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STUDY OF PHARMACOKINETIC AND PHARMACODYNAMIC MECHANISMS OF DRUG RESISTANCE AND THEIR MODULATION IN NON-SMALL CELL LUNG CANCER

Doctoral dissertation (article-based)

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AUTHOR'S DECLARATION

I declare that this work is my original authorial work, which I prepared independently (under the guidance of my supervisor: Assoc. Prof. RNDr. Jakub Hofman, Ph.D.). All literature and other sources, from which I drew during the processing, are listed in the list of used literature and properly cited in the thesis. The work was not used to obtain a different or the same title.

In Hradec Králové Yu Zhang, M.Sc.

Date: …… ……..

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ABSTRACT

Lung cancer represents one of the most threatening malignancies, which is attributed by its leading morbidity and mortality among all cancer types. Pharmacological interventions have played impressive roles in the clinical management of non-small cell lung cancer (NSCLC) with the outstanding improvements on patients' survival. Nevertheless, the inevitable emergence of drug resistance severely diminishes their efficacies.

Traditional chemotherapeutic drugs have been introduced in the treatment of NSCLC decades ago. Countless studies showed that the emergence of multidrug resistance (MDR) is deeply associated with two pharmacokinetic factors: (1) increased drug efflux via ATP-binding cassette (ABC) transporters and (2) enhanced drug deactivation by biotransformation enzymes, e.g., cytochromes P450 (CYPs). Previously, we and others have demonstrated that several novel targeted agents can synergistically modulate pharmacokinetic MDR by their interactions with ABC transporters/CYPs and their own anti-cancer activity. Thus, in the first part of our work, we characterized whether selected novel drugs used/intended for the NSCLC therapy could work as such dual-activity chemosensitizers. Four (tepotinib, sonidegib, talazoparib and encorafenib) out of seven tested drugs have gotten confirmed their MDR-modulating roles using *in vitro*, *in silico* as well as *ex vivo* models derived from NSCLC patients' tumor biopsies.

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKis) have revolutionized the pharmacotherapy of NSCLC. Numerous studies have described the mechanisms of drug resistance to EGFR-TKi therapy. However, their results are highly fragmented and lack the evaluation of their potential translation into effective resistance-combatting strategies. Thus, in the second part of this thesis, we aimed to provide a

comprehensive view on this issue. Drug-resistant cell lines were established by at least 10 month-lasting stepwise selection with the first/second/third-generation EGFR-TKis (gefitinib, dacomitinib, osimertinib, respectively). Using various methodologies, including global proteomic analysis, we reported that (1) EGFR-TKi-resistant cell lines exhibit higher invasive levels than the corresponding EGFR-TKi-sensitive variants, (2) cross-resistance is the intrinsic feature of EGFR-TKis, (3) extracellular matrix (ECM)-related signaling, cancer stem cells (CSC)-related pathways, anti-apoptotic protein BCL-2 and efflux transporter ABCG2 universally participates in the development of EGFR-TKi resistance, (4) these signaling pathways/proteins could be the potential targets for synergistic reversal of EGFR-TKi resistance and (5) Hedgehog pathway as well as ECM-related signaling might be important for the lung carcinogenesis.

In conclusion, we have conducted a complex investigation on drug resistance and its modulation within NSCLC. We have provided important findings that might be eventually translated into the effective and safe drug combination regimens beneficial for NSCLC patients suffering from drug-resistant tumors.

ABSTRAKT

Karcinom plic představuje jedno z nejnebezpečnějších zhoubných onemocnění, což se vysvětluje jeho nejvyšší morbiditou a mortalitou mezi všemi typy rakoviny. Farmakologické intervence sehrály v klinické léčbě nemalobuněčného karcinomu plic (NSCLC) působivou roli a výrazně zlepšily přežití pacientů. Nicméně nevyhnutelný vznik lékové rezistence jejich účinnost vážně snižuje.

Tradiční chemoterapeutika byla do léčby NSCLC zavedena již před několika desetiletími. Nesčetné studie ukázaly, že vznik mnohočetné lékové rezistence (MDR) je hluboce spojen se dvěma farmakokinetickými faktory: (1) zvýšený eflux léčiv prostřednictvím transportérů ABC (ATP-binding cassette) a (2) zvýšená deaktivace léčiv biotransformačními enzymy, např. cytochromy P450 (CYPy). Již dříve jsme spolu s dalšími prokázali, že několik nových cílených léčiv může synergicky modulovat farmakokinetickou MDR prostřednictvím kombinace interakcí s ABC transportéry/CYPy a vlastní protinádorové aktivity. V první části naší práce jsme tedy studovali, zda vybraná nová léčiva používaná/určená pro terapii NSCLC mohou fungovat jako tyto chemosenzitizéry s duální aktivitou. U čtyř (tepotinib, sonidegib, talazoparib a enkorafenib) ze sedmi testovaných léčiv jsme potvrdili jejich MDR-modulační role pomocí *in vitro*, *in silico* i *ex vivo* modelů odvozených z nádorových biopsií pacientů s NSCLC.

Tyrosinkinázové inhibitory cílící na receptor pro epidermální růstový faktor (EGFR-TKis) způsobily revoluci ve farmakoterapii NSCLC. Četné studie popsaly mechanismy lékové rezistence k léčbě EGFR-TKi. Jejich výsledky jsou však značně roztříštěné a chybí zhodnocení jejich možného převedení do formy účinných strategií bojujících proti rezistenci. Ve druhé části této práce jsme si proto kladli za cíl poskytnout

komplexní pohled na tuto problematiku. Lékově rezistentní buněčné linie byly vytvořeny nejméně 10 měsíců trvající postupnou selekcí s EGFR-TKis první/druhé/třetí generace (gefitinib, dacomitinib, resp. osimertinib). S využitím různých metodik, včetně globální proteomické analýzy, jsme zjistili, že (1) buněčné linie rezistentní na EGFR-TKis vykazují vyšší invazivitu než odpovídající citlivé varianty, (2) zkřížená rezistence je přirozenou vlastností EGFR-TKis, (3) signalizace související s extracelulární matrix (ECM), dráhy související s nádorovými kmenovými buňkami (CSC), anti-apoptotický protein BCL-2 a efluxní transportér ABCG2 se univerzálně podílejí na vzniku rezistence k EGFR-TKi, (4) tyto signální dráhy/proteiny by mohly být potenciálními cíli pro synergické zvrácení rezistence vůči EGFR-TKis a (5) Hedgehog dráha i signalizace související s ECM by mohly být důležité pro plicní karcinogenezi.

Závěrem lze říci, že jsme provedli komplexní výzkum týkající se lékové rezistence a její modulace v rámci NSCLC. Přinesli jsme důležité poznatky, které by se v budoucnu mohly promítnout do účinných a bezpečných kombinací léků prospěšných pro pacienty trpící lékově rezistentními NSCLC nádory.

LIST OF ABBREVIATIONS

ABC: ATP-binding cassette

ABCB1: P-glycoprotein

ABCC1: multidrug resistance protein 1

ABCG2: breast cancer resistance protein

ALK: anaplastic lymphoma kinase

BER: base excision repair

BRAF: v-Raf murine sarcoma viral oncogene homolog B

CSC: cancer stem cell

CYPs: cytochrome P450s

DDIs: drug-drug interactions

ECM: extracellular matrix

EGFR-TKis: epidermal growth factor receptor-tyrosine kinase inhibitors

EMT: epithelial–mesenchymal transition

FAK: focal adhesion kinase

FN: fibronectin

HER: human epidermal growth factor receptor

HRR: homologous recombination repair

IAPs: inhibitors of apoptosis proteins

KRAS: Kirsten rat sarcoma

MDR: multidrug resistance

MET: mesenchymal-epithelial transition

MMPs: matrix metalloproteinases

NBDs: nucleotide (ATP) binding domains

NER: nucleotide excision repair

NHEJ: non-homologous end joining

NICD: Notch intracellular domain

NSCLC: non-small cell lung cancer

ORR: objective response rate

OS: overall survival

PARP: poly (adenosine diphosphate-ribose) polymerase

PD-1: programmed cell death 1

PFS: progression-free survival

RET: rearranged during transfection

ROS1: ROS proto-oncogene 1

SCLC: small cell lung cancer

TMDs: transmembrane domains

TME: tumor microenvironment

TNF: tumor necrosis factor

TRAIL: TNF-related apoptosis-inducing ligand

YAP1: yes-associated protein 1

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1. INTRODUCTION

Due to its leading morbidity and mortality, lung cancer has undeniably become one of the most threatening malignancy types [1]. Around 80 % – 85 % of lung cancer cases were clinically characterized as non-small cell lung cancer (NSCLC). Apart from surgery and radiation therapy, pharmacological interventions, which mainly include classical chemotherapy and targeted therapy, have been playing crucial roles in the lung cancer managements [2]. Although these drug-based treatments brought outstanding benefits for patients, the emergence of drug resistance has become a tremendous roadblock on the path to successful anti-cancer practice [3].

Multidrug resistance (MDR) is triggered by pharmacokinetic and pharmacodynamic mechanisms. The pharmacokinetic ones, which mainly include drug efflux by ATP-binding cassette (ABC) transporters and drug deactivation by drug metabolizing enzymes, result in the reduction of active drug's concentration in cancer cells [3]. Pharmacodynamic mechanisms mediating drug resistance mainly include the aberration of drug targets and the dysregulation of resistance-related signaling pathways [4]. Although existing studies have described both mechanisms, scientists and physicians have failed to translate the existing knowledge into the effective strategies for combating drug resistance. Therefore, in the present study, we systemically investigated the roles of pharmacokinetic and pharmacodynamic mechanisms in drug resistance and their modulation in *in vitro* and *ex vivo* NSCLC models.

2. THEORETICAL BACKGROUND

2.1. Non-small cell lung cancer and its pharmacological therapies

2.1.1. Basic characterization of non-small cell lung cancer

Cancer persists as a formidable menace, casting a shadow over the human health. Among all the cancer types, lung cancer is undoubtedly the most threatening one. Annually, an estimated 2.2 million individuals are newly diagnosed with lung cancer, meanwhile around 1.8 million deaths are attributed to this terrifying disease. It threatens humans regardless of gender differences. Compared with other cancers, the morbidity of lung cancer ranks the second in men and women in US. Moreover, lung cancer poses the leading cancer-caused death in both genders (**Fig. 1**) [5].

Estimated New Cases

Males				Females		
Prostate	288,300	29%		Breast	297,790	31%
Lung & bronchus	117,550	12%		Lung & bronchus	120,790	13%
Colon & rectum	81,860	8%		Colon & rectum	71,160	8%
Urinary bladder	62,420	6%		Uterine corpus	66,200	7%
Melanoma of the skin	58,120	6%		Melanoma of the skin	39,490	4%
Kidney & renal pelvis	52,360	5%		Non-Hodgkin lymphoma	35,670	4%
Non-Hodgkin lymphoma	44,880	4%		Thyroid	31,180	3%
Oral cavity & pharynx	39,290	4%		Pancreas	30,920	3%
Leukemia	35,670	4%		Kidney & renal pelvis	29,440	3%
Pancreas	33,130	3%		Leukemia	23,940	3%
All Sites	**1,010,310**	**100%**		**All Sites**	**948,000**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	67,160	21%		Lung & bronchus	59,910	21%
Prostate	34,700	11%		Breast	43,170	15%
Colon & rectum	28,470	9%		Colon & rectum	24,080	8%
Pancreas	26,620	8%		Pancreas	23,930	8%
Liver & intrahepatic bile duct	19,000	6%		Ovary	13,270	5%
Leukemia	13,900	4%		Uterine corpus	13,030	5%
Esophagus	12,920	4%		Liver & intrahepatic bile duct	10,380	4%
Urinary bladder	12,160	4%		Leukemia	9,810	3%
Non-Hodgkin lymphoma	11,780	4%		Non-Hodgkin lymphoma	8,400	3%
Brain & other nervous system	11,020	3%		Brain & other nervous system	7,970	3%
All Sites	**322,080**	**100%**		**All Sites**	**287,740**	**100%**

Fig. 1. The estimated distribution of morbidity and mortality of top 10 common cancer types in both sexes. The scheme was adopted from Siegel et al. [5].

Based on cell morphology, lung cancer can be categorized into NSCLC and small cell lung cancer (SCLC). The former variant accounts for approximately 80 – 85 % of the overall lung cancer cases. Considering its enormous incidence and mortality rates, NSCLC has become the disease of interest in this thesis. For further histological classification, NSCLC can be divided into adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma [6]. The treatment for NSCLC mainly depends on tumor's stage and histological subtype as well as patient's conditions. Generally, these approaches include surgery, radiotherapy, chemotherapy (traditional anti-cancer agents and novel targeted drugs), immunotherapy and their combinations [7]. Notably, classical chemotherapy represented by platinum-based drugs and novel targeted therapy represented by epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKis) have greatly improved NSCLC-suffering patients' progression-free survival (PFS) and overall survival (OS) [8]. However, after long-term chemotherapy, the majority of patients unfortunately develop drug resistance, resulting in greatly reduced drug efficacy, tumor relapse, metastasis and even patients' death [9].

2.1.2. Conventional chemotherapy in non-small cell lung cancer

Surgical resection of cancerous tissue is undeniably the principal approach in the treatment of stage I to stage III NSCLC. However, due to the inability of complete elimination of the pathologic tissue, patients often experience cancer recurrence after surgery. Pharmacological interventions have played remarkable roles in the post-surgical regimens of stage II and stage III disease, the standard managements for stage IV disease and the substitutive treatments for the patients, who are not able/suitable/willing to undergo surgery [10,11].

The application of chemotherapy in the clinical management of lung cancer can be traced back to the 1970s (**Fig. 2**). However, the early cytostatic agents (e.g., methotrexate and doxorubicin) did not lead to satisfactory clinical outcomes. Subsequently, until the early 2000s, several chemotherapeutics have been gradually introduced into the clinical treatments for NSCLC, exhibiting improvements in patients' PFS and OS. These agents include platinum analogues, taxanes, vinorelbine, gemcitabine, etoposide, etc. [12-14]. In 1995, a milestone meta-analysis provided powerful evidence that platinum-based therapies in NSCLC could superiorly improve the patients' OS in comparison with the best supportive care group [15]. With the emergence of this evidence, platinum-based drugs in combination with other cytotoxic agents were proposed as a gold standard for the treatment of advanced NSCLC [10,12,16]. Another meaningful and interesting finding was concluded from the clinical evaluation of combination of cisplatin plus pemetrexed in NSCLC. Results showed that pemetrexed is more effective in lung adenocarcinoma rather than in lung squamous cell carcinoma, suggesting that the histopathological differences could lead to varying outcomes in NSCLC patients receiving chemotherapies [17].

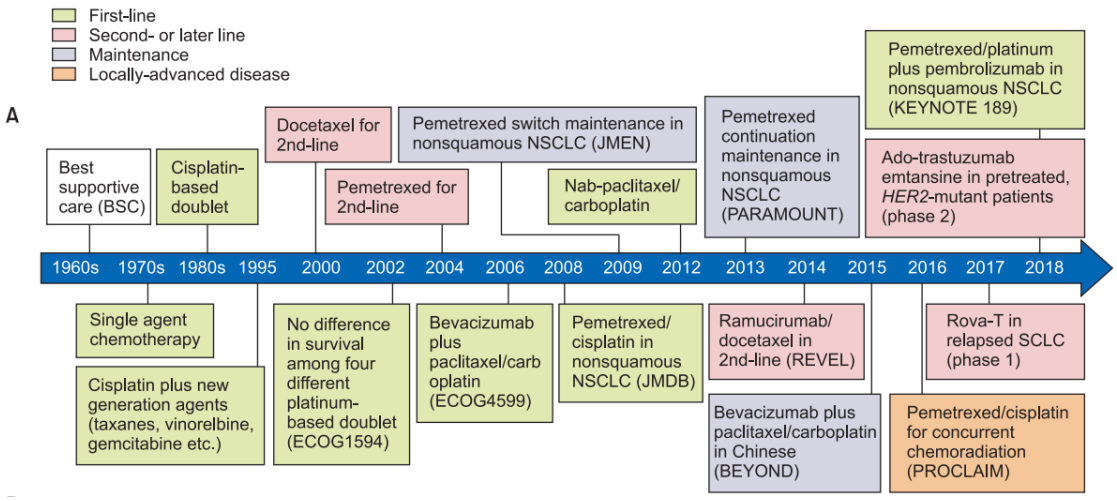

Fig. 2. The timeline of chemotherapies' introduction in the management of lung cancer. NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; The scheme was adopted from Lee et al. [12].

Despite the poor patients' tolerance to conventional cytotoxic drugs, the benefits brought by these agents are irreplaceable. As the research continues, modern approaches (such as small-molecule targeted therapies, immunotherapy and biologicals inhibiting angiogenesis) are being developed to combat NSCLC without producing high systemic toxicities. Moreover, considerable number of clinical studies showed that combining classical chemotherapy with novel approaches could decrease the risk of side effects, meanwhile maintaining or even enhancing drug's anti-NSCLC efficacy [12,18].

2.1.3. Novel targeted therapy in non-small cell lung cancer

As mentioned above, although classical chemotherapy has brought great benefits for NSCLC patients, its accompanying severe side effects represent a crucial problem. As a result, since the 1990s, pharmacologists have been seeking for the new approach to combat NSCLC with high efficiency and low toxicity. By genomic profiling of cancer tissues from NSCLC patients, the vast majority of patients were detected with overexpression or/and mutations of several oncogenes. Intensive studies were conducted on these highlighted members for investigating their cancer-promoting roles. After obtaining a substantial number of exciting findings, some of the oncogenes, such as *Kirsten rat sarcoma* (*KRAS*), *EGFR*, *anaplastic lymphoma kinase* (*ALK*) and *mesenchymal-epithelial transition* (*MET*), were successfully identified as druggable targets for NSCLC treatments [19,20]. To date, dozens of targeted drugs have been approved by the FDA for the treatment of different oncogene-driven NSCLC (**Fig. 3**). Besides, a number of potential candidates are currently undergoing preclinical or clinical trials [21].

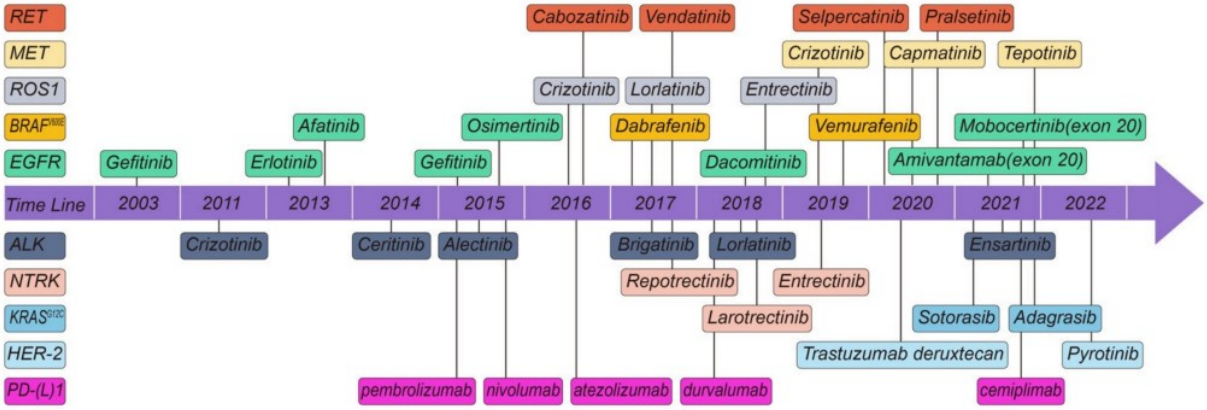

Fig. 3. The timeline of targeted therapy drugs, which were approved by FDA for the treatment of NSCLC. The scheme was adopted from Guo et al. [21].

In 2003, gefitinib (an EGFR inhibitor), the first targeted therapeutic agent for NSCLC, was approved by FDA [22]. It also symbolized a new era in the clinical treatment of this cancer type. EGFR is a transmembrane tyrosine kinase receptor, which serves as an upstream molecule of various intracellular signaling pathways governing a variety of cellular biological activities. In tumor cells, activation of EGFR signaling pathway promote tumorigenesis and proliferation, inhibits apoptosis and regulates autophagy and metabolism [23]. In NSCLC, approximately 17 % of patients harbor mutations in *EGFR* gene (**Fig. 4**) [20]. Interestingly, this prevalence in Asian population is substantially higher, with a staggering 62 % of cases exhibiting such phenomenon [24]. Until now, three generations of EGFR-TKis have been approved for the treatment of NSCLC. Compared to traditional chemotherapeutic agents, EGFR-TKis have substantially improved the median PFS and OS in NSCLC patients [25]. However, the 20-years journey of EGFR-TKi development is closely linked to the emergence of acquired drug resistance in NSCLC. The causing mechanisms and further details are described in the section **2.2.2.1.**

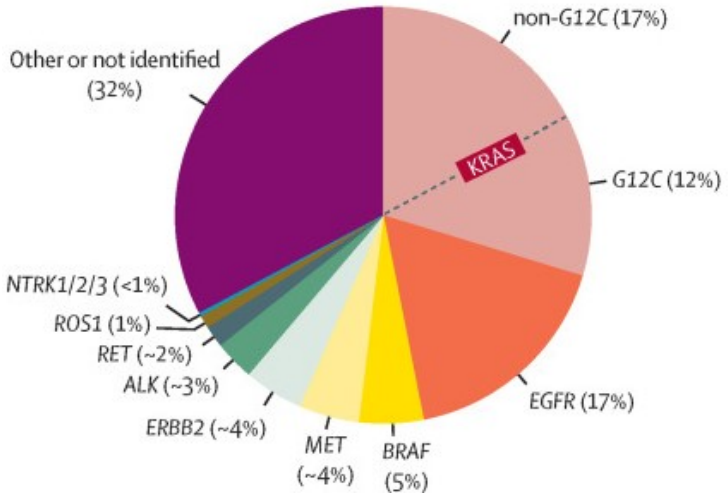

Fig. 4. Frequency of mutated oncogenes' presence in 4064 metastatic NSCLC patients [26]. The scheme was adopted from Thai et al. [20].

Another important genetic phenomenon is *ALK* gene rearrangement, which occurs in around 3 % of NSCLC patients (**Fig. 4**) [20]. Similar to EGFR signaling, transmembrane tyrosine kinase receptor ALK activates its downstream pathways to regulate tumor proliferation, invasion, migration, angiogenesis and suppress cellular apoptosis [27]. Crizotinib, approved in 2011 by FDA, was the first oral ALK-TKi using for the treatment of locally advanced or metastatic NSCLC with positive *ALK* fusion [28]. Compared with cytotoxic agent group, NSCLC patients enrolled in crizotinib group exhibited better outcomes in terms of median PFS and objective response rate (ORR) [29,30]. However, similar to EGFR-TKis, almost all patients administrated with crizotinib eventually developed acquired drug resistance [31]. To address this problem, the second generation of ALK-TKis, including ceritinib, alectinib and brigatinib, were respectively approved for the treatments *ALK*-positive NSCLC to overcome crizotinib resistance or enhance the efficacies after crizotinib pre-treatment [32]. In recent years, a third generation of ALK-TKi, lorlatinib, was also approved by FDA for the clinical application in *ALK*-positive NSCLC [33]. Besides, another third-generation drug,

ensartinib, is currently undergoing phase III clinical evaluation for the *ALK*-positive NSCLC (NCT05341583).

Other important oncogenes include *KRAS*, *v-Raf murine sarcoma viral oncogene homolog B* (*BRAF*), *MET*, *rearranged during transfection* (*RET*), *ROS proto-oncogene 1* (*ROS1*), etc. (**Fig. 4**) [20]. Targeted therapies developed for mentioned genes and their mutant forms have also exhibited remarkable efficacies in the treatment of NSCLC. Mutations in *KRAS* are commonly detected in NSCLC patients. Although dysregulation of *KRAS* has been proved to promote cancer cell proliferation, invasion and migration, it had been long-term considered as an undruggable target due to the lack of deep pockets for interacting with specific inhibitors [34,35]. Encouragingly, the efforts from pharmacologists have been successfully translated into the approval of sotorasib (in 2021) and adagrasib (in 2022) for the treatment of *KRAS G12C*-mutated NSCLC by FDA [36,37]. Another successful attempt is the development of MET inhibitors, capmatinib and tepotinib, which received the approval from FDA for the indication of metastatic NSCLC harboring *MET* exon 14 skipping in 2020 and 2021, respectively [38]. Besides, remarkable progress has been achieved in the development of RET inhibitors. For example, pralsetinib and selpercatinib, were granted accelerated and regular approval by FDA for combating *RET* fusion-positive NSCLC [39,40].

2.2. Drug resistance in the treatment of cancer

Undeniably, pharmacological interventions have brought superior benefits to the survival of patients suffering from NSCLC. Unfortunately, drug resistance inevitably occurs in almost all patients, regardless of the use of conventional chemotherapy or novel targeted therapy [31,41,42]. During tumor treatment, the emergence of drug resistance

not only greatly diminishes the therapeutic effects of drugs, but also contributes to invasion, metastasis and even causes patients' death. It is estimated that above 90 % of mortality cases of tumor patients are triggered by drug resistance [3]. The mechanisms of anti-cancer drug resistance can be broadly divided into two categories: target-dependent and target-independent manners. The former refers to the alterations of the drug target itself (usually mutation and/or overexpression), which negatively affects drug-target binding. In contrast, the latter involves various processes associated with interactions of anti-cancer drugs with cancer cells, such as drug transport and metabolism, DNA damage repair, regulation of cell proliferation/death pathways, regulation of oncogene signaling or tumor microenvironment (TME), etc [43]. In this thesis, we mainly focused on drug resistance to (1) traditional chemotherapeutics and (2) the well-established novel targeted therapy (EGFR-TKis) in NSCLC.

2.2.1. Pharmacokinetic mechanisms of drug resistance

2.2.1.1. ATP-binding cassette transporters

ABC transporters are a group of transmembrane proteins, which consists of 49 identified members in human [44]. These members are categorized into seven subfamilies, namely ABCA to ABCG, in accordance with their amino acid sequences. Generally, the majority of ABC transporters (e.g., P-glycoprotein, also called P-gp or ABCB1) consist of two transmembrane domains (TMDs) and two nucleotide (ATP) binding domains (NBDs). However, the number of TMD and NBD might be different in some special cases. For instance, breast cancer resistance protein (BCRP or ABCG2) has only one TMD and one NBD, while multidrug resistance protein 1 (MRP1 or ABCC1) contains three TMDs, two NBDs and one intracellular linker region. From a structure-function perspective, the

NBD, localized in the cytoplasm, is highly conservated and can hydrolyze ATP to generate energy for transporting substrates. On the contrary, the specificities of the substrates recognized by ABC transporters are determined by the hydrophobic and structurally distinct TMDs, which can cross cell membrane and form the channels to allow specific substrate efflux [45,46].

The expression of ABC transporters can be detected in various tissues, organs and body barriers. Their core physiological function is to transport endogenous or exogenous substances (mostly toxins) out of the cell thereby protectively maintaining the homeostasis of the interior milieu [47]. At the same time, they play important role in pharmacokinetics (governing drug-substrates' absorption, distribution and excretion) and pharmacokinetic drug-drug interactions (DDIs). It has been concluded that ABCB1, ABCG2 and ABCC2 actively participate in clinical DDIs, affecting the plasma levels of cytotoxic antibiotics, camptothecins, antihistamines and digoxin [48,49]. Particularly, the necessity of investigating the possible interactions between novel drugs with ABCB1 and ABCG2 has been emphasized by FDA and EMA [50,51].

Aberrant expression/function of ABC transporters has been proven to be related to a wide range of human diseases, e.g., cystic fibrosis, Alzheimer's disease, dyslipidemia and type 2 diabetes [52]. In addition, numerous studies have reported that ABC transporters are frequently overexpressed during carcinogenesis. Furthermore, some of the members were suggested to promote cancer invasion and metastasis via the crosstalk with other signaling pathways [53]. Importantly, ABC transporters can pump anti-cancer drugs out of the cancer cells, thus decreasing drugs' intracellular concentration, and thus eventually help cells to harbor the MDR property. Among all 49 members, ABCB1, ABCG2 and ABCC1 have been well-described to effectively establish MDR in cancer *in*

vitro and *in vivo* [54,55]. Unfortunately, a large proportion of anti-cancer drugs have been identified as victims of ABC transporter-mediated MDR, including but not limited to taxanes, anthracyclines, topoisomerase II inhibitors, camptothecins and novel targeted drugs [55,56].

2.2.1.2. Cytochrome P450s

The vast majority of drugs undergo the metabolic process after they enter the body. The main purpose of two-phase drug metabolism is to convert the lipophilic substrate to hydrophilic compound, yielding the highly water-soluble products that are more vulnerable to excretion [57].

Cytochrome P450s (CYPs) are the most important enzymes mediating phase I drug metabolism. From a wider perspective, CYPs catalyze approximately 75 % of the total drug metabolic events [58]. To date, a total of 57 human CYP isoforms have been identified, which are categorized into 18 families and 43 subfamilies [57]. These members are mainly distributed in mitochondria and endoplasmic reticulum, and their positive expression can be detected in all major organs throughout the body, with the highest expression in liver, intestine, lung and brain [59]. Studies have well-demonstrated the essential roles of CYPs in drug metabolism and mediating DDIs [57]. Accordingly, eight CYP isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5) are listed in the relevant guidelines as DDI sites to be tested [50,51].

It has been widely reported that CYPs are associated with cancer progression and could serve as biomarker for prognosis. The expression of CYPs does not show a constant positive/negative regulation of tumorigenesis [60]. For example, negative associations

were verified between the presence of CYP11A1 and the development of various tumor types [61]. In contrast, overexpression of CYP1B1 were found in lung, breast and liver cancer, etc. [62]. On the other hand, the relatively unambiguous conclusions have been obtained regarding the presence of CYPs and their involvement in the emergence of MDR. In cancer cells, chemotherapeutic agents might undergo the deactivation by CYPs, which reduces or even loses their anti-cancer effects [3]. These potential victims include not only conventional chemotherapeutics, but also novel targeted drugs such as imatinib and gefitinib [60,63]. Given the fact that (1) a single cancer cell can express multiple CYP isoforms and (2) isoforms from the same CYP family may have similar substrate range [64,65], most of the existing works based on multi-enzymatic cellular models do not accurately reflect the effect of single CYP isoform on MDR. Our recent studies in CYP2C8-, CYP3A4- and CYP3A5-transduced HepG2 cell models showed that overexpression of CYP3A4 can induce cellular resistance to docetaxel [66]. It is important to be aware that CYPs can also activate few chemotherapeutic agents. For example, the expression of CYP2B6 and CYP2C19 is important for the activation of cyclophosphamide [67].

2.2.1.3. Modulation of pharmacokinetic multidrug resistance – past and the future

Numerous studies have revealed the remarkable potential of the aforementioned pharmacokinetic mechanisms as targets for enhancing the efficacy of anti-cancer drugs. However, early pharmacological attempts to inhibit neither ABC transporters nor CYPs have clinically overcome drug resistance in cancer.

Since the 1980s, drugs represented by verapamil and cyclosporine A were found to counteract ABCB1-mediated MDR *in vitro* [68]. These drugs were subsequently confirmed to have inhibitory effects on ABCC1 as well. Nevertheless, the practical value of the first generation of drug transporter inhibitors was quickly dismissed due to their low activities and high systemic toxicities. To achieve the ideal outcomes, pharmacologists modified the structures of the first-generation drugs, resulting in the development of the second-generation ABC transporter inhibitors, such as dexverapamil (for ABCB1) and valspodar (for both ABCB1 and ABCC1). Indeed, these drugs exhibited greater inhibitory effects on ABCB1/ABCC1 and lower cytotoxicities. Yet, they failed to show sufficient potencies in clinical investigations and were evidenced to inhibit CYPs leading to the induction of unexpected DDIs [69,70]. In contrast, ABCG2 was discovered relatively late (in 1998), and its first functional inhibitor, fumitremorgin C, was also reported in the same year [71]. Meanwhile, the third-generation inhibitors of ABC transporters have been developed, in response to the unsatisfactory clinical effects from the second-generation MDR modulators. These agents effectively suppress the activity of ABC transporters at the nanomalor level, produce lower toxic effects and most of them do not interact with CYPs, thus causing less DDIs. These potent MDR modulators developed during this period included MK571 (targeting ABCC1, ABCC2 and ABCC4), Ko143 (targeting ABCG2), LY335979 (also called zosuquidar, targeting ABCB1), XR-9576 (also called tariquidar, targeting ABCB1 and ABCG2) and LY475776 (targeting ABCB1 and ABCC1), etc. Regrettably, follow-up studies found that many of them were substrates of ABC transporters (e.g., XR-9576) or failed to potentiate the effects of chemotherapeutic agents in oncological patients (e.g., LY335979) [70,72-74]. Noteworthy, researchers have observed the co-expression phenomena for ABCB1, ABCG2 and ABCC1 in several cancer types [75,76]. This might lead to a broader MDR

capacity in these cancer cells [77]. Although ABCB1, ABCG2 and ABCC1 are structurally different, studies showed that they show significant substrate overlap and can substitute for each other in the case of inhibiting one member [78-80]. Therefore, agents that simultaneously inhibit multiple ABC transporters would have greater potential as favorable weapons against MDR in cancer than the single transporter-targeting compounds.

In contrast, studies focusing on the regulation of MDR by targeting CYPs are scarce. Existing findings also do not indicate good application prospects. Combination therapy with ketoconazole (a CYP3A4 inhibitor) could greatly reduce the clearance of docetaxel but, at the same time, increase the risk of febrile neutropenia [81]. Other failed examples are CYP1B1 inhibitors: α-naphthoflavone and its analogues. Their clinical values against cisplatin and docetaxel resistance were hurdled by poor water solubility, low binding affinity or high systemic toxicity [82,83].

As time progressed, novel targeted drugs have been developed and gradually applied in the treatment of cancer. These agents have outstanding pharmacological effects while also inhibit the mechanisms that promote pharmacokinetic MDR (ABC transporters and/or CYPs). In combinatorial therapy, the “new-generation” dually-acting modulators could synergically enhance the anti-cancer effect of MDR victims, allowing for the reduction of administrated doses and incidence of toxic reactions. Critically important is a targeted nature of novel MDR modulators, as it ensures that the synergistic effects are developing selectively in tumor tissues. Laboratory results from us and other research teams have identified several targeted-therapy agents, such as imatinib, erlotinib, alectinib and ensartinib, which can act as dual-activity chemosensitizers against ABC transporter-mediated MDR [74,84,85]. Encouragingly, several clinical trials have been

conducted for assessing the effect of targeted agent in combination of conventional cytostatic in the treatment of several solid tumors [86].

2.2.2. Pharmacodynamic mechanisms of drug resistance

2.2.2.1. Mutations and/or amplification of drug's target

Target mutation/amplification is believed to be the most important pharmacodynamic aspect of tumor resistance toward anti-cancer drugs. The importance of this mechanism is reflected by the developmental history of several drug groups such as EGFR-TKis. In 2003 and 2005, gefitinib and erlotinib, representing the first generation of EGFR-TKis, were approved by FDA for the treatment of NSCLC, respectively [22,87]. These two inhibitors reversibly bind to the wild type of EGFR and its basic mutations with exon 19 deletion and L858R [10,88]. However, given the fact that patient did not receive significant benefits and suffered from the quick occurrence of drug resistance, the agents were regrettably withdrawn by the drug regulatory authorities [89,90]. Mechanistic studies revealed that approximately 50 - 60 % of drug-resistant patients were detected with T790M mutations in exon 20 of *EGFR*, implying that the presence of T790M mutant is likely to be associated with acquired resistance to the first-generation EGFR-TKis [91]. Thus, the second-generation EGFR-TKis (dacomitinib and afatinib) were introduced into drug-based therapies for NSCLC [92,93]. This generation of inhibitors is characterized by interacting with a broader range of human epidermal growth factor receptor (HER) family members (including EGFR and its T790M mutant) and exhibits irreversible drug-target binding [94,95]. Nevertheless, the therapeutic outcomes were not satisfactory in the majority of cases with the risk of tumor relapse [96]. In this context, the third-generation of EGFR-TKis exhibiting high selectivity and irreversible inhibition of *EGFR*-

T790M mutant were developed to respond to clinical treatment demands. Two representative drugs from this generation, osimertinib and olmutinib, were approved for the treatment of *EGFR*-T790M mutation-positive NSCLC in USA and South Korea, respectively [97,98]. The third-generation drugs offer profound improvements in PFS and OS in NSCLC patients, leading them to be recommended as the first-line agents for this disease. Frustratingly, acquired drug resistance inevitably emerges as a lingering shadow on the path to achieve successful EGFR-TKi therapies. Available studies suggested that the presence of *EGFR*-C797S mutants may be associated with developing drug resistance to the third generation of EGFR-TKis in NSCLC [99,100]. Not surprisingly, overcoming cancer drug resistance only via target-dependent mechanisms seems to have entered a dead-end cycle as new mutants continue to appear in therapeutic targets. On the other hand, taking target-independent mechanisms into account may effectively circumvent this dilemma. Hence, in the second part of the present research work, we devoted to investigating the universal target-independent mechanisms of acquired drug resistance to three generations of EGFR-TKis.

2.2.2.2. Activation of DNA repair system

A variety of classical chemotherapeutics produce DNA damage in cancer cells, while the enhancement of DNA repair pathways could antagonize their DNA-damaging effects thus protect cancer cell from drugs' stimuli. These pathways, such as base excision repair (BER), nucleotide excision repair (NER) and recombinational repair, are commonly overactivated in chemotherapy-insensitive cancer cells [101].

When cellular DNA is damaged by oxidation, deamination and alkylation, DNA glycosylases recognize and remove the damaged base, marking the initial of BER process.

The exposed abasic site is further cleaved by APE1 and PNKP. Meanwhile, poly (adenosine diphosphate-ribose) polymerase (PARP) protects the gap formed due to single strand break and recruit repair-necessary protein. Finally, double-stranded DNA is recovered by long patch (DNA polymerases δ/ε and PCNA) or short patch mechanism (DNA polymerase β, DNA ligase IIIα and XRCC1) [102]. Dysregulation of BER key enzymes (such as, APE1 and PARP) are demonstrated to clearly link with cancer MDR [103]. Encouragingly, several BER antagonists have been successfully approved into the medical market, for example, PARP inhibitors talazoparib and olaparib [104,105].

In the case of DNA damage caused by UV light or DNA cross-linking agents, NER is activated to eliminate the DNA lesioned area. NER is divided into two pathways: global genome NER and transcription-coupled NER. The former can repair the damage throughout the whole genome, which is initiated by XPC-RAD23B or UV-DDB recognizing DNA lesions. The latter begins with the binding of RNA polymerase to a DNA lesion, thus mediates rapid repairing on the transcribed strand of active genes [106]. Afterwards, the region of DNA damage is removed by the combined action of TFIIH, XPG and ERCC1 complex, RPA and XPA. Finally, the resulting DNA single strand is repaired by recruitment of DNA polymerases and DNA ligase 1/3 via RFC and PCNA [107]. Overexpression of XPF and ERCC1 are commonly observed in cisplatin-resistant cancer cells. However, no ERCC1 or XPC-inhibiting agents have been approved for anti-cancer applications [3].

Other DNA damage repair mechanisms also play important roles in promoting MDR in cancer cells. For example, recombinational repair consists of homologous recombination repair (HRR) and non-homologous end joining (NHEJ), is known to be hyperactivated in chemotherapy-resistant lung cancer and ovarian cancer [108,109].

DNA-PK and PARP are identified as crucial mediators in the process of NHEJ, while ATM, ATR, CHK 1/2 and WEE1 act as key enzymes of HRR. Apart from PARP inhibitors, agents targeting these enzymes are currently undergoing clinical trials in multiple cancer types, such as berzosertib (targeting ATR; NCT04216316), adavosertib (targeting WEE1; NCT03579316) and nedisertib (targeting DNA-PK; NCT04555577) [101,110].

2.2.2.3. Extracellular matrix-related signaling

Within TME, a variety of cell types are embedded in a supportive platform of extracellular matrix (ECM), that allows the cells to proceed with many biological behaviors. ECM is a complex environment, which consists of a number of proteins and other molecules, including collagen, fibronectin, integrin, elastin, laminin, proteoglycans etc. (**Fig. 5**). These components form a massive network by crosslinking and binding to each other, which supports and provides the structure to cells and tissues. During tumor development, ECM's composition and its mechanical properties can be altered by cellular/non-cellular factors, mainly characterized by its stiffness and degradation. Alterations in ECM also affect the cell-matrix and cell-cell communications via the interactions between the key members of ECM and cellular membrane receptors with their governed signaling pathways. Numerous studies have proven that ECM and its related signaling are deeply associated with the regulation of tumor progression, proliferation, invasion, migration, and responses to chemotherapeutics. Therefore, understanding the complex interactions between cancer cells and ECM is necessary for developing effective anti-cancer strategies.

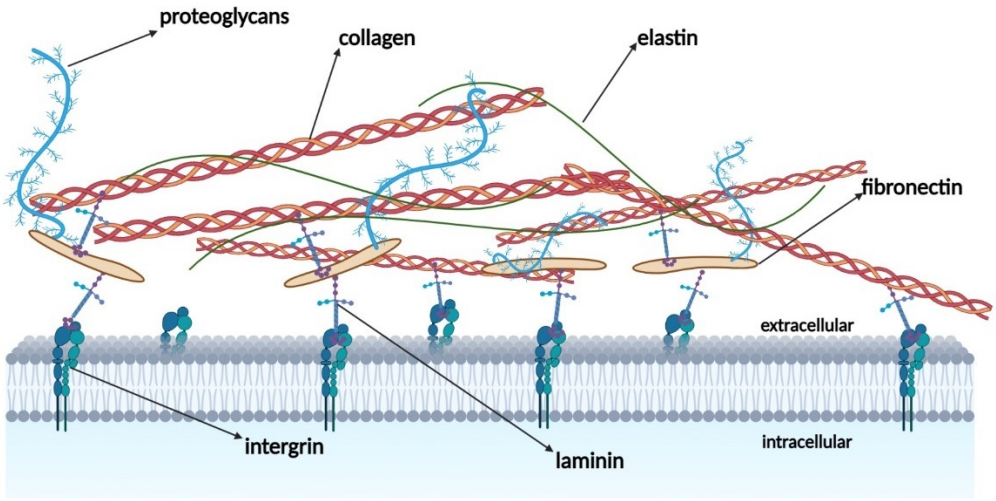

Fig.5. Structure of ECM.

Collagen is one of the most predominant members of ECM. It is composed of three left-handed helix α chains, twisting to each other to form a right-handed triple helix [111]. Among 28 discovered subtypes, collagen type I is identified as the most abundant one accounting for 90 % of organic components of the body [112]. In most of cases, collagen type I serves as a promoter in tumor development. Its upregulations are frequently recorded in various types of tumors, including NSCLC, gastric cancer, papillary thyroid cancer, breast invasive carcinoma, liver hepatocellular carcinoma etc. Besides, other studies have reported that collagen type I overexpression contributes to the increasing levels of metastasis, proliferation, angiogenesis and stemness in multiple cancer types. During tumor chemotherapy, collagen type I not only constitutes a physical protective barrier around the cells, but also activates the expression of drug resistance-related pathways or genes, thereby reducing the sensitivities of cancer cells to drug treatments [113]. It was reported that EGFR-TKi resistance in lung adenocarcinoma can be induced by collagen type I-mediated mTOR activation [114]. Another study in pancreatic cancer showed that 3D-structured collagen type I can promote gemcitabine resistance [115].

Fibronectin (FN) is a glycoprotein that consists of two monomers joined together by two disulfide bonds located at the C-terminus of the protein [116]. It is generated by cancer-associated fibroblasts and can assemble with other ECM members, including itself, thereby providing a supportive platform for various biological behaviors of the cells, especially for invading into the surrounding stroma [117,118]. It has been noted that FN can have contradictory effects on the progression of tumors. Early studies suggested that cellular FN could suppress tumor progression, while stromal FN is beneficial to tumor cell growth. As research progressed, substantial evidence proved that cellular FN could facilitate cell proliferation, angiogenesis, invasion and metastasis in various tumor types, and act as a biomarker of poor prognosis. Although there are possible explanations for this self-contradictory role of FN in tumor progression, researchers have been unable to reach a unified consensus yet [118,119]. In contrast, a positive regulatory relationship between FN overexpression and cancer drug resistance has been widely recognized, especially in lung cancer [120]. In NSCLC, the presence of FN1 has been shown to enhance the cellular resistance to chemotherapeutic agents such as cisplatin, docetaxel and EGFR-TKis [121-123].

Other ECM members also play their own irreplaceable roles in ECM assembling and regulating tumor biological activities, including drug resistance. For instance, integrin can promote cancer progression, invasion, metastasis by its intensive crosstalk with oncogenes and growth factor cytokines [124,125]. Additionally, studies also concluded that integrin could mediate the development of cancer drug resistance in an ECM- /cancer stem cell (CSC)- /immune response-dependent manner [126]. Laminin was reported to have the similar invasion-promoting function in tumor progression [127]. Its drug resistance-promoting effects also have been confirmed in various solid cancer types [128-130].

As mentioned above, ECM members can regulate the biological behavior of tumors by interacting with ECM-related signaling. ECM-related signaling pathways and their pro- or anti-tumoral functions have been widely discussed in numerous of studies. One remarkable ECM-related pathway is focal adhesion kinase (FAK) signaling. ECM components, including collagen, FN and integrin, can interact with FAK, thus activating its downstream signaling, such as Erk, PI3K, Src, JNK, etc., to promote cell growth, invasion, metastasis and drug resistance (**Fig. 6**). Encouragingly, FAK has been identified as a suitable druggable target for cancer treatments. FAK-targeting agents, such as defactinib and IN10018, have been showing promising anti-cancer outcomes in preclinical and clinical evaluations [131]. Another essential pathway highly associated with ECM is yes-associated protein 1 (YAP1) signaling. Massive number of studies have showed that high YAP1 activity positively regulates proliferation, invasion, stemness, immune escape and chemotherapy resistance in cancer cells via the crosstalk with ECM members and its related signaling (**Fig. 6**). Although the development of anti-cancer agents targeting YAP1 signaling (e.g., verteporfin) is not as impressive as hitting FAK signaling, YAP1's potential as a druggable target should not be underestimated [132]. Besides, molecules, such as PLOD1 protein, CD44 antigen, matrix metalloproteinases (MMPs), have also been well-confirmed to show close associations with ECM, therefore participating in the regulation of tumor progression and its related chemotherapy effects [133-135].

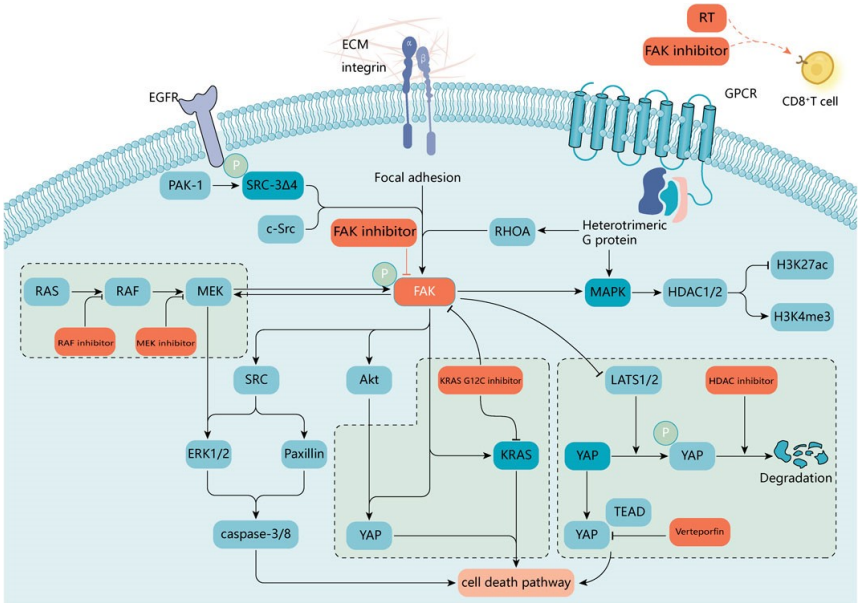

Fig. 6. FAK signaling, YAP1 signaling and their crosstalk. The scheme was adopted from Zhang et al. [136].

2.2.2.4. Cancer stem cell-related signaling

CSCs possess a remarkable ability for self-renewal and differentiation, enabling them to drive tumor initiation and recurrence [137]. Up to now, several CSC-associated molecules, such as CD44, CD133 and Ep-CAM, have been characterized as biomarkers for tumor diagnosis and prediction of treatment outcome [138]. The active roles of CSCs in the regulation of tumor angiogenesis, growth, proliferation, metastasis have been extensively reported [139]. Another crucial property of CSCs is their intrinsically lower sensitivity to chemotherapeutic agents, which can be explained by the following mechanisms: (1) the formation of CSC niches, (2) overexpression of ABC transporters, such as ABCG2 and ABCC1, (3) drug inactivation by biotransformation enzyme, (4) entering quiescence and dormancy, (5) active participation of DNA damage repair system, (6) dysregulation of survival/apoptosis-related pathways, (7) self-regeneration via

epithelial–mesenchymal transition (EMT) and (8) perishing with normal cells after receiving the treatments [140]. Hence, CSCs and their regulating factors, such as ECM, CSC-related signaling or hypoxia condition, could induce the emergence of drug resistance in tumors [141].

Many intracellular signaling pathways have been identified as CSC-related signaling, such as Wnt pathway, Notch pathway, Hedgehog pathway, PI3K pathway and NF-κB pathway [142]. Targeting Hedgehog pathway has exhibited promising prospect with several FDA-approved specific inhibitors (vismodegib, sonidegib, and glasdegib) in application for anti-cancer treatments [143]. Hedgehog pathway is mainly constituted by three different Hh ligands, surface receptor PTCH, signal transducer SMO, regulator Kif7, three types of transcription factors Gli1/2/3 and suppressor SUFU (**Fig. 7**) [144]. Aberrantly high expression level of Hedgehog pathway has been observed in various types of tumors. In tumor progression, it is involved in the regulation of tumor proliferation, metastasis, self-renewal behavior by controlling target genes' expression (e.g., CD44, c-Myc, snail and jagged-1) [141]. The roles of Notch pathway in cancer progression have been well elucidated, whereas there are still no approved Notch pathway inhibitors for cancer clinical managements [145]. The characteristic feature of the Notch pathway is the three-step cleavage of the receptor molecule. After undergoing the first cleavage by Furin in Golgi apparatus, four different Notch receptors (Notch-1/2/3/4) are assembled across the cell membrane. In total, five kinds of ligands can bind to Notch receptors, and thus, the receptors undergo the second cleavage by ADAM. Afterwards, with the third cleavage by γ-secretase, Notch intracellular domain (NICD) is generated and translocated into the nucleus. Last, in cell nucleus, NICD binds to the transcriptional factor CSL to regulate a series of genes' expression, including *Myc*, *p21*, *Cyclin D1/3*,

HER2 etc. (**Fig. 8**). Similar to Hedgehog pathway, it has been broadly implied that Notch pathway can regulate tumor progression through different aspects [146].

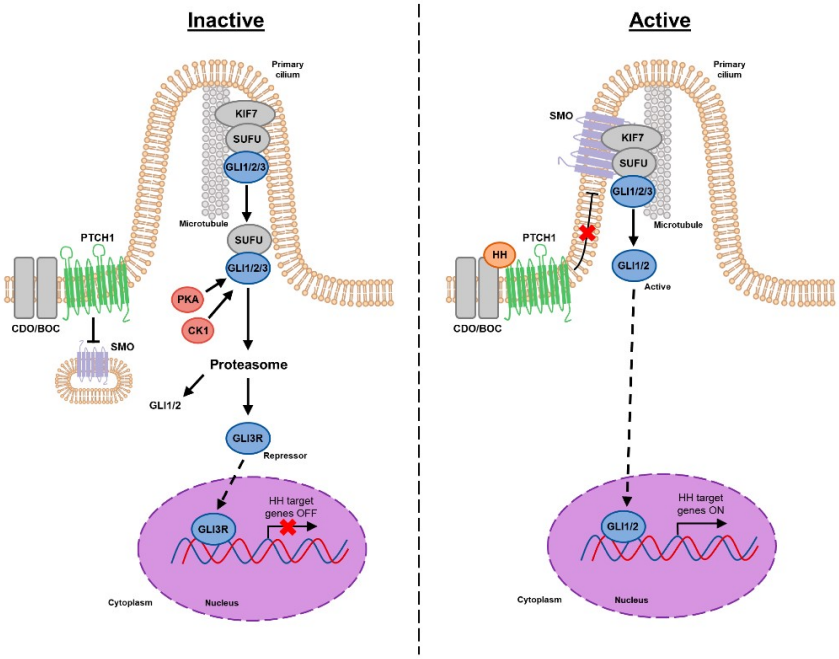

Fig. 7. The structure of Hedgehog pathway. The scheme was adopted from Doheny et al. [147].

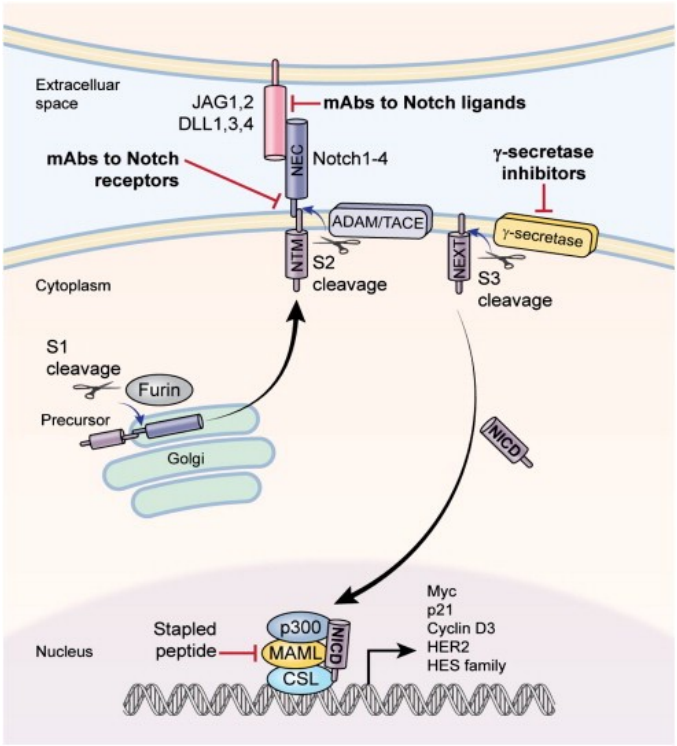

Fig. 8. The structure of Notch pathway. The scheme was adopted from Takabe et al. [146].

Moreover, both Hedgehog and Notch pathways have been verified to facilitate the development of drug resistance during lung cancer treatments [148,149]. For example, a clinical study revealed the deep association between platinum-resistance in NSCLC and hyperactivity of Hedgehog pathway [150]. Overactivation of Hedgehog pathway also led to EGFR-TKi-resistance in NSCLC cell lines [151,152]. In a similar manner, Notch pathway also plays a promotive role for emerging drug tolerance in NSCLC toward a wide range of chemotherapeutics (e.g., cisplatin, paclitaxel, gemcitabine, erlotinib, gefitinib) [153]. Although these two pathways have shown substantial potentials as promising targets for tumors treatments, there are currently no FDA-approved Hedgehog/Notch pathway-targeting drugs for treating lung cancer. In clinical trials for lung cancer, only few agents have accomplished phase I investigations, such as Notch inhibitors LY900009 and PF-03084014 [154,155], or are undergoing phase I or phase II studies, such as Hedgehog inhibitors sonidegib and vismodegib (NCT04007744; NCT04591431).

2.2.2.5. Apoptosis-related pathways

One important hallmark of drug resistant cells is resisting apoptosis, a process that is induced by the drug treatments. Therefore, dysregulation of pro- and anti-apoptotic pathways has been represented as one of the most common mechanisms causing drug resistance in tumors [156].

Cells can undergo apoptosis through extrinsic and intrinsic mechanisms, also known as death receptor pathway and mitochondrial pathway, respectively (**Fig. 9**)[157]. After membrane death receptors such as tumor necrosis factor (TNF) receptor and TNF-related apoptosis-inducing ligand (TRAIL) receptors, are stimulated by their ligands,

caspase-8 is activated. Caspase-8 activation directly induce the cleavage of caspase-3, which produces apoptosis-triggering signal. In contrast, mitochondrial pathways are activated by specific exogenous death stimulations (e.g., drug treatments). Cytochrome c is released by the regulation of BCL-2 protein family, and then forms the complex with Apaf-1 and caspase-9, consequently leading to the cleavage of caspase-3 [158]. Activation of both death receptor and mitochondrial pathways are complicated processes that are regulated by a number of signaling molecules. They appear to be mechanistically independent, but in fact affect each other through signaling molecules' interactions. Indeed, these apoptosis-associated members play important roles in the development of cancer drug resistance and its modulation. In this section, we only introduce some representative members.

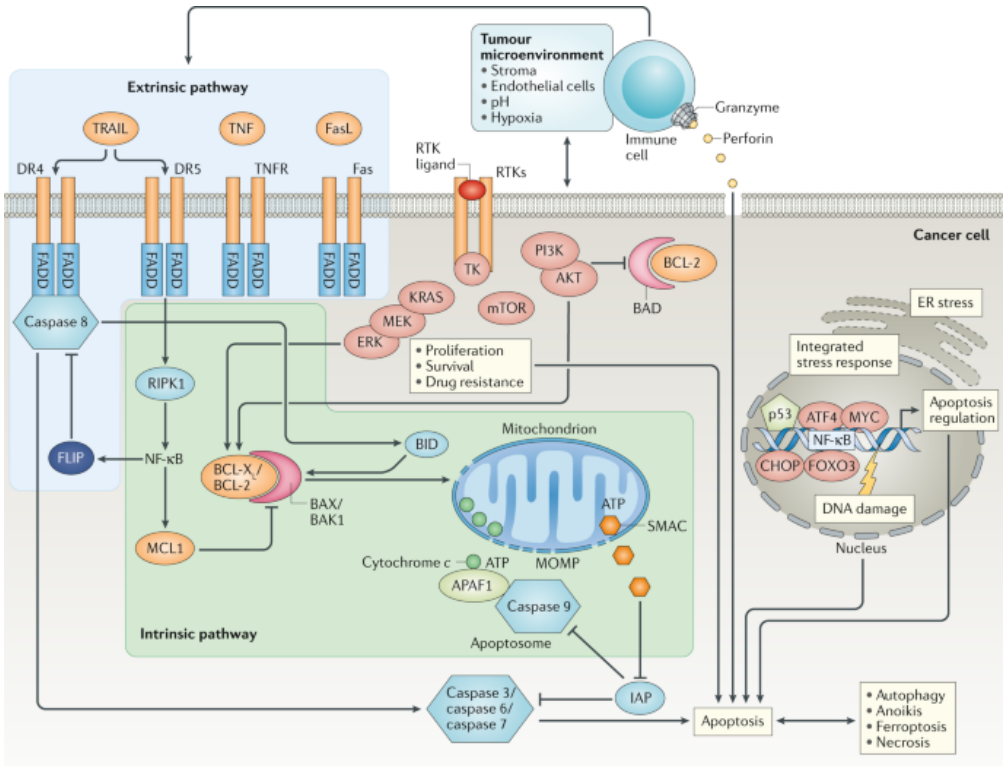

Fig. 9. Extrinsic and intrinsic pathways of apoptosis and their network. The scheme was adopted from Carneiro et. al. [157].

As mentioned above, TRAIL induces the onset of apoptosis by activating death receptor pathways, therefore, it is recognized as a pro-apoptotic member [159]. One interesting phenomenon is that TRAIL and its receptor-mediated apoptosis only influences tumor cells but not normal cells, which greatly highlighted the advantage of TRAIL-based therapies in cancer treatments [160]. In addition, TRAIL-based regimens have shown promising potential in combating chemotherapy resistance. For instance, TRAIL was proven to alleviate cisplatin resistance in urothelial cell carcinoma [161]. The antiproliferative effects of paclitaxel and cisplatin on ovarian cancer cells can be potentiated by the exposure to TRAIL [162]. Moreover, researchers have also discovered that TRAIL signaling can interact with other pathways to promote the proliferation, invasion, and metastasis of cancer cells [163]. Accordingly, several attempts have been proposed to improve TRAIL-based therapies, such as: combination with other chemotherapeutics, gene therapy or applying TRAIL sensitizers [164].

BCL-2 family is one of the most well-known and deeply-investigated regulators of cell apoptosis. This family consists of pro-apoptotic (e.g., BCL-2, BCL-xL) and anti-apoptotic (e.g., BAX, BAK, BAD, BIM) members, which are majorly localized to the outer membrane of mitochondria and the endoplasmic reticulum [165]. Studies have clarified that BCL-2 family regulates cell apoptosis process mainly by governing mitochondrial outer membrane permeabilization and releasing cytochrome c [166]. Owing to its key role in controlling apoptotic cell death, dysregulation of BCL-2 family members has been documented as one of the universal mechanisms promoting carcinogenesis and cancer chemotherapeutics tolerance [167,168]. Particularly, *BCL-2* was the first identified anti-apoptotic gene [169]. Agents developed as BCL-2 inhibitors (e.g., venetoclax, navitoclax, LP-118) have been approved or are currently under clinical investigations for various of naive/relapsed neoplasms [167,170]. However, a recent

review concluded that single administration of BCL-2 inhibitors is likely not to be efficient enough for combating solid tumors. In contrast, the drug combination approaches using BCL-2 inhibitors with other anti-cancer drugs exhibited relatively suitable anti-cancer outcomes with good pharmacokinetic profiles [171].

Both extrinsic and intrinsic apoptosis can be endogenously suppressed by inhibitors of apoptosis proteins (IAPs), which consists of eight identified members, including NAIP, cIAP1, cIAP2, XIAP, survivin, Apollon, Livin and ILP2. IAP family inhibits cellular apoptosis mainly via the direct interactions with effector caspases (caspase-3 and -7) and/or their upstream initiator caspase (caspase-9) [172,173]. Apart from the blockade of cell death, studies have also uncovered that IAPs participate in regulating cell cycle, cell division and signaling transductions [174]. In consequence, IAPs' expression levels were frequently upregulated during the development of tumors and the acquisition of drug resistance [175]. The significant roles of IAPs in tumor progression have greatly interested the scientists and clinicians to consider IAP-inhibiting strategies for cancer treatments, such as applying antisense oligonucleotides and Smac mimetics. Although several anti-cancer attempts have clinically failed when applying IAP-oriented antisense oligonucleotides or solely administrating Smac mimetics, combinatory therapies with conventional chemotherapeutics have achieved promising outcomes [175,176]. On 2020, Debio 1143, an IAP antagonist, has been granted by FDA with a Breakthrough Therapy Designation for its co-administration with cisplatin in head and neck cancer [177]. Up to now, two phase III clinical trials have been recruited for further evaluating Debio 1143's therapeutic efficacy for head and neck cancer (NCT05386550, NCT04459715).

Aside from apoptosis, cells are able to undergo other death types, which include but are not limited to autophagy, necroptosis and ferroptosis [178]. Numerous studies have clearly demonstrated that non-apoptosis-related signaling is also involved in the enhancement of cellular tolerance to classical chemotherapy, novel targeted drugs and immunotherapy. It is essential to note that modulation of these pathways in different tumor types may results in varying outcomes. Consequently, addressing the proper non-apoptosis-related signaling needs to be carefully considered in overcoming drug resistance [179-181].

3. AIM OF THE WORK

In this study, we aimed to explore the roles of pharmacokinetic and pharmacodynamic mechanisms in anti-cancer drug resistance and their modulation in NSCLC. The sub-aims are listed as below:

I. investigation on the interactions between novel targeted drugs and ABC transporters and their potentials for acting as dual-activity chemosensitizers,

II. profiling the inhibitory effects of novel targeted drugs on different CYP isoforms and their utilization for antagonizing docetaxel resistance,

III. establishment of NSCLC cell lines resistant toward drugs from different generations of EGFR-TKis and their characterization in terms of cross-resistance and invasive properties,

IV. investigation on the universal roles of pharmacodynamic and pharmacokinetic factors in the establishment of EGFR-TKi resistance and their modulation,

V. bioinformatic analysis assessing comparisons of our obtained results with publicly available databases and evaluating the implications of resistance-related signaling in the genesis of lung tumors.

4. RESULTS AND CANDIDATE'S PARTICIPATIONS

This dissertation thesis is organized as an annotated set of seven published original research articles and one submitted manuscript. The candidate is the first author of two published articles and one manuscript. All the publications/manuscripts have been accepted by or submitted to international journals with impact factor. The outlines of the publications and the candidate's contributions are listed below.

4.1. Vagiannis D., **Zhang Y.**, Novotna E., Morell A., Hofman J.: Entrectinib reverses cytostatic resistance through the inhibition of ABCB1 efflux transporter, but not the CYP3A4 drug-metabolizing enzyme. *Biochem Pharmacol* 2020; 178:114061, **$IF_{2019/2020}$ = 4.960, Q1**.

- Co-author: performance of MTT assay and qRT-PCR in NSCLC cell lines, participation in data analysis.
- Percentage of contributions: 15 %.

4.2. Vagiannis D., Budagaga Y., Morell A., **Zhang Y.**, Novotná E., Skarka A., Kammerer S., Küpper J-H., Hanke I., Rozkoš T., Hofman J.: Tepotinib Inhibits Several Drug Efflux Transporters and Biotransformation Enzymes: The Role in Drug-Drug Interactions and Targeting Cytostatic Resistance *In Vitro* and *Ex Vivo*. *Int J Mol Sci* 2021; 22(21):11936, **$IF_{2021/2022}$ = 6.208, Q2**.

- Co-author: performance of western blotting in primary culture samples, participation in data analysis and figures visualization.
- Percentage of contributions: 10 %.

4.3. Vagiannis D., **Zhang Y.**, Budagaga Y., Novotna E., Skarka A., Kammerer S., Küpper J-H., Hofman J.: Alisertib shows negligible potential for perpetrating pharmacokinetic drug-drug interactions on ABCB1, ABCG2 and cytochromes P450, but acts as dual-activity resistance modulator through the inhibition of ABCC1 transporter. *Toxicol Appl Pharmacol* 2022; 434:115823, **$IF_{2021/2022}$ = 4.460, Q2**.

- Co-author: performance of part of antiproliferative assay in NSCLC and MDCKII lines.
- Percentage of contributions: 15 %.

4.4. **Zhang Y.**, Vagiannis D., Budagaga Y., Sabet Z., Hanke I., Rozkoš T., Hofman J.: Sonidegib potentiates the cancer cells' sensitivity to cytostatic agents by functional inhibition of ABCB1 and ABCG2 *in vitro* and *ex vivo*. *Biochem Pharmacol* 2022; 199:115009, **$IF_{2021/2022}$ = 6.100, Q1.**

- First author: performance of major part of experimental work, including drug accumulation studies *in vitro*, MTT-based cytotoxicity and drug combination assay, qRT-PCR, enzyme inhibition study and western blotting for primary culture samples; participation in designing the experiments; data analysis and visualization; writing the manuscript.
- Percentage of contributions: 50 %.

4.5. Morell A., Budagaga Y., Vagiannis D., **Zhang Y.**, Laštovičková L., Novotná E., Haddad A., Haddad M., Portillo R., Hofman J., Wsól V.: Isocitrate dehydrogenase 2 inhibitor enasidenib synergizes daunorubicin cytotoxicity by targeting aldo-keto

reductase 1C3 and ATP-binding cassette transporters. *Arch Toxicol* 2022; 96(12):3265-3277, **IF$_{2021/2022}$ = 6.168, Q1.**

- Co-author: performance of MTT assay (including drug combination) in A431 cell sublines and part of qRT-PCR analysis; assisting data collection and analysis.
- Percentage of contributions: 10 %.

4.6. Sabet Z., Vagiannis D., Budagaga Y., **Zhang Y.**, Novotná E., Hanke I., Rozkoš T., Hofman J.: Talazoparib Does Not Interact with ABCB1 Transporter or Cytochrome P450s, but Modulates Multidrug Resistance Mediated by ABCC1 and ABCG2: An *In Vitro* and *Ex Vivo* Study. *Int J Mol Sci* 2022; 23(22):14338, **IF$_{2021/2022}$ = 6.208, Q2.**

- Co-author: performance of MTT assay (including drug combination) in A431 sublines, enzyme inhibition study and western blotting for primary culture samples; participation in data analysis and figure visualization.
- Percentage of contributions: 10 %.

4.7. **Zhang Y.**, Vagiannis D., Budagaga Y., Sabet Z., Hanke I., Rozkoš T., Hofman J.: Encorafenib Acts as a Dual-Activity Chemosensitizer through Its Inhibitory Effect on ABCC1 Transporter *In Vitro* and *Ex Vivo*. *Pharmaceutics* 2022; 14(12):2595, **IF$_{2021/2022}$ = 6.525, Q2.**

- First author: performance of MTT-based cytotoxicity and drug combination assay in A431 and its ABC transporter-transduced cell lines, western blotting for primary culture samples and enzyme inhibition study;

designing experimental work; preparation and finalization of figures and manuscript.

- Percentage of contributions: 45 %.

4.8. **Zhang Y.**, Gültekin O., Kupčík R., Budagaga Y., Vagiannis D., Sabet Z., Lehti K., Hofman J.: Drug resistance mechanisms across three different EGFR inhibitor generations are associated with co-targetable alterations in invasion, extracellular matrix, stemness, apoptosis and efflux transporter proteins in lung cancer. *In submission*.

- First author: performance of major part of experimental work, including 2D&3D cell culture, establishing drug-resistant cell lines, MTT-based antiproliferative and drug combination assay, ddPCR, western blotting and part of bioinformatic analysis; participation in designing the whole project; data analysis; figures visualization; writing the manuscript.
- Percentage of contributions: 40 %.

5. DESCRIPTION OF THE RESULTS

Aim I.: Investigation on the interactions between novel targeted drugs and ABC transporters and their potentials for acting as dual-activity chemosensitizers.

We started this study by investigating the interactions of several novel targeted drugs with MDR-causing ABC transporters (ABCB1, ABCG2 and ABCC1). The antagonistic abilities of the chosen drugs toward ABC transporter-mediated MDR were evaluated *in vitro* in immortalized cell lines and *ex vivo* in primary explants prepared from patients' NSCLC biopsies. All the results have been presented in seven published papers (**4.1.** – **4.7.**), which are listed in the section above.

In paper **4.1.**, **4.2.**, **4.3.**, **4.4.**, **4.5.**, **4.6.** and **4.7.**, entrectinib, tepotinib, alisertib, sonidegib, enasidenib, talazoparib and encorafenib were investigated, respectively. These drugs either have been approved or are undergoing the clinical trials for the treatment of NSCLC. Results from drug accumulation assay showed that the tested drugs exhibited varying levels of functional inhibition of selected ABC transporters. Specifically, enasidenib inhibited all tested ABC transporters (ABCB1, ABCG2 and ABCC1). Tepotinib and sonidegib showed moderate to strong inhibition of ABCB1 and ABCG2, while talazoparib potently inhibited the drug-efflux functions of ABCG2 and ABCC1. In the study focusing on entrectinib, the drug only showed the inhibitory effect on ABCB1. For alisertib and encorafenib, these two drugs selectively suppressed the drug efflux function of ABCC1.

In follow-up studies, we investigated whether the inhibitory effects of the tested drugs could modulate ABC transporter-mediated resistance to cytostatic agents (ABCB1 and ABCC1: daunorubicin; ABCG2: mitoxantrone). Drug combination assays were performed, and then the obtained data were quantified by Chou-Talalay method for

determining the combination effects. Our results proved that the tested drugs are able to synergistically enhance cytotoxicities of daunorubicin and/or mitoxantrone in ABC transporter-overexpressing cells. In paper **4.7.**, encorafenib was found to effectively antagonize ABCC1-mediated resistance not only to daunorubicin, but also to topotecan.

Considering that the phenotypic and biological properties of tumor cells are well preserved in primary cultured explants [182,183], we explored the possible clinical implications of our *in vitro* results (paper **4.2.**, **4.4.**, **4.6.** and **4.7.**) using primary tumor cells derived from NSCLC patients. Tumor biopsies were donated by patients treated at University Hospital Hradec Králové. After tumor resection and inspection by the surgeon and pathologist, respectively, we isolated primary NSCLC cells in our lab based on the published protocols with minor modifications [184-188]. Next, we evaluated the expression levels of ABCB1, ABCG2 and ABCC1 in these samples by western blotting. Subsequently, drug accumulation and drug combination assays were performed *ex vivo*. Results showed that the tested drugs can improve the anti-cancer effects of MDR victim cytostatics by functionally inhibiting highly expressed ABC transporters in primary NSCLC explants.

Additionally, we investigated whether tested drugs can be the victims of transporter-mediated resistance. Although in papers **4.2.** and **4.3.**, we proved that tepotinib and alisertib can be transported by ABCB1, comparative proliferation studies showed that the overexpression of MDR-associated ABC transporters (ABCB1/ABCG2/ABCC1) does not attenuate the antiproliferative effects of any of the drug mentioned in *Aim I.*, including tepotinib and alisertib.

Last, we evaluated the potential of the tested drugs to induce ABC transporter-mediated MDR. Gene induction studies were performed in several cancer cell lines

(Caco-2, LS174T, A549, NCI-H1299, NCI-H1975, NCI-H2228, HCC827, $KG_{1\alpha}$ and MCF-7), according to the clinical applications or trials of examined drugs. Our qRT-PCR data showed that the short-term exposure of tested drugs does not trigger significant changes in the expression of *ABCB1*, *ABCG2* and *ABCC1*.

Altogether, tested drugs were proven to effectively modulate ABC transporter-mediated cytostatic resistance by their reasonable inhibitory effects on one or more transporter(s). Encouragingly, tested drugs neither provoked the mRNA expression alterations of ABC transporters nor were positioned as MDR victims. These *in vitro* and *ex vivo* data demonstrated that tested drugs (in papers **4.1.** – **4.7.**) could be potentially exploited as dual-activity chemosensitizers in combination regimens against NSCLC.

Aim II.: Profiling the inhibitory effects of novel targeted drugs on different CYP isoforms and their utilization for antagonizing docetaxel resistance.

Another crucial pharmacokinetic factor contributing to the emergence of MDR and DDIs is the drug metabolism catalyzed by CYPs. We have previously reported that CYP3A4 diminishes cellular responsiveness to docetaxel treatment, while ensartinib counteracts docetaxel resistance by suppressing the CYP3A4 activity [66,189]. Thus, within subaim II, we described the interactions of novel targeted drugs with main CYP isoforms (CYP1A2, CYP3A4, CYP3A5, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP2D6) to investigate whether they could modulate CYP-mediated MDR and/or perpetrate clinically relevant DDIs. Relevant results have been published in paper **4.1.**, **4.2.**, **4.3.**, **4.4.**, **4.6.** and **4.7.**.

To initially characterize the inhibitory potentials of tested drugs toward the CYP isoforms mentioned above, we performed CYP inhibitory assays by using commercial Vivid CYP screening kits. In paper **4.1.**, **4.2.**, **4.3.** and **4.6.**, eight different CYP isoforms relevant for pharmacokinetic DDIs were investigated. The tested drugs exhibited different inhibitory effects on examined CYP isoforms. Specifically, entrectinib (paper **4.1.**) strongly inhibited CYP3A4, CYP3A5, CYP2C8, CYP2C9, CYP2C19 and CYP2D6. Tepotinib (paper **4.2.**) showed potent inhibitory effects on CYP3A4 and CYP2C9. Alisertib (paper **4.3.**) produced only moderate to minor inhibition of examined CYPs. Talazoparib (paper **4.6.**) exhibited negligible inhibition of all examined CYPs. In paper **4.4.** and **4.7.**, we only focused on CYP3A4 isoform. Both sonidegib (paper **4.4.**) and encorafenib (paper **4.7.**) did not suppress the activity of CYP3A4 enzyme with potential clinical relevance. To evaluate drugs' potentials for modulation of CYP3A4-mediated docetaxel resistance, we conducted P450-Glo CYP3A4 inhibitory assay in HepG2-CYP3A4 cellular model for those drugs, which showed meaningful CYP3A4 inhibition at recombinant enzyme level. Tepotinib and alisertib showed no or low inhibition of CYP3A4 isozyme at cellular level. Although a reasonable inhibitory effect was recorded for entrectinib, the drug failed to effectively sensitize HepG2-CYP3A4 cells to docetaxel treatment.

In papers **4.1.** – **4.3.**, we also investigated whether tested drugs might alter the mRNA expression levels of *CYP1A2*, *CYP2B6* and/or *CYP3A4,* and thus cause induction-based pharmacokinetic interactions. Positive upregulation was found for *CYP2B6* after exposure to alisertib. However, expressional increase did not translate into the functional changes as negative data were obtained in the following CYP2B6 enzyme activity experiments.

To sum up, we provided a comprehensive overview on the inhibitory/induction properties of tested drugs toward clinically relevant CYP enzymes. This knowledge brings important pieces of information for the use of drugs in combination with other pharmacotherapeutic agents. Besides, we brought an information about possible MDR-reversing abilities of examined drugs associated with the blocking of CYP3A4 activity; unfortunately, none of the tested drugs exhibited potential for combating CYP3A4-mediated resistance to docetaxel.

Aim III.: Establishment of NSCLC cell lines resistant toward drugs from different generations of EGFR-TKis and their characterization in terms of cross-resistance and invasive properties.

In fact, the occurrence of acquired drug resistance in tumors is a process that requires long-term and repeated drug exposure and involves multiple causing mechanisms that can be used as targets for overcoming MDR. Therefore, establishment and subsequent analysis of drug-resistant cell lines have become an irreplaceable approach for investigating the resistance drivers. During the use of EGFR-TKi-based therapies in management of NSCLC, clinicians have been inevitably facing the emergence of drug resistance, which sharply overshadowed the outstanding efficacies of drugs from all the generations of EGFR-TKis [42]. To comprehensively describe this phenomenon and provide possible strategies for surmounting this hurdle, we set up *Aim III. – Aim V.* in the present work. All the relevant methodologies and results have been described in manuscript **4.8.**.

To start this systematic study, we selected candidates from each generation of EGFR-TKis (1st: gefitinib; 2nd: dacomitinib; 3rd: osimertinib). HCC827 and NCI-H1975

cell lines were chosen with respect to the expression of specific *EGFR* mutations, which are identified as the targets of selected candidates. Drug-resistant cell lines were established by stepwise selection method. After at least 10-months drug exposure, we successfully generated three different drug-resistant cell lines that are strongly and stably tolerant to gefitinib (HCC827/GEF), dacomitinib (NCI-H1975/DAC) and osimertinib (NCI-H1975/OSI), respectively.

Compared to the regular 2D cell culture, 3D cell culture enables a more realistic mimicking of the pathophysiological microenvironment associated with tumor growth. To evaluate the invasive potentials of our tested cells, we established the protocol for 3D cell culture in our lab based on the collaboration with Prof. Kaisa Lehti's group (Karolinska Institutet, Stockholm, Sweden). After cells' cultivation in collagen-rich hydrogels, we observed remarkable morphological changes in tumoroids, especially in the drug-resistant ones. Obtained results indicated that NSCLC cells exhibit stronger invasive behaviors after developing acquired resistance to all three tested EGFR-TKis.

Cross-resistance is a phenomenon, in which cancer cells, upon becoming resistant to a specific therapeutic agent, simultaneously exhibit tolerance to other drugs from the same pharmacodynamic group [190]. Our data revealed that cross-resistance universally occurs between drugs from the same generation of EGFR-TKis.

Aim IV.: Investigation on the universal roles of pharmacodynamic and pharmacokinetic factors in the establishment of EGFR-TKi resistance and their modulation.

The emergence of acquired drug resistance is a complex process driven by multiple mechanisms. To uncover the possible target-independent mechanisms of EGFR-

TKi resistance, we conducted the global proteomic analysis in the cooperation with Rudolf Kupčík, Ph.D. (University Hospital Hradec Králové, Hradec Králové, Czech Republic). In total 1786 – 1793 proteins were identified and quantified in tested drug-resistant cells and corresponding parental cells. Then, proteomic data were proceeded with pathway analysis using Enrichr and Metascape (collaboration with Prof. Kaisa Lehti's group). Remarkably, ECM-related signaling showed significant alterations in all three drug-resistant variants, indicating its possible universal participation in the acquisition of EGFR-TKi resistance in NSCLC cells.

With respect to our proteomic data and its pathway analysis, we investigated whether ECM-related signaling could be truly implicated in the development of EGFR-TKi resistance and might represent a target for its modulation. First, western blotting results showed that two ECM-related signaling pathway members (FAK and YAP1) were significantly upregulated in EGFR-TKi-tolerant cell lines. Then, drug combination studies and Chou-Talalay assay were performed to evaluate whether FAK and YAP1 could serve as the promising targets for antagonizing EGFR-TKi resistance. As a result, inhibition of FAK and YAP1 synergically enhanced the antiproliferative effects of EGFR-TKis in our established drug-resistant cell lines. Another crucial information conveyed from proteomic analysis is that FN1 might be the key ECM-associated member for mediating dacomitinib and osimertinib resistance. Thus, we examined the protein expression of several essential ECM members (e.g., FN1, CD44, PLOD1, MMP2). Abnormal upregulation of FN1 were observed in both NCI-H1975/DAC and NCI-H1975/OSI, compared to the parental NCI-H1975. Knocking down the expression of FN1 effectively sensitized drug-resistant cells to dacomitinib and osimertinib treatments, respectively. Therefore, we concluded that ECM and its associated signaling actively and universally participate in establishing drug tolerance toward three generations of EGFR-

TKis in NSCLC. Last, apart from resistance reversal studies, we also investigate whether ECM-related signaling (FAK and YAP1) could be responsible for gaining hyperinvasive behaviors in EGFR-TKi-resistant cells. Interestingly, suppressing FAK signaling, rather than YAP1 signaling in dacomitinib- and osimertinib-resistant tumoroids effectively counteracted their invasive activities.

CSCs have been proved as important factor intrinsically forming drug-resistant tumors. Their activities are strongly regulated by ECM-related signaling [191]. Considering that CD44 (a CSCs marker) was overexpressed in tested drug-resistant cells, we investigated the roles of CSC-related pathways in the establishment of EGFR-TKi resistance. Western blotting results showed that the expression levels of Hedgehog and Notch signaling were significantly increased both in dacomitinib- and osimertinib-resistant cells. Following resistance reversal studies revealed that blocking Hedgehog and Notch pathways can overcome NSCLC cells' resistance to both dacomitinib and osimertinib. These findings proved that CSC-related pathways can positively regulate the development of EGFR-TKi resistance in NSCLC cells. Suppressing CSC-related pathways could universally attenuate drug tolerance to the second and third generations of EGFR-TKis.

One of the crucial reasons allowing for cancer cells survival following the exposure to anti-cancer drugs, is the dysregulation of apoptosis-associated signaling [192]. In this work, we examined six different pro-apoptotic/anti-apoptotic genes' expressions by performing ddPCR assay. Notably, the mRNA levels of *BCL2* were significantly increased in HCC827/GEF, NCI-H1975/DAC and NCI-H1975/OSI cells. This finding was additionally confirmed in western blotting data. By conducting resistance reversal studies, we found that inhibiting BCL-2 can synergistically potentiate the anti-cancer

effects of dacomitinib and osimertinib in NCI-H1975/DAC and NCI-H1975/OSI, respectively. However, in gefitinib-resistant HCC827 cells, suppressing BCL-2 only produced negligible effects on alleviating drug resistance. To sum up, these data suggested that BCL-2 protein may play a target role in combating drug resistance to higher generations of EGFR-TKis.

Pharmacokinetic mechanisms also play an important role in the development of cancer drug resistance [3]. Among three MDR-involved ABC transporters, ABCG2 was universally overexpressed in all our established EGFR-TKi-resistant cell lines. Functional inhibition of ABCG2 produced dacomitinib and osimertinib re-sensitizing effects in drug-resistant cells but failed to antagonize gefitinib resistance. Therefore, ABCG2 was identified as a key pharmacokinetic factor influencing the emergence of dacomitinib and osimertinib resistance in NSCLC cells.

Taken together, we presented several universal mechanisms of EGFR-TKi resistance that were of pharmacodynamic (ECM- and CSC-related signaling, BCL-2 protein) and pharmacokinetic (ABCG2 transporters) nature. Besides, our work has shown the potential of targeting signaling pathways FAK, YAP1, FN1, Notch, Hedgehog, BCL-2 and ABCG2 as effective means for combating resistance to EGFR-TKis.

Aim V.: Bioinformatic analysis assessing comparisons of our obtained results with publicly available databases and evaluating the implications of resistance-related signaling in the genesis of lung tumors.

Research on cancer drug resistance has been also extensively conducted by other teams. Comparing our proteomic results with the data from similar studies allowed us to

evaluate the universality of our findings. Hence, we conducted additional analysis for open-accessed transcriptomic data from gefitinib-resistant HCC827 cells (GSE122005) and osimertinib-resistant NCI-H1975 cells (GSE200893), both of which were sourced from different laboratories [193,194]. Pathway analysis indicated significant differences in the predominant resistance-inducing mechanisms for the same EGFR-TKi-resistant models originating from different laboratories. Despite this, ECM-related signaling was repeatedly highlighted for its universal role in regulating EGFR-TKi resistance.

In the last part, we investigated whether revealed resistance-associated signaling could participate in the lung tumorigenesis. An open-accessed database TNMplot was employed for comparing the gene expression level between healthy and tumor tissue [195]. Results showed that abnormal upregulations of *PTK2*, *YAP1* and *SMO* were frequently observed in lung tumors. We additionally performed pathway analysis in database GSE19804 [196]. During lung cancer development, pathways associated with ECM, cell migration and proliferation might be actively involved. Collectively, our bioinformatic data showed the contributive effects of ECM-related and Hedgehog signaling in lung tumorigenesis, suggesting their potentials to act as the novel targets for NSCLC treatments.

6. SUMMARY, CONCLUSIONS AND PERSPECTIVES

Lung cancer represents a formidable malignancy, which is primarily attributed to its unfavorable prognosis in patients. Non-small cell lung cancer is the most frequently diagnosed type of lung cancer. Although significant progress has been achieved in pharmacological interventions for NSCLC, the emergence of drug resistance remains a persistent shadow on the clinical success of classical chemotherapies and novel targeted regimens [197,198]. Numerous studies have well-described the mechanisms of cancer drug resistance. Unfortunately, existing findings are usually missing the converting of theoretical results into practical actions against this problem. Moreover, exploring the universal mechanisms of drug resistance is highly meaningful for scientists and clinicians to surpass this obstacle. In the present work, we aimed to investigate the possible modulatory roles of novel targeted anti-NSCLC drugs in pharmacokinetic resistance to classical cytostatics. In addition, we systematically elucidated the shared pharmacokinetic and pharmacodynamic mechanisms of acquired drug resistance among three generations of EGFR-TKis. Last, we also evaluated whether these involved mechanisms could be targetable for reversing EGFR-TKi resistance in NSCLC.

ABC transporters (ABCB1, ABCG2 and ABCC1) mediate cellular MDR by pumping out the substrates, including a variety of chemotherapeutics, thus diminishing drugs' antiproliferative effects on cancer cells [199,200]. In the first part, we provided theoretical supports for utilizing novel targeted drugs as dual-activity chemosensitizers against ABC transporter-mediated MDR. One striking highlight in this work is the intensive employment of NSCLC patient-derived primary culture cells, which has allowed us to confirm *in vitro* outcomes. Remarkably, we concluded that four tested candidates (tepotinib, sonidegib, talazoparib and encorafenib) could work as favorable

dual-activity agents in the treatments for ABC transporter-overexpressing NSCLC. Due to the timing, we did not conduct *ex vivo* studies for enasidenib, entrectinib and alisertib.

It is well-known that the inhibition of single ABC transporter can be compensated by the activity of another one, which can be explained by the overlapping substrate affinity of ABCB1, ABCG2 and ABCC1 [77]. Hence, simultaneous targeting of multiple transporters seems to be important for effective combatting of MDR. In this thesis, some of the targeted drugs were proved to regulate MDR by inhibiting two or even three transporters, which underlines their values as potential chemosensitizers. In NSCLC, ABCC1 has the most likelihood to be detected for overexpression among all ABC transporter members [201]. We demonstrated the potent inhibitory effects of alisertib, enasidenib, talazoparib and encorafenib toward ABCC1. After clinical evaluation, we believe our findings may open up more choices for the clinical treatments of NSCLC with high-level of ABCC1 expression. Notably, selecting proper MDR modulators according to the expressional profile of ABC transporters in patient's tumor tissue is necessary to successfully overcome chemotherapy resistance. This thought is in line with the trend toward precision medicine and personalized treatment. The majority of clinical trials evaluating ABC transporter inhibitors have failed in the past, most likely due to the neglection of transporter expression assessment in the patient's tumor tissue. As a result, clinical studies should be conducted selectively in the patients with high expression of ABC transporters, in order to precisely reflect the chemosensitizing potential of tested agents [202,203]. In addition, the modulator's original target should be co-expressed with transporter(s) in the tumor tissue to allow for the full synergy and projecting its dual-activity modulatory abilities.

Pharmacokinetic MDR can be also mediated by the metabolic deactivation of drugs, which is mainly catalyzed by CYPs. Although the importance of CYPs as sites for clinical DDIs has been well-known, their participation in regulating tumor's responsiveness to chemotherapeutic drugs was poorly described. Previously, we have proven that docetaxel resistance can be mediated by the presence of CYP3A4, while ensartinib could alleviate this phenomenon via the inhibition of CYP3A4 [66,189]. In this thesis, some of the tested novel targeted drugs showed favorable inhibition of CYP3A4 in cellular/non-cellular models. Nevertheless, none of them was eventually confirmed to reverse CYP3A4-caused tolerance to docetaxel. In fact, drugs that simultaneously act on both transporters and metabolic enzymes, such as ensartinib, might be very helpful for reversal of drug resistance in NSCLC, providing antagonism of pharmacokinetic MDR mechanisms in a complex fashion. Overexpression of CYP3A4 is commonly examined in NSCLC compared to paracancerous or normal tissues, which supports this idea [204,205]. Despite the fact that the investigated drugs within this thesis does not possess properties similar to ensartinib, we still look forward to the successful development/revelation of novel agents that modulate pharmacokinetic MDR through multiple mechanisms in the near future.

Along with synthetic drugs, accumulating number of studies has revealed that natural compounds can also achieve chemosensitization in cancer cells by acting on ABC transporter(s)/CYP3A4, without producing severe toxic effects. Promising MDR-antagonizing outcomes from these compounds have been documented in a number of pre-clinical studies, such as tetrandrine (inhibiting ABCB1), acacetin (inhibiting ABCB1 and ABCG2), resveratrol (inhibiting ABCB1, ABCG2 and ABCC1) and fucoxanthin (downregulating CYP3A4) [206]. Nevertheless, the application of synthetic/natural agents still encounters various obstacles, such as poor water solubility, low bioavailability,

high systemic toxicity, etc. Recently, the rapid development of nanotechnology has provided a variety of carriers for effective and selective anti-cancer drug delivery. Some nanoparticles can also modulate the function of ABC transporters/CYPs to alleviate drug resistance in cancers [207-209]. Accordingly, we can expect that the combination of nanomaterial-based drug delivery systems and precision medicine-based pharmacological therapies will be the groundbreaking strategy to reverse/prevent the occurrence of pharmacokinetic MDR. This technology can also provide the upgrade for dual-activity modulator strategy, thus eliminating the most crucial obstacle represented by the systemic toxicity evoked by the interaction(s) in the ADME-related organs.

The emergence of drug resistance also hinders the path of defeating NSCLC by EGFR-TKi targeted therapy, regardless of the drug's generation [42]. Since the drug resistance usually occurs after receiving long-term treatments and involves multiple mechanisms, we decided to simulate the process of developing drug resistance during the clinical treatment. Instead of using alternative approaches (e.g., pulsed selection and gene editing), drug resistant cell lines generated by long-term stepwise selection could consider both the persistence of resistance-associated phenotypes and the intricate interplay between diverse mechanisms [210].

We found that drug resistant NSCLC cells are not only tolerant to a single EGFR-TKi, but also have impaired responsiveness to drugs from the same generation. Unfortunately, cross-resistance phenomenon may also expand to the whole group of EGFR-TKis [211,212]. Noteworthy, cross resistance in cancer targeted therapy can be also found for HER2 inhibitors, CDK4/6 inhibitors, androgen receptor inhibitors and MAPK inhibitors [190]. This finding is very meaningful as it directly indicates that simply replacing drugs within the same drug group to combat drug resistance would be

in vain. Clinicians should keep this in mind and choose the proper alternative strategy to avoid further tumor progression and unnecessary patient's affection by adverse effects.

After the series of experimental investigations, we have uncovered that targeting ECM-related signaling (FAK, YAP1 and FN1), CSC-related pathways (Hedgehog and Notch), anti-apoptotic protein (BCL-2) and drug efflux transporter (ABCG2) could universally modulate EGFR-TKi resistance in NSCLC. Inhibiting FAK signaling was also able to attenuate the hyper-invasivity in NSCLC cells. Furthermore, we suggested that ECM-related and Hedgehog pathways might play promotive roles not only in EGFR-TKi resistance, but also in lung carcinogenesis. These findings shed light on the potential of targeting ECM-related signaling/Hedgehog pathway for the treatment of both EGFR-TKi-resistant and naive NSCLC. To date, promising progress has been achieved in developing anti-NSCLC agents targeting ECM-related and Hedgehog pathways. For example, sonidegib and vismodegib (Hedgehog pathway inhibitors) are ongoing phase I and II clinical evaluation, respectively (NCT04007744; NCT04591431). Defactinib, an inhibitor targeting FAK signaling, is currently undergoing phase I clinical trial for NSCLC (NCT03875820). Other candidates acting on these targets have also shown promising NSCLC-combating effects in preclinical studies. Moreover, abnormal activations of ECM-related signaling and Hedgehog pathway have also been frequently reported in other solid cancer, further suggesting their druggable values in anti-cancer management [147,213,214].

Synergy is an essential phenomenon behind the clinical success of many combined chemotherapy regimens. This effect allows for the reduction of the administered doses while retaining or even improving the therapeutic activity of the drugs, thereby mitigating the occurrence of adverse reactions [215]. In this thesis, we have

proposed several synergistic drug combinations that are potentially suitable to counteract conventional/novel anti-cancer drug resistance. Although we have included *ex vivo* model to increase the potential clinical impact of our results; however, it still has significant limits such as the lack of immune reaction's presence or lack of other conditions that are specific for the whole-organism level. Therefore, it is worthy to state that the verification of our results by *in vivo* testing will be essential. Tumor xenografts in animals are a suitable models for such investigation [216]. At present, our team is collaborating with Prof. Ming-Sound Tsao (Princess Margaret Cancer Centre, Toronto, Canada) regarding the establishment of xenograft models. We believe that the upcoming *in vivo* results combined with *in vitro* and *ex vivo* findings will offer a solid and reliable foundation for the subsequent clinical trials.

Strategies for the treatment of NSCLC and overcoming MDR are not limited to the approaches presented in this thesis. Immunotherapy, exemplified by the inhibition of programmed cell death protein 1 (PD-1, also called CD279), has become an attractive path for tumor therapy and/or combating MDR. This approach works by modulating the patient's immune system to mount the immune response against the cancer cells (or drug-resistant variants). Several drugs, such as nivolumab, pembrolizumab and atezolizumab, have been approved by FDA as anti-NSCLC agents [217]. However, drug resistance still has arisen as the major problem for conducting immunotherapy alone. Hence, it is worthwhile to advocate the combination of immunotherapy and chemotherapy/targeted therapy to mitigate drug resistance. Some successful cases have been applied for treating NSCLC (e.g., pembrolizumab plus carboplatin and pemetrexed; bevacizumab plus carboplatin and paclitaxel) [218,219]. Another promising approach to treat NSCLC or counteract drug resistance is through the regulation of specific pathways of programmed cell death. Ferroptosis, a unique type of necrosis driven by the free iron, has been recently

highlighted as a novel approach for anti-cancer treatments. In NSCLC, inducing ferroptosis has been demonstrated to overcome chemotherapy resistance, targeted therapy resistance and immunotherapy resistance. However, deeper descriptions on its mechanisms and biomarkers as well as the development of drug candidates specifically inducing ferroptosis in tumors are still required for introducing this strategy into clinical practice [181,220]. Besides, physical approaches, such as thermal, ultrasound, and photodynamic therapies can improve drug delivery into cancer cells, and thus combat drug resistance [221]. Finally, rapid development in the field of artificial intelligence can also assist pharmacologists and clinicians to design the possible antagonist of MDR, predict the outcome from non-surgical treatments and maximally optimize the setup for personalized regimens [222].

Taken together, drug resistance remains a persistent but not invincible challenge that accompanies NSCLC treatment. Cleverly designed drug combination regimens currently appear to be an optimal strategy to address this issue. We hope that, upon completion of *in vivo* studies and clinical trials, at least part of our results can be translated into safe and effective therapies to break through the shadow of drug resistance, ultimately yielding benefits for NSCLC patients.

7. LIST OF OTHER OUTPUTS OF THE CANDIDATE

7.1. Oral presentations

First author

- **ZHANG Y**, BUDAGAGA Y, SABET Z, VAGIANNIS D, HOFMAN J. Roles of cancer stem cell- and apoptosis-related pathways in the development of acquired resistance to EGFR inhibitors. *12th Postgraduate and Postdoc Conference* (2022), Hradec Králové, Czech Republic

- **ZHANG Y**, BUDAGAGA Y, VAGIANNIS D, SABET Z, HOFMAN J. The establishment of acquired resistance to EGFR inhibitors is associated with the expressional changes in cancer stem cells- and apoptosis-related pathways. *7th International Conference on Cancer Research & Drug Development* (2022), Baltimore, MD, United States and Online

- **ZHANG Y**, GÜLTEKIN O, KUPČÍK R, VAGIANNIS D, SABET Z, BUDAGAGA Y, LEHTI K, HOFMAN J. Extracellular matrix-related signaling participates in the development of resistance to EGFR-targeting tyrosine kinase inhibitors. *13th Postgraduate and Postdoc Conference* (2023), Hradec Králové, Czech Republic

Co-author

- BUDAGAGA Y, VAGIANNIS D, **ZHANG Y**, SKARKA A, HOFMAN J. Tepotinib reverses multidrug resistance by inhibiting the efflux function

of ABCB1 and ABCG2 transporters. *11th Postgraduate and Postdoc Conference* (2021), Hradec Králové, Czech Republic

- SABET Z, VAGIANNIS D, **ZHANG Y**, BUDAGAGA Y, SKARKA A, HOFMAN J. Alisertib acts as a dual activity resistance modulator through the inhibition of ABCC1 transporter. *12th Postgraduate and Postdoc Conference* (2022), Hradec Králové, Czech Republic

- SABET Z, VAGIANNIS D, BUDAGAGA Y, **ZHANG Y**, NOVOTNÁ E, HANKE I, ROZKOŠ T, HOFMAN J. Talazoparib effectively modulates pharmacokinetic resistance mediated by ABCG2 and ABCC1: an *in vitro* and *ex vivo* study. *13th Postgraduate and Postdoc Conference* (2023), Hradec Králové, Czech Republic

7.2. Poster presentations

First author

- **ZHANG Y**, VAGIANNIS D, BUDAGAGA Y, SKARKA A, STAUD F, HOFMAN J., Tepotinib inhibits ABCB1 and ABCG2 efflux transporters and overcomes transporter-mediated cytostatic resistance. *EPHAR 2021: 8th European Virtual Congress of Pharmacology* (2021), Online

- **ZHANG Y**, VAGIANNIS D, BUDAGAGA Y, SABET Z, HOFMAN J. Sonidegib sensitizes cancer cells to cytotoxic agents by inhibiting drug efflux functions of ABCB1 and ABCG2 *in vitro*. *2nd International*

Transmembrane Transporter Society Meeting (2022), Copenhagen, Denmark

Co-author

- BUDAGAGA Y, VAGIANNIS D, MORELL A, **ZHANG Y**, HANKE I, ROZKOŠ T, HOFMAN J. Tepotinib modulates transporter-mediated resistance in *ex vivo* primary lung tumor cells. *EPHAR 2021: 8th European Virtual Congress of Pharmacology* (2021), Online

- BUDAGAGA Y, VAGIANNIS D, **ZHANG Y**, SABET Z, HANKE I, ROZKOŠ T, HOFMAN J. Study on the functional activities of MDR-associated ABC transporters in the *ex vivo* lung tumor explants. *2nd International Transmembrane Transporter Society Meeting* (2022), Copenhagen, Denmark

- SABET Z, BUDAGAGA Y, **ZHANG Y**, VAGIANNIS D, HANKE I, ROZKOŠ T, HOFMAN J. Sonidegib acts as a pharmacokinetic resistance modulator in patient-derived NSCLC explants *ex vivo*. *2nd International Transmembrane Transporter Society Meeting* (2022), Copenhagen, Denmark

- SABET Z, **ZHANG Y**, BUDAGAGA Y, VAGIANNIS D, KUPČÍK R, HOFMAN J. The role of cancer stem cells- and apoptosis-related pathways in acquired EGFR-TKis resistance. *EMBO workshop: Cancer*

cell signaling: Linking molecular knowledge to cancer therapy (2022), Cavtat, Croatia

7.3. Grant projects

Principal investigator

- *START/MED/070* - Unraveling pharmacodynamic principles of acquired resistance to epidermal growth factor receptor inhibitors (2021 - 2023) - Grant Schemes at CU

- *GAUK 334120/C* - The role of pharmacokinetic interactions of new targeted drugs in the modulation of efficacy of cytotoxic drugs in non-small cell lung carcinoma (2020) - Grant Agency of Charles University

Co-investigator

- *GAUK 334120/C* - The role of pharmacokinetic interactions of new targeted drugs in the modulation of efficacy of cytotoxic drugs in non-small cell lung carcinoma (2021 - 2022) - Grant Agency of Charles University

- *GAUK 102121/C* - Implementation of ex vivo and in vivo lung tumor models for the evaluation of clinical value of dual drug resistance modulators (2021 - 2023) - Grant Agency of Charles University

- *GAČR 20-20414Y* - Study on the role of novel targeted breast and lung anticancer drugs in the phenomenon of pharmacokinetic drug resistance (2020 - 2022) - Czech Science Foundation

7.4. Diploma theses, in which candidate was a consultant

- Author: Mgr. Lucie Malečová

  Title: Study of the effect of ABC drug transporters' overexpression on the antiproliferative capacities of selected conventional cytostatics. [Defended in June 2022]

- Author: Natalia Trajlinková

  Title: Study of the effect of OCT1 and OCT2 drug transporters' overexpression on the antiproliferative capacities of platinum cytostatics.

7.5. Teaching activities

- lecturer of practical courses of Pharmacokinetics (summer semester 2021)
- lecturer of practical courses of Pharmacokinetics (summer semester 2023)

7.6. Scientific experience abroad

- 3.5 month-lasting research stay at the Department of Microbiology, Tumor and Cell Biology (Prof. Kaisa Lehti), Karolinska Institutet, Stockholm, Sweden; 2022

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