Dipartimento di medicina clinica e prevenzione

Role of the drug transporters in the multidrug resistance affecting bosutinib treatment

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Abstract

The multidrug resistance (MDR) has been identified in the 70s as a major cause of resistance to treatment with several unrelated agents. In the following 40 years MDR has been characterized in dept, and the molecular causes at the basis of this drug resistance are now largely known. The drug transporters play a central role in MDR, as the alteration of their expression levels is strictly related with MDR development. Down-regulation of uptake drug transporters and/or up-regulation of efflux drug transporters correlate with lower intracellular drug concentration, reduced activity on the target, and with resistance to treatment. The tyrosine kinase inhibitor (TKI) imatinib, currently the first line therapy for chronic myeloid leukemia (CML), showed MDR in presence of altered expression levels of OCT-1 (in patients), P-gp and BCRP (*in vitro*). The possible indication of OCT-1 levels as a prognostic factor for imatinib treatment is currently under debate. The hypothesis would be to increase the daily dose in patients with low expression levels of OCT-1.

The emerging therapeutic options for CML, mainly represented by new tyrosine kinase inhibitors nilotinib, dasatinib and bosutinib, offer a valid alternative in presence of imatinib MDR. Indeed, nilotinib and dasatinib show a different uptake/efflux pattern compared to imatinib, and it could be hypothesized that their efficacy could be unaffected by the drug transporters expression levels. The identification of drug transporters involved in new tyrosine kinase inhibitors is actually an active research field.

My PhD project focused on the identification of uptake/efflux mechanisms for a new TKI, bosutinib. First I produced cell lines overexpressing and overexpressing plus silencing the three main drug transporter. Then I evaluated the reliability of the cell models. Then I identified which transporter could be involved in bosutinib uptake/efflux, and finally I studied the biological and molecular relevance of the interaction between bosutinib and drug transporters.

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General Introduction

Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a progressive myeloproliferative disorder characterized by the altered production of granulocytes[1]. This pathology was identified in 1841 by Bennet in Wales and Virchow in Germany[2, 3]. The incidence in adult population is 1 to 2 cases per 100.000. CML in children is rare, while in adults represent the 15% of the whole leukemia cases. The median age of onset is 50-60 years[4, 5]. CML can be divided in three different phases named chronic phase, accelerated phase and blast crisis[6-9].

At the time of diagnosis of CML, 90% patients are in chronic phase. Chronic phase is characterized by slight increased number of mature granulocytes in the peripheral blood due to altered hematopoiesis. The bone marrow shows the accumulation of fully differentiated cells, indicating that hematopoiesis is increased but functional. Patients in chronic phase are usually asymptomatic (50%), or they show mild non-specific symptoms as fatigue, weight loss, unusual bleeding, sweats, anemia and splenomegaly[10]. At this phase, the identification of CML in patients is mainly due to the presence of Philadelphia chromosome, described in detail in the next section. Thus, only molecular analysis can establish the presence of the pathology. The chronic phase persist for a median interval of five years without a specific treatment. After this period, patients eventually progress in accelerated phase and then in blast crisis[9, 11, 12].

Accelerated phase is mainly characterized by a partial maturation arrest, with increased number of blasts both in peripheral blood and in bone marrow. The symptoms

of this phase are splenomegaly, thrombocytosis and leukocytosis. During the accelerated phase, therapy for treatment of CML can still be effective, although with a decreased rate of success than in chronic phase[13-16]. However, the accelerated phase is tipically very short, and it is difficult to identify differences between this phase and the blast phase. Accelerated phase is a sort of intermediate phase with some features of chronic and some features of blastic phases.

The last phase of disease is the blast crisis, characterized by a massive presence of non-mature blasts both in peripheral blood and in bone marrow. In this fase, the myeloid differentiation is abrupted. The median survival of patients in blast crisis is 6 months. Notably, some patients evolve directly from chronic phase to blast crisis without showing the accelerated phase[17-21]. Most of the therapeutic treatment showed to be ineffective during this phase, suggesting a molecular heterogeneity involving additional mutations that render ineffective therapies commonly used in chronic phase.

The transition between the three phases is still poorly understood. Genetic and epigenetic changes have been proposed. The lead hypothesis is that the presence of aberrant tyrosine kinase Bcr-Abl drives the leukemic cell toward the accumulation of genetic changes, responsible for the leukemic progression[22-24].

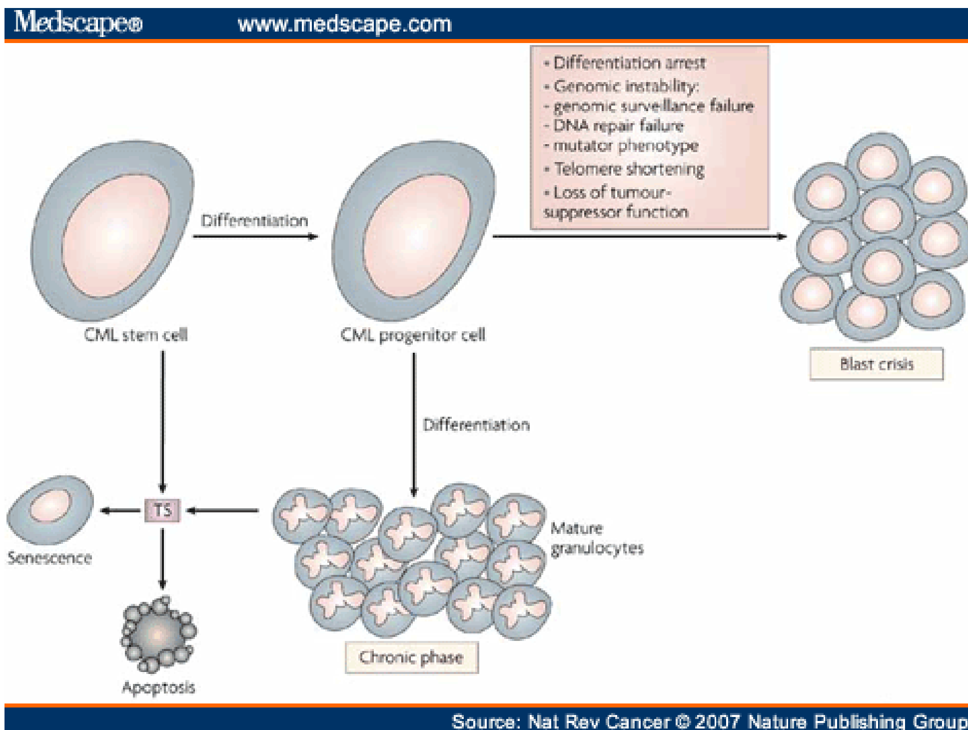

Fig. 1: **The three pathological phases of CML.**

The Philadelphia chromosome

In 1960, Nowell and Hungerford described the presence of a small chromosome in primary cells obtained from CML patients[25]. Later, this chromosome was named the Philadelphia chromosome (Ph), and it was correlated with CML pathology[26].

The best known and characterized type of chronic myeloid leukemia is the Ph^+ CML. Ph^+ CML patients show a balanced, reciprocal translocation between chromosome 9 and chromosome 22. The molecular characterization of Philadelphia

chromosome was helped by the improvement in molecular biology techniques after 1980. A seminal discover was that ABL gene, normally located on chromosome 9, was found in chromosome 22 in CML patients[27-32].

ABL gene is normally an ubiquitously expressed non-receptor tyrosine kinase of 145 kDa. ABL exerts its function by shuttling between the cytoplasm and the nucleus. ABL is mainly involved in the cytoskeleton remodeling during cell differentiation, cell division and cell adhesion. Once activated, ABL localizes in actin structures, when it phosphorylates its effectors CRK, CRKL and DOK1, proteins controlling cytoskeleton dynamics. Other ABL physiological activities include DNA repair regulation by apoptosis induction, if the DNA damage is too severe to be repaired and induction of cell cycle arrest in G1 phase[33-36].

The first description of breakpoint cluster region (BCR) on chromosome 22(q11) was done in 1984 by Groffen and colleagues[31, 37, 38]. This region is now known as a part of larger gene defined BCR gene[39]. BCR gene encodes for the ubiquitously expressed BCR protein (160kDa). Knock out mice for BCR demonstrated that BCR functions do not interfere with normal cell physiology.

In 1985, Witte and colleagues noticed that the Abl protein in K562 cell derived from CML patients was bigger than normal Abl protein and showed an increased activity[40]. Eli Canaani and colleague finally described the presence of BCR-ABL fusion transcripts in leukemic cells[41].

The mechanism responsible of the reciprocal balanced translocation t(9;22)(q34;q11) is still unclear. The principal hypothesis is that the chromosomal rearrangement could be due to the proximity of the two chromosomes between the S and G2 phases of the cell cycle. Another hypothesis accounts a role for homology regions between the two genes, such as Alu. Environmental causes (e.g. exposition to radiation) have been documented by epidemiological studies[42].

BCR-ABL contains nuclear localization signals, but the aberrant protein can be found only within the cytoplasm, where it exerts its oncogenic potential. Different breakpoint regions give rise to different BCR-ABL isoforms. From a functional point of view, only the isoforms due to altered BCR length are important for the pathology. The three isoforms originated by different breakpoint are named p230, p210 and p190, basing of the different transcript length. The p230 isoform has less oncogenic potential if compared to the other isoforms; the p210 isoform is associated with chronic myeloid leukemia; the p190 isoform is associated with acute lymphoblastic leukemia (ALL)[43].

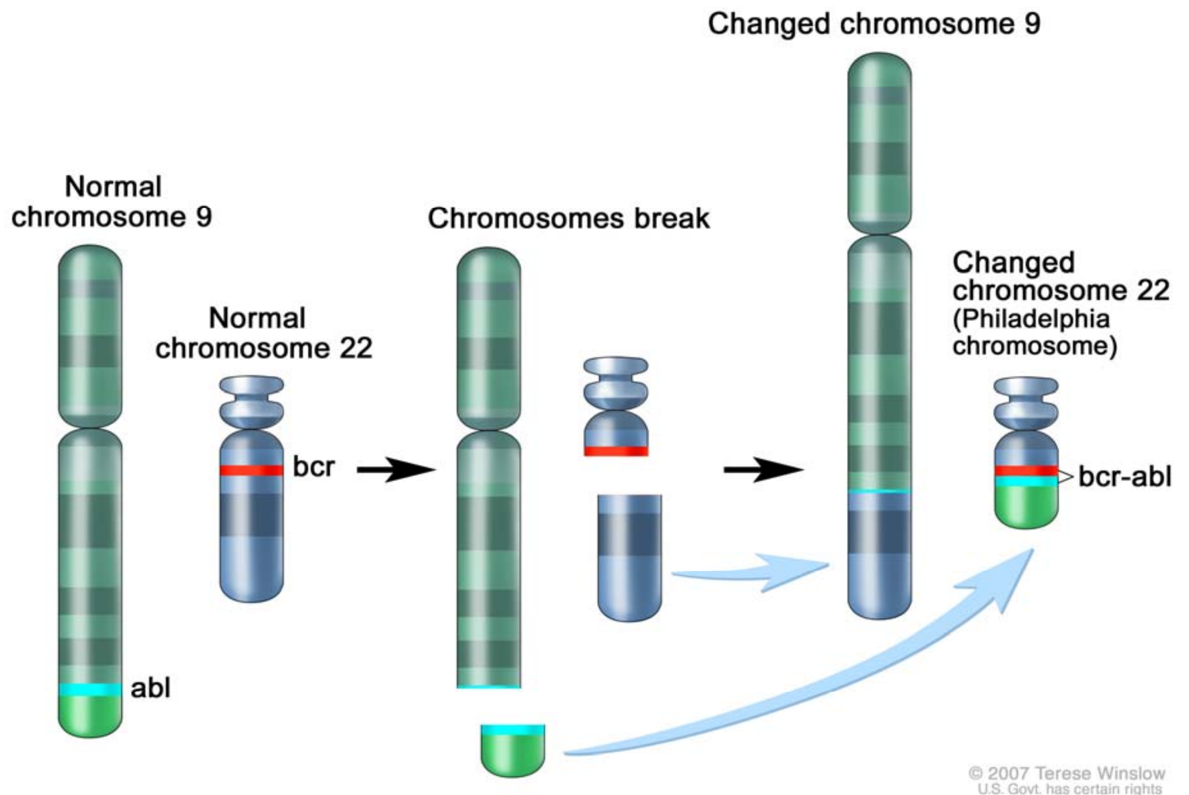

Fig 2: the balanced translocation originating Philadelphia chromosome.

BCR-ABL activity

The fusion with BCR gives to ABL new regulatory domains and motifs, such as grow factor receptor-bound 2(GRB2) SH-2 binding site. The presence of these additional domain and motifs increases the tyrosine kinase activity of ABL. The kinase activity is exerted on several downstream effectors, and thus different pathways are involved after BCR-ABL activation. Globally, the constitutively activated kinase activity

of BCR-ABL is responsible for induction of proliferation, promotion of survival and inhibition of apoptosis, even in absence of specific signals. Moreover, BCR-ABL alters the cell adhesion and the cell homing within the bone niche[44]. In the following sub-paragrafs, the main pathways involved in CML pathogenesis are depicted in detail.

RAS pathway

Ras proteins are small GTPases that act as molecular switches, transducing signals from activated receptors to downstream effectors to regulate cell proliferation, survival and differentiation. Ras small GTPases are activated in many hematopoietic growth factor signalling and in hematological malignancies, but their role in haematopoiesis and leukemogenesis is not completely known. The BCR portion is fundamental for the interaction of BCR-ABL with the growth factor receptor-binding protein (GRB2)/Gab2 complex, via the GRB2-binding Y177 site in the BCR portion of BCR-ABL that shows a high affinity-binding for the Grb2 SH2 domain when phosphorylated. The interaction of BCR-ABL with GRB2/Gab2 and the phosphorylation of SHC lead to enhanced activity of the guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor SOS, which promotes the accumulation of the active GTP-bound form of RAS. Activated Ras recruits phosphotidylinositol-3 kinase (PI3-K) and thus the downstream pathway.

The importance of RAS-dependent signalling for the phenotype of BCR-ABL/expressing cells is supported by the observation that by the use of antisense

strategies, expression of dominant negative molecules or chemical inhibitors causing a downregulation of RAS pathway suppresses proliferation and sensitizes cells to apoptosis stimuli. Additionally, in a mouse model of CML, in which the disease is induced by injection of cells transfected with BCR-ABL in a retroviral vector[45, 46], the mutation of Y177F induced a T cell leukemia and lymphoma after a prolonged latent period[47-49]. The importance of Y177, linking BCR-ABL to the Ras signaling, suggests that Ras plays a critical role in the pathogenesis of CML.

PI-3K/Akt pathway

The PI3k/Akt pathway is constitutively active in CML cells and plays a major role in cell survival. It has been demonstrated that PI3 kinase activity is regulated by BCR-ABL and is required for the growth of CML cells[50]. On activation, Akt phosphorylates key survival proteins such as the transcription factor regulator glycogen synthase kinase 3b (GSK3b) which in turn regulates Mcl-1 and other downstream proteins, resulting in decreased cell susceptibility to apoptosis[51]. The BCR-ABL oncoprotein forms multimeric complexes with PI3 kinase and the adapter proteins Cbl and CrkL[52] that ultimately lead to the activation of the serine-threonine kinase Akt[53]. Activation of Akt causes a proliferative effect down-regulating the cell cycle inhibitor p27[54] and causes also an anti-apoptotic effect inducing the phosphorylation of the pro-apoptotic protein Bad, thus preventing Bad-mediated inactivation of the anti-apoptotic protein Bcl-XL.

Survival signals mediated by the cytokine IL-3 are transduced into the cell via the PI3/Akt kinase pathway. BCR-ABL activity can thus substitute for the survival signals provided by IL-3 supply.

The MAP Kinase pathway

MAPK signaling consists of a three-kinase cascade module composed of a MAPK kinase kinase (MAPKKK or MEKK) that activates a MAPK kinase (MAPKK, MKK or MEK) which ultimately activates a MAPK enzyme. In mammals, it is possible to define four distinctly regulated groups of MAPKs regulated by specific MAPKKs: ERK1/2 regulated by MEK1/2, p38 by MKK3/6, JNKs by MKK4/7 (JNKK1/2) and ERK5 by MEK578. BCR/ABL can activate the MEK1/2- ERK1/2 signalling pathway thanks to the autophosphorylation of tyrosine 177 that generates a binding site for the adapter molecule Grb-227[55, 56]. Grb-2 associates with the Sos protein stimulating the conversion of the GDPRas to the active GTP-Ras[55]. Ras is thus able to activate Raf-1 which can act on the MEK1/2-ERK1/2 proteins that ultimately activate gene transcription. Additionally, it has been shown that Ras can be activated by Shc, CrkL and Dok adapter proteins, which are known to be BCR/ABL substrates[55]. The relevance of Ras Crkl-dependent activation is questionable because it has been shown that it is important for BCR/ABL transformation of fibroblasts, but it is not required for myeloid cells transformation[57, 58]. Although ERK2 activation has not been detected in Ba/F3 cells transfected with a temperature sensitive p210BCR/ABL molecule[59], another study demonstrated that BCR/ABL activates the Ras-Raf-MEK1/2-ERK1/2

pathway in haematopoietic cells[56]. The importance of the MAP-kinase pathway in the BCR/ABL mediated transformation is emphasized by the discovery that the oncoprotein activates also the JNK/SAPK-MAPK pathway[60]. Interestingly, the p38-MAPK pathway has been shown to be activated by IFN-α in primary cells from CML patients and in BCR/ABL-positive cells[61]. It is therefore possible that the oncoprotein negatively regulate this pathway and that IFN-α, which is used as a pharmacological treatment for CML could overcome this inhibition leading to an antiproliferative effect.

The JAK-STAT pathway

Constitutive phosphorylation of the STAT family of transcription factors has been detected in BCR/ABL-positive cell lines[62] and in primary CML cells[63]. In physiological conditions STAT proteins are phosphorylated downstream of Janus kinase (JAK) activation[64], while BCR/ABL appears to phosphorylate directly multiple STAT family members[65]. It has been demonstrated that the activation of STAT5 by p210BCR/ABL contributes to the malignant transformation of haematological cell lines[66], being responsible for the enhanced transcription of the anti-apoptotic BCL-XL protein[67, 68].

The MYC pathway

The MYC gene is over-expressed in many human malignancies[69]. In physiological conditions MYC is a transcription factor; its targets were recently identified[70] and include genes involved in cell cycle regulation (e.g. cyclin-dependent kinase 4 known as CDK4) and genes involved in apoptosis (e.g. prohibitin known as PHB).

The link between BCR-ABL and MYC induction is not well understood, although the induction of MYC expression caused by v-Abl has been already described. This model proposes that v-Abl initiates a cascade of phosphorylation involving Ras, Raf, cyclindependent kinases (cdks) and the transcription factor E2F that ultimately activates and binds to MYC promoter[71].

Experiments with rat fibroblasts showed that MYC could be activated downstream of BCR-ABL and that it was involved in the cellular transformation. Moreover, in mouse bone marrow cells the expression of a dominant negative form of MYC blocked the BCR/ABL transforming potential[72], confirming the role of MYC activation in CML oncogenesis.

More recently, BCR-ABL has been shown to activate survivin, an important regulator of cell growth and survival[73, 74], but the precise molecular mechanisms behind its expression and consequences in CML cells remain unclear. BCR-ABL promotes survivin expression and its cytoplasmic accumulation. The increase of survivin was largely controlled at the transcriptional level through a mechanism mediated by

JAK2/PI3K signal pathways that activated c-Myc, leading to transactivation of surviving promoter. Dynamic down-regulation of survivin was a key event involved in imatinibGeneral induced cell death while forced expression of survivin partially counteracted imatinib's effect on cell survival. Additionally, short hairpin RNA-mediated silencing of survivin or c-Myc inhibits the colony formation of K562 cells in soft agar culture system, suggesting an important role for c-myc transcriptional network in BCR-ABL-mediated cell transformation and survival[75].

Altered cellular adhesion

In CML, HSCs exhibit reduced adhesion to stromal cells and to the extracellular matrix of the bone marrow[76], causing a loss of regulatory signals that are normally supplied to haematopoietic progenitors. Cell surface receptors of the integrin family have an important role in transmitting signals from the extracellular environment. In particular the β1-integrins, during haematopoiesis, function as negative regulators of cell proliferation[77, 78]. It has been shown that in CML cells, the activity of β1-integrins is diminished, in part explaining the impaired binding of oncogenic cells to the bone marrow stroma[76, 79]. It is also known that integrins are connected, via their cytoplasmic tail, with various cytoskeletal proteins including the F-actin which can also complex with BCR-ABL[80]. Since BCR-ABL has been shown to interact with cytoskeletal proteins, it is thus possible that these interactions could influence cytoskeletal and integrin functions. All these effects could also lead to the release of

premature CML cells from the bone marrow, contributing to the accumulation of undifferentiated cells in the peripheral blood.

Apoptosis and Autophagy

When the cells acquire DNA damage, the damage-sensiting proteins recognize the error and stimulate repair, but when the damage is too great the cell may instead signal to undergo apoptosis. This prevents the replication of potentially harmful mutations, and evading apoptosis is one of the hallmarks of cancer. Another of the known roles of BCRABL is the inhibition of apoptosis. The resistance of K562 cells to apoptosis is a consequence of BCR-ABL expression, as antisense oligonucleotide treatments against BCR-ABL induce apoptosis[81, 82].

Growth-factor-dependent human or murine cell lines transfected with exogenous BCRABL do not undergo apoptosis after growth factor withdrawal[70, 83]. Furthermore, cell lines transfected with BCR-ABL have been reported to show an increased resistance to DNA-damage induced apoptosis[84, 85]. Cells expressing a high level of BCRABL expressed constitutively high level of p53, p21 and Bax, and low level of Bcl-2, and cytotoxic insult did not alter these levels. The reason is that high levels of BCR-ABL prevent the translocation of the pro-apoptotic proteins Bax and Bad to the mitochondria, the late mitochondrial depolarization and the Caspase 9 and 3 processing[86]. It has been shown that the cytochrome-C release from mitochondria is apparently blocked in BCR-ABL expressing cell lines[87]. In agreement with this, there is over-expression of the anti-apoptotic protein BCL-2[53, 88] and silencing of the pro-apoptotic BCL-2 family member BIM[89]. It seems that the inhibition of BIM expression is directly dependent

upon the phosphorylation of the transcription factor FoxO3A. FoxO3A was found to be inhibited through phosphorylation in the presence of the active BCR-ABL, thus being unable to activate BIM transcription[89, 90].

It has been demonstrated that BCR-ABL localization is critical for its anti-apoptotic potential[91]. The oncoprotein has a different subcellular localization compared to the normal counterpart ABL. In fact ABL is found in both the nucleus and the cytoplasm, shuttling between these two components, while BCR-ABL is exclusively cytoplasmic.

Interestingly, nuclear ABL is a positive inducer of apoptosis, while cytoplasmic BCRABL induces proliferation. It has been demonstrated that nuclear entrapment of BCRABL, by treating cells with the nuclear export inhibitor Leptomycin B (LMB), results in apoptosis induction, suggesting an important role of BCR-ABL localization for tumorigenic potential[91].

Autophagy is a highly conserved catabolic process for the elimination and recycling of organelles and macromolecules, characterized by the formation of double-membrane vesicles called autophagosomes. To date, the function of autophagy in cell differentiation is poorly documented. The treatment of BCR-ABL cells with specific anti-cancer agents, INNO-406, a second-generation BCR-ABL TK inhibitor, induced the BCR-ABL cells death by autophagy[92], representing an alternative to the apoptosis in the cases in which the mechanisms of programmed cell death is not functionally active.

BCR-ABL and the DNA damage and repair

The appearance of chromosomal abnormalities in patients with BC-CML has led to many attempts to elucidate the mechanisms by which BCR-ABL affects DNA damage and repair.

Several common themes emerge from previous studies[53, 93]: the presence of BCR-ABL increases the genomic and chromosomal instability in cells, although the increase is modest. But after a long period of BCR-ABL expression, the frequency of appearance of DNA damage increases. BCR-ABL has been shown to induce the production of reactive oxygen species, which cause oxidative damage and mutation[94-96].

Briefly, DNA damage mechanisms could lead to single-nucleotide alterations, singlestrand breaks or double-strand breaks (DBSs). Single-strand breaks are prone to generate DSBs and the two will be considered together here. Single-nucleotide alterations are repaired by mismatch repair (MMR) or by nucleotide excision repair (NER) mechanisms.

Strand breaks are repaired by either high-fidelity homologous recombination when a sister chromatin is available as a template (during the S or G2 phase of the cell cycle), or by non-homologous end joining (NHEJ), which may lead to short deletions in the repaired strands.

DNA mutations can occur as the result of several situations; when there are mutations in single-nucleotide repair pathways, mutations in proteins necessary for the

complex process of DNA DSB recognition and repair or, alternatively, when there is a failure of cell cycle checkpoints that allows subsequent replication of damaged DNA. The latter may occur because of defects in sensing DNA damage or defects in proteins necessary to execute the cell cycle arrest. Effects of BCR–ABL on all of these pathways have been described and summarized in the figure from Burke and Carroll's review[97].

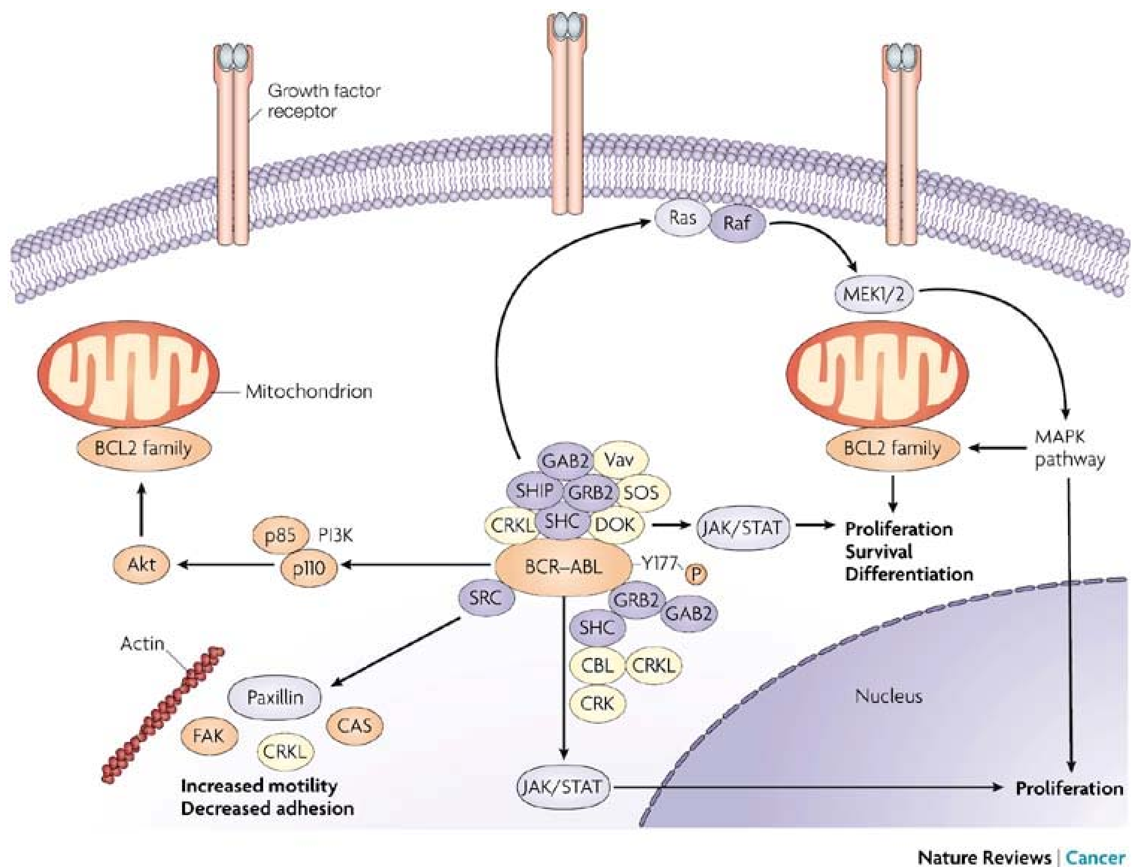

Fig 3: The molecular pathways dependent on bcr/abl activation.

CML therapy

Efficacy

The treatment of Ph^+ CML patients dramatically changed after the discovery of the central role of BCR-ABL in the pathology[98]. The link between BCR-ABL and the pathology development led to the hypothesis of a targeted strategy to avoid the molecular activity of BCR-ABL within the cells and thus preventing the propagation of leukemic cells.

Before the development of this targeted strategy, CML patients were treated with different therapies commonly unable to eradicate the pathology. Thus, the parameters of efficacy of CML patients treatment was defined even before the advent of BCR-ABL inhibitors. Here, the three main parameters are summarized as follow:

- Hematological response (HR). This parameter is based on the number of white cells, red cells and platelets. The spleen size is also evaluated. When this number is normal or close to normal, there is a complete hematological response (CHR). If there is an alteration of blood cells count, there is a partial hematological response (PHR). Blood counts are performed 2 times per weeks until a CHR is achieved, then once every three months.
- Cytogenetic response (CR). This parameter is the most used to asses the efficacy of the therapy in CML patients. The number of Ph^+ cells is evaluated

after treatment, to asses the presence and the number of leukemic cells. The cytogenetic analysis is based on a standard karyotype with 20 metaphases. The bone marrow aspiration and If there are no Ph^+ cells, there is a complete cytogenetic response (CCyR). If there are less than 33% Ph^+ cells, there is a major cytogenetic response (MCyR), while in presence of Ph^+ cells between 33% and 66%, there is a minor cytogenetic response (MyCyR). Fluorescent in situ hybridization (FISH) has been proposed as a method for the cytogenetic response. Although this technique is more sensitive compared to the conventional cytogenetic assessment, the major limitation is the background levels of false positive results. The cytogenetic test is performed first at 3 months, then every 6 months until a CCR is achieved.

- Molecular response (MR). The molecular response is evaluated basing on the levels of BCR-ABL chimeric mRNA, detected using the reverse transcription polymerase chain reaction (RT-PCR). The complete molecular response (CMR) is achieved when there are no detectable levels of BCR-ABL chimeric mRNA. The major molecular response (MMR) is achieved when there is a 3-log reduction of BCR-ABL chimeric mRNA levels compared to pretreatment levels. The levels of MR are evaluated every three months in CML patients.

Up to date, the molecular response evaluation seems to be the best option to asses the response of CML patients to treatment. Indeed, its sensitivity is much more higher than the one of HR and CR. In addition, BCR-ABL chimeric mRNA levels measured in peripheral blood show a good correlation with levels measured in the bone

marrow. The major limitation of this parameter is the wide variability of results obtained from different laboratories. Thus, a standardization of the techniques is required. In 2005, an International Scale of measurement was proposed at the consensus meeting at National Institute of Health (NIH, Bethesda).

All together, the three parameters allow to define the optimal, suboptimal or failed response to treatment[99]. The identification of the response rate will be analyzed more in detail for each treatment option in the next sections.

Pre-imatinib treatments

The history of CML treatment starts in the late years of 19th century. The first therapeutic option reported was the arsenic. For several years, however, this treatment option did not enter in the routine for the treatment of CML.

In the early years of 20th century, radiotherapy was tested as a therapeutic option with better results compared to arsenic. Indeed, radiotherapy remained the standard therapy for more than 50 years[100].

Around 1950, alkylating agents were rapidly developed, and by the 1960s they had displaced radiotherapy in the CML treatment. The first alkylating agent introduced in CML therapy was busulfan, a drug acting on primitive stem cells[101]. Better results were obtained by using hydroxycarbamide (hydroxyurea). Hydroxycarbamide is a ribonucleotide reductase inhibitor, and it exerts its action on late myeloid progenitors,

thus explaining the improved efficacy if compared to busulfan[102]. Anyway, these two agents did not have the capacity to induce Ph negativity within standard dose treatment. Better results were obtained by gaining the daily dose, but this gave remarkable side effects such as aplasia[103].

In the early 1980s, interferon –alfa (IFN-α) was introduced as a therapeutic option. IFN-α was the first therapeutic treatment able to induce a Ph negative hematopoiesis without bone marrow transplant. Unfortunately, this was the case of a minority of patients. Between them, a few achieved a complete and durable Ph negative hematopoiesis. Nevertheless, IFN-α displaced busulfan and hydroxiurea as the first option for CML treatment[104, 105].

Some years before, however, Goldman JM and colleagues demonstrated that untreated CML patients had enough stem cells within the peripheral blood to restore an autologous bone marrow after severe chemotherapy or chemoradiotherapy[106]. Although this method gave a complete or partial Ph negative hematopoiesis in the short term, often patients went back to blast crisis within few months. Focusing on the transplant, several attempts had been done to transfuse bone marrow from healthy donors, following cyclophosphamide and total body irradiation[107]. In 1979 Fefer and colleagues successfully tried a bone marrow transfusion coming from the twins of the patients. In this case, the treatment was successful as the hematopoiesis became stably Ph negative[108]. From that point, the transplant became a valid option, although the main limitation was the very high rate of mortality, ranging between 10% and 20% after 1 year and upgrading to 40% at 5 years[109].

As mentioned at the beginning of this chapter, the identification of the constitutively activated aberrant tyrosine kinase BCR-ABL as a key regulator of Ph+ CML pathology development allowed the investigators to hypothesize a more targeted and successful therapy strategy. In the next chapter, the advent of drug targeting the activity of BCR-ABL is described in detail.

Development of Imatinib, a tyrosine kinase inhibitor

The first attempt to inhibit the kinase activity of BCR-ABL was done by Anafi and colleagues in 1992. This group reported that tyrphostin, a compound related to erbstatin, was able to inhibit BCR-ABL activity[110].

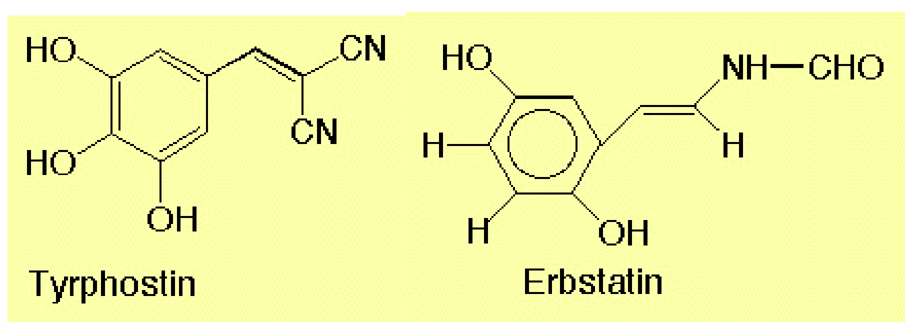

Fig. 4: Tyrphostin and erbstatin, the first compounds used as bcr-abl inhibitors.

Tyrphostin became the lead compound for the development of new bcr-abl kinase activity inhibitors, and between them AG568, AG957 and AG1112 were identified

as the most potent inhibitors. The three drugs exerted a potent inhibitory activity on BCR-ABL in K562 cells (K562,a cell line derived from a Ph^+ CML patient, is commonly one of the best used *in vitro* model of CML)[111]. Growth inhibition of cells was detected within a micromolar range. The activity of tyrphostins on BCR-ABL is related with their interaction with ATP binding pocket. Although the promising data in vitro, this compounds were not further developed for clinical studies. Other compounds showing an inhibitory activity on the tyrosine kinase activity of bcr-abl were herbimycin A[112] and genistein[113]. However, also these compounds were studied only in vitro[114].

In the late 1980s, scientist at Ciba Ceigy (now Novartis, Basel, Switzerland) started to characterize compounds with inhibitory activity on protein kinases in general. In particular, they identified a 2-phenylaminopyrimidine derivative as a lead compound for the inhibition of the activity of protein kinase C (PKC)[115]. Although this compound had low activity and poor specificity, derivates were produced and characterized on its basis[116]. In particular, four functional groups were added to the starting structure:

1) 3'-pyridil group at the 3' position of the pyrimidine. This modification allowed to improve the cellular activity.

2) Benzamide group at the phenyl ring. This modification allowed to increase the activity against tyrosine kinases.

3) "flag-methyl" group at the 6-position of the phenyl ring. This modification allowed to further enhance the activity against tyrosine kinases.

4) The highly polar side chain N-methylpiperazine to improve solubility and oral bioavailability.

Between several compounds tested, STI571 (formerly known as CGP57148B; now imatinib mesylate, Gleevec or Glivec, Novartis, Basel, Switzerland) emerged as a promising compound for further pre-clinical and clinical development. The best target of STI571 was found to be the tyrosine kinase BCR-ABL[117-119].

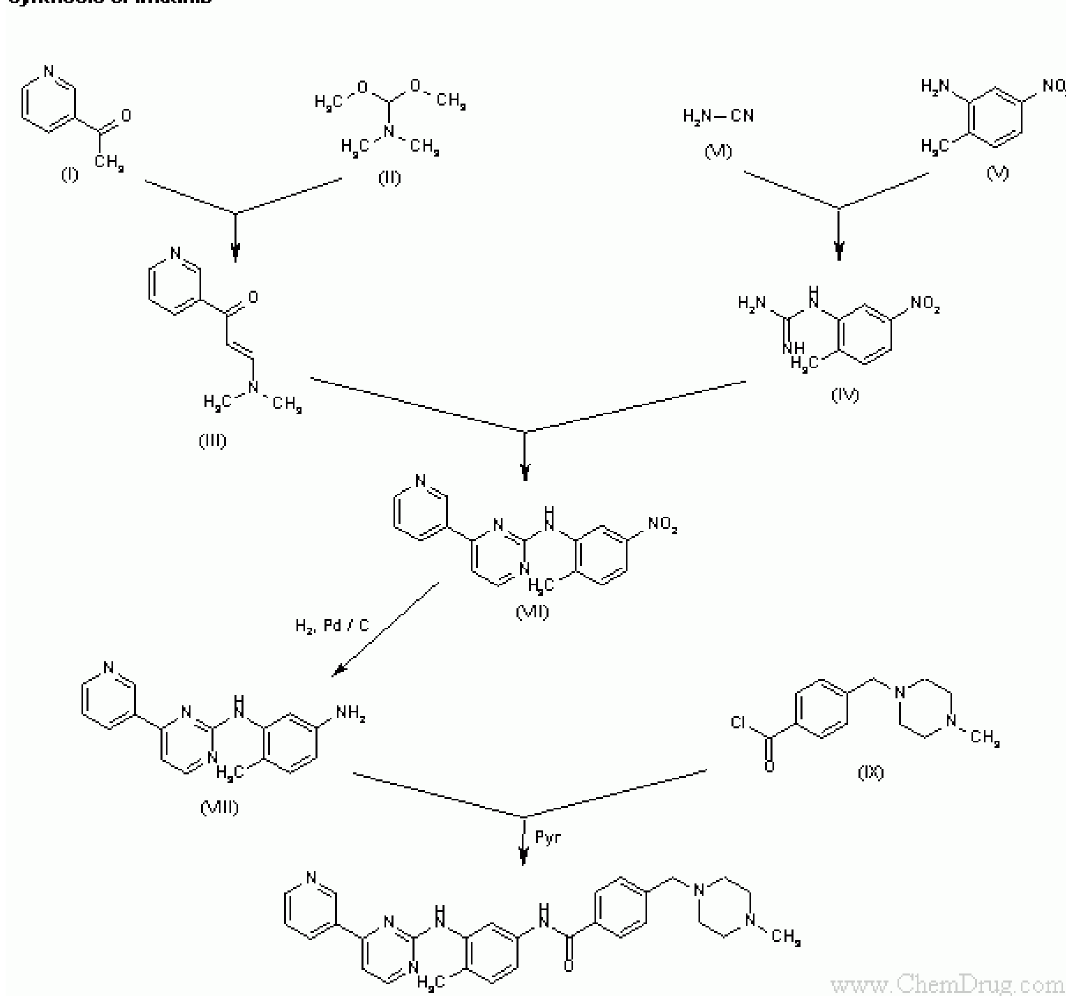

Fig. 5: synthesis of imatinib

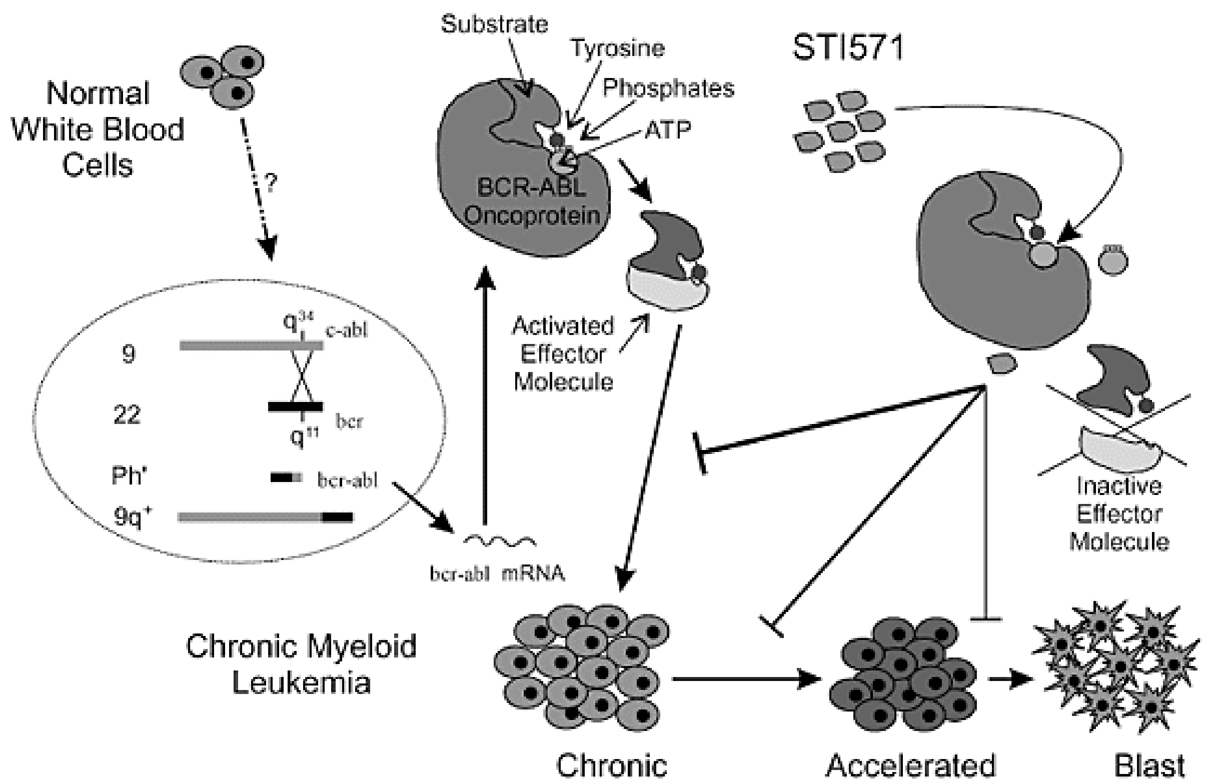

Fig. 6: mechanism of action of imatinib

Imatinib development

Pre-clinical studies

The pre-clinical characterization of imatinib as an inhibitor of BCR-ABL started immediately after. The first aim was the assessment of imatinib activity over a wider

range of tyrosine kinases. The kinase activity inhibition was assessed mainly with the ELISA assay: the degree of phosphorylation of kinases was evaluated in presence of imatinib.

Imatinib was found to be active on the following kinases, with different degrees of efficacy and potency:

- Strong activity ABL in various forms, including BCR-ABL;
- Activity on platelet derived growth factor receptor (PDGFR);
- Activity on KIT receptor;

On the counterpart, weak or no activity was found in several kinases, indicating a high selectivity for the targets:

- Vascular endothelial grow factor receptors (VEGF-R1 and VEGF-R2);
- Fibroblast growth factor receptor 1 (FGF-R1);
- Other kinases such as c-MET, IGF-1, FLT3, SRC and JAK2.

The results obtained with kinase assays were also confirmed in *in vitro* models[120]. This step was crucial to define the efficacy of imatinib to penetrate the cytoplasmic membrane and to act on the target also in the cytoplasmic environment. In detail, the inhibitory activity of imatinib for BCR-ABL, measured as IC50 of leukemic cell death, was found to be between 0.1 and 0.35μM. Moreover, also primary leukemic cells obtained from Ph^+ CML patients showed to be sensitive to the activity of imatinib.

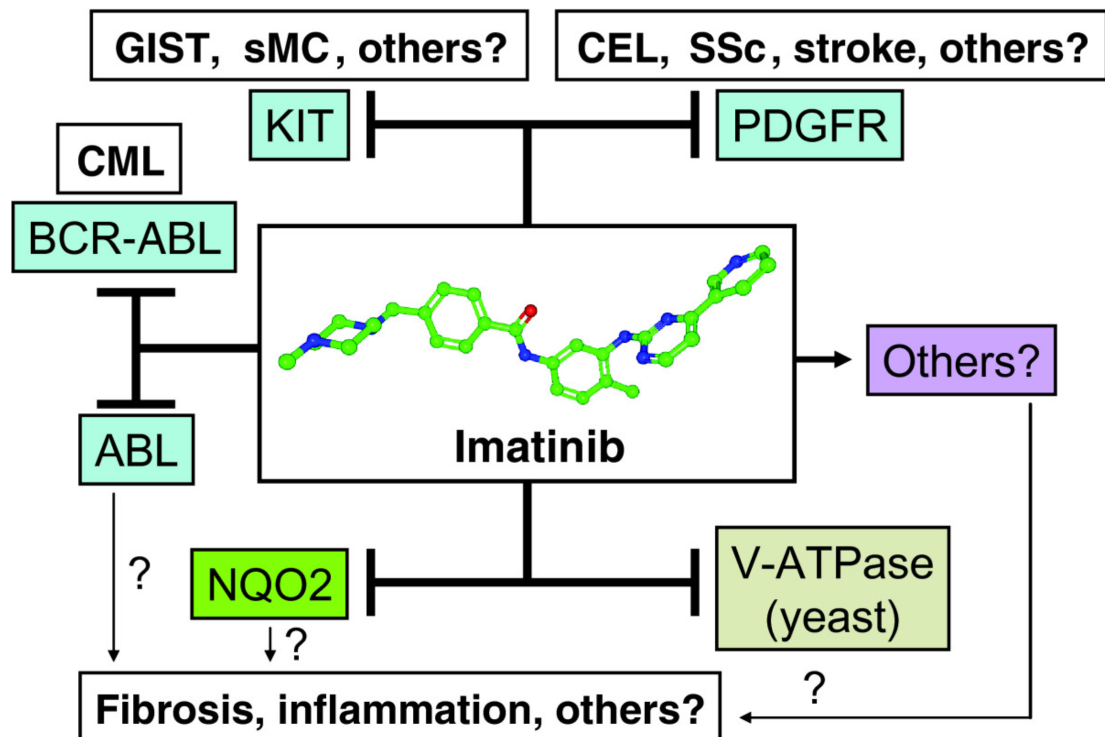

Fig. 7: molecular targets of imatinib.

Other studies suggested that the inhibition of BCR-ABL by imatinib was able to induce apoptosis in leukemic cells[119]. Thus, apoptosis induction was the mechanism of leukemic cell death observed in leukemic cell lines and primary leukemic cell lines *in vitro.*

The characterization was also done *in vivo*. 32D cells transformed with BCR-ABL were injected into syngeneic mice. Inhibition of tumor growth was strictly related with the dose of imatinib. Similar results were obtained with the injection of KU812

cells, a Ph^+ lineage. The effect was specific for BCR-ABL, because no tumor growth reduction was detected in the same experiment done with U397, a Ph^- myeloid cell line[121].

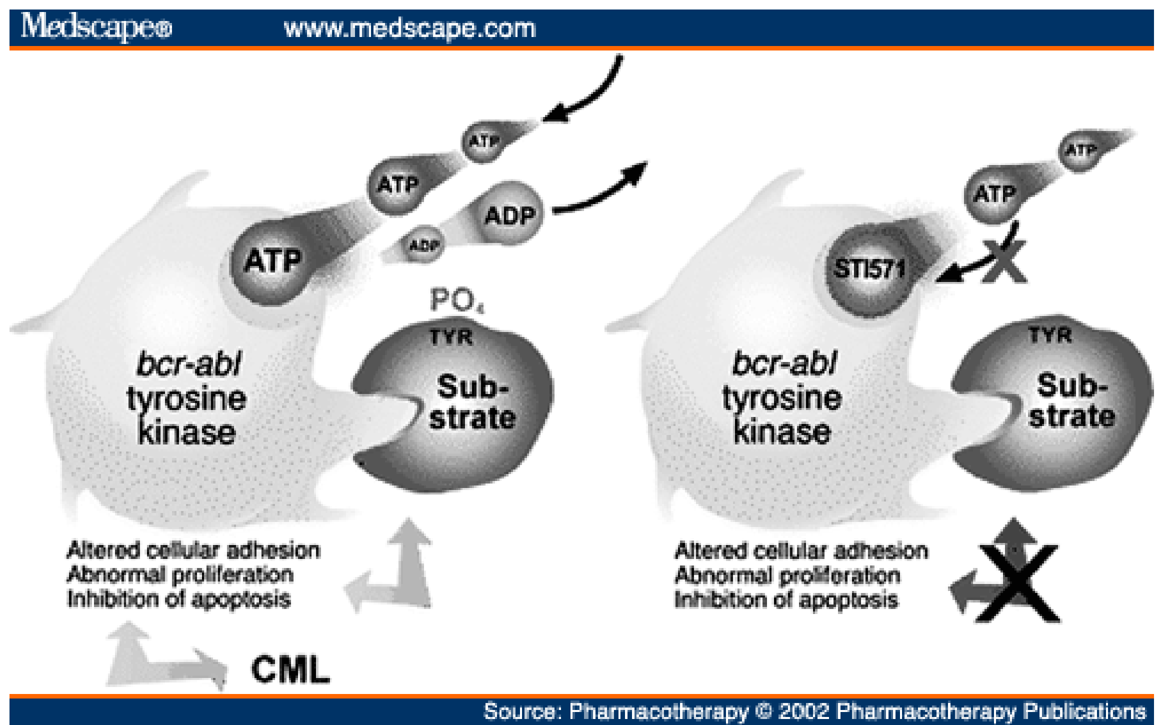

Fig. 8: overall cellular effects of imatinib.

Clinical studies

Due to the promising data obtained with pre-clinical characterization[122], imatinib entered in clinical trials in 1998. The main focus of phase 1 trial was the determination of the correct dose to be used for a correct balance between efficacy and toxicity. Besides, imatinib activity in CML patients was also evaluated. For the trials, patients in CML-CP who had failed IFN-α treatment were recruited. Taken together, the results of this phase indicated that imatinib was tolerated without dose-limiting toxicity. Moreover, the optimal daily dose was 400mg, the same dose actually in use. The study was then expanded to patients with relapsed or refractory Ph^+ ALL and CML patients in blast crisis. Unfortunately, the vast majority of patients failed to achieve a CHR stable in time, undergoing to relapse within few months[123]. This outcome suggested that BP CML, differently from CP CML, is characterized by an high rate of heterogeneity and it is not solely dependent from bcr-abl for the pathology development.

In phase 2 clinical trial, imatinib was used as single agent for CML. This phase confirmed the results of phase one and allowed the approval by the Food and Drug Administration for the use of imatinib for the treatment of CML patients who had previously failed the IFN-α based therapy[124, 125].

In phase 3 trial, imatinib was compared with IFN-α in terms of treatment efficacy. Once again, imatinib confirmed to be the best therapy option in terms of CHR, MCR, CCR and progression-free survival[126-130].

After imatinib approval as a first option for the treatment of CML patients, clinical studies focused on the hypothesis of a combined use of imatinib with other therapeutic agents such as IFN-α. All together, these studies indicated that the response to treatment is achieved earlier in combined therapies, but also that the toxicity is markedly increased. However, strategies for the combined therapy are still under study[131, 132].

Imatinib as a frontline therapy for CML

Up to date, imatinib has been the frontline therapy for CML patients for more than 10 years. The International Randomized study of interferon and imatinib (IRIS) is the most important source to understand the deep change that imatinib introduced in the therapeutic management of CML patients. In this study, the results obtained with imatinib are compared with those of interferon in a long term period. Briefly, at 7 years of follow-up, patients treated with imatinib showed hematological and cytogenetic responses, low progression to both accelerated phase and blast crisis, and good levels of overall survival rate (85%). On the counterpart, rates of relapse and progression after treatment are low, with a overall event-free survival of 81% and transformation-free survival to accelerated phase or blast crisis of 93%. The different response to imatinib or interferon treatment is now difficult to evaluate, because 64% patients initially in the cohort of interferon shifted to the imatinib therapy[133-136].

The striking importance of imatinib in modern medicine is that patients showing CCyR after 2 years of imatinib treatment, have a normal duration of life. This result has never been achieved by any drug or treatment[137].

Resistance to imatinib

Imatinib has been a milestone in the treatment of chronic myeloid leukemia and, in general, for the improvement of the knowledge of tumor pathogenesis. In addition, this drug is now considered as the starting point for the development of targeted strategies, and it could also facilitate the identification of action mechanisms and limitations of anti-cancer drugs.

Although the result achieved are excellent, the main question that come out since the beginning was why some patients were insensitive to imatinib treatment[138]. As depicted in the last section, 15% of patients did not achieve overall survival, and the first aim of clinicians was the definition of response to imatinib. For this purpose, the European Leukemia Network (ELN) and the National Comprehensive Cancer Network have proposed two separate guidelines to help clinicians in the diagnosis and management of CML patients. Both classifications defined the response to imatinib treatment as optimal, suboptimal and failure of therapy. The definitions are based on hematological and cytogenetic responses in a time-dependent manner. Patients showing a suboptimal response or a treatment failure are defined as primarily resistant to imatinib. Other patients show an initial optimal response to imatinib, but they develop a subsequent resistance to treatment. In these cases, the resistance is defined as secondary or acquired. Moreover, the overall rate of patients showing primary or

secondary resistance to imatinib is still on debate and it is a widely accepted opinion that their number could be underestimated[139].

Once again, imatinib targeted therapy allowed to improve the knowledge of drug development. Indeed, the selective activity of imatinib on BCR-ABL allowed to hypothesize several resistance mechanisms that, in general, could be responsible of the reduction of inhibitory activity on the tyrosine kinase. First of all, it was thought that the binding between imatinib and the kinase ABL domain could have been impaired. This is now known as the most important cause of resistance to imatinib in patients[140]. The impairment of drug-target binding mainly occurs for the presence of point mutations in BCR-ABL gene sequence. Point mutations can be linked with an alteration in the amino-acidic sequence of the protein, thus changing the overall structure of BCR-ABL[141, 142].

Several methods were used to define the reasons of resistance to imatinib treatment. As usual, these methods were both pre-clinical and clinical. Usually, all the Ph^+ cell lines are sensitive to imatinib with 0.5μM as a maximum dose. An exception is represented by KCL22 and SD-1 cell lines, in which other additional mechanisms have been described. However, several groups tried to establish reliable *in vitro* models to study the resistance. The best option has been found to be the treatment with imatinib at increasing concentrations. This method requires several months, and it ended up with cell lines showing a marked resistance to imatinib, with an IC50 up to 5μM[143].

Several causes were identified for the *in vitro* resistance of imatinib[138]. The most common mechanism was the significant increase of both mRNA and protein from

bcr-abl gene[144, 145]. Sometimes these findings were associated with gene amplification or duplication. Additional events were increased expression of drug transporters, responsible of imatinib efflux outside the cells[146], or increased expression of proteins involved in alternative cellular pathways such as SRC kinases[147]. Point mutations within ABL kinase were rarely found as a cause for in vitro resistance development.

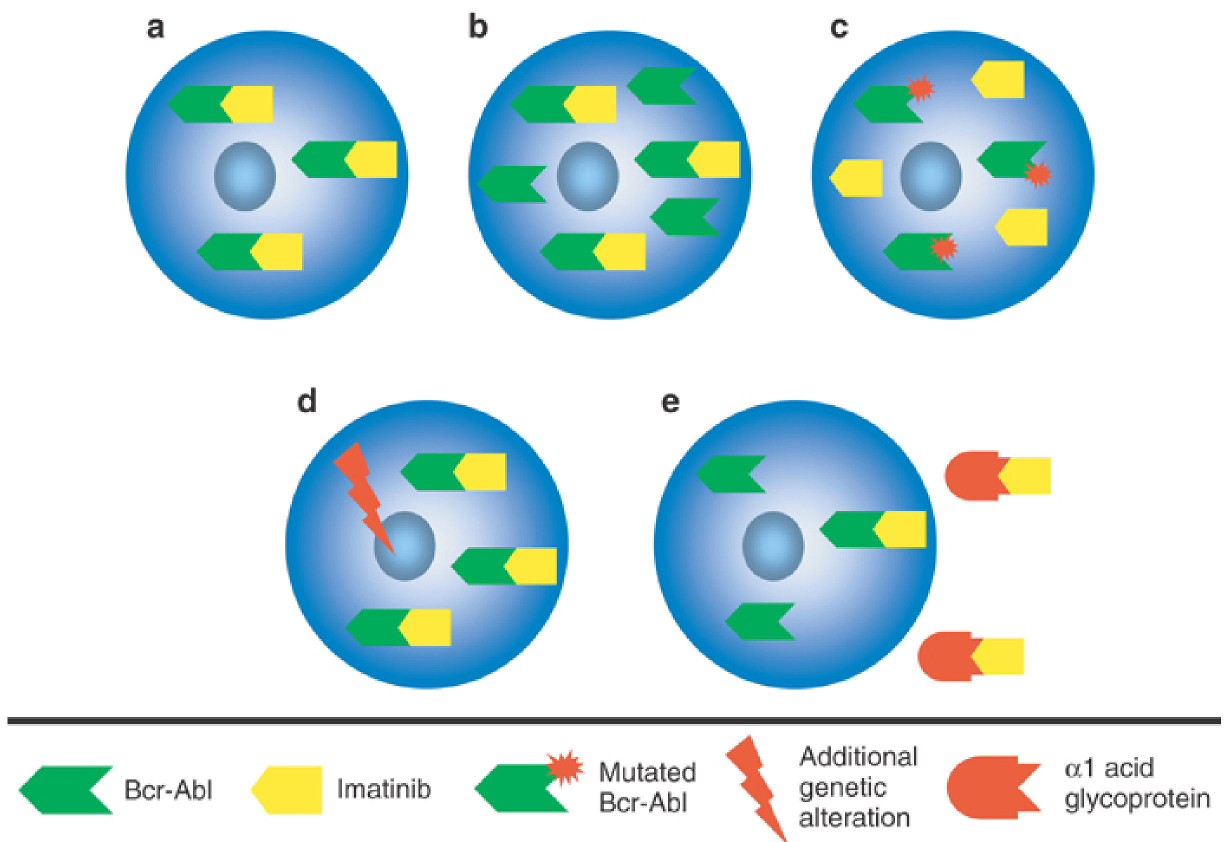

Fig. 9: examples of alterations linked to resistance to imatinib treatment.

However, clinical research showed a markedly different outcome. The most relevant cause of *in vitro* resistance, the increased expression of mRNA and protein encoded by bcr-abl gene, was found to be only marginally involved in clinical resistance to imatinib. Moreover, point mutations within BCR-ABL structure were found to be the most relevant cause of resistance development to imatinib therapy, affecting 50% to 90% patients showing secondary resistance[141]. Indeed, crystal structures developed to characterize the binding between ABL and BCR-ABL with imatinib allowed to define the more relevant clusters of protein mutations impairing the binding with imatinib, and thus related with imatinib resistance[148].

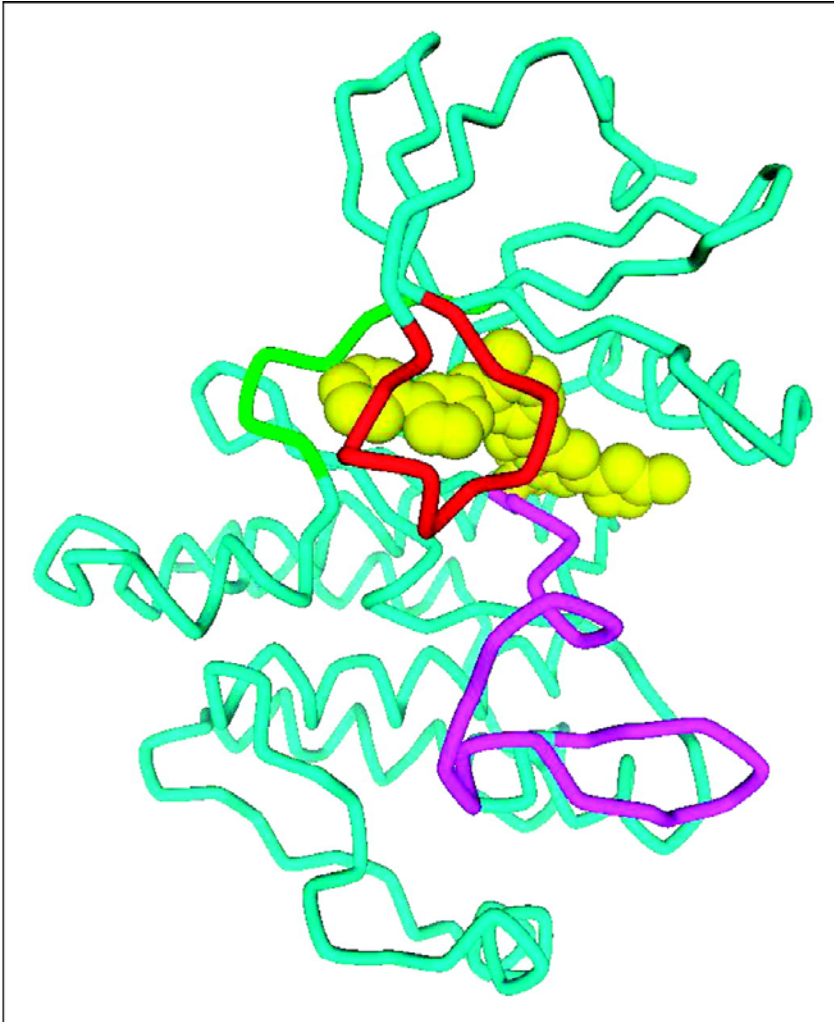

Fig 10: 3D structure of imatinib/bcr-abl complex.

The mutations can affect several aminoacids, but the more relevant can be distinguished in four main clusters: P-loop mutation, T315 mutations, M351 and A-loop mutations[149].

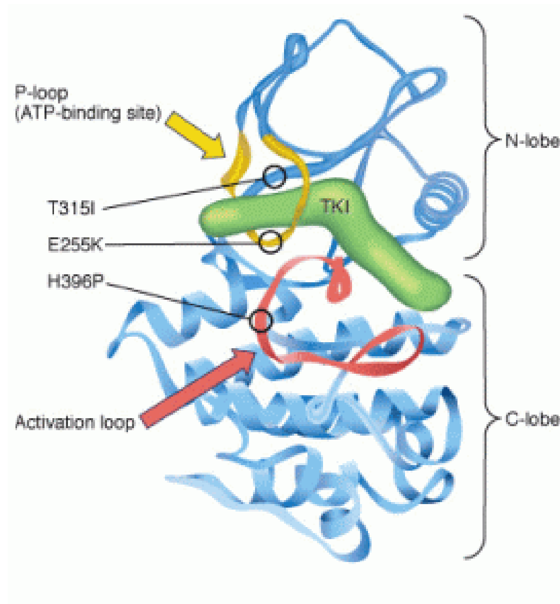

Fig. 11: common sites related to resistance in 3D structure of imatinib/bcr-abl

The P-loop domain starts from amino-acid 244 and ends at the amino-acid 255. This protein portion is usually responsible for the accommodation of the phosphate group of ATP. The interaction of imatinib with BCR-ABL is stabilized by an hydrogen bond between Y253 and N322, and by the formation of an hydrophobic pocket for the drug. If the Y253 is mutated, the hydrogen bond is disrupted. In addition, P-loop is responsible for the shift toward the active BCR-ABL conformation that is not suitable for imatinib binding. Moreover, some mutations are relevant only in the cytoplasmic environment. All together, mutations affecting the P-loop domain are responsible for a 70 up to 100 fold increase of imatinib IC50[150].

Mutations affecting T315 are the most common cause of clinical imatinib resistance. T315 forms an hydrogen bond with imatinib. The most common T315 mutation, the shift from threonine to isoleucine, leads to hydrogen bond disruption and also to allosteric interference with the drug binding. T315I is currently related with a poor outcome to imatinib treatment, as the resistance related with this mutation was not circumvented even by new generation tyrosine kinase inhibitors[151].

The crystal structure allowed to understand why mutations of M351 were related with resistance development. By interacting with ABL SH2 domain, M351 helps the stabilization of the auto-inhibited conformation of BCR-ABL. The presence of a mutation leads to the disruption of the interaction between SH2 and the kinase domain, thus resulting in a shift toward the active conformation. As explained above, imatinib can bind only to the inactive conformation, and this explains the development of resistance correlated with this M351 point mutation[152].

A-loop domain is the amino-acidic portion between the aminoacid 381 and the aminoacid 402. The kinase domain is influenced by the position of the A-loop. The N-terminal portion of the domain is composed by three amino-acids, aspartate-pnenylalanine-glycine (DFG) motif. This motif is highly conserved in all the tyrosine kinases. In the open position, A-loop moves from the catalytic center of the kinase, allowing D381 to bind Mg^{2+}, which coordinates the phosphate group of ATP. The C-terminal portion of the A-loop has a sterical role in substrates binding. In general, relevant mutations within A-loop domain preserve the kinase in an active conformation, thus preventing imatinib binding[153].

Although point mutations within catalytic domain of BCR-ABL have proven to be to most relevant cause of resistance to imatinib treatment, other molecular mechanisms have been described both in cellular models and in patients.

First of all, increased levels of BCR-ABL mRNA and protein levels were confirmed as a resistance cause also in patients. As described above, this cause was identified in vitro but failed to be translated in patients. leCoutre *et al.*, demonstrated that genetic duplication of bcr-abl gene was responsible of increased levels of transcript and protein[154]. The increased levels of the drug target correlated with an unsuccessful treatment with imatinib, unless with an increase of the daily dose[138, 154].

Another possible reason for resistance development is related with pharmacokinetics. Many chemotherapeutics show a correlation between plasma levels and drug efficacy. The same evaluation was done in CML patients treated with imatinib. Attempts were focused on the definition of the time required for leukemic cell apoptosis after treatment, and to assess the optimal imatinib concentration for a complete inhibitory activity on BCR-ABL. Few groups found a correlation between low plasma levels of imatinib and development of resistance. However, whether imatinib plasma concentrations are altered in patients showing resistance to imatinib is still under study and debatable [155-157].

Another putative resistance mechanism is the up-regulation of pathways alternative to BCR-ABL, involved in the tumor development[158]. This hypothesis is still under study, due to the lacking of systems that mimic CML without BCR-ABL, both *in vitro* and *in vivo*. However, hypothesis for the definition of pathways involved in the

resistance development have been advanced. The most characterized pathway is actually the one involving Src family kinases (SFK). The interaction between BCR-ABL and SFKs is direct, and the outcome is the opening of BCR-ABL structure in an active conformation, impairing imatinib binding. Moreover, phosphorylation of SH2 and SH3 domains of BCR-ABL by SFKs could determine an increased activation of BCR-ABL, relating again with a limited activity of imatinib at standard doses. The hypothesis of a resistance linked with SFKs up-regulation suggests a functional advantage of dual Src/Abl kinase inhibitors such as dasatinib and bosutinib. However, clinical data about this issue are still lacking. Other pathways potentiated by BCR-ABL activity are the Ras/Raf/Mek kinase pathway, Grb-2/Erk pathway, and Jak/STAT pathway. Also the involvement of altered regulation of these pathways has been only suggested, but still with no clinical results available[159-161].

Epigenetic modifications have been proposed to be involved in the increase of resistance to imatinib. Also in this case, clinical data have been controversial. At a molecular level, epigenetic modifications can result in the alteration of acetylation pattern of non-histone proteins. The reduction of acetylation can be linked with a reduction of the transcription of tumor suppressors, and thus it can be directly involved in the abnormal increase of cellular proliferation and reduction of apoptosis in leukemic cells. Taken together, the overall effect is the development of resistance to imatinib treatment. In vitro experiments both in cell lines or in primary cultures demonstrated the synergistic activity of histone de-acetylase inhibitors and tyrosine kinase inhibitors. As for the other causes of resistance, the translation to clinical studies gave poor results,

with a moderate restoration of sensitivity in resistant cells after imatinib and decitabine (an histone de-acetylase inhibitor) exposure[162-164].

The last mechanism of resistance to imatinib treatment is well described in literature, and it is named multidrug resistance (MDR)[146]. Briefly, this resistance is associated with altered levels or point mutation affecting drug transporters. Drug transporters are involved in the uptake or in the efflux of several substrates including drugs and xenobiotics. An impairment in the uptake or in the efflux of imatinib can result in a decrease of intracellular imatinib concentration, and thus with resistance to standard dose treatment with imatinib. Multidrug resistance will be described more in detail in a dedicated section, due to the relevance of the matter for my PhD thesis.

Alternative therapeutic options for CML

Second and third generation tyrosine kinase inhibitors

The lesson learned from imatinib led to the development of other tyrosine kinase inhibitors. All together, the new inhibitors were defined as second generation inhibitors, because their advent followed imatinib. The desired features of new tyrosine kinase inhibitors were increased potency and increased selectivity. Moreover, this new class of agents was expected to overcome the major limitation affecting imatinib treatment: the development of resistance.

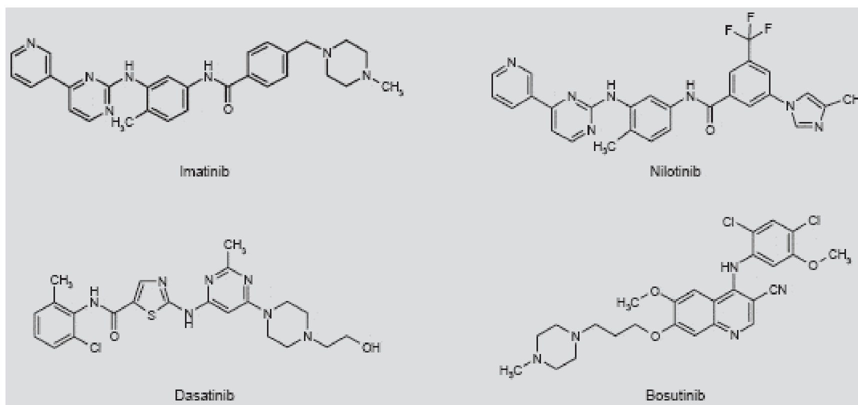

Fig 12: structure of the tyrosine kinase inhibitors imatinib, nilotinib, dasatinib and bosutinib.

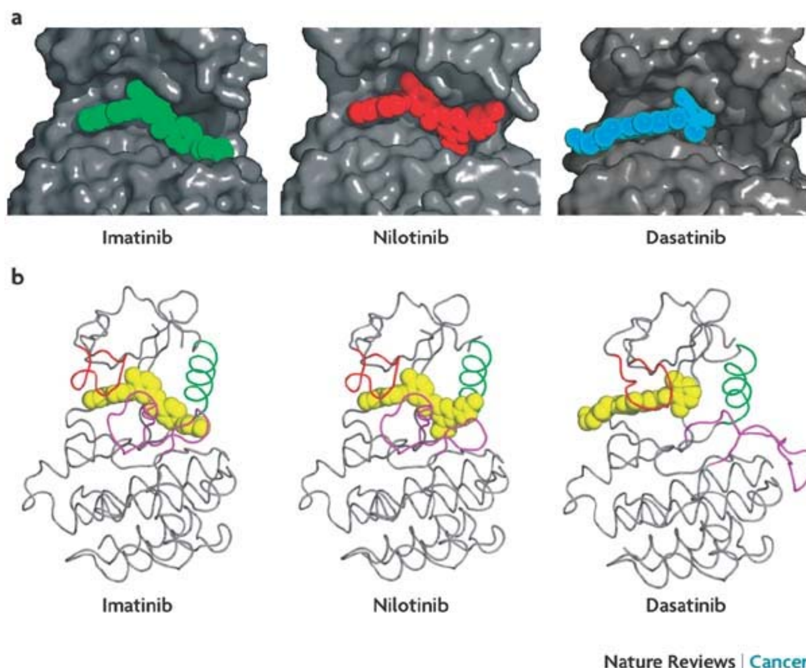

Fig. 13: interaction of imatinib, nilotinib and dasatinib with bcr-abl (3D structure).

Dasatinib was the first second generation inhibitor developed after identification of primary or secondary resistance affecting imatinib efficacy. Dasatinib is currently an oral BCR-ABL inhibitor, as it was approved in 2006 for the treatment of CML patients showing resistance to imatinib and Ph^+ ALL patients[165, 166]. Dasatinib resulted as the most promising inhibitor from a screening of series of substituted 2-(aminopyridil)- and 2-(aminopyrimidil)thiazole-5-carboxamides. Subsequent *in vitro* and *in vivo* characterizations confirmed that dasatinib had a potent inhibitory activity on BCR-ABL. Dasatinib was also identified as an inhibitor of Src kinases. Thus it is classified as a dual Src/Abl kinase inhibitor. The most important difference between dasatinib and imatinib is that the first one is active both on the inactive closed conformation of BCR-ABL and

on the active, open conformation. As described above, imatinib is able to bind BCR-ABL only if the kinase is in an inactive conformation. Dasatinib ability to bind the open active conformation elucidates also its activity on Src kinases. Indeed, Src kinases have a structural conformation similar to BCR-ABL in open conformation. Other substrates of dasatinib are c-Kit and PDGF receptor β. Dasatinib showed a more potent activity on bcr-abl compared to imatinib, with a 325fold increase on wild type bcr-abl. Moreover, dasatinib is active on several (but not all) bcr-abl mutants showing resistance to imatinib[167-171].

The other second generation inhibitor of bcr-abl activity currently approved is nilotinib (Novartis, Basel, Switzerland). FDA approval arrived in 2007, and now the inhibitor is routinely used newly diagnosed CP and AP CML patients. Nilotinib development was based on the crystal structure data of imatinib complexed with Abl, and it was selected after a screening. Nilotinib shows an increased potency on BCR-ABL (20-50 fold compared to imatinib) and improved selectivity, as it binds to Abl kinase domain with only 4 hydrogen bonds. Similarly to imatinib, nilotinib binds only the inactive conformation of BCR-ABL, but after the binding nilotinib results in a more potent activity because of the introduction of structural changes that overall improve the binding. This inhibitor also showed to be active on BCR-ABL mutants resistant to imatinib treatment. However, particular mutations such as T315I cause resistance also to nilotinib[172-176].

Taken together, dasatinib and nilotinib solved the problem of resistance in about 50% of patients previously resistant to imatinib.

With the advent of valid options to imatinib treatment, an important question have been whether the new tyrosine kinase inhibitors could substitute imatinib as a first line treatment[177]. Another therapeutic option would be the co-treatment of patients with imatinib and a second generation tyrosine kinase inhibitors or with other agents. However, the debate is actually open and only long term studies will help to assess which is the best front line therapy to be used[178, 179].

Alternative strategies

Currently, several therapeutic options are under study to circumvent resistance to treatment to imatinib but also to the newer inhibitors dasatinib and nilotinib. The most challenging cause of resistance is the mutation T315I within the kinase domain of BCR-ABL[180]. This mutation is related with resistance development to all the three inhibitors approved by FDA, and it can be found in a large cohort of patients showing resistance to imatinib and to second generation tyrosine kinase inhibitors. The different strategies to treat resistance patients render difficult to evaluate which is the overall incidence of T315I in resistance patients. However, it has been suggested that this mutation could be the resistance cause of up to 15% patients previously treated with imatinib. Imatinib, nilotinib and dasatinib show very similar structure, thus explaining why they are all ineffective to inhibit T315I BCR-ABL[181].

New strategies to overcome this problem are third generation tyrosine kinase inhibitors, Aurora kinase inhibitors, switch pocket inhibitors and apoptosis modulator.

Here, a brief report of the most promising agents is presented. Bosutinib is a third generation tyrosine kinase inhibitor, but it will be discussed in detail in a separate section.

Third generation TKIs

One of the most promising agents is ponatinib (previously AP24534. Ariad, Cambridge, MA, USA). Ponatinib is a multi-kinase inhibitor, and it is active also on T315I BCR-ABL. Ponatinib should enter soon in phase II clinical trial, to evaluate the efficacy in CP-CML patients[182].

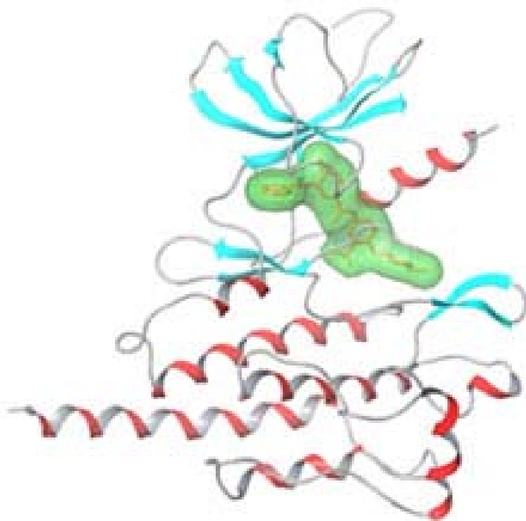

Fig. 14: 3D structure of ponatinib.

INNO-406 (previously NS-187. CytRx, Los Angeles, CA, USA) is a dual Abl/Lyn inhibitor showing a 25-55 fold increased inhibitory activity on BCR-ABL compared to

imatinib. It is inactive on T315I BCR-ABL mutants, but it shows activity against BCR-ABL with mutation within the P-loop, such as F317. Phase II studies of the inhibitor should start briefly[183]

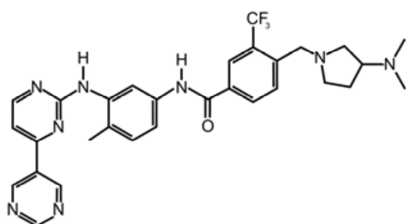

Fig. 15: chemical structure of INNO-406.

XL228 (Exelixis, South San Francisco, CA, USA) is another multi-kinase inhibitor acting on a wide range of substrates. It is considered as a promising agent for CML treatment mainly because of its activity against T315I BCR-ABL. Currently the inhibitor is in Phase I clinical trial[184].

Aurora kinase inhibitors

Aurora kinases are supposed to play a central role for the presence of karyotipic alterations, typical feature of leukemic cells. In detail, the transition from G2 to cytokinesis is regulated by several kinases, globally known as mitotic kinases. Aurora kinases A and B are the prominent components of the mitotic kinases, their function being the control of centrosome duplication, chromosome alignment, and mitotic

checkpoint arrest. Given the physiological relevance of Aurora kinases, it is easy to understand that inhibitors of Aurora kinases could have a limited clinical use due to severe adverse events linked with aberrant mitosis[185].

However, several Aurora kinases inhibitors have been described as acting also on other kinases, in particular on Abl kinase. Between other compounds, the most promising Aurora kinases inhibitors for CML treatment are danusertib (previously PHA-739358. Nerviano Medical Sciences, Milan, Italy)[186], KW-2449 (Kyowa Hakko Kirin Pharma, Tokyo, Japan)[187] and AT9283 (Astex, Cambridge, England)[188].

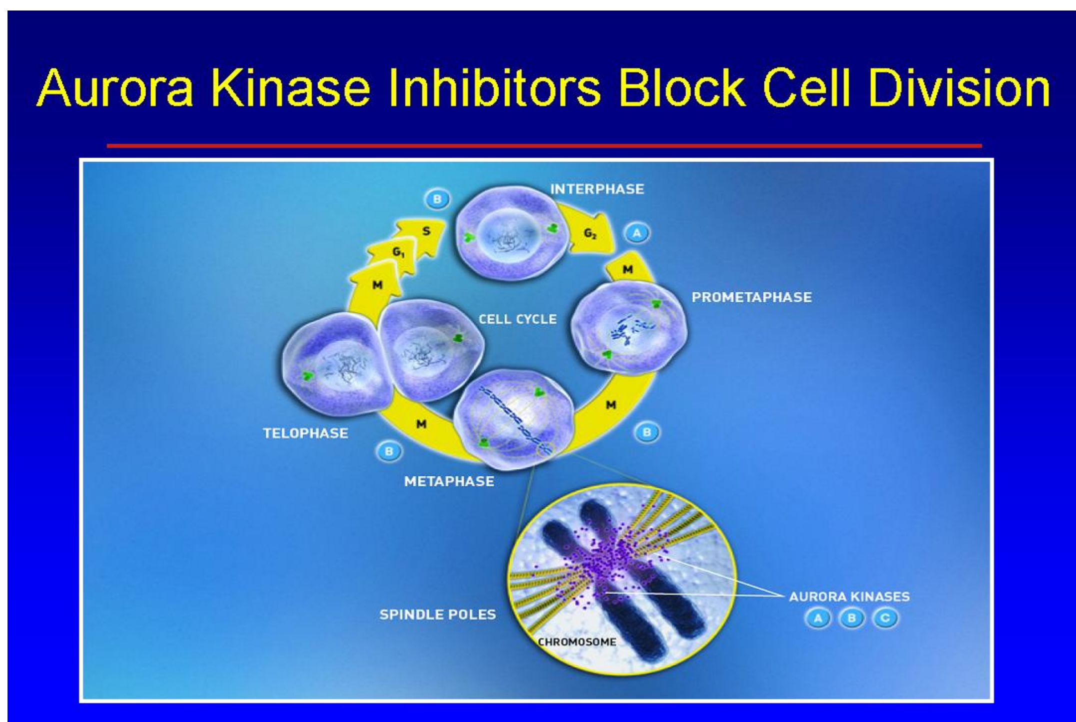

Fig. 16: mechanism of action of Aurora kinase inhibitors.

Switch pocket inhibitors

This recent class of inhibitors was developed starting from the concept of inhibiting BCR-ABL without binding to ATP pocket. The switch pocket inhibitors bind to residues that the kinase requires to shift to the open, active conformation. Thus, presence of mutations as T315I (as well as other mutations typically limiting tyrosine kinase inhibitors activity) should not interfere with the inhibitory activity of this class of compounds[189]. Actually, the lead compound DCC-2036 (Deciphera, Lawrence, KS, USA) has been pre-clinically tested with success on various mutants of Abl kinase, including T315I. A phase I clinical trial has been recently started to study the efficacy of this drug[190].

Apoptosis modulators

Apoptosis modulators are the last class of drugs under study for the treatment of patients resistant to imatinib and to second generation tyrosine kinase inhibitors. Omacetaxine (Omapro, previously Homoharringtonine. ChemGenex, Victoria, Australia) is an agent that increase protein synthesis by binding on 80S ribosome and interfering with chain elongation. Furthermore, it causes disruption of mitochondrial membrane, with consequent cytocrome C release and induction of apoptosis[191]. Another pro-apoptotic mechanisms may include the alteration of stability of MCL-1, an anti-apoptotic factor. This class of agents is promising for patients that showed resistance to tyrosine

kinase inhibitors treatment, because the modulators focus on different targets. Currently, a phase II/III clinical trial is going on with good results.

Bosutinib

As described previously, bosutinib is a dual Src/Abl inhibitor[192]. The development of Src inhibitors was started because of the central role of Src in several tumors. The following application of Src inhibitors in CML was hypothesized due to structural similarity of the Src and Abl kinase domains[193].

Four Src inhibitors were developed: dasatinib (previously described), saracatinib (previously AZD0530. Astra Zeneca), bosutinib (previously described) and KX2-391 (previously KX01. Kinex)[194]. The first three agents were considered for Abl inhibition, because they are ATP competitors within Src kinase domain, and they could exert the same activity in Abl kinase domain. Dasatinib is currently approved for the treatment of CML patients resistant or insensitive to imatinib, and phase III clinical trials are ongoing to assess dasatinib or bosutinib use a front-line therapy for CML.

The first attempts to characterize bosutinib activity were published in 2001. Bosutinib was thought to be a selective Src inhibitor, although several studies had demonstrated that the drug was able to inhibit several kinases[192]. Puttini M. et al. demonstrated in 2006 that bosutinib was active not only against Src, but also against other kinases including Csk, the kinase that regulates Src activity thorough phosphorylation[195]. The lacking of high selectivity could be seen as a limitation of

bosutinib and other Src/Abl inhibitors. However, if the clinical setting is appropriate, the wide activity of the dual inhibitors allows using them in several kind of tumors. On the other hand, side effects need to be tightly monitored.

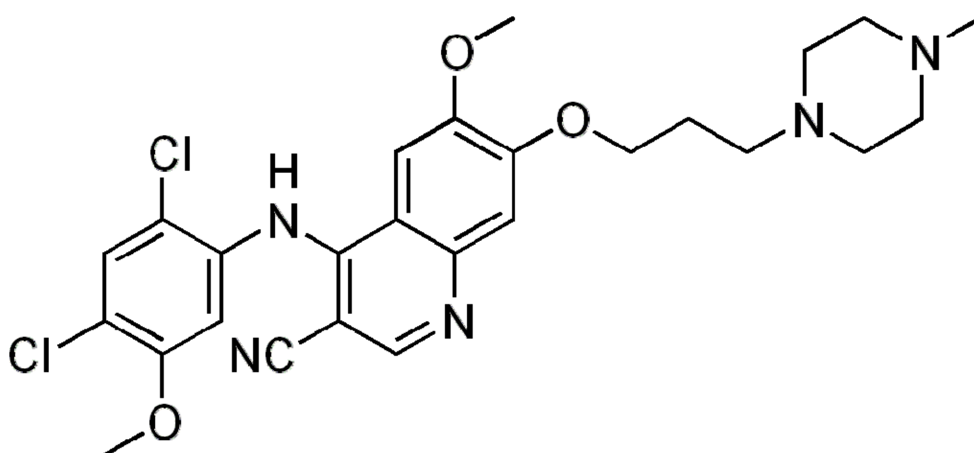

Fig. 17: bosutinib chemical structure.

The clinical characterization of dual inhibitors activity is easier for Abl than for Src, and this is due to a better defined pathology (CML) with a unique molecular target for drugs. The other advantage is represented by CML biomarkers availability that allows a direct evaluation of inhibitory effects of the drugs. Thus, clinical development of dual inhibitors has been much more rapid for CML treatment than for tumors in which Src is involved. However, bosutinib is currently under study as a kinase inhibitor for solid tumors.

The pre-clinical development of bosutinib started from a classic drug screening for the inhibition of Src. The lead compound selected was a 4-anilino-3-

quinolinecarbonitrile. After kinase activity and *in vitro* characterization, functional groups were attached to improve solubility. Bosutinib activity against Abl was disclosed in 2003, after the screening of the inhibitor activity in several tumor cell lines. Three Ph^+ cell lines were tested: KU812, K562 and Meg-01. Bosutinib showed a marked anti-proliferative activity in these cell lines, while no activity was detected in other leukemia cell lines except HSB2, a T-cell leukemia cell line. For instance, anti-proliferative activity in KU812 had an IC50 of 5nM, thus much more higher than micromolar activity seen in most cell lines. Kinase assay showed a Bcr-Abl inhibition IC50 of 1.4nM, even higher than the 3.5nM inhibition seen against Src activity. Moreover, bosutinib was also active against several Bcr-Abl mutated form, and in vivo studies confirmed that bosutinib was able to induce regression of K562 CML tumors inoculated in mice. All together, these findings suggested a clinical role for the treatment of CML patients for bosutinib[196]. Puttini M. et al. found that bosutinib inhibited proliferation and induced apoptosis in cell lines selected for the resistance to imatinib. Moreover, bosutinib was active on Bcr-Abl mutants as Y253F, E255K and D276G, but not on T315I[195].

An important element to be considered is how the inhibitors act on the signaling downstream of Bcr-Abl. A wide activity extended to other molecular targets involved in the pathology can correlate with increased potency of treatment. The key elements of the pathways activated by Bcr-Abl in CML are, between others, Bcr-Abl auto-phosphorylation at Y245, Crkl phosphorylation at Y207, STAT5 phosphorylation at Y694 and Src family kinase Lyn phosphorylation at Y397. Bosutinib showed in vitro activity against all of these targets, thus justifying the nanomolar activity on Ph^+ cells.

The activity on primitive and committed CML progenitors was also studied for bosutinib. In CML patients, the inhibitor showed to inhibit the proliferation of both these cells, while a little effect was seen in normal cells. However, focusing on the apoptotic induction, bosutinib showed to be partially ineffective in CML committed progenitors and totally ineffective in primitive cells[197].

A study by Redaelli S. et al. compared the activity of imatinib, nilotinib, dasatinib and bosutinib against a panel of Bcr-Abl mutants with marked clinical relevance. The activity of each inhibitor on wt Bcr-Abl was used for normalization, thus the activity on the mutants was expressed as a ratio indicating the activity of the inhibitors. The levels of activity were divided in sensitive, resistant and highly resistant. The main differences were found in P-loop mutants, because they showed overall sensitivity to bosutinib and dasatinib but not to imatinib and nilotinib. This panel represents a good basis for the evaluation of the best therapy in patients with Bcr-Abl mutations, but it still requires the support of clinical observations[198].

		IC_{50} fold increase (WT = 1)			
		Bosutinib	Imatinib	Dasatinib	Nilotinib
	Parental	38.31	10.78	> 50	38.43
	WT	1	1	1	1
P-LOOP	L248V	2.97	3.54	5.11	2.80
	G250E	4.31	6.86	4.45	4.56
	Q252H	0.81	1.39	3.05	2.64
	Y253F	0.96	3.58	1.58	3.23
	E255K	9.47	6.02	5.61	6.69
	E255V	5.53	16.99	3.44	10.31
C-Helix	D276G	0.60	2.18	1.44	2.00
	E279K	0.95	3.55	1.64	2.05
ATP binding region (drug contact sites)	V299L	26.10	1.54	8.65	1.34
	T315I	45.42	17.50	75.03	39.41
	F317L	2.42	2.60	4.46	2.22
SH2-contact	M351T	0.70	1.76	0.88	0.44
Substrate binding region (drug contact sites)	F359V	0.93	2.86	1.49	5.16
A-LOOP	L384M	0.47	1.28	2.21	2.33
	H396P	0.43	2.43	1.07	2.41
	H396R	0.81	3.91	1.63	3.10
	G398R	1.16	0.35	0.69	0.49
C terminal lobe	F486S	2.31	8.10	3.04	1.85

Sensitive	≤ 2
Moderately resistant	2.01-4
Resistant	4.01-10
Highly resistant	> 10

Fig. 18: comparison of imatinib, nilotinib, dasatinib and bosutinib activities on bcr-abl mutants.

The further step of bosutinib characterization was the assessment of its activity on several kinases, even structurally unrelated. In particular, a study by Kinaxo identified five major kinase groups of action for bosutinib: Eph receptors, Sterile 20

kinases, Trk family, Tec family and Axl family. Moreover, bosutinib was identified as a potent inhibitor of mutated forms of EGFR[199].

In summary, bosutinib is currently in phase III clinical trial for use in front-line treatment of CML, in phase II clinical trial for use in ER^+ breast cancer in combination with other agents, and in phase II clinical trial in triple negative breast cancer in combination with cytotoxic agents.

Multidrug resistance

Multidrug resistance concept was introduced in literature in 1970[200]. However, this kind of resistance was already known since the introduction of chemotherapy against infectious diseases and of chemotherapy with cytotoxic agents for neoplastic diseases. Although the molecular reason of this resistance was unveiled 40 years ago, activity of different agents for the treatment of different pathologies were often characterized by an initial sensitivity to treatment followed by acquired resistance within months. To overcome secondary resistance, a common strategy was to use drugs with similar activity and different chemical structures. However, this strategy was very often ineffective. The poor outcome to treatment with structurally unrelated compounds was defined multidrug resistance (MDR). Also tyrosine kinase inhibitors show this typical

resistance, and nowadays the MDR still represents a major cause of resistance to anticancer therapies[201, 202].

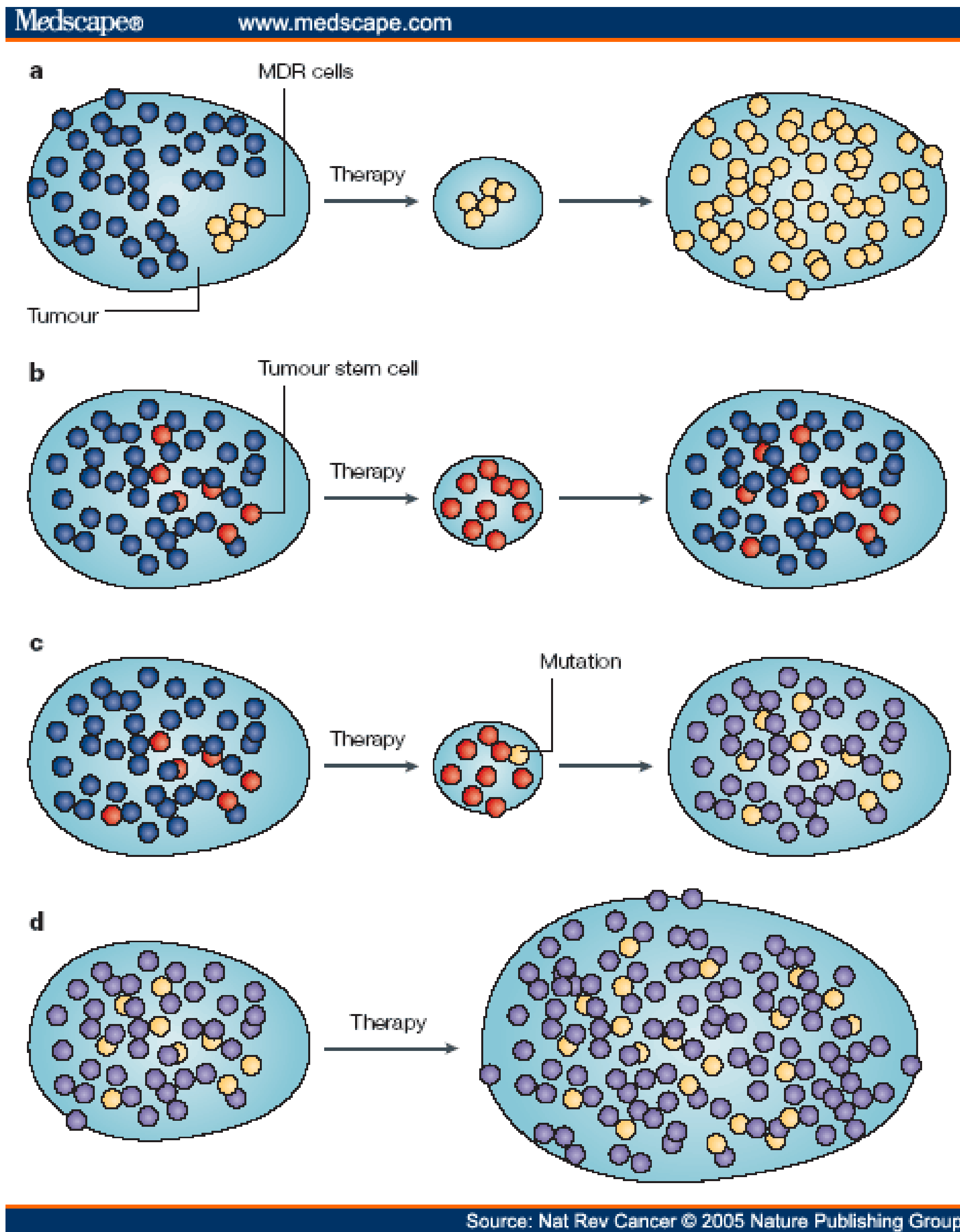

Fig. 19: multidrug resistance affecting anti-cancer therapies.

One strategy to overcome MDR is the treatment with an higher drug dose. However, the main problem of this strategy is that several anticancer agents show a low therapeutic index. The therapeutic index correlates the drug concentration needed for the therapeutic effect and the drug concentration that causes toxic effects. Thus, several agents show a limited range for the increase of concentration. MDR is thus an important limitation for the employment of these drugs.

Due to the resistance to a broad range of different drugs, several mechanisms involving alterations in pharmacokinetics can be hypothesized. These mechanisms include inefficient drug application, low metabolic activity (only in case of pro-drugs), alterations in plasma pharmacokinetics, altered tumour microenvironment and altered activity of barriers such as blood brain barrier. However, MDR is mainly due to the alteration of cellular mechanisms involved in the uptake and in the efflux of drugs.

In addition to multidrug resistance associated with pharmacokinetics alterations, it is now well known a multidrug resistance linked with cellular mechanism. As mentioned above, the dramatic change in the study of multidrug resistance started 40 years ago. The key point was the development of reliable in vitro models based on the multidrug resistance phenotype. In general, cell lines resistant to treatment were developed by treating cells with increasing concentrations of a specific drug, over a long period. The treatment ended with the selection of highly resistant cells, and often the resistance was extended also to treatment with structurally or functionally unrelated drugs. This situation corresponds with the multidrug resistance phenotype. The first MDR cell line was selected after treating a rodent cell line with daunorubicin. Nowadays,

several cell lines show a multidrug resistant phenotype, thus allowing to have several in vitro models for drugs characterization[203].

Drug transporters

The major cause of MDR in cell lines corresponds to the first one identified in vitro[204]. The common feature of MDR models was the increased expression of the drug transporter glycoprotein P (P-gp, MDR1) encoded by ABCB1 gene (also known as MDR1 gene). The correlation between MDR phenotype and over-expression of P-gp was identified in 1976. P-gp is a membrane transporter responsible for the efflux of several unrelated compounds, including drugs and xenobiotics. However, the panel of P-gp substrates is wide and include also endogenous substances[205]. The expression of P-gp is physiological as this transporter represents a protection mechanism against toxic agents for cells. It is easy to understand what happens in MDR cells. Cells treated with high levels of toxic compounds respond by increasing the expression of the efflux transporters for such compounds. The increased efflux of drugs outside cells correlates with lower intracellular concentration levels, and thus with decreased activity on the molecular drug target. This chain of events ends with the development of multidrug resistance to several drugs structurally and functionally unrelated, that share a common mechanism of efflux from cells[206]. Besides, P-gp is also expressed in on nuclear and vesicular membranes. Thus, drugs with nuclear targets show a decreased concentration within the nucleus, while all the drugs are partially sequestered within vescicolae[207].

This MDR phenotype was defined “classical”, and it is defined as a phenotype showing resistance to product-related anticancer agents described in Tab 1. The classical MDR is distinguished by atypical MDR, which is characterized by an MDR with physiological expression levels of P-gp[208].

Table 1. Selected drugs of the “classical” MDR spectrum and “classical” MDR modulators.

Drugs transported by MDR1/P-gp	“Classical” MDR modulators
Anthracyclines: daunorubicin, doxorubicin	*Calcium channel blockers:* verapamil, nifedipine, azidopine, dihydropyridines
Vinca alkaloids: vinblastine, vincristine, vindesine	*Immunosuppressants and derivatives:* cyclosporin A, valspodar (PSC833), tacrolimus
Epipodophyllotoxines: etoposide, teniposide	*Antiarrhythmics:* quinine, quinidine, amiodarone
Antibiotics: actinomycin D, dactinomycin, mitomycin C	*Antihypertensives:* reserpine, yohimbine
Taxanes: paclitaxel	*Antibiotics:* hydrophobic cephalosporins
Others: colchicine, topotecan, valinomyycin, puromycin, emetine, digoxin, imatinib	*Steroid hormones and derivatives:* progesterone, tamoxifen
Many other hydrophobic amphipatic drugs and derivatives	*HIV protease inhibitors:* sequinavir, indinavir, retanavir
	Herbal constituents: curcumin
	Others: elacridar (GF120918), zosuquidar (LY335979), tariquidar (XR9576), laniquidar (R101933)

Tab 1: List of Substrates and modulators of P-gp

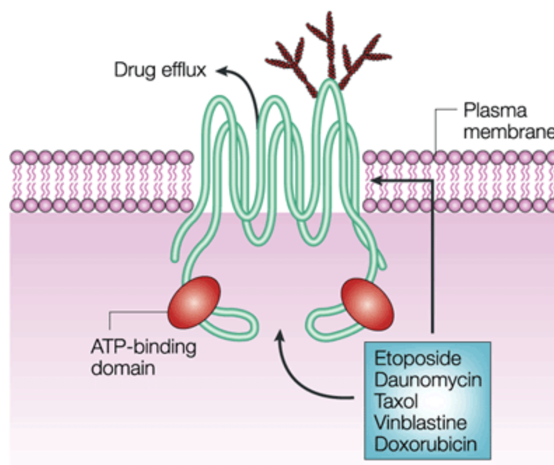

Fig. 20: ABC transporters-mediated drug efflux.

After the first identification, P-gp was identified as the cause of MDR in several other in vitro models of MDR. Currently, P-gp over-expression is considered as one of the main molecular cause of MDR.

Human P-gp is a protein composed by 1280 amino acids, with a molecular weight of 170 kDa. Physiologically is over-expressed in tissues with excretory or secretory functions. In tumours derived from such tissues P-gp in over-expressed, causing a primary multidrug-resistance phenotype. Also in other kind of cancers P-gp can be over-expressed thus causing multidrug resistance with poor treatment outcome in patients[209].

The identification of the role of the drug transporter P-gp in the MDR allowed to hypothesize that other drug transporters could be involved in the same kind of resistance. P-gp is the best characterized component of a superfamily named adenosine triphosphate (ATP)- binding cassette (ABC) transporters[210]. The name of the superfamily is due to a common structural element of the protein members. A consensus sequence of 215 amino acids is highly conserved and it is called ATP-binding cassette or nuclear binding domain (NBD). The ABC domain contains two peptide motifs called glycin-rich Walker A- and the hydrophobic Walker B-motif. The two motifs are involved in ATP binding, and they are present in all nucleotide-binding proteins. The ATP signature is a third domain, unique within ABC domains. However, proteins carrying NBD are not restricted to drug transporters, but share the common feature to couple the phosphate bond energy of ATP to many cellular processes. To be complete, the structure of ABC proteins requires also another component, the trans-

membrane domain (TMD) that is hydrophobic and embedded in membranes. Usually, the TMD is composed of at least six trans-membranes (TM) α-helices. The TMD is responsible of the specificity for the substrates of the transporter. Overall, it seems that the minimal structural requirement for an active ABC protein is the presence of two NBD domain and two TMD domain $[TMD\text{-}NBD]_2$. Usually, the complete structure is encoded by one or two genes.

After whole human genome sequencing, 48 ABC drug transporters were identified and divided in seven subfamilies basing on their phylogenetic characteristics: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG. At least 17 drug transporters were linked with MDR in vitro. However, the original classification of classical or atypical MDR has been maintained over years, indicating the primary importance of P-gp in MDR[211].

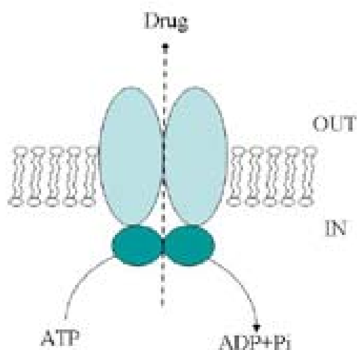

Fig. 21: ABC transporters. ATP dependence for the efflux activity.

A different class of drug transporters is responsible for the uptake of substrates by the cells. In this case, multidrug resistance is linked with a down-regulation of the corresponding genes. This situation causes a decreased uptake of drugs, and thus a reduction of intracellular concentration. Alterations of drug transporters involved in the uptake are supposed to be relevant in the primary resistance and not in the secondary resistance. While the over-expression of ABC transporters is guided by the cells to extrude toxic substances, the expression levels of drug transporters can be physiologically different between patients. The best known and characterized uptake drug transporters belong to SLC22 family[212]. This family of drug transporters include a wide collection of different transporters, such as organic cation transporters (OCTs), organic anion transporters (OATs) and zwitterion/cation transporters (OCTNs). This classification is based on the chemical structure of the substrates, but the wideness of the family is confirmed even considering the transport mechanisms, that can be a symport, and antiport or an uniport. SLC22 family components have been found in different species form bacteria to higher eukaryotes. SLC22 transporters are defined as polyspecific, as they transport a wide range of unrelated compounds, with different molecular weight, structure and function.

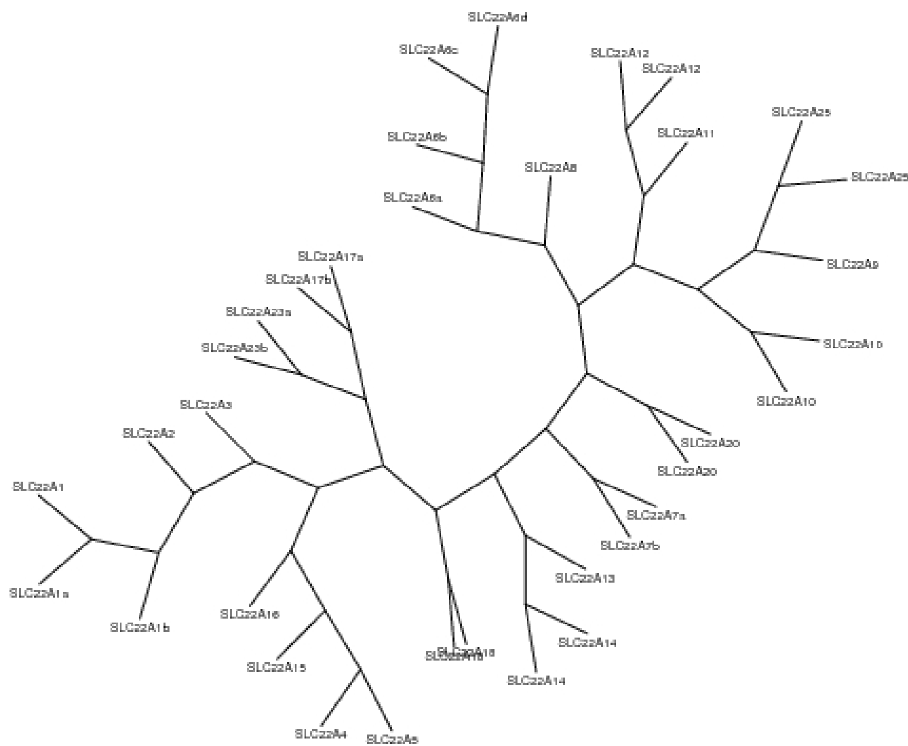

Fig. 22: the SLC22 family.

The first member of the SLC22 family was identified in 1994, and it is called Organic Cation Transporter 1 (OCT1, SLC22A1)[213]. OCT1 is the best characterized drug transporter of the family because of its importance in pharmacokinetics of several drugs. As for the ABC transporters, also OCT1 and SLC22 members in general exhibit a typical topology within the cytoplasmic membrane. OCT1 has 12 α-helical trans-membrane domains (TMDs), an intracellular N-terminus, a large glycosilated extracellular loop between TMDs 1 and 2, a large intracellular loop with phosphorylation sites between TMDs 6 and 7, and an intracellular C-terminus[214].

OCT1 is characterized by a wide distribution in several human tissues. The liver, the kidney and the small intestine show a massive expression of the transporter, but the last can be found also in epithelial cells and in neurons[215]. The broad OCT1 expression is relevant for the pharmacokinetics, because altered levels of the transporter can be linked with a decreased absorption of the drug. In the therapy of cancer, however, it is important to clarify that cancer cells are often characterized by a down-regulation of OCT-1, whit consequent decreased intracellular concentration of anti-cancer agents that are substrates of the transporter.

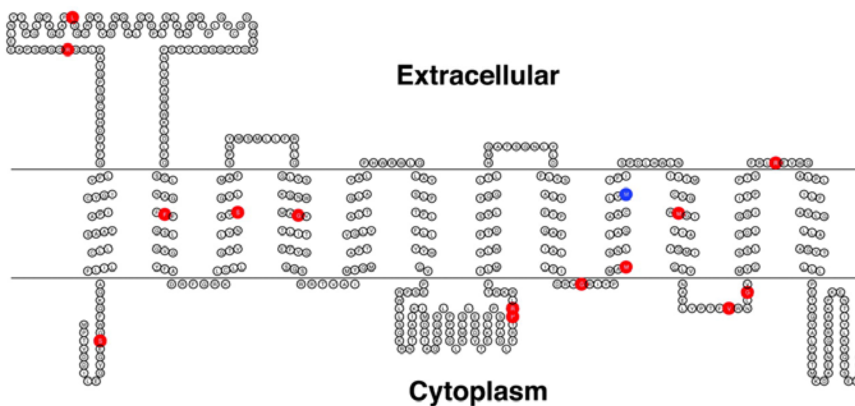

Fig. 23: schematic structure of OCT-1.

It is important to underline that OCT-1 is inhibited by a conspicuous number of agents, and it is believed that many OCT-1 substrates are also able to act as inhibitors of the drug transporter. The substrates of OCT-1 are between 8 and 500Å of diameter,

and usually they are organic cations or weak bases positively charged at physiological pH. However, OCT-1 can also transport non-charged compounds. As the other members of SLC22 family, also OCT-1 is responsible for the transport of endogenous compounds, drugs and xenobiotics[214].

Drug transporters and imatinib

The advent of imatinib as a targeted therapy for chronic myeloid leukaemia allowed to define better mechanisms involved in resistance to treatment. This was due to the “clean” model of action of imatinib, where the drug has a specific molecular target. In clinics, point mutations within the kinase domain of BCR-ABL turned out to be the most relevant cause of resistance to imatinib treatment.

However, multidrug resistance was immediately hypothesized as a putative candidate for resistance to imatinib, both primary or secondary. Pre-clinical studies confirmed that trans-membrane protein were active or passive transporters of imatinib, and thus that the alteration of their expression levels or structure could be related with a decreased intracellular concentration of imatinib[216]. Alterations in the drug transporter system can be relevant in pharmacokinetics or in cell biology. Several pre-clinical studies led to the conclusion that in anti-cancer therapy the cell biology-derived MDR is more relevant. Indeed, cancer cells are able to develop several mechanisms of protection against drugs, and the over-expression of efflux drug transporters can be considered a part of the adaptation to the drug therapy[217]. Thus, it can be suggested

that a patient treated with imatinib could show increased levels of expression of P-gp and/or BCRP in cancer cells and not in other organs where the two transporters are expressed at normal, physiological levels. The idea is that, after drug exposure, tumour cells induce increased expression of efflux drug transporters, thus preserving against apoptotic activity promoted by imatinib. This model is strongly supported by in-vitro models, where cells sensitive to imatinib, after long-time exposure to the drug become resistant and show higher expression levels of P-gp and BCRP.

The situation is markedly different for the drug transporters deputed for imatinib uptake. OCT-1 has a recognized prominent role in this mechanisms, and it is currently under debate whether could be considered as a prognostic factor for imatinib treatment[218, 219]. It has been suggested that OCT-1 could be involved in primary resistance to imatinib. Indeed, decreased levels of the drug transporters are related with a decreased intracellular concentration of imatinib within the cancer less, with consequent reduced activity against BCR-ABL (the outcome is similar to the one observed with increased levels of efflux drug transporters). Altered expression levels of OCT-1 are supposed to be due to an intra-individual difference and not to an adaptation mechanism[220]. In this case, down-regulation of OCT-1 could affect both the pharmacokinetics and the tumour cell biology, leading to an overall reduction of plasma and intracellular concentration of imatinib.

In vitro studies showed the prominent role of P-gp in the development of multidrug resistance to imatinib treatment. However, translational medicine showed that only OCT-1 expression levels seem to be important for the therapy efficacy.

However, the importance of efflux drug transporter in patients is still under debate. Evidences indicate that efflux drug transporters could be over-expressed in leukemic progenitors, thus indicating a further mechanism of protection of cancer stem cells during the targeted therapy. In particular, several reports indicate BCRP as the key drug transporter protecting cancer stem cells from imatinib activity[221].

Imatinib was also indicated as an inhibitor of drug transporter. This identification led to hypothesize that the sensitivity of cells over-expressing drug transporters could be restored with the inhibitory activity of imatinib against drug transporter activity. This scenario could have been an explanation of the lacking of clinical importance of P-gp in terms of multidrug resistance, following the P-gp role in multidrug resistance in vitro. However, recent works indicated that imatinib can inhibit the efflux activity of P-gp or BCRP only at high concentration, much more superior than those used in clinical routine. Thus, the inhibition of drug transporter activity is present but it has no clinical relevance[222].

All together, the data so far identified indicate that multidrug resistance in imatinib treatment is a mechanism that need to be considered, although other resistance mechanisms show prominent roles in patients. The identification of multidrug resistance affecting imatinib treatment suggested new therapeutic strategies for clinicians. First, the introduction of evaluation of expression levels of drug transporters before the therapy. In particular, low expression levels of OCT-1 could suggest a therapy with an increased daily dose of imatinib. Second, the alternative therapeutic agents against BCR-ABL could represent a valid option for patients showing multidrug resistance to

imatinib. Indeed, both pre-clinical and clinical studies focused on the identification of drug transporters involved in nilotinib and dasatinib uptake or efflux.

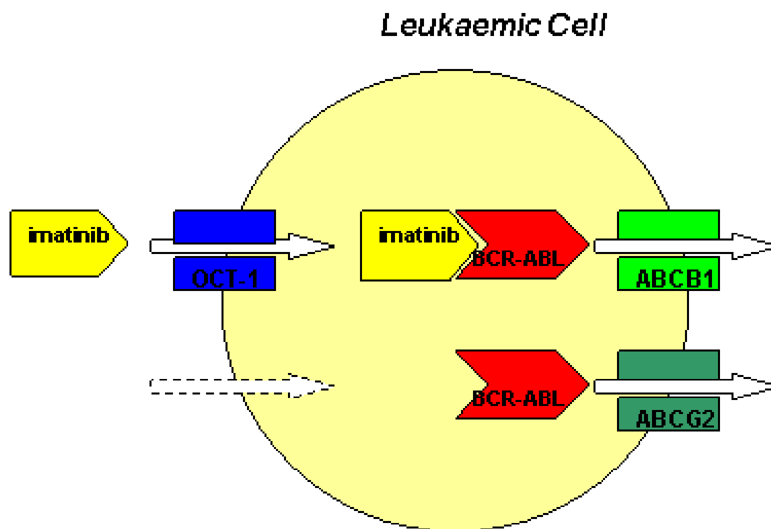

Fig. 24: summary of the drug transporters role in imatinib multidrug resistance.

PhD project aim

The aim of my PhD project was the identification of the role of the drug transporters P-gp, BCRP and OCT1 in bosutinib efflux and uptake. We selected these three transporters because of their involvement in imatinib cellular metabolism. We characterized the interaction of bosutinib with the three transporters, then we explained the functional meaning at a biological and molecular level of altered expression of transporters involved in bosutinib uptake/efflux. For the next future, an *in-vivo* experiment is planned to evaluate if a K562DOX cells show resistance to bosutinib treatment even after a transplant.

As bosutinib can be considered as an alternative to imatinib for the treatment of chronic myeloid leukaemia, the final goal should be the use of bosutinib instead of imatinib in patients showing altered levels of drug transporters not involved in bosutinib cellular metabolism.

Matherials and Methods

Chemical compounds and reagents. bosutinib (SKI-606), and C-14 radiolabeled bosutinib (C-14 bosutinib) were obtained from Pfizer Inc, New York, NY, USA. Both bosutinib and C-14 bosutinib were dissolved in DMSO to obtain a 10mM stock solution. imatinib (STI-571, gleevec) was synthesized by Enrico Rosso, MD, University of Venice, Italy. Imatinib was dissolved in water to obtain a 10mM stock solution.

Doxorubicin (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in DMSO to obtain a final concentration of 10mM. Geneticin and puromycin, purchased by Euroclone (Milan, Italy), were both dissolved in water to obtain stock solutions of 400mg/mL and 25mg/mL, respectively. The three compounds were used for the selection of cell lines.

Verapamil hydrochloride (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in ethanol to obtain a final concentration of 100mM and used as selective P-gp inhibitor. Amantadine hydrochloride (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in ethanol to obtain a final concentration of 1M and used as selective Oct1 inhibitor. Fumitremorgin C (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in DMSO to obtain a final concentration of 1mM and used as selective BCRP inhibitor. Verapamil, amantadine and fumitremorgin C were used for the selective inhibition of drug transporters.

Rhodamine 123 (Sigma-Aldrich, Saint Louis, MO, USA), 4-Di-2-ASP (Invitrogen, Carlsbad, CA, USA), Pheophorbide A (Frontier Scientific, Logan, UT, USA) were

dissolved in DMSO to obtain a final concentration of 2mM, 10mM and 1mM respectively. These compounds were used to assess the functionality of over-expressed drug transporters, being known fluorescent substrates detectable with flow cytometry.

Cell lines and cell cultures. K562DOX (kind gift of JP Marie, Université Pierre et Marie Curie, Paris), K562DOX/sh P-gp (formerly K562Dox-siMDR/MM; kind gift of E. Gunsilius, Innsbruck Medical University, Austria), K562Oct1, K562Oct1/sh Oct1, K562BCRP and K562BCRP/sh BCRP cell lines were derived from the leukemic BCR-ABL^{+} cell line K562S (K562 imatinib sensitive).

To obtain K562DOX cell line, K562S cells were treated with increasing concentrations of Doxorubicin, allowing the selection of K562S cells expressing high levels of P-gp[223]. Moreover, the over-expression of P-gp is considered as an adaptation mechanism to reduce the intracellular concentration of the chemo toxic Doxorubicin. Once the cell line has been established, the over-expression is stable and the selective pressure was kept by adding 1μM Doxorubicin once/month.

Also K562DOX/sh P-gp were obtained from other groups. Briefly, K562DOX/sh P-gp cell line was derived from K562DOX cells with a transposon-based vector system for silencing, allowing a stable P-gp silencing[224]. In these cells, the selective pressure was kept by constantly adding adding Geneticin.

K562Oct1 cell line was obtained with a stable transfection by electroporation with pcDNA3.1 plasmid carrying Oct1 gene coding sequence (kind gift of Prof. R. Clark and A. Giannoudis, Royal Liverpool University hospital, UK)[225].

BCRP transcript was isolated from the carrier plasmid pCMV6-XL5 obtained from Origene (Rockville, MD) and cloned in a pcDNA3.1 plasmid. K562BCRP cell line was obtained with a stable transfection by electroporation with pcDNA3.1 plasmid carrying BCRP gene coding sequence. K562Oct1 and K562BCRP cell lines were kept under selective pressure by adding Geneticin.

For the stable silencing we required plasmids encoding for resistance to an antibiotic different from Geneticin. We identified that Mission shRNA plasmids form Sigma-Aldrich (Sant Louis, MO, USA) encoded the gene for resistance to Puromycin. Thus we used Mission shRNA plasmids to establish stable silencing of Oct1 and BCRP in K562Oct1 and K562BCRP, respectively. For each of the two drug transporters we tested five different shRNA sequences obtained in silico. In order to improve the silencing rate, we also set up a sub-cloning starting from cells transfected with the plasmid showing higher rates of silencing.

Also K562Oct1 K562BCRP cell lines were sub-cloned to obtain homogeneous cell populations. This step was evident after the functional assay based on flow cytometry (depicted in detail above), that showed the heterogeneity of the two populations for the functional expression of drug transporters. All the sub-cloning were set on a 96-well plate, with 0.3 cells/200µl/well kept under selective pressure with the antibiotics.

All cell lines were grown in standard conditions with RPMI 1640 medium (Lonza Cambrex, East Rutherford, NJ, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Euroclone, Milan, Italy), 2mM L-glutamine, 100 units/mL Penicillin G, 80μg/mL gentamicin and 20mM Hepes. Cell lines were kept in selective pressure with the following reagents: 1μM Doxorubicin for K562DOX, 1μM Doxorubicin + 1mg/mL geneticin for K562DOX/sh P-gp, 1mg/mL geneticin for K562Oct1 and K562BCRP and 1mg/mL geneticin + 1μM Puromycin for K562Oct1/sh Oct1 and K562BCRP/sh BCRP.

Retro-transcription and qPCR. RNA extraction from all the cell lines was performed with Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the protocol suggested. After quantification, RNA was retro-transcribed to obtain cDNA with TaqMan kit (Applied Biosystems, Foster City, CA, USA), according to the standard protocol. Real time qPCR was performed to assess the transcription levels of P-gp, Oct1 and BCRP in all the cell lines. The following primers were used for the analysis: P-gp for: 5’-TGGAGGAAGACATGACCAGG-3’; P-gp rev: 5’-CAAGACCTCTTCAGCTACTGC-3’; Oct1 for: 5’-GGGCAGCCTGCCTCGTCATG-3’; Oct1 rev: 5’-ACCTCCCTCAGCCTGAAGAC-3’; BCRP for: 5’-TTAGGATTGAAGCCAAAGG-3’; BCRP rev: 5’-TAGGCAATTGTGAGGAAAATA-3’. The housekeeping gene GAPDH was used for intra-sample normalization.

Immnunoblotting. For the evaluation of the expression levels of the drug transporters, 10^7 cells were lysed with a specific protocol for membrane proteins purification. Briefly, K562DOX, K562DOX/sh P-gp, K562Oct1, K562Oct1/sh Oct1 and K562S were lysed adding boiling 125mM Tris-HCL pH 6.8, SDS 2% solution supplemented with protease inhibitors. After resuspension and further boiling, samples were sonicated and Laemmli buffer was added. K562BCRP, K562BCRP/sh BCRP and K562S were lysed using an hypotonic lysis buffer 100mM KCl, 2mM MgCl1, 100mM Tris-HCl (pH 7.4), 1% SDS supplemented with protease inhibitors. After sonication, Laemmli buffer was added and lysates were heated at 60°C for 1h. This particular protocol without the final boiling was used to avoid BCRP precipitation. Indeed, this drug transporter carries a glycosylation site that can be linked with protein precipitation.

Samples were loaded on SDS-PAGE, transferred to nitrocellulose and probed with different antibodies: monoclonal P-gp antibody C494 (Abcam, Cambridge UK); polyclonal Oct-1 antibody AB1 (Sigma-Aldrich, Saint Louis MO, USA) monoclonal BCRP antibody BXP-21 (Enzo Life Sciences, AG, Lausen, Switzerland), polyclonal actin antibody (Sigma Aldrich, Saint Louis MO, USA). The secondary anti-mouse and anti-rabbit antibodies were obtained by Biorad, Hercules, CA, USA.

For the evaluation of phosphorylation levels of BCR-ABL, K562DOX and K562S cells were treated with serial dilutions of bosutinib for 8 hours at standard conditions. Eventually, a pre-treatment of 1 hour with 5μM Verapamil was done to inhibit the P-gp activity. 10^7cells were lysed using a standard protocol described previously[195]. Samples were loaded on SDS-PAGE, transferred to nitrocellulose and probed with anti-

phosphotyrosine antibody (clone 4G10, Millipore, Billerica MA, USA). Bcr-Abl levels were probed with anti-c-abl (K12 clone, Santa Cruz Biotechnology, Santa Cruz CA, USA).

FACS analysis. Functional activity of P-gp, Oct1 and BCRP was evaluated measuring the intracellular accumulation of known transporter substrates: Rhodamine 123 for P-gp, 4-Di-2-ASP for Oct1 and Pheophorbide A for BCRP, in presence or absence of the selective transporter inhibitors. 10^6 cells were incubated for 30minutes with fluorescence substrates and, where indicated, a 2 hours pre-treatment with drug transporters inhibitors was also performed. Cells were washed twice with ice cold PBS before FACS analysis. Rhodamine 123 and 4-Di-2-ASP incorporation was determined with the BD FACSort (Becton Dickinson, San Jose CA); Pheophorbide A incorporation was determined with the BD FACScanto (Becton Dickinson, San Jose CA).

Intracellular uptake and retention assay (IUR). Incorporation of C-14 radiolabeled bosutinib (C-14 bosutinib) was evaluated in all the cell lines in presence or in absence of specific drug transporter inhibitors. 10^6 cells were resuspended in 2mL of complete medium. Drug transporters inhibitors were added at selected concentrations (described above) for 2h. C-14 bosutinib 1µM was added for an additional hour of incubation. Cells were washed trice with 1mL ice-cold PBS, resuspended in 30µL PBS

and spotted on a membrane filter. Radioactivity levels were detected on a 1450 MicroBeta Trilux β-counter (Perkin Elmer, Waltham MA).

Proliferation and co-treatment assay. 10^4 K562DOX, K562DOX/sh P-gp and K562S were seeded in a 96 well plate. imatinib and bosutinib were added at increasing concentrations. For the co-treatment assay, imatinib and bosutinib were used at serial concentrations, while the P-gp inhibitor Verapamil was added at 4 different concentrations below its IC50. For both assays, tritiated thymidine was added after 72hours, cells were then harvested and the levels of tritiated thymidine were evaluated by a 1450 MicroBeta Trilux β-counter (Perkin Elmer, Waltham MA).

Statistical analysis. All the statistical analysis, data and graph elaborations were run on GraphPad software analysis program (Prism, San Diego, CA).

Results

Characterization of the cell lines. We obtained K562DOX and K562DOX/sh P-gp form other groups, while we established stable transfections by electroporation to obtain K562Oct1, K562Oct1/sh Oct1, K562BCRP and K562BCRP/sh BCRP. All the cell lines were obtained starting from K562S cell line, characterized by low levels of P-gp, Oct1 and BCRP drug transporters. Further details about the production of the cell lines are depicted in detail in the Methods section.

The characterization of all the cell lines was required before further experiments on bosutinib transport. We decided to include also K562DOX and K562DOX/sh P-gp cell lines in these analysis, even in the expression transcript levels were already known from literature. To assess the expression level of the three drug transporters in the cell lines, we performed RT-PCR analysis as well as immunoblotting. As expected, transcript levels analysis showed an increased expression of P-gp (Fig. 25), Oct-1 (Fig. 26), BCRP (Fig. 27) in K562DOX, K562Oct1 and K562BCRP respectively, in comparison to K562S parental cells. In particular, P-gp levels in K562DOX were approximately 2800-fold, Oct1 levels in K562Oct1 approximately 2100-fold and BCRP approximately 190-fold higher than in parental cells. Silencing performed in the same overexpressing cell lines was able to successfully reduce the transcript levels of the different transporters (figure 1A). For Oct1 and BCRP transporters, the transfection with a sub-set of five plasmid carrying shRNA sequences was followed by a further a sub-cloning to improve the silencing rate of the transcript of interest. The sub-cloning was performed starting from the most promising plasmid in terms of silencing rate. For each transporter, 15

sub-clones were tested with a Real time qPCR, and the best sub-clone was selected to establish the cell line. In detail, K562DOX/sh P-gp showed a 87.9% decrease of P-gp levels, K562Oct1/sh Oct1 showed a 99.9% decrease of Oct1 levels, and K562BCRP/sh BCRP showed a 99.9% decrease of BCRP levels if compared to the corresponding overexpressing cells. Interestingly, K562BCRP/sh BCRP cells showed BCRP expression levels even lower than K562S, meaning that also endogenous BCRP was silenced.

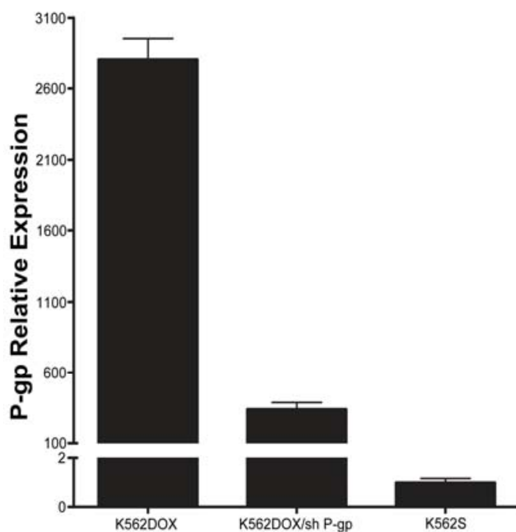

Fig. 25: *Evaluation of P-gp expression by Real time qPCR. Housekeeping GAPDH was used for intra sample normalization. Expression levels were normalized over the expression levels in K562S. Results are the average of three independent experiments ±SD. The statistical differences between expression levels of overexpressing or silenced cells and K562S cells were calculated with two-tailed unpaired t-student's t-test, and p-value of 0.05 was chosen as the limit of statistical significance.*

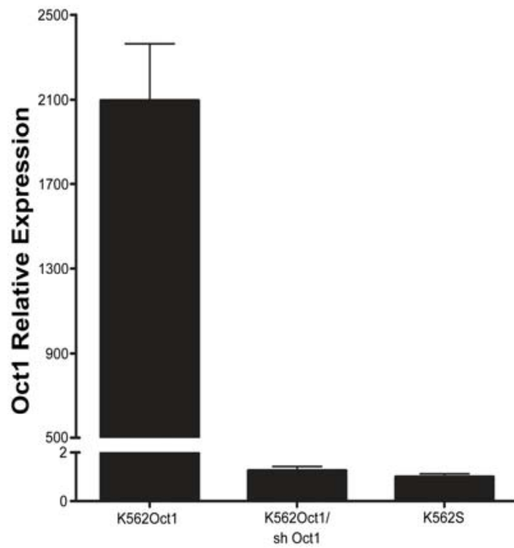

Fig. 26: *Evaluation of Oct-1 expression by Real time qPCR.*

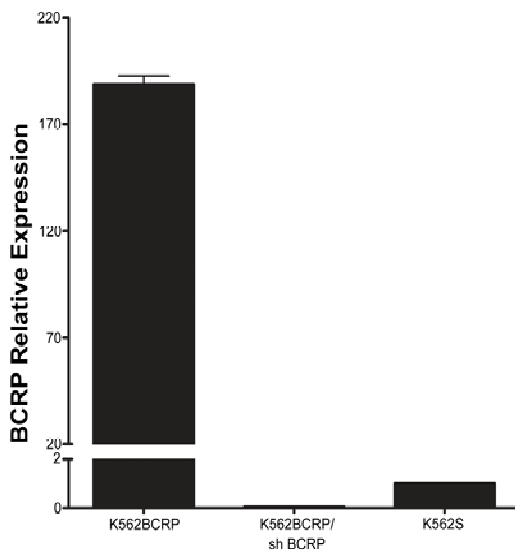

Fig. 27: *Evaluation of BCRP expression by Real time qPCR.*

We also evaluated the expression level of each transporter in each cell line to exclude modifications in the expression's pattern others than the desired one (Fig. 28, 29 and 30).

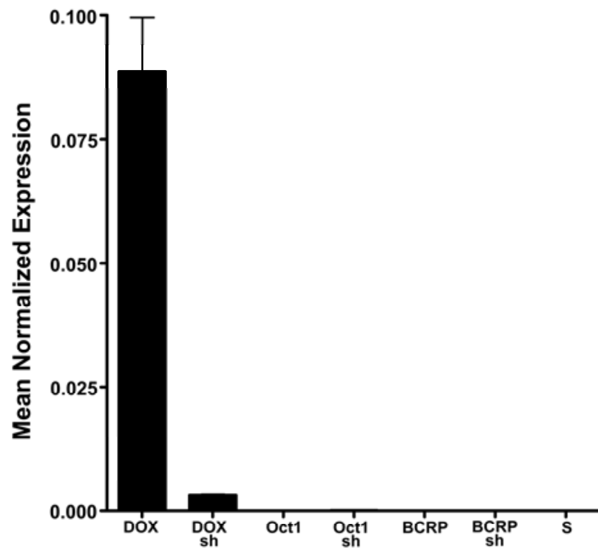

Fig. 28: *Real Time qPCR to evaluate the expression level of P-gp (normalized on GAPDH) in all the cell lines used in the work.*

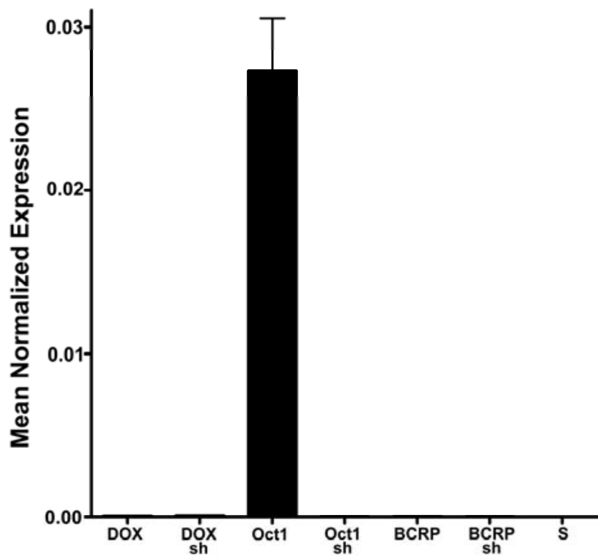

Fig. 29: *Real Time qPCR to evaluate the expression level of Oct-1 (normalized on GAPDH) in all the cell lines used in the work.*

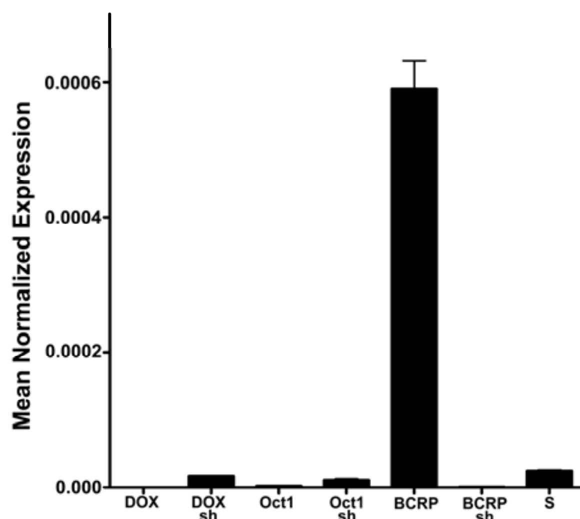

Fig. 30: *Real Time qPCR to evaluate the expression level of BCRP (normalized on GAPDH) in all the cell lines used in the work.*

For the evaluation of drug transporters protein levels we set up an immunoblotting analysis. To purify the proteins from the membrane we required specific methods for the production of lysates. A standard protocol of lysate production commonly allows a good analysis of cytoplasmic proteins, with a centrifugation step to separate cytoplasmic protein from cell membranes and other impurities. However, this protocol can be related with the loss of membrane or transmembrane proteins that are strongly bound to the membrane and thus are kept in the pellet (discarded after centrifugation). This limitation occurred also in the preparation of lysates to evaluate drug transporters protein levels.

Thus we set up a specific protocol for drug transporters. P-gp and Oct1 proteins only required the addiction of a boiling lysis buffer to the whole lysate. The details of composition of the lysis buffer are depicted in the methods section. After the addition of

Laemmli buffer, the final step was boiling the samples before loading them on the acrylamide gel. The protocol used for BCRP was more particular. BCRP is a glycosylated protein, and the presence of the glycosylated portion facilitates the precipitation of the protein at high temperatures. Thus, temperatures higher than 60°C were avoided. In literature we found that for BCRP purification an hypertonic buffer was suggested. The presence of high concentrations of salts induces the disruption of the membranes, thus mimicking the thermal shock used for the purification of P-gp and Oct1. Details about the composition of the hypertonic buffer are depicted in the methods section. After the addition of this buffer to the whole lysates and the addition of Laemmli buffer, the samples were kept for one hour at 60°C and then loaded on acrylamide gel. To have a comparable result, the K562S cell lysates were prepared following the two protocols.

Immunoblotting analysis performed with the specific antibodies on cell lysates confirmed the results obtained in Real-Time PCR (Fig 31, 32 and 33). The three cell lines overexpressing drug transporters showed a marked protein expression if compared to silenced and K562S cell lines.

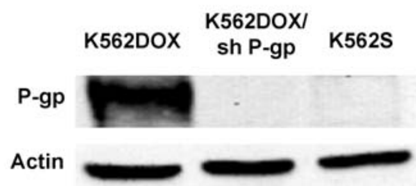

Fig. 31: Evaluation of P-gp expression levels by immunoblotting performed on whole cell lysate of overexpressing, silenced and K562S cell lines. Actin was used as a loading control.

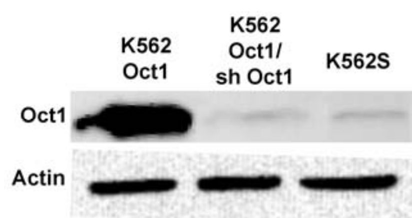

Fig. 32: Evaluation of Oct-1 expression levels by immunoblotting.

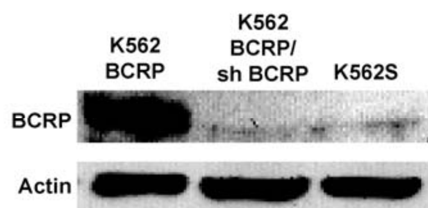

Fig. 33: Evaluation of BCRP expression levels by immunoblotting.

Functional Characterization. The over-expression of drug transporters required an additional functional characterization. Functional over-expression of transmembrane proteins is commonly more difficult than the one of cytoplasmic protein. After the translation into a full length protein, the drug transporters require also specific signals to be correctly directed to and incorporated in the cytoplasmic membranes. Thus, we

found in literature that a specific drug transporter assay could be performed to check the functionality of the proteins over-expressed.

The functionality assay was developed basing the existence of known fluorescent substrates of drug transporters. The treatment of the cell lines with the specific fluorescent substrate, followed by a flow cytometry analysis, allows to detect the levels of substrate incorporation of each cell line. The assay was not quantitative, but the comparison between different cell lines gave an indication about the functional activity of the transporters. In detail, we hypothesised that K562DOX and K562BCRP cells should be characterized by lower levels of substrates incorporation compared to K562S cells. Indeed, higher expression levels of functional efflux transporters should facilitate the exit outside the cells of the substrates. On the counterpart, Oct1 over-expression was supposed to be linked with an increased uptake of ASP, thus resulting in higher incorporation levels of the substrate compared to K562S levels.

Thus we used the fluorescent substrates Rhodamine 123 (Rho 123) for P-gp [226, 227] (Fig. 34), 4-Di-2-ASP (ASP) for Oct1 [228, 229] (Fig. 35) and Pheophorbide A (PhA) for BCRP [230, 231] (Fig. 36). While Rho 123 and PhA had been already used from other groups in similar assays, ASP had been only used in fluorescent microscopy. We decided to use ASP for the flow-cytometry incorporation assay to have comparable results for the three drug transporters instead of testing the functionality of Oct1 using Oct1 radio-labeled substrates extensively depicted in literature.

For each drug transporter, we analyzed the cellular incorporation of the fluorescent substrate in overexpressing, silenced and parental K562S cells. Over-expressing cells

analyzed were either pre-treated or untreated with specific drug transporter inhibitors. FACS analysis confirmed that the cells overexpressing either P-gp or BCRP show a decreased intracellular concentration of the fluorescent substrates Rho 123 and PhA, respectively. As expected, K562S showed a severe increase in the intracellular concentration of fluorescent substrates. In addition, incorporation levels similar to K562S were obtained in silenced cells as well as in cells treated with specific drug transporter inhibitors: Verapamil was used as selective P-gp inhibitor [232], while Fumitremorgin C selectively blocks BCRP [233]. According to the role of Oct1 in the uptake of ASP, K562Oct1 cells showed higher ASP incorporation compared to K562S. Both silencing of Oct1 and treatment with the specific Oct1 inhibitor Amantadine [234] led to ASP incorporation levels similar to K562S.

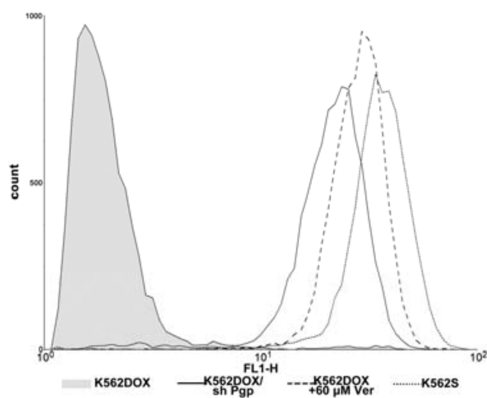

Fig. 34: *Intracellular incorporation of Rhodamine 123 was evaluated by FACS analysis. The shaded area correspond to the overexpressing cell line, the solid line to the silenced cell line, the dotted line to the overexpressing cell lines pre-treated with drug transporter inhibitor, shortly dashed line to K562S.*

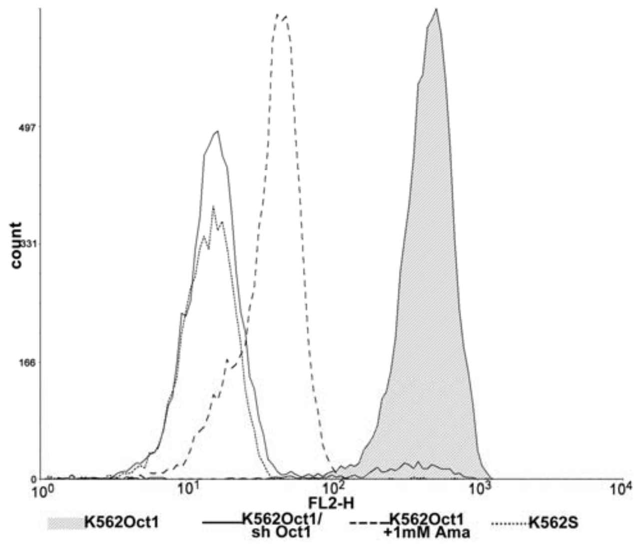

Fig. 35: *Intracellular incorporation of 2-Di-4-ASP was evaluated by FACS analysis.*

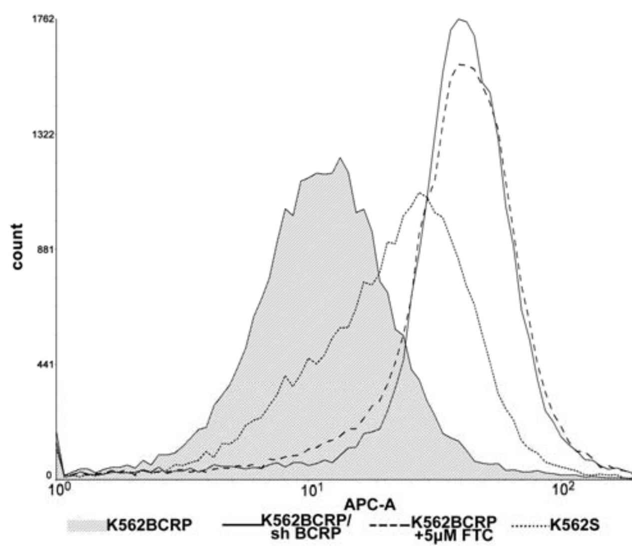

Fig. 36: *Intracellular incorporation of Pheophorbide A was evaluated by FACS analysis.*

The results depicted above allowed understanding that the drug transporters over-expressed and over-expressed and silenced in cell lines were functional. A previous experiment was run on K562Oct1 and K562BCRP cells obtained after transfection with no sub-cloning. In this case, the results showed the heterogeneity of cell lines after transfection, because one cell line often showed two picks, showing a wide population carrying the plasmid but not encoding for a functional transporter. After this results we decided to sub-clone also the over-expressing cell lines (K562Oct1 and K562BCRP), with the same method used to improve the silencing in K562Oct1/sh Oct1 and K562BCRp/sh BCRP cell lines. Thus, the resulted discussed above were obtained after the sub-cloning described.

All together, the data reported on figures 1-12 show that the cells are characterized by a different functional expression of drug transporters.

IUR assay for C-14 Bosutinib cellular incorporation. Recently, IUR was used to characterize the interaction of imatinib, nilotinib and dasatinib with drug transporters [235-238]. The assay requires the presence of the specific drug with a radio-labeled carbonium. The radio-labeled drug is produced during the synthesis, were instead of Carbon 12 is added a Carbon 14 at a specific position. Thus, 3-D structure remains the same but the compound is now radio-labeled, allowing the detection of β-ray emission. Commonly, in the common IUR protocol the radio-labeled compound is mixed with the un-labeled compound to reach a sufficient concentration. We decided to avoid the introduction of un-labeled compound to obtain more potent signal from the β-counter.

We obtained the C-14 bosutinib directly from Pfizer. However, the protocol commonly used consist of the treatment of cells with radiolabeled compound alone or with a mixture of radio-labeled and un-labeled compound. For the study of tyrosine kinase inhibitor, the incubation time at 37°C is 3 hours, enough for the uptake-efflux mechanisms but not for the induction of apoptosis exerted by the drug. After this incubation, sample were kept on ice to avoid ATP-dependent efflux by P-gp or BCRP. 3 washes with ice-cold PBS allowed to discard the excess of C-14 bosutinib kept in the surnatant.

We used a similar protocol to study the correlation between the expression of functional drug transporters and the intracellular concentration of C-14 bosutinib. First, we studied C-14 bosutinib incorporation in over-expressed, silenced and K562S cells of each transporter (Fig. 37, 38 and 39). While Oct1 and BCRP sets of cells did not show any relevant difference in C-14 bosutinib incorporation, K562DOX showed a statistically significant decrease in the incorporation of C-14 bosutinib compared to K562S (p value 0.0003). K562DOX/sh P-gp showed an intermediate behaviour (p value 0.0006).

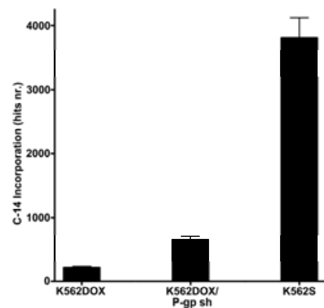

Fig. 37: *Intracellular incorporation of C-14 bosutinib is reported as the number of hits obtained from beta-counter. The results derive from three independent experiments ± SD. The statistical difference between overexpressing or silenced cells and K562S was calculated with two-tailed unpaired t-student's t-test, and p-value of 0.05 was chosen as the limit of statistical significance.*

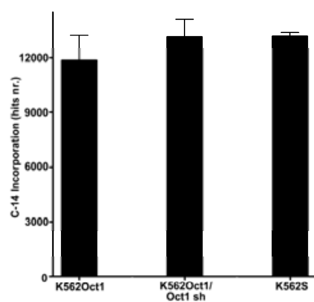

Fig. 38: *Intracellular incorporation of C-14 bosutinib in Oct1 subset of cells.*

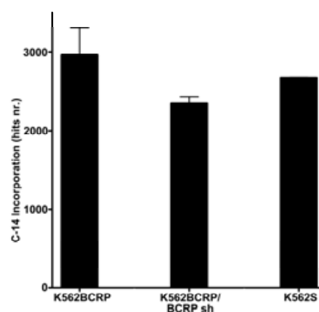

Fig. 39: *Intracellular incorporation of C-14 bosutinib in BCRP subset of cells.*

We then evaluated the levels of C-14 bosutinib incorporation after treatment with specific drug transporters inhibitors (Fig 40, 41 and 42). While Oct1 and BCRP subsets of cells did not show significant differences between overexpressing, silenced and K562S, K562DOX treated with Verapamil were characterized by a massive increase of intracellular C-14 bosutinib (~5.7 fold) compared to the corresponding untreated cells (p value <0.0001). K562DOX/sh P-gp had only a moderate increase (~2.4 fold) (p value 0.0008), while K562S showed a moderate decrease probably due to non specific toxic effect of Verapamil. The explanation of the result obtained for P-gp is that the over-expression of the drug transporters is linked with an increased efflux of C-14 bosutinib outside the cells, thus identifying the drug as a substrate of the drug transporter. After the treatment with Verapamil, the P-gp inhibitor, over-expressing cells go back to the K562S situation because the excess of protein expressed is blocked by the activity of Verapamil, thus restoring higher concentration of C-14 bosutinib.

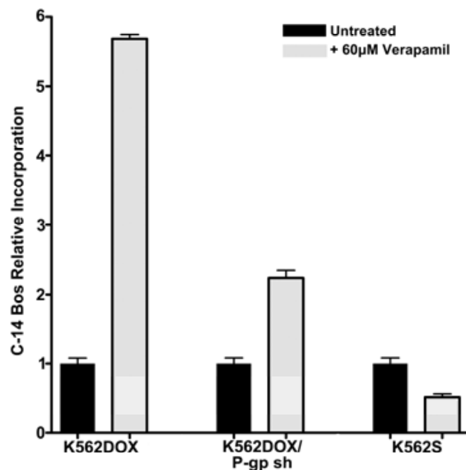

Fig. 40: *C-14 bosutinib intracellular accumulation after drug transporters inhibition (P-gp subset). The cells were pre-treated with specific drug transporters inhibitors. C-14*

bosutinib incorporation obtained with the pre-treatment was normalized over C-14 bosutinib incorporation in the same cell line without the pre-treatment. Results are an average of three independent experiments. The statistical difference between untreated and pre-treated samples within each cell line was calculated with two-tailed unpaired t-student's t-test, and p-value of 0.05 was chosen as the limit of statistical significance.

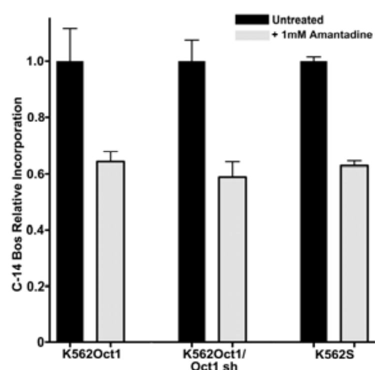

Fig. 41: *C-14 bosutinib intracellular accumulation after drug transporters inhibition (Oct1 subset).*

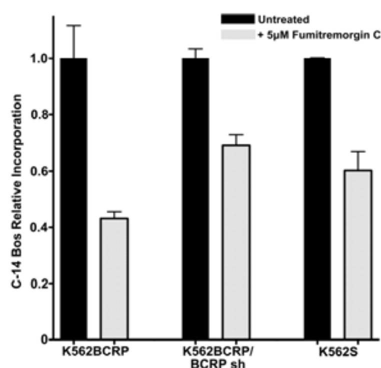

Fig. 42: *C-14 bosutinib intracellular accumulation after drug transporters inhibition (BCRP subset).*

IUR assay showed a first evidence that only P-gp, and not Oct1 and BCRP, might be involved in active bosutinib transport. Moreover, P-gp inhibition by Verapamil is able to partially avoid the efflux of Bosutinib.

K562DOX resistance to bosutinib and restoration of sensitivity by Verapamil. To further prove the involvement of P-gp in bosutinib efflux, we evaluated bosutinib IC50 in K562DOX, K562DOX/sh P-gp and K562S (Fig. 43). We expected that K562DOX cells should be characterized by the increased resistance to bosutinib treatment as well to imatinib, the we used as a positive control in this assay. Indeed, we showed with IUR that high expression levels of P-gp are related with low intracellular concentration of bosutinib. Thus, higher starting concentration of bosutinib (strictly related with IC50) should be required in K562DOX to balance the increased activity of P-gp. The positive control Imatinib was used because it is already known as a P-gp substrate. The results confirmed what expected both for Imatinib and bosutinib.

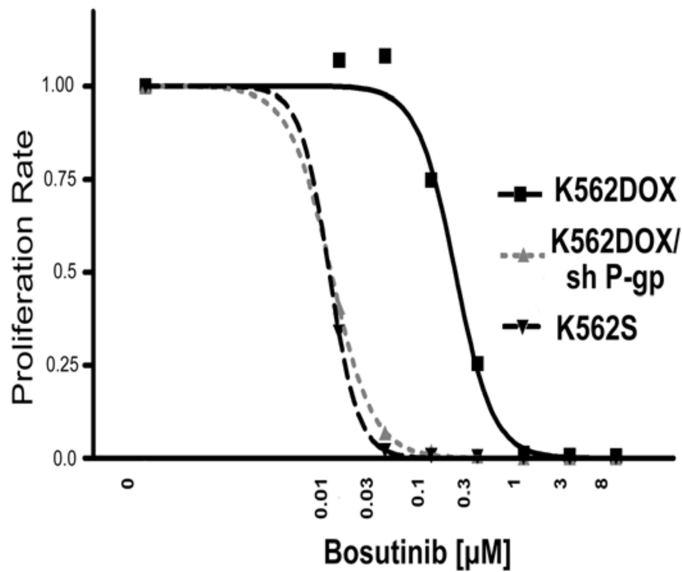

Fig. 43*: Proliferation assay on K562DOX, K562DOX/sh P-gp and K562S. Cells were cultured for 72h in presence of increasing concentrations of either bosutinib and incorporation of tritiated thymidine was evaluated. The data reported were obtained from a single experiment, but the results were confirmed in three separate experiments.*

Upon thymidine incorporation assay, bosutinib IC50 showed a 20-fold increase in K562DOX compared to either silenced and K562S cells. As a control we also analyzed imatinib IC50 changes in the same cell lines and we observed a similar increase (fold change ~15.9), as expected by the fact that imatinib is a known P-gp substrate (Fig. 44).

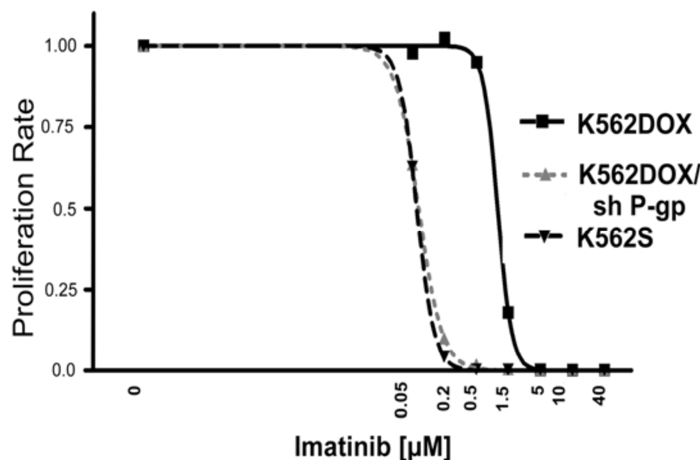

Fig. 44: *Proliferation assay on K562DOX, K562DOX/sh P-gp and K562S. Cells were cultured for 72h in presence of increasing concentrations of imatinib and incorporation of tritiated thymidine was evaluated. The data reported were obtained from a single experiment, but the results were confirmed in three separate experiments.*

To test if inhibition of P-gp corresponded to a restoration of K562DOX sensitivity to imatinib or bosutinib treatment, we co-treated K562DOX and K562S with Verapamil and either bosutinib or imatinib (Fig. 45 and 46). The background idea is that Verapamil is able to down-regulate the activity of P-gp, restoring intracellular concentration of bosutinib and thus reducing the bosutinib IC50 in K562DOX cells. On the counterpart, K562S cells are characterized by low levels of P-gp expression. In such cells, the introduction of Verapamil should be ineffective for the intracellular concentration of bosutinib.

We used increasing concentrations of bosutinib or imatinib and 3 fixed concentrations of Verapamil below its IC50, to avoid toxicity. A control without

Verapamil was included in the experiment. In absence of Verapamil, a major IC50 shift can be seen between K562DOX and K562S cells. The situation changes after the introduction of Verapamil even at low concentrations, with a massive reduction of K562DOX cells bosutinib IC50 and no effect on K562S cells bosutinib IC50. As expected, both bosutinib and imatinib IC50s showed a severe decrease in K562DOX in presence of Verapamil, even at the lowest concentration tested (p values <0.005). K562S IC50 was not affected by the presence of Verapamil.

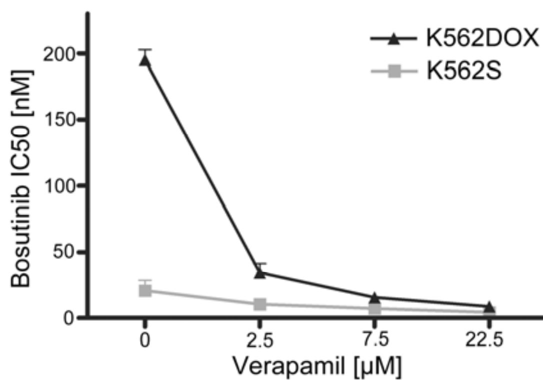

***Fig. 45**: Co-treatment of K562DOX and K562S cells with Verapamil and bosutinib. Verapamil was used at three selected concentrations,. Bosutinib was used within the same ranges used in Fig 19. In the figure, the y axis reports means + SD of bosutinib or imatinib IC50s. the data reported are an average of three separate experiments. Non-linear regression was used to evaluate IC50s.*

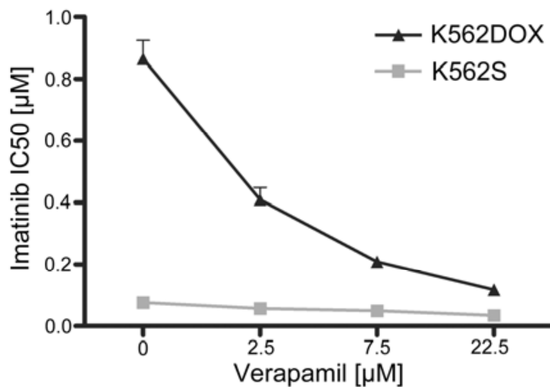

***Fig. 46**: Co-treatment of K562DOX and K562S cells with Verapamil and imatinib.*

All together, the results indicate that P-gp overespression is related with an increase of resistance to bosutinib treatment. Furthermore, inhibition of P-gp is able to restore the sensitivity of P-gp overexpressing cells to bosutinib treatment.

Bosutinib intracellular concentration levels correlate with bcr-abl activity inhibition. Bosutinib, as well as the other tyrosine kinase inhibitors, exerts a pro-apoptotic activity by directly modulating bcr-abl phosphorylation[192, 195]. To evaluate the activity and the activation levels of bcr-abl, several molecular markers were identified. The most commonly used marker is the bcr-abl molecular target Crkl. The levels of phosphorylation of Crkl gives a direct indication of the levels of activation of bcr-abl. However, there are several other targets with similar function in the analysis of bcr-abl activity. We decided to evaluate directly the levels of auto-phosphorylation of bcr-abl, because we considered this evaluation as the most direct to determine the effects of bosutinib and the correlation between P-gp expression levels, intracellular

concentration of bosutinib and levels of activation of bcr-abl. To characterize the direct effect of a decreased intracellular bosutinib concentration on Ph^+ cells, we studied the phosphorylation levels of bcr-abl in K562DOX and K562S cells (fig. 47). Cells were treated for 8 hours with increasing bosutinib concentrations ranging from 0 to 80nM and the phosphorylation levels of bcr-abl in cell lysates were evaluated by immunoblotting. We decided to keep the range 0-80nM of bosutinib concentration because only bcr-abl auto-phosphorylation levels of K562S should be decreased, while K562DOX bcr-abl auto-phosphorylation levels should remain unvaried even at 80nM bosutinib, considering that K562DOX showed a bosutinib IC50 at 200nM. As expected, at high bosutinib concentrations bcr-abl phosphorylation levels were decreased in K562S but not in K562DOX.

The following experiment started form the hypothesis that P-gp inhibition by Verapamil could restore bosutinib activity on bcr-abl auto-phosphorylation in K562DOX cells, even at 80nM or lower. When the cells were co-treated with the P-gp inhibitor Verapamil, a massive decrease of phosphorylation levels was observed also in K562DOX cells. K562S cells did not show any difference in presence or in absence of Verapamil.

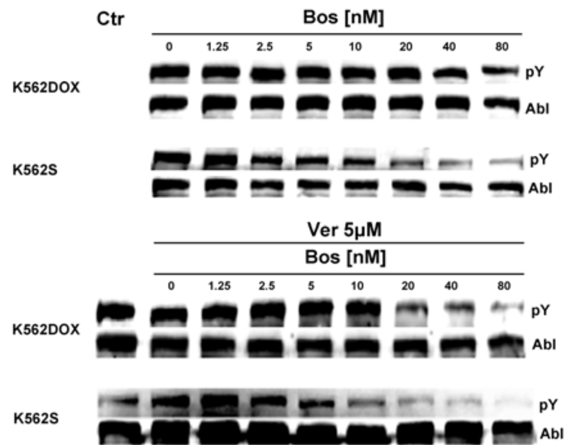

***Fig. 47**: Evaluation of bcr-abl phosphorylation levels in K562DOX and K562S cell lines, upon bosutinib treatment with or without Verapamil co-treatment. Immunoblotting analysis was performed on whole cell lysates .Abl was used as loading control.*

Thus the decreased intracellular concentration of bosutinib, due to P-gp over-expression, results in a decreased bosutinib activity on bcr-abl phosphorylation. Inhibition of P-gp activity by Verapamil is able to reconstitute higher intracellular bosutinib levels, resulting in an increased de-phosphorylating activity on bcr-abl.

Discussion

The development of second and third generation tyrosine kinase inhibitors (dasatinib, nilotinib, bosutinib) was aimed to overcome resistance affecting patients after imatinib treatment failure. Resistance to imatinib treatment is largely due to the presence of point mutations within bcr-abl kinase domain[141], but other mechanisms were also linked to resistance to imatinib treatment: bcr-abl gene amplification[239], bcr-abl gene duplication[240], drug plasma concentration[241], alternative signalling pathway activation[242], epigenetic modifications[162] and multidrug resistance due to drug cellular uptake/efflux impairment[243]. Many reports underline the different transport pattern of nilotinib and dasatinib compared to imatinib, thus the two second generation inhibitors were suggested as a valid treatment option to circumvent multidrug resistance to imatinib[236].

Multidrug resistance affecting bosutinib has been poorly investigated[244]. In 2009, Hegedus C et al developed a new mass spectrometry in vitro assay to evaluate intracellular concentration of TKIs. This assay, coupled with the analysis of bcr-abl phosphorylation, allowed the group to conclude that nilotinib and dasatinib are substrates of BCRP, and that the over-expression of this transporter is involved in the development of resistance. P-gp is also responsible for nilotinib and dasatinib transport, but the over-expression of this transporter is responsible only for the development of resistance to dasatinib. In this study, the bosutinib multidrug resistance characterization is a novel data. Authors conclude that P-gp and BCRP are not involved in the transport of Bosutinib[244].

In our work, we analyzed the interaction between bosutinib and three drug transporters mainly involved in imatinib transport: P-gp, BCRP or Oct1.

We based our analysis on the Bcr-Abl positive cell line K562S, characterized by a low expression level of all the three transporters. This feature allowed us to successfully modulate the expression of each transporter. As expected, the overexpressing cell lines were characterized by higher levels of transcript of functional protein, while overexpressing + silencing cells were characterized by a restoration of lower levels of transcript and functional protein. Moreover, the inhibition of drug transporter activity by drug transporter inhibitors was able to restore a drug transporter activity similar to K562S cells.

Intracellular uptake and retention (IUR) assay has been extensively used to study multidrug resistance for imatinib, nilotinib and dasatinib[236, 237, 245, 246]. The treatment of cells with radio-labeled bosutinib allowed to directly measure the incorporation rate of the radio-labeled drug, which is structurally identical to un-labeled bosutinib. Thus we relied on this assay to define if bosutinib is transported outside or inside the cells by P-gp, BCRP and Oct1. Only P-gp subset of cells showed different intracellular levels of C-14 bosutinib, with a severe decrease of incorporation in K562DOX if compared to K562S. K562DOX/sh P-gp showed an intermediate behaviour, according to its intermediate P-gp expression levels. This result suggests that P-gp could be responsible of bosutinib efflux outside the cells, thus leading to a decrease of intracellular bosutinib levels. K562BCRP and K562Oct1 cells showed C-14 bosutinib incorporation levels similar to K562S,

indicating that the two transporters should not be involved in bosutinib efflux and uptake, respectively.

According to our hypothesis, P-gp inhibition in K562DOX cells should result in an increase of intracellular C-14 bosutinib. As expected, the treatment with Verapamil led to a marked increase of intracellular C-14 bosutinib levels, The slight decrease in bosutinib levels observed in K562S cells upon Verapamil treatment is probably due to non specific toxic effect of Verapamil. On the counterpart, Oct1 and BCRP inhibition by, respectively, Amantadine and Fumitremorgin C did not show any significant difference in the intracellular C-14 bosutinib incorporation between overexpressing, and K562S cells, thus confirming that BCRP and Oct1 are not involved in C-14 bosutinib efflux or uptake. All together, these data indicate that bosutinib is a substrate of P-gp and not of BCRP or Oct1. The decreased intracellular levels of bosutinib in K562DOX correlates with the changes in IC50 observed in proliferation assay. As predicted, K562DOX showed an higher IC50 compared to K562S, both for bosutinib and imatinib used as known P-gp substrate. K562DOX/sh P-gp showed an IC50 similar to K562S IC50. This data confirm that the decreased intracellular concentration of bosutinib, due to P-gp over-expression, is related to the increase of resistance to treatment. Accordingly, inhibition of P-gp activity by Verapamil is able to restore the sensitivity to bosutinib and imatinib treatments in K562DOX cells. On the counterpart, K562S bosutinib or imatinib IC50s were not significantly affected by the presence of Verapamil. Thus, in vitro K562DOX resistance to bosutinib is directly related with the increased levels of P-gp.

Since bcr-abl auto-phosphorylation is a direct marker of bcr-abl molecular activity[247], we evaluated the levels of bcr-abl phosphorylation in K562DOX and K562S treated with bosutinib. K562DOX bcr-abl auto-phosphorylation levels are not influenced even at the highest dose tested, while K562S cells show a dose-dependent decrease in phosphorylation signal. This is an expected result considering K562DOX and K562S bosutinib IC50 obtained by the proliferation assay. The treatment of K562DOX with Verapamil is able to restore the sensitivity to bosutinib. The data indicate that lower intracellular concentration of bosutinib, due to P-gp over-expression, results in a decreased inhibitory activity on bcr-abl. This correlates with the increase of resistance to bosutinib treatment in K562DOX cells.

In summary, we show here that bosutinib cellular concentration are affected by the over-expression of the efflux transporter P-gp. The reduced retention of bosutinib inside the cells in turns reflects on a reduction of its bcr-abl inhibitory activity, thus leading to resistance development. Our data suggest that the analysis of P-gp expression levels might be helpful in the treatment decision for the patients that exhibit resistance to bosutinib treatment. Indeed, drug transporters could be considered as prognostic factors for the treatment of chronic myeloid leukemia. Actually Oct1 has been proposed as a prognostic factor for the treatment with imatinib. Low levels of expression of this drug transporters are related with poor outcome at standard concentrations, thus suggesting an increased daily dose of imatinib. The characterization of multidrug resistance of other tyrosine kinase

inhibitors gives a further advantage, because indicates which is the best therapeutic option in presence of altered expression levels of drug transporters increase.

Moreover, drug transporter expression levels are growing as prognostic factors before the treatment with Tyrosine kinase inhibitors. Indeed, a routinary analysis in clinics could directly indicate which could be the best tyrosine kinase inhibitor to overcome multidrug resistance.

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