Development of kinase inhibitors and activity-based probes

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1 General introduction

Kinases

A wide array of intracellular signaling events in eukaryotic cells, including metabolism, growth, division, differentiation, motility, organelle trafficking, membrane transport, muscle contraction, immunity, learning and memory, are mediated by protein phosphorylation.[1] The diversity of processes regulated by this post-translationalmodification is reflected by the large number of protein kinases (about 600) encoded by the human genome.[2] Protein kinases are related by their conserved homologous kinase domains; a catalytic core that consists of 250 – 350 residues.[3] The site of phosphate transfer is located between a N-terminal lobe composed of a β-sheet and a

single α-helix (the "C-helix") and a larger C-terminal lobe consisting of α-helices, connected by a linker referred to as the hinge region (Figure 1A). Within the conserved, narrow and hydrophobic ATP-binding pocket, the adenine ring of ATP makes a number of hydrophobic contacts in the ATP-binding cleft and forms at least one hydrogen bond with the backbone of the hinge region.[4] The triphosphate group of ATP points out of the adenosine pocket towards the hydrophilic medium. When the substrate (peptide, lipid or carbohydrate) binds to its kinase, a set of conserved residues within the kinase catalytic domain catalyses the transfer of the terminal γ-phosphate of ATP to the hydroxyl oxygen of Ser, Thr or Tyr residue of the substrate. Phosphorylation may result in significant changes in either structural features or function of the target protein (Figure 1B).

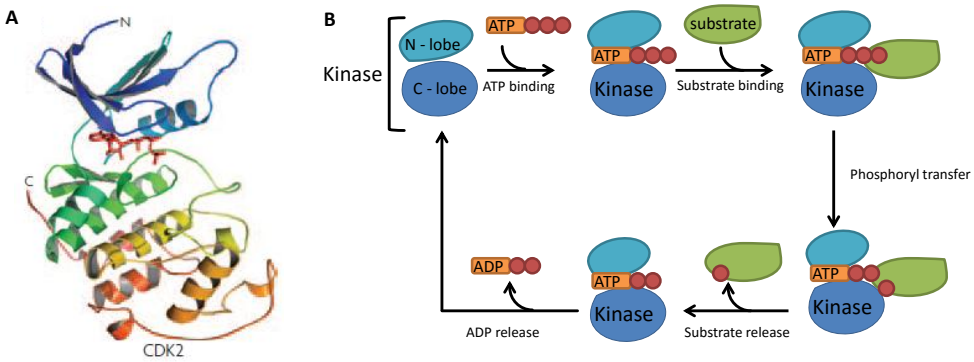

Figure 1. A) In general, protein kinases consist of a N-terminal (blue) and a C-terminal lobe (green, yellow, orange). The crystal structure of cyclin-dependent kinase 2 (CDK2) (Protein Data Bank (PDB) ID: 1QMZ) shows the representative fold. ATP is modeled bound in the cleft (red ball and stick model). B) The general catalytic cycle for substrate phosphorylation by a kinase.

The kinase superfamily consists of four major families, namely AGC, Ca^{2+}/calmodulin-dependent protein kinases (CaMK), CMGC and tyrosine kinases (TK), and four minor families, namely casein kinase 1 (CK1), STE, receptor guanylate cyclase (RGC) and TK-like (TKL) kinase.[5]

The AGC group of kinases comprises more than 60 members including the cyclic-nucleotide-dependent family protein kinases A (PKA) and G (PKG), the protein kinase C (PKC) family, the β-andrenergic receptor kinase (βARK) family, the ribosomal S6 kinase family, and other close relatives. In general, this group of kinases tends to be basic amino acid-directed enzymes by phosphorylating at Ser/Thr residues, which are near Arg and Lys. The AGC kinases participate in a variety of well-known signaling processes. They mediate cyclic AMP signaling, the response to insulin, protection against apoptosis, diacylglycerol signaling and control of protein translation.[6, 7]

The CaMK group protein kinases are Ser/Thr protein kinases. Almost all are activated by Ca^{2+}/calmodulin binding to a small domain located near the COOH-terminal to the catalytic domain. CaMKs are particularly abundant in the brain and the nervous system, where they are involved in synaptic development and plasticity, gene transcription, muscle contraction and learning and memory processes by targeting various factors including structural proteins, ion channels and protein pumps.[8, 9,10]

CMCG is a major category of Ser/Thr kinases. Mostly, CMCG members phosphorylate substrates at proline-rich sites. This group includes the protein kinases cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK3), CDC-like kinase (CLK). These kinases are known for controlling the cell cycle, activity of human tumor suppressors and cell-fate decisions.[11,12]

The protein tyrosine kinase family phosphorylates, in contrast to the families described above, on Tyr residues. Family members play a role in mitogenesis, differentiation and development, angiogenesis, cell cycle control, growth control, cell survival and apoptosis, transcriptional regulation, and glucose uptake.[13]

TKL kinases are a diverse group of Ser/Thr protein kinases with sequence similarity to TK, but lacking TK-specific motifs.[1] These kinases are involved in programmed cell death, growth and immune system.[14]

CK1 represents an unique group within the superfamily of Ser/Thr kinases. They have been implicated in the control of cytoplasmic and nuclear processes, including DNA replication and repair.[15,16]

Members of the STE kinase family include the homologues of yeast sterile 7, sterile 11 and sterile 20 kinases. This group of kinases prefers to phosphorylate serine and threonine residues with an arginine residue N-terminal to the phosphorylation site. They form the MAPK cascade and transduce signals from the cell surface to the nucleus.[17]

The kinases of the RGC family contain an active guanylate cyclase domain, which generates the cGMP second messenger. This second messenger is a key signaling molecule that in turn modulates the activity of cGMP dependent kinases, ion channels, and phosphodiesterases. They also contain a catalytically inactive kinase domain, which appears to have a regulatory domain.[18] RGCs have attracted considerable interest due to their roles in the cardiovascular and nervous systems, bone development and vision.[19]

Kinases as drug targets

Since protein kinases are key components of most signal transduction networks, this makes them therapeutically relevant drug targets and interesting to explore their biological roles and functions in detail. A few kinases together with their inhibitors relevant to studies described in this thesis are highlighted below.

PKB/AKT1 and FLT3

AKT, also known as protein kinase B (PKB), is an intracellular anti-apoptotic serine/threonine kinase belonging to the AGC family of kinases. Three isoforms of AKT are known to exist, namely AKT1, AKT2 and AKT3. These isoforms exhibit about 90% sequence identity in the kinase domain and 97-100% sequence identity in the ATP-binding site.[20] PKB/AKT is a key component of the PI3K-AKT-mTor kinase pathway, which is responsible for cell proliferation, survival, cell growth, apoptosis and metabolism.[21]
Full activation of PKB is a multi-step process that uses the PI3K signaling pathway. First, extracellular ligands (hormones and growth factors) bind to receptor tyrosine kinase (RTK), which in turn forms homo-oligomers.[22] In response, phosphatidylinositol-3 kinase (PI3K) is activated through binding to phosphotyrosine residues in the receptor and phosphorylates in turn phophoinositides (PI) to generate phosphatidylinositol 3,4,5 triphosphate (PIP3). PIP3 organizes PKB/AKT, PDK1 and Ser473-kinase on the plasma membrane, where PKB/AKT becomes phosphorylated and activated. Then, the activated PKB/AKT translocates from the cytosol to the nucleus (Figure 2).[23, 24,25]

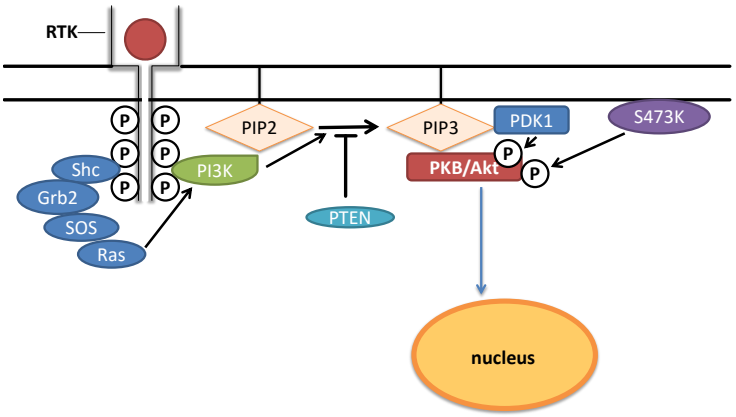

Figure 2. Schematic representation of PKB activation.

PKB/AKT is recognized as an important therapeutic target for the treatment of human cancers, such as breast, ovarian, prostate and pancreatic cancers,[26] as well as diabetes.[27] In addition, one of the three PKB/AKT isomers has been identified as a potential target to prevent intracellular growth of *Salmonella typhymurium* and *Mycobacterium tuberculosis.* Kuijl *et al.* have shown that inhibition of PKB/AKT1 resulted in less infection, which makes it a potential antibiotic drug target.[28] However, the major bottleneck is to find selective PKB/AKT1 inhibitors, since elimination of both AKT1 and AKT2 is lethal whereas genetic elimination of AKT1 had no impact on the health of the mice.

Studies have revealed that the PKA inhibitor H-89 (**1**, Figure 3) has a higher selectivity towards AKT1 then AKT2, which makes this compound an interesting lead compound. Many analogues of H-89 have been synthesized, however a nanomolar inhibitor needs still to be developed.

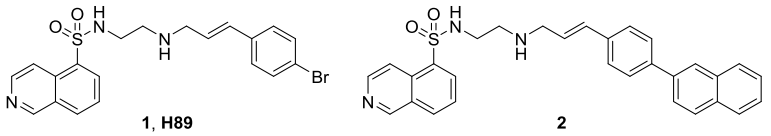

Figure 3. Structures of lead compounds **1** (PKB/AKT1) and **2** (FLT3).

Within many synthesized analogues of H-89, one interesting compound that was identified comprises naphthalene analogue **2** (Figure 3). This bulky aromatic compound showed hardly any PKB/AKT1 inhibition, but inhibited another interesting kinase named FMS-like tyrosine kinase 3 (FLT3) with an IC_{50} of 1.01 µM. FLT3 is a type III receptor tyrosine kinase and consists of five immunoglobulin-like domains in the extracellular region, a juxtamembrane domain, a tyrosine kinase domain separated by a kinase insert domain and a C-terminal domain in the intracellular region.[29] This kinase is expressed on the surface of normal hematopoietic stem/progenitor cells and plays a well-established role in normal growth, survival and differentiation of hematopoietic precursor cells. Like other RTKs, upon binding of FLT3 ligand (FL), which is expressed by bone marrow stroma cells, the FLT3 receptor dimerizes at the plasma membrane (Figure 2).[30] This leads to autophosphorylation and activation of multitude downstream effector signaling cascades, including Ras/Mek, PI3K/AKT/mTor, and STAT-5 pathways. All these pathways play important roles in the promotion of cell cycle progression, inhibition of apoptosis, and activation of differentation. FLT3 is an important key regulator in multiple hematopoietic pathways, and mutated FLT3 causes altered mechanisms of cellular proliferation and apoptosis that promote cell survival thereby conferring a substantial growth advantage to leukemic stem and progenitor cells. FLT3 mutations are mostly found in patients with acute myeloid leukemia (AML)[31,32,33], myelodysplasia (MDS),[34,35] and acute lymphoblastic leukemia (ALL).[36,37] Potent and selective FLT3 inhibitors may form the starting point for therapeutics for these diseases.

Bruton's tyrosine kinase (BTK)

Another tyrosine kinase that plays a major role in the pathogenesis of hematological malignancies, such as chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL), and autoimmune diseases as well is Bruton's tyrosine kinase (BTK).[38] BTK is a member of the Tec tyrosine kinase family and is expressed in most hematopoietic cells

such as B-cells, mast cells and microphages, except in T-cells and plasma cells. It is predominantly situated in the cytosol and has several conserved domains from the N-terminus: pleckstrin homology (PH), Tec homology (TH), Src homology 2 and 3 (SH2 and SH3) and a carboxyl-terminal regulatory and catalytic region that contains the tyrosine kinase (TK) or SH1 domains.[39] BTK functions downstream of multiple receptors including growth factors, B-cell antigen, chemokine and innate immune receptors. BTK initiates a diverse range of cellular processes, such as cell proliferation, survival, differentiation, motility, angiogenesis, cytokine production and antigen presentation.[40]

From the many pathways, the B-cell receptor (BCR) signaling pathway is crucial for the pathogenesis of many B-cell malignancies. BCR plays a fundamental role in B-cell proliferation, differentiation and function including production of antibodies.[41] It serves as an antigen receptor, which consists of a surface transmembrane immunoglobulin (Ig) receptor associated with the Ig-alpha (CD79a) and Ig-beta (CD79b) heterodimers.[42] In normal B-cells, antigen binding to the receptor leads to receptor aggregation and recruits other kinases such spleen tyrosine kinase (SYK) and LYN kinases. These in turn, phosphorylate the receptor's cytoplasmic tyrosine-based activation motifs (ITAMs) on the cytoplasmic Ig domains of the receptor. ITAM phosphorylation sets off a cascade of downstream events including activation of downstream kinases, adaptor molecules, and generation of second messengers. SYK propagates the signal by activation of PI3K, which stimulates the production of PIP3. Once an adequate amount of PIP3 is produced, BTK is mobilized to the plasma membrane. At this site, BTK is activated by phosphorylation at the Y551 site by the Src family kinases, especially LYN and FYN. BTK amplifies the signal via phosphorylation of phopholipase gamma C2 (PLCγ2), mobilization of calcium secondary messengers, PKC, and finally activation of MAP kinase pathways, nuclear factor of activated T cells (NFAT) and nuclear factor κB (NFκB). These signals regulate patterns of gene expression necessary for B cell survival and proliferation (Figure 4).[40, 42]

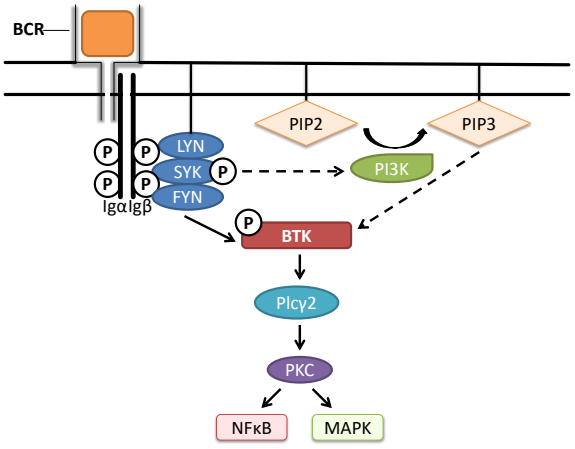

Figure 4. Schematic representation of BCR/BTK signaling pathway.

Among the many kinases involved in BCR signaling, BTK is a unique therapeutic target for treatment of B-cell malignancies. This is due to the fact that BTK inhibition leads to inhibition of NFκB DNA binding, reduction of integrin-mediated cell adhesion and migration, limitation of cell production of chemokines and, ultimately, apoptosis. The first-in-class BTK-inhibitor ibrutinib (Imbruvica®, PCI-32765, **3**, Figure 5) has recently been approved for the treatment of Waldenström's macroglobulinemia (WM), MCL and CLL by the FDA.[43,44,45,46] Apart from its clinical efficacy, ibrutinib is of interest because of its mechanism of action. Ibrutinib irreversibly blocks BTK activity through covalent modification of Cys481 within the enzyme ATP-binding pocket following conjugate addition of the cysteine thiol to the acrylamide moiety in **3** (Figure 5), an event that prevents phosphorylation of Tyr223, an essential step for BTK activation.[47] Even though the action of this drug has been studied in detail for BTK, it would also be interesting to investigate whether this drug has other cellular targets. This will give insight into the mechanism of side effects.

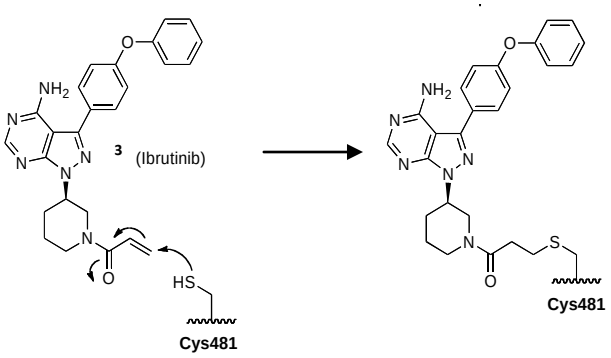

Figure 5. Covalent binding of ibrutinib (**3**) to Cys481 in the active site of BTK.

Dasatinib as inhibitor for Bcr-Abl

Chronic myeloid leukemia (CML) is a hematological malignancy, which is defined to be Philadelphia chromosome/Bcr-Abl positive. In 1960, the Philadelphia chromosome was first discovered in cell cultures from CML patients.[48] A Philadelphia chromosome is a truncated version of chromosome 22 produced by a reciprocal translocation between the Abl gene on chromosome 9 and the breakpoint cluster region (BCR) on chromosome 22, which leads to the formation of the novel Bcr Abelson tyrosine kinase (Bcr-Abl) fusion gene. This fusion kinase possesses the tyrosine kinase activity of Abl, which is constitutively active in the cytosol. As a result, the downstream signaling pathways are dysregulated and proliferation and survival of leukemic cells are increased.[49]
BCR is a serine/threonine kinase with several interactions domains for proteins such as actin, lipids and GTP. C-Abl protein, which has a molecular weight of 140 kDa, shuttles

between the nucleus and cytoplasm. It is involved in many processes such as regulation of cell growth and survival, oxidative stress reaction, response to DNA damage and regulation of actin dynamics and cell migration. Specific breakpoints in the chromosomal translocation lead to distinct forms of Bcr-Abl protein: p185 Bcr-Abl, p210 Bcr-Abl and P230 Bcr-Abl. Each type is found in different types of leukemia; the p210 form is associatied with CML, whereas p230 and p185 are associated with neutrophilic leukemia and acute lympoblastic leukemia, respectively.[50]

Up to now, there are three generation of BCR-Abl inhibitors approved by the FDA. One of the many inhibitors is dasatinib (**4**), which is a dual-specificity Abl- and Src-family kinases inhibitor having a substituted thiazole-carboxamide (Figure 6). However, the molecule is not selective for Bcr-Abl and Src, since it has been successively reported that this compound also inhibits other TKs.[48] This information provides a rationale for investigating it in other hematologic malignancies, such as CLL.

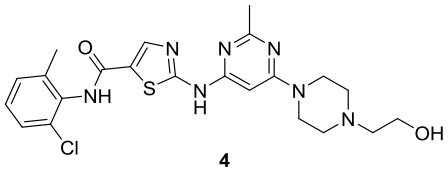

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Figure 6. Structure of dasatinib (**4**).

Finally, it should be mentioned that kinase inhibitors are important not just for the treatment of diseases, but also as reagents for gaining understanding about the physiological roles of protein kinases and additional targets that cause side effects or synergy. In these cases, the use of chemo-proteomic tools, also known as activity-based probes (ABPs), for performing activity-based protein profiling (ABPP) comes to a rescue. These chemical tools need to bind or have affinity for the target(s) of interest and contain a reporter group, such as the fluorescent group Bodipy-FL or biotin for detection, quantification, and/or enrichment/identification of labeled proteins. In cases where enzyme activities in living cells or organisms are subject of study, the use of reporter groups with a small, latent chemical handle (alkyne, azide or norbornene) may be a solution, because bulky reporter groups may hamper cell permeability feature of an ABP.

Since the inhibitors (H-89, ibrutinib and dasatinib) that are discussed above bind (non)covalently to their specific subset (or family) of catalytically related enzymes, these compounds are interesting candidates to turn into an affinity- or activity-based probe. This will provide more insight into the additional drug targets and mechanism of the various types of cancers, diabetes and immune diseases.

Aim and outline of this thesis

The central role of protein kinases in many signal transduction pathways has generated intense interest in targeting these enzymes for a wide range of diseases. This thesis focuses on both the development of more potent kinase inhibitors and the development of ABPs to study the roles of kinases, inhibitor selectivity profile and the additional targets of known kinase inhibitors.

In **Chapter 2**, an overview will be provided about three kinase profiling methods that are published in literature, namely activity-based protein profiling, photoaffinity protein profiling and affinity-based protein profiling. The fundaments of these profiling methods will be explained in detail.

More potent inhibitors towards FLT3 are needed to treat certain types of leukemia. **Chapter 3** describes the design and synthesis of 102 isoquinolinesulfonamide compounds, which share the same core as H-89 (**1**). The variations between these related compounds were created by substitution of the phenyl bromide with bulky (hetereo)aromatic groups at the ortho, meta or para phenyl site under Suzuki coupling conditions.

The biological activities of these 102 compounds together with 137 structurally related isoquinolinesulfonamides were evaluated in **Chapter 4**. IC_{50} and K_i values towards the kinases PKA, AKT1, AKT2 and FLT3 are given. Based on these findings, structure-activity relationships (SAR) are discussed and the most active and selective inhibitors shown.

In the second part of the thesis, three ways to label kinases are outlined. In **Chapter 5** the synthesis of two direct (Bodipy-FL and Bodipy-TMR) and three two-step labeling ibrutinib-based probes (azide, alkyne and norbornene) are discussed. These probes are used to label BTK and act as activity-based probes, since they bind covalently to BTK after a nucleophilic attack of the thiol-group in the active site of BTK. Furthermore, labeling of BTK in Ramos cells and lysate is visualized on SDS-PAGE gel.

Chapter 6 focuses on the synthesis of photoaffinity-based probes for PKB/AKT1 and PKA. These core of the probes are based on the PKA inhibitor H-89 (**1**) and they also contain a diazerine group as the photoaffinity group, which bind covalently to its target after exposure to UV. In addition, these probes also contain an azide ligation handle, which is useful for visualization and/or enrichment of proteins. Labeling of recombinant PKA and AKT1 kinases and competition assays will be shown.

A third way to label kinases is by affinity-based protein profiling, which is discussed in **Chapter 7**. The synthesis of an affinity-based probe based on dasatinib, which is immobilized on NHS-activated beads, will be discussed. Furthermore, information about the targets of this probe will be provided by Western blot analysis.

Finally, **Chapter 8** provides a summary of the results gained in this thesis and projects some future prospects.

References

[1] J. A. Ubersax and J. E. Ferrell Jr, *Nature Rev. Mol. Cell. Biol.*, 2007, **8**, 530.

[2] P. Blume-Jensen and T. Hunter, *Nature*, 2001, **411**, 355.

[3] S. K. Hanks and T. Hunter, *Faseb. J.*, 1995, **9**, 576.

[4] J. A. Endicott, M. E. M. Noble and L. N. Johnson, *Annu. Rev. Biochem.*, 2012, **81**, 587.

[5] E. D. G. Fleuren, L. Zhang, J. Wu and R. J. Daly, *Nature Rev. Canc.*, 2016, **16**, 83.

[6] R. T. Peterson and S. L. Schreiber, *Curr. Biol.*, 1999, **9**, R521.

[7] J. M. Arencibia, D. Pastor-Flores, A. F. Bauer, J. O. Schulze and R. M. Biondi, *Biochim. Biophys. Act.*, 2013, **1834**, 1302.

[8] G. A. Wayman, Y-S Lee, H. Tokumitsu, A. Silva and T. R. Soderling, *Neuron.*, 2008, **56**, 914.

[9] G. A. Wayman, H. Tokumitsu, M. A. Davare and T. R. Soderling, *Cell Calcium*, 2011, **50**, 1.

[10] K. A. Skelding, J. A. P. Rostas and N. M. Verrills, *Cell Cycle*, 2011, **10**, 631.

[11] N. Kannan and A. F. Neuwald, *Prot. Sci.*, 2004, **13**, 2059.

[12] M. Varjosalo,k S. Keskitalo, A. van Drogen, H. Nurkkala, A. Vichalkovski, R. Aebersold and M. Gstaiger, *Cell. Rep.*, 2013, **3**, 1306.

[13] F. A. Al-Obeidi, J. J. Wu and K. S. Lam, *Biopolymers,* 1998, **47**, 197.

[14] M. E. Handley, J. Rasaiyaah, B. M. Chain and D. R. Katz, 2007, **88**, 111.

[15] U. Knippschild, A. Gocht, S. Wolff, N. Huber, J. Lohler and M. Stoter, *Cell. Sign.*, 2005, **17**, 675.

[16] K. J. Fish, A. Cegielska, M. E. Getman, G. M. Landes and D. M. Virshup , *J. Biol. Chem.*, 1995, **270**, 14875.

[17] G. Zhu, K. Kuijl, Y. Liu, V. Codrea, J. Herrero, and S. Shaw, *J. Biol. Chem.,* 2005, **280**, 36372.

[18] L. R. Potter, *Pharmacol. Ther.*, 2011, **130**, 71.

[19] P. S. Padayatti, P. Pattanaik, X. Ma and F. van den Akker, *Pharm. Ther.*, 2004, **104**, 83.

[20] N. C. Kallan, K. L. Spencer, J. F. Blake, R. Xu, J. Heizer, J. R. Bencsik, I. S. Mitchell, S. L. Gloor, M. Martinson, T. Risom, S. D. Gross, T. H. Morales, W-I Wu, G. P. A. Vigers, B. J. Brandhuber and N. J. Skelton, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 2410.

[21] T. G. Davies, M. L. Verdonk, B. Graham, S. Saalau-Bethell, C. C. F. Hamlett, T. McHardy, I. Collins, M. D. Garrett, P. Workman, S. J. Woodhead, H. Jhoti and D. Barford, *J. Mol. Biol.*, 2007, **367**, 882.

[22] T. O. Chan, S. E. Rittenhouse and P. N. Tsichlis, *Annu. Rev. Biochem.*, 1999, **68**, 965.

[23] E. S. Kandel and N. Hay, *Exp. Cell. Res.*, 1999, **253** , 210.

[24] M. Hanada, J. Feng and B. A. Hemmings, *Biochim. Biophys. Acta.*, 2004, **1697**, 3.

[25] J. Downward, *Curr. Opin. Cell. Biol.*, 1998, **10**, 262.

[26] H. Reuveni, N. Livnah, T. Geiger, S. Klein, O. Ohne, I. Cohen, M. Benhar, G. Gellerman and A. Levitzki, *Biochem*., 2002, **41**, 10304.

[27] L. Logie, A. J. Ruiz-Alcaraz, M. Keane, Y. L. Woods, J. Bain, R. Marquez, D. R. Alessi and C. Sutherland, *Diabetes*, 2007, **56**, 2218.

[28] C. Kuijl, N. D. L. Savage, M. Marsman , A. W. Tuin, L. Janssen, D. A. Egan, M. Ketema, R. van den Nieuwendijk, S. J. F. van den Eeden, A. Geluk, A. Poot, G. van der Marel, R. L. Beijersbergen, H. S. Overkleeft, T. H. M. Ottenhof and J. Neefjes, *Nature*, 2007, **450**, 725.

[29] J. Nagoya, *J. Med. Sci.*, 2015, **77**, 7.

[30] S. A. Wander, M. J. Levis and A. T. Fathi, *Ther. Adv. Hematol.*, 2014, **5**, 65.

[31] H. Kiyoi, M. Towatari, S. Yokota, M. Hamaguchi, R. Ohno, H. Saito and T. Naoe, *Leukemia,*, 1998, **12**, 1333.

[32] D. L. Stirewalt, K. J. Kopecky, S. Meshinchi, F. R. Appelbaum, M. L. Slovak, C. L. Willman and J. P. Radich, *Blood*, 2001, **97**, 3589.

[33] S. Meshinchi, W. G. Woods, D. L. Stirewalt, D. A. Sweetser, J. D. Buckley, T. K. Tjoa, I. D. Bernstein and J. P. Radich, *Blood*, 2001, **97**, 89.

[34] S. Horiike, S. Yokota, M. Nakao, T. Iwai, Y. Sasai, H. Kaneko, M. Taniwaki, K. Kashima, H. Fujii. T. Abe and S. Misawa, *Leukemia*, 1997, **11**, 1442.

[35] S. Yokota, H. Kiyoi, M. Nakao, T. Iwai, S. Misawa, T. Okuda, Y. Sonoda, T. Abe, K. Kahsima, Y. Matsuo and T. Naoe, *Leukemia*, 1997, **11**, 1605.

[36] C. Thiede, C. Steudel, B. Mohr, M. Schaich, U. Schakel, U. Platzbecker, M. Wermke, M. Bornhauser, M. Ritter, A. Neubauer, G. Ehninger and T. Illmer, *Blood*, 2002, **99**, 4326.

[37] Y. Yamamoto, H. Kiyoi, Y. Nakano, R. Suzuki, Y. Kodera, S. Miyawaki. N. Asou, K. Kuriyama, F. Yagasaki, C. Shimazaki, H. Akiyama, K. Saito, M. Nishimura, T. Motoji, K. Shinagawa, A. Takeshita, H. Saito, R. Ueda, R. Ohno and T. Naoe, *Blood*, 2001, **97**, 2434.

[38] U. Gaygo, M. Fung, F. Clow, S. Sun, E. Faust, S. Price, D. James, M. Doyle, S. Bari and S. H. Zhuang, *Ann. N. Y. Acad. Sci.*, 2015, **1358**, 82.

[39] A. Akinleye, M. Furqan and O. Adekunle, *Clin. Lymph. Myel. Leuk.*, 2014, **14**, 253.

[40] M. S. Davids and J. R. Brown, *Leuk. Lymphoma*, 2012, **53**, 2362.

[41] A. Novero, P. M. Ravella, Y. Chen, G. Dous and D. Liu, *Exp. Hematol. Onc.*, 2014, **3**, 4.

[42] A. Aalipour and R. H. Advani, *Brit. J. Haematol.*, 2013, **163**, 436.

[43] F. Cameron and M. Sanford, *Drugs*, 2014, **74**, 263.

[44] US Food and Drug Administration, Imbruvica, 2013, (Accessed 9 December 2013).

[45] L. A. Honigberg, A. M. Smith, M. Sirisawas, E. Verner, D. Loury, B. Chang, S. Li, Z. Pan, D. H. Thamm, R. A. Miller and J. J. Buggy, *PNAS*, 2010, **107**, 13075 – 13080.

[46] Z. Pan, H. Scheerens, S-J Li, B. E. Schultz, P. A. Sprengeler, L. C. Burrill, R. V. Mendonca, M. D. Seeney, K. C. K. Scott, P. G. Grothaus, D. A. Jeffery, J. M. Spoerke, L. A. Honigberg, P. R. Young, S. A. Dalrymple and J. T. Palmer, *Chem. Med. Chem.*, 2007, **2**, 58.

[47] L. A. Honigberg, A. M. Smith, M. Sirisawad, E. Verner, D. Loury, B. Chang, S. Li, Z. Pan, D. H. Thamm, R. A. Miller and J. J. Buggy, *PNAS*, 2010, **117**, 13075.

[48] K. Yang and L.-W. Fu, *Crit. Rev.* Onc*. Hematol.*, 2015, **93**, 277.

[49] A. Desogus, S. Schenone, C. Brullo, C. Tintori and F. Musumeci, *Exp. Opin. Ther. Pat.*, 2015, **25**, 397.

[50] T. S. Ross and V. E. Mgbemena, *Mol. Cell. Oncol.*, 2014, **1**, e963450.