Discovery of BUB1 kinase inhibitors for the treatment of cancer

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General introduction

The spindle assembly checkpoint (SAC)

Eukaryotic cell division is divided into four phases: gap 1 (G_1), synthesis (S), gap 2 (G_2) and mitosis (M) (**Figure 1.1**). In the G_1 phase, cells are metabolically active and grow. In order for a cell to divide, all genetic information needs to be copied and this process occurs during the S phase. In subsequent G_2 phase, cells prepare for mitosis in which duplicated chromosomes are equally divided between the two daughter cells after which the cell cycle starts over. Mitosis itself is subdivided into five distinct phases: prophase, prometaphase, metaphase, anaphase and telophase (**Figure 1.1**). During prophase, chromosomes are highly condensed, centrosomes separate and nuclear envelope breakdown occurs.[1] During prometaphase, the spindle poles are connected to kinetochores, which are located at the centromeres of sister chromatids, and chromosomes align at the spindle equator, which is referred to as the metaphase.[1] Sister chromatids are separated and pulled towards opposite spindle poles during anaphase and in the final stage, the telophase, new nuclear envelopes are formed and DNA decondenses.[1]

The spindle assembly checkpoint (SAC) is one of the cell cycle checkpoints and is active during the prometaphase of mitosis.[2] During this mitotic phase, microtubules must form stable connections between the kinetochores of sister chromatids and spindle poles in a bi-oriented fashion (**Figure 1.2**). Proper kinetochore-microtubule connections are crucial for genomic integrity since mitotic progression with erroneous connections may lead to aneuploid cells which, in turn, might contribute to tumorigenesis.[3] The SAC is responsible for monitoring unattached kinetochores and prevents mitotic progression to the anaphase until fully satisfied (**Figure 1.2**). The anaphase is initiated by the anaphase-promoting complex/cyclosome (APC/C) which requires co-factor CDC20 (cell-division-cycle 20 homologue) to be active.[4] Once activated, APC/CDC20 targets Cyclin B and Securin for proteasomal degradation.[5] Cyclin B degradation inactivates CDK1 (cyclin-dependent kinase 1), which promotes mitotic exit, and destruction of Securin activates Separase, which in turn is required for cleaving Cohesin that holds sister chromatids together. The mitotic checkpoint complex (MCC), consisting of BUBR1 (budding uninhibited by benzimidazole-related 1), BUB3 (budding uninhibited by benzimidazole 3), MAD2 (mitotic arrest deficient 2) and CDC20, inhibits APC/C activation and is therefore crucial for inducing a mitotic arrest.[6] During this arrest, incorrect kinetochore-microtubule attachments can be corrected and unattached kinetochores can be attached to the mitotic spindle. SAC activation by unattached kinetochores results in the recruitment of SAC proteins and kinetochores might act as catalytic platforms to accelerate the production of the MCC.[2] Due to the importance of the MCC to arrest mitosis, the proteins of this complex belong to the core SAC proteins in addition to MAD1, which forms a stable complex with MAD2, as well as several kinases that are required to amplify SAC signaling and rate of MCC formation, including Aurora B, MPS1 (monopolar spindle 1) and BUB1 (budding uninhibited by benzimidazole 1).[2] Other important mitotic proteins that regulate SAC activity include proteins of the RZZ (ROD-ZW10-ZWILCH) complex as well as kinases CDK1-cyclin-B and PLK1 (polo-like kinase 1), among others.[2]

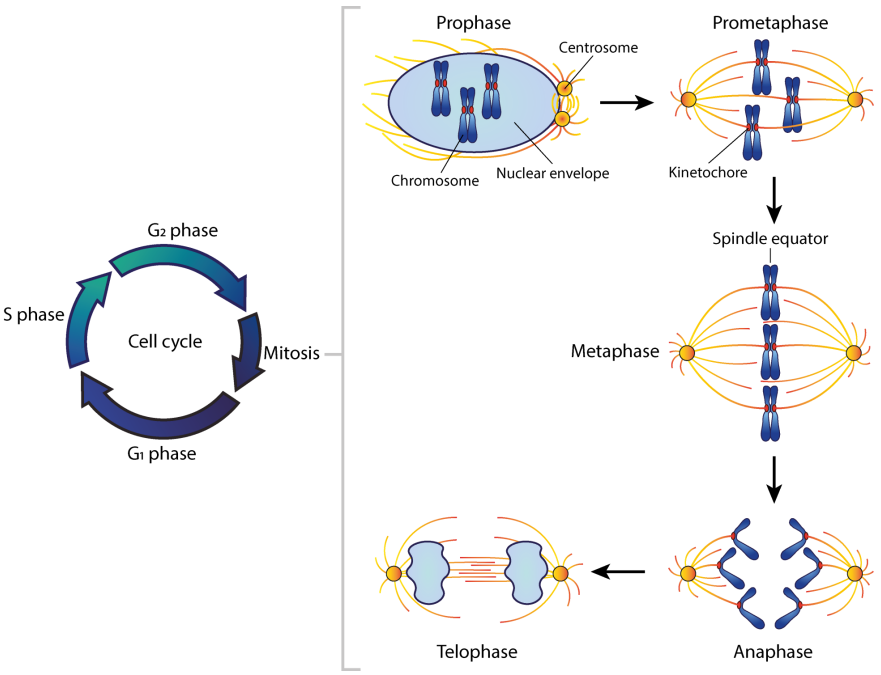


Figure 1.1 | Phases of the eukaryotic cell cycle (left) and schematic representation of mitotic phases (right).

Targeting the SAC has been suggested as potential strategy to kill cancer cells, since many cancer cells suffer from a weakened checkpoint.[3,7] Interference with these diminished checkpoints further disrupts SAC signaling which eventually results in cell death due to severe chromosomal instability.[3] Previously, it has been shown that small interfering RNA (siRNA)-mediated interference with SAC kinases BUBR1 and MPS1 sensitized cancer cells to low doses of paclitaxel in a synergistic fashion.[8,9] MPS1 depletion did not sensitize untransformed human fibroblasts, suggesting a preference for killing cancer cells.[9] Potential SAC kinase targets for small molecule inhibitors include Aurora B, MPS1 and BUB1.

Kinases as drug target

Protein kinases are required for proper SAC functioning and are therefore key players of mitosis.[10] Protein kinases are part of a large enzyme family of over 500 members, referred to as the human kinome[11], which catalyze the transfer of the γ-phosphate of ATP to side chains of serine, threonine and tyrosine residues of substrate proteins. Physiological functions of phosphorylation include enzyme activation, enzyme inhibition, protein localization, protein stabilization and protein degradation.[12] Protein kinases are therefore key regulators of cellular processes. The catalytic domain of kinases, referred to as the kinase domain, is structurally similar across the kinome and contains several conserved elements. The kinase domain consists of two major subdomains, the N-terminal lobe (N-lobe) and C-terminal lobe (C-lobe), which are connected via the so called hinge region (**Figure 1.3**).[12] Whereas the N-lobe predominantly consists of β-strands (β1-β5) and an α-helix (αC-helix), the C-lobe

primarily consists of α-helices.[13] ATP binds in the active site of a kinase which is located in between the two lobes. Binding interactions include hydrogen bonds between the adenine core of ATP and the amide backbone of the hinge region, ionic interactions with a conserved lysine from β3 which is the link between the α- and β-phosphate of ATP and a conserved glutamate present in the αC-helix, additional ionic interactions between the β- and γ-phosphate and the Mg^{2+} ion which is bound to the aspartate of the conserved DFG (Asp-Phe-Gly) motif as well as ionic interactions between the β- and γ-phosphate with glycine residues of the glycine-rich loop present between β1 and β2 (**Figure 1.3**).[14]

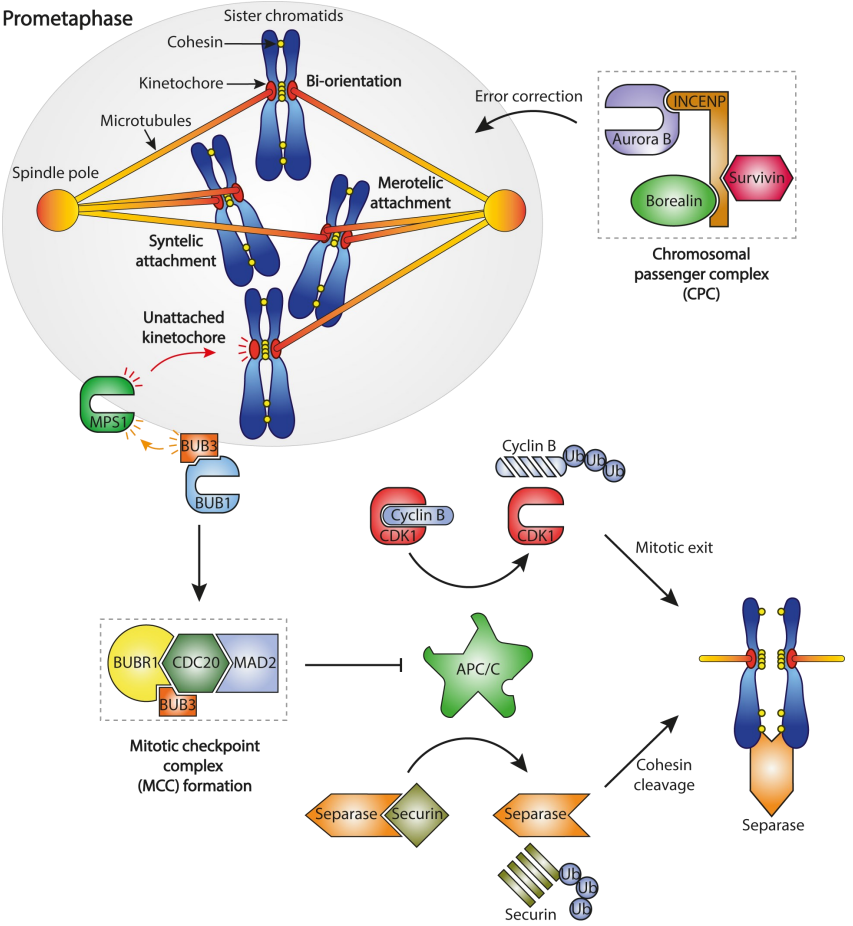


Figure 1.2 | Simplified representation of the prometaphase with different types of kinetochore-microtubule attachments. Unattached kinetochores trigger the recruitment of MPS1, which in turn recruits the BUB1-BUB3 complex. Multiple SAC proteins are subsequently recruited to unattached kinetochores resulting in the formation of the mitotic checkpoint complex (MCC) which consists of BUBR1, BUB3, CDC20 and MAD2. The MCC inhibits the activation of APC/C (which requires CDC20 to be active) thereby causing a mitotic arrest required to correct syntelic and merotelic attachment errors and connect unattached kinetochores to the spindle poles in a bi-oriented fashion. Upon activation of APC/CDC20, Cyclin B and Securin are targeted for proteasomal degradation by polyubiquitination. Cyclin B degradation inactivates CDK1 which promotes mitotic exit. Degradation of Securin activates Separase which cleaves Cohesin that holds sister chromatids together. The chromosomal passenger complex (CPC), consisting of Aurora B, INCENP, Borealin and Survivin, is responsible for correcting attachment errors.[2,15,16]

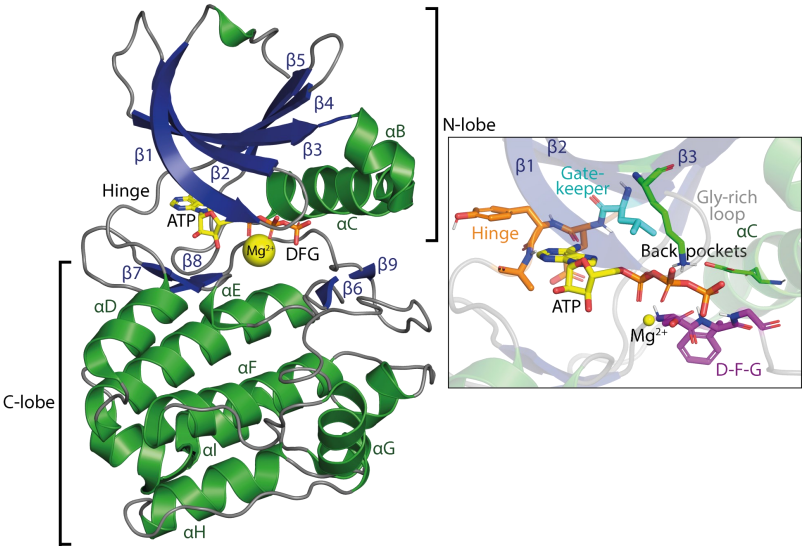


Figure 1.3 | Representation of the general structure of kinases (left) and an enlarged view of ATP binding (right). The crystal structure used for this figure represents Aurora A in complex with ATP (PDB code: 5dn3).[17] Figure generated using PyMOL.[18]

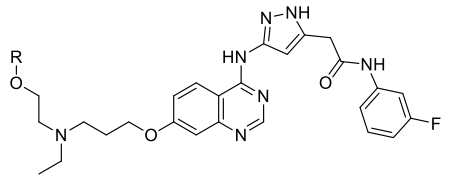
Deregulation of kinase activity, for example due to gene alterations, kinase overexpression or mutations that enhance kinase activity, has been implicated in the pathogenesis of human diseases, including cancer.[19] Counteracting undesired kinase signaling can be achieved by small molecule kinase inhibitors. The majority of kinase inhibitors developed to date bind the ATP-binding pocket and selectivity for a particular kinase can be achieved by exploiting small structural differences to other kinases as well as occupation of kinase back pockets.[20,21] The access to these back pockets is dependent on the conserved lysine in β3 as well as the size of the gatekeeper residue, which resides next to the hinge region (**Figure 1.3**).[12] Off-target activity may contribute to pharmacological side-effects, however, targeting multiple kinases, an phenomenon known as polypharmacology, can also favor efficacy. Kinases have been shown to be prominent drug targets with currently more than 70 kinase inhibitors approved for clinical use.[22] Since the approval of Gleevec, the first kinase inhibitor, in 2001, approval of drugs targeting kinases have steadily increased from about one per year in the period 2001-2006 to almost nine per year in the period 2017-2020.[22] The majority of these kinase inhibitors, about 84%, is used for the treatment of cancer.[22] Besides established kinase targets, new kinase targets have emerged for clinical evaluation, among which are kinases of the spindle assembly checkpoint, including Aurora B and MPS1.[23–26]

Aurora kinase B (Aurora B)

Aurora kinase B belongs to the aurora kinase family which consists of aurora kinase (Aurora) A, B and C. Despite their homology, aurora kinases have distinctive functions.[27,28] Whereas Aurora A functions in centrosome maturation, separation and bipolar spindle assembly, Aurora C is hypothesized to more closely resemble functions of Aurora B, but Aurora C is present in germ cells.[29] Aurora B is the enzymatic component of the chromosomal passenger

complex (CPC) which fulfills important roles in mitotic progression.[16] In addition to Aurora B, the CPC is formed by INCENP (inner centromere protein), Borealin and Survivin. The CPC is, via Aurora B activity, responsible for correcting erroneous, i.e. syntelic[30] and merotelic[31], kinetochore-microtubule attachments (**Figure 1.2**). In addition, Aurora B activity is required for the recruitment of key SAC proteins to kinetochores, among which are MPS1, BUBR1, BUB1.[16,32–34] Aurora B is hypothesized to function upstream of the SAC and is suggested to contribute to SAC activity which is independent from its function in error correction.[35,36] Furthermore, in late mitosis, Aurora B has been shown to be involved in cytokinesis.[37]

Aurora B has been found to be overexpressed in a multitude of cancers which is associated with poor prognoses.[38] Therefore, inhibition of its kinase function is thought to have therapeutic potential. Several aurora kinase inhibitors have reached clinical trials, however, only a limited number of these compounds are selective for Aurora B over Aurora A.[39–41] Barasertib (AZD-1152, a prodrug, **Figure 1.4**)[42], which shows over 3,000-fold selectivity over Aurora A, potently inhibited proliferation of several hematologic malignant cell lines, which could be enhanced by tubulin depolymerizing agent vincristine.[43] In addition, barasertib potently inhibited growth of human colon, lung and hematologic tumors in mouse xenografts by inducing polyploidy and concurrent apoptosis.[44] Although lacking efficacy in solid tumors during phase I[45], the inhibitor reached a phase II clinical trial for the treatment of acute myeloid leukemia (AML) and showed an improved objective complete response rate.[46] However, due to an inconvenient route of administration (7-day infusion), a nanoparticle encapsulating AZD-2811 (the active inhibitor of prodrug AZD-1152, **Figure 1.4**) was developed which allowed for continuous drug release for over a week, showed lower toxicity and increased efficacy in multiple xenograft models.[23] AZD-2811 is currently in clinical trials for small-cell lung cancer (phase II) and AML (phase I/II). Overall, these data support clinical proof of concept for Aurora B inhibition, however, with neutropenia being the most common on-target dose-limiting toxicity, the therapeutic window for Aurora B inhibition may be small.[47]



AZD-1152 R = PO_3H_2
AZD-2811 R = H

Figure 1.4 | Chemical structure of barasertib (AZD-1152, a prodrug) and corresponding Aurora B inhibitor AZD-2811.

Monopolar spindle 1 (MPS1)

MPS1, also known as TTK (threonine tyrosine kinase), is recruited to unattached kinetochores and initiates the SAC response.[48,49] MPS1 phosphorylates KNL1 (kinetochore scaffold 1), a member of the KMN (KNL1-MIS12-NDC80) network[50] which is essential for both microtubule binding and SAC signaling.[51] Phosphorylated KNL1 is recognized by BUB3, which in complex with BUB1, results in kinetochore recruitment of BUB1 (**Figure 1.2**).[52] In addition, like Aurora B, MPS1 is thought to be involved in correction of kinetochore-microtubule attachments errors.[34,53] Furthermore, MPS1 catalytic activity is required for the formation and/or stability of the MCC[53–55] and is important for maintaining SAC signaling.[36,56]

MPS1 is overexpressed in several tumors and its gene is part of a genetic signature associated with chromosomal instability in human cancers.[57,58] MPS1 inhibition with small molecule inhibitors is therefore studied as a potential strategy to kill cancer cells. Several MPS1 inhibitors have been developed of which a few reached phase I or phase I/II clinical trials.[59] In multiple studies of animal models, single agent therapy using MPS1 inhibitors only showed efficacy when administered near the maximum tolerated dose (MTD), indicating a small therapeutic window.[60–63] However, doses below the MTD synergistically enhanced the efficacy of taxanes (i.e. docetaxel, paclitaxel) in mouse xenograft models and this combination therapy was better tolerated.[25,62] Similarly, MPS1 inhibitors currently in clinical trials, including BOS-172722[24], BAY-1161909[25], BAY-1217389[25] and CFI-402257[26], are investigated in combination with paclitaxel.

Budding uninhibited by benzimidazole 1 (BUB1)

BUB1 was first identified in mutant strains of budding yeast *Saccharomyces cerevisiae* in which cell cycles at mitosis failed to arrest upon loss of microtubule function by benzimidazole.[64] Three years later, the *BUB1* gene and its product were characterized.[65] Human BUB1 is a 1085-residue protein which contains several structural elements, some of which are highly important for SAC function of BUB1, whereas the contribution to SAC signaling of others are not yet fully understood. These elements include the TPR (tetratricopeptide repeat) motif, BUB3-binding domain, R1LM (BUBR1 localization motif), RZZ binding domain, CD1 (conserved domain 1) region, ABBA (present in Cyclin A, BUBR1, BUB1 and Acm1) motif, KEN (lysine(K)-glutamate(E)-asparagine(N)) boxes, N-terminal extension and the kinase domain (**Figure 1.5**). The TPR motif[15], which although allows for the interaction with KNL1 (a kinetochore protein required for BUB1 localization[66]), is dispensable for kinetochore localization of BUB1.[67] The BUB3-binding domain, also known as the GLEBS (Gle20-binding site) motif, is responsible for binding BUB3 which is crucial for kinetochore recruitment of BUB1.[67] The R1LM was found to recruit BUBR1 to kinetochores, however, this domain was not found to be essential for SAC signaling.[68] In contrast, removal of the R1LM was found to increase SAC strength, suggesting BUB1-mediated BUBR1 recruitment to kinetochores might be required for SAC silencing.[68] CD1 is phosphorylated by MPS1 which enables the binding of MAD1.[69] Disturbance of MAD1 binding to BUB1 is detrimental for the

SAC, suggesting that this interaction is important for kinetochore localization of MAD1.[70] The RZZ binding domain is important for efficient kinetochore localization of the RZZ complex.[68] This domain has overlap with the CD1 domain of BUB1 (**Figure 1.5**) and it is thought that BUB1-mediated MAD1 recruitment is highly integrated with the RZZ complex, since the RZZ complex has been shown to localize MAD1-MAD2 to kinetochores.[68,71] The ABBA motif (also called Phe box) is required to activate the SAC by proper recruitment of CDC20 to unattached kinetochores.[72] Since the ABBA motif is close to CD1 of BUB1 (**Figure 1.5**), BUB1-mediated recruitment of CDC20 to kinetochores might bring CDC20 close to MAD1-MAD2 which may facilitate MCC formation.[73] BUB1 contains two KEN boxes which, together with the ABBA motif, are important for binding of CDC20 and is required for CDC20 phosphorylation to inhibit APC/C.[74,75] In addition, BUB1 facilitates binding of PLK1 to CDC20 allowing both kinases to phosphorylate several residues of CDC20 which, in turn, inhibits APC/CDC20 and thereby contribute to SAC signaling.[76] The N-terminal extension is required for BUB1 kinase activity[74] and the kinase domain was found to interact with CENP-F (centromere protein F) which is necessary for kinetochore recruitment of CENP-F.[77] The C-terminal tail of BUB1's kinase domain, but not its kinase activity, was reported to be important for chromosome alignment.[78] Kinase activity of BUB1 was found to phosphorylate histone H2A at threonine 120 which results in the centromere localization of Shugoshin 1 (SGO1).[79] SGO1 localization, in turn, recruits the CPC subunit Borealin.[80] CPC recruitment is enhanced by Haspin-mediated phosphorylation of histone H3 at threonine 3, resulting in the binding of CPC subunit Survivin which is important for activation of Aurora B and checkpoint signaling.[81,82] In addition, SGO1 protects centromeric cohesion which reveals a role of BUB1 in controlling sister chromatid cohesion through SGO1.[83,84] Despite histone H2A being a clear phosphorylation target of BUB1, the importance of the kinase function of BUB1 in the SAC is still under debate.[10,85,86] Therefore, the current hypothesis is that BUB1's kinase activity contributes to the strength of SAC signaling.[10,85,86]

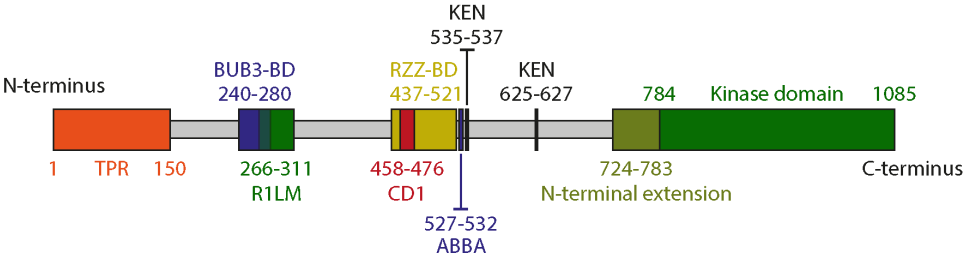


Figure 1.5 | Schematic representation of structural domains and motifs of human BUB1. TRP, tetratricopeptide repeat; BUB3-BD, BUB3 binding domain (also known as GLEBS (Gle20-binding site) motif); R1LM, BUBR1 localization motif; RZZ-BD, RZZ-binding domain; CD1, conserved domain 1; ABBA, also present in Cyclin A, BUBR1, BUB1 and yeast Acm1; KEN, lysine(K)-glutamate(E)-asparagine(N).[67–69,72,74,85]

Like Aurora B and MPS1, BUB1 is overexpressed in numerous human cancers and often correlates with poorer prognoses.[87–91] Recently, two structurally related BUB1 inhibitors were published, BAY-320 and BAY-524 (**Figure 1.6**), which were the first optimized BUB1 inhibitors reported.[92] The effect of BUB1 inhibition was compared to siRNA-mediated BUB1 depletion in aneuploid HeLa and diploid RPE1 cells. BAY-320 (3 μM) or BAY-524 (7 μM) resulted in

about 80% reduction of both H2A phosphorylation and centromeric levels of SGO1 and SGO2. In addition, BUB1 inhibition reduced centromeric levels of CPC components Aurora B, Borealin and INCENP by about 50% and also the activity of Aurora B was reduced. In contrast to siRNA-mediated BUB1 depletion, BUB1 inhibition did not significantly alter MAD1, MAD2 and BUBR1 kinetochore levels, which is in line with reports mentioned above and indicates that kinetochore recruitment of these proteins is independent on BUB1 kinase activity. Based on all data, Baron *et al*. hypothesized that BUB1 protein is predominantly required for the SAC and that its kinase activity is largely dispensable.[92] However, they found that BUB1 inhibition sensitized cells to low doses (1–4 nM) paclitaxel, which particularly affected the aneuploid HeLa cells, whereas diploid RPE1 cells were less affected. More recently, BAY1816032 (**Figure 1.6**) was published as an optimized lead BUB1 inhibitor.[93] BAY1816032 was found to synergistically inhibit cell proliferation of several cancer cell lines, including triple-negative breast cancer (TNBC) cells, when combined with paclitaxel or docetaxel. In addition, cell proliferation was synergistically inhibited when combined with ATR kinase inhibitor AZ20[94] in ATM-proficient cells, which are both protein kinases involved in the DNA damage response.[95] Furthermore, a synergistic effect on cell proliferation was observed when BAY1816032 was combined with several PARP inhibitors. Efficacy of BAY1816032, with or without paclitaxel, was investigated in mouse xenografts using TNBC cells (SUM-149) as a model system. Whereas paclitaxel initially reduced tumor growth, BAY1816032 did not show efficacy as single agent. In contrast, BAY1816032 combined with paclitaxel outperformed the efficacy of paclitaxel single agent therapy. Treatments were found to be well tolerated and no treatment related effects were observed in toxicologic studies on rats and dogs at concentrations up to 20-fold (rat) and 7-fold (dog) above efficacious concentrations in mice. Overall, these data suggest a clinical proof of concept for BUB1 inhibition combined with taxanes.

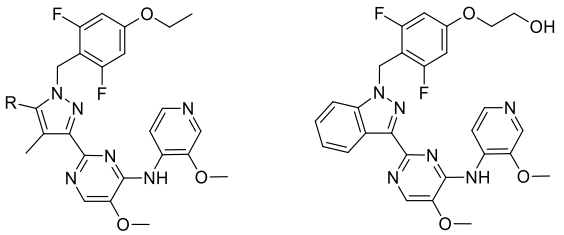


Figure 1.6 | Chemical structure of BUB1 inhibitors BAY-320, BAY-524 and BAY1816032.

Unlike Aurora B and MPS1 inhibitors, BUB1 kinase inhibition seems to be much better tolerated with respect to adverse effects in preclinical models. However, BUB1 inhibition by BAY1816032 lacked *in vivo* efficacy as single agent. The reasons for this lack of efficacy are unclear at the moment, but could be due to the extent of BUB1 inhibition which may be below the threshold required to induce a significant effect. The spindle assembly checkpoint

has been found to be extraordinarily sensitive and even a single unattached kinetochore allows for a mitotic arrest in vertebrate cells.[96] In addition, it was recently found that knocking-out BUB1 by CRISPR/Cas9 could induce alternatively spliced BUB1 mRNA which recovered SAC function.[97,98] CRISPR/Cas9-mediated BUB1 knockout was estimated to still express about 4% of BUB1, which was suggested to be sufficient for normal checkpoint activity.[71,98] Only when CRISPR/Cas9 knockout was combined with BUB1 siRNA, the SAC was significantly impaired.[71,98] Although these observations probably impacts the scaffolding function of BUB1 more significantly, the same might be true for its kinase activity. Therefore, more potent BUB1 inhibitors may be required to allow for single agent efficacy. In addition, targeted therapy may result in acquired resistance, in which mutations in the target protein can prevent inhibitor binding.[99] Alternative chemotypes may still be active on the mutated protein, which therefore supports the need for drug discovery (**Box 1.1**) of new BUB1 inhibitors. Finally, single agent efficacy may also be obtained by a mixed inhibition profile of BUB1 and other kinases.

Box 1.1 | Drug discovery.

The development of a small molecule drug (or a new chemical entity) is a laborious and expensive trajectory. The average costs for a successful drug discovery program is estimated to be $1.8 billion and requires approximately 13.5 years.[100] The drug discovery process follows several sequential phases starting with target selection and progresses towards clinical evaluation of the drug candidate (**Figure 1.7**). The first phase, target selection, requires a target that is 'druggable', meaning that it should be accessible to a potential drug molecule which, upon binding, can induce a biological response.[101] The strategy of target selection can be categorized into two subclasses: a speculative research target and innovative improvement.[102] A speculative research target is a new target for which therapeutic utility has not yet been proven by existing drugs. The speculative target is based on, for example, information obtained from samples derived from patients with a particular disease or targets hypothesized to be drivers of human diseases. In contrast, the innovative improvement approach aims to improve the performance of an existing drug by focusing on a target that is already known to have therapeutic utility. Protein families currently targeted by drugs include G-protein coupled receptors (GPCRs), nuclear receptors, ion channels and enzymes such as kinases.[103] Target selection is followed by hit identification which aims to find an appropriate starting point for further drug discovery. Hit identification strategies are diverse[101] and the choice for a particular strategy depends on the available information about the target of interest and molecules that are known to bind this target, as well as available assays to determine binding affinity, accessibility to the protein target and expertise of the people contributing to the project. Identified hits are subsequently resynthesized and evaluated to confirm their activity. Confirmed hits, in turn, are subjected to extensive hit to lead optimization during which the structure-activity relationship is investigated by iterative rounds of designing and synthesizing new molecules followed by evaluation of their activity.[104] Hits are initially optimized on *in vitro* activity while keeping properties such as molecular weight, lipophilicity, number of hydrogen bond donors/acceptors, rotatable bonds and polar surface area into account.[105,106] Once sufficient *in vitro* potency is achieved, optimized hits are further profiled for selectivity, cellular target engagement and cellular activity. In addition, pharmacokinetic properties such as absorption, distribution, metabolism and excretion (ADME) are investigated by *in vitro* assays which allow for assessment of plasma- and metabolic stability, aqueous solubility, plasma protein binding, cell permeability, among others.[104] ADME properties can subsequently be optimized, when required, to obtain lead compounds. Lead compounds are subsequently investigated *in vivo* during which the pharmacokinetic (PK) profile is investigated. The PK parameters investigated include

clearance, volume of distribution, half-life and bioavailability.[104] When required, lead optimization is used to address unfavorable PK profiles. Once acceptable PK profiles are achieved, optimized leads can proceed to animal models representative for the disease under investigation. In these models proof of efficacy and safety are required before drug candidates may enter clinical evaluation in humans.[104] During clinical evaluation drug candidates are tested in humans for the first time and evaluation is divided into four clinical phases (phase I – IV).[104] Phase I clinical trials are aimed at determining safety, maximum tolerated doses and to assess dose limiting toxicities in a small group of healthy or diseased volunteers. Phase II trials are usually performed on a larger group of patients who have the disease under investigation and aim to investigate preliminary efficacy of the candidate drug as well as to determine the dose for phase III studies. Safety is still carefully monitored due to higher exposures to the drug. Usually, phase III clinical trials consist of two or three studies in which efficacy and safety need to be confirmed on a broad patient population. Once successfully completed, marketing approval will be provided. Marketing approval commonly requires additional surveillance studies which are referred to as phase IV trials.

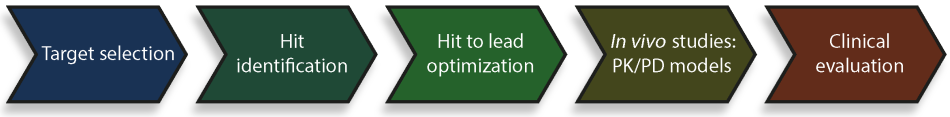


Figure 1.7 | Simplified scheme of the different phases in drug discovery.

Aim and outline

The aim of the research described in this thesis is to discover and optimize novel inhibitors of the spindle assembly checkpoint kinase BUB1 to study its biological role in cancer cell proliferation.

Chapter 2 describes the results of a high-throughput screen which was used as hit identification strategy in the search for novel chemical scaffolds as BUB1 inhibitors. The activity of over 50,000 compounds was assessed in a primary screen and subsequent confirmation screen, deselection assay and dose-response measurements yielded a qualified hit list of 25 compounds. Resynthesis of four prioritized hits confirmed their activity and provided excellent starting points for further drug discovery.

Chapter 3 continues with hit optimization of one of the confirmed hits: OSI-420. Synthesis and biochemical evaluation of structural analogues provided insight into the structure-activity relationship and resulted in optimization of lipophilicity (cLogP) as well as lipophilic efficiency (LipE).

Chapter 4 describes the hit optimization of another confirmed hit, AT-9283. A comprehensive investigation of the structure-activity relationship by synthesis and biochemical evaluation of AT-9283 derivatives resulted in highly potent BUB1 inhibitors with sub-nanomolar biochemical activity, good lipophilicity and excellent lipophilic efficiency. In addition, a co-crystal structure of one of the inhibitors revealed the binding mode of this molecule in the kinase domain of BUB1.

Chapter 5 presents the development of an assay which allows for assessment of cellular BUB1 target engagement. A previously published chloro-fluoroacetamide probe,

reported to show off-target activity on BUB1, as well as structural analogues were synthesized and investigated for their potential as BUB1 probes. Probe binding was studied by mutating the proposed nucleophilic cysteine of BUB1 and provided evidence for the amino acid responsible for covalent bond formation. One of the probes allowed for dose- and time-dependent BUB1 labeling in living cells. Labeling could dose-dependently be outcompeted with published BUB1 inhibitor BAY1816032 which provided proof of principle for the use of this assay to study cellular BUB1 target engagement.

Chapter 6 focusses on the biological profiling of the most potent benzimidazole-based inhibitors discovered in Chapter 4. Several *in vitro* absorption, distribution, metabolism and excretion (ADME) assays were performed to study drug-likeness. Cellular BUB1 target engagement was measured and activities of the compounds on cell proliferation was assessed. In addition, the *in vitro* selectivity profile was studied in a broad panel of kinases and, finally, the antiproliferative activity in a large panel of cancer cell lines was investigated of the most promising inhibitor. Overall, these assays revealed two lead BUB1 inhibitors, ROB433 and ROB464, with favorable drug-like properties.

Chapter 7 summarizes the research described in this thesis and provides future directions.

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