# Novel kinase inhibitor compounds for combination cancer therapy

Thesis for doctoral degree (Ph.D.)

**Gyulavári, Pál**

Doctoral School of Pharmaceutical Sciences
Semmelweis University

| | |
|---|---|
| Supervisor: | Vántus, Tibor Ph.D. |
| Opponents: | Tábi, Tamás Ph.D. |
| | Wunderlich, Lívius Ph.D. |
| Chair of examination board: | Tretter, László Ph.D., D.Sc. |
| Members of examination board: | Balla, András Ph.D. |
| | Molnár, Tamás Ph.D. |

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Contents

List of abbreviations

| | |
|---|---|
| ADME | absorption, distribution, metabolism, excretion |
| AKI(s) | Aurora kinase inhibitor(s) |
| ALL | acute lymphoblastic leukaemia |
| AML | acute myeloid leukaemia |
| ATCC | American Type Culture Collection |
| BRCA1 | breast cancer 1 (protein) |
| CI | combination index |
| CIN | chromosomal instability |
| CPC | chromosomal passenger complex |
| c-Met | hepatocyte growth factor receptor |
| DMSO | dimethyl sulfoxide |
| DNA | desoxyribonucleic acid |
| DTT | dithiothreitol |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EMT | Epithelial-mesenchymal transition |
| EVL | Extended Validation Library™ |
| Fa | fraction affected (=activity) |
| FAK | focal adhesion kinase |
| FBS | foetal bovine serum |
| FDA | food and drug administration (US office) |
| GnRH | gonadotropin-releasing hormone |
| GPCR | G protein-coupled receptor |
| HEPES | 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (buffer) |
| HER2 | human epidermal growth factor receptor 2 |
| HGF | hepatocyte growth factor |
| HRP | horseradish peroxidase |
| $IC_{50}$ | inhibitory concentration at 50% effect |
| INCENP | inner centromere protein |

| | |
|---|---|
| KI(s) | kinase inhibitor(s) |
| $K_M$ | Michaelis-Menten constant |
| KMN | *K*nl1 - *M*is12 - *N*dc80 (protein complex) |
| MAPK | mitogen-activated protein kinase |
| MC | mitotic checkpoint |
| MR | master regulator (protein) |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-<br>2,5-diphenyltetrazolium bromide |
| NCL | Nested Chemical Library™ |
| NSCLC | non-small cell lung cancer |
| PARP | poly (ADP-ribose) polymerase |
| PBS | phosphate-buffered saline (solution) |
| PDGF | platelet-derived growth factor |
| PDGFR | platelet-derived growth factor receptor |
| PI3K | Phosphoinositide 3-kinase |
| PI | propidium-iodide |
| PMSF | phenylmethylsulfonyl fluoride |
| PVDF | polyvinylidene-difluoride |
| rt | room temperature |
| RTKs | receptor tyrosine kinase(s) |
| SAR | structure-activity relationship |
| TAMRA | carboxytetramethylrhodamine |
| TBST | tris buffered saline (solution) with 0.1% TWEEN 20 |
| TKI(s) | tyrosine kinase inhibitor(s) |
| VEGF | vascular-endothelial growth factor |
| VEGFR | vascular-endothelial growth factor receptor |

# 1. Introduction

## 1.1. Cancer

### 1.1.1. On cancer in general

Abnormal tissue growth – *tumour* – is a common phenomenon in multicellular organisms. While benign tumours usually cause no harm, malignant tumours – *cancers* (from the Greek word for crab) – have the ability to detach from the originating tissue. These invading *cancer cells* then spread across the body via the blood and lymphatic current and create new tumours elsewhere – called *metastases*.[1] These cancerous bodies crowd out normal cells and cause dysfunction of the invaded organs which eventually leads to the death of the patient. Nowadays cancer became one of the most prevalent cause of death in both developing (mainly because of environmental risks) and developed countries (mainly because of longer lifespan).[2, 3, 4]

Cancers are highly heterogeneous in many aspects:

- Regarding the tissue of origin, cancers can be classified into numerous histological types (carcinoma, leukaemia, melanoma etc.) that gives valuable information at the first glance.[5]
- Cancer cells from different parts of a given tumour tend to utilize different metabolism.[6]
- Like healthy cells, cancer cells might also differ in their potency to differentiate into specialised (cancer) cell types. This notion is an ever hotter topic – see the theory of cancer stem cells.[7]
- Furthermore, there are always non-cancer cells in a tumour: endothelial cells build up the well-known amorphous vasculature of tumours,[8] cancer-associated fibroblasts surround and interact with epithelial cancer cells[9,10] also immune cells infiltrate tumours and either hinder or promote cancer progression.[11] These cells constitute the *tumour microenvironment* that also able to modify drug response.[12]
- However, in the last thirty years it has became clear, that various *genetic alterations* and mainly the resulted *malfunctioning proteins* – increased or lost activity – stimulate cancer cells to divide ceaselessly.[13] The heterogeneity set up by these diverse genetic alterations not only explains the development of cancers but also conveys firm clues for therapy as well.[14,15] Targeted pharmacological inhibition of the malfunctioning proteins

has already improved many cancer patient's life expectancy.[16] However, the list of potential target proteins is far from complete, and also many proved targets still lack an approved drug.

### 1.1.2. Cancer as a genetic disease

Genetic alterations occur naturally (mainly during DNA replication)[17] but certain environmental factors – *carcinogens* (like viral infections, certain chemical compounds and high-frequency electromagnetic radiation (gamma and UVB rays)) – are able to rise their number through damaging the DNA. This way carcinogens raise the probability that a normal cell turns into a cancer cell.[18] Some cancer-promoting genetic alterations can be inherited as well.[19]

If too many DNA damages are accumulated, normal cells commit suicide – called *apoptosis* – or cease dividing for ever – called *senescence* –, in order to prevent the formation of genetic alterations that might lead to uncontrolled cell proliferation.[20] If that does not happen and the DNA damage causes permanent genetic alteration that is able to abnormally stimulate, "*drive*" the proliferation of a cell, a cancer cell is born.[21]

Regarding their origin, cancer driver genetic alterations can be:

a) *Mutations*, alterations in the DNA sequence affecting rather few nucleotides (substitution, insertion, deletion). The majority of them affect no regulator or protein coding regions. A *silent mutation* does so, but without altering any function or protein sequence. Even mutation of a protein coding region that leads to amino acid change usually does not alter the function of the encoded protein – they are *passenger mutations*. However, if a controller region is spoiled the mutation results in an over- or under-expressed protein (and so *increased* or *decreased* activity of the pool of that protein).[22] Similarly, if the swapped amino acid is crucial for protein function the mutation results in a malfunctioning translated protein with increased or decreased activity on its own.[23] In both cases the affected gene becomes a *mut-driver gene*.

b) *chromosomal abnormalities* of various scale are alterations in the DNA sequence affecting larger chromosomal segments (translocation, duplication or deletion) or even loss/gain of whole chromosomes compared to normal number – later phenomenon called *aneuploidy*. They might cause gene copy-number alterations (*amplification* or *deletion*) and new *fusion genes*.[24] These alterations are also a source of over-, under-expressed or fusion proteins with increased activity.[21]

c) *Epigenetic alterations* are actually not genetic phenomena since they affect no DNA sequence[25] but usually affect the regulator regions of genes – resulting in over- or under-expressed proteins. The affected genes are *epi-driver genes*.[26]

d) *Aberrant RNA processing and splicing* might also result in over- or under-expressed proteins.[27]

Point c) and d) constitute the most elusive kind of genetic alterations which are hard to analyse by conventional sequencing methods. Therefore, most studies focus on sheer sequence alterations of DNA, especially mutations.

Regarding function, driver genes fall into two groups:

I) many genes and proteins stimulating normal cell growth, division and differentiation are *proto-oncogenes*. Provided a genetic alteration affects them, their activity increases and they become *oncogenes*. Oncogenes endow the cell with selective growth advantage compared to normal cells of the same tissue.[21]

II) genes and proteins hindering cell growth, division and differentiation are *tumour suppressors*. Many of them induce apoptosis or senescence and their loss of function is which endows the affected cell with selective growth advantage.[21]

Regarding effect, drivers concert seven important *hallmarks* of cancer cells.[28] The first two are fundamental – so called *enabling* – characteristic in the progression of a cancer. The following five phenomena usually occur later but there is no invariant order of them and not every cancer cell displays all of them – e.g. benign tumours typically lack point 5).[29]

1) *sustained proliferative signalling* is usually a result of increased activity of an oncogene, e.g. due to gene mutation or protein overexpression in case of EGFR (epidermal growth factor receptor) or due to gene amplification in case of c-Met (hepatocyte growth factor receptor).

2) *evasion of apoptosis* – mainly due to decreased activity of tumour suppressors.

However, as cancer cells continue to divide and the tumour mass grows, new challenges immediately arise. These obstacles surely eliminate most incipient cancers – or at least keep them in a few-cell, undetectable and harmless state:

3) *preserving telomeres.* Telomere sequences protect chromosomes from stochastic breakage and fusion while shortening with each cell division.[30] A critical length of telomeres induce apoptosis or senescence. For incipient cancer cells these repeated

breakage-fusion cycles create new – sometimes driver – genetic alterations.[31] This is the well-known CIN (chromosomal instability) that further fosters – and eventually becomes the major source of – genetic heterogeneity found in cancers[32] and indicates poor prognosis.[33] Of course high CIN can be detrimental to cancer cells,[34] so after a while telomere restoring enzymes – telomerases – are acitvated in about 90% of cancers.[35]

4) *induction of angiogenesis*. As the tumour mass reaches a critical volume, it needs blood vessels to efficiently obtain nutrients and oxygen – similarly to healthy tissues. For this purpose, cancer cells need to express or increase the activity of pro-angiogenic molecules like: VEGFR (vascular-endothelial growth factor receptor), PDGFR (platelet-derived growth factor receptor) and their natural ligands VEGF (vascular-endothelial growth factor) and PDGF (platelet-derived growth factor).[36]

5) *invasion and formation of metastasis*. EMT (epithelial-mesenchymal transition) is the phenomenon when some differentiated epithelial cells break the cell-cell junctions and penetrate the basal membrane. The EMT program is normally active in embryonic cells[37] or during wound healing.[38] Cancers of epithelial origin often activate genes and proteins promoting EMT, detach from the basal membrane and invade neighbouring tissues.[39] Apart from stochastic endogen cellular processes[40] hypoxic tumour environment[41] and certain drugs can also induce EMT.[42] Unfortunately in cancer cells with active EMT program also anti-apoptotic signals are evoked and they become more resilient to treatment.[43] However, many cancer cells do not survive amidst the shearing forces of blood or lymphatic current and only a fraction or them manage to colonise distant tissues.[44] Established metastases then independently evolve to a new tumour.[45] According to the latest studies, there are no solid "metastasis genes or mutations" and metastasis occurs at a very early stage during cancer development.[46]

6) *Evading the immune system* – solid cancers are known to be infiltrated by cells of innate and adaptive immunity: "tumours are wounds that never heal".[47] In fact, evading immune destruction by selection of less-immunogenic clones (displaying altered cell membrane proteins) might be an important step in cancer development, at which many incipient cancers fail.[48] Paradoxically, immune destruction of cancer cells and the accompanying inflammation have tumour-promoting effect as well, because of the

secretion of angiogenic and survival factors and the breaking down of intracellular matrix (easing invasion).[11]

7) *Reprogramming energy metabolism.* Incipient cancers often lack oxygen (see point 4), thus they switch off oxidative phosphorylation and use only glycolysis to ferment glucose to lactate. Surprisingly, very often well-oxygenised cancers behave the same way – the phenomenon is called aerob glycolysis or Wartburg effect.[6] The rationale is that upregulated glycolysis produces more intermediers ("building blocks") for intensive cell growth. Also proliferation-inducing oncogenes are known to activate aerob glycolysis.[49]

### 1.1.3. Comprehending the genetic heterogeneity of cancers

The elevated number of genetic alterations found in a tumour mass originates from increased cell proliferation and CIN. It is important to note that there are no invariant or consensus genetic alterations in cancers of any histological type, rather more abundant ones in a given sample.[50] Different parts of the same tumour (intratumoral heterogeneity) and even metastases of the very same primordial tumour (intrametastatic heterogeneity) harbour different genetic alterations.[51] This genetic heterogeneity provides the pool for the "natural selection" of cancer cells by the physiological obstacles mentioned in chapter 2.1.2. (hallmarks 1-7). Only those cancer cells form a life-threatening metastatic cancer in the long run, which acquire enough drivers to overcome most of these obstacles and continue proliferating – this is a real evolutionary process. Unfortunately recent pharmacological therapies cannot exterminate 100% of cancer cells, there are always survivors. So drugs further stimulate this evolutionary process,[52] selecting drug resistant cancer clones which eventually results in the relapse of the patient in most of the cases.[53]

Vogelstein et al. defined 138 mut-driver genes according to mutation frequencies (54 oncogenes and 71 tumour suppressors) which are responsible for the growth of most human cancers. A regular cancer accumulates 0-6 driver mutations during several years or even decades before the diagnosis.[21] Besides, an average cancer harbours a huge number of passenger mutations, cancers from fast-renewing tissues the most (e.g. up to 80000 in melanoma[54]).[55] Actually, >99% of all genetic alterations detected in human cancers are merely passengers.[21]

While this model is clear and well-corroborated, it does not really address other genetic alterations than mutations (see points b), c) and d) in chapter 1.1.2.).[56] For example, 20% of all human cancers express malfunctioning proteins that regulate epigenetic modifications.[57] Since epigenetic alterations are early phenomena in cancer[58] their therapeutic reversal is very enticing.[59]

The notion that huge percentage of cancers do not harbour unambiguous drivers led to the theory of *mini-drivers*. Instead of a few drivers with great impact, a multi-step, continuous model of cancer development has been proposed by Castro-Giner et al.[60] They say that many functions of a cancer cell are the result of numerous, redundant mini-drivers. Rather than occasionally gaining a major driver mutation, perpetual accumulation of mutations with modest effect provide the selective growth advantage eventually.[61]

According to the concept of *mutator mutations*, the malfunction of DNA replication and damage repair machinery might be the primary alterations which rise the number of genetic alterations and drive cancer progression.[62] Upon a selection pressure (like any anti-cancer drug) a possibly advantageous mutation arises sooner in cells with elevated mutation rate. The notion that cancers sometimes harbour the advantageous mutations, e.g. drug-resistant clones, already prior to therapy seems to corroborate this.[63] This theory is based on the preconception that normal mutation rate in rapidly dividing cells is not enough to gather so many mutations. On the other hand, it is known that there are slow-dividing cancer cells.[64] Furthermore, erroneous DNA damage repair increases CIN (which is a double-edged sword for cancer cells), so its therapeutic inhibition may be beneficial.[65]

According to another aspect, a few *MR* (*master regulator*) proteins form small, autoregulated modules called cancer checkpoints.[66] These checkpoints integrate the effect of heterogeneous genetic alterations (drivers) to a more defined cancerous cell homeostasis. Moreover, MRs themselves can malfunction due to post-translational modifications and drive cancer formation, indicating that rather protein abundance and activity data are needed instead of DNA mutation analysis to understand cancer function.[67]

These seemingly irreconcilable theories well represent that we have just began to untangle the roots of the most complex human disease. Nevertheless, the central role of genetic alterations seems to be fundamental in every model so far.

## 1.2. Kinases

### 1.2.1. Kinases as part of signal transduction pathways

In living cells information flow is nothing more, but induced conformational alteration upon the physical interaction of molecules. Kinases are proteins with an enzymatic activity that are able to transfer a phosphoryl group ($PO_3^{2-}$) from ATP to their substrates (lipids, carbohydrates or proteins).[68] The phosphorylation reaction is highly substrate-specific: in case of protein kinases, there is a consensus amino acid sequence in the protein substrate that should surround the phosphorylatable residue (a tyrosine, serine or threonine).[69] This transfer then alters the conformation of the substrate protein and activates or impedes a specific function of it.[70] Kinases are not active all the time – in fact they are mostly switched off. Furthermore the "on" and "off" states are non-binary, rather multi-step.[71] The level of kinase activity might be influenced by phosphorylation (by another kinase or by themselves – later called *autophosphorylation*), or by binding a ligand molecule, a scaffold protein or another kinase domain of the same type.[71] Finally, the effect of kinases is compensated by phosphatase enzymes which constantly remove the phosphoryl groups from the substrate molecules – so the conformation altering effect of kinases is mostly transient.[72]

Every protein kinase has a similar conserved structural module – called *domain* – that possesses kinase activity: it consists of a smaller amino-terminal and a larger carboxy-terminal lobe connected by a so called *hinge*. These two lobes form the MgATP-binding cleft (for ATP to coordinate its β and γ phosphate groups a $Mg^{2+}$ ion is always needed) while the protein substrate bounds mainly to the carboxy-terminal lobe.[71] Inside the amino-terminal lobe there is the *αC helix* – an important inner switch of activity – and inside the carboxy-terminal lobe there is the *activation segment*.[73] The activation segment of the carboxy-terminal lobe has extended or closed conformation which is one factor influencing the activity of the kinase. It begins with a "*DFG*" motif – the aspartate residue D binds the crucial $Mg^{2+}$ ion[68]– and its end interacts with the phosphorylatable serine / threonine / tyrosine residue of the substrate protein.[74] There is

also a phosphorylatable residue in the activation segment, the phosphorylation of which is usually needed for enzyme activation.[71] The exception is EGFR family kinases.[75]

If the protein substrate of a kinase is also a kinase, a kinase cascade formed. Kinase cascades with multiple members are common types of *signalling pathways*. Signalling pathways are the means of signal transduction from receptors in the plasma membrane to transcription factors inside the nucleus.[76] Signalling pathways amplify the signal up to a ~hundred fold[77] and by cross-talking they form an elaborate information processing network inside every cell.[78] In the end transcription factors regulate transcription of genes and the resulting proteins influence various cell functions: transcription of further genes and metabolism, growth, division, motility or apoptosis of cells.[68]

Unprovoked increased activity of certain protein kinases – so corrupted information flow – is common in many human illnesses (diabetes, cardiovascular-, nervous- and inflammatory diseases and cancer) and their inhibition proved to efficiently mitigate the symptoms, so they have become the leading drug targets in the past two decades.[79]

### 1.2.2. Kinases as drivers of cancers

Given their central role in the regulation of so many cellular functions it is not surprising that many protein kinases are common drivers of cancers.[80] As far back as 1952 Williams-Ashman and Kennedy noticed that cancer cells usually more actively phosphorylate than healthy ones.[81] Increased activity of a protein kinase overdrives the signal transduction pathway in which it is situated or – in case of the effector kinases – directly stimulates oncogenic cellular functions.[82] Increased activity can be the result of a) various genetic alterations mentioned in chapter 1.1.2., b) increased paracrine or autocrine stimulation by ligands – in case of receptor-kinases, c) decreased phosphatase activity and d) increased structural stability due to elevated amount of chaperones (like HSP90).[83]

The human genome encodes 538 protein kinases[84] and of the 54 oncogenes in Vogelstein's model 31 are protein kinases.[21] Unfortunately, only mut-driver kinases or kinase fusion genes can be detected by DNA sequencing, the aforementioned other reasons of increased activity are by proteome analysis only.[66]

#### 1.2.2.1. Aurora kinases

Every cell is the result of a previous cell division. Cells that are not in a quiescent state (phase *G0*) continuously synthesize all their components and grow in volume (phase *G1*). During phase *S* also the DNA content (chromosomes) and the *centrosome* are duplicated. In phase *G2* the cell continues to grow and prepares to the division itself, phase *M* (*mitosis*). The most delicate process in mitosis is the equal distribution of the duplicated chromosomes to the daughter cells (Figure 1). Centrosome contains *centrioles* and is the centrum of the microtubule scaffold system of cells.[85] During mitosis microtubule spindles build up to connect the two centrosomes (*polar* microtubules) while some run to the cell membrane (*astral* microtubules) – these will excert the force that physically separates daughter cells. Other microtubule spindle fibers reach the pinch of the duplicated and condensed chromosomes – called *centromeres* – and join to the complex network of proteins there – called *kinetochores*.[86] When correctly aligned, these spindles pull *sister chromatids* evenly into distinct daughter cells.[87] Ideally only one microtubule spindle should bind to one kinetochore and each sister chromatid to ones emanating from opposite centrosomes.[88] Every other possibilities – if not corrected – cause *aneuploidy*, that is one form of CIN.[89, 90]

Three cell cycle checkpoints – intricate systems of feedback signalling at important phase transitions – assess the condition of the cell and let continue cell cycle only when certain progresses are completed.[91] They are the G1/S checkpoint, the G2/M or DNA damage checkpoint and the MC (mitotic checkpoint).[92] The MC ensures equal distribution of chromosomes into daughter cells: since microtubule-kinetochore bonds created and break stochastically, the MC hinders sister chromatid segregation until all attachments are normal.[93]

Aurora kinases are key effector kinases of cell division.[94] They regulate maturation, duplication and separation of the centrosome, likewise proper mitotic spindle assembly and microtubule-chromosome attachment, furthermore separation of daughter cells – *cytokinesis* – itself.[95]

In humans the centrosome-associated Aurora kinase is denoted A, while the chromosome-associated paralogue B.[96]

The third Aurora kinase 'C' orchestrates cell division of gametocytes.[97] Aurora C has similar role to B[98] and is overexpressed in several cancer cell lines.[99] However, data

regarding its real significance in cancer is scarce, so it will not be discussed in this study.

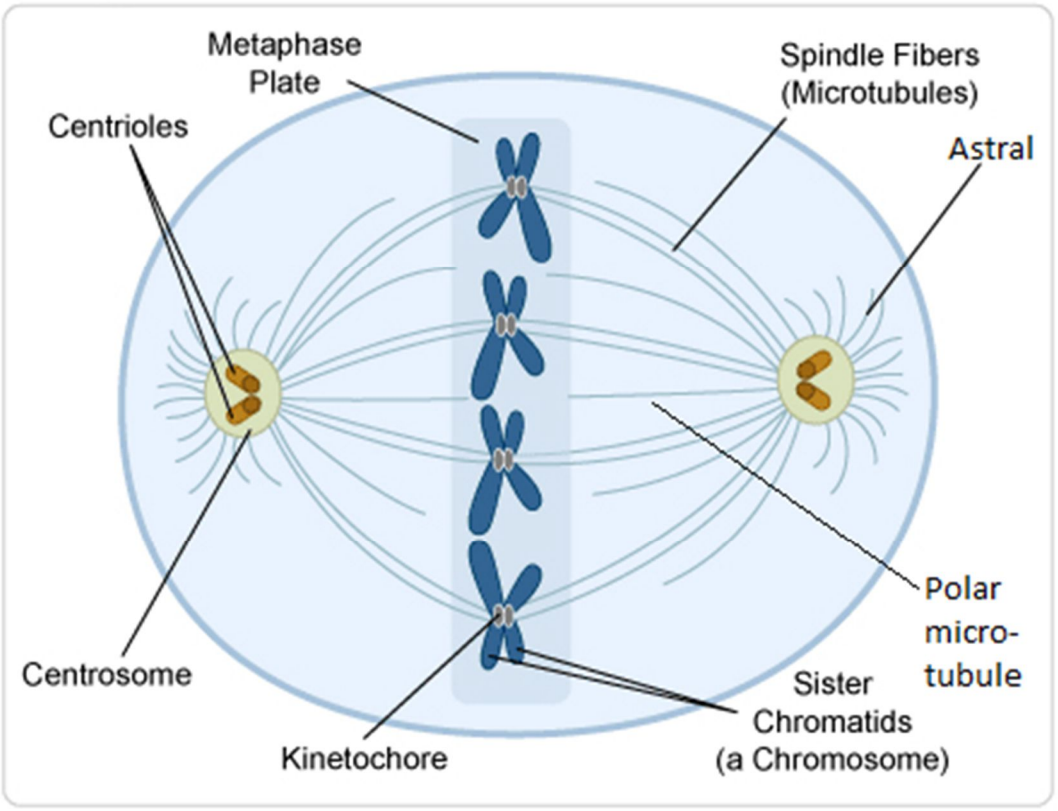



**Figure 1.** Scheme of a cell in metaphase. Duplicated chromosomes are arranged to the midsection of the dividing cell – called *metaphase plate*. Modification of picture from: [https://www.emedicalprep.com/study-material/biology/cell-structure-functions/cell-cycle-cell-division]

**Aurora A**

The serine-threonine kinase Aurora A is expressed predominantly during mitosis in every human cell where it is localized at the centrosomes[100] and transiently along the spindle microtubules.[101]

Basically, function of Aurora A is regulated by expression and autophosphorylation but several other signals also impact its activity: hypoxic conditions[102] or well-known driver kinases like PI3K (Phosphoinositide-3-kinase)[103], BCR-ABL[104] and HER2[105] (human epidermal growth factor receptor 2) activate Aurora A kinase whereas Chrf[106] and p53[107] tumour suppressors promote its degradation.

The activation of Aurora A is a multi-step process, and besides (auto-)phosphorylation, it requires the interaction of protein TPX2.[108] Activated Aurora A – directly or indirectly – stimulates all major intracellular signalling pathways: MAPK,[109] PI3K/Akt[110] and NF-κB.[111] However, the most important role of Aurora A is to facilitate the G2/M phase transition:[112] it phosphorylates the aforementioned p53 tumour suppressor[113] and negatively regulates its function. In turn, p53 represses transcription of *AURKA*.[114] Aurora A also phosphorylates the PLK kinase[115] and activates key structure proteins that orchestrate maturation, duplication[116] and separation of the centrosome.[117] Later, during mitosis the main role of Aurora A is to stabilize microtubule spindles[118] and indirectly to ensure the stabile biorientation of chromosomes.[119] At the end of mitosis Aurora A also triggers the completion of M/G1 transition, the "mitotic exit".[120] What is more, its degradation is crucial for the proper separation of daughter cells – called *cytokinesis*.[121]

**Role of Aurora A kinase in cancer**

Evidences point Aurora A as a biomarker of cancerous cell growth. The *AURKA* gene is located in a chromosome region that is frequently amplified in cancer.[122] Indeed, besides *AURKA* gene amplification (that means Aurora A protein overexpression and so increased activity) transcriptional and posttranslational modifications all can increase Aurora A activity.[123] Elevated Aurora A activity is a common phenomenon in several cancers like ones of the digestive tract,[124] head and neck squamous cell carcinomas,[125] ovarian cancer,[126] bladder cancer,[127] cervical cancer[128] and is associated with shorter cancer patient survival.

Indeed, increased Aurora A activity influences many hallmarks of cancer formation:

Hallmark 1), proliferation. Aurora A has some non-mitotic functions: it is able to phosphorylate important signalling proteins which relay proliferation signal.[129] Unfortunately cancer cells might express Aurora A in any phase[130] in which case it fosters cell proliferation and induces resistance to cytotoxic therapy.[131]

Hallmark 2), anti-apoptosis. Aurora A directly activates anti-apoptotic signalling[111] and so confers resistance to many anti-cancer drugs.[132]

Hallmark 3), genomic instability. As mentioned above, Aurora A facilitates cell phase transitions. Provided Aurora A has increased activity, it indirectly abrogates the G2/M DNA damage checkpoint.[133] More importantly, increased Aurora A activity might result

in more than two centrosomes (and so multipolar spindles) and cytokinesis failure[134] all of those leading to aneuploidy. This way Aurora A directly contributes to CIN and confers resistance to drugs which interfere with microtubule dynamics.[135]

Hallmark 4) As a consequence of Aurora A activity VEGF expression is upregulated and angiogenesis is stimulated in the tumour mass.[136]

Hallmark 5) Aurora A promotes EMT as well,[105] through activation of several proto-oncogenes like AKT,[137] MAPK (mitogen-activated protein kinase),[138] Coffilin-F-actin,[103] Src,[139, 140] FAK (focal adhesion kinase),[140] Rap-1A[141] and NM23-H1.[142] Aurora A-induced PI3K/Akt signalling also confers resistance to many cytotoxic drugs.[132]

While overexpressed Aurora A protein causes multipolar spindles, cytokinesis failure, thus chromosomal aberrations, its transcriptional silencing impairs centrosome maturation and separation, leading to monopolar spindles, delayed mitotic entry,[143] activation of the MC and thus inhibition of cell proliferation. Silencing of Aurora A induced apoptosis in some experiments,[144] it is still not clear whether Aurora A is a *bona fide* driver.[134] However, since inhibition of Aurora A kinase activity hinders cell division it might be a useful therapeutic target in cancer.[145]

**Aurora B**

After the discovery of Aurora A, a paralogue serine-threonine kinase was identified in many organisms attached to the condensed chromosomes.[146] The new kinase, Aurora B, is also activated by autophosphorylation and regulated by a complex network:[147] for example BubR1,[148] or Mad2 – if overexpressed[149]– counteracts Aurora B function while Bub1[150] and the MAPK pathway[151] activates Aurora B.

Together with proteins INCENP (inner centromere protein),[152] Survivin and Borealin,[153] Aurora B constitutes the highly important CPC (chromosomal passenger complex).[147] The CPC is located at the kinetochores during the first part of mitosis, and then relocates to the microtubule spindle during the last steps.[154] The CPC ensures three delicate tasks during mitosis:

1) condensation of the chromosomes through phosphorylation of histone H3 by Aurora B[155, 156]

2) correct sister chromatid segregation.[157] Aurora B phosphorylates the KMN (Knl1 - Mis12 - Ndc80) protein network, that part of the kinetochore which directly connects to

microtubules.[158, 159] Phosphorylation destabilizes and breaks up erroneous microtubule-chromosome connections which are always weaker than correct ones.[160] On the freed kinetochores new, stabile attachments can build up and in the end only functional connections remain (each sister chromatid is connected to only one of the two centrosomes) that ensures equal segregation of chromosomes. This way Aurora B is an important constituent of the MC.[161] Furthermore, Aurora B directly facilitates MC and chromosome segregation through activation of Mps1 kinase[162] and Hec1 protein,[163] as well.

3) Cytokinesis. As the dividing cell is pulled apart by bipolar microtubule spindles, tension increases on kinetochores of bioriented chromosomes that separate CPC and thus Aurora B from there.[164] The CPC then migrates to the half-section of the microtubule spindle – called *midzone* – and concerts cytokinesis.[165] Assembly of the midzone protein complex on the microtubule spindle will mark the point where cytokinesis will occur.[166, 167] At the end of mitosis Aurora B protein is degraded just like Aurora A.[168]

**Role of Aurora B kinase in cancer**

Currently no mutation is known in any genes of the CPC proteins. *AURKB* gene amplification, or altered promoter methylation have not been reported either.[169] In human cancer cells level of Aurora B protein is often reduced, e.g. by simultaneous deletion of *AURKB* and *TP53* genes.[170] Since p53protein is able to arrest cell-cycle at the G2/M checkpoint in case of genetic alterations, absence of these two central regulator proteins might contribute CIN.[171] The apoptotic regulator Mad2 protein – if overexpressed – also able to reduce level of Aurora B protein.[149]

While reduced level of Aurora B is not linked to carcinogenesis, overexpression in many cancer cell lines and cancer types[172] is explicitly associated with aneuploidy[173] and poor prognosis.[174] The reason is that overexpression means increased Aurora B activity, over-phosphorylation the aforementioned KMN network and histone H3.[173] These false signals give rise to CIN through three mechanisms: a) accumulation of impaired microtubule-kinetochore connections leads to chromosome segregation problems and aneuploidy;[175] b) cytokinesis failure gives rise to monstrous, multinucleated cells with amplified centrosomes which leads to mal-attachments in the

next mitosis and fosters aneuploidy even further;[90] c) premature sister chromatid separation – that is poorly understood yet.[176]

It is possible though, that in many experiments the elevated level of Aurora B might have been rather the result of increased proliferation itself, since it is predominantly expressed during phase G2 and mitosis.[100] Also, Aurora B is overexpressed together with many other proteins regulating cell division – so it is not entirely clear yet, to what extent increased Aurora B activity contributes to CIN.[169] Although loss of INCENP, Borealin and Survivin also impairs error correction and cytokinesis, there is no strong evidence that omission of any CPC components indeed increase segregation errors in mouse models.[177] Nevertheless, if overexpressed Aurora B is only an accompanyment phenomenon, it is still an important one because druggable by KIs (kinase inhibitors – see chapter 1.3.4.) – unlike Survivin or Borealin.

It is worth to note that loss of Aurora B function results in very similar phenomena to increased activity[178] and can be also detrimental to cells,[179] so there is an optimal level of increased Aurora B activity (see also point 3 in chapter 1.1.2.).[34, 175] Therefore it was hypothesised that further increasing the number of missegregations in cancer cell might be therapeutically favourable, but hard to carry out.[180] On the other hand, depletion of Aurora B protein or inhibition of its kinase activity prevents cytokinesis, results in multi-nucleated polyploid cells and ultimately leads to apoptosis of normal and cancerous cells. Since kinase activity of Aurora B can be inhibited by designed small-molecules, it qualifies as a potential drug target in cancer.[181, 182]

Still, it is not clear whether increased Aurora B activity is a cause or a consequence[183, 170] – many claim that Aurora A is the better target.[184, 185] Indeed, while Aurora A is overexpressed in rapidly proliferating glioblastoma[186] and breast[187] cancer cells and correlated with poor outcome, B is not. Furthermore, inhibition of Aurora B caused neutropenia in some clinical trials.[188]

Yet, since perpetual proliferation is the very essence of cancer and the number of *druggable* (see chapter 1.3.3.) proteins regulating it is limited, pharmacological inhibition of both Auroras remains a possible approach.[189, 190]

#### 1.2.2.2. EGFR

EGFR was the first RTK discovered,[191] and is also one of the most studied kinase.[192] It makes up the EGFR family with HER2, HER3 and HER4.[193] The constitution of EGFR follows the standard build of RTKs (receptor tyrosine kinases): extracellular domains bind the ligand (receptor part) and facilitate dimerization. Linked to them through a short transmembrane segment the intracellular tyrosine kinase domain activates downstream proteins.[194]

EGFR exists as an inactive monomer in the cell membrane of most epithelial cells[194] and activated when its extracellular domains bind one of its specific ligands e.g. EGF (epidermal growth factor).[195] Two activated receptor monomers then able to form a dimer – called *homodimer* if two EGFRs, or *heterodimers* if different members of the EGFR family constitute it.[196] Upon dimerization the two intracellular kinase domains get into proximity and form an asymmetric dimer, in which one kinase domain is the allosteric activator of the other.[197] The activated kinase domain then phosphorylates the C-terminal cytoplasmic tail of its own (*autophosphorylation*) and of the other receptor's (*transphosphorylation*) on several tyrosine residues.[198]

Activated EGFR dimers internalized by endocytosis and either degraded or recycled.[199] However, simultaneously a signalling platform builds up on the phosphorylated C-terminal tails[200] that serve as an origo for many signalling pathways[201] encompassing circa 122 proteins:

a) the RAS-RAF-MEK-ERK (also called MAPK) pathway,[202, 203]

b) the PI3K-AKT-mTOR cascade,[204]

c) the PLC-γ1-PKC pathway,[205]

d) the Jak-STAT pathway,[206]

e) and the NOTCH pathway.[207]

Eventually most signalling pathway activates transcription factors that effectuate the signal coming from EGFR.[196] This way EGFR is able to positively regulate most cellular processes: metabolism, growth, motility, differentiation, survival (anti-apoptosis), migration (EMT) and angiogenesis.[208, 209] Nevertheless, the most striking effect of EGFR activity is the one on proliferation: it drives cells past the G1/S checkpoint during cell cycle.[210] But how can a single receptor regulate so many pathways? First, different ligands of EGFR[211] and the different pH of the internalised

vesicules[212] both seem to trigger distinct downstream pathways.[213] However, the main source of this diversity is heterodimerisation.[214] EGFR family members are able to form heterodimers also with other RTKs, such as c-Met. It is worth to note though that vast majority of these observations happened in cancer cells with overexpressed RTKs,[215] for example EGFR–c-Met heterodimers are present in hepatoma (liver cancer) cells but not in normal hepatocytes.[216]

Furthermore, under certain stimuli EGFR can translocate to the nucleus where it phosphorylates nuclear proteins like histone H4[217] and directly associates with transcription factors[218] and activates genes like *AURKA*.[219] The effect of these functions is also enhanced cell proliferation.[220]

**Role of EGFR in cancer**

The first relationship of receptor overexpression and cancer formation was demonstrated with EGFR[221] so EGFR is also one of the first proven drivers. Indeed, active EGFR promotes many processes, all favourable for cancer cells (see hallmarks in chapter 1.1.2.):

Hallmark 1) fosters continuous cell division,[222]

Hallmark 2) promotes cell survival,[223]

Hallmark 4) elevates the expression level of angiogenic factors and receptors,[224]

Hallmark 5) induces EMT which triggers metastasis but also confers resistance to EGFR TKIs (tyrosine kinase inhibitors – see chapter 1.3.4.2.).[225]

Of course, EGFR and the activated pathways also increase EGFR TKI drug resistance of cancer cells without activation of the EMT process.[226, 227] E.g. heterodimerisation fosters TKI resistance, because a TKI-inhibited EGFR kinase domain is still able to act as an allosteric activator for c-Met.[228] Another possible mechanism of EGFR TKI resistance is the increase of the activity of another signalling component that drives the same pathways as EGFR, like KRAS.[229] In case of NSCLC (non-small cell lung cancer) the appearance of RTK c-Met can be such a phenomenon. Nuclear localisation of EGFR is also particularly common in cancer[230] where it confers resistance to radio-, cytotoxic [231] and EGFR TKI therapy.[232]

Increased activity of EGFR can have many origins:

1) Ligands of EGFR are often overexpressed in human cancers, most prominently EGF that triggers increased EGFR activity.[233] Then elevated EGFR activity further facilitates EGFR expression in a positive feedback loop.[234]

2) Methylation of the *EGFR* gene promoter increases translation and EGFR protein overexpression.[235, 236]

3) The *EGFR* gene is often amplificated (that leads to protein overexpression and increased activity)[237, 238] or mutated.[239] These mutations stabilize ligand-independent homo/heterodimers,[208] facilitate evasion of endocytosis (and the frequency of degradation, so "switching off" of the receptor),[240] or constitutively activate the kinase domain itself. The EGFR kinase domain mutations can be classified into activating and resistance mutations according their main impact on the cancer cell. *Activating* EGFR mutations (e.g. point mutation L858R or various deletions right before the αC helix ($EGFR_{Del}$)) increase and sustain phosphorylation (thus activity) of the receptor without ligand stimulation.[241] This way the cancer cell becomes addicted to the activity of EGFR but simultaneously more sensitive to EGFR TKIs. Therefore these mutations are also called sensitising mutations and their presence and inhibition greatly improves patient survival.[242, 243] Unfortunately, cancer becomes *resistant* in time and most patients relapse. Among the various reasons[244, 245] new, secondary EGFR mutations are often the cause,[246] like T790M residue exchange (see chapter 1.3.4.2.) that does not reduces the affinity of EGFR to the TKI but enhances its catalytic activity.[247] The T790M accounts for approximately half of all secondary, resistance mutations.[248] Unfortunately the T790M mutation is sometimes present in the cancer before treatment; moreover it can also be an inherited polymorphism.[19]

According to all these notions EGFR qualifies as a proto-oncogene[249] *in vitro*[250] and in many human cancer histotypes: carcinomas,[251] sarcomas,[252] gliomas[253] and non-small cell lung cancer NSCLC.[254]

#### 1.2.2.3. c-Met

C-Met is a RTK similar to EGFR and also situated in the plasma membrane. C-Met has one exclusive ligand, HGF (hepatocyte growth factor).[255] When two c-Met monomers bind one HGF with their extracellular domains, they form a dimer and the intracellular kinase domains phosphorylate the C-terminal tails of each other.[256] The active c-Met

(hetero- or homo-) dimer then activates signal transduction pathways, many common ones with EGFR.[257]

Whereas in adults c-Met is expressed by many tissue types (e.g. liver, pancreas, prostate, kidney), its function is more vital during embryonic development and wound healing where it drives cell migration and normal EMT process.[258]

**Role of c-Met in cancer**

Increased c-Met activity can be a result of stronger-than normal autocrine / paracrine HGF stimulus or c-Met protein overexpression – latter sometimes due to *MET* gene amplification[259]– and is present in many cancer types with poor prognosis.[260] Selective inhibition of c-Met is able to beat some cancer cell lines, and *MET* amplificated gastric or NSCLC patients respond to the c-Met–ALK dual inhibitor crizotinib. So in these examples c-Met seems to function as a driver.[261] However, it is hard to appropriately select patients for c-Met targeted therapy[262] because activity of other RTKs (e.g. EGFR) are usually also increased[257] and they are able to form heterodimers. In other words increased c-Met activity is rarely a standalone phenomenon.[256] Furthermore, activating mutations of c-Met are rare.[263]

It is rather important that increased c-Met activity is a source of secondary resistance to EGFR TKIs[264] and cytotoxic drugs,[265] most of all in NSCLC.[266] Similarly to resistance mutations of EGFR, erroneous presence and increased activity of c-Met sometimes occur before EGFR TKI treatment.[267] In both of those cases, simultaneous inhibition of c-Met and EGFR restores sensitivity to EGFR TKIs *in vitro*.[268, 269] This topic is still hot,[270] since after a while cancer cell lines become resistant to c-Met inhibitors as well - which foreshadows the clinical fate of c-Met inhibitor drugs.[271, 272]

## 1.3. Targeted cancer therapy

### 1.3.1. Types of targeted agents

Surgery is the most obvious and also the oldest approach to cure cancer. It proves to be remarkably effective in case of some types of cancer, but has its limitations.[273] In the past ~50 years conventional cytotoxic and radiotherapy have emerged and still represent an important force of anti-cancer efforts.[274] Their common mechanism of action is to interrupt the division process at some point that induces apoptosis of the affected cells. Applied systemically, cytotoxic therapies act on every dividing cell – regardless they

are healthy or cancerous.[275] Therefore most of their side-effects are derived from the malfunction of fast-dividing tissues and are quite harsh: skin rush, hair loss, digestion problems, immunosuppression, myelosuppression, mucositis and hepatotoxicity.[276] Unfortunately even this rude, generic intervention can't keep cancer from acquiring resistance[277] through various means e.g. overexpressing transporters that expel the cytotoxic drug.[278] This principal problem facilitated the development of further alternatives: hormone,[279] immuno-,[280] gene[281] and targeted therapy.

Targeted therapies are designed to interfere with the very driver oncogene(s) of the given cancer type, patient, or single tumour itself. Of course healthy cells are also affected since they harbour proto-oncogenes but they are not "addicted" to them and therefore less sensitive to their loss – as coined by Weinstein et al.[282] Unfortunately, resistance occurs with targeted agents as well[283] and they not necessarily increase survival much better than cytotoxic drugs.[284] Furthermore the number of available approved drugs is very limited even against proven drivers. It is also important to note that targeted therapies are useless without equally developed diagnostic tools, since only those patients benefit from a targeted agent who harbour the given oncogene.[285] Four types of targeted agents exist up to day: monoclonal antibodies,[286] aptamers,[287] immunotoxines[288] and small molecule inhibitors. Regarding the topic of this Thesis the fourth type will be specified in the followings.

### 1.3.2. Properties of small molecule KIs

So called small molecule inhibitors are low molecular weight organic compounds that typically contain several heterocycles. They are not easily biodegradable, so can maintain an effective serum concentration for longer periods. Contrary to monoclonal antibodies which exclusively bind to extracellular domains of transmembrane proteins, small molecule inhibitors freely diffuse through cell membranes without active transport and can inhibit intracellular targets as well. The drawback of this is that small molecules are not targeted by themselves. That is, they can reach almost every protein in the body so inhibit their target no matter it is in a healthy cell or a cancerous one. However, they can be conjugated to targeting moieties like GnRH (gonadotropin-releasing hormone),[289] carbon nanotubes[290] or embedded into liposomes[291] to direct their spatial distribution in the body.

Most small molecules are developed to inhibit protein kinases because they are often drivers in cancers and relatively easily druggable (see chapter 1.3.3.) by structural analogues of ATP.[292] Unfortunately many of the driver proteins e.g. most convergent nodes of pathways are transcription factors with no enzyme activity to inhibit.[293] While the most widely used targeted agents against kinases are monoclonal antibodies, small molecule KIs are close second.[294]

Since ATP is a highly conserved energy currency of all living things, it came as a surprise that analogues of ATP can have enzyme specificity at all. Indeed, the ATP-binding pocket of protein kinases is highly conserved, but the surrounding (mostly hydrophobic) side-pockets are quite unique to the particular enzyme that enables remarkable selectivity of ATP analogue small molecule KIs.[295]

When small molecule KIs attach to the surface of the target protein they disturb conformation of the enzyme and block its activity. This can be achieved through several ways.[73] Basically, KIs are classificated according to the activation state of the kinase target they bind:

Type I and II inhibitors are ATP *competitive*. They all reversibly occupy the ATP-binding pocket thus have to compete with high intracellular ATP concentration. They prefer different positions of the αC helix and DGF sequence – so active (DFG-in) or inactive (DFG-out) conformations, respectively – and utilize the back/front hydrophobic side-pockets depending on their type. Type I inhibitors bind without regard to the conformation of the kinase, but might induce either DFG-in or -out states. Whereas type II inhibitors specifically recognise the DFG-out state. Examples of Type I or II inhibitors are VX-680, MLN8054, MLN8237, erlotinib and crizotinib.[296]

Type III allosteric inhibitors occupy a pocket close to the ATP-binding pocket -thus they are *uncompetitive* or *noncompetitive* inhibitors of ATP.[297]

Type IV allosteric inhibitors occupy a pocket far from the ATP-binding pocket.

Type V inhibitors are *bivalent* inhibitors because they are able to bind to two different regions of the protein kinase domain at the same time.[298]

Type VI inhibitors are *irreversible*: they occupy the ATP-binding pocket like type I inhibitors but harbour a reactive moiety that binds covalently to a suitable residue of the kinase. This way the targeted kinase protein becomes permanently disabled.[299]

Protein KIs usually bind to their target enzyme by forming 1-3 hydrogen bonds with the hinge residues and also interacting with residues of the ATP-binding site and the hydrophobic pockets. A significant amino acid residue of the ATP-binding site is the gatekeeper residue (e.g. threonine 790 in case of EGFR) that usually shrinks a hydrophobic pocket and hinders the attachment of KIs.[300] The effectiveness of a reversible inhibitor can be described with the dissociation constant and the $IC_{50}$ value – later is the inhibitor concentration required to elicit half of the maximum effect.

The number of diseases targeted by KIs is increasing: inflammatory and autoimmune diseases,[301] hypertension, Parkinson's disease. However, most KIs are designed purposely for cancer treatment.[302]

Protein KIs have generally good toxicity profile[303] but some patients experience quite harsh side-effects.[304] Still, targeted agents do not prolong life greatly compared to conventional cytotoxic drugs.[305] After the initial response[306] resistance occurs to nearly all KIs in a few months or years.

### 1.3.3. Development of small molecule KIs

The two preconditions of anti-cancer drug development are:[307]

- a validated drug target, practically a malfunctioning protein that is a proven driver of cancer. Furthermore, it has to be *druggable* – that is it has to be accessible by e.g. small molecule drugs and should have a specific function that can be inhibited upon binding with the drug.
- finely adjusted, reliable assays that provide useable data.

Provided these are given, the time-honoured first step of drug development is the screening of numerous compounds against the targeted kinase. Large molecule libraries contain several thousands of compounds and usually have a multi-layer structure – like the NCL™ (Nested Chemical Library™) of Vichem Ltd. (Figure 2). In case of focused or knowledge-based screening only a smaller subset of the molecule library is checked that is likely to have activity – like the EVL™ (Extended Validation Library™) of Vichem Ltd.[308] There are of course more modern approaches to drug development, e.g. *in-silico* modelling and design is getting more and more invaluable. Except molecular docking none of them were used in the Thesis, therefore not discussed here.

The tool of screening is mostly an *in vitro* assay where a recombinant kinase represents the target. This approach focuses only on the interaction of compound and kinase.

However, it is possible to utilize more expensive and time consuming cell-based assays on cell lines driven by the particular target. In that case some information is acquired also on the metabolism and secretion of the compound in a living cell.[309] This is important because many compounds that effective in *in vitro* enzyme assay fail in cellular tests due to a number of conditions modifying their effect (e.g. enzymatic degradation or susceptibility to drug efflux pumps).[310] However, in this case subsequent assays are even more important to confirm mechanism of action of compounds.[311] Whether enzyme or cell-based assays are the best to begin is still an open question.[312] Finally, the outputs of a screening process – called *hits* – have verified activity on the given target. Dose-response curves of hits usually obtained as soon as possible to get $IC_{50}$ values which enable refined comparison of compounds.

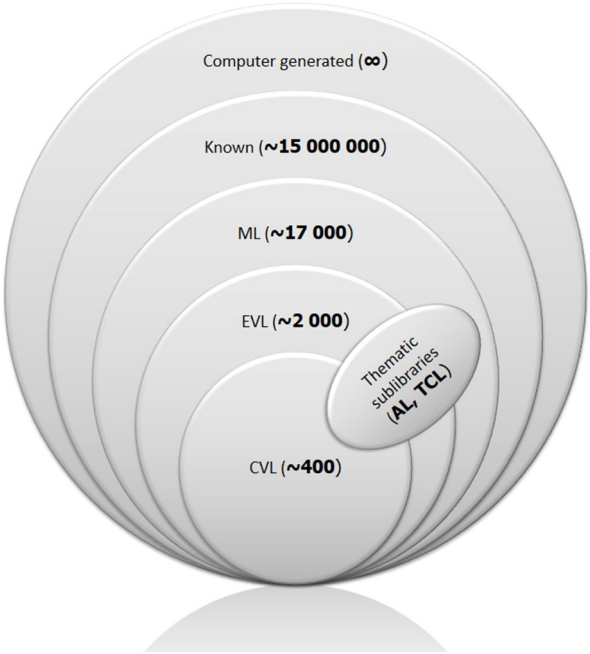



**Figure 2.** Build-up of the Nested Chemical Library (NCL™) of Vichem Ltd. [https://vichemchemie.com/nested-chemical-library-ncl]

The second step of drug development is the hit to lead phase. Hits undergo many further functional assays to test their "drug-likeness": *pharmacokinetic* properties like the aforementioned membrane permeability, and ADME (absorption, distribution, metabolism, excretion) parameters. Also solubility and drug selectivity measurements commenced in this phase. The aim of SAR (structure-activity relationship) study is to

define essential substituents associated with activity. Small molecules usually designed according to Lipinski's rule of five[313] and considered drug-like if they possess these features:

- have molecular weights of less than 500 Dalton (g/mol)
- have a clogP value (a measure of membrane permeability) not greater than 5.
- have no more than 5 hydrogen bond donors
- have a maximum of 10 hydrogen bond acceptors

Promising molecules are addressed to the next step: lead optimization. During this phase the aim is to maintain favourable properties while improving deficiencies through modification of the structure. For this purpose new analogues are synthetized. After additional rounds of pharmacokinetic and *in vivo* pharmacodynamic assays, a clinical candidate is declared.

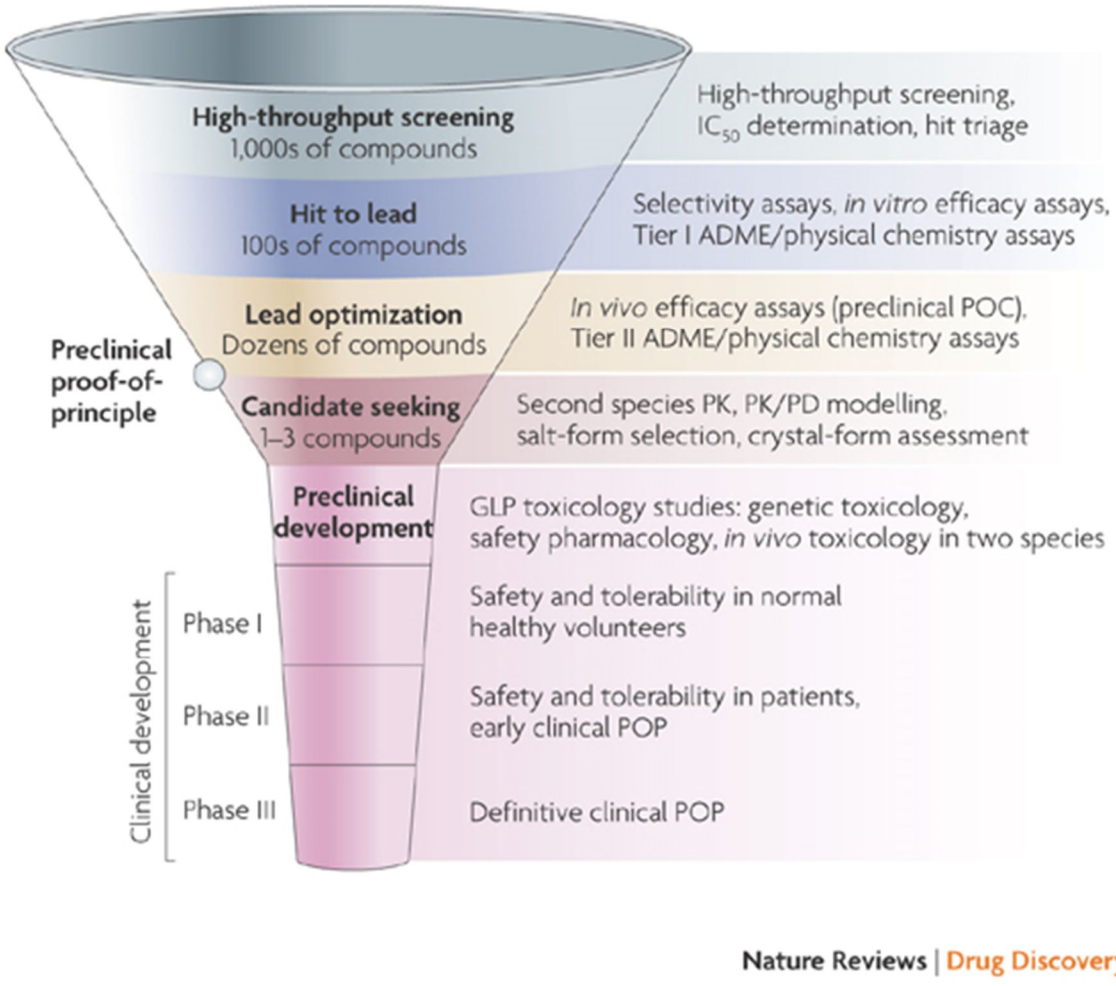



**Figure 3.** Scheme of the drug discovery and development process. The diameter of the funnel represents the number of molecules involved at the particular level.

Up to $10^6$ molecules have to be screened to find one or two clinical candidates. Attrition of compounds in the clinical phases is much lower – approximately 1 in 10 reaches the market. In turn, the cost of clinical trials is much higher than of the previous preclinical tests (Figure 3).

### 1.3.4. Examples for small molecule KIs

#### 1.3.4.1. Aurora KIs

Inhibition of Aurora kinases affects all dividing cells like conventional cytotoxic drugs therefore similar systemic effects are expected. The rationale of aurora inhibition lies in the fact that their activity tends to be increased in cancer cells (see chapter 1.2.2.1.). AKIs (Aurora kinase inhibitors) can be more-or-less paralogue selective or pan-AKIs. The major cellular phenotypic response of dual Aurora A and B inhibitors is consistent with inhibition of Aurora B, in other words inhibition of Aurora B has dominant phenotype.[314] So it was hypothesized for a long time that these dual inhibitors mediate their anti-cancer activity through inhibition of Aurora B activity.[315, 316] Now there are quite selective Aurora A inhibitors that also able to induce apoptosis. The most notable AKIs that reached phase II up to date are:

- VX-680 (tozasertib, MK-0457, Figure 4/A) is a type I small molecule inhibitor that promotes DFG-out conformation of Aurora kinases[317] A and B – so it is a pan-AKI. VX-680 efficiently abrogated the growth of tumour xenografts in animal models[318] but failed in clinical trial phase II due to frequent adverse events and low efficiacy.[319]
- MLN8054 (Figure 4/B) and MLN8237[320] (alisertib, Figure 4/C) are both type I inhibitors, promoting DFG-out state. Since Aurora A is more likely a driver, so MLN 8054 was developed by Millennium Pharmaceuticals (now Takeda Oncology Company) in 2007 to be selective to Aurora A. MLN8054 decreases proliferation of cancer cell lines in *in vitro* cell culture and in xenografts.[321] Unfortunately in phase I study MLN8054 caused somnolescence in patients with advanced solid cancers because of off-target $GABA_A$ receptor ($GABA_AR$)-binding.[322] After minimal modification of the structure of MLN8054 a new analogue, namely MLN8237 was developed. MLN8237 has similar pharmacokinetic properties to MLN8054 and quite the same $GABA_AR$-binding but has increased affinity to Aurora A.[320] Several clinical trials have been commenced with MLN8237 alone[323, 324, 325] or in combination with other

drugs[326, 327, 328, 325] but only one proceeded to phase III so far, and even that one was terminated in 2015[329] because of harsh general cytotoxicity. However, applying it more carefully for the treatment of selected patients and using more precise dosing MLN8237 is worth for further investigation.[330, 331] So recently new trials have been started with MLN8237. [www.clinicaltrials.gov]

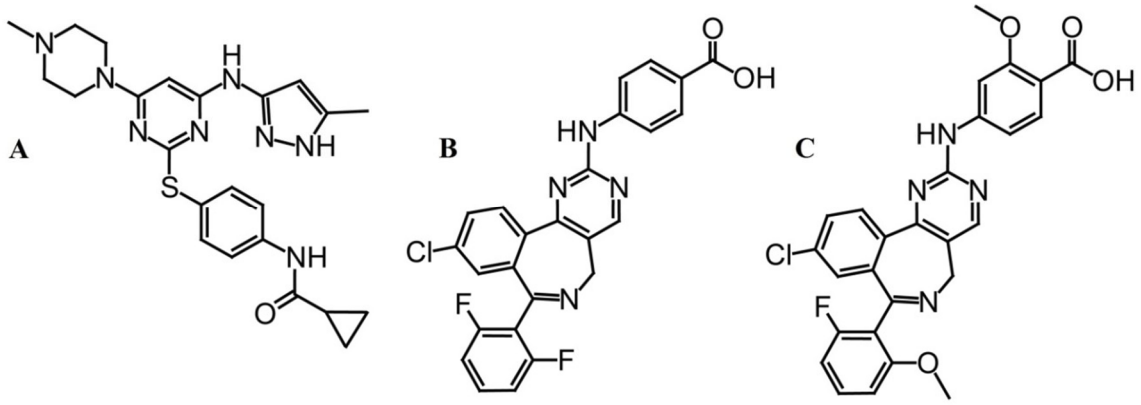



**Figure 4.** Chemical structure of reference compounds A) VX-680, B) MLN8054 and C) MLN8237

- AZD1152 (barasertib) is a dedicated Aurora B inhibitor which induces apoptosis in human ALL (acute lymphoblastic leukaemia)[332] and AML (acute myeloid leukaemia)[333] cell lines. After several phase I studies AZD1152 was evaluated in two phase II trials with randomized AML patients. Despite frequent adverse events[334] approximately 35% of patients had complete cancer remission compared to 11.5% in case of the conventional cytotoxic drug cytosine arabinoside.[335] AZD1152 showed transient toxicity and modest response in ~20% of B-cell lymphoma patients, but further phases as monotherapy were not encouraged in the report.[336]

- AT9283 (type I, promotes DFG-in) is rather a multi-kinase inhibitor with considerable effect on Aurora A/B, JAK2/3 and ABL1 kinases.[337] After several phase I studies AT9283 failed in phase I/II trial due to lack of clinical response.[338]

- ENMD-2076 (type I, promotes DGF-in) inhibits FLT3/4, RET, Aurora A and VEGFR3 kinases in the low nanomolar range. Unfortunately it failed in clinical phase II trial against ovarian clear cell carcinoma because of low efficiacy.[339] However, ENMD-2076 provided benefit for 17% of advanced or metastatic triple-negative breast cancer patients with moderate adverse effects.[340]

- PHA-739358 (danusertib, type I, promotes DGF-out) is basically also a pan-AKI with slightly stronger effect on Aurora B than Aurora A. Two phase II trials were performed with PHA-739358. In the first study toxicity was tolerable and ~13% of prostate cancer patients had complete remission after failing to respond to Docetaxel.[341] In the second study patients with various types of cancer (including NSCLC) had manageable adverse effects but also minimal progression.[342] Currently there is no sign of any new study with PHA-739358.

- SU-6668 (orantinib) is a potent inhibitor of several kinases including Aurora A and B.[343] Phase I/II study of SU-6668 for advanced hepatocellular carcinoma was completed with promising results[344] but a phase III study in combination with transcatheter arterial chemoembolization in advanced hepatocellular carcinoma was terminated because the treatment did not improve overall survival of patients at all.[345]

- MK-5108 is a highly selective Aurora A inhibitor (type I, promotes DGF-out). Despite modest effect in monotherapy, anti-cancer activity of MK-5108 was significantly enhanced when combined with taxane-based cytotoxic drugs in preclinical studies.[346] However, phase I study was terminated due to high toxicity when administered in combination with docetaxel.[347]

To sum up, at least 30 AKIs have been evaluated as cancer therapeutics in the last few decades[348, 349, 350, 351, 188] yet, there is still no approved AKI in the market. The possible reasons are diverse. For example one huge handicap of AKI therapy is the lack of markers (including Aurora A or B expression levels themselves) which significantly correlate with their anti-cancer activity.[188] Therefore patients cannot be selected to AKI trials (about how important is to have selected patients for trials of targeted agents see next chapter on erlotinib and gefitinib). Furthermore, it might be possible that selective Aurora inhibition alone is not eligible for cancer monotherapy at all:

1) Inhibiting cell division is a strategy very alike to conventional cytotoxic drugs. Although cancer cells are more susceptible to it, side-effects still common (see history of AKIs above).

2) Preclinical experiments suggest that Aurora inhibition might induce cancer cell senescence, that would result in disease stabilization instead of remission.[352]

3) Response to Aurora inhibitors is slow because certain cycle of cell divisions should occur beforehand (see also chapter 4, Figure 10).[353]

4) Interacting protein partners of Aurora kinases (TPX2 and INCENP) influence whether the kinase is in a DFG-in or -out state and so alter the paralogue selectivity and efficacy of AKIs accordingly.[354]

There is also no multi-kinase inhibitor with strong effect on Aurora kinases which succeeded in clinical trials so far. However, many of the aforementioned studies suggest using AKIs in combination with conventional cytotoxic drugs.[355] Since concurrent treatment with targeted agents is on the rise, also AKIs have already been combined with other small molecules (see 1.3.5.).

### 1.3.4.2. EGFR inhibitors

EGFR inhibitors were among the first targeted small molecule KIs designed and approved. The most notable examples are:

- erlotinib (Genentech, approved by the FDA (food and drug administration of the US) in 2004) and gefitinib (AstraZeneca, FDA approved in 2003, withdrawn in 2005, approved again in 2015) were the first line of EGFR TKIs. Initial clinical trials of erlotinib and gefitinib were conducted on unselected patient cohorts therefore failed to show significant response.[356] It turned out soon that they give longer survival compared to cytotoxic drugs only in patients harbouring certain activating EGFR mutations like EGFR L858R and $EGFR_{Del}$),.[242, 357] It later turned out to be a general phenomenon: various EGFR mutations have very different sensitivity to a given TKI.[358] Unfortunately, even among patients initially responding to erlotinib or gefitinib resistance occurs in time, resulting in a median overall survival of only 27 months.[359] So second-generation EGFR TKIs were developed to overcome resistance mutation T790M – all of them are irreversible, class VI inhibitors:

- afatinib has been the third FDA-approved EGFR TKI, it is active against both activating and resistance mutant EGFR.[360]

- neratinib (HKI-272) is active against certain resistance EGFR mutations but not against common ones.[361] In a phase II study neratinib in combination with temsirolimus (a cytotoxic drug) produced responses in ~19% of patients.[362] Nertatinib has been approved for adjuvant therapy. [https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&varApplNo=208051]

- PF-00299804 (dacomitinib) is an experimental irreversible inhibitor of EGFR with which several phase III trials were conducted so far. One of them ended in 2017 and

found PF-00299804 a little bit more effective than gefitinib for NSCLC. [https://www.medpagetoday.com/MeetingCoverage/ASCO/65818] [http://www.medscape.com/viewarticle/881192)]

The third generation of approved EGFR TKIs were also designed to inhibit activating and resistance mutations but without inhibiting wild-type EGFR:

- osimertinib was approved in 2017 by the FDA.[363]
- olmutinib is a type IV EGFR inhibitor approved only in South Korea.[364]
- EGF816 (nazartinib) is still in experimental phase.[365]

The new step of the never-ending race, a new secondary EGFR mutation (C797S) was found recently in one patient that restores resistance to osimertinib[366, 367] and olmutinib.[368] Strange enough, this mutation appeared to be sensitive to first-generation EGFR TKIs alone or in combination with a third-generation one.[369]

While inhibitors of resistance mutant EGFR already exist, if *MET* gene amplification (and overexpression of c-Met) is diagnosed, patients have poor prospects. Therefore it is highly desirable to develop dual EGFR–c-Met inhibitors (like compound **34**)[370] or assess the feasibility of EGFR and c-Met inhibitor drugs in combination.

#### 1.3.4.3. c-Met inhibitors

Inhibitors of c-Met are less numerous than AKIs or EGFR TKIs:

- crizotinib. Up to date the ALK/ROS1 inhibitor Crizotinib (Pfizer, FDA-approved in 2011) is the only marketed drug with significant potency on c-Met.[371] Crizotinib was designed to be a selective c-Met inhibitor[372] but was approved for the treatment of EML4-ALK fusion protein-driven NSCLCs (5% of all NSCLC patients). The overall response rate is 57% and resistance occurs with a median of ~10 months. A dozen of mutations can cause resistance to crizotinib but strikingly most of them don't affect the sequence or abundance of EML4-ALK protein.[373]
- BMS-777607 (Bistrol-Myers Squibb) is an effective inhibitor of c-Met, RON and AXL kinases.[374] BMS-777607 proved to be effective against gastric cancer xenografts *in vivo*[375] but failed in Phase I/II trials on patients with advanced or metastatic solid tumours [clinicaltrials.gov].

### 1.3.5. Combinatorial therapy

Conventional cytotoxic drugs of different mechanism of action were first designed for monotherapy, but it turned out soon (as far as 1960) that their combination boosts the anti-cancer effect in many cases.[376] Similarly, first KIs (regarding the topic of the Thesis predominantly the combination of KIs will be discussed in the followings) were designed to be exclusively selective for the targeted kinase, but this task turned out to be difficult. Differences in the side pockets of the ATP-binding pocket are not so huge to allow designing a 100% selective inhibitor for any kinase. Therefore most current KIs have a more or less wide spectrum of targets.[377]

Unfortunately, KI monotherapies often result in the resistance of cancer cells because they tend to harbour more than one driver at the moment of diagnosis and if not, they easily collect new ones when treated with drugs due to CIN.[378] Thus, multi-target KIs would be rather desirable. However, due to the differences of side pockets it is almost impossible to design a multi-target, ATP-analogue KI for two (or more) arbitrary kinases. It is much easier in case of evolutionarily related kinases (like members of the EGFR family) than distant ones, (like EGFR and c-Met).[370] Since driver kinases in a cancer cell seldom related in structure, this condition highly limits the use of multi-kinase KIs as anti-cancer drugs.

Another approach is to use KIs in combination. Theoretically any two or three kinases could be targeted this way, in fact toxicity frequently limits the applicability of otherwise successful combinations.[53, 379] There are further reasons why combining targeted agents in general is more challenging than conventional cytotoxic drugs[380]:

- their mechanism of action is more complex and thus not completely understood,
- there is a lack of standardised preclinical and clinical tools to assess target effects,
- conventional methodology of clinical trials might not be suitable for combination therapies,
- regulatory and intellectual property circumstances are not favourable for the commercialisation of drug combinations,
- finally, drug combinations are expected to have higher price for healthcare systems and patients.[381]

So up till now there is no approved combination of targeted agents, they are typically applied together with traditional cytotoxic drugs.[382] At the same time, results of clinical

trials are enticing because combination of targeted agents also have some compelling advantages:[383, 384]

- existing drugs can be approved for several new indications as part of a combination, which also means more available new therapy. Considering that the growing expenses of development more and more delay approval of new drugs, it really is good news.
- it is possible to assess the most effective (see synergism soon) drug cocktail on the given driver set. This approach – called *personalised medicine* – promises maximal therapeutic effect with minimal side-effect,
- the most substantial property of combination therapy is that it can forego and overcome drug resistance by targeting multiple drivers[306, 293] and multiple pathways.[385]

It is worth to note that while occurrence of drivers – either prior to treatment or as secondary resistance – is heterogeneous, it has recurrent patterns that help to design effective drug combinations.[293] Accordingly, the possible setups for combinatorial therapy might be (in case of two drivers):

- inhibition of the same driver with two drugs – resistance easily emerge in this case.[386]
- inhibition of multiple nodes in the same pathway – it is better because more than one driver in the same pathway is rare,[387, 388] it rather occurs as drug-induced resistance.[389, 390]
- inhibition of components of parallel signalling pathways which are typically utilized by cancer cells to bypass monotherapy, like c-Met amplification and overexpression upon EGFR TKI therapy,[391, 392] or GPCR (G protein-coupled receptor) activation upon MAPK inhibition.[393]

So called *synthetic lethal* interaction of certain protein targets offers an exceptionally favourable – albeit rare – opportunity for drug combinations. The term "synthetic lethality" means that inhibition of either protein causes no harm to cancer cells but both induce apoptosis. For example defect of a tumour suppressor (e.g. BRCA1 – breast cancer 1) endows another protein (e.g. PARP – poly (ADP-ribose) polymerase) to be essential for cancer cell survival and the concomitant inhibition of this second enzyme induces strong apoptosis.[394] According to a recent study Aurora A kinase inhibition is synthetic lethal with loss of the RB1 tumour suppressor gene.[395] Also EGFR and c-Met can act as synthetic lethal pairs in some circumstances.[269]

Several mathematical models exist to assess the effectivity of a given drug combination. The method of Chou and Talalay[396] is the most widely used nowadays. According to this model a combination of two drugs (each one at an exact concentration) has a CI (combination index) value that indicates whether synergy, additive effect or antagonism arises at the given concentrations. Synergy is desirable, because it typically means high effect at low doses – so less drug burden for the patient (and presumably less severe side effects).

Last, but not least it is crucial to know the individual drivers present in the given cancer before commencing combinatorial therapy. Sometimes even the combination of 2-3 drugs to block 2-5 pathways are needed to kill all cancer cells in cellular experiments. On the other hand, some of these combinations work at extraordinarily low doses (but still at low CI values) – as it was observed in promising in-house experiments (data not shown). Whether these results will apply to more complex *in vivo* systems is of course yet to decide.

Also AKIs have already been combined with many targeted agents. For the scope of the Thesis the following combination partners are particularly important:

- EGFR inhibitors[397]
- Src inhibitiors,[398, 399]
- PI3K/mTOR pathway inhibitors[400]
- histone deacetylase inhibitors.[401], [402]
- farnesyl transferase inhibitors[403]
- proteasome inhibitors [https://clinicaltrials.gov]

## 2. Aims of the Thesis

The general aim of my work was to progress the field of targeted drug development. Considering the central role of Aurora kinases in cell division and cancer, the lack of approved AKIs is perplexing. In the molecule library of Vichem Ltd. a small molecule family was found to have promising effect on Aurora kinases. The compounds are based on a benzotiophene-3-carboxamide scaffold, unprecedented among published AKIs. Therefore in the followings I had one major and two secondary objectives:

I) To corroborate the AKI potency of the benzotiophene-3-carboxamide derivatives. To achieve this, biochemical (*in vitro* enzyme assays), computational (*in silico* molecular docking) and various cellular assays (cell viability measurement, flow cytometry, fluorescence microscopy and western blot) were utilized. In the end a lead molecule was selected.

II) To achieve better understanding of Aurora kinase inhibition using the benzotiophene-3-carboxamide derivatives. Therefore structure-activity relationship (SAR) and Aurora paralogue selectivity of the compounds were monitored.

III) To test the lead AKI compound in combination with experimental or approved targeted agents. Six of the applied combinations were already published, one was an original idea and one was performed by using another in-house inhibitor.

## 3. Materials & Methods

### Compounds

The benzotiophene-3-carboxamide based AKIs (compound **1**-**33**) and the EGFR–c-Met dual inhibitor (compound **34**) were designed, synthesised and provided by the Vichem Chemie Ltd. (Budapest, Hungary). The reference compounds VX-680, MLN8054, erlotinib, crizotinib were purchased from Selleck Chemicals LLC (USA) and Sigma-Aldrich, respectively. All compounds were solved in anhydrous DMSO (dimethyl sulfoxide), stored at rt (room temperature) and their purity was verified by HPLC every three months.

The Molecular Library of Vichem Ltd. possesses more than 17000 chemical entities collected around 110 core structures, majority of them original, patentable compounds. The EVL™ encompasses ~2000 carefully chosen compounds as a representative set of the whole Molecule Library.

### General cell culturing protocol and cancer cell lines

HCT 116 and HT-29 human colon carcinoma cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD, U.S.A.), primer fibroblast cells were isolated in-house. HCT 116 was maintained in McCoy's 5A, HT-29 in RPMI and primer fibroblasts in DMEM cell culture medium supplemented with 10% (V/V) FBS (foetal bovine serum). All media contained antibiotics (MycoZap™ Plus-CL, Lonza Group Ltd., Switzerland). All cell lines were cultured at 37°C, in a humidified, 5% $CO_2$ incubator. Cell culture media containing FBS and antibiotics are referred as "complete media".

Routine passaging and seeding to multi-well plates for experiments was performed with typsinisation: cell culture was washed with sterile PBS (phosphate-buffered saline), then incubated with 0.1% trypsine-EDTA solution (Lonza) for 10-15 min at 37°C, in a humidified, 5% $CO_2$ incubator. Detached cells were resuspended with excess amount of complete medium and pelleted by centrifugation (300x g, rt). The pellet was resuspended in 1 ml complete medium and 50 µl of it was mixed with equal amount of 0.4% (m/V) trypan-blue solution. Cell number in the stained sample was counted with Bürker-chamber.

**MTT cell viability assay**

For MTT measurements 8000 cells were seeded into each well of a 96-well plate in 150 µl complete medium. Cells were let to attach overnight at 37°C in a humidified, 5% $CO_2$ incubator. Four-fold concentrated dilutions of drugs were added to the wells – each in 50 µl. The concentration of DMSO was always kept at maximum 0.5% (V/V). For the determination of $IC_{50}$ values three-fold serial dilutions were created starting from 10 µM. After further 48 h incubation the treatment medium was removed and 50 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (2 mg/ml in PBS) was added to each well. Plates were incubated (1.5 h, 37°C), MTT solution was carefully removed and crystalline formazan was solubilized with 200 µl detection solution (2-propanol, 1 mM HCl and 10% (V/V) Triton X-100). Absorbance was measured with a Synergy 2 plate reader (BioTek), at wavelengths 570 and 635 nm. The 635 nm data (reference wavelength) was subtracted from 570 nm data (test wavelength) and results were used to calculate normalised cell viability data compared to DMSO treated positive and cell-free negative control wells. Using these data $IC_{50}$ values were determinated with Excel (Microsoft) and XLfit 5.1.0 (IDBS, Surrey, UK) software.

***In vitro* inhibition of recombinant kinase activity**

Active, recombinant Aurora A and B enzymes were incubated with ATP, fluorescent dye-conjugated peptide substrate and compounds of various concentrations in a suitable buffer solution.

Constitution of Aurora A reaction buffer was: 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid) pH 7.5, 1 mM DTT (dithiothreitol), 2 mM $MgCl_2$ and 0.01% (V/V) TWEEN 20 as detergent. TAMRA-PKAtide (5TAMRA-GRTGRRNSI-$NH_2$, Sigma) was used as substrate at a final concentration of 400 nM. The final concentration of ATP was 8.3 µM ($K_{M[ATP]}$) and 8 nM for the Aurora A recombinant kinase (Proteros Biostructures).

Constitution of Aurora B reaction buffer was: 20 mM HEPES pH 8.5, 1 mM DTT, 2 mM $MgCl_2$ and 0.01% (V/V) BriJ35 as detergent. TAMRA-PKAtide (5TAMRA-GRTGRRNSI-$NH_2$, Sigma) was used as substrate at a final concentration of 400 nM. The final ATP concentration was 125 µM ($K_{M[ATP]}$ for Aurora B). Aurora B recombinant kinase (SignalChem, lot: E021-1) concentration was 4 nM.

Enzyme activity was assayed in 384 well microtiter plates (Corning 3676). Reaction time was 30 min for Aurora A and 1 h for Aurora B, at rt. Arrest of enzyme reaction and detection of the phosphorylated peptide substrate was performed by IMAP detection mixture (100% (V/V) IMAP Binding Buffer A, 1/400 IMAP Binding reagent, Molecular Devices). The fundament of IMAP assay is that phosphorylated peptides bind with high affinity to metal ions ($M^{3+}$) immobilized on the surface of nano-scale beads. The phosphorylated peptide substrates are conjugated with fluorophores (like 5TAMRA – carboxytetramethylrhodamine). Upon binding to the bead the degrees of freedom of the peptide and the fluorophore decreases and do not spoil polarisation of the illuminating fluorescent light (Figure 5). Fluorescence polarization and fluorescence intensity measurements were performed using an Analyst GT Multimode Reader (Molecular Devices). Quantification of enzyme activity values was done compared to positive and negative controls. Preliminary screens were run at 10 µM [ATP]. For $IC_{50}$ determination the $K_{M[ATP]}$ (Michaelis-Menten constant) values were determined for both enzymes and enzyme reactions were run at the calculated [ATP] – see exact values above. Determination of $IC_{50}$ values were made with Excel (Microsoft) and XLfit 5.1.0 (IDBS, Surrey, UK) software.

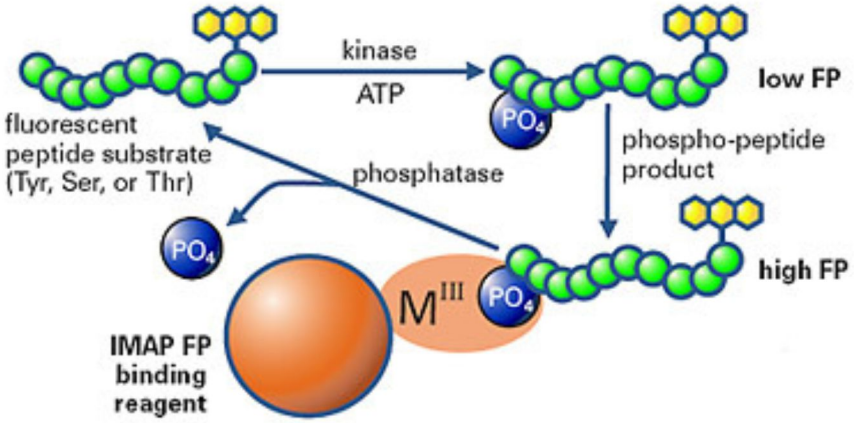



**Figure 5.** Scheme of IMAP technology.
[http://www.moleculardevices.com/pages/reagents/imap_intro.html]

**Flow cytometry methods**

For both staining methods cancer cells were seeded into 24 well plates and let to attach overnight at 37°C, in a humidified, 5% $CO_2$ incubator. Next day culture medium was changed to medium containing reference and in-house compounds and cells were

treated at the concentration and for the time indicated, respectively. After treatment supernatants were collected together with trypsinized cells. The proportion of fluorescent cell populations was detected with a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences). Sample evaluation was performed also with CellQuest Pro and Excel (Microsoft) software.

**- PI (propidium-iodide) staining**

Cell suspensions were centrifuged (250x g, 4 min, 4°C) and fixed with ethanol (70%, -20°C). After at least 24 h (but never more than 72 h) cells were pelleted (250x g, 4 min, 4°C), resuspended in 300 µl apoptosis buffer (200 mM $Na_2HPO_4$, 200 mM citric acid pH 7.8) containing 100 µg/ml RNase A (Sigma), incubated (30 min, rt) and supplemented with PI at 10 µg/ml final concentration. After additional 5 minutes of incubation samples were run on the flow cytometer.

**- PI staining and Annexin V labelling**

Trypsinized cell suspensions were centrifuged (200 x g, 10 min, rt) and washed once with great volume of PBS. Cell pellets were incubated with 100 µl PBS containing Annexin V-FLUOS conjugate (20 min, rt, dark) at the recommended concentration (ROCHE, Ref.: 11 828 681 001). After staining, cells were pelleted again (250 x g, 4 min, 4°C) and resuspended in 300 µl PBS containing PI at 10 µg/ml final concentration. After additional 5 minutes of incubation samples were run through the flow cytometer.

**SDS-PAGE and western blot analysis**

Cancer cells were seeded into 60 mm Petri dishes in complete medium and let to grow until 90% confluency. Then media were changed to fresh complete media with indicated compound concentrations. Cells were incubated with the compounds for 3 h (37°C in a humidified, 5% $CO_2$ incubator) then washed with PBS and lysed at 4°C with ice-cold RIPA buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1% (V/V) NP-40, 0.5% (V/V) sodium deoxycholate, 0.1% (V/V) SDS, 2 mM EDTA, 2 mM EGTA, supplemented right before use with 1 mM DTT, 1 mM sodium orthovanadate, 200 µM PMSF (phenylmethylsulfonyl fluoride) and 0.5% (V/V) protease inhibitor cocktail (Calbiochem). Cell lysates were scraped with rubber policeman, pipetted into Eppendorf tubes, sonicated for 4 x 10 seconds and incubated in ice for additional 20 minutes. Lysates were centrifuged (10000x g, 15 min, 4°C) and protein concentration of the

supernatants were determined according to Bradford method (#500-0207 Bio-Rad). Finally, lysates were mixed with loading buffer (5x concentrated, 62.5 mM Tris-HCl pH 6.8, 2% (m/V) SDS, 10% (V/V) glycerol, 50 mM DTT, 0.01% (m/V) bromophenol blue) and denatured by boiling (5 min, 100°C). Sample volumes containing 4-80 µg protein were separated with constant 130 V by using 10% SDS-PAGE at rt, and transferred with constant 400 mA to PVDF (polyvinylidene-difluoride) membranes (#162-0177 Bio-Rad) at 4°C. Membranes were blocked in TBST (tris buffered saline with 0.1% TWEEN 20) supplemented with 5% (m/V) skimmed milk (1 h, rt), probed with primary antibodies at 1:1000 (TBST with 1% (m/V) BSA, overnight, 4°C), washed three times with TBST (10 min, rt) and incubated with HRP-conjugated (horseradish peroxidase) secondary antibodies (anti-rabbit 1:2000, anti-mouse 1:4000) in TBST supplemented with 1% (m/V) BSA for 1 h at rt. After washing three times (TBST, 10 min, rt) proteins of interest were visualized with chemiluminescence reagent (1-10 min, rt, Western Lightning Plus-ECL, PerkinElmer) on CL-XPosure Films (Thermo Scientific, MA, USA). Primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA): Aurora A (#4718), phospho-Aurora A/B/C (#2914), Aurora B (#3094), Histone H3 (#3638), phospho-Histone H3 (#3377) and from Sigma-Aldrich (St. Louis, MO, USA): Tubulin (T9026). HRP-conjugated secondary antibodies were purchased from Cell Signaling Technologies: anti-rabbit (#7074) and anti-mouse (#7076).

**Fluorescence microscopy**

HT-29 cells were seeded to 96 well Ibidi $\mu$-plate (89626) at 10000 cells/well density in 250 µl complete medium. After 24 h medium was removed and cultures were treated with indicated inhibitor concentrations or vehicle (DMSO) dissolved in 250 µl complete medium and incubated for additional 24 h at 37°C in a humidified, 5% $CO_2$ incubator. At the end of the treatment cells were washed with 250 µl PBS, fixed with 150 µl 4% (V/V) formalin solution (10 min, rt) and washed twice with PBS (10 min, rt). Then cells were permeabilized by 150 µl PBS supplemented with 0.1% Triton X-100 detergent (10 min, rt) and washed twice with PBS for (10 min, rt). Prepared cells were incubated with anti-tubulin antibody (1:10000, Sigma T9026) dissolved in PBS supplemented with 10% (m/V) BSA (overnight, 4°C). Samples were washed with PBS once for 1 min

and three times for 10 min (rt) then incubated with Alexa 488-conjugated secondary antibody (1:500, Life Technologies A11001) dissolved in PBS supplemented with 10% (m/V) BSA (1 h, rt).

Samples were washed with PBS once for 1 min and three times for 10 min then nuclei were stained with 150 µl PBS containing 1 µg/ml DAPI (10 min, rt). After removing DAPI solution, cells were covered with 200 µl PBS and observed with Zeiss Axiovert 200M fluorescence microscope and AxioVision 3.1 software. Images were uniformly taken by using the 63x oil-immersion objective and filter set 25 for DAPI (excitation filter TBP 400/495/570 nm, mirror FT 410/505/584 nm, emission filter TBP 460/530/610 nm) and filter set 10 for Alexa 488 (excitation filter BP 450-490 nm, mirror 510 nm, emission filter BP 515-565 nm). Merged images were created by FIJI software.

**Drug combination experiments**

For drug combination studies cell viability was measured with MTT assay as described above. All compounds were applied in either monotherapy and also in combination at a constant ratio of 1:1 as a serial three-fold dilution starting from 30 µM. Mean cell viability data were transformed to be between 0 and 1 as required by the CompuSyn® software. Therefore mean values equal to or above 1 were set to 0.99 and mean values equal to or under 0 to 0.005. Transformed cell viability data of monotherapy and combination treatments were compared using CompuSyn® v1.0 software (ComboSyn Inc.) and CI (combination index) values were calculated. Only the CI value at the $IC_{50}$ value (0.5 Fa – fraction affected) of a given combination was considered. In practice $CI < 1$ indicates synergistic, $CI = 1$ additive and $CI > 1$ antagonistic effects, respectively. A more refined classification to interpret the CI values provided by CompuSyn® is shown in Table 1.[404] Accordingly, in this Thesis CI values under 0.7 were considered synergism.

**Table 1.** Ranges of CI values calculated by CompuSyn® software and their description.

| Range of CI | Description |
|---|---|
| < 0.1 | very strong synergism |
| 0.1 – 0.3 | strong synergism |
| 0.3 – 0.7 | synergism |
| 0.7 – 0.85 | moderate synergism |
| 0.85 – 0.90 | slight synergism |
| 0.90 – 1.10 | nearly additive |
| 1.10 – 1.20 | slight antagonism |
| 1.20 – 1.45 | moderate antagonism |
| 1.45 – 3.30 | antagonism |
| 3.30 – 10 | strong antagonism |
| > 10 | very strong antagonism |

**Statistical analysis**

Cell viability, enzyme inhibition and apoptosis induction data are expressed as mean value ± standard deviation. Flow cytometry data were analysed by Student's *t*-test (two-sided, unpaired) using Excel software. Statistical significance was defined as $p < 0.05$. Recombinant kinase inhibition measurements were evaluated by calculating the Z' value: $Z'=1-((3SD_{max}+3SD_{min})/(AV_{max}-AV_{min}))$ where $SD_{max}$ is the standard deviation of the positive, $SD_{min}$ is of the negative control, $AV_{max}$ is the mean value of the positive and $AV_{min}$ is of the negative controls. Only measurements of a Z' value higher than 0.5 were accepted for evaluation.

**Docking methods**

For the *in silico* modelling the previously determined crystal structures of Aurora A (PDB ID: 4J8M) and Aurora B-INCENP (PDB ID: 4AF3) proteins were used. All calculations were carried out with the modules of Schrödinger Suites 2015-3 (Schrödinger, LLC, New York, NY) in Maestro. Before docking in-house compounds, the proteins were prepared by removing water molecules and adding hydrogens to the residues with Protein Preparation Wizard. After performing restrained minimization using OPLS_2005 force field, the grid box were centred at the bound ligands of the crystal structures. The 3D structure of the ligand was determined by LigPrep at pH 7.4 by using OPLS_2005 force field.

The binding modes of ligands were identified by Induced Fit docking using Extended Sampling protocol. The best binding poses were chosen for further investigation based on the IFD Score, the docking score, and visual inspection of poses of the docked ligand. All *in silico* molecular modelling were performed by Marcell Krekó at Vichem Chemie Ltd.

**Solubility measurements:**

DMSO stock compound solutions of 5 mM were diluted in DMSO (control) or phosphate buffer (pH 7.4 and pH 2.0) to a 120 µM final concentration. These samples were incubated for 24 hours at rt followed by centrifugation (3700 rpm, 30 min, rt). Next, 40 µl of the supernatants were injected into RP-HPLC and the AUC (Area Unit under the Curve) values were measured on a sample specific wavelength. AUC value of every buffered sample was divided by the AUC value of the DMSO control sample at the same wavelength. Gradient elution: eluent A – 0.1% formic acid in water, eluent B – MeCN. The column was XBridge C18 3.5 µm 4.6 x 50 mm. All work was performed by Eszter Illyés and Zsófia Czudor at Vichem Ltd.

## 4. Results

### Selecting the eight best benzothiophene-3-carboxamide derivatives in cell viability assay

During preliminary, *in vitro* recombinant Aurora A and B enzyme inhibition tests more than 100 benzothiophene-3-carboxamide derivatives were measured at 10 μM ATP concentration (data not shown). Many of those compounds had promising effect (% value) on both kinases. All of them had variable substituents at three positions ($R^1$, $R^2$ and $R^3$) as presented on Figure 6.

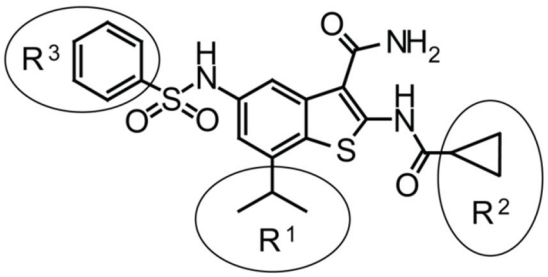
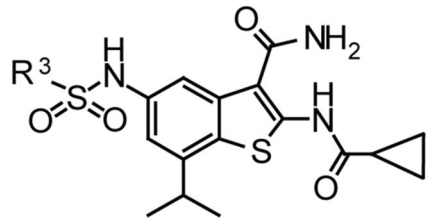



**Figure 6.** Chemical structure of compound **1**. Circles indicate the three important substituents and sites of difference in the benzothiophene-3-carboxamide compound family.

According to the preliminary enzyme inhibition data I assessed the $IC_{50}$ value of 84 selected benzothiophene-3-carboxamide derivatives with MTT cell viability assay (48 h treatment). As model cancer cell line HCT 116 cells were chosen. Since compounds differing in side group $R^2$ have parallel synthesis paths, cyclopropanoyl-amino and methylureido series can be distinguished. Following the logic of chemistry and for clarity I will discuss the cell viability $IC_{50}$ data in two sections.

SAR of the 84 selected benzothiophene-3-carboxamide derivatives was quite coherent. The substance of SAR observations is presented with 33 molecules which inhibited the viability of HCT 116 cells most (Table 2 and 3). For the analysis of SAR see chapter 5, Discussion. According to cell viability data I choose compounds **9**, **11**, **17**, **21**, **22**, **25**, **28** and **31** with an $IC_{50}$ value equal to or below 0.6 μM for further investigations. The cellular effect of these eight compounds was comparable to, or surpassed the pan-AKI VX-680 and the Aurora A inhibitor MLN8054 in MTT cell viability assay.

**Table 2.** The cyclopropanoyl-amino series. Core structure and inhibition of cell viability of compounds **1-8** on HCT 116 cell line using MTT assay. $IC_{50}$ values are the mean of at least three independent experiments ± standard deviation (SD).



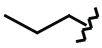
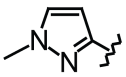
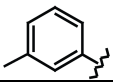
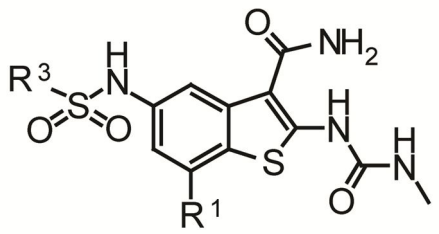
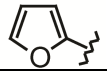
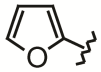
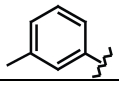
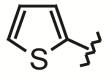
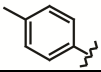
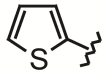
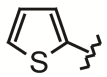
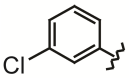
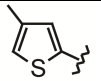
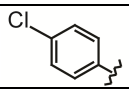
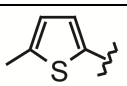
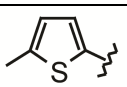
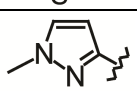
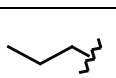
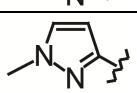
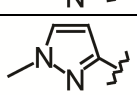
| Compound | $R^3$ | $IC_{50}$ ± SD [µM] |
|---|---|---|
| **1** |  | 1.388 ± 0.964 |
| **2** |  | 9.982 ± 0.032 |
| **3** | $CH_3$ | 1.391 ± 0.291 |
| **4** |  | 2.356 ± 0.320 |
| **5** |  | 6.195 ± 1.104 |
| **6** |  | 4.582 ± 1.027 |
| **7** |  | 0.764 ± 0.226 |
| **8** |  | 9.009 ± 0.994 |

**Table 3.** The methylureido series. Core structure and cell viability inhibition of compounds **9-33** as well as VX-680 and MLN8054 as reference AKIs on HCT 116 cell line using MTT assay. $IC_{50}$ values are the mean of at least three independent experiments ± standard deviation (SD). Cpd = compound.



| Cpd | $R^1$ | $R^3$ | $IC_{50}$ ± SD [µM] | Cpd | $R^1$ | $R^3$ | $IC_{50}$ ± SD [µM] |
|---|---|---|---|---|---|---|---|
| **9** |  |  | 0.313 ± 0.164 | **23** |  |  | 1.297 ± 0.484 |
| **10** |  |  | 1.774 ± 0.778 | **24** | $CH_3$ |  | 0.952 ± 0.188 |
| **11** |  |  | 0.474 ±0.341 | **25** |  |  | 0.287 ± 0.167 |
| **12** |  |  | 3.802 ± 1.168 | **26** | $CH_3$ |  | 0.790 ± 0.073 |
| **13** |  |  | 3.407 ± 1.408 | **27** |  |  | 1.532 ± 0.413 |
| **14** |  |  | 1.024 ± 0.422 | **28** |  |  | 0.338 ± 0.170 |
| **15** |  |  | 6.997 ± 3.133 | **29** |  |  | 2.326 ± 1.014 |
| **16** |  | $CH_3$ | 1.465 ± 0.222 | **30** | $CH_3$ |  | 2.356 ± 0.218 |
| **17** |  |  | 0.600 ± 0.053 | **31** |  |  | 0.556 ± 0.288 |
| **18** |  |  | 0.773 ± 0.288 | **32** | $CH_3$ |  | 9.779 ± 0.240 |
| **19** |  |  | 2.579 ± 0.425 | **33** |  |  | 1.185 ± 0.429 |
| **20** |  |  | 0.946 ± 0.352 | VX-680 | | | 0.449 ± 0.149 |
| **21** |  |  | 0.295 ± 0.085 | MLN8054 | | | 0.850 ± 0.070 |
| **22** |  |  | 0.411 ± 0.224 | | | | |

***In vitro* recombinant Aurora A and B kinase inhibition assay**

I measured the $IC_{50}$ values of compounds **9**, **11**, **17**, **21**, **22**, **25**, **28** and **31** in *in vitro* recombinant Aurora A and B kinase inhibition assays. For $IC_{50}$ measurements ATP concentrations were adjusted to the $K_{M[ATP]}$ value of the particular enzyme. As reference compound the pan-AKI VX-680 was used. According to the $IC_{50}$ values most of the compounds performed better than VX-680 on both kinases. At the same time, $IC_{50}$ values of the eight compounds were very similar, the exceptions being **21** and **31** with an even better effect on Aurora A than on Aurora B (Table 4).

**Table 4.** $IC_{50}$ values of the hit compounds in *in vitro* recombinant Aurora A and B kinase assay. Values are the mean of at least three independent experiments ± standard deviation (SD).

| Compound | Enzymatic assay $IC_{50}$ ± SD [μM] | |
|---|---|---|
| | Aurora A | Aurora B |
| **9** | 0.037 ± 0.017 | 0.035 ± 0.014 |
| **11** | 0.095 ± 0.015 | 0.046 ± 0.026 |
| **17** | 0.024 ± 0.012 | 0.038 ± 0.011 |
| **21** | 0.009 ± 0.003 | 0.023 ± 0.016 |
| **22** | 0.021 ± 0.007 | 0.011 ± 0.006 |
| **25** | 0.032 ± 0.017 | 0.029 ± 0.014 |
| **28** | 0.080 ± 0.037 | 0.048 ± 0.023 |
| **31** | 0.005 ± 0.001 | 0.017 ± 0.009 |
| VX-680 | 0.127 ± 0.038 | 0.043 ± 0.031 |

**Assessing DNA content by flow cytometry**

Since all eight selected compounds inhibited Aurora B in the recombinant kinase assay I measured the ratio of multinucleated (mostly octaploid – 8n) cell population that failed cytokinesis upon compound treatment. All compounds were applied uniformly at 100 nM and for 24 h. Then DNA content was stained with PI and analysed by using flow cytometer. According to the measurements, none of the compounds induced apoptosis (indicated by percentage of cells with attenuated DNA content – the subG1 fraction) compared to vehicle (DMSO) treated control. However, appearance of octaploid cells was explicit in case of VX-680 and in-house compounds **9**, **11**, **25**, **28** and **31** (Figure 7).

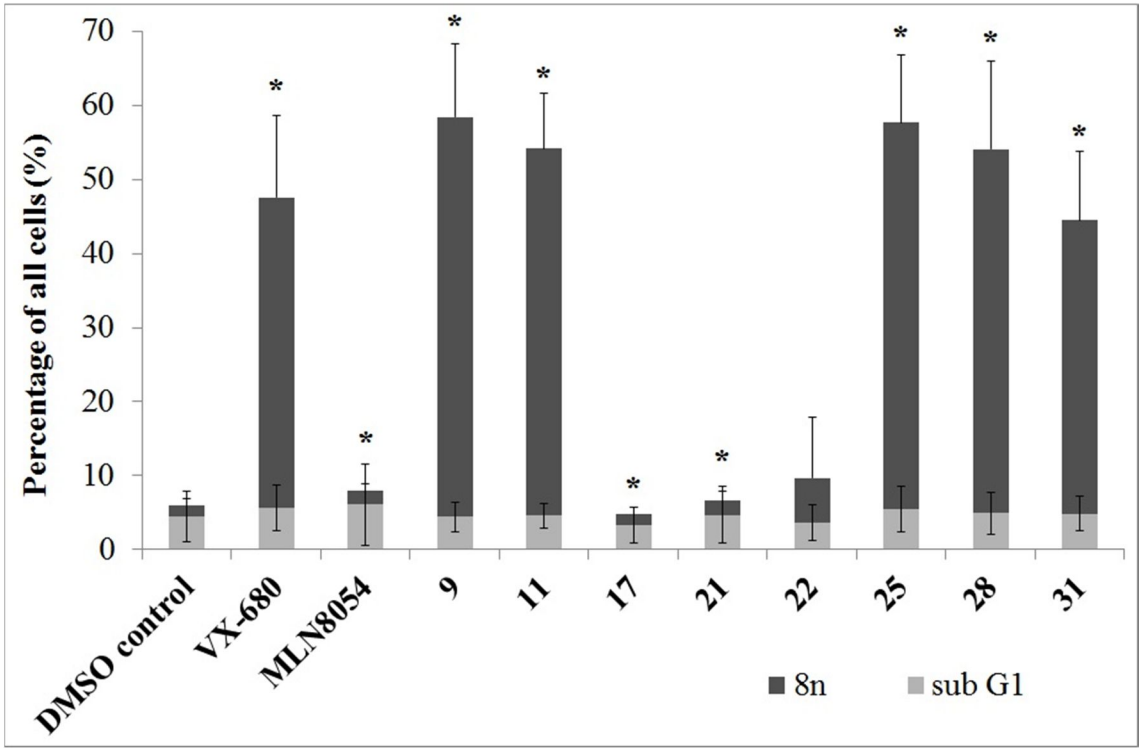



**Figure 7.** HCT 116 cells treated with reference AKIs and eight selected in-house compounds at 100 nM for 24 h. Apoptotic and octaploid cell fractions are depicted as a percentage of total cell population. Treatment groups were replicated at least three times. Error bars represent standard deviation (SD). *denotes significant difference compared to DMSO control ($p < 0.05$) according to Student's *t*-test.

These treatments were repeated also at 1 µM compound concentration. The same two reference AKIs, and two in-house compounds were chosen: **25** that increased ploidity and **21** that did not. Interestingly, at 1 µM also previously ineffective compounds like MLN8054 or **21** inhibited cytokinesis and created octaploid cells. Whereas compounds that were effective inhibitors of cytokinesis at 100 nM – like VX-680 or **25** – showed a slightly decreased effect (Figure 8).

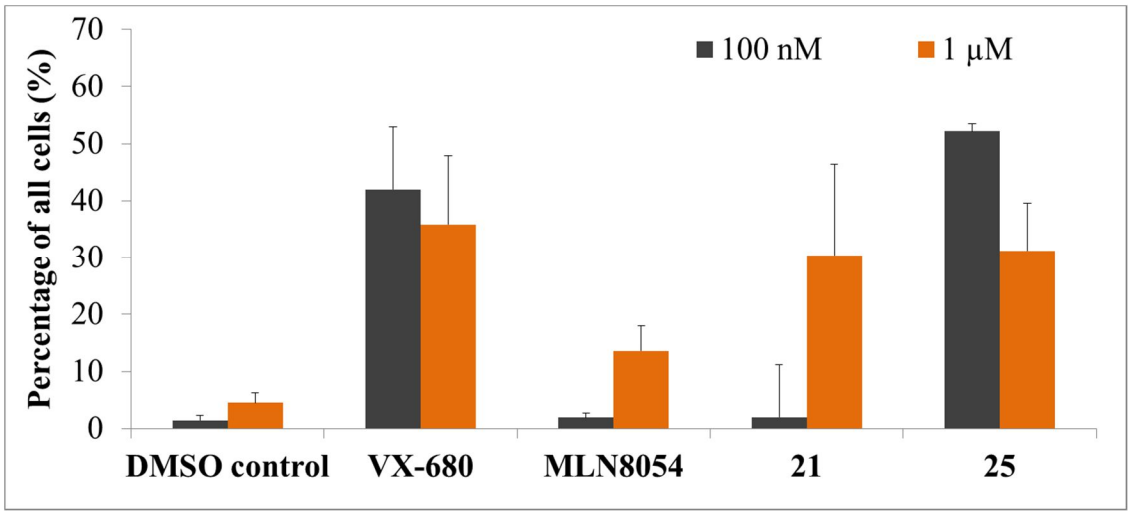



**Figure 8.** HCT 116 cells were treated with AKIs and in-house compounds at 100 nM or 1 µM for 24 h. Octaploid cell fraction is depicted as a percentage of total cell population. Treatment groups were replicated at least two times; error bars represent standard deviation (SD).

**Assessing DNA content by fluorescence microscopy**

I treated HT-29 cells with reference compounds VX-680, MLN8054, MLN8237 and in-house compounds **21** and **25**. Cell morphology was observed by using fluorescence microscope (Figure 9/1 and 9/2). Similarly to flow cytometry results VX-680 and **25** inhibited cytokinesis already at 100 nM and as a result larger-than-normal cells with two or more nuclei appeared in the culture. Compounds **21**, MLN8054 and MLN8237 had no such effect at 100 nM, nor did DMSO. However, at 1 µM also **21** and MLN8237 created multinucleated cells, while MLN8054 still did not.

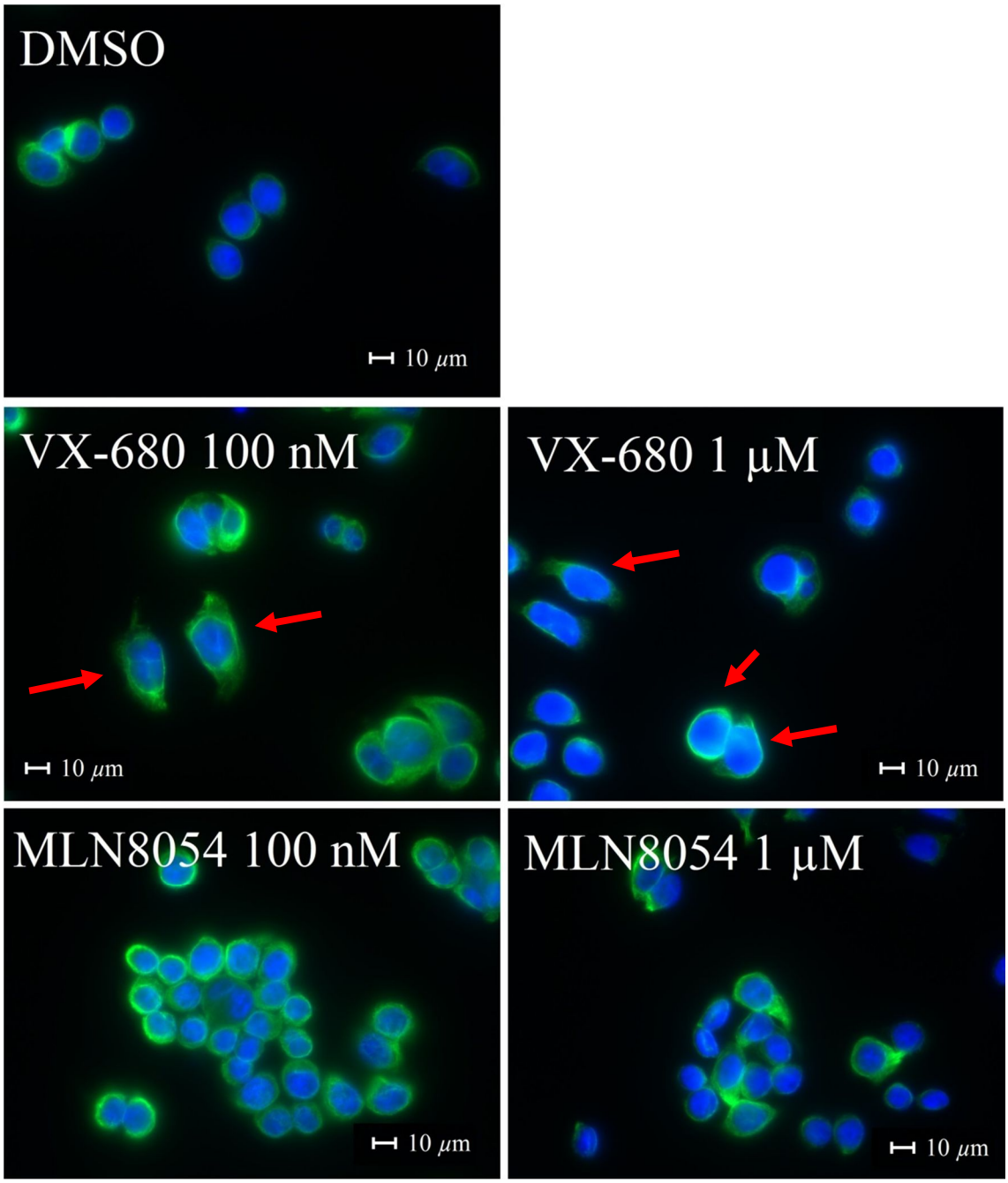



**Figure 9/1.** Merged images of HT-29 cells treated with vehicle (DMSO) or reference AKIs VX-680 and MLN8054 at 100 nM or 1 μM for 24 h. Blue (DAPI) staining indicates nuclei, green (Alexa488) tubulin. Pictures were taken at 63x magnification. Red arrows point to cells with at least two nuclei.

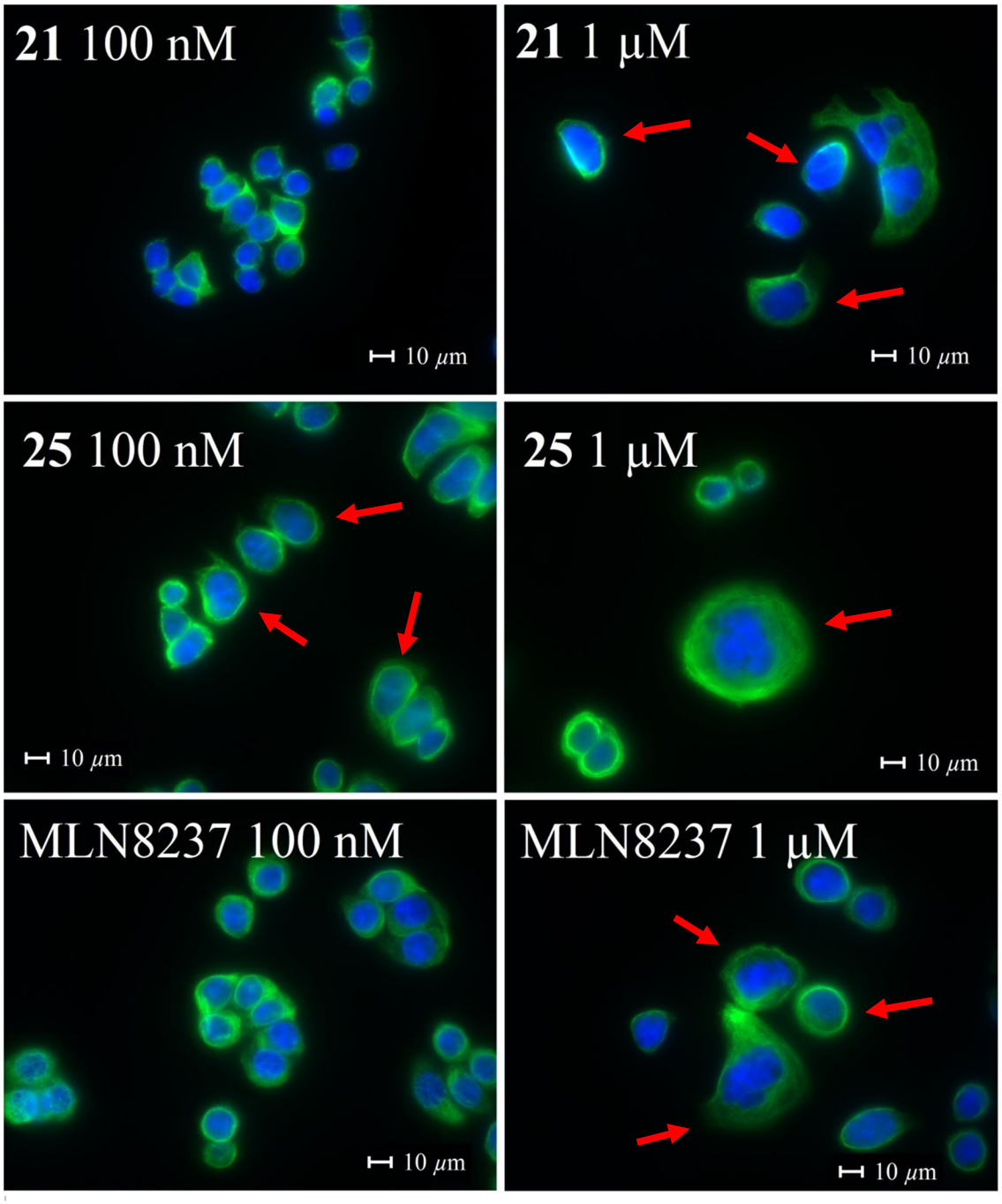



**Figure 9/2.** Merged images of HT-29 cells treated with in-house compounds **21**, **25** and reference AKI MLN8237 at 100 nM or 1 μM for 24 h. Blue (DAPI) staining indicates nuclei, green (Alexa488) tubulin. Pictures were taken at 63x magnification. Red arrows point to cells with at least two nuclei.

**Assessing induction of apoptosis by flow cytometry**

I used PI and Annexin V-FLUOS dual staining to measure the apoptotic fraction of compound treated HCT 116 cells. Intriguingly, considerable percentage of apoptotic cells were detected only after 72 h treatment in case of some compounds (Figure 10 and Table 5). Moreover, the pattern was the same as observed in case of DNA content: VX-680 and compounds **9**, **11**, **25**, **28** induced substantial apoptosis while MLN 8054, **17**, **21**, **22** only a moderate one (Figure 11). The most effective in-house compound was **25** that – like VX-680 – induced apoptosis in almost 20% of the cells. Again, the experiment was repeated also at 1 μM compound concentration. The results were similar to single PI staining: at 1 μM also previously ineffective compounds – like **21** – induced apoptosis (Figure 12).

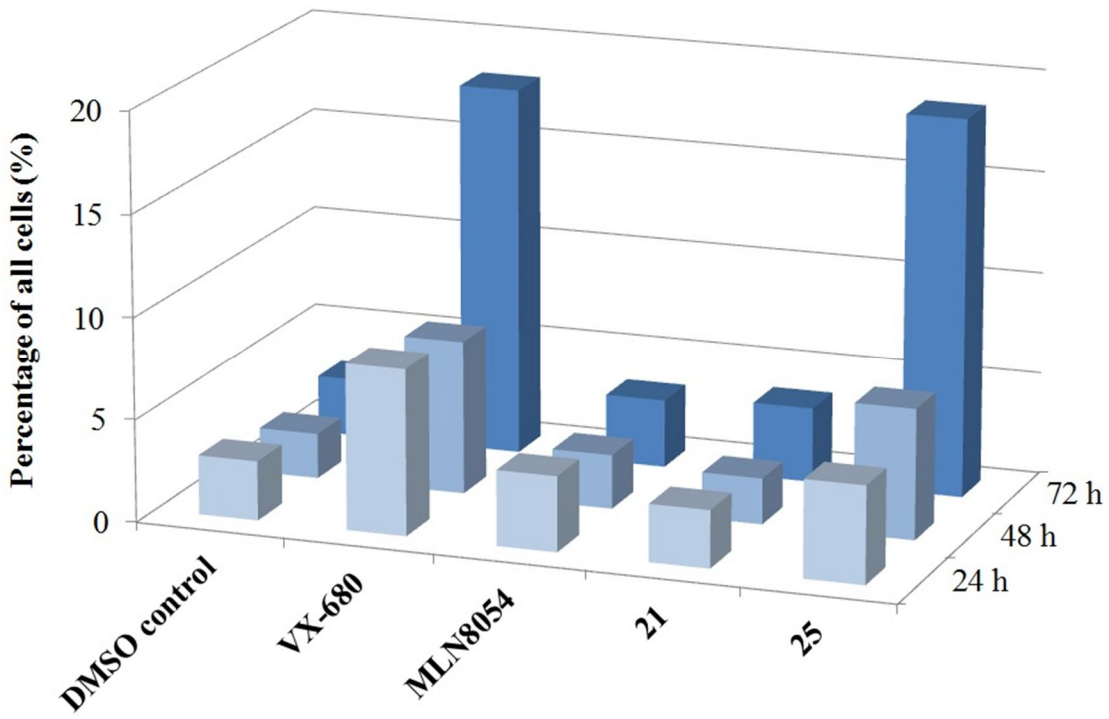



**Figure 10.** Apoptotic fraction of HCT 116 cells measured by PI and Annexin V-FLUOS staining after 24, 48 and 72 h treatment at 100 nM inhibitor concentration uniformly. DMSO – vehicle control. Values are the mean of at least two independent experiments.

**Table 5.** Apoptotic fraction of HCT 116 cells measured by PI and Annexin V-FLUOS staining after 24, 48 and 72 h treatment at 100 nM inhibitor concentration uniformly. DMSO – vehicle control. Mean values ± standard deviation (SD) of the data depicted on Figure 10.

| | DMSO control | VX-680 | MLN8054 | **21** | **25** |
|---|---|---|---|---|---|
| 24 h | 2.90 ± 1.08 | 8.13 ± 5.76 | 3.69 ± 1.56 | 2.82 ± 1.17 | 4.77 ± 1.65 |
| 48 h | 2.20 ± 0.49 | 7.51 ± 1.03 | 2.64 ± 0.81 | 2.25 ± 0.50 | 6.38 ± 0.97 |
| 72 h | 2.84 ± 0.01 | 18.13 ± 1.29 | 3.33 ± 0.33 | 3.66 ± 1.28 | 18.58 ± 0.94 |

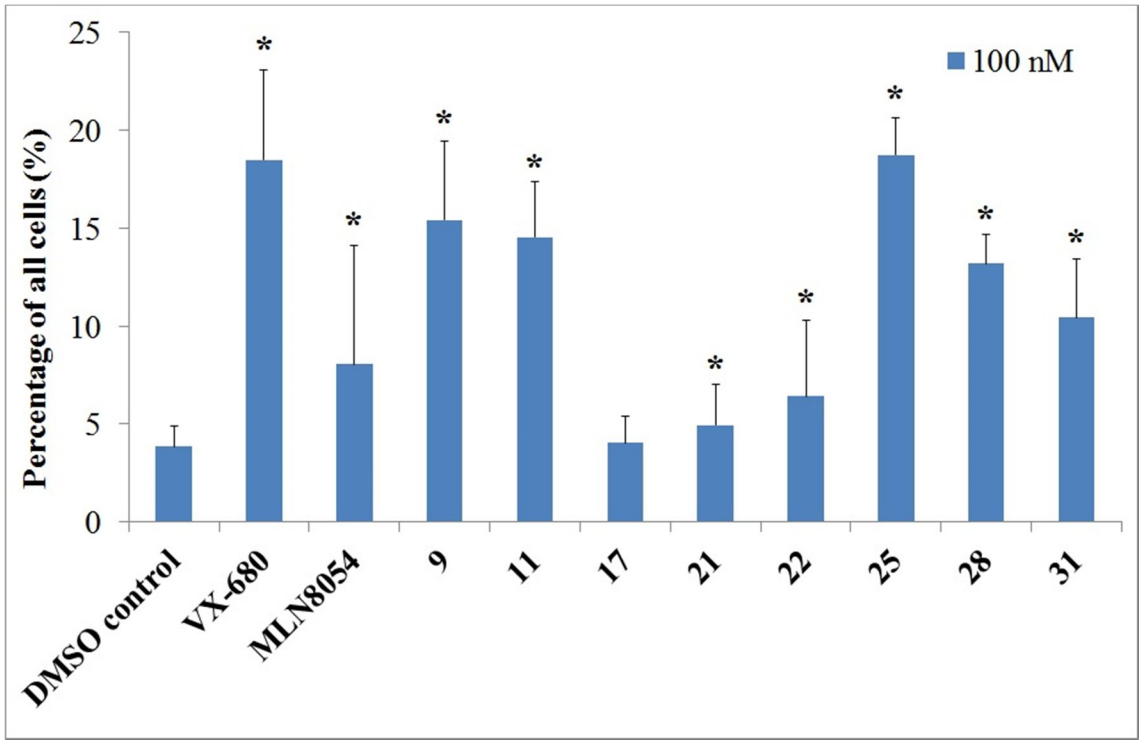



**Figure 11.** Fraction of HCT 116 cells undergoing apoptosis measured by PI and Annexin V-FLUOS staining after 72 h treatment. Reference compounds VX-680 and MLN8054 and eight selected in-house compounds were used at 100 nM. DMSO – vehicle control. Values are the mean of at least four independent experiments. Error bars represent standard deviation (SD), *denotes significant difference compared to DMSO control ($p < 0.05$) according to Student's *t*-test.

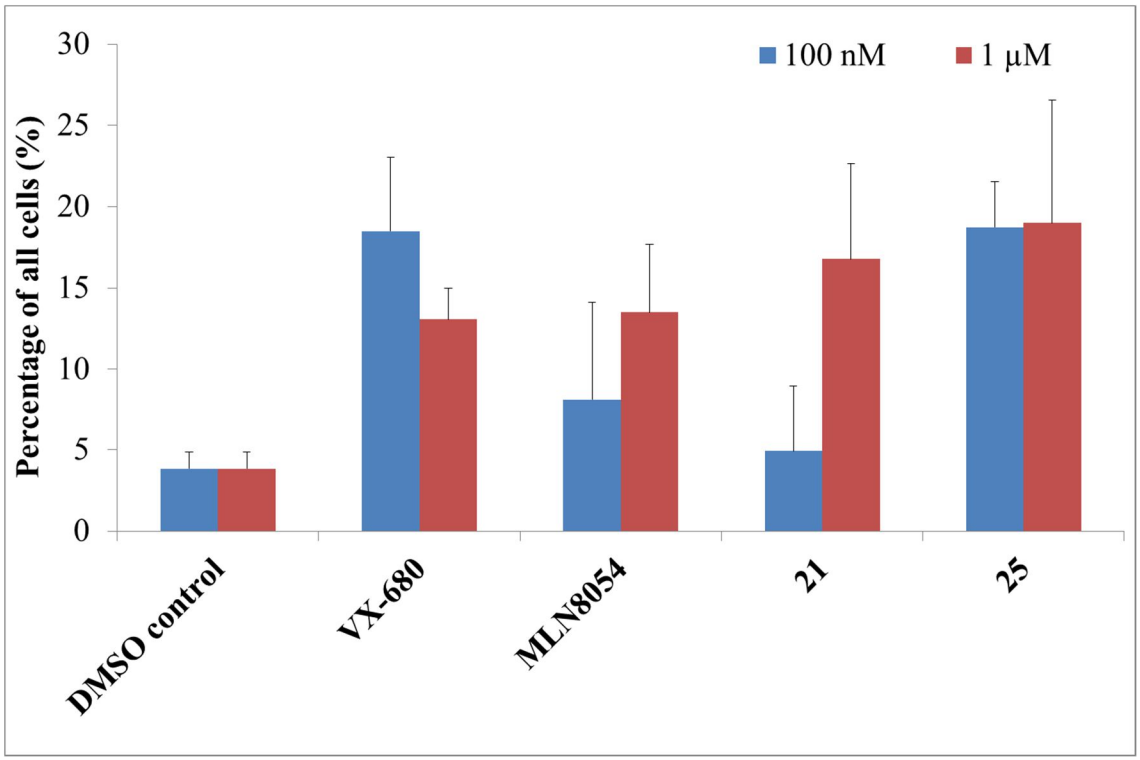



**Figure 12.** Fraction of HCT 116 cells undergoing apoptosis measured by PI and Annexin V-FLUOS staining after 72 h treatment. Reference compounds VX-680 and MLN8054 and selected in-house compounds **21** and **25** were used at 100 nM or 1 µM, respectively. DMSO – vehicle control. Values are the mean of at least two independent experiments; error bars represent standard deviation (SD).

**Testing cell viability inhibition of primer fibroblast cell culture**

According to cell viability and apoptosis measurements I choose **25** as the primary hit molecule of the eight selected benzotiophene-3-carboxamides. However, compound **21** was also studied in some of the following experiments just for comparison (Figure 13).

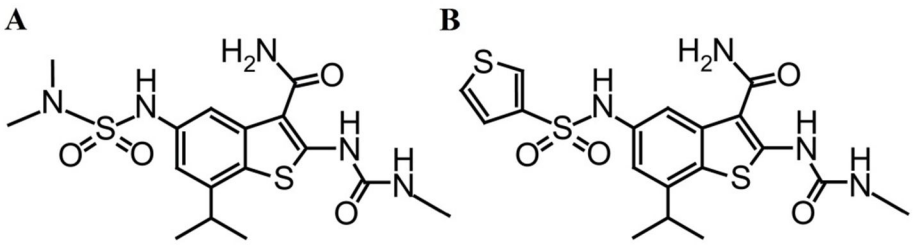



**Figure 13.** Chemical structure of benzotiophene-3-carboxamide derivatives A) **21** and B) **25**.

To make sure that these most important compounds do not affect viability of healthy cells also primer fibroblasts were treated with them. All circumstances were the same as in the HCT 116 experiments except compound concentration which was fixed at 1 μM. Data clearly present that none of the compounds influenced viability of fibroblast cells considerably. (Figure 14)

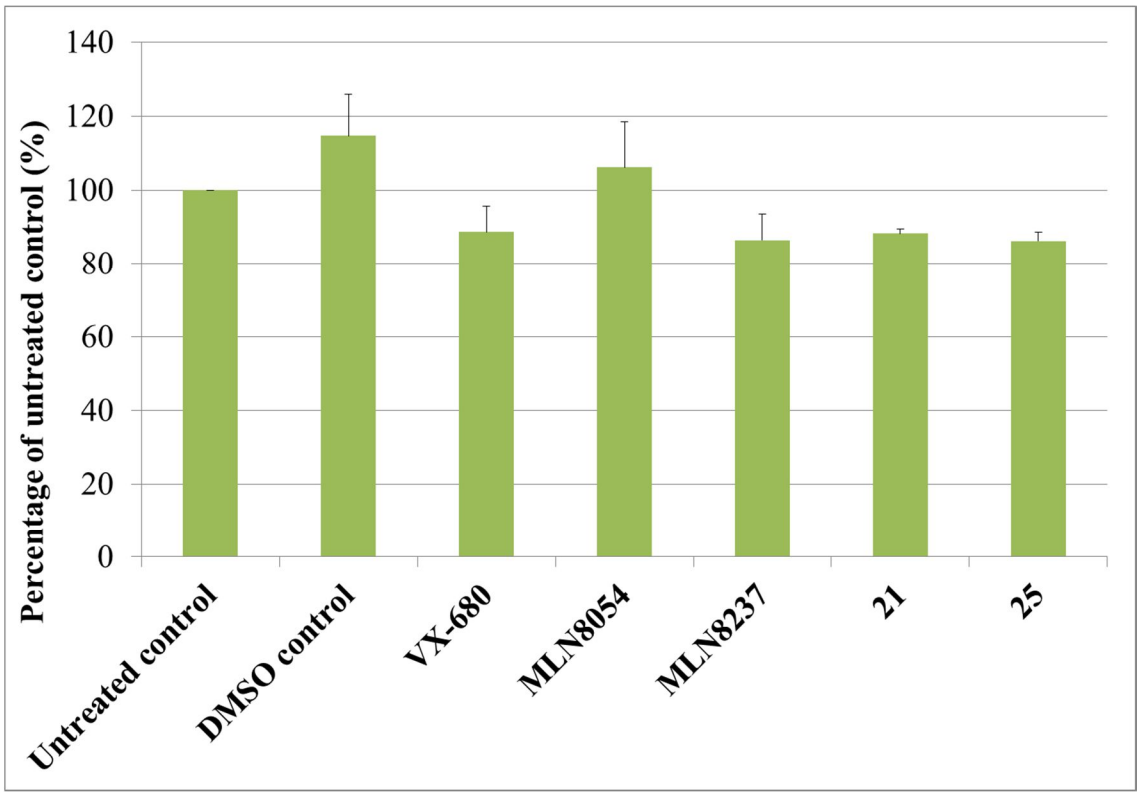



**Figure 14.** Cell viability of primer fibroblast cells after 48 hours compound treatment. Reference AKIs were VX-680, MLN8054 and MLN8237, in-house compounds were **21** and **25**. An MTT assay, vehicle control was DMSO. Percentage (%) values are the mean of two independent experiments ± standard deviation (SD).

**Western blot analysis**

To explore mechanism of action of in-house compounds **21** and **25** in the HCT 116 cells we performed Western blot experiments. According to levels of autophosphorylated Aurora A and B, **21** inhibited Aurora A activity completely at 500 nM while Aurora B partially at 1 μM. So in cells **21** behaves similarly to reference compound MLN8237, a selective Aurora A inhibitor (Figure 15). At the same time **25** decreased activity of both Aurora kinases equipotent to VX-680, already at 100 nM. Also Histone H3 Serine10

phosphorilation was diminished by **25** at 100 nM which is the sure sign of absent Aurora B function. Meanwhile total protein levels of Aurora A, Aurora B and Histone H3 remained unchanged at all concentrations (Figure 16). Therefore **25** is considered a potent, pan-AKI – unlike **21** which proved to be less potent but selective to Aurora A.

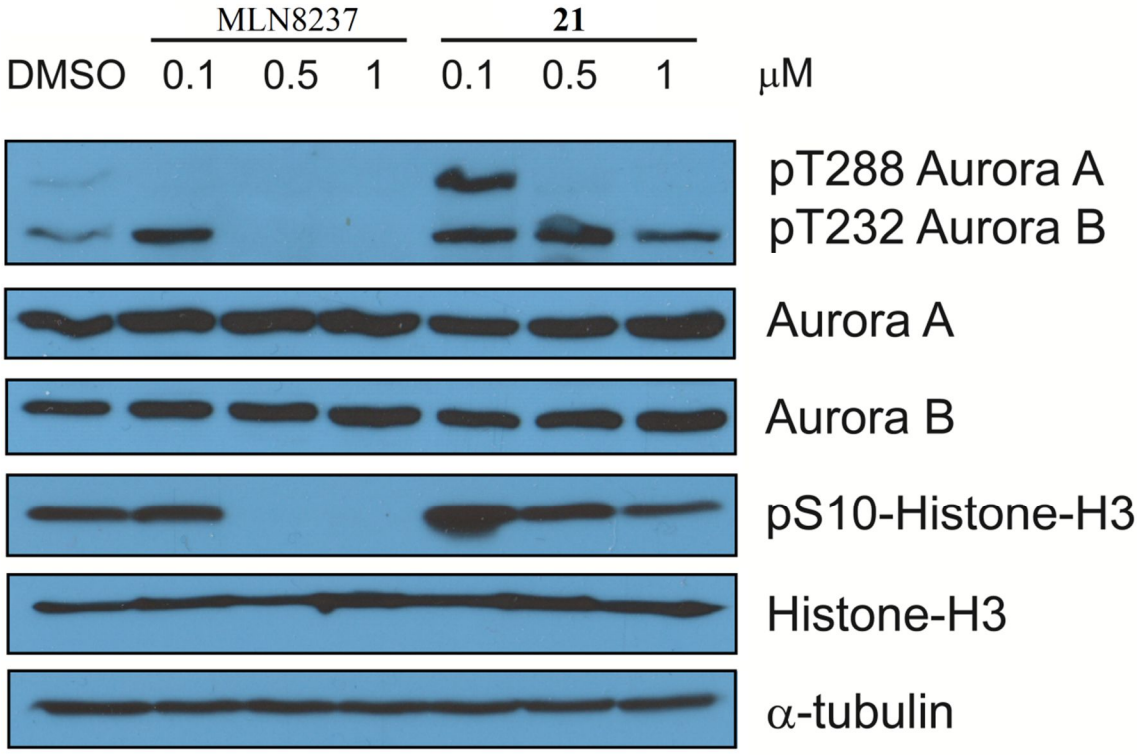



**Figure 15.** Cellular total protein level and phosphorylation status of Aurora kinases A, B and Histone H3 after 3 h treatment at three concentrations of reference compound MLN8237 and **21**. Representative blot of three independent experiments on HCT 116 cells.

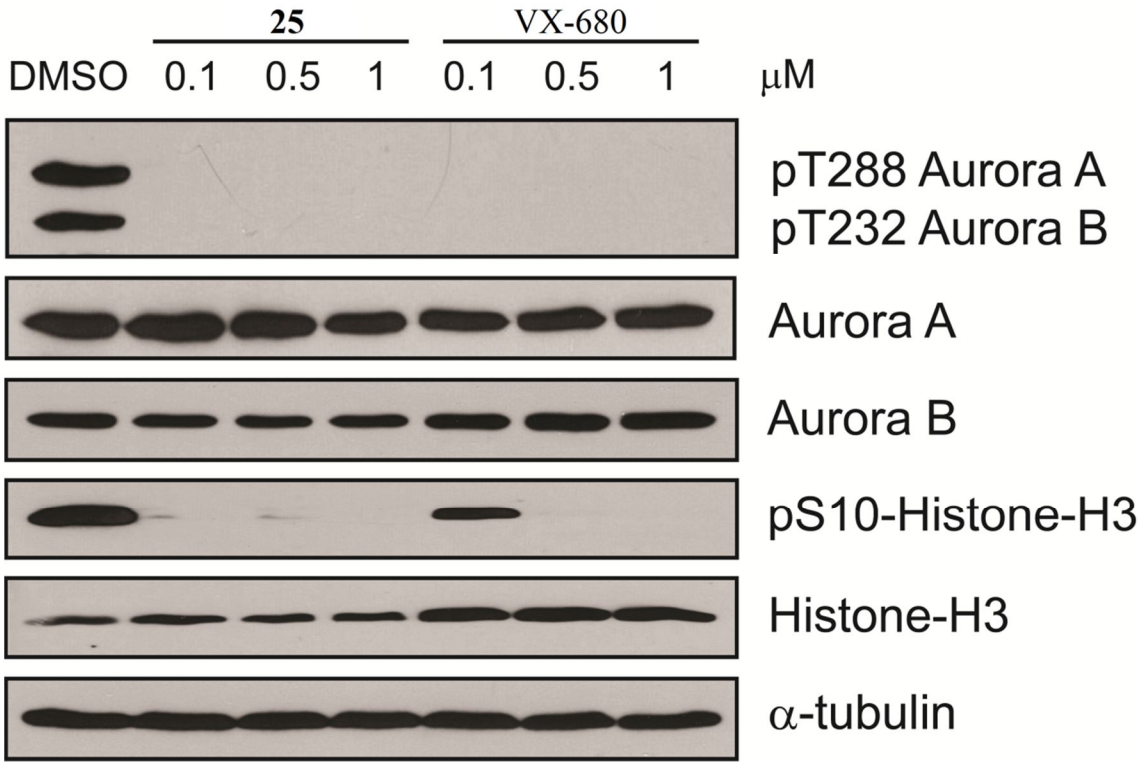



**Figure 16.** Cellular total protein level and phosphorylation status of Aurora kinases A, B and Histone H3 after 3 h treatment at three concentrations of **25** and VX-680 a pan-AKI. Representative blot of three independent experiments on HCT 116 cells.

**Development of EGFR–c-Met dual inhibitors.**

In one of our alternative project also focusing to signal transduction therapy we developed an EGFR–c-Met dual TKI. The aim was to decrease the viability of activating mutant EGFR-driven but simultaneously EGFR TKI resistant NSCLC cell lines.[370] First, five *in vitro* recombinant kinase assays were adjusted and optimised (including $K_{M[ATP]}$ determination): wild-type EGFR ($EGFR_{wt}$), activating mutant EGFRs ($EGFR_{L858R}$ and $EGFR_{Del}$), activating and resistant mutant EGFR ($EGFR_{L858R/T790M}$) and c-Met. Then extensive screening of the EVL™ of Vichem Ltd. was performed by using these five enzymes. As a result few compounds were identified based on a novel 'N-[4-(quinolin-4-yloxy)-phenyl]-biarylsulfonamide' core structure. Sulfonamides were tested on relevant NSCLC cell lines, as well. During lead optimization several further derivatives were synthetized and tested in the same recombinant kinase and cellular assays. The reference inhibitor for EGFR was erlotinib, for c-Met crizotinib and BMS-777607. The new sulphonamide derivatives could have

been divided roughly to two subgroups: compounds inhibiting rather EGFR varieties or rather c-Met. Only one compound, **34** had low enough $IC_{50}$ values on wild-type or activating mutant EGFRs and c-Met kinases (Figure 17). No potent inhibitor of the resistant mutant variety of EGFR ($EGFR_{L858R/T790M}$) was found in the EVL™ or among the new derivatives. In a recombinant kinase-based ATP-competitivity assay **34** proved to be a dedicated type I-II inhibitor that occupied the ATP-binding pocket of both $EGFR_{wt}$ and c-Met. During further cell-based experiments **34** indeed reduced EGFR and c-Met autophosphorylation (thus activity), abrogated downstream signalling pathways and induced apoptosis at an extent comparable to reference inhibitor erlotinib.

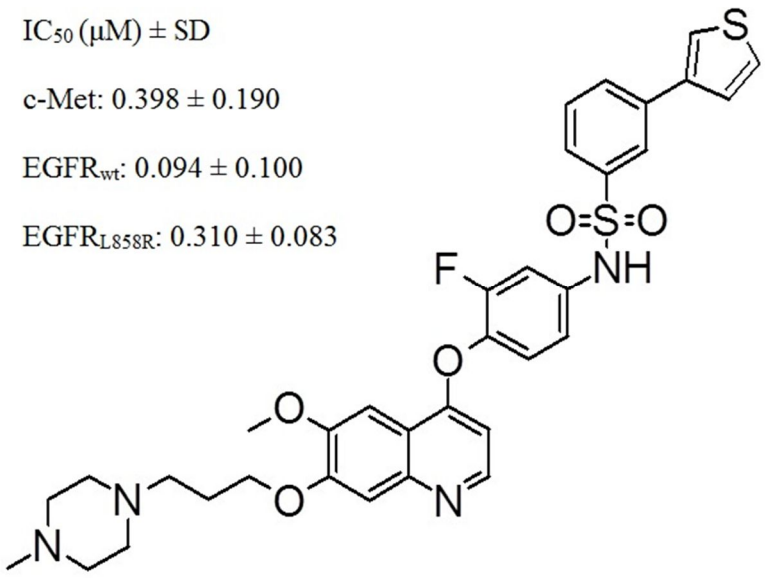



**Figure 17.** Chemical structure and *in vitro* recombinant c-Met, $EGFR_{wt}$ and $EGFR_{L858R}$ enzyme inhibition values of compound **34**. $IC_{50}$ values are the mean of at least three independent experiments ± standard deviation (SD).

**Drug combination experiments**

I examined the potency of the two best in-house compounds from the AKI and the EGFR–c-Met TKI development projects in concurrent treatment. HCT 116 and HT-29 colon carcinoma cell lines were treated with **25** or VX-680 in combination with other targeted agents that were already known to synergise with AKIs, and compound **34** (Table 6). Cell viability was measured using MTT assay and CI values were calculated

by using the data. According to the CI values 9 of the 32 combinations showed synergism (0.7 > CI > 0.3) and 13 strong or very strong synergism (0.3 > CI). Both cell lines were sensitive to similar drug combinations and both **25** and VX-680 behaved alike. So the effect of **25** showed the pattern of a functional AKI in this setting.

**Table 6.** Effect of **25** or VX-680 on cell viability in combination with various targeted agents. Mean inhibition values of at least three independent experiments were used to calculate CI by CompuSyn® software. Yellow marking indicates combinations with synergism (0.7 > CI > 0.3), orange with strong synergism (0.3 > CI > 0.1) and red with very strong synergism (0.1 > CI).

| Cell line | HCT 116 | | HT-29 | |
|---|---|---|---|---|
| Compound | **25** | VX-680 | **25** | VX-680 |
| GSK2126458 | 1.042 | 0.511 | 1.044 | 6.330 |
| Erlotinib | 0.250 | 0.503 | 0.075 | 0.125 |
| Trichostatin A | 0.195 | 0.321 | 0.185 | 0.111 |
| Dasatinib | 1.000 | 0.561 | 0.073 | 0.097 |
| Lonafarnib | 0.755 | 1.114 | 0.198 | 0.340 |
| Carfilzomib | 113.078 | 669.014 | 0.050 | 1.827 |
| Crizotinib | 0.136 | 0.207 | 0.102 | 0.212 |
| **34** | 0.393 | 0.308 | 0.395 | 0.836 |

***In silico* compound docking**

We utilized Schrödinger software for *in silico* docking to investigate the Aurora kinase-binding mode of **25** (Figure 18). We can sum up our observations in six points:

1) The 3-carboxamide moiety forms hydrogen bonds with the -NH and -CO groups of the protein backbone hinge region (A213 and E211 in Aurora A; A157 and E155 in Aurora B).

2) The nitrogens of $R^2$ also form hydrogen bonds with the backbone carbonyl oxygen of the aforementioned alanines. Many KIs form hydrogen bond with the hinge region,[300] which is also important in case of AKIs.[405]

3) The oxygen of the sulphonamide group interacts with the catalytic lysine residue (K162 in Aurora A and K106 in Aurora B).

4) Further hydrogen bonds were detected with residues D274 (part of the DFG-motif), R137 and R220 in Aurora A, and with A217 in Aurora B.

5) In case of Aurora B a π-π stacking is present between the benzothiophene ring and F88.

6) According to Schrödinger software the docking score – that is the binding energy – of **25** (-10.894 kcal/mol for Aurora A and -9.054 kcal/mol for Aurora B) is comparable to of VX-680 (-10.587 kcal/mol for Aurora A and -9.224 kcal/mol for Aurora B).

All these observations corroborate that **25** indeed fits into the ATP-binding cleft of both Aurora A and B kinases, forms several secondary chemical bonds there and has a binding affinity comparable to VX-680.

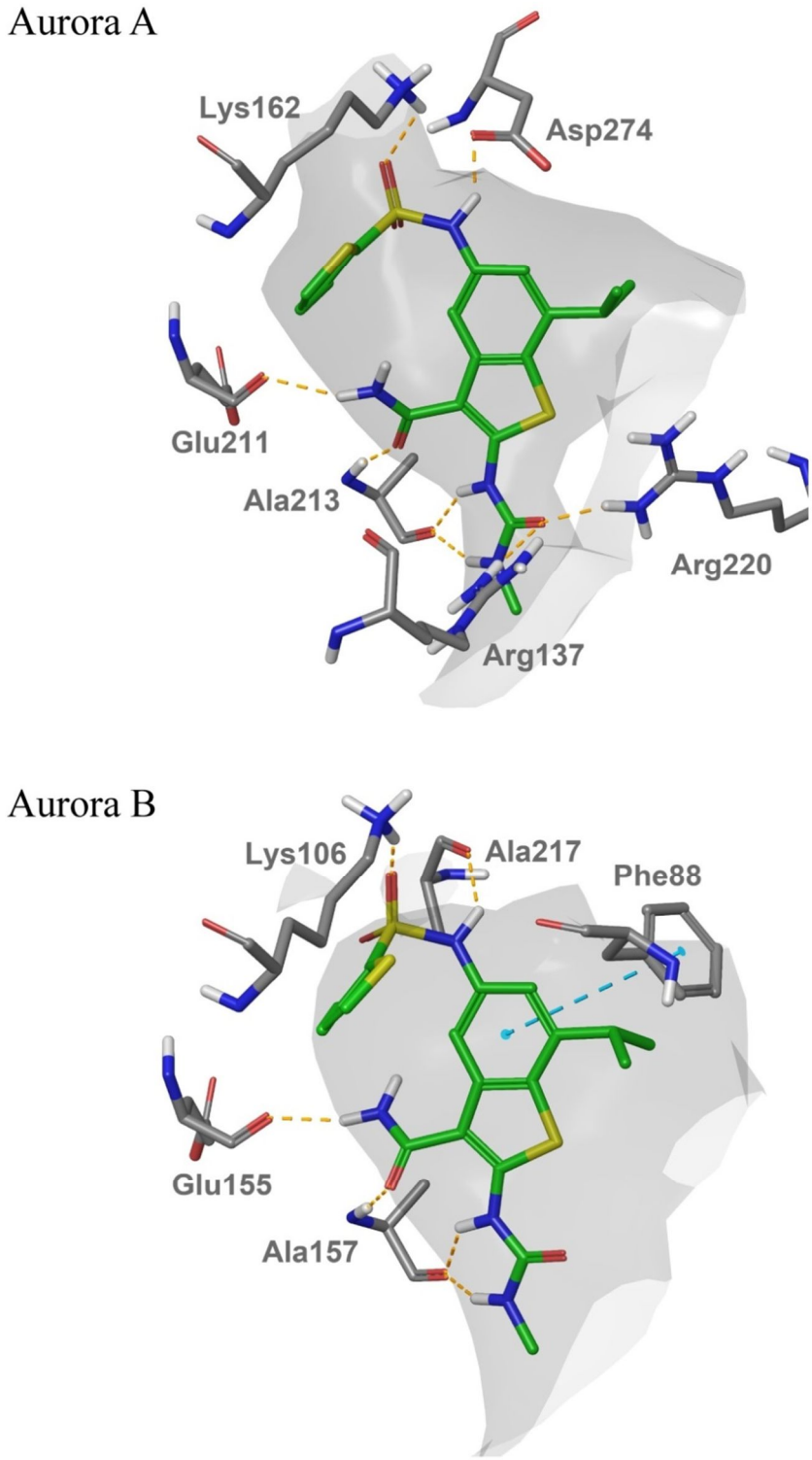



**Figure 18.** Molecular docking of **25** into the ATP-binding site of Aurora A (PBD ID: 4J8M) and Aurora B-INCENP (PDB ID: 4AF3) crystal structures. Interacting residues are grey while **25** highlighted green. H-bonds are indicated by yellow, while π-π stacking by blue dashed lines.

**Solubility measurements**

Solubility of few benzotiophene-3-carboxamide compounds was measured at two physiologically relevant pH values (Table 7). Generally, in-house compounds had poor solubility at both pH values compared to reference compounds. However, the solubility limit of most compounds was higher than 1 μM which was applied in the most important experiments. Concentrations higher than 1 μM were only used during cell viability and drug combination experiments (30 μM, 10 μM and 3 μM, respectively). Note that **21** is more soluble than **25** at both pH values.

**Table 7.** Solubility values of some benzotiophene-3-carboxamides at pH 7.4 and pH 2. Seven of the eight selected compounds are marked with grey. All values are the means of two independent measurements.

| | Solubility (μM; max 120) | |
|---|---|---|
| Compound | pH 7.4 | pH 2.0 |
| **9** | 1.7 | 1.3 |
| **11** | 3.6 | 2.6 |
| **14** | 0.0 | 0.0 |
| **17** | 13.6 | 11.4 |
| **18** | 3.0 | 2.8 |
| **20** | 4.6 | 4.3 |
| **21** | 13.1 | 8.0 |
| **23** | 6.7 | 3.3 |
| **24** | 3.9 | 1.1 |
| **25** | 1.9 | 1.8 |
| **26** | 12.8 | 5.3 |
| **27** | 1.1 | 0.0 |
| **28** | 1.4 | 1.6 |
| **31** | 6.2 | 4.2 |
| **33** | 4.8 | 3.7 |
| VX-680 | 120 | 120 |
| MLN8054 | 98.25 | 5.80 |

**Kinase selectivity panel**

Inhibitor potency of compounds **21** and **25** was assessed on the most important driver kinases to approximate their *in vivo* selectivity (Table 8/1 and 8/2). All work was performed by Proteros GmbH. Both compounds inhibit quite a few kinases beside Aurora A and B, so rather qualify as multi-kinase inhibitors.

**Table 8/1.** *In vitro* recombinant kinase inhibition values (%) of **21** (at 1 μM) and **25** (at 10 μM) on 36 kinases. Data of Aurora A kinase is marked with grey. Order of kinases in the table is set according to the inhibition values of **25**. ND – not determined.

| Kinase | **21** | **25** |
|---|---|---|
| AXL | 37.21 | 112.17 |
| VEGFR2 | 68.15 | 108.71 |
| c-Src | 94.39 | 104.99 |
| Aurora A | 114.04 | 100.39 |
| ABL | 88.79 | 100.35 |
| *J*AK3 | 54.64 | 99.63 |
| RET | 97.17 | 98.37 |
| PAK4 | 11.19 | 95.67 |
| TrkA | 46.85 | 95.35 |
| PDGFR-β | 61.29 | 88.03 |
| DDR1 | 75.92 | 84.43 |
| FGFR3 | 14.42 | 81.62 |
| PLK3 | ND | 78.73 |
| c-Kit | 28.88 | 71.58 |
| CHK1 | 16.41 | 62.84 |
| FLT3 | 46.63 | 56.82 |

**Table 8/2.** *In vitro* recombinant kinase inhibition values (%) of **21** (at 1 μM) and **25** (at 10 μM) on 36 kinases. Order of kinases in the table is set according to the inhibition values of **25**. ND – not determined.

| Kinase | **21** | **25** |
|---|---|---|
| B-RAF | -0.30 | 53.47 |
| c-Met | 2.76 | 47.44 |
| CDK4/CycD1 | 27.00 | 44.13 |
| ErbB2 | ND | 35.87 |
| TIE2 | 0.75 | 35.69 |
| PIM1 | 41.60 | 35.68 |
| CDK2/CYCA | 22.77 | 33.85 |
| INSR | 3.00 | 28.46 |
| PAK1 | 5.28 | 26.73 |
| PKCα | 17.94 | 21.44 |
| CSK | 19.54 | 17.27 |
| SYK | 51.10 | 16.56 |
| IKK-β | 30.67 | 16.20 |
| IRAK4 | 8.14 | 15.96 |
| AKT1 | ND | 13.63 |
| ZIPK (DABK3) | 39.62 | 12.93 |
| MAPK-ERK1 | 17.64 | 12.38 |
| JNK1 | ND | 12.16 |
| ROCK2 | 15.67 | 11.82 |
| mTOR | 3.66 | 8.62 |

## 5. Discussion

The common denominator of conventional cytotoxic cancer therapeutic approaches is to destroy dividing cells, regardless they are cancerous or healthy. Next-generation drugs are much more precise – they target the very protein that malfunctions and drives the given cancer. Unfortunately, even the targeting of such a strong, proven driver like EGFR leads fast to resistance. So it is worth to consider simultaneous inhibition of multiple drivers and even non-driver kinases to beat cancer.

Aurora kinases are fundamental, conserved regulators of every eukaryotic cell division. While their inhibition seems to have a general, non-targeted effect like conventional cytotoxic therapeutics, it is also well known that Aurora kinases often have increased activity in various cancer types. They do not seem to be drivers, but due to their ubiquity (they expressed in every dividing human cell) and central role (the essence of cancer cells is perpetual division) they remain promising drug targets for mono- or combination therapies. However, the development of AKIs proved to be a hard task.

Therefore I surveyed a family of benzotiophene-3-carboxamide derivatives as potential AKIs in the laboratories of Vichem Ltd. and the Pathobiochemistry Research Group at Semmelweis University. All the experimental work in the Thesis was performed by me, unless indicated else in the Materials and Methods (chapter 3) or Results (chapter 4) sections. I used various methods available to me so I got data diverse enough to summarise the project and make some interesting statements.

### Preliminary experiments

Recombinant Aurora A and B kinases were purchased by Vichem Ltd. and after adjusting their optimal buffer conditions used to screen the EVL™ to find potential new AKIs. These preliminary tests provided only % inhibition values and were performed uniformly at 10 μM ATP concentration which is suboptimal for most kinases and unfavourable for inhibitors of some types. Therefore these percentage inhibition values are not too precise. Yet, benzotiophene-3-carboxamide derivatives were unambiguously identified as compound family with promising AKI properties during this first medium-throughput screening step.

The NCL™ of Vichem Ltd. contains almost 200 benzothiophene-3-carboxamide derivatives, about half of which had preliminary Aurora A and B enzyme inhibition data at the time I entered the project. Since many of them were not effective inhibitors of either Aurora kinase, I considered their structure (as a preliminary SAR study) and excluded many compounds from the following studies.

**The $IC_{50}$ determination using cellular screen.**

As we have seen, inhibition of Aurora kinases directly hinders proliferation of cells: lack of Aurora A compromises mitotic progression, while inhibition of Aurora B abrogates cytokinesis and induces polyploidy. Both phenomena result in reduced cell number, loss of Aurora B function even marked phenotypic alteration. Therefore I decided to determine the $IC_{50}$ values of 84 selected benzotiophene-3-carboxamide compounds on cancer cell lines first, instead of kinase assay. This reverse logic is rarely utilized, but given the well-known discrepancy of kinase and cellular screen data, has its advantages.[406] This way $IC_{50}$ values not only permit precise ranking of compounds but also provide some information on the behaviour of inhibitors inside human cells. For the following cell-based experiments I choose two colon carcinoma cell lines, both of which express elevated level of Aurora A[407] and frequently utilized for testing AKIs: HCT 116[408, 320] and HT-29[409, 410]. Regarding the driver constitution of this two cell lines:

- both cell lines harbour wild-type EGFR,[242]
- VEGFR is overexpressesed in HCT 116 but nearly absent in HT-29.[411]
- HCT 116 harbours mutant K-RAS and PI3K [Broad Institute Cancer Cell Line Encyclopedia].[412] while both kinases are wild-type in HT-29.
- In turn, mutant form of BRAF [Broad Institute Cancer Cell Line Encyclopedia] and p53 (R273H – decreased activity) proteins are expressed in HT-29.[413]

**SAR study of cell viability inhibition data.**

Even the smallest alteration in the structure of a small molecule may profoundly alter its physicochemical properties (like solubility or membrane permeability) and so pharmacokinetics – that is the fate of drug in the organism (ADME criteria) and pharmacodynamics – that is the effect the drug exerts on the organism. According to the cell viability $IC_{50}$ data I could make several intriguing assertions regarding the effect of

substituents ($R^1$, $R^2$ and $R^3$) on activity. I discuss these relationships using a group of thirty-three representative compounds that also include the most effective benzotiophene-3-carboxamides. First, let's see the cyclopropanoyl-amino series (Chapter 4, Table 2) where $R^3$ – the only variable substituent – is represented with aryl, alkyl, cycloalkyl and heteroaryl groups:

- Compared to compound **1** ($R^3$ phenyl) only **7** ($R^3$ methylpyrazol) augmented cell viability inhibition.
- The lack of delocalized electron pairs in the cyclohexyl group greatly reduced activity of **2** compared to **1**.
- Similarly, hydrophobic alkyl side chains (**3** methyl, **4** ethyl, **5** propyl) were disadvantageous – the longer the chain, the greater extent.

In case of the methlyamine series (Chapter 4, Table 3) $R^3$ substituents were more diverse.

- The single change of group $R^2$ to methylureido significantly increased cell viability inhibition in many cases: **1** vs. **9**, **4** vs. **17**, **5** vs. **18**, **6** vs. **25**, **7** vs. **31**, **8** vs. **11**. Molecular docking reveals the underlying mechanism: the nitrogens of the methylureido group form two hydrogen bonds with the hinge region of Aurora kinases while the cyclopropanoyl-amino group presumably only one.
- In case of methyl substituents of $R^3$ there was no significant difference in the biological effect of the cyclopropanoyl-amino (**3**) or the methylureido (**16**) derivatives.
- Among alkyl substituents of $R^3$ ethyl (**17**) was the best compared to longer (**18**, **20**) or branching (**19**) ones. Though, even **17** did not approximated the cellular effect of **9**.
- Substitution on the $R^3$ benzene ring of **9** resulted in roughly similar $IC_{50}$ only when the methyl group was at meta position (**11**).
- A secondary or tertiary amine side chain at $R^3$ (**22** and **21**) was equally effective to **9** and definitely more favourable than the alkyl chains of similar shape and size (**20** and **19**).
- Evaluating the heterocyclic substituents at position $R^3$, the 2-furan derivative (**23**) had weaker cell viability inhibition effect than the 2-thiophene (**25**) or the methylpyrazole (**31**) one.

- Similarly to the $R^3$-phenyl substitution, a methyl group on the thiophene ring was tolerated only at „meta" position (**28**), but not „para" (**29**) – considering the longer radius of the sulphur atom.
- Swapping the $R^1$ isopropyl group to methyl or cyclopropyl group consistently abolished the cellular effect (**23** vs. **24**, **25** vs. **26** and **27**, **31** vs. **32** and **33**). Unfortunately *in silico* docking does not reveal the reason behind this phenomenon.

It would have been interesting to further study SAR but I decided not to, because:

1) cell viability screen of another cell line and binding energies of *in silico* docking scores of these thirty-three compounds could not corroborate these observations (Data not shown).

2) due to the aforementioned reasons cellular screen is not an ideal model system for SAR studies. One can never be sure whether the observed cellular effect is due to altered potency of enzyme inhibition or metabolism/secretion of the drug.

3) My topmost goal was to corroborate the AKI potency of benzotiophene-3-carboxamides and characterise a hit compound.

**Corroborating KI potency in *in vitro* assay.**

According to the cell viability assay I selected the eight most potent compounds (**9**, **11**, **17**, **21**, **22**, **25**, **28** and **31**) with an $IC_{50}$ value below 0.6 μM on HCT 116 cells. As the first subsequent assay I determined the Aurora A and B kinase inhibition $IC_{50}$ values of these eight benzotiophene-3-carboxamide derivatives. For the $IC_{50}$ determination ATP concentrations were set to the $K_{M[ATP]}$ value of each kinase. The $K_{M[ATP]}$ values had to be measured in a separate set of experiments. The $K_{M[ATP]}$ value relates to the affinity of ATP to the particular kinase. Running the kinase assay at the $K_{M[ATP]}$ concentration has the advantage of making the $IC_{50}$ values of different inhibitors (type I, II, etc.) comparable.[414] According to the optimized *in vitro* recombinant kinase assay all eight compounds were comparably effective to or better than reference compound VX-680. Furthermore, most compounds proved to be equally effective Aurora A and B KIs, the only exceptions being compound **21** and **31** that were one order of magnitude more efficient on Aurora A than on B. While **21** had the second lowest $IC_{50}$ on HCT 116 cells (identical to **25**), **31** was considerably less effective. At the same time, despite their identical potency on Aurora B *in vitro*, **31** induced apoptosis at 100 nM but **21** not.

These facts point to the existence of unknown mechanisms modulating cellular effect of benzotiophene-3-carboxamides. Regarding compound **25**, western blot and i*n silico* docking experiments also hint to its similar affinity to both Aurora kinases. Binding poses of **25** with lowest docking score and binding energy in the ATP-binding sites of both Aurora A and B kinases were almost identical. This result is not surprising, since the structure and the ATP-binding pockets of Aurora A and B kinases is very alike (also the difference in their regulated substrates is rather the consequence of different cellular localisation and protein partners and not substrate selectivity), that is why highly isoform selective (type I or II) AKIs are rare.[415]

**Analysing rise of DNA content and number of nuclei.**

As described in chapter 2.2.2.1., appearance of multinucleated, polyploid cells is a hallmark of Aurora B inhibition. Since all eight selected compounds proved to be effective Aurora B inhibitors *in vitro* I was curious whether they indeed reduced cell viability through Aurora B inhibition and cytokinesis failure. Therefore I quantified DNA content of compound treated HCT 116 cells by flow cytometry and took fluorescence microscopic images to visualize multiplication of nuclei.

PI staining of ethanol-fixed cells reveals the amount of DNA in a flow cytometer. This way proportion of cell populations can be measured: G0 or G1 (with two series of chromosomes – 2n), G2/M (four series – 4n), multinucleated (8n and above) and apoptotic ones (called *subG1* population – less than 2n). Of course within the 4n population cytokinesis-inhibited cells cannot be distinguished from normal ones in G2/M. The average duplication time of cancer cells is around one day so I decided to treat for 24 h to ensure the appearance of 8n population. It was less time than applied for cell viability determination. I applied compounds in these experiments uniformly at 100 nM – this meant slightly higher concentration than their enzymatic $IC_{50}$ values but a lower one than their cellular $IC_{50}$ values. This way I suspected that Aurora kinases are already inhibited, but the possibility of off-target effects is minimal. Furthermore, this concentration is below the solubility limit of most compounds that are unfortunately rather low, compared to reference inhibitors VX-680 and MLN8054 (Chapter 4, Table 7). This experimental setting proved to be optimal, because no compounds induced apoptosis during the treatment but I could observe signs of marked cytokinesis failure

and rise of multinucleated cells in case of some compounds (like **25**), with both flow cytometry and fluorescence microscopy, respectively. The eight selected compounds could have been divided to cytokinesis inhibitors and non-inhibitors at 100 nM. Interestingly, this property did not correlate with cellular or enzymatic $IC_{50}$ values. For example **21** and **25** or **17** and **31** were equally effective on HCT 116 cells yet, only **25** and **31** inhibited cytokinesis at 100 nM. This notion again highlights that enzymatic data sometimes loosely correlate to cellular results because former miss differences in off-target effect and physicochemical properties.

It is well-known that selective inhibitors of Aurora A tend to inhibit also Aurora B at higher concentrations.[416] Therefore I was curious whether the ineffective in-house compounds became effective at higher concentrations. So I repeated these experiments with the two reference compounds, the cytokinesis disruptor **25** and the non-disruptor **21** at 1 μM, as well. Indeed, at the higher concentration also MLN8054 and **21** inhibited cytokinesis and created octaploid cells, but **21** much more. Whereas, compounds that were effective inhibitors of cytokinesis at 100 nM – like VX-680 or **25** – showed only a slightly decreased effect.

According to fluorescence microscopic images MLN8054 proved to be a more selective Aurora A inhibitor than MLN8237 – latter induced more multinucleated cells at 1 uM. This observation is in concert with flow cytometry experiments, where the effect of **21** improved more with increased concentration than of MLN8054. Therefore the Aurora kinase selectivity of **21** might be more similar to MLN8327 than MLN8054.

**Proving induction of apoptosis.**

According to scientific literature multinucleated cell state triggers apoptosis in time. To check this phenomenon I performed double PI and Annexin V-FLUOS staining with HCT 116 cells. Phosphatidylserine is a lipid situated exclusively in the inner plasma membrane of human cells. Flipping out of phosphatidylserine is a sign of apoptosis. The protein Annexin V is a specific binding partner of phosphatidylserine and utilized to mark cells undergoing apoptosis. First, I performed treatments at 100 nM for 24 (like in case of single PI staining) or 48 (like in case of cell viability measurements) hours but did not experience elevated level of apoptosis. Only after 72 could I detect considerable percentage of apoptotic cells in case of any compounds. This suggests that treated

cancer cells went through several cell divisions lacking cytokinesis without apoptosis induction. When those enormous, multinucleated cells finally underwent apoptosis, I observed two interesting correspondences:

1) only hit compounds harbouring benzene or heterocyclic side groups at position $R^3$ (**9**, **11**, **25**, **28**, **31**) and the pan-Aurora inhibitor VX-680 induced apoptosis at 100 nM, whereas compounds bearing alkyl or alkyl-amine functions at position $R^3$ (**17**, **21**, **22**) and the dedicated Aurora A inhibitor MLN8054 did not. (It is worth to note here, that according to the published effect of MLN8054, at 250 nM it is able to induce weak apoptosis of HCT 116 cells already after 24 hours.[321]) Therefore, I hypothesized that compounds **17**, **21** and **22** – in spite of their excellent effect on Aurora B *in vitro* – did not inhibit Aurora B in cancer cells at 100 nM.

2) This pattern of apoptosis induction was exactly the same as observed during DNA content analysis: only those compounds induced apoptosis which ones inhibited cytokinesis at 100 nM (VX-680, **9**, **11**, **25**, **28**, **31**). Moreover, at 1 μM – in consonance with DNA content analysis – also compounds with alkyl or alkyl-amine $R^3$ groups (like **21**) and MLN8054 induced apoptosis.

These data underline that the new in-house compounds can be divided to inhibitors which blocked cytokinesis and induced apoptosis at low concentrations (like **25**) and inhibitors which do neither (like **21**).

**Corroborating the link between elevated DNA content and apoptosis.**

To further confirm that multinuclear cell state and apoptosis are the result of Aurora inhibition effect of benzotiophene-3-carboxamides we performed western blot experiments. Only two in-house compounds were tested: **21**, a less potent, and **25**, the most effective cytokinesis-blocking and apoptosis-inducing inhibitor.

It came as a surprise that **21** proved to be an even weaker Aurora A and B inhibitor than MLN8237 in cells. At the same time **21** seemed to be selective to Aurora A, similarly to MLN8237 and in agreement with the *in vitro* kinase assay data. These observations somewhat contradicted the ones seen in fluorescence microscopy – where both compounds had similar potency. It is worth to emphasize though that treatment time was only 3 h in case of western blot experiments – opposed to 1 day in case of fluorescence microscopy. It is possible that even the weak Aurora B inhibition effect of

**21** was enough to arrest cytokinesis and induce multinucleated cells in the long run. Another reason might be of course the different off-target profile of MLN8237[320] and **21**.

On the other hand, western blot analysis corroborated that both Aurora A and B are indeed the target of **25** in cancer cells. **25** diminishes phosphorylation (and so activity) of Aurora kinases even at 100 nM – better than **21** or MLN8237 and equally potent to VX-680. So basically both **21** and **25** act as a pan-AKI, **21** just needs more than five-fold higher concentration to inhibit Aurora A and ten-fold to inhibit Aurora B. However, both compounds had similar potency on Aurora B in *in vitro* kinase assay and **21** is more soluble than **25** which is counterintuitive. The reason of this discrepancy – again – might be their different physicochemical properties, metabolism and secretion in cancer cells. Unfortunately we had no possibility to determine any of these properties.

**Final speculations about the properties of benzotiophene-3-carboxamides**

After the admittedly incomplete characterisation of benzotiophene-3-carboxamides it is worth to contemplate the whole picture again:

The cell viability inhibition $IC_{50}$ value of **25** is higher than the one in *in vitro* kinase assay. WB also corroborates that at the cellular $IC_{50}$ values (300 nM, 3 h) both Aurora kinases were already blocked. While in flow cytometry apoptosis started only after 72 h, compound concentration was also less (100 nM). Whether apoptosis starts at 300 nM already after 48 h (or less) is not sure. It is well-known though that the MTT method cannot differentiate why the treated cell culture is less "viable". Phenomena like cytokinesis inhibition and polyploidy (in case of Aurora B inhibition), or apoptosis of dividing cells (due to inhibition of Aurora A or off-target kinases) or simply ceased cell division (quiescence – G0, or senescence) all can give the same decrease in cell viability. That is why MTT assay is less and less utilised nowadays. Therefore, besides Aurora kinases another source of cell viability inhibition observed at the cellular $IC_{50}$ value of **25** is very likely due to off-target kinases. Main off-targets of **25** are receptor-kinases (AXL, VEGFR2, PDGFR-b, DDR1) known to malfunction in many cancer types (e.g. VEGFR2 in HCT 116 – see above), or c-Src whose simultaneous inhibition with aurora kinases is synergistic.[417] According to their kinase inhibition profile both **21** and **25** have more off-targets than VX-680. At first glance **21** seems to be the more

selective inhibitor to Aurora A, but the fact that it was screened at 1 μM while **25** is at 10 μM surely makes comparison hard. Of course, it cannot be excluded that cell viability inhibition effect of **25** is also a consequence of its Aurora A inhibition at the cellular $IC_{50}$ value (300 nM).

In case of **21** another explanation is needed. **21** was just as potent in cell viability experiments as **25**, albeit it starts to block Aurora A only at 500 nM (maybe less) and Aurora B at 1 μM. So at the cellular $IC_{50}$ value of **21** (~300 nM) Aurora B is not inhibited. Considering the alike *in vitro* kinase inhibition values of **21** and **25**, a feasible explanation might be to the decreased potency of **21** in cell-based assays its attenuated intracellular concentration (lower permeability or more active metabolism or higher susceptibility to drug-efflux pumps). However, the fact that MLN8237 blocks Aurora A at 100 nM after 3 hours but its more paralogue selective derivative (MLN8054) does not induce apoptosis even after 72 hours does not support the role of Aurora A inhibition in our system. MLN8054 (and presumably also MLN8237) is reported to induce weak apoptosis at 250 nM even after 24 hours. The same phenomenon might be the case regarding **21**. According to the kinase selectivity panel, Aurora A is the main target of **21**. At its cellular $IC_{50}$ value the effect of **21** might be the result of selective Aurora A inhibition. Unfortunately excessive investigation of a less effective compound was not priority during our work, so I did not perform apoptosis measurements at 300 or 500 nM. Besides the possible effect of **21** on Aurora A at the cellular $IC_{50}$ value, the influence of off-target effects might be equally important (like in case of **25** – considering their similar structure).

Fortunately, other Aurora A selective inhibitors may give some clue to assess the importance of Aurora A inhibition in my experiments. For example in HCT 116 cell-based experiments the Aurora A inhibitor ENMD-2076 had an $IC_{50}$ value of 200 nM and inhibited cellular Aurora A from 200 nM and Aurora B from 1 uM. So Aurora A inhibition was sufficient to induce apoptosis in that case. At the same time, ENMD-2076 is also a multi-kinase inhibitor – it has activity also on VEGFR and Src (similarly to **25** – which might point to the similar ATP-binding pockets of these kinases) former being a driver of HCT 116. So off-target inhibition surely adds to the effect of ENMD-2076.[418]

However, in xenograft experiments effective doses of MLN8054 and MLN8237 transiently also inhibited histone H3 phosphorylation – so Aurora B as well.[419] Also MLN8054 is selective to Aurora A at 1 μM and induces weak apoptosis even at 250 nM after 24 hours, but inhibits also Aurora B at 4 μM in HCT 116 cells.[321]

Likely, MK-5108 is more potent on Aurora A (0.04 nM) than on Aurora B (~10 nM) *in vitro*, still in cell viability experiments first signs of apoptosis rose only 48 hours later and after 72 hours also histone H3 phosphorylation decreased – a sure sign of decreased Aurora B activity.[420]

These aforementioned examples highlight that even in case of the most selective Aurora A inhibitors, the influence of Aurora B cannot be excluded – particularly in *in vivo* animal models where treatments usually longer. Furthermore, it is worth to mention again, that also Aurora A inhibition takes ~18 hours to show any effect: cells first exit mitosis and undergo cytokinesis then apoptosis.[421] Meanwhile also off-target inhibition of Aurora B has a chance to take effect. Provided further advances in the field of AKI development underline Aurora A as the better target, then **21** might serve as an origo for further work.

However, in our experimental systems decreased activity of Aurora B precisely accompanied apoptosis, while of Aurora A did not. Therefore I concluded that **25** was the lead molecule of all benotiophene-3-carboxamides in the NCL™ of Vichem Ltd. So in the last experiment only **25** was applied.

**Trying out compound 25 in drug combination experiments.**

As discussed earlier, combination of anti-cancer drugs is nowadays a very promising therapeutic approach. However, assessing whether a drug combination has better than additive effect – that is, the two drugs synergize – is a surprisingly difficult mathematical question.[422] In the last decades several models were developed to answer this question and quantify experimental results.[423] The most widely accepted algorithm is defined by Chou and Talalay.[424, 425] They also developed a free software tool – called CompuSyn®– based on their algorithm. So in the last round of experiments I tested the lead AKI **25** and the reference compound VX-680 in a concurrent treatment with other targeted agents. Eight drugs were applied alone or in 1:1 combination to HCT 116 and HT-29 colon carcinoma cell lines. The first six drugs were previously reported to

synergize with Aurora inhibitors (see chapter 1.3.5.) so they served as point of reference. Crizotinib (as a c-Met–ALK dual inhibitor) and – obviously – the in-house compound **34** have never been combined with AKIs before. I used two cell lines to test the influence of different mutational background on the results.

- GSK2126458, a PI3K/mTOR inhibitor
- Erlotinib, an EGFR inhibitor
- Trichostatin A, a Class I and II histone deacetylase inhibitor
- Dasatinib, a BCR/Abl and Src family KI
- Lonafarnib, a farnesyltransferase inhibitor
- Carfilzomib, a proteasome inhibitor
- Crizotinib, the ALK, ROS1 and c-Met inhibitor.
- Compound **34**, the in-house EGFR–c-Met dual inhibitor

According to the calculated CI values I concluded that **25** behaves very similarly to VX-680 in the drug combination setups. Both AKI showed synergism with most drugs – I could reassert most of the previously reported drug combinations. In case of GSK2126458 and Lonafarnib not all cell line and AKI combination proved to be synergistic. Moreover in case of combinations with Carfilzomib, I observed strong antagonism on HCT 116 cells: the extraordinarily high CI value seems to be an outlier at first glance. However, high CI values like this are interpretable, since the antagonism scale for CI values is from 1 to infinity.[396] The reason of this high CI value is the fact that Carfilzomib is an extremely efficient compound that reduced viability of HCT 116 cells with 93% even at 1.5 nM as monotherapy (HT-29 cells were not as sensitive). In combination with an AKI the sum effect decreased and its $IC_{50}$ value was similar to the other combination pairs'. Therefore the huge difference in the effect of Carfilzomib mono- and combination therapy resulted in a high CI value. On the other hand I experienced weak antagonism and an unambiguous synergism on HT-29 cells. It is hard to unravel the mechanisms underlying these results. While both cell lines express elevated level of Aurora A, their p53 status is different: the HCT 116 cells express wild-type p53, HT-29 cells a R273H mutant one.[413] It is known that cancer cell lines harbouring mutant or overexpressed p53 are more sensitive to AKIs – particularly Aurora A selective ones.[426] Aurora B inhibition induces polyploidy and apoptosis regardless of p53 status. Whereas the Aurora A selective inhibitor MK-8745 (a

derivative of MK-5108) induces apoptosis in case of wild-type p53 and polyploidy in case of mutant one.[421] However, not every mutation of p53 created equal: R280K mutation increases Aurora A expression while R175H does not.[114] Furthermore p53 null subclones of HCT 116 cells are not sensitive to inhibition of proteasome at all.[427] So the p53 status (R273H mutant) might explain why HT-29 cells were not as sensitive to Carfilzomib monotherapy and why I got lower CI values for the combinations. What sure is that the p53 mutant HT-29 was more sensitive to VX-680 monotherapy than the wild-type p53 HCT 116 cell line in my experiments, which explains why more combinations showed very strong synergism on the former.

The only drug combination which had no precedent in the scientific literature is the AKI and c-Met inhibitor pair – certain results only point to the feasibility of it.[428] Since the CI values of AKI and crizotinib combination were appealing in every setup, I was the first to report the potency of this combination.

As we have seen, both the inhibition of EGFR (by erlotinib) and c-Met (by crizotinib) had a synergistic effect with AKI treatment. So I hypothesized whether the in-house EGFR–c-Met dual inhibitor **34** would also synergise with the AKIs. As I expected, combinations of either **25** or VX-680 with **34** proved to be more effective than using either agent alone.

So in the drug combination experiments the lead molecule **25** proved its AKI properties again. Furthermore, my observations underline the observation that that despite same histology (colon carcinoma) and common genetic alterations (in this case Aurora A overexpression), the different mutational background of cancer cells might profoundly alter response to a given drug combination.

Generally, I can conclude that the preclinical studies presented in the Thesis confirm the AKI properties of benzotiophene-3-carboxamide derivatives. If these molecules will form a basis of further AKI development, of course further experiments (particularly *in vivo* animal models) will be needed. Until that time the selected lead molecule, compound **25** remains the most potent dual AKI of this compound family.

## 6. Conclusions

According to my results and the auxiliary experiments I can make the following assertions:

I) The completely novel benzothiophene-3-carboxamide scaffold is indeed a promising structure for the further development of AKIs. Many benzothiophene-3-carboxamide derivatives inhibit Aurora A and B kinase function in *in vitro* assays, abrogate viability and induce apoptosis of human colon cancer cells at concentrations comparable to reference compounds.

II) Inhibition of Aurora B kinase and the resulting cytokinesis disruption and multinuclear cell state always coincided with apoptosis induction in HCT 116 cells. Some of our in-house compounds and published inhibitors selective to Aurora A inhibit Aurora B and induce apoptosis only at higher concentrations. Therefore disrupting the function of Aurora B is an indispensable property of benzotiophene-3-carboxamides to achieve anti-cancer effect in our experiments.

III) Compound **25** is a drug-like multi-kinase inhibitor with strong AKI properties and qualifies as the lead molecule of the benzotiophene-3-carboxamide derivative compounds of Vichem Ltd.

IV) Also in combination with various targeted agents **25** behaves like an AKI. I demonstrated the first time that the combination of a c-Met–ALK inhibitor and an AKI can be synergistic in some circumstances.

## 7. Summary

Cancer is one of the most devastating disease for developed societies. In the last few decades cancer research made enormous achievements in the field of understanding its molecular drivers, diagnosing their alteration at an early stage and inhibiting their function in a targeted way. However, a comprehensive and reliable model of the network of cancer drivers is still missing and the importance of individual drivers is not always clear. Also the repertoire of targeted drugs is very limited. Therefore targeted therapies usually fail to cure cancer due to various acquired resistance mechanisms. Aurora kinases A and B provide a perfect example: while they don't seem to be infallible drivers, their activity is crucial for cell proliferation and frequently increased in cancer cells. Despite many efforts to design specific Aurora kinase inhibitors, most compounds have failed in clinical trials and there is still no marketed drug of this kind.

During a drug development project I excessively investigated a family of small molecules based on a completely new, benzotiophene-3-carboxamide core structure. Many benzotiophene-3-carboxamide compounds inhibited Aurora A and B kinases *in vitro*, triggered morphological alterations typical for Aurora B inhibition and reduced cancer cell viability inducing apoptosis. The most effective, lead compound performed equally well to reference aurora kinase inhibitors in all *in vitro*, *in silico* and cellular tests.

One current trend to improve targeted therapies is the simultaneous inhibition of more than one drivers by multi-target drugs or drug combinations. I also proved that the lead compound in combination therapy experiments gives similar results to published reference Aurora kinase inhibitors.

Accordingly, the lead in-house benzotiophene-3-carboxamide compound proves to be a potent Aurora kinase inhibitor and qualifies as a new, promising candidate for further anti-cancer drug development.

## 8. Összefoglalás

A rákos elfajulások a fejlett országok legmagasabb morbiditású betegségei közé tartoznak. Az elmúlt évtizedek hatalmas fejlődést hoztak a rák molekuláris hátterének megértésében, korai diagnózisában és célzott terápiás gátlásában. Továbbra sincs azonban átfogó és megbízható hálózatos modell ezen molekuláris elváltozásokról és az egyes hibák szerepe sem mindig egyértelmű. A rendelkezésre álló célzott hatóanyagok száma is korlátozott. Ezért sajnos a jelenleg alkalmazott célzott terápiák túlnyomó többsége ellen idővel változatos rezisztencia mechanizmusok alakulnak ki a rákos elfajulásban. Jó példák erre az Auróra A és B kinázok: bár nem tartoznak a leginkább rákkeltő molekuláris elváltozások közé, funkciójuk elengedhetetlen a sejtosztódáshoz és aktivitásuk gyakran emelkedett a rákos szövetekben. Az elmúlt másfél évtizedben számtalan specifikus hatóanyagot fejlesztettek ki a gátlásukra, de sajnos túlnyomó többségük elbukott a klinikai vizsgálatok során, tehát még mindig nincs engedélyezett Auróra gátló gyógyszer a piacon.

A Vichem Kft.-vel együttműködésben végzett gyógyszerfejlesztési munka keretein belül részletesen megvizsgáltam egy új, benzotiofén-3-karboxamid alapvázú hatóanyag családot, mint ígéretes Auróra kinázgátlókat. Kísérleteim eredményei azt mutatták, hogy több benzotiofén-3-karboxamid származék valóban gátolta az Auróra A és B kinázok működését *in vitro*. A molekulák egy része pedig kifejezetten az Auróra B kináz gátlásra jellemző sejtmorfológiai változásokat hozott létre és apoptózis indukálásán keresztül gátolta a vastagbélráksejtek életképességét. A leghatékonyabb vegyület a referencia Auróra kinázgátlókkal egyformán hatékonynak bizonyult minden *in vitro*, *in silico* és ráksejtvonal alapú vizsgálatban.

A célzott terápiák hatékonyság növelésének egyik iránya több molekuláris elváltozás egyidejű gátlása többszörös támadáspontú hatóanyagokkal vagy több egyszeres támadáspontú hatóanyag kombinációjával. Ennek szellemében a leghatékonyabb vegyületet több célzott hatóanyaggal is kombináltam a sejtes vizsgálatokban és ismét a referencia Auróra kinázgátlókhoz nagyon hasonló hatásokat tapasztaltam.

Összefoglalva, a leghatékonyabb benzotiofén-3-karboxamid származék minden szempontból hatékony Auróra kinázgátlónak bizonyult és új, ígéretes kiindulási alapja lehet további rákellenes gyógyszerhatóanyag fejlesztési munkáknak.

## 9. List of references


1. Guan X. (2015) Cancer metastases: challenges and opportunities. Acta Pharm Sin B, 5: 402-418.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. (2015) Global cancer statistics, 2012. CA Cancer J Clin, 65: 87-108.
3. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer, 136: E359-386.
4. Persano L, Zagoura D, Louisse J, Pistollato F. (2015) Role of environmental chemicals, processed food derivatives, and nutrients in the induction of carcinogenesis. Stem Cells Dev, 24: 2337-2352.
5. El-Bolkainy MN. (2016) Golden rules in practice of cancer pathology. African Journal of Urology, 22: 137-140.
6. (1931) The metabolism of tumours. Investigations from the Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Edited by Otto Warburg, Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Translated from the German edition, with accounts of additional recent researches, by Frank Dickens, M.A., Ph.D., whole-time worker for the Medical Research Council, Courtauld Institute of Biochemistry, Middlesex Hospital, London. Demy 8vo. Pp. 327 + xxix. Illustrated. 1930. London: Constable & Co. Ltd. 40s. net. BJS, 19: 168-168.
7. Shukla G, Khera HK, Srivastava AK, Khare P, Patidar R, Saxena R. (2017) Therapeutic Potential, Challenges and Future Perspective of Cancer Stem Cells in Translational Oncology: A Critical Review. Curr Stem Cell Res Ther, 12: 207-224.
8. Zecchin A, Borgers G, Carmeliet P. (2015) Endothelial cells and cancer cells: metabolic partners in crime? Curr Opin Hematol, 22: 234-242.
9. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. (2015) Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. Cancers (Basel), 7: 2443-2458.

10. Martinez-Outschoorn UE, Lisanti MP, Sotgia F. (2014) Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth. Semin Cancer Biol, 25: 47-60.
11. Shalapour S, Karin M. (2015) Immunity, inflammation, and cancer: an eternal fight between good and evil. J Clin Invest, 125: 3347-3355.
12. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, Davis A, Mongare MM, Gould J, Frederick DT, Cooper ZA, Chapman PB, Solit DB, Ribas A, Lo RS, Flaherty KT, Ogino S, Wargo JA, Golub TR. (2012) Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature, 487: 500-504.
13. Salk JJ, Fox EJ, Loeb LA. (2010) Mutational heterogeneity in human cancers: origin and consequences. Annu Rev Pathol, 5: 51-75.
14. Prasad V. (2016) Perspective: The precision-oncology illusion. Nature, 537: S63.
15. Biankin AV. (2017) The road to precision oncology. Nat Genet, 49: 320-321.
16. Stockley TL, Oza AM, Berman HK, Leighl NB, Knox JJ, Shepherd FA, Chen EX, Krzyzanowska MK, Dhani N, Joshua AM, Tsao MS, Serra S, Clarke B, Roehrl MH, Zhang T, Sukhai MA, Califaretti N, Trinkaus M, Shaw P, van der Kwast T, Wang L, Virtanen C, Kim RH, Razak AR, Hansen AR, Yu C, Pugh TJ, Kamel-Reid S, Siu LL, Bedard PL. (2016) Molecular profiling of advanced solid tumors and patient outcomes with genotype-matched clinical trials: the Princess Margaret IMPACT/COMPACT trial. Genome Med, 8: 109.
17. Tomasetti C, Li L, Vogelstein B. (2017) Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Science, 355: 1330-1334.
18. Loeb LA, Harris CC. (2008) Advances in chemical carcinogenesis: a historical review and prospective. Cancer Res, 68: 6863-6872.
19. Inukai M, Toyooka S, Ito S, Asano H, Ichihara S, Soh J, Suehisa H, Ouchida M, Aoe K, Aoe M, Kiura K, Shimizu N, Date H. (2006) Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. Cancer Res, 66: 7854-7858.

20. Jonchere B, Vetillard A, Toutain B, Guette C, Coqueret O. (2016) [Contribution to tumor escape and chemotherapy response: A choice between senescence and apoptosis in heterogeneous tumors]. Bull Cancer, 103: 73-86.
21. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW. (2013) Cancer genome landscapes. Science, 339: 1546-1558.
22. Piraino SW, Furney SJ. (2016) Beyond the exome: the role of non-coding somatic mutations in cancer. Ann Oncol, 27: 240-248.
23. Poulos RC, Sloane MA, Hesson LB, Wong JW. (2015) The search for cis-regulatory driver mutations in cancer genomes. Oncotarget, 6: 32509-32525.
24. Mertens F, Johansson B, Fioretos T, Mitelman F. (2015) The emerging complexity of gene fusions in cancer. Nat Rev Cancer, 15: 371-381.
25. Flavahan WA, Gaskell E, Bernstein BE. (2017) Epigenetic plasticity and the hallmarks of cancer. Science, 357.
26. Chatterjee A, Rodger EJ, Eccles MR. (2018) Epigenetic drivers of tumourigenesis and cancer metastasis. Semin Cancer Biol, 51: 149-159.
27. Sveen A, Kilpinen S, Ruusulehto A, Lothe RA, Skotheim RI. (2016) Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. Oncogene, 35: 2413-2427.
28. Hanahan D, Weinberg RA. (2011) Hallmarks of cancer: the next generation. Cell, 144: 646-674.
29. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmuller G, Klein CA. (2008) Systemic spread is an early step in breast cancer. Cancer Cell, 13: 58-68.
30. Artandi SE, DePinho RA. (2010) Telomeres and telomerase in cancer. Carcinogenesis, 31: 9-18.
31. Bastians H. (2015) Causes of Chromosomal Instability. Recent Results Cancer Res, 200: 95-113.
32. Tanaka K, Hirota T. (2016) Chromosomal instability: A common feature and a therapeutic target of cancer. Biochim Biophys Acta, 1866: 64-75.
33. Sheltzer JM. (2013) A transcriptional and metabolic signature of primary aneuploidy is present in chromosomally unstable cancer cells and informs clinical prognosis. Cancer Res, 73: 6401-6412.

34. Silk AD, Zasadil LM, Holland AJ, Vitre B, Cleveland DW, Weaver BA. (2013) Chromosome missegregation rate predicts whether aneuploidy will promote or suppress tumors. Proc Natl Acad Sci U S A, 110: E4134-4141.
35. Dabas N, Byrnes DM, Rosa AM, Eller MS, Grichnik JM. (2012) Diagnostic role of chromosomal instability in melanoma. J Skin Cancer, 2012: 914267.
36. Zuazo-Gaztelu I, Casanovas O. (2018) Unraveling the Role of Angiogenesis in Cancer Ecosystems. Front Oncol, 8: 248.
37. Thiery JP. (2003) Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol, 15: 740-746.
38. Sundaram GM, Quah S, Sampath P. (2018) Cancer: the dark side of wound healing. FEBS J.
39. Thiery JP. (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer, 2: 442-454.
40. Izumchenko E, Chang X, Michailidi C, Kagohara L, Ravi R, Paz K, Brait M, Hoque MO, Ling S, Bedi A, Sidransky D. (2014) The TGFbeta-miR200-MIG6 pathway orchestrates the EMT-associated kinase switch that induces resistance to EGFR inhibitors. Cancer Res, 74: 3995-4005.
41. Nurwidya F, Takahashi F, Kobayashi I, Murakami A, Kato M, Minakata K, Nara T, Hashimoto M, Yagishita S, Baskoro H, Hidayat M, Shimada N, Takahashi K. (2014) Treatment with insulin-like growth factor 1 receptor inhibitor reverses hypoxia-induced epithelial-mesenchymal transition in non-small cell lung cancer. Biochem Biophys Res Commun, 455: 332-338.
42. Kurokawa M, Ise N, Omi K, Goishi K, Higashiyama S. (2013) Cisplatin influences acquisition of resistance to molecular-targeted agents through epithelial-mesenchymal transition-like changes. Cancer Sci, 104: 904-911.
43. Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I, Nieto MA. (2004) Snail blocks the cell cycle and confers resistance to cell death. Genes Dev, 18: 1131-1143.
44. Bernards R, Weinberg RA. (2002) Metastasis genes: A progression puzzle. Nature, 418: 823.
45. Klein CA. (2009) Parallel progression of primary tumours and metastases. Nat Rev Cancer, 9: 302-312.

46. Somarelli JA, Ware KE, Kostadinov R, Robinson JM, Amri H, Abu-Asab M, Fourie N, Diogo R, Swofford D, Townsend JP. (2017) PhyloOncology: Understanding cancer through phylogenetic analysis. Biochim Biophys Acta Rev Cancer, 1867: 101-108.
47. Schafer M, Werner S. (2008) Cancer as an overhealing wound: an old hypothesis revisited. Nat Rev Mol Cell Biol, 9: 628-638.
48. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ. (2008) Immune-mediated dormancy: an equilibrium with cancer. J Leukoc Biol, 84: 988-993.
49. Kroemer G, Pouyssegur J. (2008) Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell, 13: 472-482.
50. Fox EJ, Salk JJ, Loeb LA. (2009) Cancer genome sequencing--an interim analysis. Cancer Res, 69: 4948-4950.
51. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, Morsberger LA, Latimer C, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal SA, Leroy C, Jia M, Menzies A, Butler AP, Teague JW, Griffin CA, Burton J, Swerdlow H, Quail MA, Stratton MR, Iacobuzio-Donahue C, Futreal PA. (2010) The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature, 467: 1109-1113.
52. Bozic I, Reiter JG, Allen B, Antal T, Chatterjee K, Shah P, Moon YS, Yaqubie A, Kelly N, Le DT, Lipson EJ, Chapman PB, Diaz LA, Jr., Vogelstein B, Nowak MA. (2013) Evolutionary dynamics of cancer in response to targeted combination therapy. Elife, 2: e00747.
53. Al-Lazikani B, Banerji U, Workman P. (2012) Combinatorial drug therapy for cancer in the post-genomic era. Nat Biotechnol, 30: 679-692.
54. Berger MF, Hodis E, Heffernan TP, Deribe YL, Lawrence MS, Protopopov A, Ivanova E, Watson IR, Nickerson E, Ghosh P, Zhang H, Zeid R, Ren X, Cibulskis K, Sivachenko AY, Wagle N, Sucker A, Sougnez C, Onofrio R, Ambrogio L, Auclair D, Fennell T, Carter SL, Drier Y, Stojanov P, Singer MA, Voet D, Jing R, Saksena G, Barretina J, Ramos AH, Pugh TJ, Stransky N, Parkin M, Winckler W, Mahan S, Ardlie K, Baldwin J, Wargo J, Schadendorf D, Meyerson M, Gabriel SB, Golub TR, Wagner SN, Lander ES, Getz G, Chin

L, Garraway LA. (2012) Melanoma genome sequencing reveals frequent PREX2 mutations. Nature, 485: 502-506.

55. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, Ding M, Bamford S, Cole C, Ward S, Kok CY, Jia M, De T, Teague JW, Stratton MR, McDermott U, Campbell PJ. (2015) COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res, 43: D805-811.
56. Beggs AD, Jones A, El-Bahrawy M, Abulafi M, Hodgson SV, Tomlinson IP. (2013) Whole-genome methylation analysis of benign and malignant colorectal tumours. J Pathol, 229: 697-704.
57. Pfister SX, Ashworth A. (2017) Marked for death: targeting epigenetic changes in cancer. Nat Rev Drug Discov, 16: 241-263.
58. Vicente-Duenas C, Hauer J, Cobaleda C, Borkhardt A, Sanchez-Garcia I. (2018) Epigenetic Priming in Cancer Initiation. Trends Cancer, 4: 408-417.
59. Strauss J, Figg WD. (2016) Using Epigenetic Therapy to Overcome Chemotherapy Resistance. Anticancer Res, 36: 1-4.
60. Castro-Giner F, Ratcliffe P, Tomlinson I. (2015) The mini-driver model of polygenic cancer evolution. Nat Rev Cancer, 15: 680-685.
61. Garraway LA, Lander ES. (2013) Lessons from the cancer genome. Cell, 153: 17-37.
62. Fox EJ, Prindle MJ, Loeb LA. (2013) Do mutator mutations fuel tumorigenesis? Cancer Metastasis Rev, 32: 353-361.
63. Ramos P, Bentires-Alj M. (2015) Mechanism-based cancer therapy: resistance to therapy, therapy for resistance. Oncogene, 34: 3617-3626.
64. Giuffrida D, Rogers IM. (2010) Targeting cancer stem cell lines as a new treatment of human cancer. Recent Pat Anticancer Drug Discov, 5: 205-218.
65. Gavande NS, VanderVere-Carozza PS, Hinshaw HD, Jalal SI, Sears CR, Pawelczak KS, Turchi JJ. (2016) DNA repair targeted therapy: The past or future of cancer treatment? Pharmacol Ther, 160: 65-83.
66. Califano A, Alvarez MJ. (2017) The recurrent architecture of tumour initiation, progression and drug sensitivity. Nat Rev Cancer, 17: 116-130.

67. Lin F, Li Z, Hua Y, Lim YP. (2016) Proteomic profiling predicts drug response to novel targeted anticancer therapeutics. Expert Rev Proteomics, 13: 411-420.
68. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. (2002) The protein kinase complement of the human genome. Science, 298: 1912-1934.
69. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell, 127: 635-648.
70. Krebs EG. (1985) The phosphorylation of proteins: a major mechanism for biological regulation. Fourteenth Sir Frederick Gowland Hopkins memorial lecture. Biochem Soc Trans, 13: 813-820.
71. Taylor SS, Keshwani MM, Steichen JM, Kornev AP. (2012) Evolution of the eukaryotic protein kinases as dynamic molecular switches. Philos Trans R Soc Lond B Biol Sci, 367: 2517-2528.
72. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T. (2004) Protein tyrosine phosphatases in the human genome. Cell, 117: 699-711.
73. Roskoski R, Jr. (2016) Classification of small molecule protein kinase inhibitors based upon the structures of their drug-enzyme complexes. Pharmacol Res, 103: 26-48.
74. Taylor SS, Radzio-Andzelm E, Hunter T. (1995) How do protein kinases discriminate between serine/threonine and tyrosine? Structural insights from the insulin receptor protein-tyrosine kinase. Faseb j, 9: 1255-1266.
75. Roskoski R, Jr. (2014) ErbB/HER protein-tyrosine kinases: Structures and small molecule inhibitors. Pharmacol Res, 87: 42-59.
76. Stadtman ER, Chock PB. (1978) Interconvertible enzyme cascades in metabolic regulation. Curr Top Cell Regul, 13: 53-95.
77. Fujioka A, Terai K, Itoh RE, Aoki K, Nakamura T, Kuroda S, Nishida E, Matsuda M. (2006) Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes. J Biol Chem, 281: 8917-8926.
78. Mendoza MC, Er EE, Blenis J. (2011) The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends Biochem Sci, 36: 320-328.

79. Cohen P. (2002) Protein kinases--the major drug targets of the twenty-first century? Nat Rev Drug Discov, 1: 309-315.
80. Blume-Jensen P, Hunter T. (2001) Oncogenic kinase signalling. Nature, 411: 355-365.
81. Williams-Ashman HG, Kennedy EP. (1952) Oxidative phosphorylation catalyzed by cytoplasmic particles isolated from malignant tissues. Cancer Res, 12: 415-421.
82. Tsai CJ, Nussinov R. (2013) The molecular basis of targeting protein kinases in cancer therapeutics. Semin Cancer Biol, 23: 235-242.
83. Drake JM, Lee JK, Witte ON. (2014) Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer. Mol Cell Biol, 34: 1722-1732.
84. Gosal G, Kochut KJ, Kannan N. (2011) ProKinO: an ontology for integrative analysis of protein kinases in cancer. PLoS One, 6: e28782.
85. Bornens M, Azimzadeh J. (2007) Origin and evolution of the centrosome. Adv Exp Med Biol, 607: 119-129.
86. D'Assoro AB, Lingle WL, Salisbury JL. (2002) Centrosome amplification and the development of cancer. Oncogene, 21: 6146-6153.
87. Saka Y, Giuraniuc CV, Ohkura H. (2015) Accurate chromosome segregation by probabilistic self-organisation. BMC Biol, 13: 65.
88. Cheeseman IM, Desai A. (2008) Molecular architecture of the kinetochore-microtubule interface. Nat Rev Mol Cell Biol, 9: 33-46.
89. Santaguida S, Amon A. (2015) Short- and long-term effects of chromosome mis-segregation and aneuploidy. Nat Rev Mol Cell Biol, 16: 473-485.
90. Silkworth WT, Nardi IK, Scholl LM, Cimini D. (2009) Multipolar spindle pole coalescence is a major source of kinetochore mis-attachment and chromosome mis-segregation in cancer cells. PLoS One, 4: e6564.
91. Malumbres M, Barbacid M. (2009) Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer, 9: 153-166.
92. Hartwell LH, Weinert TA. (1989) Checkpoints: controls that ensure the order of cell cycle events. Science, 246: 629-634.
93. Foley EA, Kapoor TM. (2013) Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. Nat Rev Mol Cell Biol, 14: 25-37.

94. Katayama H, Brinkley WR, Sen S. (2003) The Aurora kinases: role in cell transformation and tumorigenesis. Cancer Metastasis Rev, 22: 451-464.
95. Carmena M, Earnshaw WC. (2003) The cellular geography of aurora kinases. Nat Rev Mol Cell Biol, 4: 842-854.
96. Nigg EA. (2001) Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol, 2: 21-32.
97. Tang CJ, Lin CY, Tang TK. (2006) Dynamic localization and functional implications of Aurora-C kinase during male mouse meiosis. Dev Biol, 290: 398-410.
98. Balboula AZ, Schindler K. (2014) Selective disruption of aurora C kinase reveals distinct functions from aurora B kinase during meiosis in mouse oocytes. PLoS Genet, 10: e1004194.
99. Lin BW, Wang YC, Chang-Liao PY, Lin YJ, Yang ST, Tsou JH, Chang KC, Liu YW, Tseng JT, Lee CT, Lee JC, Hung LY. (2014) Overexpression of Aurora-C interferes with the spindle checkpoint by promoting the degradation of Aurora-B. Cell Death Dis, 5: e1106.
100. Kimura M, Kotani S, Hattori T, Sumi N, Yoshioka T, Todokoro K, Okano Y. (1997) Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. J Biol Chem, 272: 13766-13771.
101. Stenoien DL, Sen S, Mancini MA, Brinkley BR. (2003) Dynamic association of a tumor amplified kinase, Aurora-A, with the centrosome and mitotic spindle. Cell Motil Cytoskeleton, 55: 134-146.
102. Cui SY, Huang JY, Chen YT, Song HZ, Huang GC, De W, Wang R, Chen LB. (2013) The role of Aurora A in hypoxia-inducible factor 1alpha-promoting malignant phenotypes of hepatocelluar carcinoma. Cell Cycle, 12: 2849-2866.
103. Wang LH, Xiang J, Yan M, Zhang Y, Zhao Y, Yue CF, Xu J, Zheng FM, Chen JN, Kang Z, Chen TS, Xing D, Liu Q. (2010) The mitotic kinase Aurora-A induces mammary cell migration and breast cancer metastasis by activating the Cofilin-F-actin pathway. Cancer Res, 70: 9118-9128.

104. Yang J, Ikezoe T, Nishioka C, Udaka K, Yokoyama A. (2014) Bcr-Abl activates AURKA and AURKB in chronic myeloid leukemia cells via AKT signaling. Int J Cancer, 134: 1183-1194.

105. D'Assoro AB, Liu T, Quatraro C, Amato A, Opyrchal M, Leontovich A, Ikeda Y, Ohmine S, Lingle W, Suman V, Ecsedy J, Iankov I, Di Leonardo A, Ayers-Inglers J, Degnim A, Billadeau D, McCubrey J, Ingle J, Salisbury JL, Galanis E. (2014) The mitotic kinase Aurora--a promotes distant metastases by inducing epithelial-to-mesenchymal transition in ERalpha(+) breast cancer cells. Oncogene, 33: 599-610.

106. Privette LM, Petty EM. (2008) CHFR: A Novel Mitotic Checkpoint Protein and Regulator of Tumorigenesis. Transl Oncol, 1: 57-64.

107. Wu CC, Yang TY, Yu CT, Phan L, Ivan C, Sood AK, Hsu SL, Lee MH. (2012) p53 negatively regulates Aurora A via both transcriptional and posttranslational regulation. Cell Cycle, 11: 3433-3442.

108. Dodson CA, Bayliss R. (2012) Activation of Aurora-A kinase by protein partner binding and phosphorylation are independent and synergistic. J Biol Chem, 287: 1150-1157.

109. Dos Santos EO, Carneiro-Lobo TC, Aoki MN, Levantini E, Basseres DS. (2016) Aurora kinase targeting in lung cancer reduces KRAS-induced transformation. Mol Cancer, 15: 12.

110. Zou Z, Yuan Z, Zhang Q, Long Z, Chen J, Tang Z, Zhu Y, Chen S, Xu J, Yan M, Wang J, Liu Q. (2012) Aurora kinase A inhibition-induced autophagy triggers drug resistance in breast cancer cells. Autophagy, 8: 1798-1810.

111. Mazzera L, Lombardi G, Abeltino M, Ricca M, Donofrio G, Giuliani N, Cantoni AM, Corradi A, Bonati A, Lunghi P. (2013) Aurora and IKK kinases cooperatively interact to protect multiple myeloma cells from Apo2L/TRAIL. Blood, 122: 2641-2653.

112. Marumoto T, Hirota T, Morisaki T, Kunitoku N, Zhang D, Ichikawa Y, Sasayama T, Kuninaka S, Mimori T, Tamaki N, Kimura M, Okano Y, Saya H. (2002) Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells. Genes Cells, 7: 1173-1182.

113. Liu Q, Kaneko S, Yang L, Feldman RI, Nicosia SV, Chen J, Cheng JQ. (2004) Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. J Biol Chem, 279: 52175-52182.
114. Yang TY, Teng CJ, Lin TC, Chen KC, Hsu SL, Wu CC. (2018) Transcriptional repression of Aurora-A gene by wild-type p53 through directly binding to its promoter with histone deacetylase 1 and mSin3a. Int J Cancer, 142: 92-108.
115. Seki A, Coppinger JA, Jang CY, Yates JR, Fang G. (2008) Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. Science, 320: 1655-1658.
116. Barr AR, Gergely F. (2007) Aurora-A: the maker and breaker of spindle poles. J Cell Sci, 120: 2987-2996.
117. Giet R, Uzbekov R, Cubizolles F, Le Guellec K, Prigent C. (1999) The Xenopus laevis aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XlEg5. J Biol Chem, 274: 15005-15013.
118. Kinoshita K, Noetzel TL, Pelletier L, Mechtler K, Drechsel DN, Schwager A, Lee M, Raff JW, Hyman AA. (2005) Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. J Cell Biol, 170: 1047-1055.
119. Kim Y, Holland AJ, Lan W, Cleveland DW. (2010) Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E. Cell, 142: 444-455.
120. Chow C, Wong N, Pagano M, Lun SW, Nakayama KI, Nakayama K, Lo KW. (2012) Regulation of APC/CCdc20 activity by RASSF1A-APC/CCdc20 circuitry. Oncogene, 31: 1975-1987.
121. Floyd S, Pines J, Lindon C. (2008) APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. Curr Biol, 18: 1649-1658.
122. Ewart-Toland A, Briassouli P, de Koning JP, Mao JH, Yuan J, Chan F, MacCarthy-Morrogh L, Ponder BA, Nagase H, Burn J, Ball S, Almeida M, Linardopoulos S, Balmain A. (2003) Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. Nat Genet, 34: 403-412.

123. Kitajima S, Kudo Y, Ogawa I, Tatsuka M, Kawai H, Pagano M, Takata T. (2007) Constitutive phosphorylation of aurora-a on ser51 induces its stabilization and consequent overexpression in cancer. PLoS One, 2: e944.

124. Wang J, Yang S, Zhang H, Song Y, Zhang X, Qian H, Han X, Shi Y. (2011) Aurora-A as an independent molecular prognostic marker in gastric cancer. Oncol Rep, 26: 23-32.

125. Reiter R, Gais P, Jutting U, Steuer-Vogt MK, Pickhard A, Bink K, Rauser S, Lassmann S, Hofler H, Werner M, Walch A. (2006) Aurora kinase A messenger RNA overexpression is correlated with tumor progression and shortened survival in head and neck squamous cell carcinoma. Clin Cancer Res, 12: 5136-5141.

126. Lassmann S, Shen Y, Jutting U, Wiehle P, Walch A, Gitsch G, Hasenburg A, Werner M. (2007) Predictive value of Aurora-A/STK15 expression for late stage epithelial ovarian cancer patients treated by adjuvant chemotherapy. Clin Cancer Res, 13: 4083-4091.

127. Sen S, Zhou H, Zhang RD, Yoon DS, Vakar-Lopez F, Ito S, Jiang F, Johnston D, Grossman HB, Ruifrok AC, Katz RL, Brinkley W, Czerniak B. (2002) Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J Natl Cancer Inst, 94: 1320-1329.

128. Twu NF, Yuan CC, Yen MS, Lai CR, Chao KC, Wang PH, Wu HH, Chen YJ. (2009) Expression of Aurora kinase A and B in normal and malignant cervical tissue: high Aurora A kinase expression in squamous cervical cancer. Eur J Obstet Gynecol Reprod Biol, 142: 57-63.

129. Taga M, Hirooka E, Ouchi T. (2009) Essential roles of mTOR/Akt pathway in Aurora-A cell transformation. Int J Biol Sci, 5: 444-450.

130. Burum-Auensen E, De Angelis PM, Schjolberg AR, Kravik KL, Aure M, Clausen OP. (2007) Subcellular localization of the spindle proteins Aurora A, Mad2, and BUBR1 assessed by immunohistochemistry. J Histochem Cytochem, 55: 477-486.

131. Yang H, He L, Kruk P, Nicosia SV, Cheng JQ. (2006) Aurora-A induces cell survival and chemoresistance by activation of Akt through a p53-dependent manner in ovarian cancer cells. Int J Cancer, 119: 2304-2312.

132. Saiprasad G, Chitra P, Manikandan R, Sudhandiran G. (2014) Hesperidin induces apoptosis and triggers autophagic markers through inhibition of Aurora-A mediated phosphoinositide-3-kinase/Akt/mammalian target of rapamycin and glycogen synthase kinase-3 beta signalling cascades in experimental colon carcinogenesis. Eur J Cancer, 50: 2489-2507.
133. Stark GR, Taylor WR. (2006) Control of the G2/M transition. Mol Biotechnol, 32: 227-248.
134. Nikonova AS, Astsaturov I, Serebriiskii IG, Dunbrack RL, Jr., Golemis EA. (2013) Aurora A kinase (AURKA) in normal and pathological cell division. Cell Mol Life Sci, 70: 661-687.
135. Anand S, Penrhyn-Lowe S, Venkitaraman AR. (2003) AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell, 3: 51-62.
136. Romain C, Paul P, Kim KW, Lee S, Qiao J, Chung DH. (2014) Targeting Aurora kinase-A downregulates cell proliferation and angiogenesis in neuroblastoma. J Pediatr Surg, 49: 159-165.
137. Guan Z, Wang XR, Zhu XF, Huang XF, Xu J, Wang LH, Wan XB, Long ZJ, Liu JN, Feng GK, Huang W, Zeng YX, Chen FJ, Liu Q. (2007) Aurora-A, a negative prognostic marker, increases migration and decreases radiosensitivity in cancer cells. Cancer Res, 67: 10436-10444.
138. Wan XB, Long ZJ, Yan M, Xu J, Xia LP, Liu L, Zhao Y, Huang XF, Wang XR, Zhu XF, Hong MH, Liu Q. (2008) Inhibition of Aurora-A suppresses epithelial-mesenchymal transition and invasion by downregulating MAPK in nasopharyngeal carcinoma cells. Carcinogenesis, 29: 1930-1937.
139. Do TV, Xiao F, Bickel LE, Klein-Szanto AJ, Pathak HB, Hua X, Howe C, O'Brien SW, Maglaty M, Ecsedy JA, Litwin S, Golemis EA, Schilder RJ, Godwin AK, Connolly DC. (2014) Aurora kinase A mediates epithelial ovarian cancer cell migration and adhesion. Oncogene, 33: 539-549.
140. Mahankali M, Henkels KM, Speranza F, Gomez-Cambronero J. (2015) A non-mitotic role for Aurora kinase A as a direct activator of cell migration upon interaction with PLD, FAK and Src. J Cell Sci, 128: 516-526.

141. Chen CH, Chuang HC, Huang CC, Fang FM, Huang HY, Tsai HT, Su LJ, Shiu LY, Leu S, Chien CY. (2013) Overexpression of Rap-1A indicates a poor prognosis for oral cavity squamous cell carcinoma and promotes tumor cell invasion via Aurora-A modulation. Am J Pathol, 182: 516-528.
142. Du J, Hannon GJ. (2002) The centrosomal kinase Aurora-A/STK15 interacts with a putative tumor suppressor NM23-H1. Nucleic Acids Res, 30: 5465-5475.
143. Glover DM, Leibowitz MH, McLean DA, Parry H. (1995) Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. Cell, 81: 95-105.
144. Du J, Hannon GJ. (2004) Suppression of p160ROCK bypasses cell cycle arrest after Aurora-A/STK15 depletion. Proc Natl Acad Sci U S A, 101: 8975-8980.
145. D'Assoro AB, Haddad T, Galanis E. (2015) Aurora-A Kinase as a Promising Therapeutic Target in Cancer. Front Oncol, 5: 295.
146. Giet R, Prigent C. (1999) Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. J Cell Sci, 112 ( Pt 21): 3591-3601.
147. Carmena M, Wheelock M, Funabiki H, Earnshaw WC. (2012) The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. Nat Rev Mol Cell Biol, 13: 789-803.
148. Suijkerbuijk SJ, Vleugel M, Teixeira A, Kops GJ. (2012) Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments. Dev Cell, 23: 745-755.
149. Sotillo R, Hernando E, Diaz-Rodriguez E, Teruya-Feldstein J, Cordon-Cardo C, Lowe SW, Benezra R. (2007) Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. Cancer Cell, 11: 9-23.
150. Ricke RM, Jeganathan KB, van Deursen JM. (2011) Bub1 overexpression induces aneuploidy and tumor formation through Aurora B kinase hyperactivation. J Cell Biol, 193: 1049-1064.
151. Xu L, Liu T, Han F, Zong Z, Wang G, Yu B, Zhang J. (2012) AURKB and MAPK involvement in the regulation of the early stages of mouse zygote development. Sci China Life Sci, 55: 47-56.

152. Wheatley SP, Carvalho A, Vagnarelli P, Earnshaw WC. (2001) INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. Curr Biol, 11: 886-890.
153. Gassmann R, Carvalho A, Henzing AJ, Ruchaud S, Hudson DF, Honda R, Nigg EA, Gerloff DL, Earnshaw WC. (2004) Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. J Cell Biol, 166: 179-191.
154. Gohard FH, St-Cyr DJ, Tyers M, Earnshaw WC. (2014) Targeting the INCENP IN-box-Aurora B interaction to inhibit CPC activity in vivo. Open Biol, 4: 140163.
155. Adams RR, Maiato H, Earnshaw WC, Carmena M. (2001) Essential roles of Drosophila inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. J Cell Biol, 153: 865-880.
156. Giet R, Glover DM. (2001) Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. J Cell Biol, 152: 669-682.
157. Cimini D, Wan X, Hirel CB, Salmon ED. (2006) Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors. Curr Biol, 16: 1711-1718.
158. Varma D, Salmon ED. (2012) The KMN protein network--chief conductors of the kinetochore orchestra. J Cell Sci, 125: 5927-5936.
159. van der Waal MS, Hengeveld RC, van der Horst A, Lens SM. (2012) Cell division control by the Chromosomal Passenger Complex. Exp Cell Res, 318: 1407-1420.
160. Lampson MA, Cheeseman IM. (2011) Sensing centromere tension: Aurora B and the regulation of kinetochore function. Trends Cell Biol, 21: 133-140.
161. Loncarek J, Kisurina-Evgenieva O, Vinogradova T, Hergert P, La Terra S, Kapoor TM, Khodjakov A. (2007) The centromere geometry essential for keeping mitosis error free is controlled by spindle forces. Nature, 450: 745-749.

162. Saurin AT, van der Waal MS, Medema RH, Lens SM, Kops GJ. (2011) Aurora B potentiates Mps1 activation to ensure rapid checkpoint establishment at the onset of mitosis. Nat Commun, 2: 316.

163. Zhu T, Dou Z, Qin B, Jin C, Wang X, Xu L, Wang Z, Zhu L, Liu F, Gao X, Ke Y, Wang Z, Aikhionbare F, Fu C, Ding X, Yao X. (2013) Phosphorylation of microtubule-binding protein Hec1 by mitotic kinase Aurora B specifies spindle checkpoint kinase Mps1 signaling at the kinetochore. J Biol Chem, 288: 36149-36159.

164. Welburn JP, Vleugel M, Liu D, Yates JR, 3rd, Lampson MA, Fukagawa T, Cheeseman IM. (2010) Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface. Mol Cell, 38: 383-392.

165. Sampath SC, Ohi R, Leismann O, Salic A, Pozniakovski A, Funabiki H. (2004) The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. Cell, 118: 187-202.

166. Straight AF, Field CM. (2000) Microtubules, membranes and cytokinesis. Curr Biol, 10: R760-770.

167. Lens SM, Voest EE, Medema RH. (2010) Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer, 10: 825-841.

168. Nguyen HG, Chinnappan D, Urano T, Ravid K. (2005) Mechanism of Aurora-B degradation and its dependency on intact KEN and A-boxes: identification of an aneuploidy-promoting property. Mol Cell Biol, 25: 4977-4992.

169. Thiru P, Kern DM, McKinley KL, Monda JK, Rago F, Su KC, Tsinman T, Yarar D, Bell GW, Cheeseman IM. (2014) Kinetochore genes are coordinately up-regulated in human tumors as part of a FoxM1-related cell division program. Mol Biol Cell, 25: 1983-1994.

170. Hegyi K, Egervari K, Sandor Z, Mehes G. (2012) Aurora kinase B expression in breast carcinoma: cell kinetic and genetic aspects. Pathobiology, 79: 314-322.

171. Thompson SL, Compton DA. (2010) Proliferation of aneuploid human cells is limited by a p53-dependent mechanism. J Cell Biol, 188: 369-381.

172. Hegyi K, Mehes G. (2012) Mitotic failures in cancer: Aurora B kinase and its potential role in the development of aneuploidy. Pathol Oncol Res, 18: 761-769.

173. Ota T, Suto S, Katayama H, Han ZB, Suzuki F, Maeda M, Tanino M, Terada Y, Tatsuka M. (2002) Increased mitotic phosphorylation of histone H3 attributable to AIM-1/Aurora-B overexpression contributes to chromosome number instability. Cancer Res, 62: 5168-5177.
174. Carter SL, Eklund AC, Kohane IS, Harris LN, Szallasi Z. (2006) A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nat Genet, 38: 1043-1048.
175. Munoz-Barrera M, Monje-Casas F. (2014) Increased Aurora B activity causes continuous disruption of kinetochore-microtubule attachments and spindle instability. Proc Natl Acad Sci U S A, 111: E3996-4005.
176. Nguyen HG, Makitalo M, Yang D, Chinnappan D, St Hilaire C, Ravid K. (2009) Deregulated Aurora-B induced tetraploidy promotes tumorigenesis. FASEB J, 23: 2741-2748.
177. Honda R, Korner R, Nigg EA. (2003) Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. Mol Biol Cell, 14: 3325-3341.
178. Hindriksen S, Meppelink A, Lens SM. (2015) Functionality of the chromosomal passenger complex in cancer. Biochem Soc Trans, 43: 23-32.
179. Fernandez-Miranda G, Trakala M, Martin J, Escobar B, Gonzalez A, Ghyselinck NB, Ortega S, Canamero M, Perez de Castro I, Malumbres M. (2011) Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development. Development, 138: 2661-2672.
180. Janssen A, Kops GJ, Medema RH. (2009) Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. Proc Natl Acad Sci U S A, 106: 19108-19113.
181. Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL, Peters JM. (2003) The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J Cell Biol, 161: 281-294.
182. Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T, Mortlock A, Keen N, Taylor SS. (2003) Aurora B couples chromosome alignment with

anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol, 161: 267-280.

183. Lin ZZ, Jeng YM, Hu FC, Pan HW, Tsao HW, Lai PL, Lee PH, Cheng AL, Hsu HC. (2010) Significance of Aurora B overexpression in hepatocellular carcinoma. Aurora B Overexpression in HCC. BMC Cancer, 10: 461.

184. Malumbres M, Perez de Castro I. (2014) Aurora kinase A inhibitors: promising agents in antitumoral therapy. Expert Opin Ther Targets, 18: 1377-1393.

185. Subramaniyan B, Jagadeesan K, Ramakrishnan S, Mathan G. (2016) Targeting the interaction of Aurora kinases and SIRT1 mediated by Wnt signaling pathway in colorectal cancer: A critical review. Biomed Pharmacother, 82: 413-424.

186. Samaras V, Stamatelli A, Samaras E, Arnaoutoglou C, Arnaoutoglou M, Stergiou I, Konstantopoulou P, Varsos V, Karameris A, Barbatis C. (2009) Comparative immunohistochemical analysis of aurora-A and aurora-B expression in human glioblastomas. Associations with proliferative activity and clinicopathological features. Pathol Res Pract, 205: 765-773.

187. Nadler Y, Camp RL, Schwartz C, Rimm DL, Kluger HM, Kluger Y. (2008) Expression of Aurora A (but not Aurora B) is predictive of survival in breast cancer. Clin Cancer Res, 14: 4455-4462.

188. Falchook GS, Bastida CC, Kurzrock R. (2015) Aurora Kinase Inhibitors in Oncology Clinical Trials: Current State of the Progress. Semin Oncol, 42: 832-848.

189. Schmit TL, Ahmad N. (2007) Regulation of mitosis via mitotic kinases: new opportunities for cancer management. Mol Cancer Ther, 6: 1920-1931.

190. Tang A, Gao K, Chu L, Zhang R, Yang J, Zheng J. (2017) Aurora kinases: novel therapy targets in cancers. Oncotarget, 8: 23937-23954.

191. Ushiro H, Cohen S. (1980) Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J Biol Chem, 255: 8363-8365.

192. Cohen S, Ushiro H, Stoscheck C, Chinkers M. (1982) A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J Biol Chem, 257: 1523-1531.

193. Pines G, Kostler WJ, Yarden Y. (2010) Oncogenic mutant forms of EGFR: lessons in signal transduction and targets for cancer therapy. FEBS Lett, 584: 2699-2706.
194. Roskoski R, Jr. (2014) The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol Res, 79: 34-74.
195. Singh B, Carpenter G, Coffey RJ. (2016) EGF receptor ligands: recent advances. F1000Res, 5.
196. Lemmon MA, Schlessinger J. (2010) Cell signaling by receptor tyrosine kinases. Cell, 141: 1117-1134.
197. Stamos J, Sliwkowski MX, Eigenbrot C. (2002) Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J Biol Chem, 277: 46265-46272.
198. Kovacs E, Das R, Wang Q, Collier TS, Cantor A, Huang Y, Wong K, Mirza A, Barros T, Grob P, Jura N, Bose R, Kuriyan J. (2015) Analysis of the Role of the C-Terminal Tail in the Regulation of the Epidermal Growth Factor Receptor. Mol Cell Biol, 35: 3083-3102.
199. Roepstorff K, Grandal MV, Henriksen L, Knudsen SL, Lerdrup M, Grovdal L, Willumsen BM, van Deurs B. (2009) Differential effects of EGFR ligands on endocytic sorting of the receptor. Traffic, 10: 1115-1127.
200. Holowka D, Baird B. (2017) Mechanisms of epidermal growth factor receptor signaling as characterized by patterned ligand activation and mutational analysis. Biochim Biophys Acta, 1859: 1430-1435.
201. Oda K, Matsuoka Y, Funahashi A, Kitano H. (2005) A comprehensive pathway map of epidermal growth factor receptor signaling. Mol Syst Biol, 1: 2005.0010.
202. Morrison DK. (2012) MAP kinase pathways. Cold Spring Harb Perspect Biol, 4.
203. Fang JY, Richardson BC. (2005) The MAPK signalling pathways and colorectal cancer. Lancet Oncol, 6: 322-327.
204. Yip PY. (2015) Phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway in non-small cell lung cancer. Transl Lung Cancer Res, 4: 165-176.
205. Lattanzio R, Piantelli M, Falasca M. (2013) Role of phospholipase C in cell invasion and metastasis. Adv Biol Regul, 53: 309-318.

206. Quesnelle KM, Boehm AL, Grandis JR. (2007) STAT-mediated EGFR signaling in cancer. J Cell Biochem, 102: 311-319.
207. Chen X, Xiao W, Liu X, Zeng M, Luo L, Wu M, Ye S, Liu Y. (2014) Blockade of Jagged/Notch pathway abrogates transforming growth factor beta2-induced epithelial-mesenchymal transition in human retinal pigment epithelium cells. Curr Mol Med, 14: 523-534.
208. Endres NF, Barros T, Cantor AJ, Kuriyan J. (2014) Emerging concepts in the regulation of the EGF receptor and other receptor tyrosine kinases. Trends Biochem Sci, 39: 437-446.
209. Lemmon MA, Schlessinger J, Ferguson KM. (2014) The EGFR family: not so prototypical receptor tyrosine kinases. Cold Spring Harb Perspect Biol, 6: a020768.
210. Pennock S, Wang Z. (2003) Stimulation of cell proliferation by endosomal epidermal growth factor receptor as revealed through two distinct phases of signaling. Mol Cell Biol, 23: 5803-5815.
211. Singh AB, Harris RC. (2005) Autocrine, paracrine and juxtacrine signaling by EGFR ligands. Cell Signal, 17: 1183-1193.
212. French AR, Tadaki DK, Niyogi SK, Lauffenburger DA. (1995) Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. J Biol Chem, 270: 4334-4340.
213. Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ, 2nd. (2009) Functional selectivity of EGF family peptide growth factors: implications for cancer. Pharmacol Ther, 122: 1-8.
214. Olayioye MA, Neve RM, Lane HA, Hynes NE. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J, 19: 3159-3167.
215. Kennedy SP, Hastings JF, Han JZ, Croucher DR. (2016) The Under-Appreciated Promiscuity of the Epidermal Growth Factor Receptor Family. Front Cell Dev Biol, 4: 88.

216. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. (2000) Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem, 275: 8806-8811.

217. Chou RH, Wang YN, Hsieh YH, Li LY, Xia W, Chang WC, Chang LC, Cheng CC, Lai CC, Hsu JL, Chang WJ, Chiang SY, Lee HJ, Liao HW, Chuang PH, Chen HY, Wang HL, Kuo SC, Chen CH, Yu YL, Hung MC. (2014) EGFR modulates DNA synthesis and repair through Tyr phosphorylation of histone H4. Dev Cell, 30: 224-237.

218. Wang YN, Yamaguchi H, Hsu JM, Hung MC. (2010) Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. Oncogene, 29: 3997-4006.

219. Lee HH, Wang YN, Hung MC. (2015) Non-canonical signaling mode of the epidermal growth factor receptor family. Am J Cancer Res, 5: 2944-2958.

220. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L, Hung MC. (2001) Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol, 3: 802-808.

221. Thompson DM, Gill GN. (1985) The EGF receptor: structure, regulation and potential role in malignancy. Cancer Surv, 4: 767-788.

222. Reissmann PT, Koga H, Figlin RA, Holmes EC, Slamon DJ. (1999) Amplification and overexpression of the cyclin D1 and epidermal growth factor receptor genes in non-small-cell lung cancer. Lung Cancer Study Group. J Cancer Res Clin Oncol, 125: 61-70.

223. Dey N, De P, Leyland-Jones B. (2017) PI3K-AKT-mTOR inhibitors in breast cancers: From tumor cell signaling to clinical trials. Pharmacol Ther, 175: 91-106.

224. Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ. (2000) Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. Cancer Res, 60: 2926-2935.

225. Jakobsen KR, Demuth C, Sorensen BS, Nielsen AL. (2016) The role of epithelial to mesenchymal transition in resistance to epidermal growth factor

receptor tyrosine kinase inhibitors in non-small cell lung cancer. Transl Lung Cancer Res, 5: 172-182.

226. Normanno N, De Luca A, Maiello MR, Campiglio M, Napolitano M, Mancino M, Carotenuto A, Viglietto G, Menard S. (2006) The MEK/MAPK pathway is involved in the resistance of breast cancer cells to the EGFR tyrosine kinase inhibitor gefitinib. J Cell Physiol, 207: 420-427.

227. Xie M, He CS, Wei SH, Zhang L. (2013) Notch-1 contributes to epidermal growth factor receptor tyrosine kinase inhibitor acquired resistance in non-small cell lung cancer in vitro and in vivo. Eur J Cancer, 49: 3559-3572.

228. Tanizaki J, Okamoto I, Sakai K, Nakagawa K. (2011) Differential roles of trans-phosphorylated EGFR, HER2, HER3, and RET as heterodimerisation partners of MET in lung cancer with MET amplification. Br J Cancer, 105: 807-813.

229. Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, Zakowski MF, Heelan RT, Kris MG, Varmus HE. (2005) KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med, 2: e17.

230. Lo HW, Xia W, Wei Y, Ali-Seyed M, Huang SF, Hung MC. (2005) Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer. Cancer Res, 65: 338-348.

231. Liccardi G, Hartley JA, Hochhauser D. (2011) EGFR nuclear translocation modulates DNA repair following cisplatin and ionizing radiation treatment. Cancer Res, 71: 1103-1114.

232. Huang WC, Chen YJ, Li LY, Wei YL, Hsu SC, Tsai SL, Chiu PC, Huang WP, Wang YN, Chen CH, Chang WC, Chang WC, Chen AJ, Tsai CH, Hung MC. (2011) Nuclear translocation of epidermal growth factor receptor by Akt-dependent phosphorylation enhances breast cancer-resistant protein expression in gefitinib-resistant cells. J Biol Chem, 286: 20558-20568.

233. Hodkinson PS, Mackinnon A, Sethi T. (2008) Targeting growth factors in lung cancer. Chest, 133: 1209-1216.

234. Clark AJ, Ishii S, Richert N, Merlino GT, Pastan I. (1985) Epidermal growth factor regulates the expression of its own receptor. Proc Natl Acad Sci U S A, 82: 8374-8378.

235. Li J, Jia XF, Liu J, Liu JJ, Zhao HB. (2015) Relationship of EGFR DNA methylation with the severity of non-small cell lung cancer. Genet Mol Res, 14: 11915-11923.
236. McInerney JM, Wilson MA, Strand KJ, Chrysogelos SA. (2001) A strong intronic enhancer element of the EGFR gene is preferentially active in high EGFR expressing breast cancer cells. J Cell Biochem, 80: 538-549.
237. Gaber R, Watermann I, Kugler C, Reinmuth N, Huber RM, Schnabel PA, Vollmer E, Reck M, Goldmann T. (2014) Correlation of EGFR expression, gene copy number and clinicopathological status in NSCLC. Diagn Pathol, 9: 165.
238. Suzuki S, Dobashi Y, Sakurai H, Nishikawa K, Hanawa M, Ooi A. (2005) Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas. An immunohistochemical and fluorescence in situ hybridization study. Cancer, 103: 1265-1273.
239. Sharma SV, Bell DW, Settleman J, Haber DA. (2007) Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer, 7: 169-181.
240. Gan HK, Cvrljevic AN, Johns TG. (2013) The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. FEBS J, 280: 5350-5370.
241. Yasuda H, Kobayashi S, Costa DB. (2012) EGFR exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications. Lancet Oncol, 13: e23-31.
242. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med, 350: 2129-2139.
243. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science, 304: 1497-1500.
244. Ware KE, Hinz TK, Kleczko E, Singleton KR, Marek LA, Helfrich BA, Cummings CT, Graham DK, Astling D, Tan AC, Heasley LE. (2013) A

mechanism of resistance to gefitinib mediated by cellular reprogramming and the acquisition of an FGF2-FGFR1 autocrine growth loop. Oncogenesis, 2: e39.

245. Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, Hashida S, Maki Y, Ichihara E, Asano H, Tsukuda K, Takigawa N, Kiura K, Gazdar AF, Lam WL, Miyoshi S. (2013) Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells. Cancer Res, 73: 3051-3061.

246. Costa C, Molina MA, Drozdowskyj A, Gimenez-Capitan A, Bertran-Alamillo J, Karachaliou N, Gervais R, Massuti B, Wei J, Moran T, Majem M, Felip E, Carcereny E, Garcia-Campelo R, Viteri S, Taron M, Ono M, Giannikopoulos P, Bivona T, Rosell R. (2014) The impact of EGFR T790M mutations and BIM mRNA expression on outcome in patients with EGFR-mutant NSCLC treated with erlotinib or chemotherapy in the randomized phase III EURTAC trial. Clin Cancer Res, 20: 2001-2010.

247. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, Meyerson M, Eck MJ. (2008) The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc Natl Acad Sci U S A, 105: 2070-2075.

248. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, Kris MG, Miller VA, Ladanyi M, Riely GJ. (2013) Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res, 19: 2240-2247.

249. Nicholson RI, Gee JM, Harper ME. (2001) EGFR and cancer prognosis. Eur J Cancer, 37 Suppl 4: S9-15.

250. Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR. (1987) Epidermal-growth-factor-dependent transformation by a human EGF receptor proto-oncogene. Science, 238: 1408-1410.

251. Cowley GP, Smith JA, Gusterson BA. (1986) Increased EGF receptors on human squamous carcinoma cell lines. Br J Cancer, 53: 223-229.

252. Gusterson B, Cowley G, McIlhinney J, Ozanne B, Fisher C, Reeves B. (1985) Evidence for increased epidermal growth factor receptors in human sarcomas. Int J Cancer, 36: 689-693.

253. Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B. (1987) Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. Proc Natl Acad Sci U S A, 84: 6899-6903.
254. Cheng L, Zhang S, Alexander R, Yao Y, MacLennan GT, Pan CX, Huang J, Wang M, Montironi R, Lopez-Beltran A. (2011) The landscape of EGFR pathways and personalized management of non-small-cell lung cancer. Future Oncol, 7: 519-541.
255. Gentile A, Trusolino L, Comoglio PM. (2008) The Met tyrosine kinase receptor in development and cancer. Cancer Metastasis Rev, 27: 85-94.
256. Viticchie G, Muller PAJ. (2015) c-Met and Other Cell Surface Molecules: Interaction, Activation and Functional Consequences. Biomedicines, 3: 46-70.
257. Organ SL, Tsao MS. (2011) An overview of the c-MET signaling pathway. Ther Adv Med Oncol, 3: S7-S19.
258. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. (2012) Targeting MET in cancer: rationale and progress. Nat Rev Cancer, 12: 89-103.
259. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Janne PA. (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science, 316: 1039-1043.
260. Peters S, Adjei AA. (2012) MET: a promising anticancer therapeutic target. Nat Rev Clin Oncol, 9: 314-326.
261. Kawakami H, Okamoto I, Okamoto W, Tanizaki J, Nakagawa K, Nishio K. (2014) Targeting MET Amplification as a New Oncogenic Driver. Cancers (Basel), 6: 1540-1552.
262. Hughes VS, Siemann DW. (2018) Have Clinical Trials Properly Assessed c-Met Inhibitors? Trends Cancer, 4: 94-97.
263. Pilotto S, Carbognin L, Karachaliou N, Ma PC, Rosell R, Tortora G, Bria E. (2017) Tracking MET de-addiction in lung cancer: A road towards the oncogenic target. Cancer Treat Rev, 60: 1-11.

264. Cappuzzo F, Janne PA, Skokan M, Finocchiaro G, Rossi E, Ligorio C, Zucali PA, Terracciano L, Toschi L, Roncalli M, Destro A, Incarbone M, Alloisio M, Santoro A, Varella-Garcia M. (2009) MET increased gene copy number and primary resistance to gefitinib therapy in non-small-cell lung cancer patients. Ann Oncol, 20: 298-304.

265. Krumbach R, Schuler J, Hofmann M, Giesemann T, Fiebig HH, Beckers T. (2011) Primary resistance to cetuximab in a panel of patient-derived tumour xenograft models: activation of MET as one mechanism for drug resistance. Eur J Cancer, 47: 1231-1243.

266. Benedettini E, Sholl LM, Peyton M, Reilly J, Ware C, Davis L, Vena N, Bailey D, Yeap BY, Fiorentino M, Ligon AH, Pan BS, Richon V, Minna JD, Gazdar AF, Draetta G, Bosari S, Chirieac LR, Lutterbach B, Loda M. (2010) Met activation in non-small cell lung cancer is associated with de novo resistance to EGFR inhibitors and the development of brain metastasis. Am J Pathol, 177: 415-423.

267. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, Toschi L, Rogers A, Mok T, Sequist L, Lindeman NI, Murphy C, Akhavanfard S, Yeap BY, Xiao Y, Capelletti M, Iafrate AJ, Lee C, Christensen JG, Engelman JA, Janne PA. (2010) Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell, 17: 77-88.

268. Van Der Steen N, Pauwels P, Gil-Bazo I, Castanon E, Raez L, Cappuzzo F, Rolfo C. (2015) cMET in NSCLC: Can We Cut off the Head of the Hydra? From the Pathway to the Resistance. Cancers (Basel), 7: 556-573.

269. Yi YW, You K, Bae EJ, Kwak SJ, Seong YS, Bae I. (2015) Dual inhibition of EGFR and MET induces synthetic lethality in triple-negative breast cancer cells through downregulation of ribosomal protein S6. Int J Oncol, 47: 122-132.

270. Drilon A, Cappuzzo F, Ou SI, Camidge DR. (2017) Targeting MET in Lung Cancer: Will Expectations Finally Be MET? J Thorac Oncol, 12: 15-26.

271. Qi J, McTigue MA, Rogers A, Lifshits E, Christensen JG, Janne PA, Engelman JA. (2011) Multiple mutations and bypass mechanisms can contribute to development of acquired resistance to MET inhibitors. Cancer Res, 71: 1081-1091.

272. McDermott U, Pusapati RV, Christensen JG, Gray NS, Settleman J. (2010) Acquired resistance of non-small cell lung cancer cells to MET kinase inhibition is mediated by a switch to epidermal growth factor receptor dependency. Cancer Res, 70: 1625-1634.
273. Wyld L, Audisio RA, Poston GJ. (2015) The evolution of cancer surgery and future perspectives. Nat Rev Clin Oncol, 12: 115-124.
274. DeVita VT, Jr., Chu E. (2008) A history of cancer chemotherapy. Cancer Res, 68: 8643-8653.
275. Lind MJ. (2011) Principles of cytotoxic chemotherapy. Medicine, 39: 711-716.
276. Pearce A, Haas M, Viney R, Pearson SA, Haywood P, Brown C, Ward R. (2017) Incidence and severity of self-reported chemotherapy side effects in routine care: A prospective cohort study. PLoS One, 12: e0184360.
277. Klement GL. (2016) Eco-evolution of cancer resistance. Sci Transl Med, 8: 327fs325.
278. Vadlapatla RK, Vadlapudi AD, Pal D, Mitra AK. (2013) Mechanisms of drug resistance in cancer chemotherapy: coordinated role and regulation of efflux transporters and metabolizing enzymes. Curr Pharm Des, 19: 7126-7140.
279. Huggins C, Hodges CV. (2002) Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. J Urol, 167: 948-951; discussion 952.
280. Littman DR. (2015) Releasing the Brakes on Cancer Immunotherapy. Cell, 162: 1186-1190.
281. Wirth T, Yla-Herttuala S. (2014) Gene Therapy Used in Cancer Treatment. Biomedicines, 2: 149-162.
282. Weinstein IB. (2002) Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science, 297: 63-64.
283. Khamisipour G, Jadidi-Niaragh F, Jahromi AS, Zandi K, Hojjat-Farsangi M. (2016) Mechanisms of tumor cell resistance to the current targeted-therapy agents. Tumour Biol, 37: 10021-10039.
284. Camidge DR. (2014) Targeted therapy vs chemotherapy: which has had more impact on survival in lung cancer? Does targeted therapy make patients live

longer? Hard to prove, but impossible to ignore. Clin Adv Hematol Oncol, 12: 763-766.

285. Le Tourneau C, Delord JP, Goncalves A, Gavoille C, Dubot C, Isambert N, Campone M, Tredan O, Massiani MA, Mauborgne C, Armanet S, Servant N, Bieche I, Bernard V, Gentien D, Jezequel P, Attignon V, Boyault S, Vincent-Salomon A, Servois V, Sablin MP, Kamal M, Paoletti X, investigators S. (2015) Molecularly targeted therapy based on tumour molecular profiling versus conventional therapy for advanced cancer (SHIVA): a multicentre, open-label, proof-of-concept, randomised, controlled phase 2 trial. Lancet Oncol, 16: 1324-1334.

286. Li GN, Wang SP, Xue X, Qu XJ, Liu HP. (2013) Monoclonal antibody-related drugs for cancer therapy. Drug Discov Ther, 7: 178-184.

287. Szeitner Z, Andras J, Gyurcsanyi RE, Meszaros T. (2014) Is less more? Lessons from aptamer selection strategies. J Pharm Biomed Anal, 101: 58-65.

288. Dosio F, Stella B, Cerioni S, Gastaldi D, Arpicco S. (2014) Advances in anticancer antibody-drug conjugates and immunotoxins. Recent Pat Anticancer Drug Discov, 9: 35-65.

289. Muranyi J, Gyulavari P, Varga A, Bokonyi G, Tanai H, Vantus T, Pap D, Ludanyi K, Mezo G, Keri G. (2016) Synthesis, characterization and systematic comparison of FITC-labelled GnRH-I, -II and -III analogues on various tumour cells. J Pept Sci, 22: 552-560.

290. Son KH, Hong JH, Lee JW. (2016) Carbon nanotubes as cancer therapeutic carriers and mediators. Int J Nanomedicine, 11: 5163-5185.

291. Tanka-Salamon A, Bota A, Wacha A, Mihaly J, Lovas M, Kolev K. (2017) Structure and Function of Trypsin-Loaded Fibrinolytic Liposomes. Biomed Res Int, 2017: 5130495.

292. Gross S, Rahal R, Stransky N, Lengauer C, Hoeflich KP. (2015) Targeting cancer with kinase inhibitors. J Clin Invest, 125: 1780-1789.

293. Konieczkowski DJ, Johannessen CM, Garraway LA. (2018) A Convergence-Based Framework for Cancer Drug Resistance. Cancer Cell, 33: 801-815.

294. Baudino TA. (2015) Targeted Cancer Therapy: The Next Generation of Cancer Treatment. Curr Drug Discov Technol, 12: 3-20.

295. Sawa M. (2008) Strategies for the design of selective protein kinase inhibitors. Mini Rev Med Chem, 8: 1291-1297.
296. Dar AC, Shokat KM. (2011) The evolution of protein kinase inhibitors from antagonists to agonists of cellular signaling. Annu Rev Biochem, 80: 769-795.
297. Monod J, Changeux JP, Jacob F. (1963) Allosteric proteins and cellular control systems. J Mol Biol, 6: 306-329.
298. Lamba V, Ghosh I. (2012) New directions in targeting protein kinases: focusing upon true allosteric and bivalent inhibitors. Curr Pharm Des, 18: 2936-2945.
299. Singh J, Petter RC, Baillie TA, Whitty A. (2011) The resurgence of covalent drugs. Nat Rev Drug Discov, 10: 307-317.
300. Zuccotto F, Ardini E, Casale E, Angiolini M. (2010) Through the "gatekeeper door": exploiting the active kinase conformation. J Med Chem, 53: 2681-2694.
301. Patterson H, Nibbs R, McInnes I, Siebert S. (2014) Protein kinase inhibitors in the treatment of inflammatory and autoimmune diseases. Clin Exp Immunol, 176: 1-10.
302. Levitzki A, Klein S. (2010) Signal transduction therapy of cancer. Mol Aspects Med, 31: 287-329.
303. Widakowich C, de Castro G, Jr., de Azambuja E, Dinh P, Awada A. (2007) Review: side effects of approved molecular targeted therapies in solid cancers. Oncologist, 12: 1443-1455.
304. Curry JL, Torres-Cabala CA, Kim KB, Tetzlaff MT, Duvic M, Tsai KY, Hong DS, Prieto VG. (2014) Dermatologic toxicities to targeted cancer therapy: shared clinical and histologic adverse skin reactions. Int J Dermatol, 53: 376-384.
305. Kantarjian HM, Fojo T, Mathisen M, Zwelling LA. (2013) Cancer drugs in the United States: Justum Pretium--the just price. J Clin Oncol, 31: 3600-3604.
306. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. (2013) Cancer drug resistance: an evolving paradigm. Nat Rev Cancer, 13: 714-726.
307. Hughes JP, Rees S, Kalindjian SB, Philpott KL. (2011) Principles of early drug discovery. Br J Pharmacol, 162: 1239-1249.
308. Boppana K, Dubey PK, Jagarlapudi SA, Vadivelan S, Rambabu G. (2009) Knowledge based identification of MAO-B selective inhibitors using

pharmacophore and structure based virtual screening models. Eur J Med Chem, 44: 3584-3590.

309. Michelini E, Cevenini L, Mezzanotte L, Coppa A, Roda A. (2010) Cell-based assays: fuelling drug discovery. Anal Bioanal Chem, 398: 227-238.

310. Deu E, Verdoes M, Bogyo M. (2012) New approaches for dissecting protease functions to improve probe development and drug discovery. Nat Struct Mol Biol, 19: 9-16.

311. Fox S, Farr-Jones S, Sopchak L, Boggs A, Nicely HW, Khoury R, Biros M. (2006) High-throughput screening: update on practices and success. J Biomol Screen, 11: 864-869.

312. Moore K, Rees S. (2001) Cell-based versus isolated target screening: how lucky do you feel? J Biomol Screen, 6: 69-74.

313. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev, 46: 3-26.

314. Yang H, Burke T, Dempsey J, Diaz B, Collins E, Toth J, Beckmann R, Ye X. (2005) Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase. FEBS Lett, 579: 3385-3391.

315. Keen N, Taylor S. (2004) Aurora-kinase inhibitors as anticancer agents. Nat Rev Cancer, 4: 927-936.

316. Mortlock AA, Keen NJ, Jung FH, Heron NM, Foote KM, Wilkinson RW, Green S. (2005) Progress in the development of selective inhibitors of aurora kinases. Curr Top Med Chem, 5: 807-821.

317. Cheetham GM, Charlton PA, Golec JM, Pollard JR. (2007) Structural basis for potent inhibition of the Aurora kinases and a T315I multi-drug resistant mutant form of Abl kinase by VX-680. Cancer Lett, 251: 323-329.

318. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, Miller KM. (2004) VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat Med, 10: 262-267.

319. Seymour JF, Kim DW, Rubin E, Haregewoin A, Clark J, Watson P, Hughes T, Dufva I, Jimenez JL, Mahon FX, Rousselot P, Cortes J, Martinelli G,

Papayannidis C, Nagler A, Giles FJ. (2014) A phase 2 study of MK-0457 in patients with BCR-ABL T315I mutant chronic myelogenous leukemia and philadelphia chromosome-positive acute lymphoblastic leukemia. Blood Cancer J, 4: e238.

320. Sells TB, Chau R, Ecsedy JA, Gershman RE, Hoar K, Huck J, Janowick DA, Kadambi VJ, LeRoy PJ, Stirling M, Stroud SG, Vos TJ, Weatherhead GS, Wysong DR, Zhang M, Balani SK, Bolen JB, Manfredi MG, Claiborne CF. (2015) MLN8054 and Alisertib (MLN8237): Discovery of Selective Oral Aurora A Inhibitors. ACS Med Chem Lett, 6: 630-634.

321. Manfredi MG, Ecsedy JA, Meetze KA, Balani SK, Burenkova O, Chen W, Galvin KM, Hoar KM, Huck JJ, LeRoy PJ, Ray ET, Sells TB, Stringer B, Stroud SG, Vos TJ, Weatherhead GS, Wysong DR, Zhang M, Bolen JB, Claiborne CF. (2007) Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. Proc Natl Acad Sci U S A, 104: 4106-4111.

322. Dees EC, Infante JR, Cohen RB, O'Neil BH, Jones S, von Mehren M, Danaee H, Lee Y, Ecsedy J, Manfredi M, Galvin K, Stringer B, Liu H, Eton O, Fingert H, Burris H. (2011) Phase 1 study of MLN8054, a selective inhibitor of Aurora A kinase in patients with advanced solid tumors. Cancer Chemother Pharmacol, 67: 945-954.

323. Barr PM, Li H, Spier C, Mahadevan D, LeBlanc M, Ul Haq M, Huber BD, Flowers CR, Wagner-Johnston ND, Horwitz SM, Fisher RI, Cheson BD, Smith SM, Kahl BS, Bartlett NL, Friedberg JW. (2015) Phase II Intergroup Trial of Alisertib in Relapsed and Refractory Peripheral T-Cell Lymphoma and Transformed Mycosis Fungoides: SWOG 1108. J Clin Oncol, 33: 2399-2404.

324. Dickson MA, Mahoney MR, Tap WD, D'Angelo SP, Keohan ML, Van Tine BA, Agulnik M, Horvath LE, Nair JS, Schwartz GK. (2016) Phase II study of MLN8237 (Alisertib) in advanced/metastatic sarcoma. Ann Oncol, 27: 1855-1860.

325. Melichar B, Adenis A, Lockhart AC, Bennouna J, Dees EC, Kayaleh O, Obermannova R, DeMichele A, Zatloukal P, Zhang B, Ullmann CD, Schusterbauer C. (2015) Safety and activity of alisertib, an investigational aurora kinase A inhibitor, in patients with breast cancer, small-cell lung cancer,

non-small-cell lung cancer, head and neck squamous-cell carcinoma, and gastro-oesophageal adenocarcinoma: a five-arm phase 2 study. Lancet Oncol, 16: 395-405.

326. Huck JJ, Zhang M, Mettetal J, Chakravarty A, Venkatakrishnan K, Zhou X, Kleinfield R, Hyer ML, Kannan K, Shinde V, Dorner A, Manfredi MG, Shyu WC, Ecsedy JA. (2014) Translational exposure-efficacy modeling to optimize the dose and schedule of taxanes combined with the investigational Aurora A kinase inhibitor MLN8237 (alisertib). Mol Cancer Ther, 13: 2170-2183.

327. Matulonis UA, Sharma S, Ghamande S, Gordon MS, Del Prete SA, Ray-Coquard I, Kutarska E, Liu H, Fingert H, Zhou X, Danaee H, Schilder RJ. (2012) Phase II study of MLN8237 (alisertib), an investigational Aurora A kinase inhibitor, in patients with platinum-resistant or -refractory epithelial ovarian, fallopian tube, or primary peritoneal carcinoma. Gynecol Oncol, 127: 63-69.

328. Goldberg SL, Fenaux P, Craig MD, Gyan E, Lister J, Kassis J, Pigneux A, Schiller GJ, Jung J, Jane Leonard E, Fingert H, Westervelt P. (2014) An exploratory phase 2 study of investigational Aurora A kinase inhibitor alisertib (MLN8237) in acute myelogenous leukemia and myelodysplastic syndromes. Leuk Res Rep, 3: 58-61.

329. (2016) First Multicenter, Randomized Phase 3 Study in Patients (Pts) With Relapsed/Refractory (R/R) Peripheral T-Cell Lymphoma (PTCL): Alisertib (MLN8237) Versus Investigator's Choice (LUMIERE trial; NCT01482962). Clin Adv Hematol Oncol, 14: 12-13.

330. Tayyar Y, Jubair L, Fallaha S, McMillan NAJ. (2017) Critical risk-benefit assessment of the novel anti-cancer aurora a kinase inhibitor alisertib (MLN8237): A comprehensive review of the clinical data. Crit Rev Oncol Hematol, 119: 59-65.

331. Liewer S, Huddleston A. (2018) Alisertib: a review of pharmacokinetics, efficacy and toxicity in patients with hematologic malignancies and solid tumors. Expert Opin Investig Drugs, 27: 105-112.

332. Yang J, Ikezoe T, Nishioka C, Tasaka T, Taniguchi A, Kuwayama Y, Komatsu N, Bandobashi K, Togitani K, Koeffler HP, Taguchi H, Yokoyama A. (2007)

AZD1152, a novel and selective aurora B kinase inhibitor, induces growth arrest, apoptosis, and sensitization for tubulin depolymerizing agent or topoisomerase II inhibitor in human acute leukemia cells in vitro and in vivo. Blood, 110: 2034-2040.

333. Grundy M, Seedhouse C, Shang S, Richardson J, Russell N, Pallis M. (2010) The FLT3 internal tandem duplication mutation is a secondary target of the aurora B kinase inhibitor AZD1152-HQPA in acute myelogenous leukemia cells. Mol Cancer Ther, 9: 661-672.

334. Lowenberg B, Muus P, Ossenkoppele G, Rousselot P, Cahn JY, Ifrah N, Martinelli G, Amadori S, Berman E, Sonneveld P, Jongen-Lavrencic M, Rigaudeau S, Stockman P, Goudie A, Faderl S, Jabbour E, Kantarjian H. (2011) Phase 1/2 study to assess the safety, efficacy, and pharmacokinetics of barasertib (AZD1152) in patients with advanced acute myeloid leukemia. Blood, 118: 6030-6036.

335. Kantarjian HM, Martinelli G, Jabbour EJ, Quintas-Cardama A, Ando K, Bay JO, Wei A, Gropper S, Papayannidis C, Owen K, Pike L, Schmitt N, Stockman PK, Giagounidis A. (2013) Stage I of a phase 2 study assessing the efficacy, safety, and tolerability of barasertib (AZD1152) versus low-dose cytosine arabinoside in elderly patients with acute myeloid leukemia. Cancer, 119: 2611-2619.

336. Collins GP, Eyre TA, Linton KM, Radford J, Vallance GD, Soilleux E, Hatton C. (2015) A phase II trial of AZD1152 in relapsed/refractory diffuse large B-cell lymphoma. Br J Haematol, 170: 886-890.

337. Tanaka R, Squires MS, Kimura S, Yokota A, Nagao R, Yamauchi T, Takeuchi M, Yao H, Reule M, Smyth T, Lyons JF, Thompson NT, Ashihara E, Ottmann OG, Maekawa T. (2010) Activity of the multitargeted kinase inhibitor, AT9283, in imatinib-resistant BCR-ABL-positive leukemic cells. Blood, 116: 2089-2095.

338. Vormoor B, Veal GJ, Griffin MJ, Boddy AV, Irving J, Minto L, Case M, Banerji U, Swales KE, Tall JR, Moore AS, Toguchi M, Acton G, Dyer K, Schwab C, Harrison CJ, Grainger JD, Lancaster D, Kearns P, Hargrave D, Vormoor J. (2017) A phase I/II trial of AT9283, a selective inhibitor of aurora kinase in children with relapsed or refractory acute leukemia: challenges to run early phase clinical trials for children with leukemia. Pediatr Blood Cancer, 64.

339. Lheureux S, Tinker AV, Clarke BA, Ghatage P, Welch S, Weberpals JI, Dhani NC, Butler MO, Tonkin K, Tan Q, Tan DSP, Brooks K, Ramsahai J, Wang L, Pham NA, Shaw PA, Tsao MS, Garg S, Stockley TL, Oza AM. (2018) A clinical and molecular Phase II trial of oral ENMD-2076 in ovarian clear cell carcinoma (OCCC): A study of the Princess Margaret Phase II Consortium. Clin Cancer Res.

340. Diamond JR, Eckhardt SG, Pitts TM, van Bokhoven A, Aisner D, Gustafson DL, Capasso A, Sams S, Kabos P, Zolman K, Colvin T, Elias AD, Storniolo AM, Schneider BP, Gao D, Tentler JJ, Borges VF, Miller KD. (2018) A phase II clinical trial of the Aurora and angiogenic kinase inhibitor ENMD-2076 for previously treated, advanced, or metastatic triple-negative breast cancer. Breast Cancer Res, 20: 82.

341. Meulenbeld HJ, Bleuse JP, Vinci EM, Raymond E, Vitali G, Santoro A, Dogliotti L, Berardi R, Cappuzzo F, Tagawa ST, Sternberg CN, Jannuzzo MG, Mariani M, Petroccione A, de Wit R. (2013) Randomized phase II study of danusertib in patients with metastatic castration-resistant prostate cancer after docetaxel failure. BJU Int, 111: 44-52.

342. Schoffski P, Besse B, Gauler T, de Jonge MJ, Scambia G, Santoro A, Davite C, Jannuzzo MG, Petroccione A, Delord JP. (2015) Efficacy and safety of biweekly i.v. administrations of the Aurora kinase inhibitor danusertib hydrochloride in independent cohorts of patients with advanced or metastatic breast, ovarian, colorectal, pancreatic, small-cell and non-small-cell lung cancer: a multi-tumour, multi-institutional phase II study. Ann Oncol, 26: 598-607.

343. Laird AD, Vajkoczy P, Shawver LK, Thurnher A, Liang C, Mohammadi M, Schlessinger J, Ullrich A, Hubbard SR, Blake RA, Fong TA, Strawn LM, Sun L, Tang C, Hawtin R, Tang F, Shenoy N, Hirth KP, McMahon G, Cherrington. (2000) SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. Cancer Res, 60: 4152-4160.

344. Kanai F, Yoshida H, Tateishi R, Sato S, Kawabe T, Obi S, Kondo Y, Taniguchi M, Tagawa K, Ikeda M, Morizane C, Okusaka T, Arioka H, Shiina S, Omata M. (2011) A phase I/II trial of the oral antiangiogenic agent TSU-68 in patients with advanced hepatocellular carcinoma. Cancer Chemother Pharmacol, 67: 315-324.

345. Kudo M, Cheng AL, Park JW, Park JH, Liang PC, Hidaka H, Izumi N, Heo J, Lee YJ, Sheen IS, Chiu CF, Arioka H, Morita S, Arai Y. (2018) Orantinib versus placebo combined with transcatheter arterial chemoembolisation in patients with unresectable hepatocellular carcinoma (ORIENTAL): a randomised, double-blind, placebo-controlled, multicentre, phase 3 study. Lancet Gastroenterol Hepatol, 3: 37-46.

346. Shimomura T, Hasako S, Nakatsuru Y, Mita T, Ichikawa K, Kodera T, Sakai T, Nambu T, Miyamoto M, Takahashi I, Miki S, Kawanishi N, Ohkubo M, Kotani H, Iwasawa Y. (2010) MK-5108, a highly selective Aurora-A kinase inhibitor, shows antitumor activity alone and in combination with docetaxel. Mol Cancer Ther, 9: 157-166.

347. Amin M, Minton SE, LoRusso PM, Krishnamurthi SS, Pickett CA, Lunceford J, Hille D, Mauro D, Stein MN, Wang-Gillam A, Trull L, Lockhart AC. (2016) A phase I study of MK-5108, an oral aurora a kinase inhibitor, administered both as monotherapy and in combination with docetaxel, in patients with advanced or refractory solid tumors. Invest New Drugs, 34: 84-95.

348. Kollareddy M, Zheleva D, Dzubak P, Brahmkshatriya PS, Lepsik M, Hajduch M. (2012) Aurora kinase inhibitors: progress towards the clinic. Invest New Drugs, 30: 2411-2432.

349. Cicenas J. (2016) The Aurora kinase inhibitors in cancer research and therapy. J Cancer Res Clin Oncol, 142: 1995-2012.

350. Bavetsias V, Linardopoulos S. (2015) Aurora Kinase Inhibitors: Current Status and Outlook. Front Oncol, 5: 278.

351. Cicenas J, Cicenas E. (2016) Multi-kinase inhibitors, AURKs and cancer. Med Oncol, 33: 43.

352. Liu Y, Hawkins OE, Su Y, Vilgelm AE, Sobolik T, Thu YM, Kantrow S, Splittgerber RC, Short S, Amiri KI, Ecsedy JA, Sosman JA, Kelley MC, Richmond A. (2013) Targeting aurora kinases limits tumour growth through DNA damage-mediated senescence and blockade of NF-kappaB impairs this drug-induced senescence. EMBO Mol Med, 5: 149-166.

353. Boss DS, Witteveen PO, van der Sar J, Lolkema MP, Voest EE, Stockman PK, Ataman O, Wilson D, Das S, Schellens JH. (2011) Clinical evaluation of

AZD1152, an i.v. inhibitor of Aurora B kinase, in patients with solid malignant tumors. Ann Oncol, 22: 431-437.

354. Lake EW, Muretta JM, Thompson AR, Rasmussen DM, Majumdar A, Faber EB, Ruff EF, Thomas DD, Levinson NM. (2018) Quantitative conformational profiling of kinase inhibitors reveals origins of selectivity for Aurora kinase activation states. Proc Natl Acad Sci U S A, 115: E11894-E11903.

355. Porcelli L, Guida G, Quatrale AE, Cocco T, Sidella L, Maida I, Iacobazzi RM, Ferretta A, Stolfa DA, Strippoli S, Guida S, Tommasi S, Guida M, Azzariti A. (2015) Aurora kinase B inhibition reduces the proliferation of metastatic melanoma cells and enhances the response to chemotherapy. J Transl Med, 13: 26.

356. Twombly R. (2005) Failing survival advantage in crucial trial, future of Iressa is in jeopardy. J Natl Cancer Inst, 97: 249-250.

357. Pao W, Miller VA. (2005) Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. J Clin Oncol, 23: 2556-2568.

358. Kobayashi Y, Mitsudomi T. (2016) Not all epidermal growth factor receptor mutations in lung cancer are created equal: Perspectives for individualized treatment strategy. Cancer Sci, 107: 1179-1186.

359. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, Majem M, Lopez-Vivanco G, Isla D, Provencio M, Insa A, Massuti B, Gonzalez-Larriba JL, Paz-Ares L, Bover I, Garcia-Campelo R, Moreno MA, Catot S, Rolfo C, Reguart N, Palmero R, Sanchez JM, Bastus R, Mayo C, Bertran-Alamillo J, Molina MA, Sanchez JJ, Taron M, Spanish Lung Cancer G. (2009) Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med, 361: 958-967.

360. Joshi M, Rizvi SM, Belani CP. (2015) Afatinib for the treatment of metastatic non-small cell lung cancer. Cancer Manag Res, 7: 75-82.

361. Sequist LV, Besse B, Lynch TJ, Miller VA, Wong KK, Gitlitz B, Eaton K, Zacharchuk C, Freyman A, Powell C, Ananthakrishnan R, Quinn S, Soria JC. (2010) Neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor: results of a phase II trial in patients with advanced non-small-cell lung cancer. J Clin Oncol, 28: 3076-3083.

362. Gandhi L, Besse B, Mazieres J, Waqar S, Cortot A, Barlesi F, Quoix E, Otterson G, Ettinger D, Horn L, Moro-Sibilot D, Socinski M, Gold K, Gray J, Oton A, Heist RS, Costa D, McCulloch L, Bebchuk J, Bryce R, Kris M. (2017) MA04.02 Neratinib ± Temsirolimus in HER2-Mutant Lung Cancers: An International, Randomized Phase II Study. Journal of Thoracic Oncology, 12: S358-S359.

363. Janne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, Ahn MJ, Kim SW, Su WC, Horn L, Haggstrom D, Felip E, Kim JH, Frewer P, Cantarini M, Brown KH, Dickinson PA, Ghiorghiu S, Ranson M. (2015) AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med, 372: 1689-1699.

364. Park K, Lee J-S, Lee KH, Kim J-H, Cho BC, Min YJ, Cho JY, Han J-Y, Kim B-S, Kim J-S, Lee DH, Kang JH, Cho EK, Kim H-G, Lee KH, Kim HK, Jang I-J, Kim H-Y, Son J, Kim D-W. (2016) BI 1482694 (HM61713), an EGFR mutant-specific inhibitor, in T790M+ NSCLC: Efficacy and safety at the RP2D. Journal of Clinical Oncology, 34: 9055-9055.

365. Lelais G, Epple R, Marsilje TH, Long YO, McNeill M, Chen B, Lu W, Anumolu J, Badiger S, Bursulaya B, DiDonato M, Fong R, Juarez J, Li J, Manuia M, Mason DE, Gordon P, Groessl T, Johnson K, Jia Y, Kasibhatla S, Li C, Isbell J, Spraggon G, Bender S, Michellys PY. (2016) Discovery of (R,E)-N-(7-Chloro-1-(1-[4-(dimethylamino)but-2-enoyl]azepan-3-yl)-1H-benzo[d]imid azol-2-yl)-2-methylisonicotinamide (EGF816), a Novel, Potent, and WT Sparing Covalent Inhibitor of Oncogenic (L858R, ex19del) and Resistant (T790M) EGFR Mutants for the Treatment of EGFR Mutant Non-Small-Cell Lung Cancers. J Med Chem, 59: 6671-6689.

366. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, Lai Z, Markovets A, Vivancos A, Kuang Y, Ercan D, Matthews SE, Cantarini M, Barrett JC, Janne PA, Oxnard GR. (2015) Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. Nat Med, 21: 560-562.

367. Minari R, Bordi P, Tiseo M. (2016) Third-generation epidermal growth factor receptor-tyrosine kinase inhibitors in T790M-positive non-small cell lung

cancer: review on emerged mechanisms of resistance. Transl Lung Cancer Res, 5: 695-708.

368. Passaro A, Guerini-Rocco E, Pochesci A, Vacirca D, Spitaleri G, Catania CM, Rappa A, Barberis M, de Marinis F. (2017) Targeting EGFR T790M mutation in NSCLC: From biology to evaluation and treatment. Pharmacol Res, 117: 406-415.

369. Niederst MJ, Hu H, Mulvey HE, Lockerman EL, Garcia AR, Piotrowska Z, Sequist LV, Engelman JA. (2015) The Allelic Context of the C797S Mutation Acquired upon Treatment with Third-Generation EGFR Inhibitors Impacts Sensitivity to Subsequent Treatment Strategies. Clin Cancer Res, 21: 3924-3933.

370. Szokol B, Gyulavari P, Kurko I, Baska F, Szantai-Kis C, Greff Z, Orfi Z, Petak I, Penzes K, Torka R, Ullrich A, Orfi L, Vantus T, Keri G. (2014) Discovery and Biological Evaluation of Novel Dual EGFR/c-Met Inhibitors. ACS Med Chem Lett, 5: 298-303.

371. Kazandjian D, Blumenthal GM, Chen HY, He K, Patel M, Justice R, Keegan P, Pazdur R. (2014) FDA approval summary: crizotinib for the treatment of metastatic non-small cell lung cancer with anaplastic lymphoma kinase rearrangements. Oncologist, 19: e5-11.

372. Roskoski R, Jr. (2013) The preclinical profile of crizotinib for the treatment of non-small-cell lung cancer and other neoplastic disorders. Expert Opin Drug Discov, 8: 1165-1179.

373. Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, Jessop NA, Wain JC, Yeo AT, Benes C, Drew L, Saeh JC, Crosby K, Sequist LV, Iafrate AJ, Engelman JA. (2012) Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. Sci Transl Med, 4: 120ra117.

374. Zeng JY, Sharma S, Zhou YQ, Yao HP, Hu X, Zhang R, Wang MH. (2014) Synergistic activities of MET/RON inhibitor BMS-777607 and mTOR inhibitor AZD8055 to polyploid cells derived from pancreatic cancer and cancer stem cells. Mol Cancer Ther, 13: 37-48.

375. Schroeder GM, An Y, Cai ZW, Chen XT, Clark C, Cornelius LA, Dai J, Gullo-Brown J, Gupta A, Henley B, Hunt JT, Jeyaseelan R, Kamath A, Kim K, Lippy J, Lombardo LJ, Manne V, Oppenheimer S, Sack JS, Schmidt RJ, Shen G,

Stefanski K, Tokarski JS, Trainor GL, Wautlet BS, Wei D, Williams DK, Zhang Y, Zhang Y, Fargnoli J, Borzilleri RM. (2009) Discovery of N-(4-(2-amino-3-chloropyridin-4-yloxy)-3-fluorophenyl)-4-ethoxy-1-(4-fluorophenyl )-2-oxo-1,2-dihydropyridine-3-carboxamide (BMS-777607), a selective and orally efficacious inhibitor of the Met kinase superfamily. J Med Chem, 52: 1251-1254.

376. Galmarini D, Galmarini CM, Galmarini FC. (2012) Cancer chemotherapy: a critical analysis of its 60 years of history. Crit Rev Oncol Hematol, 84: 181-199.
377. Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallares G, Hocker M, Treiber DK, Zarrinkar PP. (2011) Comprehensive analysis of kinase inhibitor selectivity. Nat Biotechnol, 29: 1046-1051.
378. Lackner MR, Wilson TR, Settleman J. (2012) Mechanisms of acquired resistance to targeted cancer therapies. Future Oncol, 8: 999-1014.
379. Park SR, Davis M, Doroshow JH, Kummar S. (2013) Safety and feasibility of targeted agent combinations in solid tumours. Nat Rev Clin Oncol, 10: 154-168.
380. Kummar S, Chen HX, Wright J, Holbeck S, Millin MD, Tomaszewski J, Zweibel J, Collins J, Doroshow JH. (2010) Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements. Nat Rev Drug Discov, 9: 843-856.
381. Morrison C. (2015) 'Financial toxicity' looms as cancer combinations proliferate. Nat Biotechnol, 33: 783-784.
382. Sun J, Wei Q, Zhou Y, Wang J, Liu Q, Xu H. (2017) A systematic analysis of FDA-approved anticancer drugs. BMC Syst Biol, 11: 87.
383. Blackwell KL, Burstein HJ, Storniolo AM, Rugo HS, Sledge G, Aktan G, Ellis C, Florance A, Vukelja S, Bischoff J, Baselga J, O'Shaughnessy J. (2012) Overall survival benefit with lapatinib in combination with trastuzumab for patients with human epidermal growth factor receptor 2-positive metastatic breast cancer: final results from the EGF104900 Study. J Clin Oncol, 30: 2585-2592.
384. Johnson DB, Flaherty KT, Weber JS, Infante JR, Kim KB, Kefford RF, Hamid O, Schuchter L, Cebon J, Sharfman WH, McWilliams RR, Sznol M, Lawrence DP, Gibney GT, Burris HA, 3rd, Falchook GS, Algazi A, Lewis K, Long GV,

Patel K, Ibrahim N, Sun P, Little S, Cunningham E, Sosman JA, Daud A, Gonzalez R. (2014) Combined BRAF (Dabrafenib) and MEK inhibition (Trametinib) in patients with BRAFV600-mutant melanoma experiencing progression with single-agent BRAF inhibitor. J Clin Oncol, 32: 3697-3704.

385. Yap TA, Omlin A, de Bono JS. (2013) Development of therapeutic combinations targeting major cancer signaling pathways. J Clin Oncol, 31: 1592-1605.

386. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, Lichinitser M, Dummer R, Grange F, Mortier L, Chiarion-Sileni V, Drucis K, Krajsova I, Hauschild A, Lorigan P, Wolter P, Long GV, Flaherty K, Nathan P, Ribas A, Martin AM, Sun P, Crist W, Legos J, Rubin SD, Little SM, Schadendorf D. (2015) Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med, 372: 30-39.

387. Ciriello G, Cerami E, Sander C, Schultz N. (2012) Mutual exclusivity analysis identifies oncogenic network modules. Genome Res, 22: 398-406.

388. Yeang CH, McCormick F, Levine A. (2008) Combinatorial patterns of somatic gene mutations in cancer. FASEB J, 22: 2605-2622.

389. Girotti MR, Pedersen M, Sanchez-Laorden B, Viros A, Turajlic S, Niculescu-Duvaz D, Zambon A, Sinclair J, Hayes A, Gore M, Lorigan P, Springer C, Larkin J, Jorgensen C, Marais R. (2013) Inhibiting EGF receptor or SRC family kinase signaling overcomes BRAF inhibitor resistance in melanoma. Cancer Discov, 3: 158-167.

390. Van Emburgh BO, Arena S, Siravegna G, Lazzari L, Crisafulli G, Corti G, Mussolin B, Baldi F, Buscarino M, Bartolini A, Valtorta E, Vidal J, Bellosillo B, Germano G, Pietrantonio F, Ponzetti A, Albanell J, Siena S, Sartore-Bianchi A, Di Nicolantonio F, Montagut C, Bardelli A. (2016) Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. Nat Commun, 7: 13665.

391. Bardelli A, Corso S, Bertotti A, Hobor S, Valtorta E, Siravegna G, Sartore-Bianchi A, Scala E, Cassingena A, Zecchin D, Apicella M, Migliardi G, Galimi F, Lauricella C, Zanon C, Perera T, Veronese S, Corti G, Amatu A, Gambacorta M, Diaz LA, Jr., Sausen M, Velculescu VE, Comoglio P, Trusolino L, Di

Nicolantonio F, Giordano S, Siena S. (2013) Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer Discov, 3: 658-673.

392. Niederst MJ, Engelman JA. (2013) Bypass mechanisms of resistance to receptor tyrosine kinase inhibition in lung cancer. Sci Signal, 6: re6.

393. Johannessen CM, Johnson LA, Piccioni F, Townes A, Frederick DT, Donahue MK, Narayan R, Flaherty KT, Wargo JA, Root DE, Garraway LA. (2013) A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. Nature, 504: 138-142.

394. Ashworth A, Lord CJ. (2018) Synthetic lethal therapies for cancer: what's next after PARP inhibitors? Nat Rev Clin Oncol, 15: 564-576.

395. Gong X, Du J, Parsons SH, Merzoug FF, Webster Y, Iversen PW, Chio LC, Van Horn RD, Lin X, Blosser W, Han B, Jin S, Yao S, Bian H, Ficklin C, Fan L, Kapoor A, Antonysamy S, Mc Nulty AM, Froning K, Manglicmot D, Pustilnik A, Weichert K, Wasserman SR, Dowless M, Marugan C, Baquero C, Lallena MJ, Eastman SW, Hui YH, Dieter MZ, Doman T, Chu S, Qian HR, Ye XS, Barda DA, Plowman GD, Reinhard C, Campbell RM, Henry JR, Buchanan SG. (2019) Aurora A Kinase Inhibition Is Synthetic Lethal with Loss of the RB1 Tumor Suppressor Gene. Cancer Discov, 9: 248-263.

396. Ashton JC. (2015) Drug combination studies and their synergy quantification using the Chou-Talalay method--letter. Cancer Res, 75: 2400.

397. Burtness B, Bauman JE, Galloway T. (2013) Novel targets in HPV-negative head and neck cancer: overcoming resistance to EGFR inhibition. Lancet Oncol, 14: e302-309.

398. Petersen W, Liu J, Yuan L, Zhang H, Schneiderjan M, Cho YJ, MacDonald TJ. (2014) Dasatinib suppression of medulloblastoma survival and migration is markedly enhanced by combining treatment with the aurora kinase inhibitor AT9283. Cancer Lett, 354: 68-76.

399. Ratushny V, Pathak HB, Beeharry N, Tikhmyanova N, Xiao F, Li T, Litwin S, Connolly DC, Yen TJ, Weiner LM, Godwin AK, Golemis EA. (2012) Dual inhibition of SRC and Aurora kinases induces postmitotic attachment defects and cell death. Oncogene, 31: 1217-1227.

400. Brewer Savannah KJ, Demicco EG, Lusby K, Ghadimi MP, Belousov R, Young E, Zhang Y, Huang KL, Lazar AJ, Hunt KK, Pollock RE, Creighton CJ, Anderson ML, Lev D. (2012) Dual targeting of mTOR and aurora-A kinase for the treatment of uterine Leiomyosarcoma. Clin Cancer Res, 18: 4633-4645.

401. Paller CJ, Wissing MD, Mendonca J, Sharma A, Kim E, Kim HS, Kortenhorst MS, Gerber S, Rosen M, Shaikh F, Zahurak ML, Rudek MA, Hammers H, Rudin CM, Carducci MA, Kachhap SK. (2014) Combining the pan-aurora kinase inhibitor AMG 900 with histone deacetylase inhibitors enhances antitumor activity in prostate cancer. Cancer Med, 3: 1322-1335.

402. Muscal JA, Scorsone KA, Zhang L, Ecsedy JA, Berg SL. (2013) Additive effects of vorinostat and MLN8237 in pediatric leukemia, medulloblastoma, and neuroblastoma cell lines. Invest New Drugs, 31: 39-45.

403. Fei F, Lim M, Schmidhuber S, Moll J, Groffen J, Heisterkamp N. (2012) Treatment of human pre-B acute lymphoblastic leukemia with the Aurora kinase inhibitor PHA-739358 (Danusertib). Mol Cancer, 11: 42.

404. Chou TC. (2008) Preclinical versus clinical drug combination studies. Leuk Lymphoma, 49: 2059-2080.

405. Warner SL, Bashyam S, Vankayalapati H, Bearss DJ, Han H, Mahadevan D, Von Hoff DD, Hurley LH. (2006) Identification of a lead small-molecule inhibitor of the Aurora kinases using a structure-assisted, fragment-based approach. Mol Cancer Ther, 5: 1764-1773.

406. Geuns-Meyer S, Cee VJ, Deak HL, Du B, Hodous BL, Nguyen HN, Olivieri PR, Schenkel LB, Vaida KR, Andrews P, Bak A, Be X, Beltran PJ, Bush TL, Chaves MK, Chung G, Dai Y, Eden P, Hanestad K, Huang L, Lin MH, Tang J, Ziegler B, Radinsky R, Kendall R, Patel VF, Payton M. (2015) Discovery of N-(4-(3-(2-aminopyrimidin-4-yl)pyridin-2-yloxy)phenyl)-4-(4-methylthiophen-2-yl)p hthalazin-1-amine (AMG 900), a highly selective, orally bioavailable inhibitor of aurora kinases with activity against multidrug-resistant cancer cell lines. J Med Chem, 58: 5189-5207.

407. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S. (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet, 20: 189-193.

408. Azzariti A, Bocci G, Porcelli L, Fioravanti A, Sini P, Simone GM, Quatrale AE, Chiarappa P, Mangia A, Sebastian S, Del Bufalo D, Del Tacca M, Paradiso A. (2011) Aurora B kinase inhibitor AZD1152: determinants of action and ability to enhance chemotherapeutics effectiveness in pancreatic and colon cancer. Br J Cancer, 104: 769-780.

409. Chiang CC, Lin YH, Lin SF, Lai CL, Liu C, Wei WY, Yang SC, Wang RW, Teng LW, Chuang SH, Chang JM, Yuan TT, Lee YS, Chen P, Chi WK, Yang JY, Huang HJ, Liao CB, Huang JJ. (2010) Discovery of pyrrole-indoline-2-ones as Aurora kinase inhibitors with a different inhibition profile. J Med Chem, 53: 5929-5941.

410. Arbitrario JP, Belmont BJ, Evanchik MJ, Flanagan WM, Fucini RV, Hansen SK, Harris SO, Hashash A, Hoch U, Hogan JN, Howlett AR, Jacobs JW, Lam JW, Ritchie SC, Romanowski MJ, Silverman JA, Stockett DE, Teague JN, Zimmerman KM, Taverna P. (2010) SNS-314, a pan-Aurora kinase inhibitor, shows potent anti-tumor activity and dosing flexibility in vivo. Cancer Chemother Pharmacol, 65: 707-717.

411. Shimizu M, Shirakami Y, Sakai H, Yasuda Y, Kubota M, Adachi S, Tsurumi H, Hara Y, Moriwaki H. (2010) (-)-Epigallocatechin gallate inhibits growth and activation of the VEGF/VEGFR axis in human colorectal cancer cells. Chem Biol Interact, 185: 247-252.

412. Buck E, Eyzaguirre A, Brown E, Petti F, McCormack S, Haley JD, Iwata KK, Gibson NW, Griffin G. (2006) Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. Mol Cancer Ther, 5: 2676-2684.

413. Guo F, Chen H, Chang J, Zhang L. (2016) Mutation R273H confers p53 a stimulating effect on the IGF-1R-AKT pathway via miR-30a suppression in breast cancer. Biomed Pharmacother, 78: 335-341.

414. Janzen WP, Bernasconi P. (2009) High throughput screening. Methods and protocols, second edition. Preface. Methods Mol Biol, 565: v-vii.

415. Fu J, Bian M, Liu J, Jiang Q, Zhang C. (2009) A single amino acid change converts Aurora-A into Aurora-B-like kinase in terms of partner specificity and cellular function. Proc Natl Acad Sci U S A, 106: 6939-6944.

416. de Groot CO, Hsia JE, Anzola JV, Motamedi A, Yoon M, Wong YL, Jenkins D, Lee HJ, Martinez MB, Davis RL, Gahman TC, Desai A, Shiau AK. (2015) A Cell Biologist's Field Guide to Aurora Kinase Inhibitors. Front Oncol, 5: 285.
417. Arai R, Tsuda M, Watanabe T, Ose T, Obuse C, Maenaka K, Minami A, Ohba Y. (2012) Simultaneous inhibition of Src and Aurora kinases by SU6656 induces therapeutic synergy in human synovial sarcoma growth, invasion and angiogenesis in vivo. Eur J Cancer, 48: 2417-2430.
418. Fletcher GC, Brokx RD, Denny TA, Hembrough TA, Plum SM, Fogler WE, Sidor CF, Bray MR. (2011) ENMD-2076 is an orally active kinase inhibitor with antiangiogenic and antiproliferative mechanisms of action. Mol Cancer Ther, 10: 126-137.
419. Pollard JR, Mortimore M. (2009) Discovery and development of aurora kinase inhibitors as anticancer agents. J Med Chem, 52: 2629-2651.
420. Chinn DC, Holland WS, Mack PC. (2014) Anticancer activity of the Aurora A kinase inhibitor MK-5108 in non-small-cell lung cancer (NSCLC) in vitro as monotherapy and in combination with chemotherapies. J Cancer Res Clin Oncol, 140: 1137-1149.
421. Nair JS, Ho AL, Schwartz GK. (2012) The induction of polyploidy or apoptosis by the Aurora A kinase inhibitor MK8745 is p53-dependent. Cell Cycle, 11: 807-817.
422. Greco WR, Faessel H, Levasseur L. (1996) The search for cytotoxic synergy between anticancer agents: a case of Dorothy and the ruby slippers? J Natl Cancer Inst, 88: 699-700.
423. Greco WR, Bravo G, Parsons JC. (1995) The search for synergy: a critical review from a response surface perspective. Pharmacol Rev, 47: 331-385.
424. Chou TC. (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev, 58: 621-681.
425. Chou TC, Talalay P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul, 22: 27-55.

426. Diamond JR, Eckhardt SG, Tan AC, Newton TP, Selby HM, Brunkow KL, Kachaeva MI, Varella-Garcia M, Pitts TM, Bray MR, Fletcher GC, Tentler JJ. (2013) Predictive biomarkers of sensitivity to the aurora and angiogenic kinase inhibitor ENMD-2076 in preclinical breast cancer models. Clin Cancer Res, 19: 291-303.

427. Bychkov ML, Gasparian ME, Dolgikh DA, Kirpichnikov MP. (2014) Combination of TRAIL with bortezomib shifted apoptotic signaling from DR4 to DR5 death receptor by selective internalization and degradation of DR4. PLoS One, 9: e109756.

428. Clemenson C, Chargari C, Liu W, Mondini M, Ferte C, Burbridge MF, Cattan V, Jacquet-Bescond A, Deutsch E. (2017) The MET/AXL/FGFR Inhibitor S49076 Impairs Aurora B Activity and Improves the Antitumor Efficacy of Radiotherapy. Mol Cancer Ther, 16: 2107-2119.

## 10. List of the candidate's publications

**Publications related to the Thesis:**

Gyulavári P, Szokol B, Szabadkai I, Brauswetter D, Bánhegyi P, Varga A, Markó P, Boros S, Illyés E, Szántai-Kis Cs, Krekó M, Czudor Zs, Őrfi L (**2018**) Discovery and optimization of novel benzothiophene-3-carboxamides as highly potent inhibitors of Aurora kinases A and B. *Bioorganic & Medicinal Chemistry Letters* 28(19), 3265-3270. DOI: 10.1016/j.bmcl.2018.05.064.

Gyulavári P, Szokol B, Kurkó I, Baska F, Szántai-Kis Cs, Greff Z, Őrfi Z, Peták I, Pénzes K, Torka R, Ullrich A, Őrfi L, Vántus T and Kéri Gy (**2014**) Discovery and biological evaluation of novel dual EGFR/c-Met inhibitors. *ACS Medicinal Chemistry Letters* 5(4), 298-303.
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Szokol B, Gyulavári P, Baska F, Kurkó I, Greff Z, Szántai-Kis Cs, Őrfi Z, Peták I, Ullrich A, Vántus T, Kéri Gy and Őrfi L (**2013**) EGFR/c-Met kettősgátlók fejlesztése és biokémiai vizsgálata. *Acta Pharmaceutica Hungarica* 83: 4 pp. 121-133., 13 p.

Gyulavári P, Szántai-Kis Cs, Őrfi L, Pató J, Wáczek F, Szabadkai I, Breza N, Kéri Gy, Vántus T (**2010**) Biochemical characterization of novel EGFR and c-Met single and dual inhibitors. FEBS Journal 277: Suppl. 1 pp. 121-121., 1 p.

**Further publications:**

Szabadkai I, Torka R, Garamvölgyi R, Baska F, Gyulavári P, Boros S, Illyés E, Choidas A, Ullrich A and Őrfi L (**2018**) Discovery of N-[4-(Quinolin-4-yloxy) phenyl]-benzenesulfonamides as Novel AXL Kinase Inhibitors. *Journal of Medicinal Chemistry* 61(14), 6277-6292.
DOI: 10.1021/acs.jmedchem.8b00672.

Murányi J, Varga A, Gurbi B, Gyulavári P, Mező G, Vántus T (**2017**) In Vitro Imaging and Quantification of the Drug Targeting Efficiency of Fluorescently Labeled GnRH Analogues. *Journal of Visualized Experiments* 121 Paper: e55529.
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Murányi J, Gyulavári P, Varga A, Bökönyi Gy, Tanai H, Vántus T, Pap D, Ludányi K, Mező G and Kéri Gy (**2016**) Synthesis, characterization and systematic comparison of FITC-labelled GNRH-I, -II and -III analogues on various tumour cells. *Journal of Peptide Science* 22(8), 552-60.
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Varga A, Gyulavári P, Greff Z, Futosi K, Németh T, Simon-Szabó L, Kerekes K, Szántai-Kis Cs, Brauswetter D, Kokas M, Borbély G, Erdei A, Mócsai A, Kéri Gy, Vántus T (**2015**) Targeting vascular endothelial growth factor receptor 2 and protein kinase D1 related pathways by a multiple kinase inhibitor in angiogenesis and inflammation related processes *in vitro*. *PLoS One* 10(4), e0124234.
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