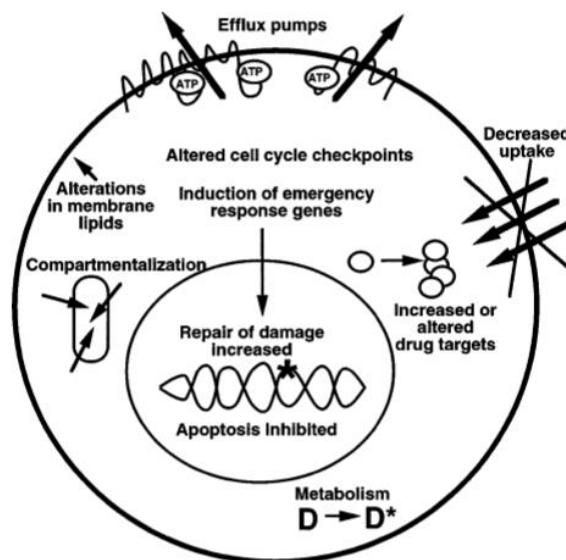
Figure 4. *This image summarizes many of the ways in which cultured cancer cells have been shown to become resistant to cytotoxic anticancer drugs.* (8)

The best known mechanisms are those that modify accumulation of drugs within cells. This is ensured by the balance between drug entry and exit mechanism. Drugs can entry the cells in several ways. (Figure 5.) (8)
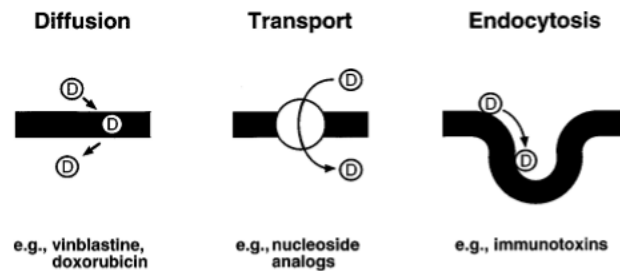
Figure 5. *Ways in which drugs can get into cells. Examples are given for the three major routes: diffusion across the plasma membrane, piggy-backing onto a receptor or transporter, and endocytosis.* (8)

Each of these mechanism has a physiological significance which has been verified by detailed uptake studies and by the observation of defects in these pathways in resistant mutants. (8) The rest of this review focuses on the mechanism of multidrug resistance resulting from alterations in the pathways of drug uptake or efflux from the cell.

5. Novel drugs based on MDR

5.1 Mechanism that increase drug efflux from cancer cells

The most well-known efflux pump is P-glycoprotein (P-gp) or alternatively multidrug transporter 1 (MDR1). (15) It is the product of MDR1 gene in the human which was one of the first members described of a large family of ATP-dependent transporters known as the ATP-binding cassette (ABC) family. (16) Its function is also to move nutrients and other biologically important molecules across plasma membranes and intracellular organelles in cells. P-gp is widely expressed in many human cancers. (17) P-gp can detect and bind a large variety of hydrophobic natural-product drugs as they enter across the plasma membrane. These drugs include many of the commonly used naturally produced anticancer drugs such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol, as well as many commonly used pharmaceuticals ranging from antiarrhythmic and antihistamines to cholesterol-lowering statins and HIV protease inhibitors. (18) Binding of these drugs results in activation of one of the ATP-binding domains, and the hydrolysis of ATP causes a major change in the shape of P-gp, which results in release of the drug into the extracellular space. (19) Hydrolysis of a second molecule of ATP is needed to restore the transporter to its original state so that it can repeat the cycle of drug binding and release. (20) Even though the detailed mechanism of action of other ABC transporters is not known, it is presumed that the ATP binding cassette acts as the engine for the transport mediated by members of this large family of transporters. Other well-known examples of efflux transporters include multi drug resistance-associated proteins (MDRs) and the breast cancer resistance protein (BCRP).

These problems of resistance, which arise mainly from the increased efflux of cancer drugs, has led researches to develop MDR inhibitors, which are able to increase the intracellular drug levels in co-application with MDR substrates by the effect of efflux pump inhibition. Verapamil, Ca^{2+} channel blocker, is the most investigated and often used as reference compound, but unfortunately in combination with actual anticancer drugs cardio toxicity has been observed. (21) In the last year intensive efforts have been taken to develop small-molecule inhibitors with a favourable resorption in contrast to complex natural compounds with high and critical molecular weights which are unfavorable also because the synthetic access to such structures is difficult and expensive. (22) (23)

5.2 Development of more effective MDR inhibitors

A rational approach of drug design was used to develop more effective MDR inhibitors on the basis of thieno[2,3-b]pyridines, which was in last decade characterized by the broad spectrum of biological activity. A structure of thieno[2,3-b]pyridines possesses cytotoxic activity, anti-inflammatory, antiviral or antibacterial activity. (24) (25) (26) Some of them are useful as hypolipoproteinemic and anti-atherosclerotic agents (27), and are accordingly of benefit as pharmaceutical agents in the treatment of autoimmune, anti-inflammatory, cardiovascular, proliferative and nociceptive conditions. The research (28) from Latvian Institute of Organic Chemistry, has shown that thieno[2,3-b]pyridine scaffold might be suitable for the linked pharmacophore approach. A model was created assuming one part of Verapamil as linker and methoxyphenyl groups as essential for pharmacophore. (Figure 6.) "As Ca^{2+} channel blocker verapamil in combination with anticancer drugs reveals cardiotoxicity (21), effect of obtained thieno[2,3-b]pyridines on cardiovascular system as well as their cytotoxicity were tested. Compounds obtaining insignificant calcium antagonist properties as side effect and LD50 >2000mg/kg were tested as potential MDR modulators." (28)

The conclusion of the research group from Latvian Institute of Organic Chemistry was that a new class of MDR inhibitors, possessing a 3-amino-thieno[2,3-b]pyridine scaffold has been discovered. Pharmacophore model was created assuming thieno[2,3-b]pyridine scaffold as linker and methoxyphenyl groups as essential for potential MDR-reversal drug. Decoration of 3-amino-thieno[2,3-b]pyridine scaffold with hydrophobic aryl groups in position 2 and 4, ester group in position 5 (reaching optimum lipophilicity), amino group in position 3 (hydrogen bond donor) and methoxyphenyl groups (bearing appropriate amount of hydrogen bond acceptors) has led to potent P-glycoprotein, multi drug resistance-associated protein and the breast cancer resistance protein inhibitors which significantly exceeded activity of Verapamil. (28)
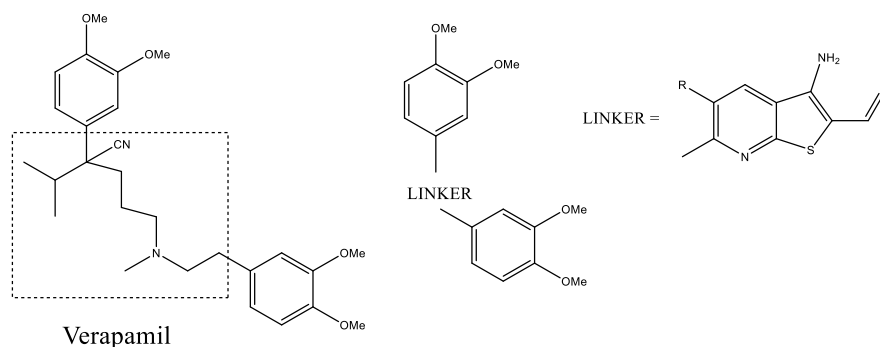

Figure 6. *Pharmacophore approach with modified linker.* (28)

Despite of this results, this analog of verapamil may not be easily transported into the cells. For that, my original plan was to synthesised new prodrugs of verapamil analog to improve the uptake via cancer-cell selectively transporter, L-type amino acid transporter 1 (LAT1). (29)

6. <u>IKKB as a potential target of anti-cancer drugs</u>

From the view of hypothetical targets of cancer cells, the research also focused on the nuclear factor- Kβ (NF-Kβ) proteins, which are a small group of heterodimeric transcription factors that play an important role in regulating the inflammatory, immune and apoptotic responses, and also the aberrant NF-Kβ signalling activity has been associated with the development of cancer, chronic inflammatory diseases and auto-immune diseases. (30) (31)

NF-Kβ is omnipresent in the cytoplasm and its activity is normally suppressed by association with inhibitor of IKKβ. (32) The intracellular NF-Kβ signalling cascade is initiated by a variety of inducers including proinflammatory cytokines TNF-α, IL-1 or endotoxins. (33) The abnormal activity to the NF-Kβ signalling pathway has been implicated in the development of a number of human diseases including cancer, auto-immune and chronic inflammatory conditions. (34) The IKKβ kinase is a complex composed of IKKα, IKKβ and NEMO (regulatory unit NF-Kβ) subunits, when the IKKβ has been shown to play the dominant role in modulating NF-Kβ activity. (35) Phosphorylated IKKβ is afterwards tagged by the E1 ubiquitin enzyme and degraded by the proteasome to liberate active to detach active NF-Kβ. Free NF-Kβ then translocates into the nucleus, where it binds to its cognate DNA site and enhances the expression of a number of genes related to the immune response, cell proliferation and survival. (36) Therefore, IKKβ represents an attractive target in the NF-kB pathway for the development of anticancer or anti-inflammatory therapeutics. (Figure 7.)

These findings led me to develop and synthesize new IKKβ inhibitor which may offer new therapeutic options for the treatment of cancer and inflammatory diseases. The aim was also to prepare an LAT1-transporter-targeted prodrug from this IKKβ inhibitor to improve its selective cellular uptake into the cancer cells.
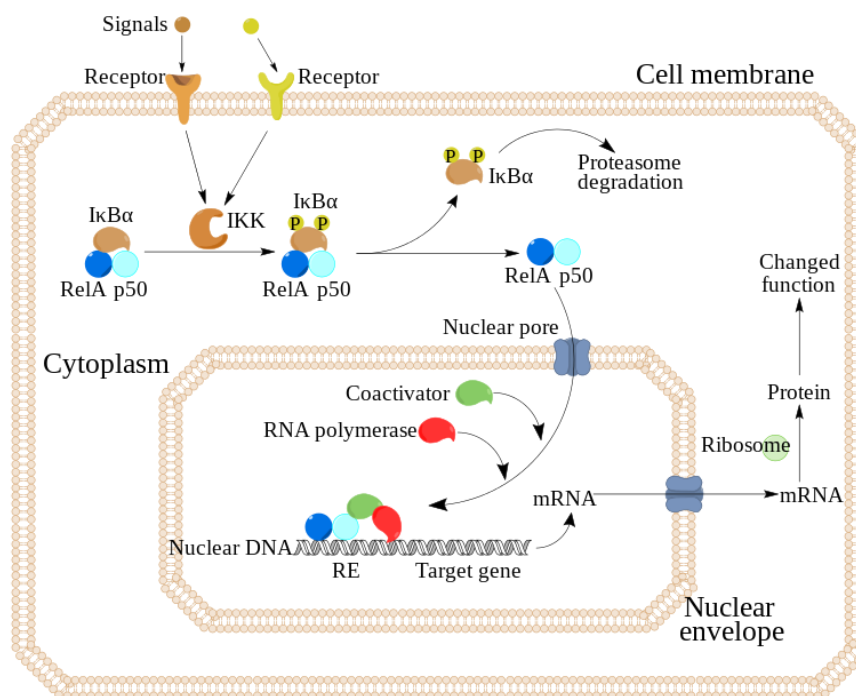
Figure 7. (37) *Mechanism of NF-Kßaction: In this figure, as an example is used the NF-Kß heterodimer between Rel and p50 proteins. Normally, in inactivated state NF-Kß is located in the cytosol complexed with the inhibitory protein IKβα. A variety of extracellular signals can activate the enzyme IKß kinase (IKKß) through the midpoint of integral membrane receptors. IKKß in sequence, phosphorylates the IKβα protein, which results in ubiquitination, disconnection of IKβα from NF-Kß, and eventual degradation of IKβα by the proteasome. The activated NF-Kßis then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-Kß complex then converts other proteins, such as coactivators and RNA polymerase, which, in turn, is translated into protein, which results in a change of cell function.* (35)

7. Gene therapy for malignant gliomas

The third part of my thesis is focused on the gene therapy for malignant gliomas, the most common primary brain tumors. Currently, the treatment consists of surgery followed by radiotherapy and chemotherapy. However, malignant gliomas almost invariably recurs, and after recurrence, the median survival is only 2-3 months. In my research I focused on ganciclovir, as a novel investigational cancer drug that requires a viral thymidine kinase to be activated (phosphorylated), and this enzyme can be deliver into the cancer cell by viral plasmid (GDEPT = gene-directed-enzyme prodrug therapy).

7.1 Ganciclovir:

Ganciclovir is an essential part of the Herpes simplex virus thymidine kinase (HSV-tk) gene therapy of malignant gliomas. (38) Gliomas are the most common primary brain tumors and most of them are malignant. Glioblastoma multiform is considered the most common and aggressive form of all brain tumors. (39) In patients with this disease, the life expectancy is only one year after diagnosis. (40) The treatment consists of a surgery and chemotherapy, but in spite of this therapy, the malignant gliomas almost invariably recur, and after that the median survival is only 2-3 months. That is why there is necessary to devise more effective therapies.

The herpes simplex thymidine kinase (HSV-tk) gene therapy with GCV represents a promising new therapeutic strategy for the treatment of recurrent malignant gliomas. (41) After surgical removal of the tumor, the adenovirus-mediated HSV-tk gene is targeted to the remaining dividing cells on the walls of the tumor cavity. (42) Tumor cells transduced with the HSV-tk gene convert GCV to a GCV monophosphate (GCV-MP), which is further phosphorylated into the cytotoxic GCV triphosphate (GCV-TP) by cellular kinases. (43) (Figure 8.)
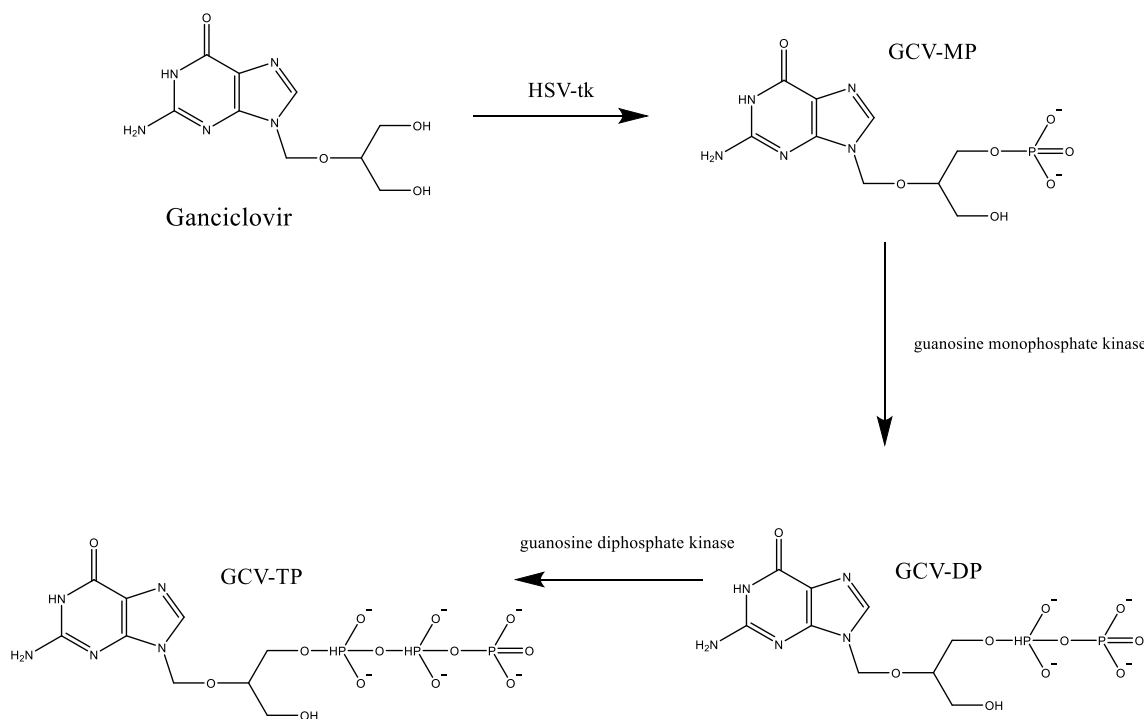

Figure 8. *Brain Pharmacokinetics of Ganciclovir* (38)

The cytotoxic GCV-TP inhibits the activity of DNA polymerase and thus prevents DNA replications, ultimately resulting in cell death. "Despite promising results in preclinical trials, at best only a modest increase in median survival has been achieved with HSV-tk/GCV gene therapy in the clinical trials conducted in patients with recurrent gliomas." (42) It has been speculated that the limited transduction efficiency of tumor cells with the HSV-tk gene and tumor heterogeneity, which challenge vector targeting and delivery, may impair the efficacy of the gene therapy concept. (44) "However, adequate and stable GCV levels are needed to achieve cytotoxic GCV-TP levels inside the tumor cells. Thus, another less well recognized reason for the limited efficacy of HSV-tk/GCV gene therapy may be the poor delivery of GCV into the tumor cells." (38)

Factors as hypoxia, intratumoral pressure gradients, and the abnormal vasculature can limit drug uptake from the systemic circulation into the extracellular spaces surrounding tumor cells. (45) These factors limit the tumor uptake of lipophilic cancer drugs, for which tumor uptake is governed by tumor perfusion rather than their permeability across cell membranes. Therefore, lipophilic cancer drugs may reach higher concentration in healthy brain than in the tumor. "In the case of polar molecules it is likely that tumor or brain uptakes are predominantly limited by poor permeability across the blood-tumor barrier or blood-brain barrier (BBB). Thus, the angiogenic and fenestrated tumor vasculature present in high-grade gliomas may result in higher drug concentrations. In the tumor than those found in healthy brain tissue. Because the GCV is a highly polar molecule, it belongs to the category of a drug with poor permeability across biologicals membranes, and the concentration of GCV in tumor likely exceeds the concentration in healthy brain. Even if GCV was able to reach the tumor extracellular fluid (ECF) across the BBB, its high polarity may limit drug uptake into the tumor cells and its next transformation into GCV-TP." (38) Therefore, small increase in its lipophilicity via a prodrug structure may help to overcome this problem.

The goal of my study was to synthesize novel substituted prodrugs of GCV, which would be more lipophilic and thus, reach higher concertation in the tumor cells where they would convert to GCV firstly by cellular esterases and secondly to GCV-MP by the HSV-tk delivered into the cancer cell in a form of a gene. Then GCV-MP would be further phosphorylated into the cytotoxic GCV-TP by cellular kinase.

8. Experimental part

8.1 List of chemical reagents

| Reagent	Manufacturer	CAS number
3,4,5-Trimethoxybenzaldehyde	Fluka	86-81-7
2-Cyanothioacetamide	Sigma Aldrich	7357-70-2
2'-Bromo-4'-methoxyacetophenon	Acros Organics	2632-13-5
4-Dimethyl(amino)pyridine (DMAP)	Fluka	1122-58-3
3-(2-furyl)-3-phenylpropan-1-amine	Sigma Aldrich	374910-04-0
Benzoylchloride	Sigma Aldrich	98-88-4
Di-tert-butyl-dicarbonate	Fluka	24424-99-5
Dioxane	Sigma Aldrich	123-91-1
Ethylacetoacetate	Acros Organics	141-97-9
Ganciclovir	Matrix Scientific	82410-32-0
L-Isoleucine	Sigma Aldrich	73-32-5
L-Lysine	Sigma Aldrich	56-871
N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)	Sigma Aldrich	25952-53-8
Piperidine	Sigma Aldrich	110-89-4
Pyridine	Riedel-de-Haën	110-86-1
Phtalic anhydride	Merck Schuchdardt	85-44-9
Sodium methoxide	Sigma Aldrich	124-41-4
Triethylamine	Riedel-de-Haën	121-44-8

8.2 Synthesis of verapamil analog
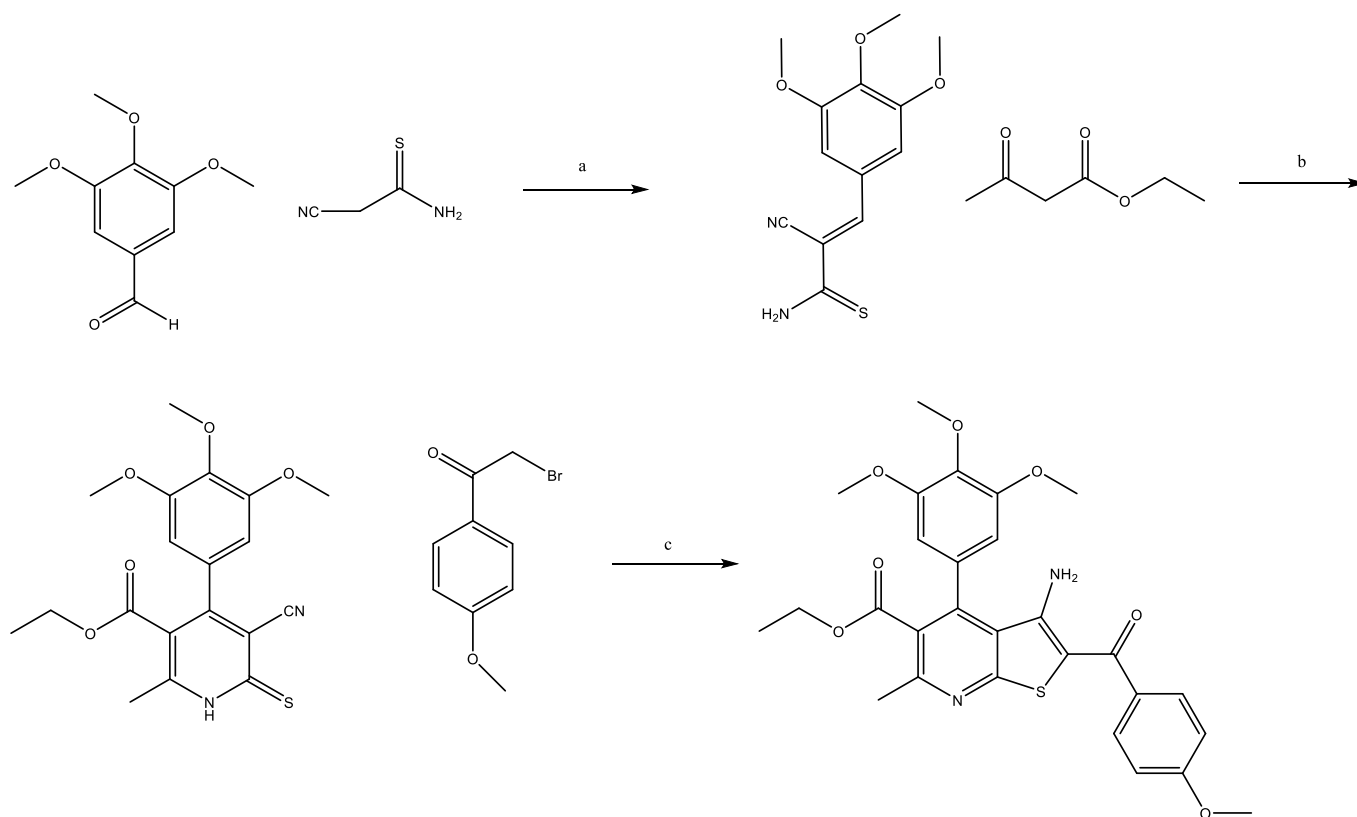
Scheme 1. Preparation of verapamil analog (KH-VK-03)

Reaction conditions: (a) TEA, EtOH; (b) piperidine, EtOH; (c) 3M NaOH (aq.), EtOH

8.2.1 Step 1 – Preparation of 2-cyano-3-(3,4,5-trimethoxyphenyl)prop-2-en-ethioamide (KH-VK-01)
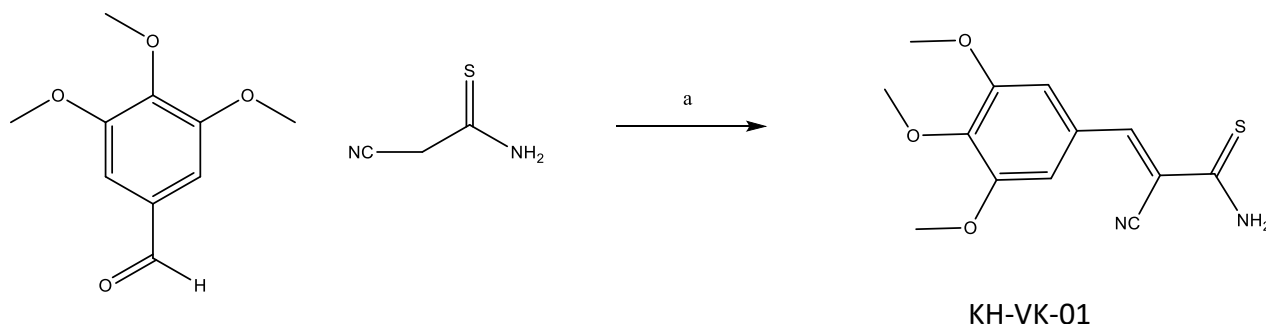
Scheme 1a. Synthesis of *KH-VK-01*

Reaction condition: (a) TEA, EtOH; reflux overnight

Compound KH-VK-01 was synthesized starting from *3,4,5*-trimethoxybenzaldehyde (0.50g; 2.55mmol) **(1)**, *2*-cyanothioacetamide(0.26g; 2.55mmol)**(2)** and triethylamine (0.13ml; 0.89 mmol)**(3)**. **(1)**, **(2)** and **(3)** were dissolved in EtOH and the reaction mixture was refluxed overnight. Then the reaction mixture was cooled on the ice-bath and let precipitated. The solution was filtrated, washed with cold EtOH and water. Afterwards the crystals was dried under high-vacuum to give a yellow solid of 0.12g (17.35%) of compound KH-VK-01.

8.2.2 Step 2 – Preparation of ethyl-5-cyano-2-methyl-6-thioxo-4-(3,4,5-trimethoxyphenyl) - 1,6-dihydropyridine-3-cyrboxylate (KH-VK-02)
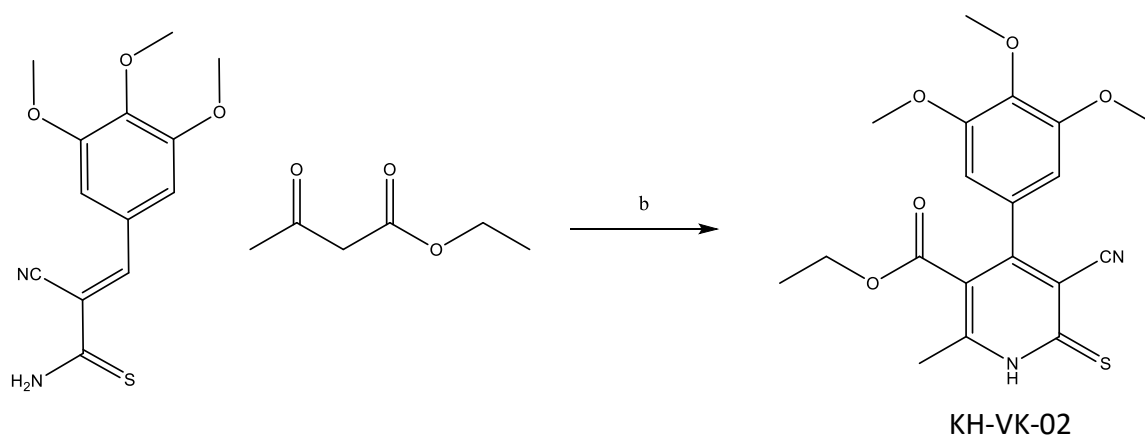
Scheme 1b. Synthesis of *KH-VK-02*

Reaction condition: (b) piperidine, EtOH; reflux overnight

Compound KH-VK-02 was synthesized from KH-VK-01 (0.123g; 0.44mmol)**(1)**, ethyl acetoacetate (0.06ml; 0.44 mmol)**(2)** and piperidine (0.02ml; 0.22mmol) **(3)**. (**1**),(**2**) and (**3**) were dissolved in EtOH and the reaction mixture was refluxed overnight. The solvent was evaporated using a rotatory evaporator and vacuum pump and the residue was purified with column chromatography (EA/PE = 1:2 to 2:1) to give a yellow liquid of 0.10g (58.8%) compound KH-VK-02.

8.2.3 Step 3 – Preparation of ethyl-3-amino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)thieno[2,3-b]pyridine-5-carboxylate (KH-VK-03)
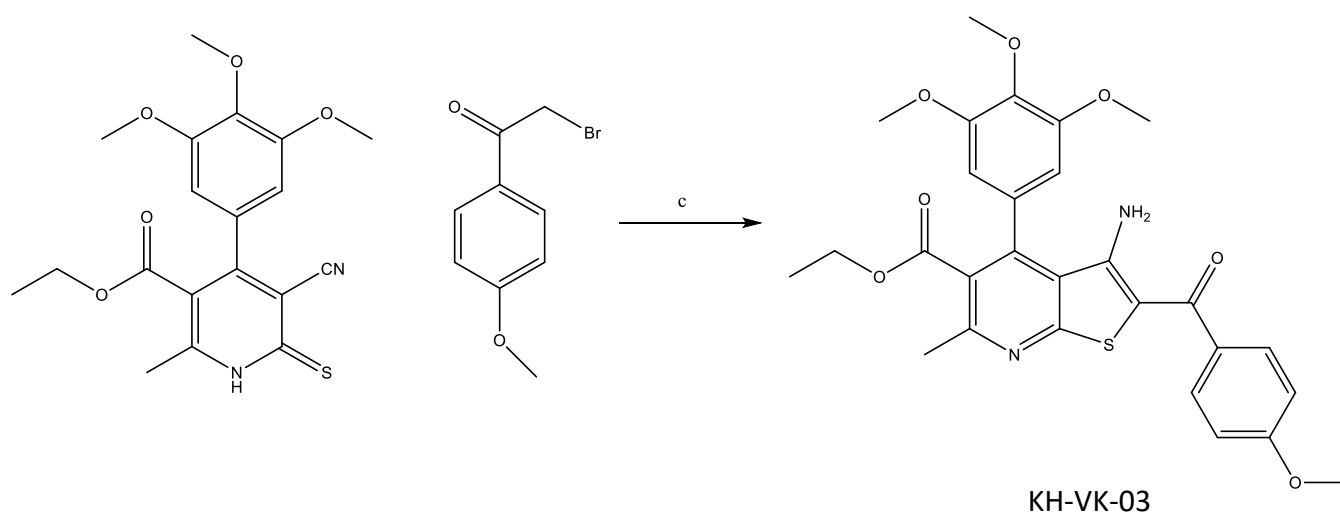
KH-VK-03

**Scheme 1c.** Synthesis of *KH-VK-03*

Reaction condition: (c) 3M NaOH, EtOH; reflux for 5-10min.; RT for 30 min.

Compound KH-VK-03 was synthesized from KH-VK-02 (0.100g; 0.26mmol) **(1)**, 2-bromo-4'-methoxyacetophenon (0.06g; 0.26mmol)**(2)** and 3M NaOH (1ml/1mmol of (**1**))**(3)**. (**1**) was dissolved in EtOH and then added 3M NaOH. The reaction mixture was refluxed for 5-10min. (2) was added to reaction mixture and refluxed additional 5 min. and then stirred at room temperature for 30min. The reaction mixture was cooled on the ice-bath and the compound let precipitate. The solution was filtrated and washed with cold EtOH and water. The crystals were dried under high-vacuum to give yellow solid of 0.04g (29.7%) of title compound KH-VK-03.

^{1}H NMR (DMSO): δ 7.87 (d,2H); 6.98 (d, 2H); 6.62 (s, 2H); -4.09 (q, 2H); 3.94 (s, 3H); 3.89 (s, 3H); 3.87 (s, 6H); 2.70 (s, 3H); 1.02 (t, 3H).

Originally my plan was to attach to promoiety to the free amine group and thus create a LAT1-transporter-targeted prodrug from this analog, but as I did not succeed to synthesize enough starting material for prodrug synthesis I could not do it. However, synthesis to prepare the desired prodrug will be continued at the University of Eastern Finland.

8.3 Synthesis of inhibitor IKKβ
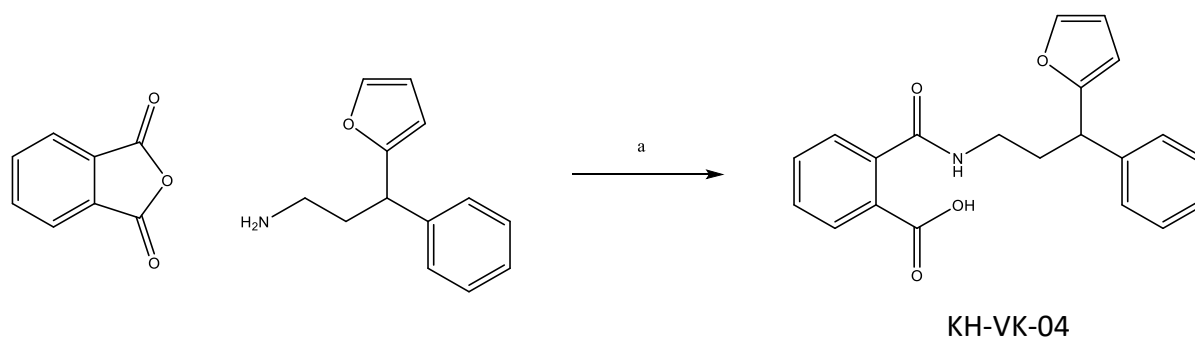
Scheme 2. Preparation of inhibitor of IKKβ (KH-VK-04)

Reaction conditions: *(a) Acetonitrile*

Compound KH-VK-04 was synthesized starting from phtalic anhydride (0.55 g, 3.73 mmol) (**1**) and 3-(2-furyl)-3-phenylpropan-1-amine (0.50 g, 2.48 mmol)(**2**). **1** and **2** were dissolved in acetonitrile (25 ml) and the mixture was stirred for 4 hours. Then additional **2** (0.55 g, 2.73 mmol) was added and the reaction mixture was stirred overnight. The solvent was evaporated using a rotatory evaporator and vacuum pump and the residue was purified with column chromatography (DCM/MeOH = 99:1 to 94:6) to give a yellow solid compound of 0.42 g (32.3 %) of the title compound KH-VK-04.

^{1}H NMR (DMSO): δ 7.77-7.75 (m, 1H); 7.58-7.55 (m, 1H); 7.52-7.48 (m, 2H); 7.39-7.37 (m, 1H); 7.32-7.30 (m, 2H); 7.27-7.25 (m, 2H); 7.23-7.21 (m, 1H); 6.39-6.38 (m, 1H); 6.26 (d, 1H); 4.14 (t, 1H); 3.17-3.06 (m, 2H); 2.30-2.24 (m, 1H); 2.09-2.03 (m, 1H).

As the next step I also tried to couple the LAT1-transporter-targeted promoiety to the free acid group of this molecule to prepare a prodrug, but I did not succeed with it, may be since it is in quite shielded position. However, synthesis to prepare the desired prodrug will be continued at the University of Eastern Finland.

8.4 Synthesis of prodrug of GCV
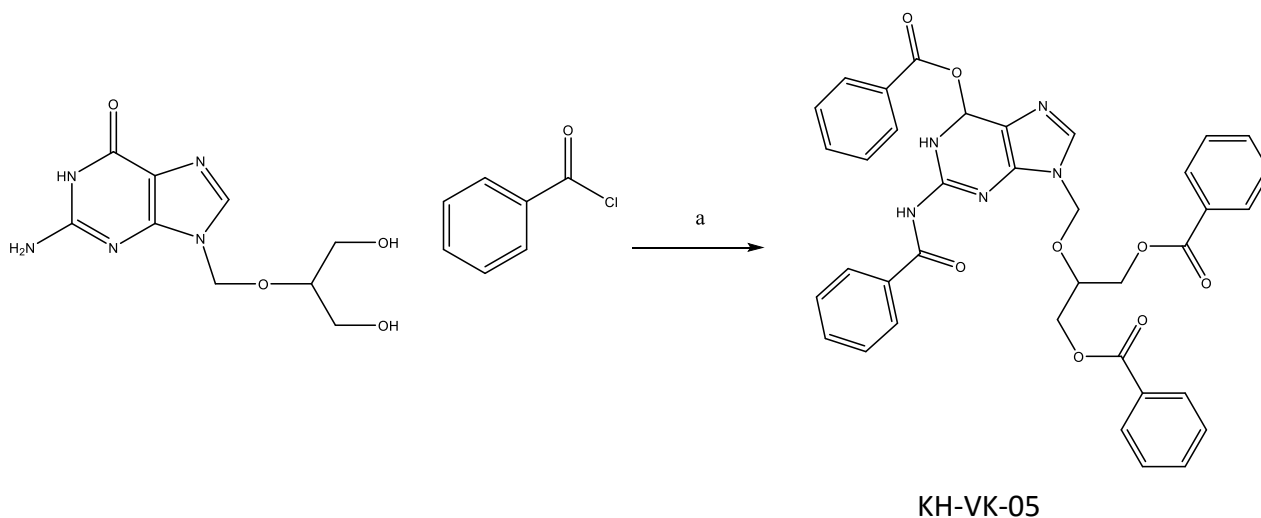
8.4.1   Synthesis of benzamidoylganciclovir (KH-VK-06)
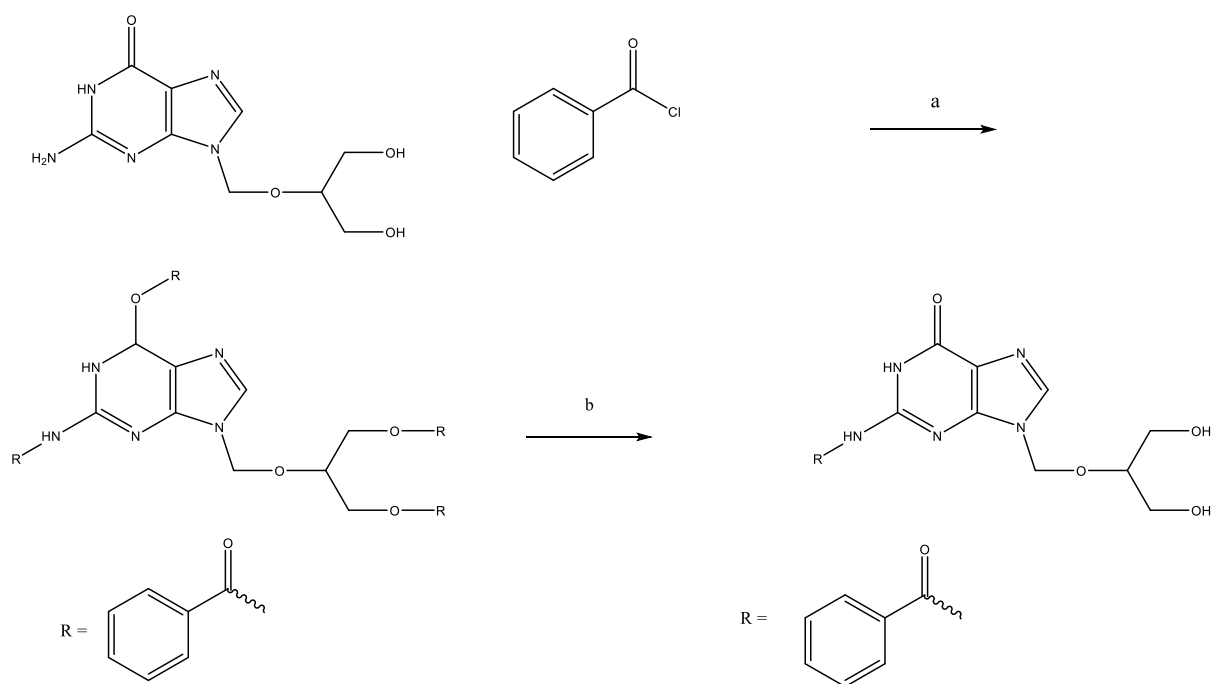
Scheme 3. Preparation prodrug of GCV – benzamidoylganciclovir (KH-VK-06)

Reaction conditions: (a) pyridine (anhydrous), Ar-atmosphere, RT overnight; (b) NaOMe, MeOH (anhydrous)

8.4.1.1 Step 1 – Preparation of 2-((2-benzamido-6-(benzoyloxy)-1,6-dihydro-9H-purin-9-yl) methoxy) propane-1,3-diyl dibenzoate (KH-VK-05)

KH-VK-05

Scheme 3a. Synthesis of *KH-VK-05*

Reaction condition: (a) pyridine (anhydrous), Ar-atmosphere, RT overnight; (b) NaOMe, MeOH (anhydrous)

Compound KH-VK-04 was synthesized starting from ganciclovir (0.100 g, 0.39 mmol) **(1)** and benzoylchloride (0.270 ml, 2.35 mmol) **(2)**. Compound **1** and **2** were dissolved in pyridine (anhydrous) (10 ml) and the mixture was stirred in Ar-atmosphere at room temperature overnight. Solvent was evaporated using a rotary evaporator and vacuum pump. The residue was redissolved in DCM (50 ml) and washed with cold 3M HCl (2x50 ml). The separated organic layers were washed first with saturated aqueous NaHCO$_3$ (50 ml) and then with brine (50 ml). The separated organic phase was dried over Na$_2$SO$_4$ and evaporated to dryness under reduced pressure to give an orange-yellow solid of 0.43 g (KH-VK-05). This material was carried to the next step without further purification.

8.4.1.2 Step 2 – Preparation of Benzamidoylganciclovir (KH-VK-06)
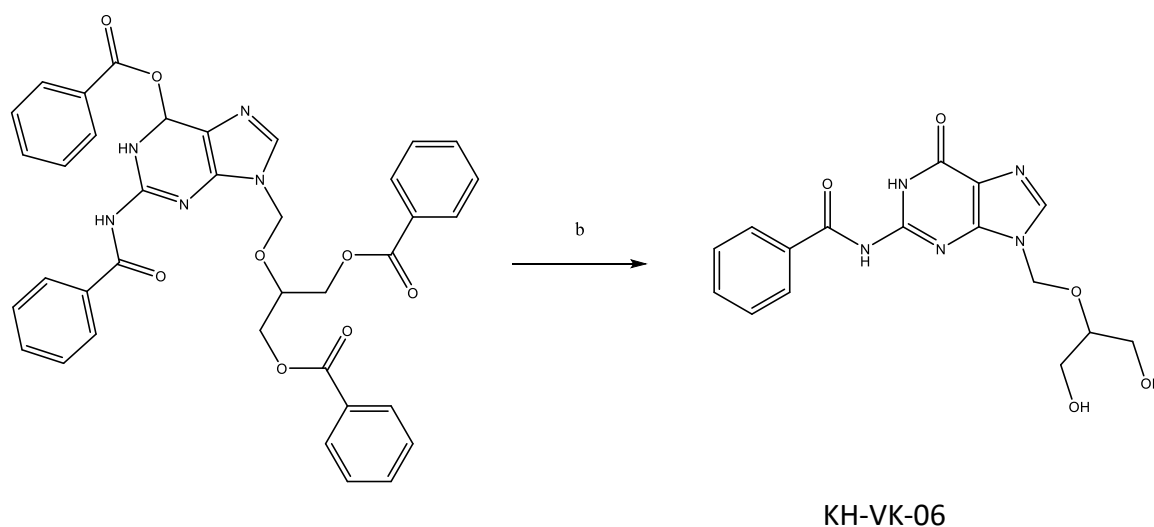
Scheme 3b. Synthesis of *KH-VK-06*

Reaction condition: (b) NaOMe, MeOH (anhydrous); RT overnight

Compound KH-VK-05 (0.430 g, 0.39 mmol)(**1**) and sodium methoxide (0.13 g, 2.34 mmol)(**2**) were dissolved in anhydrous MeOH (20 ml) and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was redissolved in anhydrous MeOH (20 ml) and stirred with sodium methoxide (0.13 g, 2.34 mmol) at room temperature for 4 hours. The solvent was evaporated using a rotary evaporator and vacuum pump and the residue was purified with column chromatography (DCM/MeOH = 95:5 to 3:7) to give a white solid of 0.14 g (100%) of title compound (KH-VK-06).

^{1}H NMR (DMSO): δ 8.50 (s, 2H); 7.69 (s, 1H); 7.29 (s, 1H); 7.14 (s, 2H); 5.42 (s, 2H); 3.56 – 3.50 (m, 1H);   3.45 – 3.24 (m, 4H);

The final compound will be used for further physicochemical and biological studies at the University of Eastern Finland.

8.4.2   Synthesis of – L-isoleucinamidoylgancoclovir (KH-VK-09)
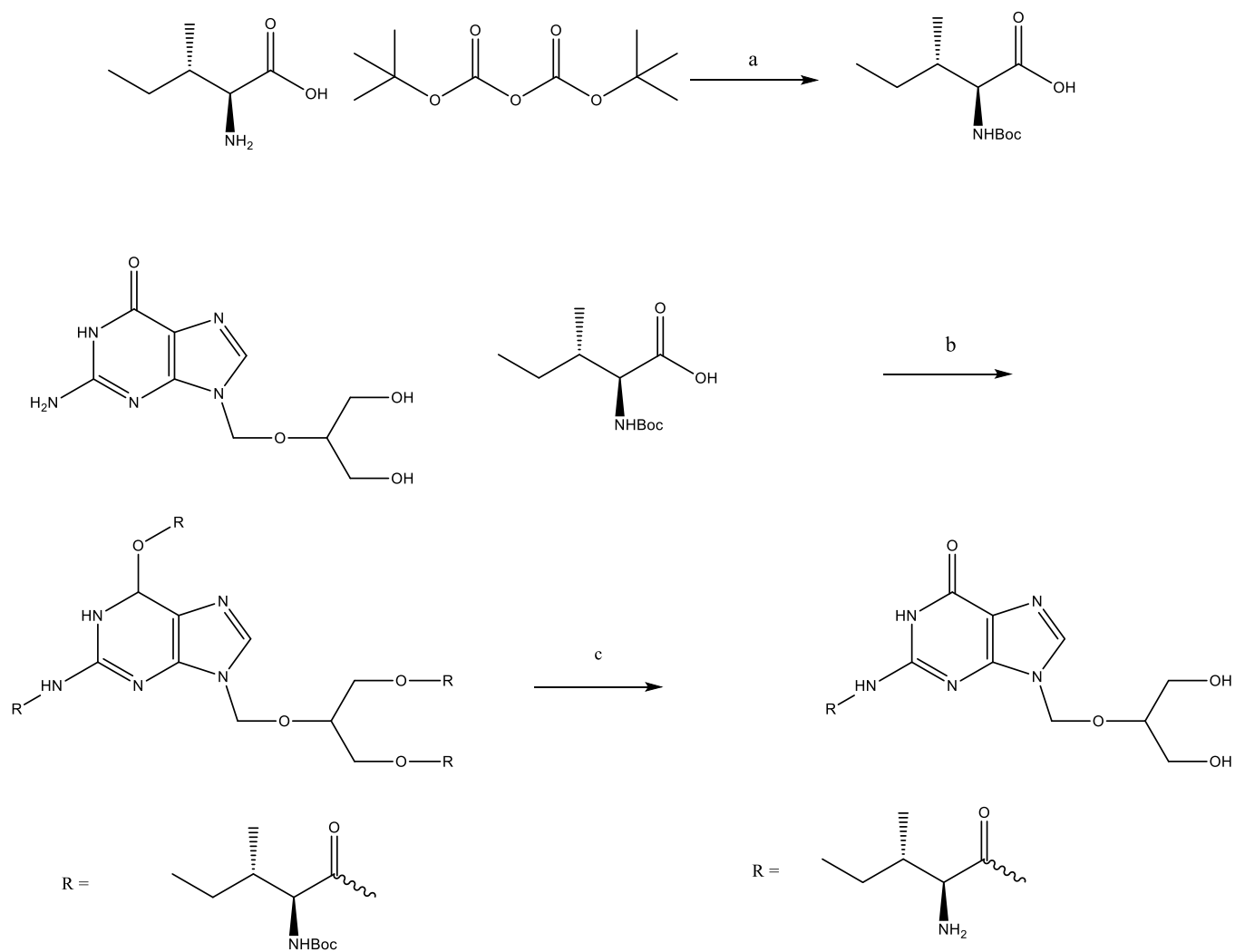
Scheme 4. Preparation prodrug of GCV – L-isoleucinamidoylgancoclovir (KH-VK-09)
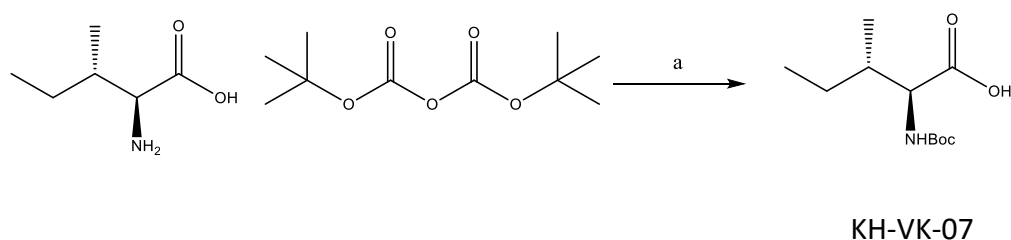
Reaction conditions: *(a) 1M NaOH, Dioxane, 70°C overnight; (b) EDC, DMAP, DMF (anhydrous), 50°C overnight; (c) NaOMe, MeOH (anhydrous)*

8.4.2.1 Step 1– Preparation of Boc-L-Isoleucine (KH-VK-07)

Scheme 4a. Synthesis of *KH-VK-07*

Reaction condition: (a) 1M NaOH, Dioxane, 70°C overnight

Compound KH-VK-07 was synthesized starting from L-isoleucine (1.0 g, 7.62 mmol)(**1**), 1M NaOH (5 ml)(**2**)and di-tert-butyl-dicarbonate(2.0 g, 9.15 mmol)(**3**). Compounds **1**, **2** and **3** were dissolved in dioxane (5 ml) and stirred at 70°C overnight. The reaction mixture was cooled on the ice-bath and acidified with 1M HCl to pH 2-3. Therefore the reaction mixture was stirred vigorously with ethyl acetate (3 x 50 ml) to extract compound. The separated organic phase was washed with water (100 ml), dried over Na_2SO_4 and evaporated to dryness under reduced pressure to give a colourless oil of 1.88 g (KH-VK-07). This material was carried to the next step without further purification.

8.4.2.2   Step 2– Preparation of 2-((6-(((*tert*-butoxycarbonyl)-L-isoleucinyl)oxy)-2-((2S,3S)-2-((tert-butoxycarbonyl)amino)-3-methylpentaamido-1,6-dihydro-9H-purin-9yl)methoxy)propane-1,3-diyl-(2S,2'S,3S,3's)-bis(2-((tert-butoxycarnobyl)amino)3-methylpentanoat (KH-VK-08)
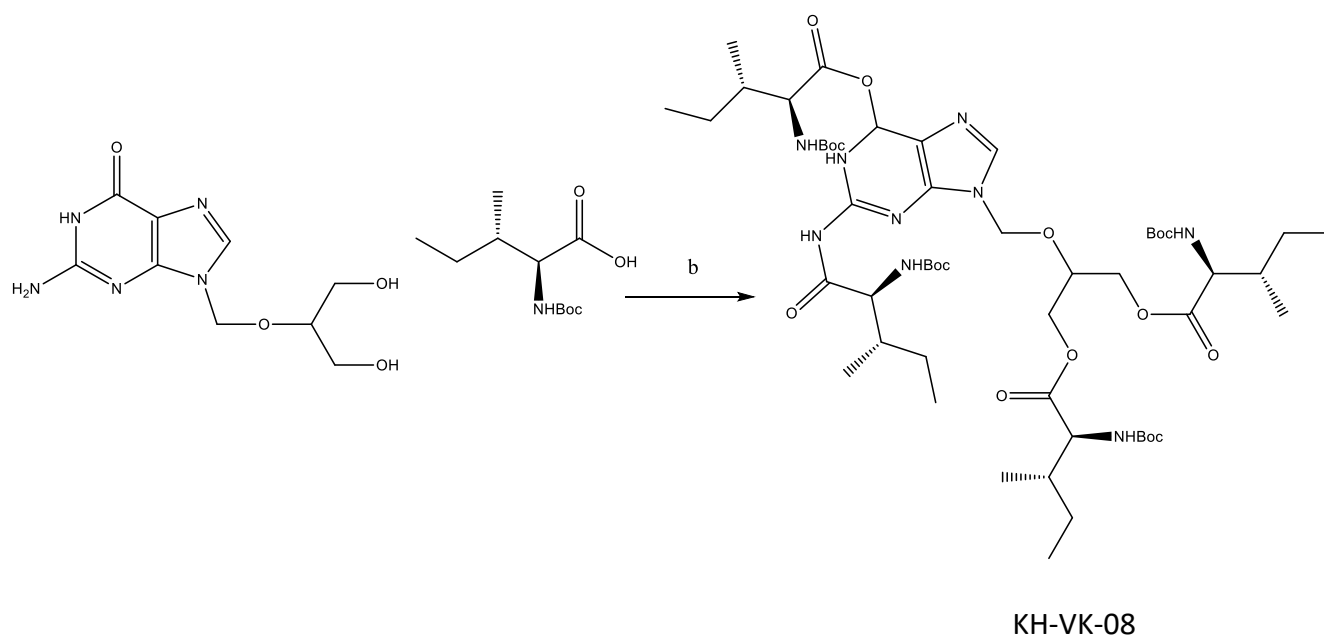
KH-VK-08

Scheme 4b. Synthesis of *KH-VK-08*

Reaction condition: (b) EDC, DMAP, DMF (anhydrous), 50°C overnight; (c) NaOMe, MeOH (anhydrous)

Compound KH-VK-06 (0.54 g, 2.35 mmol)(**1**), ganciclovir (0.10g, 0.39mmol) (**2**), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.45g, 2.35mmol) (**3**) and 4-(dimethylamino)pyridine (0.28 g, 2.35 mmol)(**4**) were dissolved in anhydrous DMF under Ar-atmosphere and stirred at 50°C overnight. The solvent was evaporated using a rotary evaporator and vacuum pump and the residue was purified with column chromatography (DCM/MeOH = 98:2 to 9:1) to give an colourless oil of 0.49 g (111.8 %) (KH-VK-08) with traces of solvents present.

8.4.2.3 Step 3– Preparation of (2S,3S)-2-amino-N-(9-(((1,3-dihydroxypropan-2-yl)oxy)methyl)-6-oxo-6,9-dihydro-*1H-purin-2-yl)-3-methylpentanamid (KH-VK-09)*
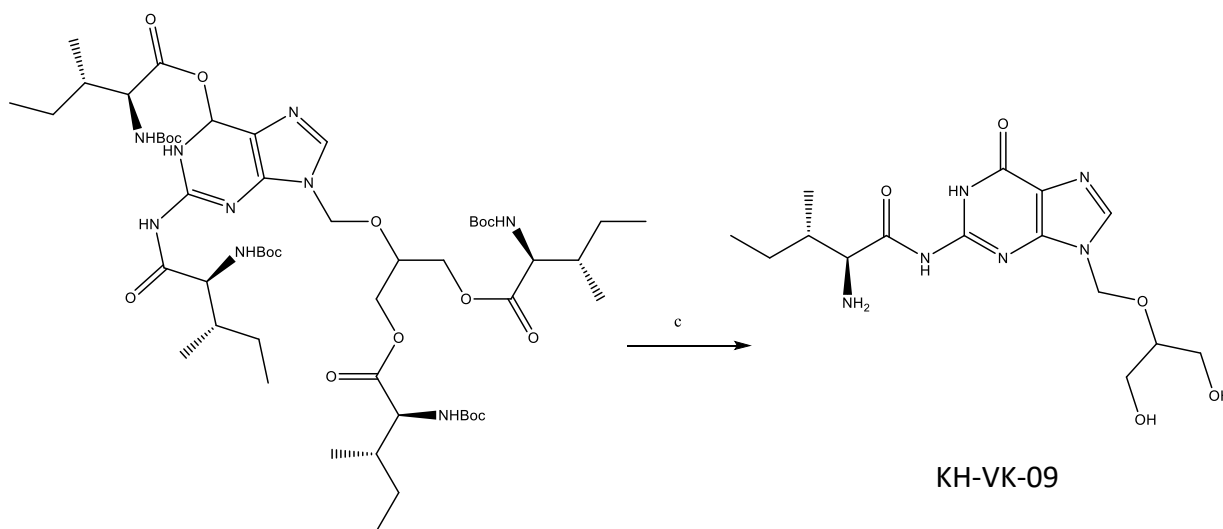
Scheme 4c. Synthesis of *KH-VK-09*

Reaction condition: (c) NaOMe, MeOH (anhydrous), RT overnight

Compound KH-VK-08 (0.49 g, 0.44 mmol)(**1**) and sodium methoxide (0.14 g, 2.63 mmol)(**2**) were dissolved in anhydrous MeOH (20 ml) and the mixture was stirred at room temperature overnight. The solvent was evaporated and residue was redissolved in anhydrous MeOH and stirred with sodium methoxide (0.14 g, 2.63 mmol) at room temperature for 4 hours. The solvent was evaporated using a rotary evaporator and vacuum pump and the residue was purified with column chromatography (DCM/MeOH = 9:1 to pure MeOH) to give an white solid of 0.08 g (46.6 %) (KH-VK-09) title compound.

^{1}H NMR (DMSO): δ 7,69 (s, 1H); 5,42 (s, 2H); 3.57-3.53 (m, 1H); 3.52-3.17 (m, 2H); 3.01-2.94 (m, 4H); 2.18-2.15 (m, 3H); 1.47 (quint, 2H); 0.97 (t, 3H)

The final compound will be used for further physicochemical and biological studies at the University of Eastern Finland.

Scheme 5. Preparation of prodrug of GCV – L-lysinamidoylganciclovir (KH-VK-11)

Reaction conditions: *(a) 2M NaOH, dioxane, 70°C overnight; (b) EDC, DMAP, MeOH (anhydrous), 70°C overnight; (c) NaOMe, MeOH (anhydrous)*

8.4.3.1 Step 1– Preparation of Boc-L-Lysine (KH-VK-10)
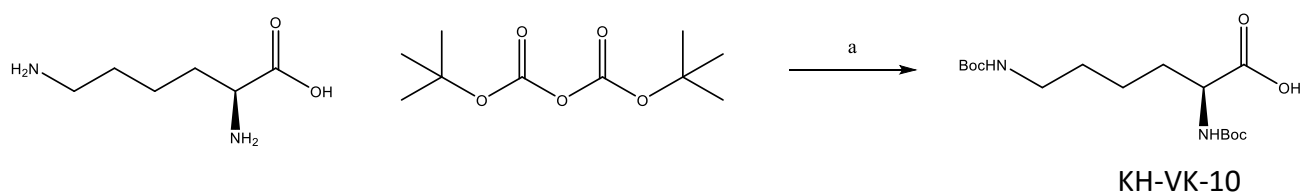
Scheme 5a. Synthesis of Boc-L-Lysine (*KH-VK-10)*

Reaction condition: 2M NaOH, dioxane, 70°C overnight

Compound KH-VK-10 was synthesized starting from L-lysine (1.0 g, 6.84 mmol)(**1**), 2M NaOH (5 ml)(**2**), di-*tert*-butyl-dicarbonate (3.58 g, 16.42 mmol)(**3**). Compounds **1**, **2** and **3** were dissolved in dioxane (5 ml) and stirred at 70°C overnight. The reaction mixture was cooled on the ice-bath and acidified with 1M HCl to pH 2-3. After that the reaction mixture was stirred vigorously with ethyl acetate (3 x 50 ml) to extract compound. The separated organic phase was washed with water (100 ml), dried over Na_2SO_4 and evaporated to dryness under reduced pressure to give a colourless sticky oil of 2.10 g (88.8%) (KH-VK-10).

8.4.3.2 Step 2– Preparation of di-tert-butyl ((5,S)-7-(2-((S)-2,6-bis((tert-butoxycarbonyl)
amino) hexanamido)9-(((1,3-dihydroxypropan-2-yl)oxy)methyl)-6,9-dihydro-1H-purin-6-
yl)-6-oxoheptane-1,5-diyl)dicarbamate (KH-VK-11)
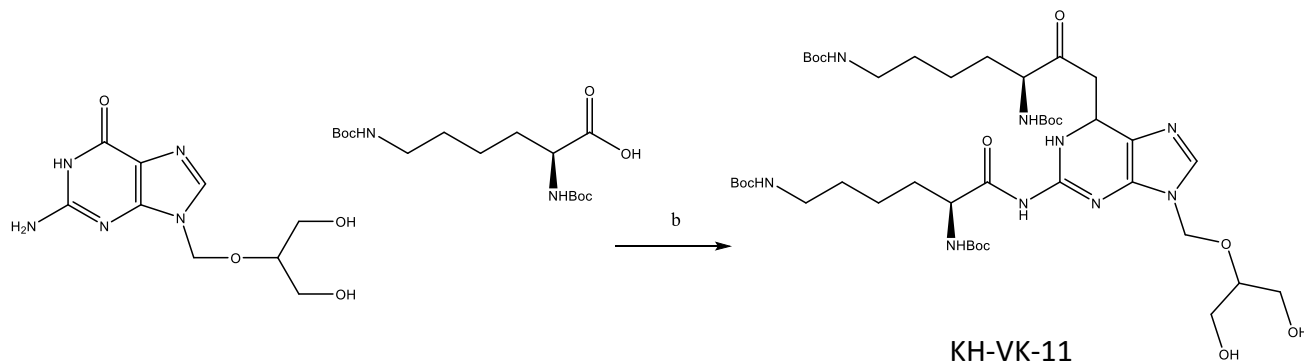
Scheme 5b. Synthesis of *KH-VK-11*

Reaction condition: (b) EDC, DMAP, DMF (anhydrous), Ar- atmosphere, 50°C overnight

Compound KH-VK-10 (0.81 g, 2.35 mmol)(**1**), ganciclovir (0.100 g, 0.39 mmol)(**2**), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (0.450g, 2.35 mmol) (**3**) and 4-(dimethylamino)pyridine (0.28 g, 2.35 mmol)(**4**) were dissolved in anhydrous DMF under Ar-atmosphere and stirred at 50°C overnight. The solvent was evaporated using a rotary evaporator and vacuum pump and the residue was purified with column chromatography (DCM/MeOH = 98:2 to 9:1) to give an white solid of 0.27g (74.86%) (KH-VK-11) of title compound.

^{1}H NMR (DMSO): δ 7.81 (s, 1H); 5.42 (s, 2H); 4.17-3.98 (m, 5H); 3.97-3.79 (m, 2H); 2.92-2.83 (m, 4H); 1.54-1.42 (m, 4H); 1.36 (s, 36H); 1.34-1.18 (m, 8H).

The original plan was to prepare monosubstituated GCV but in this reaction I couldn't manage to do it. Because in second step had cleaved two Boc-L-lysine group from –OH chain. Even though I tried to cleave the second protecting group of Boc-L-lysine from my compound, I weren't able to manage it. So there will be necessary optimize this reaction.

9. Results and discussion

The main goal of this thesis was to prepare new prodrug structures which would improve the drug delivery of anti-cancer drugs. In the first part of the thesis, I continued from the conclusion of Latvian Institute of Organic Chemistry, which interpreted that the decoration of 3-amino-thieno[2,3-b]pyridine scaffold (necessary for potential anti-resistant effect), with hydrophobic aryl groups in position 2 and 4, ester group in position 5 (reaching optimum lipophilicity), amino group in position 3 (hydrogen bond donor) and methoxyphenyl groups (bearing appropriate amount of hydrogen bond acceptors) has led to potent P-glycoprotein, multi drug resistance-associated protein and the breast cancer resistance protein inhibitors which significantly exceeded the activity of Verapamil. The aim was to prepare a transporter-targeted prodrug of verapamil analog, which would be more efficiently and selectively delivered into the cancer cells. This synthesis of verapamil has three steps. Unfortunately we were not able to optimize these reaction steps to get enough high yields and we managed to synthesize only a very small amount of this analog (**KH-VK-03**), which was not enough for the preparation of this desired prodrug.

The second part of my thesis results from the study about nuclear factor-Kβ, which has a connection to the development of cancer and autoimmune diseases. My goal was to synthesize a new potential inhibitor of IKKβ (subunit of NF-Kβ), and subsequently its transporter-targeted prodrug, which could offer new therapeutic options in the cancer therapy. The synthesis of inhibitor IKKβ was a one-step reaction and I managed to optimize this reaction and prepared the title compound (**KH-VK-04**). However, my attempts to synthesize prodrugs from IKKβ inhibitor were not successful due to lack of time, but the synthesis continued at the University of Eastern Finland afterwards.

The third part of this thesis is focused on the GCV as a new potential drug in gene therapy of malignant glioma. The main goal was to synthesize novel prodrugs of GCV which would be slightly more lipophilic and therefore reach higher concentration in tumor cells ready for the activation to GCV-TP by the HSV-tk delivered into the cancer cells via viral vector in a form of gene. The synthesis of this prodrug has two steps and I have optimized this reaction. I managed to synthesize two new prodrugs of GCV (**KH-VK-06, KH-VK-09**). In case of the third prodrug **(KH-VK-11)**, I was not able to prepare monosubstitued derivate of GCV, but

only a disubstitued derivate, because of its high instability and quick break-up back to the starting material - GCV. The optimization of this reaction is needed.

10. <u>Conclusion</u>

During my research, I managed to synthesize novel anti-cancer agents and their prodrugs. The prodrugs were designed to increase the cellular uptake of these anti-cancer drugs and be selectively transported into cancer cells via transporters that are over-expressed in cancer cells or via passive diffusion in the case of GDEPT, in which the prodrug activating enzyme is selectively delivered into cancer cells. Syntheses were quite challenging, however I managed to produce two novel prodrugs and two novel anti-cancer agents, which were highly pure (>95%) and which we could use for our later *in vivo/in vitro* studies.

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