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The Paradigm of Targeting an Oncogenic Tyrosine Kinase: Lesson from BCR-ABL

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Abstract

The aberrant tyrosine phosphorylation, either due to constitutive tyrosine kinases (TKs) or to inactivation of protein tyrosine phosphatases (PTPs), is a widespread feature of many cancerous cells. The BCR-ABL fusion protein, which arises from the Philadelphia chromosome, is a molecular distinct and peculiar trait of some kind of leukemia, namely Chronic Myeloid and Acute Lymphoblastic Leukemia, and displays constitutive tyrosine kinase activity. In the chapter, we will highlight the milestones that had led to the identification of the BCR-ABL fusion gene and its role as the only molecular pathogenic event sufficient to elicit and sustain chronic myeloid leukemia. We will also discuss the effort made to unveil the molecular mechanisms of action of the chimeric tyrosine kinase that eventually lead to aberrant cell proliferation and impaired cell-death. Furthermore, we will also review the lesson learned from the selective inhibition of BCR-ABL which currently represent a breakthrough in the treatment of several tumors characterized by defective tyrosine kinase activity.

Keywords: chromosomal translocation, fusion gene, tyrosine kinase, leukemia, tyrosine kinase inhibitors, targeted therapy

1. Introduction

The up-regulated enzymatic activity of tyrosine kinases (TKs) is one of the most frequent events in human cancers. Basically, it is attributable to three distinct molecular-genetic mechanisms covering either the overexpression, the activating mutations or eventually chromosomal translocations involving tyrosine kinase genes. Therefore, targeting the kinases harboring oncogenic properties has led to prominent changes in cancer clinical management. An outstanding contribute in achieving the goal has been offered by the BCR-ABL oncogene, whose story started more than half a century ago. In the 1960s a couple of scientists working in Philadelphia described a major chromosomal abnormality in patients affected by Chronic Myeloid Leukemia (CML) [1, 2]. The chromosomal aberration consisted of an acrocentric chromosome that was originally thought as the outcome of a chromosomal deletion. At that time, it was the first chromosomal abnormality unambiguously associated to a specific malignancy. With the improvement of the chromosomal banding techniques, it became clear that the chromosome

abnormality was a shortened chromosome 22. Among a chorus of skepticism and wonder at the beginning of the seventies that short chromosome, that it is now known as Philadelphia chromosome (Ph), was identified as the product of a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22). However, we had to wait until the eighties to know that the exact molecular consequence of the t(9;22) was a fusion gene encoding for a chimeric protein displaying constitutively tyrosine kinase activity. Altogether these discoveries delivered an outstanding message whereby a disease was tightly linked to a single oncogene, BCR-ABL. Since then, dozens of translocations have been found in other cancers, including acute promyelocytic leukemia.

BCR-ABL is a peculiar protein for several of reasons: 1) it is a chimeric protein that is encoded by a fusion gene deriving from a reciprocal chromosomal translocation; 2) it is a constitutively active tyrosine kinase eliciting oncogenic signals, 3) it has been the first oncogene associated to a disease displaying dual properties either as driver and in sustaining the neoplasm evolution, and 4) it has been the first kinase to be selectively targeted with small molecules, thus paving the way for the development of a number of tyrosine kinase inhibitors (TKIs).

In the present chapter we are going to discuss the milestones of a story, started 60 years ago, which has happily led to the selective pharmacological inhibition of BCR-ABL. Hence, CML, whose diagnosis was before a death sentence, is now successfully cured in the vast majority of the cases.

2. Is there any specific reason underlying the generation of the Ph chromosome?

Basically, every chromosomal translocations require DNA Double-strand breaks (DSBs) in two different locations and that the broken ends of non-homologous chromosomes are fused together. DNA double-strands breaks might be due to different causes (*e.g.* ionizing radiation, reactive oxygen species, DNA replication across a nick, malfunctioning of DNA metabolic enzymes such as type II DNA topoisomerase or RAG complex during illegitimate V(D)J recombination). Cells to preserve their genome integrity upon DNA damage respond by activating a repair machinery that should catalyzes the joining of the broken ends [3]. However, the outcome of the joining process leads to a variety of rearrangement. For instance, precise joining of broken ends can generate a normal chromosome. Inversions, deletions and duplications can occur when joining involves two broken ends on the same chromosome. Non-Homologous End Joining (NHEJ) is often imprecise; thus some nucleotides may be lost during the joining process. Eventually, translocations may occur when the broken ends of two non-homologous chromosomes are joined together thus leading to novel chromosomes containing part of normal chromosomes [4].

Aside these notions, currently our knowledge regarding the molecular mechanisms responsible for the reciprocal chromosomal translocation occurring between the chromosome 9 and the 22, t(9;22), generating the Philadelphia chromosome (Ph), remain still rather elusive. Fundamentally, it has been speculated that there are two plausible hypotheses. One view prefers to lean towards an entirely random “breaking and re-ligating process” occurring with relatively similar frequency between any two chromosomes within a cell. Chromosomal translocations that give and adaptive advantage are pretty rare and associated with negative consequences (*e.g.* cancer). The success of the t(9;22) can be explained by the fact that, the resulting fusion gene encodes for a protein with transforming properties conferring selective fitness advantages to the host cell. Conversely, by virtue of this, any

other chromosomal rearrangement that does not satisfy the requisite for survival and expansion will be handicapped and thus will soon disappear. Though, at this time there are no experimental indications ruling out this view it is worthwhile to notice that the juxtaposition of the ABL and BCR genes has been observed in nuclei of human hematopoietic cells over the S/G2 phases and through the whole G2 phase up to the middle of the M phase (*i.e.* metaphase stage) [5]. Ultimately, there is no conclusive evidence that DNA sequences potentially relevant to chromosomal translocations, such as the Alu repeats or Chi-like octamers, are present around the BCR-ABL rearrangement [6]. Hence, by now the only trustable conviction is that the exchange between two chromosomal positions implies that they must be physically close to each other at the time the event occurs. However, besides the external triggering events (*e.g.* ionizing radiation) the details about what are the molecular players and how they cooperate to the birth of such aberrant chromosome remain to be mechanistically elucidated.

3. What is the consequence of the t(9;22)?

The results of the chromosomal translocation occurring between the chromosome 9 and 22, t(9;22), are a longer chromosome 9 (9q+) and a smaller derivative chromosome 22, the Ph [7, 8]. By the eighties of the past century the molecular characterization of the Ph led to the identification of a novel chimeric gene, BCR-ABL, which later on has been found to encode for a chimeric protein with a constitutive tyrosine kinase activity and with potent oncogenic properties [9, 10]. The c-ABL and Breakpoint of Cluster Region (BCR) loci are localized on the long arm of the chromosome 9 and 22, respectively [11]. Depending on the different breakpoints occurring on the two chromosomes resulting in different BCR-ABL variants. Though, all BCR and c-ABL DNA breakpoints fall within intronic regions those occurring in the BCR gene are highly variable and thus responsible for defining the major differences among the different variants. The variation in the BCR part of the fusion transcript contrasts with the constant c-ABL part. Indeed, all the breakpoints so far identified within the c-ABL gene occur in a large (300-kb) region in the 5' portion of the gene, localized upstream of the exon 2, and generally falling in the intron sequences restricted between the two alternative first exons (1b and 1a). Regardless the structure of the different fusion genes the BCR exons directly fuse to the second c-ABL exon (a2). The most frequent BCR-ABL fusion variant is the p210 in which the BCR exon 13, or 14, is fused downstream of the alternative exons 1 of the c-ABL gene and thus leading to a fusion protein with approximately the first half from BCR and the remaining second half from ABL. Mostly this variant is found in CML patients accounting for approximately 95% of the BCR-ABL fusion gene in all the CML cases. A second frequent variant, p190, is found in approximately 20–30% of adult patients with Acute Lymphoblast Leukemia (ALL) [12] and, very rarely, also in Acute Myeloid Leukemia (AML) [13]. When compared to the p210 variant, in this case the breakpoint within the BCR locus is localized in the 3' half of the first BCR intron, thus encoding for a shorter BCR portion (approximately 425 aminoacids). The third most common BCR-ABL variant, p230, is the largest and is defined by a breakpoint cluster region encompassed between the exons 19 and 21. Whereas the p190 characterizes a more acute form of leukemia usually of lymphoid origin, the latter variant is peculiar of neutrophilic CML. Besides, there are additional BCR-ABL variants, though they have been observed less frequently. Interestingly, some of them are peculiar because they are the results of alternative splicing leading to truncated chimeric proteins that are all lacking tyrosine kinase activity [14]. Furthermore, in hematopoietic malignancies, the BCR gene has been identified

fused to multiple tyrosine kinases encoding genes, other than ABL, including Fibroblast Growth Factor Receptor1 (FGFR1) -t(8;22)- [15, 16], Platelet Derived Growth Factor Receptor A (PDGFRA) -t(4;22)- [17, 18], RET -t(10;22)- [19] and Jak2 -t(9;22)- [20–22] producing different fusion transcripts that are all encoding for cytoplasmic chimeric proteins displaying dysregulated tyrosine kinase enzymatic activity and oncogenic properties. The causal reason behind the commonality of BCR as fusion partner is not well understood. As we have previously discussed it has been speculated that genes such as BCR are located near chromosomal fragile sites that show breaks or gaps on metaphase chromosomes due to replication stress which are prone to breakage and translocation as result. Interestingly, though BCR fusion genes have also been detected in solid tumors, to date BCR fusion proteins that behave as cancer drivers have solely been identified in hematological cancers.

4. Structural features of the different BCR-ABL protein variants

Both c-ABL and BCR are rather large proteins with molecular sizes ranging from 145 to 160 kDa, respectively, and harboring numerous well defined structural conserved domains. The c-ABL is a non-receptor tyrosine kinase harboring several motifs that are required for its own enzymatic activity and to signal to other molecules. Intramolecular interactions occurring between the SH3 domain and the linker peptide connecting the SH2 and the tyrosine kinase domain, alongside with that occurring between the kinase and the SH2 domain, keep c-ABL in a close inactive state [23]. The central part of the protein is characterized by proline-rich (PxxP) stretches acting as docking sites for SH3 containing proteins and a DNA binding domain (DBD). Eventually the carboxy-terminal region contains an actin binding domain (ABD) which allows the interaction either with the monomeric- (G) and with the filamentous- (F) actin. Within cells, the ABL is distributed either in the nucleus and, to a lesser extent, in the cytoplasm where it plays distinct roles. The shuttling between the two compartments is steered by its nuclear-localization and nuclear-export signals and it is depending on different extracellular cues (*e.g.* cell to substratum adherence) [24]. While cytoplasmic c-ABL regulates several actin-dependent cellular processes, for example by positively controlling the filopodia exploration and the membrane ruffling [25], the nuclear c-ABL is a pivotal proapoptotic play-actor, playing a role in the cellular response to genotoxic stress (*e.g.* ionizing radiation) [26].

Alike c-ABL, BCR is a multidomains protein with a peculiarity consisting of a Dbl homology (DH) and a Rho-GAP domain that are localized in the central region and at the C-terminus of the protein, respectively. These domains act as Guanine Exchange Factor (GEF) and GTPase Activating regulatory elements (GAP) for some members of the Rho superfamily, including Cdc42, Rac1, Rac2 and RhoA. Additionally, BCR protein harbors other structural regions, including two lipid binding domains namely Pleckstrin Homology (PH) and Calcium-dependent lipid-binding domain (C2), which is localized in the central part of the protein, and an N-terminal 63 aminoacids long coiled-coil oligomerization peptide that is followed by a Serine/Threonine kinase domain. BCR expression is rather ubiquitous and enriched in brain. Differently from c-ABL, the subcellular localization of BCR is predominantly restricted to the cytoplasmic compartment [27].

The structural composition of BCR-ABL proteins may vary quite a lot depending from which fusion gene breakpoint one refer to (**Figure 1**).

However, the variation is always restricted to the BCR part, while the c-ABL part remains constant in all the different transcript variants. This is in itself an indication that c-ABL is mostly responsible for its transforming properties. Briefly, all BCR-ABL proteins share the same c-ABL part with all the prominent structural

Figure 1.
 Structural features of the different Bcr-Abl protein variants. Linear depiction of the functional motif composition of the different BCR-ABL proteins: p190, p210 and p230. CC: Coiled-coil; S/T kinase: Serine/threonine kinase; DH: Dbl-homology; PH: Pleckstrin homology; C2: Ca^{2+} -dependent membrane-targeting module; Rac-GAP: Rac GTPase; SH3: Src Homology3; SH2: Src Homology2; Tyr kinase: Tyrosine kinase; DBD: DNA binding domain; ABD: Actin binding domain; PXXP: Proline rich region, where X indicates any aminoacid.

features of c-ABL, including SH3, SH2, tyrosine kinase, proline rich regions, DNA-binding and Actin Binding Domains. By contrast, the distinguishing part is represented by the BCR peptide. In the shortest BCR-ABL variant (p190) or alternatively named p185, the BCR portion encodes for a peptide of approximately 490 amino-acids encoded by the first BCR exon, encompassing the very N-terminal coiled-coil oligomerization domain and the serine/threonine kinase domain, fused to the ABL. Conversely, in the longest BCR-ABL variant, p230, the BCR portion harbors all BCR structural domains with the exception of the GAP that is truncated. Eventually, the most common BCR-ABL variant, p210, encodes for a chimeric protein in which the BCR portion comprises the coiled-coil, Ser/Thr, Rho-GEF and PH domains. Crucial for the constitutive activation of the c-ABL tyrosine kinase is the BCR oligomerization domain that promotes either the dimerization or tetramerization of the protein [28]. In this way the BCR-ABL proteins can cross-phosphorylate each other on tyrosine residues in their kinase-activation loops. BCR-ABL phosphorylated tyrosine residues usurp the physiological functions of the normal ABL and can act as docking sites for SH2-domain containing proteins that contribute in activating downstream signaling pathways. On the whole this leads to clear readouts comprising deregulated cellular proliferation, decreased adherence of leukemia cells to the bone marrow stroma and reduced apoptotic response to mutagenic stimuli. Alike the BCR, but differently from the c-ABL protein, strikingly all the BCR-ABL chimeric proteins display a cytoplasmic localization, though all retain both the nuclear-localization and nuclear-export peptide sequences. The main reason for its cytoplasmic localization is its constitutively activated tyrosine kinase activity that thus allows to the chimeric tyrosine kinase to interact and cross talk with a number of proteins, thus exerting its leukemogenic effect. Interestingly, upon BCR-ABL pharmacological inactivation (*i.e.* Imatinib) and concurrent blocking of its nuclear export (*i.e.* leptomycin B) the protein re-localizes within the nuclear compartment and it is trapped there. Astonishingly, upon Imatinib removal and the tyrosine kinase activity of the nuclear BCR-ABL is reactivated it is converted from an antiapoptotic to proapoptotic protein thus inducing cell death [29].

5. “In-vitro” and “in-vivo” tools for the assessment of the BCR-ABL leukemogenic properties

The importance of BCR-ABL in leukemogenesis/neoplastic transformation has been examined in numerous “*in-vitro*” and “*in-vivo*” biological systems, including

immortalized fibroblast cell lines, growth-factor-dependent hematopoietic cell lines, primary bone marrow cells and mice. Though all these models represent very important tools that have significantly contributed to elucidate the molecular mechanisms of CML formation and to identify potential therapeutic targets, each of them display pros and cons either in term of their tractability and physiological relevance. Many cancer cell lines, including leukemia, have been excellent models for “*in-vitro*” studies because of their relative ease in obtaining a large number of cells for biochemical analysis, genetic manipulation and biological examinations. However, they display remarkable limitations, including their failure to recapitulate the physiology of the disease. By contrast animal models are excellent in term of physiological relevance, thus allowing to recapitulate the disease and to assess its potential evolution, but rather deficient in tractability. The product of BCR-ABL is a constitutively active tyrosine kinase that is more active than c-ABL, thus the expression of BCR-ABL transforms established mouse fibroblast cell lines, factor-dependent hematopoietic cell lines and primary bone-marrow cells. Usually, under physiological conditions normal hematopoiesis requires a strict balancing among cellular-proliferation, –growth and –survival, which are all tightly regulated by growth factors and cytokines (e.g. IL-3, IL-7, GM-CSF and erythropoietin) [30], which upon binding to their cognate receptors activate a number of intracellular signaling pathways. By making use of different cell lines it has been determined that the constitutively active BCR-ABL tyrosine kinase abrogates this growth factor dependency [31] by activating essential downstream molecules in a ligand independent manner. Hence, the expression of BCR-ABL, likewise v-ABL, confers immortalizing properties to the cells. In summary, cellular models have been extremely useful to dissect the molecular pathways activated by BCR-ABL and to determine which parts of the protein are required to confer transforming properties. Nonetheless, transgenic murine models offer additional benefits thus allowing to ascertain and further validate which parts of the protein are mandatorily required for the induction of a CML-like disease, to study the role of the environment in leukemogenesis and eventually to identify therapeutic target for pre-clinical investigations. The “*in-vivo*” convincing experimental evidence validating the leukemogenicity of BCR-ABL were provided only around the 1990s by using transgenic murine models [32, 33]. In this respect, the initial development of transgenic and knock-in murine CML models displayed major drawbacks. Indeed, the generation of conventional BCR-ABL transgenic knock-in mice, through the expression of the chimeric gene under the control of the BCR promoter, caused embryonic lethality due to the toxicity of the activated tyrosine kinase during embryonic development. Afterwards, the use of murine stem-cell retroviral vector and mice created through expression of BCR-ABL under the control of a tetracycline-responsive promoter allowed to overcome that problem and revealed that to develop a CML-like disorder it is crucial to express this oncogene in proper tissue/cell type. With the help of these models it was also shown that the expression of the p210 BCR-ABL variant in bone marrow caused a CML-like disease. Remarkably, the progression of the p210 associated disease was consistent with the apparent indolence of the human CML chronic phase. Interestingly, mice models expressing the p190 variant at levels similar to that of the p210, allowed to uncover that they displayed clinically distinct conditions consisting in a de-novo development of acute leukemia with a short period of latency [34]. Furthermore, these studies allowed to functionally dissect the BCR-ABL protein and to determine to what extent the different domains of the BCR-ABL protein are required for the onset of the different kind of leukemia. The tyrosine-kinase activity of BCR-ABL is essential for its oncogenic properties, but not sufficient. Indeed, although the transduction of v-ABL in a helper virus-containing system causes a murine hematopoietic disease it is distinct from the

CML-like syndrome elicited by BCR-ABL developing only modest splenomegaly and malignant disease of several hematopoietic cell types [35]. In addition to its tyrosine kinase domain the *in vivo* molecular dissection of the protein led to the identification of another domain that is apparently required for the induction of the CML-like disease. This domain turned out to be the SH2. Remarkably, the SH2 domain requirement is peculiar only for myeloid but not for the lymphoid leukemogenesis. Notably, this later issue has been rather debated due to some discrepancies between different studies [36, 37]. Additionally, in mice model for the induction of the chronic leukemia-like disease the Grb2 binding site (Tyr-177) is required [38].

6. Molecular mechanisms conferring oncogenic properties to BCR-ABL

Once BCR-ABL has been identified as the molecular pathogenic event in CML and other leukemia related disorders, significant effort has been addressed to unveil the molecular mechanisms of action of the chimeric tyrosine kinase through the identification of signaling pathways that are impacted by BCR-ABL. The most prominent feature of the BCR-ABL fusion protein is its potent and constitutive tyrosine kinase activity. The tyrosine phosphorylation is a vital mechanism of intracellular signal transduction, used by many growth factor receptors. Usually, approximately less than 2% of total cellular tyrosine residues are phosphorylated, and the activity of tyrosine kinases is counterbalanced by the activity of tyrosine phosphatases. In cells that express a constitutively active tyrosine kinase, this finely-tuned regulation is subverted, leading to a situation that resembles chronic growth factor stimulation.

Actually, BCR-ABL displays a tyrosine kinase activity amazingly higher than that of the c-ABL counterpart [39] and differences among the different variants have been assessed, being the p190 more potent than that of the p210 and the latter more potent than p230 [40]. Though the BCR-ABL oncoprotein can activate a large number of different signal transduction pathways they appear to target few crucial cellular functions, including increased cellular proliferation, reduced apoptosis and autophagy combined with a deregulated interaction with the bone marrow stromal cellular matrix (**Figure 2**).

Whereas in BCR-ABL transformed cell the PI3K/AKT signaling has been shown to have a pivotal role in mediating both the activation of cell survival and anti-apoptotic signaling, the activation of the Ras/Raf/MEK/ERK cascade has been implicated in the BCR-ABL-dependent uncontrolled cell growth [41]. To the latter purpose the adaptor protein Crk Like (CrkL) has shown to be an important player, being constitutively bound to and a substrate of BCR-ABL [42, 43]. Noteworthy, BCR-ABL itself, through the phosphorylated Tyr-177 can activate the Ras/Raf/MEK/ERK pathway by interacting with Grb2 which in turn recruits SOS that activates Ras [44, 45]. Eventually, Ras triggers the downstream signaling cascade leading to the activation of ERK1/2 [46]. The BCR-ABL dependent pathways leading to apoptosis resistance involve the aberrant expression of the apoptosis regulators proteins of the Bcl2 family including Mcl1, Bcl2 and BclXL along with the proapoptotic members Bim and Bad [47, 48]. Their regulation is mediated by the BCR-ABL-activated PI3K/AKT pathways [49]. The AKT-dependent phosphorylation of Bad leads to its dissociation from Bcl2 and to its sequestering by the adaptor protein 14-3-3, hence leaving less free Bad available to heterodimerize with the anti-apoptotic BclXL proteins. Therefore, more BclXL and Bcl2 remain in the cytoplasm exerting their antiapoptotic role by preserving the mitochondria outer membrane integrity. In addition, it is likely that BCR-ABL also negatively regulates c-ABL, whose function in regulating the apoptotic process is central. The constitutive

Figure 2. BCR-ABL exerts its leukemogenic effects by impacting diverse cellular processes: the constitutively active BCR-ABL tyrosine kinase triggers a numbers of signaling pathways, including the Ras/Raf/MEK/ERK and PI3K/mTORC/Akt pathways. On the whole their enhanced activation leads to increased cell-survival and -proliferation, and impaired apoptosis rate. Meanwhile, the oncogenic tyrosine kinase impacts also the cellular autophagy rate and eventually the interaction of the BCR-ABL positive leukemic cells with the stromal microenvironment.

tyrosine kinase activity of BCR-ABL impacts also on the cell-to-substratum adhesion. Indeed, the BCR-ABL-transformed cells display an impaired adhesion to the extracellular matrix. Mostly this behavior is due to the CrkL protein that is one, among many, substrate on the chimeric protein BCR-ABL [42]. Interestingly, CrkL is constitutively binds to BCR-ABL through its first SH3 domain and, at least *in-vitro*, CrkL supports and even potentiates the c-ABL tyrosine kinase activity. CrkL plays a pivotal role in adhesion and cell motility through its association with paxillin, Crk-associated substrate (Cas), Focal Adhesion Kinase (FAK) and the Cbl proto-oncogene. Furthermore, BCR-ABL itself can directly affects the actin cytoskeleton via its actin binding domain localized at its very carboxy-terminal tail and by regulating crucial proteins, such as Rho, Rac and Cdc42 responsible for the cytoskeletal actin dynamics [50–52]. The gene expression of several cell adhesion molecules encoding genes, either mediating the cell-to-substratum and cell-to-cell adhesion including the integrin subunit α -6 and the L- and P-selectins, is under the control of BCR-ABL [53, 54]. Eventually, BCR-ABL suppresses autophagy, an intracellular degradative process allowing cells to adapt to developmental changes and/or unfavorable environmental conditions. Remarkably, autophagy has been shown to provide a survival mechanism to cancer cells [55]. The BCR-ABL-mediated suppression of autophagy occurs via the PI3K/mTORC/Akt signaling pathway since by pharmacologically inhibiting the PI3K in BCR-ABL expressing cells the autophagy is induced again [56].

Alike CrkL some downstream BCR-ABL downstream effectors might play dual role, such is the case of Stat5 that is directly tyrosine phosphorylated by BCR-ABL in a JAK independent way [57, 58]. The Stat5 transcription factor mediates the transcription of several pro-survival and pro-proliferative, as well as anti-pro-apoptotic protein encoding genes [59].

Interestingly, though all BCR-ABL variant proteins are collectively characterized by constitutive and enhanced tyrosine kinase activity they still differ in their binding partners, substrates and as a consequence in their elicited signals. For example, while both p190 and p210 can activate the Ras/Raf/MEK/ERK through the Grb2/SOS complex that binds to the phosphorylated tyrosine residue at position 177 (Tyr-177), the activation of Stat5 is exclusively triggered by the p210. Conversely, the p190, when compared to the p210, shows higher affinity towards the tetrameric

Adaptor Protein Complex 2 (AP2), the adaptor protein DOK1 and the tyrosine kinase Lyn [60]. Overall, the signals triggered by the constitutively active BCR-ABL tyrosine kinase are promiscuous affecting several aspects of the components of the cellular machinery.

7. BCR-ABL inhibitors: paving the way for novel tyrosine kinase inhibitors

Amazingly, in the 1980s and 1990s both the scientific community and pharmaceutical industry were rather skeptic about the issue of pharmaceutically inhibiting protein kinases. Much of their skepticism was lying in the prevailing perception that ATP-binding competitive inhibitors would have had a rather limited target specificity to be translated into useful clinical drug. Moreover, some of the early transgenic animals, in which the genes encoding for tyrosine kinases were inactivated, displayed embryonic lethal phenotypes. Altogether these observations led to the acceptance that tyrosine kinase inhibitors would have been enormously toxic, thus inadequate either for scientific and clinical use. Last but not least, it was assumed that the selective targeting of a single defect would not be sufficient to treat a highly heterogeneous disease such cancers. However, by the end of the 1980s and the beginning of 1990s the first selective tyrosine kinase inhibitors (TKIs) were developed, tyrphostins also known as benzene malononitrile derivatives. Outstandingly, these compounds were found to effectively inhibit EGFR [61]. Afterwards, with crucial reagents in hands, including phospho-tyrosine specific antibody coupled to time-consuming approach such as high-throughput screens of chemical libraries, a team of scientists at Giba-Geigy (now Novartis) seeking for compounds with kinase inhibitory activity identified a promising class of compounds: the 2-phenylaminopirimidine series. Surprisingly, among these molecules one displayed very high selectivity towards the receptor for the platelet derived growth factor (PDGFR), ABL and the stem cell factor receptor (c-kit) [62, 63]. In the first half of 1990s a single molecule the Signal Transduction Inhibitor 571 (STI571) (Gleevec, Glivec, ImatinibTM) was shown to be the most specific at selectively suppress the growth of BCR-ABL expressing cells either from CML patients or cell lines [64]. Afterwards, following these *in-vitro* encouraging results pre-clinical data were produced with the help of BCR-ABL transgenic animal models [65, 66]. Consistent with the “*in-vitro*” evidence the animal studies showed that Imatinib treatment led to a dose dependent selective inhibition of BCR-ABL-expressing cells without significant effects against v-SRC-expressing tumors. On the whole, in 1998 these encouraging and promising data prompted a handful of scientist led by B. Druker to set-up the first clinical trial using Imatinib in CML patients. However, before clinical trials could start scientists had to overcome some difficulties concerning the toxicity of the molecule, whether targeting a single kinase would have been an effective and successful strategy and most important whether the pharmaceutical company would realize a return on its investment due to the fact that CML is a pretty rare disease and thus representing a small market. At the end of the 1990s a phase I dose escalation study using Imatinib in CML patients’ refractory to IFN α -based therapy started. Surprisingly, within one year the vast majority chronic phase patients who had failed IFN- α therapy and treated with Imatinib 300 mg once a day achieved a complete hematological response. These promising data paved the way for a phase II study and eventually in 2001, three years later after the phase I, Imatinib received the final approval from the Food and Drug Administration (FDA) [67]. The dramatic success in the treatment of CML by an inhibitor of the BCR-ABL kinase is due to a mechanism involving a single biochemical defect a

special characteristic that is missing in nearly all the other forms of malignancy. Indeed, conversely from other cancers, in which each genotype encodes diverse phenotypic traits, CML displays an unambiguous genotype–phenotype relationship. However, although most of patients responded excellently to Imatinib therapy a minority relapsed. Especially those patients with advanced CML phases initially respond to Imatinib but then progressed to accelerated or blast crisis. The reason for the relapse is straightforward: while in the patients that respond to Imatinib the BCR-ABL tyrosine kinase activity is abrogated, in those that relapse the tyrosine kinase is reactivated due to mechanisms that either prevent Imatinib to reach the target or render the target insensitive to Imatinib. A combination of approaches, including functional studies that have been then validated by the crystallization of the ABL tyrosine kinase domain with Imatinib coupled with the sequencing of the ABL tyrosine kinase domain, allowed to identify and determine critical contact points between the protein and the inhibitor [68, 69]. Indeed, most of the patients who developed Imatinib insensitivity harbor ABL tyrosine kinase point mutation, especially in the P-loop decreasing its flexibility and therefore its capability to bind to Imatinib. The resistance to Imatinib has led to the development of second generation of tyrosine kinase inhibitors (Nilotinib™, Dasatinib™ and Bosutinib™) and the boost of pharmacogenomics [70]. Imatinib is effective also in the treatment of various malignancies, other than CML. For example, it has shown significant activity in patients with Acute Lymphoblastic Leukemia Ph + (ALL Ph+) [71], in a significant proportion of people with Gastrointestinal Tumor (GIST) that harbor c-KIT mutations [72] and those disorders characterized by translocations involving the PDGFRB gene, including myeloproliferative and myelodysplastic syndromes [73, 74]. The demonstration that small molecule inhibitors could effectively treat chronic myeloid leukemia opened the door to the development of new tyrosine kinase inhibitors and to the blooming era of targeted cancer therapies (**Figure 3**).

Though cancer is the predominant indication for tyrosine kinase inhibitors (TKIs), currently the disease targets are extensively growing. For example, Tofacitinib™ is a Jak3 inhibitor that is currently approved for the treatment of rheumatoid arthritis [75, 76] and Nintedanib™ is a FGFR/multikinase inhibitor that is approved for the treatment of pulmonary fibrosis [77]. Furthermore, Pegaptanib, Ranibizumab and Aflibercept that act by inhibiting the VEGF receptor tyrosine kinase activity are currently used for the treatment of the age-related

Figure 3. The FDA approval timeline of tyrosine kinase inhibitors (TKIs): Upon the approval of the Imatinib for the treatment of CML other TKIs have been developed and nowadays small molecules TKIs are dozens. Though, originally they have been designed for neoplasms in the last decade we have also witnessed to an amazingly growth of the diseases, other than cancers, that significantly benefit from TKIs treatment.

macular degeneration, which is a common cause of visual impairment and blindness in elderly adult [78–80].

8. Conclusions

Advances in our understanding in tumor biology have encouraged not only the reassessment of the tumors classification by the site of origin in favor of molecular alterations but also in terms of oncogenic drivers (e.g. tyrosine kinases) amenable for treatment. Since Imatinib has been approved by FDA in 2001 as small molecule competing with ATP, dozens of orally effective small molecule protein kinase inhibitors have been subsequently approved. This is also due to the significantly shortening of the timelines of drug development, as it happened in the case of a record time for the Crizotinib™. The approval of Imatinib for the successful treatment of leukemia (CML) definitively chased away the notion targeting the ATP-binding sites of protein kinases was not selective or efficacious because of the large number of protein kinases, thus leading to copious side effects.

Conflict of interest

The authors declare no conflict of interest.

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