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The effect of lipid signalling pathway interference on sorafenib cytotoxic efficacy and function of efflux transporters in mouse hepatocellular carcinoma cells.

Diploma thesis

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Hradec Králové 2017 Aigul Sagandykova

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ABSTRACT

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Nowadays cancer remains one of the most challenging health issues worldwide. Chemotherapy represents one of the essential approaches in the treatment of malignant diseases. However, multidrug resistance (MDR), a multifactorial phenomenon described as a loss of sensitivity of cancer cells to several diverse chemotherapeutic agents at the same time, often compromises the therapy outcomes. A well-known cause of MDR is an increased expression or/and an enhanced activity of efflux drug transporters of ATP binding cassette (ABC) superfamily, which has been found in many types of cancer.
In the last decade, an expanding body of literature suggested a new hallmark of cancer cells – inflammation. An inflammatory microenvironment potentiates tumorigenesis and upregulation of transporters. Moreover, several observations show that ABC transporters mediate the transport of some signalling lipids. This new insight provided possibilities for novel anti-inflammation approach of cancer treatment. Compounds that target the upregulated release of arachidonic acid and its proinflammatory products leukotrienes and prostaglandins, could represent an alternative treatment. In this study we aimed to find out whether the new "theoretical" strategy of general downregulation of ABC transporters overexpression can be achieved using two experimental compounds, LBG-10119 and JJKK-048, an *N*-methyl-*D*-aspartic acid (NMDA) receptor competitive antagonist and monoacylglycerol lipase (MAGL) inhibitor respectively, each in their own way interfere in lipid signalling pathway. We hypothesised that these compounds by restraining inflammation could increase the intracellular accumulation of efflux probes and elevate the antiproliferative efficacy of sorafenib. In this work, the hypothesis was evaluated using two mouse hepatocellular carcinoma cell lines.

ABSTRAKT

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Nádorová onemocnění představují i v současné době jednu z celosvětově nejvýznamnějších terapeutických výzev. Chemoterapie dosud zůstává základním přístupem v léčbě maligních onemocnění. Její léčebné výsledky nicméně komplikuje mnohočetná léková rezistence (MDR), fenomén popsaný jako ztráta citlivosti nádorových buněk vůči širokému spektru chemoterapeutických léčiv. Dobře známou příčinou MDR je zvýšení exprese a/nebo zvýšení aktivity efluxních lékových transportérů ATP-dependentní transportérové rodiny (tzv. ABC transportérů), jež byly nalezeny v mnoha typech nádorů. V posledním desetiletí stále větší množství vědecké literatury věnuje pozornost novému znaku nádorových buněk – zánětu. Zánětlivé mikroprostředí potencuje tumorigenezi a up regulaci transportérů. Navíc řada pozorování potvrzuje, že ABC transportéry zprostředkovávají přenos signálních lipidů. Tento nový pohled vede k možnosti volby protizánětlivé léčby jako nového přístupu v nádorové léčbě. Léčiva, která cílí zvýšené uvolňování kyseliny arachidonové a jejích prozánětlivých produktů leukotrienů a prostaglandinů, představují alternativní léčbu. Cílem této práce bylo zjistit, zda nový „teoretický“ přístup pomocí downregulace nadměrně exprimovaných ABC transportérů může být dosažen použitím dvou experimentálních látek, LBG-10119, kompetitivního inhibitoru receptoru *N*-methyl-*D*-aspartátové kyseliny (NMDA) a JJKK-048, inhibitoru monoacylglycerol lipázy (MAGL), které interferují s lipidovou signální cestou. Předpokládali jsme, že omezením zánětu by tyto látky mohly zvýšit nitrobuněčnou akumulaci testovaných látek, jež jsou substráty efluxních transportérů a zvýšit též antiproliferativní působení sorafenibu. Tato hypotéza byla v této práci testována s použitím dvou myších hepatocelulárních buněčných linií.

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1. LIST OF ABBREVIATIONS

AA	arachidonic acid
ABC	ATP-binding cassette transporters
ALOX-5	arachidonate 5-lipoxygenase
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein, also known as ABCG2
cGMP	cyclic guanosine monophosphate
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
FFA	free fatty acids
GPCRs	G protein-coupled membrane receptors
GRIN1	glutamate ionotropic receptor NMDA type subunit 1 or NR1
GRIN2A	glutamate ionotropic receptor NMDA type subunit 2A or NR2A
HBSS	Hank's balanced salt solution
HCC	hepatocellular carcinoma
HPLC-MS	high-performance liquid chromatography-mass spectrometry
IC_{50}	half maximal inhibitory concentration
LPC	lysophosphatidylcholine
LTB4	leukotriene B4
LTC4	leukotriene C4
LC-MS/MS	liquid chromatography tandem-mass spectrometry

MAGL	monoacylglycerol lipase
MDR	multidrug resistance
MRP1	multidrug resistance-associated protein 1 or ABCC1
MRP2	multidrug resistance-associated protein 2 or ABCC2, also known as canalicular multispecific organic anion transporter 1 (c-MOAT)
NMDA	*N*-methyl-*D*-aspartate receptor
NSAIDs	nonsteroidal anti-inflammatory drugs
p14ARF	human (alternate reading frame) tumour suppressor gene and cell-cycle inhibitor gene
p19ARF	murine homolog of human *p14ARF* gene
p53	tumour suppressor gene, an oncogene
PAF	platelet-activating factor
PC	phosphatidylcholine
PGE2	prostaglandin E2
P-gp	P-glycoprotein, also known as ABCB1 or multidrug resistance protein 1 (MDR1)
PLA2	phospholipase A2
PXR	pregnane X receptor

2. INTRODUCTION

Cancer remains one of the greatest global health issues throughout the world (WHO, 2017). Even though there is a big progress in developing new approaches to treat tumour, it is still a source of high morbidity and mortality worldwide. Besides surgery and radiation, chemotherapy is the most prevalent treatment option for most of the tumour diseases. Despite the selectiveness of modern chemotherapeutics or specificity of intended target, there are still several barriers between chemotherapeutics and their intended efficacy. One of such barriers is the delivery of chemotherapeutics at an effective concentration to tumour tissues. Multidrug resistance (MDR) is a clinical phenomenon when tumours show or acquire resistance to many diverse chemotherapeutic agents with different chemical structures and mechanisms of action at the same time. It is still the major cause of chemotherapy failure since there is a rise in cancer-related deaths despite modern advances in the science and treatment of cancer (Lee, 2010). The clinical outcome of patients, unfortunately, remains far from expectations. The distinctive induction of ATP binding cassette (ABC) transporters responsible for drug efflux has been associated with MDR in many cancers (Minemura et al., 1999; Doyle et al., 1998). An intensive effort has been devoted to the development of ABC transporter inhibitors that might be used in combination with standard chemotherapeutic agents to reverse MDR and enhance therapeutic efficacy (Szakacs et al., 2006; Nobili et al., 2006). Three generations of so called "MDR modulators" have been developed and tested, however, they mostly failed to demonstrate sufficient efficacy, while showing toxicity in the clinical studies. Nevertheless, finding a therapeutic approach to overcome MDR still maintains its attractiveness as a tool that might help improve pharmacotherapy outcome.

On the other hand, during the past decade, an expanding body of literature has suggested a link between inflammation and cancer (Mantovani et al., 2008; Fletcher et al., 2010). It provides a new possibility to fight cancer, a new target for anticancer drugs and a potential to boost the effect of already existing drugs. Metabolic reprogramming of metabolism and proliferation are closely associated with carcinogenesis and tumour progression (DeBerardinis et al., 2008). Disorders connected with abnormal lipid metabolism have been proved to be involved in a vast range of diseases including hepatocellular carcinoma (HCC). Since the beneficial effects of statins on HCC were discovered by an accident (El-Serag et al., 2009), lipid metabolism in the liver became an object of interest. Several

studies have revealed that nonsteroidal anti-inflammatory drugs (NSAIDs), which interfere in lipid signalling pathway, improve cancer cells sensitivity to the antitumor drugs by modulation of ABC transporter activity (Hiľovská et al., 2015). The relationship of lipid metabolism and tumour progression provided possibilities for novel approaches in treating cancer.

In this project, our aim was to investigate the potential behind a hypothesis that ABC transporter expression could be downregulated in the cancer cells by interfering in lipid signalling pathway. In order to achieve our aim, we studied the effect of two experimental substances JJKK-048 and LBG-10119 on the cancer cell biology.

3. THEORETICAL PART

3.1 Cancer and hepatocellular carcinoma

Cancer remains one of the leading causes of death globally. In 2015 it was ranked second leading cause of death after cardiovascular disease (WHO, 2017). Worldwide, almost 1 in 6 death is due to cancer. Cancer mortality is expected to grow in all countries due to ageing of the population. For example, only in 2015 liver cancer burden was the reason of 788000 deaths worldwide. Primary liver cancer is the second leading cause of cancer death and the sixth most common neoplasm in the world (Global Burden of Disease Cancer Collaboration, 2015). HCC is the most common type of hepatobiliary cancer accounting for 70–90% of primary hepatic malignancies. Its incidence is increasing because of the spread of the multiple predisposing causes of HCC, including hepatitis B and C, excessive alcohol consumption and non-alcoholic fatty liver disease (Perz et al., 2006; El-Serag, 2011). Those observations call for further investigations and treatment development.

Poor prognosis and high mortality rates of HCC are due to lack of observable symptoms in the early stages of cancer, its aggressiveness leading to hepatic failure, and restricted therapeutic options in the final stages (Barman et al., 2014). The most effective treatments for advanced HCC are hepatic resection, orthotopic liver transplantation, image-guided tumour ablation and since 2008 also pharmacotherapy with sorafenib. First two options can only be applied in a small number of cases. Sorafenib was the first approved small molecule targeting anticancer drug representing a new treatment option for patients with advanced hepatocellular carcinoma. It is a multi-kinase inhibitor, which has antiproliferative and antiangiogenic activity and also inhibits cancer progression. It had proven benefits for the survival of patients diagnosed at advanced stage HCC or who progressed into advanced stage after the failure of previous treatment (Forner et al., 2012; Adler et al., 2008).

In developed multicellular organisms, cell division and death must be regulated to keep homeostasis in tissues (Pucci et al., 2003). The process of cell loss and cell gain should be in homeostatic balance in order to maintain the complex architecture of tissues, and to allow adaptation to changing conditions. This connection may be achieved through the link between the cell cycle and programmed cell death by using a joint set of factors, including, for example, oncogenes and proto-oncogenes like *Nras*, *p14* and *p53*.

Dysregulated cell cycle progression and programmed cell death are one of the most often alterations in cancer cells. The *Nras* gene is an oncogene, when mutated, has a potential to turn normal cell to cancerous. *Nras* encodes a protein called N-Ras that is involved primarily in regulating cell division (U.S National Library of Medicine, 2017). *p14ARF* (alternate reading frame) is a well-known tumour suppressor gene and a cell-cycle inhibitor. P14ARF protein has a key role in stabilization and promotion of the transcription activity of p53 in response to tumorigenic processes (Wang et al., 2006; Serrano et al., 1996). *p14ARF* gene (*p19ARF* in the mouse) is often altered in HCC and plays an important role in the pathogenesis of this neoplasm (Anzola et al., 2004). *p53* is a tumour suppressor gene, its activity stops carcinogenesis. *p53* that plays a crucial role in regulation of the cell cycle. Mutations in *p53* are the most common mutations in HCC (up to 30-60%), it is related to vascular invasion and cancer recurrence (Sheen et al., 2003). Moreover, overexpression of *p53* mutation correlates with poorer 5-year survival statistics (Yuan et al., 2006).

3.2 Drug resistance, its mechanisms, multidrug resistance and drug efflux

According to Goldman's opinion, most of the cancer-related deaths are the result of unsuccessful chemotherapy (Goldman, 2003). One of the main reasons of anticancer pharmacotherapy failure is drug resistance of cancer cells that is developed through various mechanisms. It can be acquired (because of host factors) or intrinsic (due to genetic or epigenetic factors) (Gottesman et al., 2002; Jemal et al., 2009). Drug resistance is an event that is characterised by the reduction in effectiveness of a drug in curing a disease or improving patient's symptoms. Despite the fact that in the beginning many types of cancer are susceptible to chemotherapy, over the time they may develop resistance through several mechanisms. They include increased drug efflux or decreased drug uptake (Dietel, 1991), drug target modification, enhanced repair of DNA damage, DNA mutation, drug inactivation and changes in metabolic processes that advance drug degradation and inhibition, inhibition of programmed cell death (apoptosis) (Housman et al., 2014).

The logic solution to overcome resistance would be to treat patients using various structurally, chemically and functionally different cytotoxic pharmaceuticals. Unfortunately, tumours show or acquire resistance to many diverse chemotherapeutic

agents with different chemical structures and mechanisms of action at the same time, which is known as MDR or pleiotropic resistance. The success of antineoplastic treatment depends on achieving an adequate concentration of a drug on the site of its target, which is located mostly inside the cancer cell. Nevertheless, intracellular amount of drug is greatly influenced by uptake and efflux processes by families of membrane transporters (Fukuda and Schuetz, 2012). One of the most studied and best-characterised mechanisms of MDR is modified membrane transport (Bosch and Croop, 1996).

Increased expression or/and enhanced activity of many drug transporters of the ABC transporter family was found in several types of cancer. They are responsible for efflux of many chemotherapeutic agents across the membranes from cells even against a considerable concentration gradient using ATP as the driving force (Lage, 2008). The efflux leads to a reduction of intracellular drug concentration, under the cytotoxic and apoptotic levels rendering the consequent drug insensitivity of cancer cells.

ABC transporters are large transmembrane proteins that can be found not only in cancer cells but also in many physiological tissues. Besides exogenous chemicals including drugs, they are able to translocate also a wide range of endogenous compounds, such as lipids and metabolic products (Borst and Elferink, 2002). There are 48 known members of human ABC family divided into seven (A-G) subfamilies according to their phylogenetic characteristics (Dean et al., 2001). The ABC transporters showing an important role in driving MDR in cancer cells comprise mainly P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance-associated protein 1 (MRP1) (Szakacs et al., 2006). While multidrug resistance-associated protein 2 (MRP2) plays an important role in conferring resistance to chemotherapeutic drugs in HCC (Nies et al., 2001).

3.3 An overview of human ABC family transporters included in this project

An overall description of all members of ABC superfamily transporters goes beyond the scope of this research. For this reason, only a compact summary of certain ABC transporters, which expression has been determined on the surface of used cell cultures in this study, is provided. They are P-gp, BCRP and MRP2. All of the ABC drug efflux transporters discussed here could be mainly found in the plasma membrane, where they

can efflux a wide range of structurally diverse drugs, drug conjugates and metabolites, and other compounds from the cell.

Table 1. *Summary of members of ABC transporter family, which were tested in the accumulation studies.*

Protein	Gene name	Alternative names	Exogenous substrates of the transporter (anticancer drugs)	Endogenous substrates of the transporter (cancer-related cellular substrates)
P-gp	*ABCB1*	MRP1, PGY1, GP170	5-fluorouracil, chlorambucil, colchicine, cisplatin, cytarabine, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, gefitinib, hydroxyurea, irinotecan, methotrexate, mitomycin C, mitoxantrone, paclitaxel, tamoxifen, teniposide, topotecan, vinblastine, vincristine	PAF
MRP2	*ABCC2*	cMOAT, cMRP	cisplatin, doxorubicin, epirubicin, etoposide, irinotecan, mitoxantrone, methotrexate, vinblastine, vincristine	LTC4 , PGD2 , PGA1 and PGE2
BCRP	*ABCG2*	MXR, ABCP	daunorubicin, doxorubicin, epirubicin, etoposide, gefitinib, imatinib, irinotecan, mitoxantrone, methotrexate, teniposide, topotecan, sorafenib	cGMP

Data obtained from Schinkel and Jonker, 2003; Chen et al., 2016; Sodani et al., 2012; Huang et al., 2013; de Waart et al., 2006; Fletcher et al., 2010.

3.3.1 ABCB subfamily

P-gp (also known as ABCB1**,** multidrug resistant protein 1 or MDR1) is a member of ABCB subfamily. P-gp was the first member of B subfamily to be discovered in 1976. It has been characterised to be responsible for conferring multidrug resistance upon rodent cancer cells to show reduced sensitivity to antitumor agents (Juliano and Ling, 1976). P-gp is an apical membrane transporter that has prominent expression in intestinal mucosal membrane, kidney proximal tubule epithelia, liver, placenta, and luminal blood-brain barrier, where it is a key player in defence of the body against xenotoxins and cellular toxicants (Loo et al., 2013; Chen et al., 2016). The negative side is that P-gp can interfere with the transport of drugs to their target tissues. P-gp translocates unmodified neutral or positively charged hydrophobic substances and demonstrates the efflux of a vast spectrum of drugs (Table 1) including clinically relevant antitumor pharmaceuticals such as taxol, vincristine, etoposide, daunorubicin and irinotecan amongst many others (Ambudkar et al., 2003; Borgnia et al., 1996; Eytan et al., 1994; Szakacs et al., 2006). It was initially identified that P-gp was overexpressed in cell cultures that became resistant to such anticancer agents (Schinkel and Jonker, 2003). P-gp also effluxes endogenous substrate platelet-activating factor (PAF), which has been implicated in various cancer-associated functions and signals (Raggers et al., 2001).

3.3.2 ABCC subfamily

ABCC subfamily (also known as multidrug resistance-associated protein or MRP) is the largest subfamily consisting of 13 members (ABCC1-ABCC13). Most inhibitors developed to date target P-gp; however, most known cancer-related cellular ABC transporter substrates are effluxed by the ABCC subfamily (Fletcher et al., 2010).

MRP2 (or ABCC2) is primarily expressed in important pharmacological barriers, such as the canalicular (apical) membrane of hepatocytes, enterocytes of small and large intestines and kidney proximal tubules, where its function is to be a major exporter of exogenous and endogenous compounds (Jemnitz et al., 2010). It was recently revealed by Korita et al., 2010 that the expression of MRP2 determines the efficacy of cisplatin-based treatment of patients with HCC. MRP2 also transports endogenous signalling lipids, including some prostaglandins and leukotrienes (Table 1).

3.3.3 ABCG subfamily

BCRP (or ABCG2) was classified as a second member of G subfamily of ABC transporters. Even though BCRP was discovered later than most of the efflux transporters discussed here (Doyle et al., 1998), there is already a large body of evidence that, similar to P-gp and MRP2, it mediates transport of a substantial number of very broad structurally and functionally diverse drugs; thus, BCRP has a significant effect on their general pharmacology inside the body. Originally, *ABCG2* gene was isolated from breast cancer cell line; therefore, it was named breast cancer resistance protein or *BCRP* gene. There is no indication, though, that it is expressed specifically in breast tumours or that BCRP should have a significant role in chemoresistance of breast cancer cells. BCRP expression could be found throughout the whole human body (Nishimura and Naito, 2005) and its involvement has been observed practically in all important pharmacological barriers (Jani et al., 2014). BCRP was frequently expressed in solid cancers of different origin, including in neoplasms from the digestive tract, endometrium, lung and melanoma (Diestra et al., 2002). As seen in Table 1, BCRP is responsible for efflux of a wide range of various anticancer drugs, including sorafenib, doxorubicin, irinotecan, methotrexate (Huang et al., 2013). In numerous reviews increased expression of BCRP was correlated with pessimistic prognosis (Mo and Zhang, 2012; Robey et al., 2011; Natarajan et al., 2012).

BCRP also effluxes endogenous cyclic guanosine monophosphate (cGMP), which is a cyclic nucleotide (de Wolf et al., 2007). Cyclic nucleotides have been identified as important secondary messengers downstream of G protein-coupled protein receptors (GPCRs). It has been proved that they have relevance to cancer biology (Dorsam and Gutkind, 2007).

3.4 Cancer and inflammation

According to Hanahan and Weinberg (2000), there are six essential acquired capabilities that cancer cells must have in order to survive and prosper. They are insensitivity to anti-growth signals, evasion of programmed cell death, tissue invasion and metastasis, infinite potential to replicate, independence in the development, and sustained angiogenesis. There is also an increasing evidence leading to a proposal of seventh ability, which is a contribution of cancer-related inflammation (Figure 1). As we know, the main

concept is that physiological inflammation – for example, wound healing – usually limits itself, since synthesis of anti-inflammatory cytokines follows proinflammatory cytokines closely. While an error/s in the regulation of any of the converging factors may be the reason of irregularities and finally, pathogenesis leading to neoplastic progression. Cell proliferation potentiates tumorigenesis, especially in a microenvironment rich in inflammatory cells, cytokines, chemokines, growth factors, activated stroma, and DNA-damaging agents (Coussens and Werb, 2002). There is an analogy between injury associated with wounding and cancerous tissue behaviour. For example, cell proliferation is increased while the tissue is undergoing repair; proliferation and inflammation decrease after the attacking agent is removed or the regeneration is completed. Whereas, proliferating cells that maintain DNA damage and/or mutagenic assault continue to replicate in the microenvironment rich in inflammatory cells and growth factors that support their growth. In a sense, tumours behave themselves as wounds that have failed to heal – meaning that cancer and inflammation are closely associated (Dvorak, 1986).

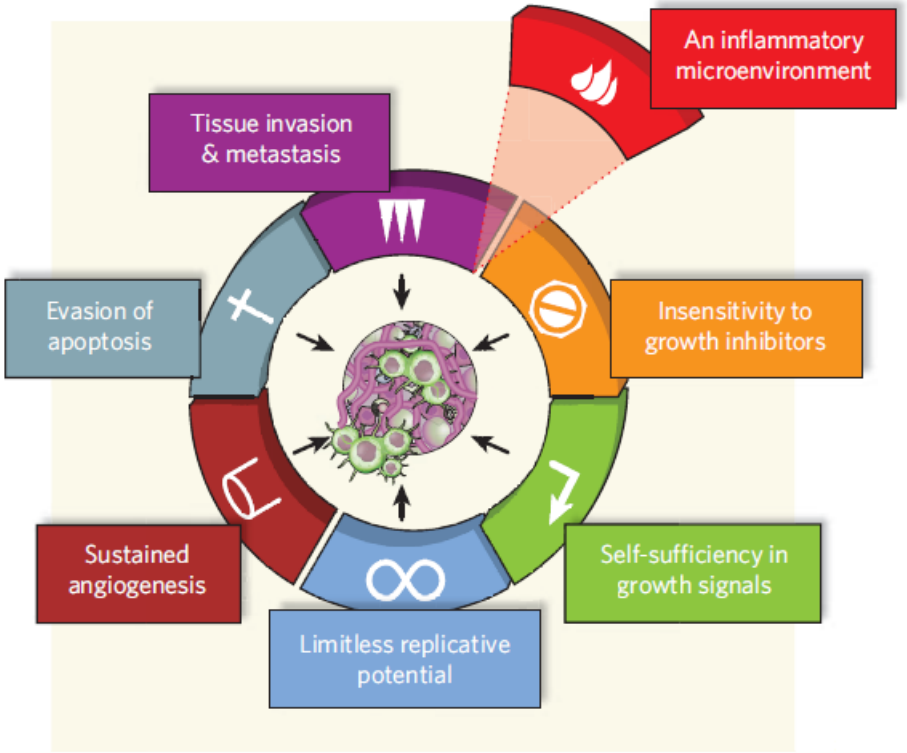

Figure 1: *Model of six essential cancer hallmarks, by Hanahan and Weinberg (2000) and a new emerging seventh hallmark – inflammation.*

Adopted from: Mantovani, 2009

Probably, one of the best proofs of the inflammation importance during neoplastic progression is shown in the study of cancer risk among patients, who are long-term users of acetylsalicylic acid and NSAIDs. Data indicate that colon cancer risk is dropped by approximately half (by 40-50%) after use of these drugs among men and women (Baron and Sandler, 2000). NSAIDs may be also used for prevention of certain types of cancer, such as oesophageal and stomach cancer (Garcia-Rodriguez and Huerta-Alvarez, 2001) and possibly against prostate and ovary cancer (Mahmud et al., 2004; Baron, 2003). The protective effect has not been confirmed for paracetamol and other antipyretics that have a different mechanism of action than anti-inflammatory agents (Baron and Sandler, 2000).

Chemoprevention property of NSAIDs is linked to their ability to inhibit cyclooxygenase-1 (COX-1) and COX-2. Cyclooxygenase is one of the key enzymes

in lipid signalling pathway; it metabolizes arachidonic acid (AA) to prostaglandins. While COX-1 is constitutively expressed in many tissues, the COX-2 isoform is almost untraceable in normal tissues but is often overexpressed in premalignant and malignant tissues, where it can present an important target for therapy (Dannenberg and Subbaramaiah, 2003). In *in vivo* studies led by Stolina et al., 2000, specific inhibition of COX-2 expression showed a significant decline in tumour progression and improved survival rate. In other studies, direct and indirect effects of COX inhibition have been proved, such as its ability to inhibit cancer cell proliferation, induce programmed cell death and restrict metastatic dissemination (Fulton et al., 2006). The presence of COX-2 in cancer is considered as an indicator of pessimistic prognosis (Ristimäki et al., 2002). A strong positive correlation between COX-2 levels and expression of P-gp has been found in studies on HCC, breast and lung cancer (Surowiak et al., 2008; Surowiak et al., 2005; Ziemann et al., 2002). Moreover, it was reported that COX-2 could be involved in the upregulation of all three P-gp, MRP1 and BCRP transporters expression (Surowiak et al., 2008; Liu et al., 2010).

3.5 Lipid signalling pathway

Taking these data into consideration, the obvious choice for multidrug-resistant cancer treatment would be the use of selective COX-2 inhibitors in conjunction with standard anticancer therapy. Unfortunately, unlike traditional NSAIDs that are dual inhibitors of two COX isoforms, selective COX-2 inhibitors inhibit platelet function thereby substantially increasing the risk of cardiovascular complications (Dannenberg and Subbaramaiah, 2003). In addition from Figure 2 it can be observed, that neither selective COX-2 inhibitors nor traditional NSAIDs do not affect other cancer-inflammation contributing products of AA – leukotrienes or PAF. Compounds that target the upregulated (not physiological) release of AA might represent an alternative.

General scheme of lipid signalling pathway can be seen in Figure 2

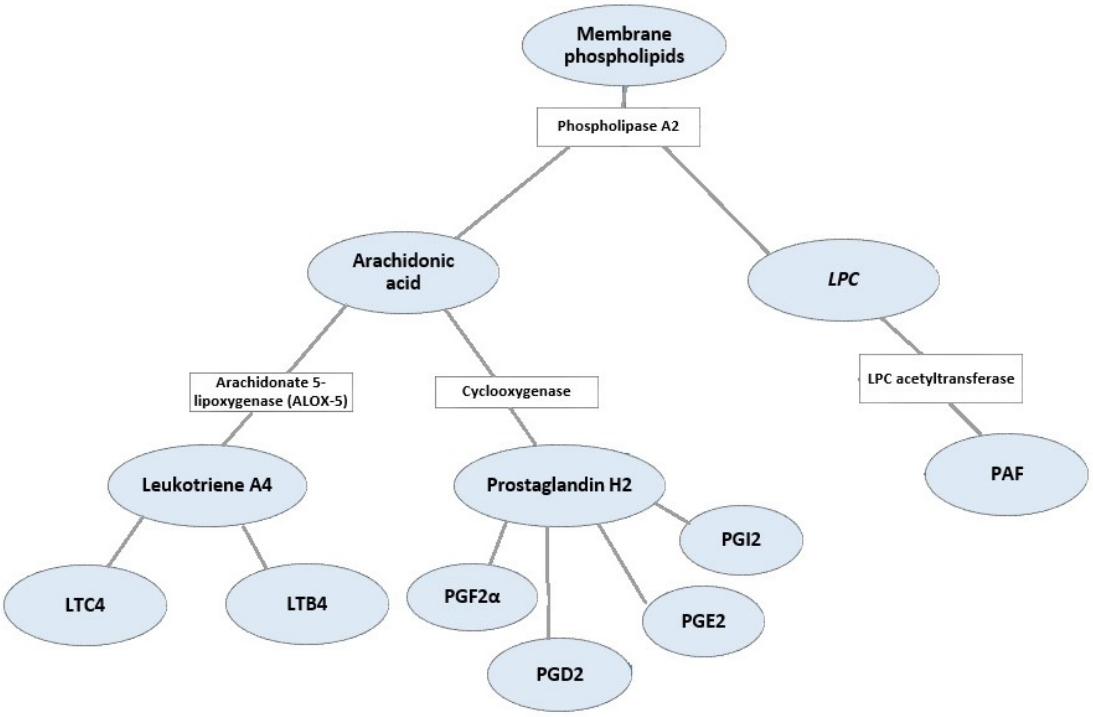

Figure 2: *Proinflammatory lipid synthesis. Eicosanoids (prostaglandins and leukotrienes) and platelet-activating factor (PAF) synthesis begins with the release of arachidonic acid (AA) and lysophosphatidylcholine (LPC) from phospholipids by the action of phospholipase A2 (PLA2). The cyclooxygenase (COX) then metabolises AA to prostaglandin H2, which is converted by the activity of specific prostaglandin isomerases to various prostaglandins. At the same time, AA is converted by arachidonate 5-lipoxygenase (ALOX-5) into leukotriene A4, which can be hydrolysed to leukotrienes C4 (LTC4) or B4 (LTB4). PAF is synthesized from LPC by LPC acetyltransferase.*

Adopted from: Dannenberg and Subbaramaiah, 2003; Fletcher et al., 2010.

The importance of ABC transporters extends beyond only drug transport to assisting inflammatory processes and further progression of cancer. In 2010 Fletcher et al. revealed that ABC family transporters contribute to cancer progression not only with MDR, but also might promote cell survival (without the influence of cytotoxic drug efflux), stem cell maintenance and growth, cell migration and invasion, and transport of different signalling molecules.

3.5.1 Prostaglandins

Regardless of the trigger for the development, most cancers support inflammatory microenvironment that advances their own proliferation and survival and supports angiogenetic and metastatic processes (Mantovani et al., 2008). Products of AA metabolism – eicosanoids (prostaglandins and leukotrienes) are known mediators of chronic inflammation. COX-2-derived prostaglandins have an impact on numerous mechanisms that have been implicated in tumorigenesis. For example, best known as a mediator of pain and inflammation, prostaglandin E2 (PGE2) has been implicated in tumorigenesis along with stimulation of cell proliferation, motility and response to cytotoxic treatment while circumventing apoptosis and hindering immune surveillance. PGE2 has also been implicated in the development of some tumours as well as the stimulation of their growth and angiogenesis, and response to cytotoxic chemotherapy. PGE2 can also take part in angiogenesis by enhancing the production of proangiogenic factors including vascular endothelial growth factor (Hanaka et al., 2009; Lin et al., 2008; Gupta and Dubois, 2001; Gasparini et al., 2003). As seen in Table 1, some members of ABC transporter family mediate the uptake of prostaglandins. For example, MDR2 mediates the transport of prostaglandins PGE2, PGD2 and PGA1 (de Waart et al., 2006; Fletcher et al., 2010). In addition, NSAIDs exert their effects on cancer cells, as discussed before, mostly by inhibiting the synthesis of prostaglandins.

It is well known that COX-2 is upregulated in tumour, thus, enhances PGE2 synthesis. To be precise, the cellular effect of PGE2 are mediated through activation of extracellular GPCRs EP1, EP2, EP3 and EP4 (Narumiya et al., 1999). Among these receptors, EP2 and EP4 are linked with oncogenesis. Several observations report that EP2 signalling stimulates growth, drives angiogenesis and resistance to apoptosis (Dannenberg and Subbaramaiah, 2003). Genetic ablation of the EP2 receptor inhibits neoplastic growth and prolongs survival in syngeneic mouse tumour models (Yang et al., 2003), reduces the progression of intestinal polyposis with reduced expression of COX-2 in polyp tissues (Sonoshita et al., 2001). While, EP4 signalling stimulation demonstrate a primary involvement in breast cancer cells migration (Timoshenko et al., 2003).

3.5.2 Leukotrienes

In comparison with prostaglandins, less information is available regarding the role of proinflammatory leukotrienes in the progression of tumour. Nonetheless, emerging information from studies implies that leukotrienes can play a substantial role in oncogenesis. Similarly, leukotrienes like prostaglandins exert their effect extracellularly via GPCRs, leading to different processes that include activation of β-catenin, which aids cell survival, replication, leucocytes invasive behaviour and secretion of pro-tumorigenic factors (Fletcher et al., 2010).

The inhibition of arachidonate 5-lipoxygenase (ALOX-5) expression and activity stimulates programmed cell death and cancer growth arrest (Hayashi et al., 2006; Meng et al., 2013; Schroeder et al., 2007). Consistent with this fact, the increased level of ALOX-5 product leukotriene B4 (LTB4) is mostly associated with carcinogenesis. For example, LTB4 level is elevated in human colon and prostate cancer (Larré et al., 2008; Dreyling et al., 1986), and the overexpression of LTB4 receptors is observed in human pancreatic cancer (Hennig et al., 2002). Recently the role of leukotriene C4 (LTC4) in promoting oxidative DNA damage was reported, which if not sufficiently repaired may contribute to genomic instability and increased mutation rates (Dvash et al., 2015). A huge number of members of the ABCC subfamily have been reported to participate in LTC4 transport: MRP1, MRP2, MRP3, MRP4 (Cui et al., 1999; Rius et al., 2008; Zeng et al., 2000 Fletcher et al., 2010).

Above all, inflammation, lipid signalling pathway and AA metabolism products in particular play an important role in the development of cancer. The molecular pathways of this cancer-related inflammation are now being studied closely, resulting in the identification of new target molecules that could lead to improved diagnosis and treatment. Potential targets that would affect both proinflammatory products of AA – leukotrienes and prostaglandins – could be an *N*-methyl-*D*-aspartate (NMDA) receptor and monoacylglycerol lipase (MAGL), which take part in the lipid signalling pathway and thus are involved in the upregulation of ABC transporter expression as well as the creation of inflammatory microenvironment.

3.5.3 Platelet-activating factor

It has been reported that PAF is involved in various tumour-associated processes and signals. PAF is a naturally occurring short chain phosphatidylcholine (PC). P-gp recognizes and transports short chain analogues of PC across the plasma membrane, including PAF (Raggers et al., 2001). There are two intracellular ways of PAF synthesis. The 'de novo' synthesis is held responsible for constitutive PAF synthesis. The remodelling' pathway accounting for stimulated PAF synthesis, involves the phospholipase A2 (PLA2) release of arachidonate (Figure 2), providing the precursor of PAF lyso-PC (LPC) and the eicosanoid precursor AA. LPC is further converted into PAF (Record et al., 1989; Raggers et al., 2001). PAF is a bioactive lipid that binds to its extracellular G protein-coupled PAF receptor. It is synthesized by a vast spectrum of cells, including circulating inflammatory cells, endothelial cells and epithelial cells (Triggiani et al., 1992). PAF participates in a wide variety of biological effects, from activation of inflammatory cells to vascular and other physiological processes. Moreover, it is also involved in diverse pathological processes, including shock, sepsis and multiple organ failure (Anderson et al., 1991). PAF has been detected in tumour tissue and its effect on tumour development has been investigated. For example, PAF is involved in angiogenetic growth and upregulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL in breast cancer (Heon Seo et al., 2006; Montrucchio et al., 1998; Bussolati et al., 2000), facilitation of metastasis in melanoma (Melnikova et al., 2006) and colorectal carcinoma (Denizot et al., 2005).

3.6 *N*-methyl-*D*-aspartic acid receptor

NMDA receptor is a glutamate receptor and an ion channel protein. It has been identified on the surface of different tumour cell lines and tumours, including glioma (Aronica et al., 2001), oral squamous cell carcinoma (Choi et al., 2004), prostate cancer (Abdul and Hoosein, 2005), osteosarcoma (Kalariti et al., 2005), gastric cancer (Liu et al., 2007) and HCC (Yamaguchi et al., 2013). NMDA receptor is a heterotetramer, which mainly consists of two obligatory GRIN1 glutamate ionotropic receptor NMDA subunits 1 (also known as NR1) and two out of the four types of regulatory subunits GRIN2A, B, C or D. The resulting complex might combine with either GRIN3A or GRIN3B, which replaces one of the GRIN2 subunits (Stepulak et al., 2014).

It has been reported that glutamate signalling is dysregulated in several different types of cancer. Glutamate is released from cancer cells, stimulating cell proliferation and thus promoting tumour progression (Seidlitz et al., 2009; Prickett and Samuels, 2012). It possibly can be due to the link between NMDA receptor stimulation and AA signalling, which, unfortunately, has not been studied in detail. It is known, however, that Ca^{2+} influx mediated by NMDA receptor activation stimulates PLA2 (Rao et al., 2007). PLA2 releases AA from phospholipids in the cell membranes, therefore providing the substrate to the COX-2 isoform and ALOX-5, which are the initial steps in catalysis of proinflammatory prostaglandins and leukotrienes (Lazarewicz et al., 1990). AA metabolites in their turn lead to upregulation of P-gp and MRP2 (Luna-Munguia et al., 2015; Bauer et al., 2008; Avemary et al., 2013). Moreover, AA molecule itself can have its own effects independent from its metabolites; some of which can have harmful consequences on a cell (Wieloch and Siesjo 1982; Yu et al. 1986; Barbour et al. 1989; Williams et al. 1989; Miller et al. 1992; Vazquez et al. 1994; Volterra 1994). New novel approach to optimize therapeutic outcome has been based on this theoretical background. Its aim to stop the release of AA rather than to stop effects of the AA metabolites. Taking into consideration tolerability problems with different approaches interfering in AA pathway, in addition to COX-2, the NMDA receptor was suggested as an alternative target (Bauer et al., 2008).

In the experimental part of this study was used the competitive antagonist of a heterodimeric subtype of NMDA (GRIN1/2A) LBG-10119. It selectively binds to GRIN2A subunit (encoded by *GRIN2A* gene). *GRIN2A* is expressed in several healthy tissues, but mainly in the brain and in the small amount in the female reproductive tissues. Our substance LBG-10119 was designed not to cross the blood-brain barrier, which means that it will not affect healthy tissues in the brain. It was reviewed by Mehrotra, 2015 that *GRIN2A* gene subunit is expressed in laryngeal, gastric cancer and HCC (Yamaguchi et al., 2013). Western blotting method proved that cell cultures used in this study express on their surface GRIN2A and GRIN1 subunits (unpublished data).

3.7 Monoacylglycerol lipase

There is also another potential target in the novel approach of cancer treatment. MAGL is a serine hydrolase that plays a significant role in lipid metabolism. It was initially characterized from adipose tissue (Karlsson et al., 1997; Labar et al., 2010). MAGL catalyses the last step in lipolysis releasing free fatty acids (FFA) and glycerol for fuel or lipid synthesis. MAGL is best recognised for terminating and inactivating the signalling mediated by endocannabinoid 2-arachidonoylglycerol. By doing so, it forms glycerol and AA and provides a substrate for COX-2 and ALOX-5, which in its turn results in a synthesis of proinflammatory and pro-tumorigenic leukotrienes and prostaglandins (Zechner et al., 2012). MAGL contributes to cancer pathogenicity by producing precursors for tumour-promoting bioactive lipids, such as prostaglandins, leukotrienes and lysophosphatidic acid (Nomura et al., 2011).

MAGL, through hydrolysis of monoacylglycerols, controls levels of FFA in cancer cells. In contrast, MAGL does not control the levels of FFA in normal healthy tissues (Nomura et al., 2008; Long et al., 2009). Therefore, MAGL inhibitors would have therapeutic potential by reducing the production of AA-derived products and consequent efflux transporters overexpression in cancer cells. Increased MAGL levels were found in different types of cancer, such as melanoma, breast, ovarian cancer and HCC (Nomura et al., 2010; Zhang et al., 2016). In addition, the upregulated expression of MAGL in HCC is associated with recurrence and poor patient prognosis (Zhu et al., 2016; Zhang et al., 2016). According to Zhang et al. MAGL activity in HCC cell lines contributes to proliferation, invasion and evasion of apoptosis; it is also closely correlated with the degree of malignancy being a prognostic indicator of cancer aggressiveness (Zhang et al., 2016). MAGL is highly increased in the aggressive form of tumour from different origin tissues.

Studies with MAGL inhibitors reported, that they show the added benefit of reducing proinflammatory lipid signalling molecules to produce anti-inflammatory and neuroprotective responses in a brain tumour. Additionally, MAGL inhibitors do not possess NSAIDs gastrointestinal toxicity, because they do not exert control over AA and prostaglandin pathways in the gastrointestinal system (Nomura et al., 2011). Actually, data from Kinsey et al., 2011 suggest that MAGL inhibition have a protective effect against gastrointestinal bleeding caused by diclofenac, a dual COX-1/COX-2

inhibitor, through CB1 cannabinoid receptor-dependent mechanisms. It was proved that MAGL also possesses pro-tumorigenic qualities that are caused by PGE2, a downstream product of MAGL (Zhang et al., 2016). Collectively, these findings imply that pharmacological inhibition of MAGL function may confer significant therapeutic benefits.

4. AIMS

The main aim of this study was to evaluate the effect of JJKK-048 and LGB-10119, the respective MAGL inhibitor and competitive antagonist of NMDA (GRIN1/2A) receptor interfering the lipid signalling pathway of cancer cells. The effect of tested substances will be evaluated in different conditions – in the presence of two different mutations of *p19ARF* and *p53* using two mouse hepatocarcinoma cell lines: Nras driven p19ARF$^{-/-}$ and Nras driven p53$^{-/-}$, respectively.

The research was designed to address in particular the following questions:

- To find out whether the new strategy could downregulate the function of ABC transporters using intracellular accumulation assay with relevant model substrates. The compounds will be analysed in three different concentrations to find out the most effective concentration enabling next step in the laboratory investigation
- To evaluate the effect of the tested compounds JJKK-048 and LGB-10119 on the antiproliferative efficacy of sorafenib.

5. EXPERIMENTAL PART

5.1 Chemicals and materials

JJKK-048 or {4-[bis-(benzo[d][1,3]dioxol-5-yl)methyl]-piperidin-1-yl}(1H-1,2,4-triazol-1-yl)methanone, was synthesized at the School of Pharmacy, University of Eastern Finland (Kuopio, Finland). CAS No: 1515855-97-6.

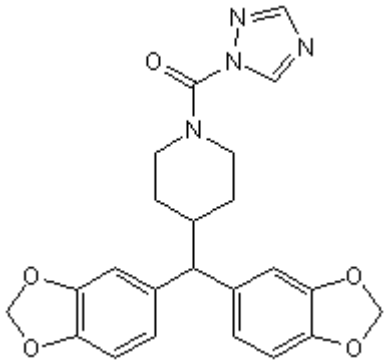

Figure 3: *Chemical structure of JJKK-048 {4-[bis-(benzo[d][1,3]dioxol-5-yl)methyl]-piperidin-1-yl}-1H-1,2,4-triazol-1-yl-methanone.*

LBG-10119 was designed and synthesized in Bunch research group (Department of Drug Design and Pharmacology, University of Copenhagen, Denmark).

Commercially available chemicals were used in the studies. The reagent grade chemical dimethylsulfoxide (DMSO) and cell culture components used, namely Dulbecco's modified eagle's medium (DMEM) (Gibco-BRL, Carlsbad, CA, USA), penicillin-streptomycin (EuroClone® S.p.A, Italy), L-glutamine (EuroClone® S.p.A, Italy). Fetal bovine serum (FBS) was from Gibco-BRL (Carlsbad, CA, USA).

Sorafenib – Cayman Chemical, USA, Ann Arbor, Michigan.

[^{3}H]-digoxin – Perkin Elmer, USA, Boston.

Fluorescein – Sigma-Aldrich, USA, Missouri.

Laboratory water was purified using a Milli-Q water purification system (Millipore, Milford, MA, USA) system.

5.2 Cell culture

5.2.1 Cell lines

For the experimental part were used Murine Nras driven $p53^{-/-}$ (knockout model for *p53* gene) and Nras driven $p19ARF^{-/-}$ (knockout model for *p19ARF* gene) hepatocarcinoma cells. The combination of mutations of *Nras* with a knockout of *p19ARF* or *p53* increases the cell proliferation and makes the phenotype more aggressive. The cell lines were a kind gift from Prof. Dr. Lars Zender, University of Tubingen (Germany). The cells were cultivated at 37°C in 5 % CO_2 in DMEM supplemented with L-glutamine (2 mM), bovine serum albumin (BSA) (10 %), penicillin (50 IU/mL), streptomycin (50 µg/mL).

5.2.2 Seeding of the cells for accumulation studies

For experimental part, the cells were seeded in a 24-well plate at a density $3x10^4$ cells per well. The cells were incubated for 24 hours before starting the experiments. On the second day after the seeding tested compounds dissolved in DMSO were added followed by 24-hour incubation. Next day the cells were used for accumulation experiment. After removal of culture medium, cells were carefully washed with pre-warmed Hank's balanced salt solution (HBSS) containing 125 mM choline chloride, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM glucose, 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7,4) and then pre-incubated with 500 µL of pre-warmed HBSS at 37°C for 10 min. The cells were then undergoing accumulation studies at the room temperature in 250 µL of uptake medium solution containing either 50 µM of fluorescein or 1 µM of sorafenib solution or 0.375 µM of [^{3}H]-digoxin in HBSS. Subsequently, the cells were washed 2 times with 500 µL of ice-cold HBSS. The cells were then lysed with 500 µl of 0.1 M NaOH.

Tested compounds dissolved in DMSO were also evaluated at 48-hour incubation time, but the results did not show a statistically significant difference compared to 24-hour incubation time.

5.3 Accumulation studies

The effect on ABC transporter function is determined by incubating mouse hepatocarcinoma cells for 24 hours with the tested compounds, followed by accumulation studies performed with relevant efflux probes, [^{3}H]-digoxin, fluorescein and sorafenib, the substrates of P-gp (Rautio et al., 2006), MRP2 (Löscher and Potschka, 2005) and BCRP (Huang et al., 2013), respectively. While sorafenib is one of two targeted therapy drugs for liver cancer approved by Food and Drug Administration (FDA) (American Cancer Society, 2017).

a) Fluorescein solution in HBSS 50 μM was added to the cells and plates were then incubated for 30 min at room temperature and protected from light. After washing with ice-cold HBSS the cells were lysed in 0.1 M NaOH, fluorescence was measured on an Envision plate reader (Perkin Elmer) using appropriate filters with excitation at 494 nm and emission at 521 nm.
b) A solution of 0.375 μM [^{3}H]-digoxin in HBSS was added to the cells and then left to accumulate for 30 minutes. The efflux was stopped by washing the cells with ice-cold HBSS. The cells were then lysed using 0.1 M NaOH. The lysate was mixed with 1 ml of Ultima Gold cocktail (PerkinElmer, Waltham, MA, USA). The radioactivity was measured by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Inc., Finland).
c) Sorafenib solution in HBSS 1 μM was added to the cells and plates were then incubated at room temperature for 30 minutes. The efflux was stopped by washing the cells in ice-cold HBSS and cells were lysed using 0.1 M NaOH. Sorafenib accumulation was measured using High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis.

5.3.1 High-performance liquid chromatography-mass spectrometry analysis of sorafenib.

An internal standard diclofenac sodium salt was purchased from Sigma-Aldrich (St Louis, Mo., USA). An Agilent 1200 Series Rapid Resolution LC System was used together with a Poroshell 120 EC-C-18 column (50 mm × 2.1 mm, 2.7 μm) for liquid chromatography prior to MS analysis of sorafenib with Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent

Technologies Inc., Wilmington, DE). The high-performance liquid chromatography eluents were water (A) containing 0.1% (v/v) formic acid and acetonitrile (B). A gradient elution with 20–90 % B was applied over 1–4 minutes, followed by 1 minute of isocratic elution with 90 % B and 3 minutes column equilibration, giving a total time of 8 minutes/injection. The eluent flow rate was 0.2 mL/min, the column temperature was 40°C and injection volume 2 µL. The following mass spectrometry conditions were used for sorafenib. Electrospray ionization, positive ion mode; drying gas (nitrogen) temperature, 300°C; drying gas flow rate, 8 L/min; nebulizer pressure, 20 psi; and capillary voltage, 3500 V. Analyte detection was performed using multiple reaction monitoring. The transitions for sorafenib and the used internal standard, diclofenac were 465.1 → 252.2, 296.1 → 250, respectively. Fragmentor voltages used for sorafenib and diclofenac were 140 V and 100 V and the collision energies were 30 V and 10 V, respectively. The pressure of the collision cell nitrogen was adjusted to 2.9 10-5 Torr. Agilent MassHunter Workstation Acquisition software (Data Acquisition for Triple Quadrupole Mass Spectrometer, version B.03.01) was used for data acquisition, and Quantitative Analysis (B.04.00) software was used for the data processing and analysis.

5.3.2 Protein quantification

The protein concentration (on each plate) was determined as mean ± SEM (n=3-6 samples) by Bio-Rad Protein Assay (EnVision, PerkinElmer, Inc.). Protein amount was assessed using calibration curve prepared as dilution row of Protein Assay Dye Reagent Concentrate (BioRad Laboratories GmbH, Germany, Munich). Cellular accumulation of fluorescein, [^{3}H]-digoxin, sorafenib was then normalized to protein amount.

5.4 Cell viability assay

The antiproliferative effect of sorafenib (1-10 µM) in the presence of tested compounds was determined in hepatocarcinoma cells employing resazurin based cell proliferation kit (*In Vitro* Toxicology Assay Kit, Sigma, St. Louis, MO, USA), which is directly proportional to aerobic respiration and cellular metabolism of cells. The Nras driven

p19ARF$^{-/-}$ cells were seeded in a 96-well plate at a density 1x10^4 cells per well. On the following day, tested compounds JJKK-048 and LBG-10119 were added at 10 μM concentration and incubated for 72 hours with an everyday change of medium (containing tested compounds). The samples were measured fluorometrically by monitoring the increase in fluorescence at a wavelength of 590 nm using an excitation wavelength of 560 nm (EnVision, PerkinElmer, Inc., Waltham, MA, USA).

5.5 Data analysis

All statistical analyses were performed using GraphPad Prism v. 7.02 software (GraphPad Software, San Diego, CA, USA) using one-way ANOVA, followed by a two-tailed Dunnett's test or Tukey's test, where appropriate. The results were considered as statistically significant with P value <0,05. All results are presented as means ± standard error of the mean (SEM) in % of control.

6. RESULTS

Analysis of the key results is divided into two parts. First part is formed by accumulation assays of [^{3}H]-digoxin, fluorescein and sorafenib in Nras driven p19ARF$^{-/-}$ and Nras driven p53$^{-/-}$ cells. The second part shows the results of cytotoxicity study with sorafenib in relation to the presence of experimental JJKK-048 and LBG-10119 compounds.

6.1 Accumulation assays

In order to reveal the possible effect of JJKK-048 and LBG-10119 in efflux transporter-mediated MDR, we measured the effect of both compounds on the cellular accumulation of [^{3}H]-digoxin, sorafenib and fluorescein, the relevant substrates of P-gp, BCRP, and MRP2 in the ABC transporters-expressing mouse hepatocellular carcinoma cell cultures (Figure 4 and 5).

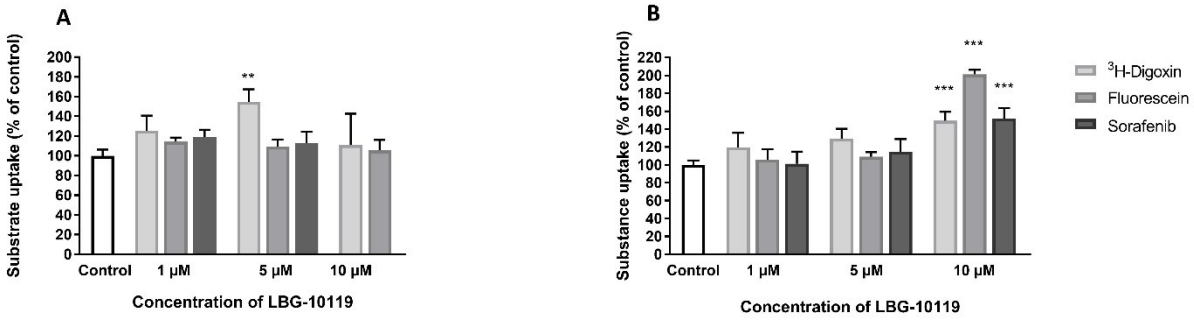

Figure 4: *Concentration-dependent effect of 24-hour incubation of LBG-10119 on BCRP substrate sorafenib, P-gp substrate [^{3}H]-digoxin, MRP2 substrate fluorescein in A) Nras driven p19ARF$^{-/-}$ and B) Nras driven p53$^{-/-}$ cells. The substrates were added and left to accumulate for 30 min. An asterisk denotes a statistically significant difference from the respective control (**P < 0.01, ***P < 0.001, one-way ANOVA, followed by Dunnett's test). Data are presented as the mean ± SEM (n=3-6 wells).*

Our data from Figure 4A indicate that LBG-10119 affects the accumulation of [^{3}H]-digoxin, but not fluorescein or sorafenib in Nras driven p19ARF$^{-/-}$ cells. Interestingly, the maximal effect was observed at 5 µM concentration, while

10 μM concentration did not cause a statistically significant increase in [^{3}H]-digoxin accumulation. Unfortunately, the results of sorafenib accumulation in Nras driven p19ARF$^{-/-}$ after incubation with 10 μM LBG-10119 was not possible to obtain due to technical issues with equipment.

While in Figure 4 LBG-10119 at 5 μM concentration significantly reversed the accumulation of [^{3}H]-digoxin in Nras driven p19ARF$^{-/-}$, the inhibition of efflux was observed only at 10 μM (but not at 5 μM) concentration in Nras driven p53$^{-/-}$. In contrast to Nras driven p19ARF$^{-/-}$ cells, 10 μM LBG-10119 significantly affected the accumulation of all three probe substances (***P < 0.001) in Nras driven p53$^{-/-}$.

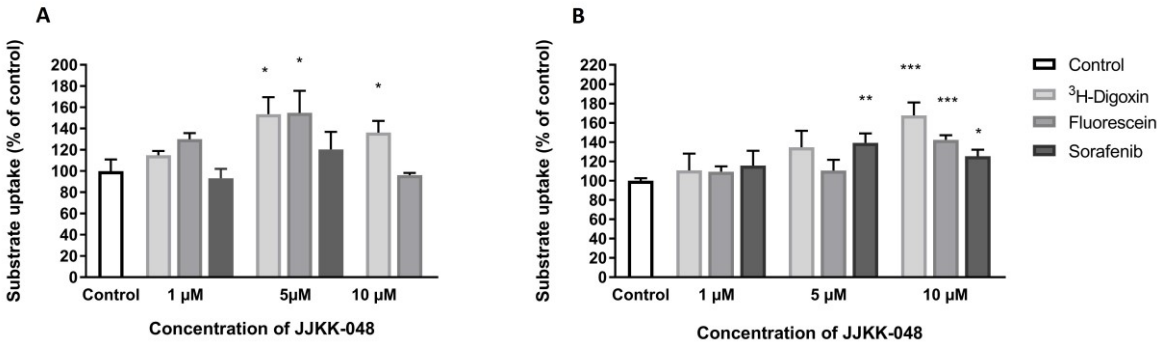

Figure 5: *Concentration-dependent effect of 24-hour incubation of JJKK-048 on BCRP substrate sorafenib, P-gp substrate [^{3}H]-digoxin, MRP2 substrate fluorescein cell accumulation in A) Nras driven p19ARF$^{-/-}$ and B) Nras driven p53$^{-/-}$ cells. The substrates were added and left to accumulate for 30 min. An asterisk denotes a statistically significant difference from the respective control (*P < 0.05, **P < 0.01,***P < 0.001, one-way ANOVA, followed by Dunnett's test). Data are presented as the mean ± SEM (n=3-6).*

It can be observed from the Figure 5A, that higher concentrations of JJKK-048 enhance intracellular accumulation of [^{3}H]-digoxin, while 10 μM concentration does not have a statistical advantage over 5 μM (*P < 0.05) in Nras driven p19ARF$^{-/-}$. The accumulation of fluorescein (Figure 5A) is affected more by 5 μM concentration of JJKK-048 (*P < 0.05) than by 10 μM. Unfortunately, results for the accumulation of sorafenib

in Nras driven p19ARF$^{-/-}$ after incubation with 10 μM JJKK-048 was not possible to obtain due to technical issues with equipment.

Figure 5B shows that 10 μM JJKK-048 statistically enhances intracellular concentration (% of respective control) of [^{3}H]-digoxin and fluorescein (***P < 0.001), while the effect on accumulation of sorafenib is less significant (*P < 0.05). In contrast to the latter, 5 μM JJKK-048 has more significant impact on the accumulation of sorafenib (**P < 0.01).

The results of the impact of 10 μM JJKK-048 and LBG-10119 on [^{3}H]-digoxin, fluorescein and sorafenib accumulation by Nras driven p53$^{-/-}$ and Nras driven p19ARF$^{-/-}$ cell lines were obtained by Dr Mikko Gynther and his team and were kindly shared to fulfil the idea and aims of this work.

To investigate further the efficiency of anticancer treatment sorafenib in the presence of potential expression inhibitors, cytotoxicity tests were conducted on the same *in vitro* model Nras driven p19ARF$^{-/-}$.

6.2 Cytotoxicity studies

In the cytotoxicity assays, we observed concentration-dependent cytotoxic effect of sorafenib showing a decrease of cell viability by 60 % at the highest tested 10 μM concentration (Figure 6). Nevertheless, none of the tested compounds LBG-10119 (Figure 6A) or JJKK-048 (Figure 6B) affected cytotoxic properties of sorafenib in Nras driven p19ARF$^{-/-}$ *in vitro* model.

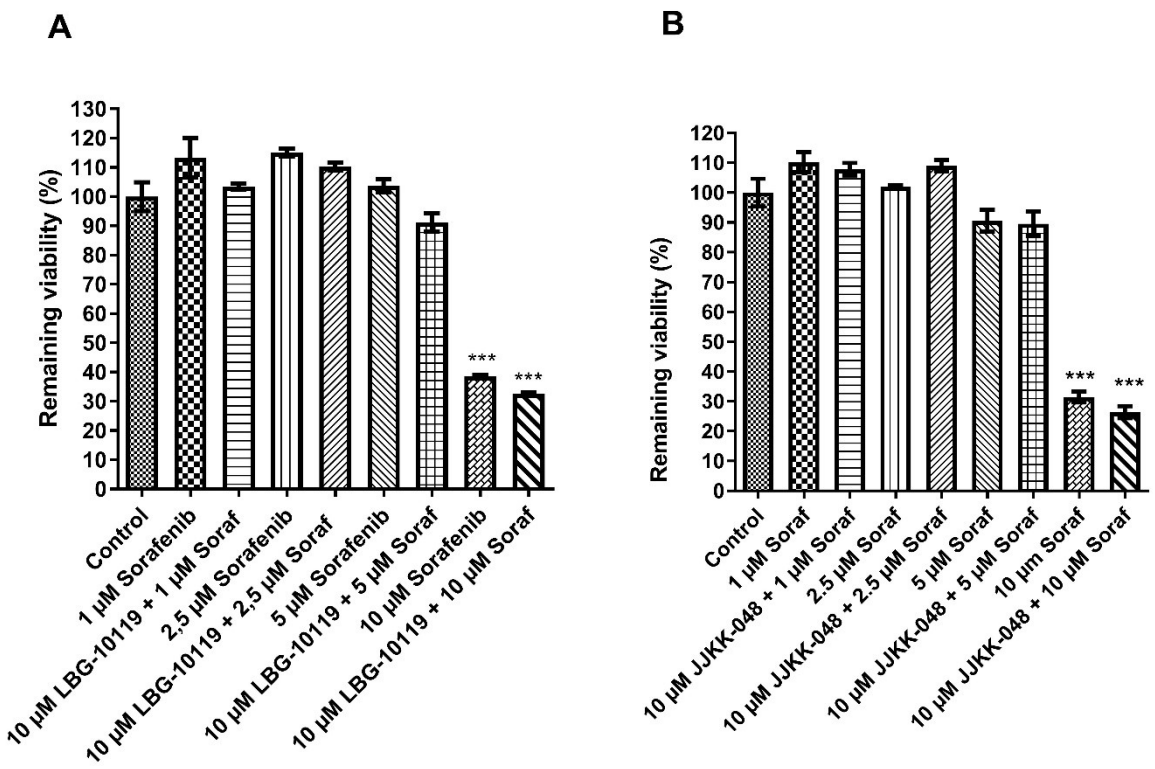

Figure 6: *The potentiating effect of JJKK-048 and LBG-10119 on cytotoxicity of sorafenib in Nras driven p19ARF$^{-/-}$ after 72 hours of incubation. The statistical differences are determined using one-way ANOVA followed by Tukey's test. ***P < 0.001. Data are presented as the mean ± SEM (n=3-4).*

7. DISCUSSION

As it was mentioned before, ever since it was proven that efflux of antineoplastic and other substrates by P-gp can be inhibited by certain compounds (Tsuruo et al., 1981), an intensive effort has been devoted to the development of ABC transporter inhibitors that might be used in combination with standard chemotherapeutic agents to reverse MDR and enhance therapeutic efficacy (Szakacs et al., 2006; Nobili et al., 2006). Unfortunately, three generations of the "MDR modulators" have failed in clinical trials, because of unintended toxicity. Direct inhibitors of ABC transporter family hardly differentiate between normal tissue and a cancerous one. They influence transporter function at basal levels in the whole body, limiting their protective function (Fromm, 2004). The result is the exposure of healthy cells to an elevated intracellular concentration of cytotoxic chemotherapeutics, hence the undesired toxicity of "MDR modulators". Nevertheless, there is still current scientific interest in overcoming the MDR.

There is also another emerging approach in increasing efficacy of anticancer treatment by selective downregulation of resistance genes in cancer cells. Nuclear receptors are a superfamily of different transcription factors that regulate the expression of their target genes by binding to sequence-specific (promoter) elements. Several nuclear receptors have been implicated in drug-induced changes in transporter expression, recognised for their significance in MDR process and implied as potential drug targets, because of their overexpression in various types of cancer (Chen et al., 2012) and their role as regulators of genes that encode phase I (e.g., cytochrome P450) and phase II (e.g., glutathione transferase) enzymes of drug metabolism (Urquhart et al., 2007; Xie and Evans, 2001). The most studied nuclear receptor is pregnane X receptor (PXR), also referred as steroid and xenobiotic receptor or pregnane-activated receptor. PXR regulates replication of both cancer and non-cancer cells, but the expression levels of PXR differ significantly between healthy and cancerous tissues. Targeted PXR antagonists are implied to improve the efficacy of chemotherapeutic treatment by inhibiting the metabolism and the efflux of anticancer pharmaceuticals (Biswas et al., 2009). However, their effects on cancer therapy and development are controversial, and finding of tissue-specific aspect of PXR is of high interest (Robbins and Chen, 2014). Thus, clinically targeting PXR as a therapeutic molecule warrants further investigations. Moreover, PXR receptor flexibility and extreme promiscuity in ligand recognition

and target gene activation make it a challenge to produce predictive ligand or structure-based computational models for PXR (Ekins et al., 2009).

The present study was a part of a research project, which strategy was to overcome chemoresistance by modulating the expression of ABC transporters by interfering in lipid signalling pathway. The aim was to establish a situation, in which cells are unable to promote both, inflammatory microenvironment and ABC transporter-mediated chemoresistance. Therefore two different compounds, JJKK-048 and LGB-110119, the inhibitors of *N*-methyl-*D*-aspartate receptor (GRIN1/2A) and monoacylglycerol lipase (MAGL), respectively, that take a part in the lipid signalling pathway in hepatocarcinoma cells, were employed.

According to previous studies, JJKK-048 is a very potent MAGL inhibitor with half maximal inhibitory concentration $IC_{50} < 0.4$ nM for rodent and human MAGL (Aaltonen et al., 2013). In our study, the inhibitory effect on the accumulation of substrates of ABC transporters was detected at much higher, 5 μM concentration, as can be seen in Figure 5. We further did not see any effect of JJKK-048 indicating potentiation of antiproliferative characteristics of sorafenib. The lack of observed effect might be explained by the fact, that MAGL expression in experimental *in vitro* models is not high enough or MAGL is not playing a significant role in a given type of mutation in cancer. Moreover, the cellular model we employed in this study was murine one and interspecies differences in the regulation of the lipid signalling pathway and expression of ABC transporters could contribute to the lack of observed effect. Nevertheless, our preliminary data show the statistically significant effect (****P < 0.001)* of JJKK-048 at 100 μM concentration in the accumulation studies of ABC transporter substrates ([^{3}H]-digoxin and fluorescein) in mouse hepatocellular carcinoma cells Nras driven p19ARF$^{-/-}$ (data not shown, unpublished data by Gynther et al.).

Recently it was proven that JJKK-048 applied at μM concentration block off-target proteins, including other serine hydrolases (unpublished data). Those off-target proteins, which are present in our *in vitro* models (Nras driven p19ARF$^{-/-}$ and Nras driven p53$^{-/-}$) in a significant amount, are now being investigated, supposedly they also can affect cancer lipid signalling pathway. In a theory, if cancer cell line would have high expression of MAGL, the effect of JJKK-048 would be possible to observe at low concentrations (at 100 nM, or even lower). For example, in experimental studies conducted by Zhang

et al. pharmacological inhibition or knockdown of MAGL succeeded in downregulation of PGE2 (Zhang et al., 2016).

In the Figure 5 JJKK-048 have an influence on sorafenib accumulation at 5 μM in Nras driven $p53^{-/-}$, while the same and a higher concentration of JJKK-048 did not show a significant effect on the accumulation of sorafenib in Nras driven $p19ARF^{-/-}$. The explanation could be behind the different mutations in *p19ARF* and *p53* in 2 cancer cell lines and thus, the different expression level of proteins (transporters).

Recently, it was found out that IC_{50} of LBG-10119 against GRIN1/2A is 15 μM (unpublished data). These data suggest that there is a need for higher concentration of LBG-10119 to show some effect. This might be the reason why the low concentration of LBG-10119 did not have a significant impact in sorafenib cytotoxicity test (Figure 6B) or accumulation assays of probe substances (Figure 4). Our preliminary data show that higher 100 μM concentration of LBG-10119 has a significant effect on sorafenib added cytotoxicity at 1 μM, 2,5 μM and 5 μM with statistical significance at $***P < 0.001$ in Nras driven $p53^{-/-}$ (data not shown, unpublished data by Gynther et al.).

In Figure 4A LBG-10119 at 5 μM significantly affected the accumulation of [^{3}H]-digoxin, but did not affect the accumulation of [^{3}H]-digoxin at 10 μM. The same situation could be noticed in Figure 5A for fluorescein at JJKK-048 concentrations of 5 μM and 10 μM. At this point, there is no clear explanation. The discrepancy in the data might be due to so far limited number and high variability of the experiments. With the gathered information, no clear conclusion could be driven. Nevertheless, this study provided the pilot estimation for further laboratory studies and experiments that will be conducted.

According to a plan the next step of our research would be the use of the same "working" conditions (test compound concentration, incubation time etc.) to quantitate the amount of efflux proteins at the cell membrane with more accurate and precise protein quantification method using Multiplexed Selected/Multiple Reaction Monitoring by liquid chromatography tandem-mass spectrometry (LC-MS/MS) (proteomics). At the time the experiments, which are part of this diploma thesis, were conducted, it was not possible to quantify proteins with LC-MS/MS because establishing and validation of the methodology were still ongoing in the laboratory. Recently the effects of JJKK-048 and LBG-10119 on efflux transporters were quantified using the LC-MS/MS approach

in the laboratory and the expression of P-gp and BCRP was downregulated in Nras driven $p53^{-/-}$ cells after 24 hours of exposure to 10 μM concentration of JJKK-048 and LBG-10119 (unpublished data).

It was hypothesised for this study that interference in the lipid signalling pathway could reduce the overexpression of ABC transporters and thus reduce efflux of anticancer agents and increase their intracellular concentration reaching applicable effective dose to have an apoptotic and cytotoxic effect. According to obtained preliminary data, 10 μM JJKK-048 increased significantly (***$P < 0.001$) antiproliferative efficacy of sorafenib at its 1 μM, 2,5 μM, 5 μM and 10 μM concentration in cytotoxicity study on the cell culture Nras $p53^{-/-}$ (unpublished data). On the other hand, Nras driven $p19ARF^{-/-}$ (Figure 6B) did not show added efficacy in the same conditions. This notion might lead to the suggestion that cancer cell line with a mutation in *p53* is more susceptible to the effect of the tested compound than cancer cell line with mutated *p19ARF*. Consistent with this suggestion, increased COX-2 levels were found in malignancies that expressed mutant protein p53 (Ristimäki et al., 2002). These findings could imply that the balance between activation of oncogenes and inactivation of tumour suppressor genes could modify the level of expression of COX-2 in tumours. Thus, the novel approach in cancer treatment is potentially applicable for cancer types, where AA and therefore, inflammation plays more prominent role in the malignant progression and growth.

8. CONCLUSION

In the present, chemotherapy remains to be one of the prominent strategies to fight a variety of solid as well as systematic malignancies in the human population (Lee et al., 2005; Chabner and Roberts, 2005). Unfortunately, MDR of cancer cells significantly reduces the efficacy of chemotherapy. One of the mechanisms of MDR origin, overexpression of efflux transporters, was closely linked with inflammatory microenvironment in cancer tissues. It opened a new possibility to overcome drug resistance by countering the elevated levels of ABC transporters superfamily with interference in lipid signalling pathway. In particular, in the beginning of AA signalling by aiming two targets: GRIN1/2A or MAGL.

Results from this study, in general, provided support for the involvement of GRIN1/2A and MAGL into the lipid signalling pathway. Therefore inhibition of these two targets helps to reduce overexpression of ABC transporters. Our results, in general, are in agreement with theoretical background. Even though gathered laboratory information shows unambiguous results, valuable data could be withdrawn from it. It would point a direction for further investigation in this field. The discovery that JJKK-048 affected off-target proteins (other serine hydrolases) in μM concentration could open new possibilities and use for experimental substances. Taking these facts into account, targeting the regulatory pathways that drive efflux transporter overexpression could be a promising alternative approach in treating tumour.

Further research needs to be done; for JJKK-048 in the cell lines with high expression of MAGL; for LBG-10119 there is a need to develop more potent analogues. It is possible to continue experiments on animal models or human cell models and proteomics method to prove further an effect of experimental substances on the expression of ABC transporters and to specify which transporters exactly they affect.

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