Identifying therapeutic targets by elucidating signaling pathways in pediatric lymphoid leukemias
van der Sligte, Naomi Eline

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Can kinomics and proteomics bridge the gap between pediatric cancers and newly designed kinase inhibitors?

Naomi E. van der Sligte
Kim R. Kampen
Eveline S.J.M. de Bont
Chapter 2

ABSTRACT

The introduction of kinase inhibitors in cancer medicine has transformed chronic myeloid leukemia from a fatal disease into a leukemia subtype with a favorable prognosis by interfering with the constitutively active kinase BCR-ABL. This success story has resulted in the development of multiple kinase inhibitors. We are currently facing significant limitations in implementing these kinase inhibitors into the clinic for the treatment of pediatric malignancies. As many hallmarks of cancer are known to be regulated by intracellular protein signaling networks, we suggest to focus on these networks to improve the implementation of kinase inhibitors. This viewpoint will provide a short overview of currently used strategies for the implementation of kinase inhibitors as well as reasons why kinase inhibitors has unfortunately not yet resulted in the widespread use for the treatment of pediatric cancers. We argue that using a future personalized medicine strategy, combining kinomics, proteomics, and drug screen approaches, the gap between pediatric cancers and the use of kinase inhibitors may be bridged.
INTRODUCTION

Cancer is the second most common cause of death among children living in developed countries. The current incidence of childhood cancers is 189.5 per million children and this incidence is increasing with approximately 0.6% each year. Although the five-year overall survival rates are ranging around 80%, every year, nearly 2000 children die due to cancer in the United States of America.

The introduction of chemotherapy for childhood leukemia in the beginning of the 1950s was a remarkable improvement for cancer research. However, it took until 1963 and early 1970 before the first patients with acute childhood leukemia and advanced Hodgkin’s lymphoma were cured using a combination of chemotherapeutics. The observed major improvements in outcome obtained over the past few decades, achieved by dose optimization and combination chemotherapy, are nowadays stagnated due to chemotherapy related toxicity. With the introduction of tyrosine kinase inhibitors (TKIs), like imatinib (Gleevec®), a new era of cancer therapy emerged. Imatinib transformed chronic myeloid leukemia (CML) from a fatal disease to a leukemic subtype with a favorable prognosis. During the last decade, a rapid increase in the development of small molecule inhibitors and monoclonal antibodies enabled the availability for therapeutic intervention. In 2014, the US Food and Drug administration (FDA) approved 41 new drugs, of which 2 protein kinase inhibitors for cancer indications (e.a., iselalisib and ceritinib, Table 1). Today, 29 protein kinase inhibitors are FDA-approved for the treatment of cancer (Table 1). Remarkably, main targets of these approved protein kinase inhibitors are limited to the BCR-ABL kinase (6 inhibitors), members of the ErbB-family receptor tyrosine kinases, especially EGFR (5 inhibitors), the ALK kinase (2 inhibitors), and the BRAF kinase (4 inhibitors), all frequently mutated in types of adult-onset cancer.

Protein kinase inhibitors suppress the activity of kinases, enzymes catalyzing protein phosphorylation by transferring phosphate groups from adenosine triphosphate (ATP) to specific proteins. Protein kinases are attractive targets for cancer therapy as the malignant transformation of cells highly depends on deregulated kinase-mediated signal transduction pathways; intracellular signaling cascades involving protein phosphorylation events regulating critical cellular processes.

Focusing on FDA-approved protein kinase inhibitors for children revealed an approval of only 3 inhibitors (Table 1). To date, several drugs that have been approved for the treatment of adult malignancies are often only prescribed off-label for the treatment of pediatric cancer patients. However, the extrapolation of clinical trial results obtained from treating adult patients towards pediatric cancer patients is often inappropriate. First, malignancies in children are different compared to adult malignancies. Secondly, medications metabolize differently in children compared to adults, resulting in unpredictable treatment responses and side effects in children. Pediatric drug testing is problematic for a number of reasons. Clinical trials in children are restricted to diseased children for who a minimal benefit of participating in the clinical trials is expected.
trial should be achieved. Furthermore, in contrast to trial participation in adults, parents and pediatricians are usually more concerned about the risks and benefits for the individual child. The most important reason why clinical trials in children have been hampered, is the limited number of patients eligible for clinical trials, since pediatric cancer is relatively rare. Moreover, as a consequence of these low patient numbers, the pharmaceutical industry is less interested in funding clinical trials in children, since pediatric clinical trials are costly and the financial profit is minimal. Nonetheless, we have to prevent that ineffective and potential harmful interventions are subjected to pediatric oncology patients before they have been properly tested.

To improve pediatric medicine, pediatric regulations came into force in the European Union in 2007 and the Pediatric Investigation Plan (PIP) was launched; a research and development program aimed at ensuring the generation of data required to determine the conditions in which a compound may be authorized to treat the pediatric population. As a reward for participating in the PIP, pharmaceutical companies gain patent extension. The introduction of these regulations has resulted in more pediatric clinical trials, an increase in available drugs authorized for pediatric indications, and prevented that children are subjected to unnecessary studies. Nevertheless, still only 3 protein kinase inhibitors are approved for the treatment of pediatric malignancies.

To summarize the current problem, on the one hand we have a multitude of small molecule inhibitors including protein kinase inhibitors (either FDA approved or still in the pipelines of pharmaceutical companies), and on the other hand we have a number of children with untreatable cancer. Since we face limitations implementing these kinase inhibitors for the treatment of pediatric malignancies, many potentially useful drugs remain unused. This viewpoint will provide (1) a short overview of study strategies, including genome and transcriptome profiling, kinome and proteome profiling, and drug screen approaches, currently used to gain insight into intracellular signaling networks which are of potential interest for the introduction of new treatment options, and (2) highlights the reasons why kinase inhibitors are unfortunately not commonly used for the treatment of pediatric cancer. Lastly, we will propose a personalized medicine strategy by combining kinomics, proteomics, and drug screens aiming to bridge the gap between pediatric cancers and the use of kinase inhibitors.
Table 1 FDA approved protein kinase inhibitors for the indication of cancer until April 1st 2015\textsuperscript{a}

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade name</th>
<th>Target</th>
<th>FDA approval date</th>
<th>FDA approved indications</th>
<th>FDA approval date for children</th>
<th>FDA approved indications for children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afatinib</td>
<td>Gilotrif</td>
<td>EGFR, HER2, HER4</td>
<td>July 2013</td>
<td>Metastatic NSCLC (EGFR\textsuperscript{+})</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Axitinib</td>
<td>Inlyta</td>
<td>VEGFR1/2/3, PDGFR, c-KIT</td>
<td>January 2012</td>
<td>Advanced RCC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bosutinib</td>
<td>Bosulif</td>
<td>Bcr-Abl, Src, Lyn, Hck</td>
<td>September 2012</td>
<td>Resistant CML (Ph\textsuperscript{+})</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cabozantinib</td>
<td>Cometriq</td>
<td>FLT3, c-KIT, c-MET, RET, VEGFR1/2/3, TrkB, Axl, Tie2</td>
<td>November 2012</td>
<td>Progressive metastatic MTC</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Crizotinib</td>
<td>Xalkori</td>
<td>ALK, c-MET, ROS1</td>
<td>August 2011</td>
<td>Metastatic NSCLC (ALK\textsuperscript{+})</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ceritinib</td>
<td>Zykadia</td>
<td>ALK, IGF-1R, InsR, ROS1</td>
<td>April 2014</td>
<td>Metastatic crizotinib resistant NSCLC (ALK\textsuperscript{+})</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Dabrafenib</td>
<td>Tafinlar</td>
<td>BRAF</td>
<td>May 2013</td>
<td>Unresectable or metastatic melanoma (BRAF V600E\textsuperscript{+})</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Sprycel</td>
<td>Bcr-Abl, Src, Lck, Yes, Fyn, c-KIT, EphA2, PDGFRb</td>
<td>June 2006</td>
<td>Imatinib resistant CML (Ph\textsuperscript{+})</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>Tarceva</td>
<td>EGFR</td>
<td>November 2004</td>
<td>Metastatic NSCLC (EGFR\textsuperscript{+})</td>
<td>Unresectable or metastatic pancreatic cancer</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 1 FDA approved protein kinase inhibitors for the indication of cancer until April 1st 2015\textsuperscript{a} (Continued)

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade</th>
<th>Target</th>
<th>FDA approval date</th>
<th>FDA approved indications</th>
<th>FDA approval date for children</th>
<th>FDA approved indications for children</th>
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</thead>
<tbody>
<tr>
<td>Everolimus</td>
<td>Afinitor</td>
<td>mTOR, FKBP12</td>
<td>March 2009</td>
<td>Unresectable SGCA (associated with TS)</td>
<td>October 2010</td>
<td>Unresectable SGCA (associated with TS)</td>
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<tr>
<td></td>
<td>(Novartis)</td>
<td></td>
<td></td>
<td>PNET</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Advanced BC (HR\textsuperscript{+}, HER2\textsuperscript{-})</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Advanced RCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Iressa</td>
<td>EGFR</td>
<td>May 2003</td>
<td>Advanced or metastatic NSCLC</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td></td>
<td>(AstraZeneca)</td>
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<tr>
<td>Ibrutinib</td>
<td>Imbruvica</td>
<td>BTK</td>
<td>November 2013</td>
<td>CLL</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td></td>
<td>(Pharmacycics and J&amp;J)</td>
<td></td>
<td></td>
<td>MCL</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Relapsed CLL</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Relapsed B-cell FL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Relapsed small lymphocytic lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idelalisib</td>
<td>Zydelig</td>
<td>PI3K</td>
<td>July 2014</td>
<td>Relapsed CLL</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(Gilead Sciences)</td>
<td></td>
<td></td>
<td>MCL</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imatinib mesylate</td>
<td>Gleevec</td>
<td>Bcr-Abl, c-KIT, PDGFR</td>
<td>May 2001</td>
<td>CML (Ph\textsuperscript{+})</td>
<td>September 2006</td>
<td>CML (Ph\textsuperscript{+})</td>
</tr>
<tr>
<td></td>
<td>(Novartis)</td>
<td></td>
<td></td>
<td>ALL (Ph\textsuperscript{+})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unresectable or metastatic GIST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lapatinib</td>
<td>Tykerb</td>
<td>EGFR, HER2</td>
<td>March 2007</td>
<td>Advanced or metastatic BC (HER2\textsuperscript{-})</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(GlaxoSmithKline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Table 1 FDA approved protein kinase inhibitors for the indication of cancer until April 1st 2015\(^a\) (Continued)

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade</th>
<th>Target</th>
<th>FDA approval date</th>
<th>FDA approved indications</th>
<th>FDA approval date for children</th>
<th>FDA approved indications for children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenvatinib</td>
<td>Lenvima (Eisai)</td>
<td>VEGFR2/3</td>
<td>February 2015</td>
<td>Recurrent, metastatic, progressive, radioactive iodine-refractory, differentiated TC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>Tasigna (Novartis)</td>
<td>Bcr-Abl, PDGFR</td>
<td>October 2007</td>
<td>CML (Ph(^+)) Imatinib resistant CML-AP and CML-BC (Ph(^+))</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Palbociclib</td>
<td>Ibrance (Pfizer)</td>
<td>CDK4, CDK6</td>
<td>February 2015</td>
<td>Advanced BC (ER(^+), HER2(^-))</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pazopanib</td>
<td>Votrient (GlaxoSmithKline)</td>
<td>VEGFR1/2/3, PDGFR, c-KIT, FGFR1/3, Lck, Fms, Ltk</td>
<td>October 2009</td>
<td>Advanced STS Advanced RCC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ponatinib</td>
<td>Iclusing (Ariad)</td>
<td>Bcr-Abl, Bcr-Abl T3151, FGFR, FLT3, VEGFR, PDGFR, Eph, Src, c-KIT, RET, Tie2</td>
<td>December 2012</td>
<td>Resistant or T3151(^+) CML (Ph(^+)) Resistant or T3151(^+) ALL (Ph(^+))</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Regorafenib</td>
<td>Stivarga (Bayer)</td>
<td>VEGFR1/2/3, Bcr-Abl, BRAF, c-KIT, PDGFR, RET, FGFR1/2, Tie2, Eph2A</td>
<td>September 2012</td>
<td>Advanced, unresectable or metastatic GIST Metastatic CRC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>Jakafi (Novartis)</td>
<td>JAK1, JAK2</td>
<td>November 2011</td>
<td>High-risk Myelofibrosis</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 1 FDA approved protein kinase inhibitors for the indication of cancer until April 1st 2015a (Continued)

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade (Company)</th>
<th>Target</th>
<th>FDA approval date</th>
<th>FDA approved indications</th>
<th>FDA approval date for children</th>
<th>FDA approved indications for children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorafenib</td>
<td>Nexavar (Bayer)</td>
<td>BRAF, c-RAF, c-KIT, FLT3, RET, VEGFR1/2/3, PDGFR</td>
<td>December 2005</td>
<td>Advanced RCC Unresectable HCC Recurrent, metastatic or progressive DTC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>Sutent (Pfizer)</td>
<td>PDGFR, VEGFR1/2/3, c-KIT, FLT3, CSF-1R, RET</td>
<td>January 2006</td>
<td>Progressive PNET Advanced RCC Progressed or imatinib resistant GIST</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Temsirolimus</td>
<td>Torisel (Wyeth Pharmaceuticals)</td>
<td>mTOR, FKBP12</td>
<td>May 2007</td>
<td>Advanced RCC</td>
<td>May 2012</td>
<td>Advanced or recurrent solid cancers</td>
</tr>
<tr>
<td>Trametinib</td>
<td>Mekinist (GlaxoSmithKline)</td>
<td>MEK1/2</td>
<td>May 2013</td>
<td>Unresectable or metastatic melanoma (BRAF V600E or V600K)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>Caprelsa (AstraZeneca)</td>
<td>EGFR, RET, VEGFR2, Brk, Tie2, EphR, Src</td>
<td>April 2011</td>
<td>Symptomatic and progressive MTC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>Zelboraf (Roche and Plexxikon)</td>
<td>A/B/C-RAF, BRAF</td>
<td>Augustus 2011</td>
<td>Unresectable or metastatic melanoma (BRAF V600E)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Vismodegib</td>
<td>Erivedge (Genentech Inc)</td>
<td>Smo</td>
<td>January 2012</td>
<td>Advanced or metastatic BCC</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

aData from http://www.fda.gov/Drugs/default.htm and http://www.fda.gov/ScienceResearch/SpecialTopics/PediatricTherapeuticsResearch/default.htm
Abbreviations Table 1: NA= Not Applicable; EGFR= Endothelial Growth Factor Receptor; HER= Human Epidermal Growth Factor Receptor; NSCLC= Non-Small-Cell Lung Cancer; VEGFR= Vascular Endothelial Growth Factor Receptor; PDGFR= Platelet Derived Growth Factor Receptor; RCC= Renal Cell Carcinoma; CML= Chronic Myeloid Leukemia; Ph= Philadelphia chromosome; FLT= Fms-like tyrosine kinase; MTC= Medullary Thyroid Cancer; ALK= Anaplastic Lymphoma Kinase; ROS1= c-Ros Oncogene 1; IGF-1R= Insuline-like Growth Factor 1 Receptor; InsR= Insulin Receptor; EphA2= Ephrin type-A receptor 2; SGCA= Subependymal Giant Cell Astrocytoma; TS= Tuberous Sclerosis; PNET= Pancreatic Neuroendocrine Tumors; BC= Breast Cancer; HR= Hormone receptor; HER2; Human Epidermal Growth Factor Receptor 2; BTK= Bruton's tyrosine kinase; CLL= Chronic Lymphoid Leukemia; MCL= Mantle Cell Lymphoma; PI3K= phosphoinositide 3-kinase; B-cell FL= Follicular B-cell non-Hodgkin Lymphoma; SLL= Small Lymphocytic Lymphoma; ALL= Acute Lymphoblastic Leukemia; GIST= Gastrointestinal Stromal Tumors; TC= Thyroid Cancer; CML-AP= Accelerated Phase CML; CML-BC= Blast Crisis CML; CDK= Cyclin Dependent Kinase; ER= Estrogen Receptor; STS= Soft Tissue Sarcoma; FGFR= Fibroblast Growth Factor Receptor; CRC= Colorectal Cancer; HCC= Hepatocellular Carcinoma; DTC= Differentiated Thyroid Carcinoma; CSF-1R= Colony Stimulating Factor 1 Receptor; EphR= Ephrin Receptor; Smo= Smoothened; BCC= Basal Cell Carcinoma.

HALLMARKS OF CANCER

Cancer can arise in different organs, tissues and cell types, all with a distinct disease presentation and outcome. Several characteristics are shared throughout different cancers. These characteristics, or key hallmarks of cancer, were established by Hanahan and Weinberg presenting the complexity and capabilities of cancer cells.¹³,¹⁴ The hallmarks of cancer comprise: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, avoiding immune destruction, tumor-promoting inflammation, genome instability and mutation, and deregulating cellular energetics. These diverse processes have in common that they provide a growth advantage of the cancer cell compared to its normal counterpart. Recent technologies such as genome-wide genetic and transcriptional analysis using next-generation sequencing revealed the mutational landscape of many adult and pediatric cancers.¹⁵-¹⁸ These thorough analyses led to the discovery that in general, pediatric cancers exhibit fewer mutations than adult cancers, and that within specific types of cancer there is a high variability of the mutations present.¹⁵ This knowledge leaves us with the thought that CML, harboring one unique and uniform driver mutation (namely, BCR-ABL), is in fact an exceptional situation. In other malignancies, the pathobiology is more complex. For example, despite intensive genome and transcriptome profiling, the majority of the pediatric acute lymphoblastic leukemia (ALL) cases remain without explanation of precise genetic etiology.¹⁸ Therefore, the question must be asked how to bridge the gap from insights in the hallmarks of cancer to the use of available kinase inhibitors that are on the market?

In addition to genetic alterations, epigenetic alterations and the influence of microenvironmental factors can contribute to oncogenesis and disease progression. Many of these alterations
ultimately support somatic cells to escape the restraints that normally withhold them from unlimited cell proliferation. This growth advantage is the net result of aberrant activated signal transduction pathways. Within cancer cells, the above mentioned hallmark capabilities are regulated by highly interconnected intracellular signaling networks. Therefore, to understand how specific kinase inhibitors may affect cancer hallmarks, more insights into the key proteins within the intracellular signaling networks to which these drugs can counteract are needed. Currently, many recent studies focus on generating insights into signal transduction networks as a final common pathway of various cancer hallmarks that are translated into cancer cell progression.

SHORT OVERVIEW OF STRATEGIES USED FOR THE IDENTIFICATION OF TREATMENT OPTIONS

Genome and transcriptome profiling

In the last decade, genome and transcriptome sequencing have improved our understanding of human cancers significantly. These cancer studies have revealed a set of 138 genes that, when altered by mutations, can promote oncogenesis. Additionally, mutations can be theoretically distinguished in “driving mutations”, mutations that confer a selective growth advantage, or “passenger mutations”, mutations that have no effect on neoplastic processes. In practice, it is difficult to determine which mutations drive oncogenesis and/or contribute to malignant transformation or progression. This is partially due to the fact that some mutations require collaborative mutations to enable oncogenic transformation. Importantly, comprehensive sequencing efforts have revealed genetic alterations that are now being treated with specific kinase inhibitors. This started with BCR-ABL inhibitors for the treatment of CML and has continued with, for example, ALK translocations and EGFR mutations in non-small-cell lung cancer and BRAF mutations in melanoma (Table 1), as well as genetic alterations currently tested in clinical trials such as FLT3-ITD and JAK mutations in pediatric leukemias. Unfortunately, although genome and transcriptome profiling has increased our understanding of oncogenesis and improved outcome for several malignancies, there are still malignancies, especially in children, in which the oncogenic alterations that drive cancer progression are largely unknown. Consequently, kinase inhibitors directed to specific driving mutations cannot be used for the treatment of most pediatric malignancies. Moreover, the combination of gene expression signatures and anticancer drug sensitivity patterns provided inconsistent results. While some cancers with known mutations expected to be sensitive towards specific inhibitors proved indeed to be highly sensitive, in other cancers, harboring the same mutation, specific inhibitors presented minor anticancer responses or respond only for a short period of time. Additionally, the group of Clevers recently showed a gene-drug association of only ~1% between individual oncogenic mutations and drug response in adult colorectal carcinoma patients. Therefore, additional study strategies are required for the identification of suitable targets for therapy.
Kinome and proteome profiling

As genetic and epigenetic alterations and / or microenvironmental factors ultimately influence the activation of intracellular signaling networks, insight into these intracellular signaling pathways might be a potent strategy to identify targetable signaling hubs for the treatment with kinase inhibitors (Figure 1). Current techniques to study protein phosphorylation include for example high-throughput techniques as reverse phase protein arrays (RPPA), more intensive analysis by mass-spectrometry or single cell probe-based flow cytometry. Additionally, the activity of kinases might be studied by for example high-throughput peptide based kinase activity arrays. We recently provided an extensive overview of the pros and cons for different proteomic techniques that aim to assess protein kinase activation and protein phosphorylation. Elucidating signaling networks to identify suitable targets for therapy might be valuable particularly for children since pediatric cancers harbor fewer mutations compared to adult cancers. In the recent years, our lab focused on using comprehensive kinome and proteome profiling to identify signaling networks as well as potential druggable targets for various pediatric malignancies. These studies showed that kinome profiling is an elegant approach to identify therapeutic targets by elucidating signaling pathways for common pediatric cancers, e.g. leukemias (ALL and acute myeloid leukemia (AML)) and brain tumors. For example, kinome and proteome profiling revealed c-AMP responsive element binding protein (CREB) activity in pediatric ALL, MEK activation in pediatric MLL-rearranged AML, Src activity in pediatric brain tumors, and a role for the Eph / ephrin signaling in pediatric medulloblastoma. All these findings were validated using in vitro cytotoxicity screens that confirmed their potential as a therapeutic target. More importantly, we showed that the combination of kinome and proteome profiling is a powerful prediction approach for signaling pathway adaptations and redundant pathway discovery upon single targeted therapy and can be used to define rational combination therapies. For example, this approach revealed activity of the MAPK and PI3K/Akt/mTOR signaling pathways in pediatric MLL-rearranged AML and predicted that a sustained PI3K/Akt/mTOR pathway activation enabled a subpopulation of cells to survive MEK inhibition.

![Image](image.png)

**Figure 1**
Shown is an illustration explaining why insight into intracellular signaling pathways might be a potent strategy to bridge the gap between pediatric malignancies and the use of available kinase inhibitors.
Drug screen approaches

Drug screening and genetic knockdown approaches, such as high throughput RNAi and kinase inhibitor screens, have been used to define kinase pathway dependence.\textsuperscript{33-37} These strategies created patient specific \textit{in vitro} sensitivity profiles against specific kinase targets by treating adult primary leukemia cells with siRNA or kinase inhibitors.\textsuperscript{35,37} Two important conclusions could be drawn based upon these results, namely (1) there is a great heterogeneity in predicted kinase targets between patients, even within similar diagnostic subgroups and (2) oncogenic mechanism for predicted therapeutic targets could not be elucidated based upon underlying genomic alterations.\textsuperscript{33,35} For example, gene-silencing identified an upregulation of receptor tyrosine kinase-like orphan (RO)R1 expression in t(1;19)-positive pediatric ALL patients, a mechanism based upon pre-B-cell receptor signaling inhibition rather than ROR1 activating mutations or aberrant transcription profiles.\textsuperscript{38} Most importantly, drug screens showed that targeting intracellular signaling pathways is a feasible therapeutic option.

As a proof of concept, Tyner and Pemovska used the results from their drug screen to treat adult leukemia patients not eligible to standard treatment options using FDA-approved kinase inhibitors \((N = 1\) and \(N = 8\), respectively).\textsuperscript{35,37} The initial results were very promising, showing rapid decreases in white blood cell counts and bone marrow blast counts in the majority of the patients. However, effects were only short lasting; within months patients relapsed after personalized kinase inhibitor treatment.\textsuperscript{35,37} Repeated drug screens, of the relapsed leukemia samples, showed resistance to the initially used kinase inhibitors as compared to their corresponding pretreatment samples.\textsuperscript{35,37} These examples illustrate why most long-term clinical results of kinase inhibitors are disappointing when using mono-therapy.\textsuperscript{39} Innate or acquired cellular resistance to kinase inhibitors are a major clinical challenge.\textsuperscript{24,35,37,40}

\section*{Resistance to Kinase Inhibitors}

Several mechanisms of cancer cell resistance to kinase inhibitors have been described. First of all, advanced alterations in the present mutation, for example new kinase domain mutations, confer resistance to kinase inhibitors by decreasing the efficiency of the inhibitor.\textsuperscript{41} A classic example is BCR-ABL1 kinase domain mutations decreasing the sensitivity to imatinib in CML.\textsuperscript{42} Secondly, newly acquired alterations might circumvent the inhibitory effect of a given drug; for instance, the accumulation of various new genetic abnormalities in CML result in the activation of signaling pathways independent of BCR-ABL activity and consequently facilitates disease progression to blast crisis.\textsuperscript{41,43,44} Similarly, mutations in MEK1 can confer resistance to BRAF inhibition.\textsuperscript{45}

Thirdly, therapy resistance can be mediated by cellular adaptations through dynamic
reprogramming, e.g. the activation of alternated routes of kinase pathway activation in response to pharmacological inhibition. Cellular adaptation by dynamic reprogramming is an important challenge for the implementation of kinase inhibitors and can occur by either the reactivation of the targeted pathway or via bypass opportunities through the activation of alternative signaling pathways. An example of reactivation of the targeted pathway is \textit{BRAF} (V600E)-positive melanoma. These cells can acquire resistance to vemurafenib by reactivating the MAPK pathway via N-RAS upregulation. Dynamic reprogramming might also result in the activation of alternative signaling pathways; for instance, by the upregulation of RTK-ligand levels that has been frequently observed following kinase inhibition and is able to activate downstream highly interconnected intracellular signaling pathways, most notably the PI3K/Akt/mTOR or MAPK signaling networks. In addition, resistance to the \textit{BRAF} inhibitor in colorectal carcinoma might be due to the activation of the PI3K/Akt/mTOR signaling pathway. Finally, intratumor heterogeneity can also restrict the implementation of targeted therapy. Intratumor heterogeneity may lead to inferior therapeutic responses to kinase inhibitors, since it has been established that the outgrowth of a therapy resistant subclone(s) can lead to refractory or relapsed disease.

**FUTURE STRATEGY FOR CLINICAL TRIALS IN PEDIATRIC ONCOLOGY**

Taken together, this viewpoint has highlighted currently used strategies for the implementation of kinase inhibitors as well as reasons why kinase inhibitors has unfortunately not yet resulted in the widespread use in pediatric cancer therapy. In this paragraph, we will propose a personalized medicine strategy attempting to improve the implementation of kinase inhibitors in pediatric cancer.

We argue that establishing the active intracellular signaling pathway networks in cancer patient samples will be a suitable strategy in deciding which kinase inhibitors (either FDA approved or in the pipelines of pharmaceutical companies) should be used to target the cancer cell (Figure 1). As previously mentioned, drug screens have initially showed short-term promising results towards this end. Further, we have demonstrated that kinome and proteome profiling is an elegant approach to identify potential druggable targets in pediatric malignancies. Additionally, we showed that this strategy is able to predict signaling pathway adaptations which can be used to define rational combination therapies, as shown for combined MEK and VEGFR-2 inhibition in pediatric MLL-rearranged AML. Combining these kinomics and proteomics study approaches with a comprehensive drug screen can define major contributing protein kinases relevant for cancer cell survival (Figure 2). Following upon initial tumor characterization, we propose to perform kinome and proteome profiling to determine networks of active signaling pathways, which enables to extract key signaling hubs and also provides insights how to predict possible cancer cell bypass mechanisms based upon signaling availability.
Chapter 2

Tumor characterization

- Morphology
- Immunophenotype / Immunohistochemistry
- Cytogenetic analysis
- Mutations and copy number alteration analyses

Kinomics

Proteomics

Drug screen

Signaling dynamics

Drug sensitivity patterns

Rational selection of combination therapies

Treatment effects on signaling pathways

Kinomics

Proteomics

Bypass signaling dynamics

Data integration

Optimal personalized treatment strategy
Figure 2
Visualization of a future personalized medicine strategy attempting to improve the implementation of kinase inhibitors in pediatric cancer. After initial tumor characterization, we propose to perform kinome and proteome profiling on patient samples, as well as subject patient cells to a drug screen including multiple kinase inhibitors (either FDA approved or in the pipelines of pharmaceutical companies) to characterize their patient specific cancer profile. Integrating these results will define rational combination therapies. In order to determine treatment effects on signaling, kinome and proteome profiles will be re-determined after in vitro treatment with potential combination therapies. Ultimately, data integration of all these multilevel study elements will result in a comprehensive network of pre-treatment active signaling pathways, putative targets for targeted therapy, and subsequent post-treatment drug induced bypass mechanisms for cellular resistance.

Additionally, cancer cells will be subjected to a drug screen containing drugs in current use for cancer treatment, drugs previously investigated in or currently undergoing clinical trials, and experimental compounds to characterize cancer cell specific drug sensitivity patterns. While drug screens are relatively easy to perform for hematological malignancies, the implementation of drug screens for solid tumors is more challenging but not impossible. Recently, it has been shown that an organoid culture platform can be used for functional drug screening assays of solid cancers. This model also reflects the polyclonality of tumors enabling a suitable predictive model to define cytotoxic responses to therapy at the level of the individual patient. Integrating the kinome and proteome profiles together with drug sensitivity profiles into one network will generate an overview of highly active signaling pathways including the corresponding putative novel targets for therapy (highlighted in the network, Figure 2). Based on this network, rational combination therapies could be defined by selecting suitable targets from different signaling pathways.

Since cellular dynamic reprogramming and intratumor heterogeneity are major challenges for the implementation of kinase inhibitors, insight into the adaptive kinome responses and subclonal resistance to kinase inhibitors is essential. By doing so, one can anticipate on mechanisms of resistance. As it has been shown that redundant signaling pathways as well as signaling profiles of minor subclones are not per se detectable at the time of diagnosis and might become more prominent after treatment with specific kinase inhibitors, it will be necessary to re-determine cellular dynamics of signaling pathway activation after in vitro treatment with selected combination therapies (Figure 2). This second network analysis of signaling pathways might reveal cellular adaptations by activating signaling events that can facilitate therapeutic resistance.

Integration of all these multilevel study elements will generate a comprehensive network of pre-treatment active signaling pathways, putative targets for targeted therapy, and subsequent post-treatment drug induced bypass mechanisms for cellular resistance. If necessary, the initial selected combination therapies can be modified, to circumvent drug-induced bypass signaling pathways and to select an optimal therapeutic strategy in advance. Extrapolation
of this proposed *in vitro* model to an *in vivo* model increases the translational feasibility of the preclinical treatment screening which is highly desirable since only 5% of the identified putative anticancer compounds present sufficient clinical activity in phase III trials. In the meantime, optimization of *in vitro* models, for example by tumor organoid cultures, is of great importance to improve preclinical models for drug testing.

One additional problem that we have to overcome is the low number of pediatric patients eligible for clinical trials. We have noticed an overlap of recurrent active signal transduction pathways within different subtypes of cancer. Furthermore, the kinase inhibitor screen of Tyner *et al.* showed no complete segregation based upon leukemia subtypes. Therefore, we propose that all children suffering from cancer without evidence-based treatment options are eligible to enroll in this study strategy. Combining different patient populations allows studying the mechanism of signal transduction adaptations and the rational design of combination therapies in a significant larger cohort of children. More importantly, a trial including children with comparable signaling dynamics will provide information about the optimal biological dose for the kinase inhibitor; the dose that produces a quantifiable effect in inhibiting the target in the cancer cells (primary endpoint). Additionally, pharmacokinetics, pharmacodynamics, side effects, and toxicity spectrum of the specific inhibitor in pediatric oncology patients should be included as important objectives. Moreover, while the primary objective of the proposed study design is: bridging the gap between pediatric cancers and newly designed kinase inhibitors to the improve survival of children without evidence-based treatment options, consequent studies regarding the long-term effects of kinase inhibitors on energy metabolism, growth and bone mineral density, gonadal function and reproduction, and cardiac health are warranted.

Finally, since the continuous development of new study approaches is essential for the implementation of targeted therapies, we expect that the proposed pre-clinical screening strategy should incorporate additional novel methods according to new developments. This integrated multilevel screen might easily be developed further to an integrated model of genome, kinome, and proteome profiling, supported with networks of cell-cell and cell-stroma interactions. In conclusion, despite initial disappointing results of kinase inhibitors in clinical trials, we propose that available kinase inhibitors holds tremendous promise for most malignancies when using novel selective combinations of therapeutic interventions. In this viewpoint, we illustrate a personalized medicine strategy combining kinomics and proteomics approaches with a comprehensive drug screen to define rational combination therapies that may bridge the gap between pediatric cancers and the implementation of kinase inhibitors.

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