**Kill one Bird with two Stones: Potential efficacy of BCR-ABL and autophagy inhibition in CML.**

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## Abstract

The introduction of imatinib in the treatment of chronic myeloid leukaemia (CML) represents the most successful example of targeted therapy in human cancer. However, leukaemic stem cells (LSCs) are insensitive to tyrosine kinase inhibitors (TKIs) and contribute to disease persistence by representing a reservoir of self-renewing cells that replenish the disease upon drug discontinuation. This has re-focused the interest of scientists towards drug combinations i.e. treating with TKIs and simultaneously targeting alternative survival mechanisms. One candidate target mechanism is autophagy, a cellular recycling process that acts as a cytoprotective shield in CML cells in response to TKI-induced stress and in other cancer cells surviving in an inhospitable microenvironment. On that basis, inhibition of autophagy has now become an exciting option for combination treatment in cancer and clinical trials have been initiated in solid and haemopoietic tumours such as CML, chronic lymphocytic leukaemia (CLL) and multiple myeloma. This review describes the biology of CML and elucidates how the molecular driver BCR-ABL led to the development of TKIs. We then discuss the molecular regulation of autophagy and the potential for autophagy inhibition as the next step in our attempt to tackle the problem of CML persistence to offer a curative option.

## Introduction

Fifty years ago a genetic link to cancer was provided by Peter C. Nowell and David Hungerford, who for the first time described an unusual small chromosome present in leukocytes from patients with chronic myeloid leukaemia (CML). This abnormality, designated as the Philadelphia (Ph) chromosome after the city in which it was discovered, was found in all malignant cells of CML patients and is now considered the hallmark of CML.[1] In 1973 Janet Rowley demonstrated that the Ph chromosome resulted from a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9:22)(q34;q11).[2] Later, it was shown that this alteration generates a fusion between c-*ABL* (human homologue of the Abelson Murine Leukaemia virus), a tyrosine kinase encoding oncogene, and *BCR* (Breakpoint Cluster Region), the function of which is still not clear.[3] The chimeric BCR-ABL protein possesses cellular transforming ability which is ascribed to the elevated tyrosine kinase (TK) activity of the molecule compared to the native c-ABL.[4-5] Further studies have established *BCR-ABL* as a leukaemogenic oncogene and both mouse models and *in vitro* assays have shown that BCR-ABL, as the sole oncogenic event, is sufficient to induce leukaemia.[6]

## Molecular mechanisms of BCR-ABL

There are three major forms of the BCR-ABL oncoprotein, depending on the break point occurring within the *BCR* gene. The most common is the p210 kDa protein (e13a2 or e14a2 transcript) that is found in the majority of CML cases and in approximately one third of adults with Ph-positive acute lymphoblastic leukaemia (ALL).[7] A p190 kDa protein (e1a2 transcript) is expressed in the remaining Ph-positive ALL patients and rarely in patients with CML. The third fusion protein (p230 kDa; e19a2 transcript) has been identified in the rare neutrophilic CML (CML-N).[8] All three major BCR-ABL fusion proteins induce a similar CML-like syndrome in mice, but differ in their ability to induce lymphoid leukaemia.[9]

***Key BCR-ABL-activated signaling pathways***

Once established as the oncogene causing CML, significant effort was invested in understanding the molecular mechanism of action of BCR-ABL. To date several signaling pathways affected by the constitutively active BCR-ABL have been identified, as well as numerous binding partners and substrates that provide a link between these pathways and the defects that characterise CML (Figure 1).

BCR-ABL mimics growth factor stimulation in many ways; activation of these signaling pathways leads to (i) increased proliferation and decreased apoptosis of haemopoietic stem and progenitor cells, giving rise to a massive increase in myeloid cell numbers, (ii) reduced growth factor-dependence, and (iii) abnormal interaction with the extra-cellular matrix and stroma, leading to premature release of immature myeloid cell into the circulation. These events, together with the fact that BCR-ABL promotes genomic instability, ultimately drive disease progression.[7]

***RAS/MAP kinase pathway***: The RAS pathway is well studied, both in normal and cancer cells, and activating mutations in *RAS* are found in many human cancers. Phosphorylation of tyrosine 177 within the BCR region of BCR-ABL allows interaction with the adapter molecule GRB2, an important factor in human haemopoietic progenitor transformation.[10] Even though BCR-ABL does not affect the level of GRB2 expression, this binding enables the formation of complexes between GRB2 and SOS (son of sevenless; a guanine-nucleotide exchange factor of RAS), leading to activation of RAS signaling.[11]

***PI3-K/AKT pathway***: Another pathway commonly activated in various cancers and a crucial event in tumourigenesis is the PI3-K/AKT pathway. [12] Activation of receptor TKs (RTKs) by growth factors or BCR-ABL expression induces activation of this pathway.[13] Many molecules downstream of AKT have been shown to be important players in mediating the leukaemogenic effects of BCR-ABL, either through activation or in-activation, including BAD, MDM2, mammalian target of rapamycin (mTOR) and the FOXO sub-class of forkhead factors (FOXO1, FOXO3a and FOXO4);[12] (i) BAD mediates its pro-apoptotic effect by binding to anti-apoptotic BCL-2 proteins and preventing their functions;[14] (ii) MDM2 is a negative regulator of p53 and has been shown to be upregulated by BCR-ABL;[15] (iii) mTOR functions mainly in the translational control of proteins involved in cellular growth, size and cycle regulation.[16] In BCR-ABL expressing cells, mTOR is active and rapamycin, an inhibitor of mTOR, suppresses the growth of BCR-ABL transformed cells.[17] mTOR also negatively regulates autophagy (*discussed later in this review*). Finally, (iv) the FOXO transcription factors are involved in cell cycle arrest and/or apoptosis.[18] BCR-ABL transformation has been shown to inhibit FOXO3a activity by maintaining its constitutive phosphorylation and cytoplasmic retention.[19]

***JAK-STAT pathway***: Another anti-apoptotic pathway activated by BCR-ABL is the JAK-STAT pathway. Recent *in vivo* experiments in mouse models have portrayed Stat5 as an indispensible factor for induction and maintenance of Bcr-Abl positive leukaemia, underlining the critical role of STAT5 in CML.[20-21] Furthermore, a recent study by Warsch and colleagues explored the role of STAT5 in the development of TK inhibitor (TKI)-resistance, demonstrating that enhanced STAT5 levels reduced sensitivity to TKIs in a STAT5-dependent, but JAK2-independent manner.[22] The role of the RAS and the JAK-STAT pathways in the cellular response to various growth

factors could explain why many growth factor dependent cell lines become independent following BCR-ABL expression.[6] Evidence has emerged illustrating an autocrine loop dependent on BCR-ABL-induced secretion of growth factors. BCR-ABL induces an interleukin-3 (IL-3) and granulocyte-colony-stimulating factor (G-CSF) positive feedback mechanism in early progenitor cells which could alter critical cell cycle regulators.[23] Autocrine production of IL-3 and G-CSF may therefore play a role in promoting cell cycle entry of primitive leukaemic stem cells (LSC) and early progenitors during the chronic phase (CP) of disease, where the G-CSF receptor is expressed.[24]

## CML treatment

***Disease course and progression***: The natural history of CML is triphasic, involving an initial chronic phase (CP), lasting 3-5 years, followed by progression either to accelerated phase (AP) and then blast crisis (BC) or directly to BC. These advanced phases of the disease carry a much poorer prognosis with median survival measured in months. CML progression is generally a gradual and complex process promoted by the accumulation of secondary genetic and epigenetic changes, rather than isolated alterations. As reviewed in Perrotti *et al*, BCR-ABL expression and activity may be significantly elevated at advanced stage via gene amplification, increased promoter activity or other less direct mechanisms, such as decreased miR-203 levels, inhibition of SHP-1 phosphatase and inactivation of PP2A.[25] BCR-ABL-driven manipulation of the transcriptome plays a key role in disease progression by activating mitogenic, anti-apoptotic and anti-differentiation mediators (e.g. MYC, JAK2, hnRNP-E2, MDM2, STAT5, BMI-1 and BCL-2), inhibiting tumour suppressors (e.g. p53 or CEBPa), or through aberrant splicing of modulators like *GSK3β* and *PYK2*.[25]

***TKIs in the management of CML***: Over the 1980s increased understanding of the mechanism of BCR-ABL as the main driver of the disease led to the development of an Abl specific TKI, imatinib (Gleevec, Glivec).[26] Preclinical studies followed by clinical trials verified the success of imatinib in specifically targeting BCR-ABL-positive cells and subsequently imatinib has been established as the standard therapy for CML.

***The problem of resistance***: The mechanisms of imatinib-resistance have been extensively investigated and it is now known that approximately 50% of patients who relapse on imatinib have *BCR-ABL* point mutations in more than 40 different amino acids within the *ABL* kinase domain.[27] These findings led to the rapid development of second generation TKIs to circumvent this problem. Nilotinib and dasatinib both demonstrate increased potency over imatinib and inhibit most imatinib resistance mutants, except T315I.[28,29] Both inhibitors have now been approved by FDA and EMEA and are used routinely and very effectively as second-line therapy for patients with resistance or intolerance to imatinib.[30-31] Interestingly, as reported by Saglio *et al* and Kantarjian *et al*, administration of nilotinib or dasatinib to newly diagnosed CML CP patients resulted in higher frequencies and faster achievement of cytogenetic and molecular responses compared to standard dose imatinib (400mg daily). By 12 months of treatment there was a trend towards reduced progression rates which achieved significance for nilotinib.[32-33] Based on these remarkable results, both drugs are now undergoing approval for first-line use in many countries. For the T315I mutant inhibitors including ponatinib (AP24534), a pan BCR-ABL inhibitor (Phase

II) and DCC-2036, a "switch-control" inhibitor (Phase I), have advanced to clinical trials.[34-37]

***Combination studies***: In an effort to make patients' responses more robust, clinical trials have been launched involving the use of TKIs in combination with other drugs that could potentially augment TKI-induced cell death. One such study combines imatinib with peginterferon alfa-2a or cytarabine and currently shows a significant advantage for the interferon combination in terms of molecular responses (overall survival and progression free survival unchanged).[38] Further smaller trials are being conducted that combine imatinib with zileuton (Phase I), panabinostat, sodium valproate (Phase I), arsenic trioxide (Phase II) or hydroxychloroquine (Phase II) and there is a Phase I evaluation of dasatinib with a SMO inhibitor underway.[39]

***The problem of persistence*:** Disease persistence refers to the presence of primitive BCR-ABL-positive CD34+ cells that are inherently insensitive to TKIs in patients who are otherwise responding well. Whilst the majority of these patients are in major molecular response with BCR-ABL transcripts detectable by Q-RT-PCR, recent data also confirm the presence of BCR-ABL expressing cells by genomic PCR in patients in complete molecular remission on TKIs.[40-41] We and others have shown that four of the currently available TKIs (imatinib, nilotinib, dasatinib and bosutinib) have strong anti-proliferative effects, but fail to induce cell death in primitive stem/progenitor cells. This may explain why primitive leukaemic cells are present in the bone marrow of patients with established complete cytogenetic response (CCR) over 5 years on imatinib.[42-46] It is also in keeping with the rapid recurrence of the ability to detect BCR-ABL transcripts in most patients with apparent complete molecular remission following imatinib discontinuation.[40,47] In recent studies by the French and Australian groups disease recurrence rates were 61% (of 100 patients) and 56% (of 18 patients), respectively, in selected patients who had undetectable disease maintained over more than 2 years prior to TKI discontinuation. These clinical observations have prompted the development of stem cell directed therapy and have raised the debate as to whether CML stem cells are oncogene addicted, i.e. if CML stem cells are dependent on BCR-ABL for survival. Recent results from Corbin *et al* and our laboratory (unpublished) suggest that CML stem cells can survive following complete BCR-ABL inhibition and are therefore not oncogene addicted.[48]
Given that BCR-ABL expressing stem cells can survive without BCR-ABL signaling, combination therapy is likely to be one way to circumvent the problem of disease persistence. However, improving existing or combining different BCR-ABL inhibitors is unlikely to tackle the problem of persisting CML stem cells that are able to survive despite full BCR-ABL inhibition.[48] Therefore, alternative survival pathways responsible for leukaemic stem cell survival following oncogene inactivation must be examined in order to apply successful stem cell directed therapy, potentially leading to patient cure. One example of such a survival pathway is macroautophagy (autophagy).[49-50]

**Molecular mechanism of autophagy**

Autophagy [self (auto)-eating (phagy)] is a recycling process that leads to sequestration and degradation of damaged proteins and other intracellular material within lysosomes (Figure 2).[51] Recycling of these intracellular constituents can serve

as an alternative source of energy during periods of metabolic stress or starvation to maintain cellular homeostasis and survival.[52]
An understanding of the molecular regulation of autophagy has been gained only in the past 10 years. Autophagy has been thoroughly examined in yeast where many autophagy-related genes (*ATG*) have been identified and several of these genes have now been shown to have mammalian orthologues. The process can be divided into sequential steps that ultimately result in breakdown of cytoplasmic material within the lysosomes (Figure 2).

***Initiation*:** It has recently been elucidated that the initiation of autophagy starts with activation of a serine/threonine kinase complex containing ULK1 and 2 (yeast Atg1), ATG13, and FIP200.[53] During glucose starvation, AMP activated protein kinase (AMPK) phosphorylates ULK1, promoting activation of autophagy. Alternatively, when nutrient sufficiency is established mTOR blocks this interaction.[54-55] Hence, mTOR acts as the main regulator of autophagy by directly inhibiting the ULK1/2/ATG13/FIP200 complex and other ATG proteins.[56] Consequently, upon mTOR inhibition (i.e. cell starvation or rapamycin treatment), the UKL1 complex dissociates from mTOR allowing it to accumulate at an isolation cup-shaped membrane, called the phagophore.[53]

***Autophagosome formation*:** The class III PI3-K (PI3-K-III) VPS34 is critical for further expansion of phagophores to autophagosomes, double-membraned vesicles which are the main mediators of autophagy.[57] VPS34 forms a complex with VPS15 and autophagy protein BECLIN1 (yeast Atg6).[51] The anti-apoptotic protein BCL-2 can interact with the BH3 domain of BECLIN1 and inhibit autophagy.[58] This interaction is reduced upon starvation, freeing BECLIN1 to activate autophagy.[59] It has been suggested that this anti-autophagy function of BCL-2 might maintain autophagy at levels that are optimal for cell survival. BCL-2 has also been shown to suppress autophagy by inhibiting cytosolic calcium elevation, which has been shown to induce autophagy.[60] UVRAG (UV irradiation resistance-associated gene) and AMBRA1 are other proteins that have been shown to interact with BECLIN1 and positively regulate autophagy, indicating that BECLIN1-dependent autophagy might be tightly regulated by BECLIN1 binding partners.[61]
Autophagosome completion is mediated by two ubiquitin-like conjugation systems: the ATG12/ATG5 and ATG8-PE [(ATG8 is also known as microtubule-associated protein 1 light chain 3; LC3), (PE; phosphatidylethanolamine)] conjugation systems, which are both essential for autophagy.[62] The ATG8-PE process is mediated by ATG4, ATG7 (E1-ubiquitin enzyme) and ATG3 (E2-ubiquitin enzyme) that perform the lipid modification of ATG8. ATG4 also cleaves the amide bond between ATG8 and PE to release the protein from membranes.[63] The ATG12/ATG5 process starts with conjugation of ATG12 to ATG5 with ATG10 acting as an E2 enzyme. The ATG12/ATG5 conjugate then interacts with ATG16 and this complex (ATG12/ATG5/ATG16) exerts an E3 enzyme-like function and regulates the ATG8-PE binding to the autophagosomal membrane.[64] LC3 (ATG8) has been investigated most thoroughly and acts now as the main marker for autophagosomes. LC3 maturation completes with the reversible conjugation of LC3-I to PE (by ATG7 and ATG3) to form LC3-II on the surface of autophagosomes.[65] This alteration is used to monitor autophagy by various assays. However, correct interpretation of LC3-I/II conversion is very important as accumulation of autophagosomes could, for example, reflect either increased autophagosome formation due to an increase in autophagic

activity, or inhibition of autophagosome turnover. Leading scientists in the field have published detailed guidelines and a standard set of criteria for monitoring autophagy that should help new investigators that join this growing field.[66]

***Maturation and degradation*:** The maturation process includes the fusion of the outer membrane of autophagosomes with lysosomes to form autolysosomes. The mechanism of this process is not very well understood but it has been shown that LAMP1/2 (lysosomal proteins), RAB7 (small GTPase) and cathepsinD/B/L are all involved in the autophagosome maturation, with RAB7 and LAMP1/2 potentially facilitating autophagosome and lysosome fusion.[67] UVRAG has also been shown to be involved in the maturation step, where it stimulates RAB7 GTPase activity and autophagosome fusion with lysosomes.[57] At the final autolysosomes, the inner single membrane of the autophagosomes and its cargo is lysed by lysosomal hydrolases, especially cathepsins and the content degraded.[68]
However, autophagy should not be viewed only as recycling machinery. This process is constantly taking place at basal levels in the majority of mammalian cells, as early as the stage of oocyte fertilisation, and is involved in a plethora of mechanisms and pathways including cell development, differentiation, homeostasis, quality control, aging, infection and immunity, neurodegeneration and cancer.[69]

## The paradoxical role of autophagy in cancer

Autophagy has been suggested to work as a double-edged sword in cancer development and progression, by inducing both tumour cell survival and death. These diverse effects depend to a large degree on the type of tumour, stage of disease and nature of treatment.[70] Basal autophagy within normal cells is pivotal since it (i) promotes genetic stability by removing damaged mitochondria that would otherwise produce ROS (reactive oxygen species) and damage the DNA, and (ii) functions as a "guardian" of the proteome and promotes adaption under changing conditions and/or stress.[69] The first strong link between autophagy and cancer was published in 1999 by Levine's laboratory showing that *BECLIN1* is a haploinsufficient tumour suppressor.[71] Subsequent studies revealed that *BECLIN1* is lost at high frequency in human breast, ovarian and prostate cancers and *BECLIN1*-heterozygous mice are prone to tumour development.[72] On the other hand, autophagy inhibition leads to enhanced apoptotic effect of irradiation, alkylating agents and arsenic trioxide in tumour cells.[70] Hence, autophagic activity may suppress tumourigenesis in healthy cells by removing damaged organelles and proteins, reducing ROS and suppressing necrosis-induced inflammation that can lead to cancer development when chronic.[73] However, it seems to have a cytoprotective role in established tumour cells and thus, its inhibition could enhance apoptosis. Greater understanding of the functional role of autophagy with regard to cancer in the future will be critical in order to enable specific autophagy inhibition/promotion in a range of tumours for therapeutic gain.

## Pharmacological inhibition of autophagy

Several inhibitors that disrupt the autophagy process are available and frequently used to study the role of autophagy in tumourigenesis and in response to cancer therapy. 3-methyladenine (3-MA) is a PI3-K-III inhibitor and therefore inhibits autophagosome formation. Wortmannin (pan PI3-K inhibitor) has also been used to inhibit autophagy at its early stage. In contrast to the PI3-K-III inhibitors, bafilomycin $A_1$, chloroquine

(CQ), hydroxychloroquine (HCQ) and $NH_4Cl$ inhibit autophagy at a later stage by preventing the fusion of autophagosomes and lysosomes.[74] Bafilomycin $A_1$ is a specific inhibitor of vacuolar-type $H^+$-ATPase, whereas CQ, HCQ and $NH_4Cl$ are lysosomotropic drugs that raise intracellular pH and therefore impair autophagosome and lysosome fusion and autophagic protein degradation. Although these drugs are not specific autophagy inhibitors and will have an effect on other pathways, they have proven very useful in studying and inhibiting autophagy in the laboratory. Autophagy inhibition in a specific manner is currently performed *in vitro* via siRNA-mediated knockdown of essential autophagy genes, such as *BECLIN1*, *ATG5* and *ATG7*. The challenge for the future is the development of autophagy specific drugs. With our ever increasing understanding of the main regulators of this key process this should not be far away. There are already some critical players that appeal as attractive drug targets, such as the kinases in the ULK1 protein complex, VPS34 and essential ATG autophagy proteins (e.g. ATG4 or ATG7), for which activity could be repressed with small inhibitory compounds.[70]

**Autophagy in CML**

BCR-ABL signaling, like growth factor activation, leads to activation of the PI3-K/AKT pathway and mTOR. In line with this, it has been demonstrated that BCR-ABL expressing mouse haemopoietic precursor cells have low basal levels of autophagy, but are highly dependent on this process.[75] Inhibition of BCR-ABL by TKIs has now been shown to not only induce apoptosis but also autophagy, a similar effect to that seen following growth factor withdrawal.[49] But the questions of (i) what level of autophagy is taking place within haemopoietic stem cells (HSCs) and (ii) what is its role remain unclear. However, two recent studies have elucidated the field. Firstly, Liu *et al* reported that FIP200 is indispensible for the function and maintenance of fetal murine HSCs in a cell-autonomous manner.[76] The hypothesis that autophagy is essential for the maintenance of HSCs was further supported by Mortensen *et al* who showed in mice that adult Atg7-deficient HSCs fail to engraft lethally irradiated recipients, clearly depicting autophagy as an essential modulator of HSCs maintenance.[77]

Many recent publications have shown that chemotherapeutic agents used in the treatment of haemopoietic malignancies, including CML, induce both apoptosis and autophagy. The ultimate fate of a cell resembles a hub that integrates all the signals from the apoptosis, autophagy, necrosis and senescence responses. Hence, the choice between death and survival depends critically on the balance between these mechanisms. However, the ultimate decision between apoptosis and survival may also differ dependent on the maturational status of the cell. As hypothesised in a recent review by Calabretta and Salomoni, TKI treatment in CML could potentially trigger different signaling pathways in primitive CD34+CD38- cells versus their progenitors, i.e. inhibition of BCR-ABL could trigger apoptosis within the progenitors but protective autophagy at the primitive stem cell level.[78]

In many situations targeting autophagy has increased the effect of the anticancer drug, although it is not yet clear whether autophagy inhibition is enhancing programmed or necrotic cell death. Nonetheless, by using mouse models it has been convincingly shown that autophagy serves as a survival pathway and its inhibition enhances p53 or drug-induced cell death in lymphoma cells.[79] This indicates that autophagy inhibitors, such as CQ, in combination with therapies designed to induce cell death, could be beneficial in the treatment of haematological neoplasms. Recently a number of

publications have linked CML with autophagy (Table 1). In 2006 it was shown that the neurotoxin crotoxin induced autophagy in K562 cells and blockade of autophagy with 3-MA and $NH_4Cl$ potentiated the neurotoxin's cytotoxicity.[80] One year later Carew *et al* showed that targeting autophagy using CQ enhanced the anticancer activity of the histone deacetylase inhibitor SAHA in imatinib-resistant primary CML cells.[81] These reports represented the first evidence that CML cells induce autophagy as a cell survival response following treatment with anticancer drugs, suggesting autophagy inhibition as a novel strategy with therapeutic implications for imatinib resistance.[82] The first evidence that inhibition of BCR-ABL activity induced autophagy came one year later when bafetinib (INNO-406), a potent c-ABL and LYN kinase inhibitor, was shown to induce autophagy in K562 cells.[83] Again, this autophagy response had a protective role as treatment with CQ enhanced cell death induced by bafetinib. A few months later, imatinib was also shown to induce autophagy in K562 cells and co-treatment with imatinib and CQ markedly enhanced imatinib-induced death.[84]

The strongest evidence that autophagy inhibition might enhance the therapeutic effects of imatinib in the treatment of CML was shown in 2009.[49-50] There we demonstrated that autophagy inhibition combined with TKIs (imatinib, dasatinib or nilotinib) resulted in near complete elimination of phenotypically and functionally defined CML stem cells. Our data were further supported by a study from Crowley *et al* who also showed that autophagy works as a cellular protection against imatinib-induced stress.[85] Despite a recently published role for ABL kinases in the regulation of late-stage autophagy, the effect in our study was specific to BCR-ABL inhibition.[86] Autophagy was not induced upon imatinib treatment in a cell line carrying the T315I mutation and ectopic expression of resistant c-ABL (T315I) mutant did not block the induction following imatinib exposure. It is likely that imatinib induces autophagy by inhibiting mTOR, the negative regulator of autophagy, and it was shown to potentially be associated with ER (endoplasmic reticulum) stress, but the exact molecular mechanism is still not clear and is being investigated in our laboratory. Importantly, our data were supported by *in vivo* experiments in a mouse model. As published by Altman *et al*, transplantation of Bcr-Abl expressing haemopoietic cells that were depleted of *Atg3* by conditional deletion, to lethally irradiated mice revealed that Bcr-Abl failed to induce leukaemia in an autophagy ablated background.[87]

All the aforementioned promising results within the CML stem cell population have led to the initiation of the Phase II CHOICES (*CH*l*O*roquine and *I*matinib *C*ombination to *E*liminate *S*tem cells) clinical trial. The CHOICES trial is a randomised trial of imatinib versus HCQ and imatinib for CML patients in major cytogenetic response (MCyR) with residual disease detectable by quantitative polymerase chain reaction (q-PCR) and is the first clinical trial using autophagy inhibition in CML.

To support the role of active mTOR in blocking autophagy in BCR-ABL expressing cells, inhibition of mTOR using OSI-027, that inhibits both mTORC1 and mTORC2 (the two separate mTOR complexes), has recently been shown to induce autophagy in K562 cells.[88] Furthermore, combination of OSI-027 with CQ resulted in increased apoptosis indicating that autophagy was acting as a survival mechanism following OSI-027 treatment.

Paradoxically, drug treatment has also been shown to induce autophagic cell death (type II apoptosis) in imatinib sensitive and imatinib resistant K562 cells.[89] Puissant *et al* showed that resveratrol (phytoalexin produced naturally by several plants) treatment induced apoptosis and autophagy through AMPK-dependent inhibition of

the mTOR pathway. Following autophagy inhibition with either bafilomycin $A_1$ or knockdown of *ATG5* or *LC3*, resveratrol-induced apoptosis was partially rescued. It was also shown that bafilomycin $A_1$ reduced resveratrol-induced cell death in primary CD34+ CML cells, with the opposite effect seen on imatinib-induced death where bafilomycin $A_1$ enhanced the imatinib effect (consistent with our previous results).[49,89] These results underline the importance of understanding the type of autophagy induction following specific types of stress and anticancer treatment so the most appropriate manipulation of autophagy can be applied for therapeutic intervention. On-going and future investigation regarding the use of autophagy regulators *in vivo* will shed light on this enigma since autophagy response differs not only among different patients and different organs within the same patient, but also among cells within the same tumour.[69] Thus, how CHOICES and other clinical trials' patients respond to combination therapy with an autophagy inhibitor, will give us essential information on whether modulation of autophagy in human tumours is a future therapeutic option of merit.

**Autophagy inhibition in the clinic**

None of the available autophagy inhibitors is specific for autophagy and hence, affect other pathways. Nevertheless, CQ and HCQ are used as anti-malarial drugs and have been approved for treatment of other diseases, e.g. rheumatoid arthritis. Since HCQ is less toxic than CQ to the retina, a number of clinical trials have now been initiated in patients with haemopoietic or solid tumours using HCQ as an autophagy inhibitor.[90-91] These trials use, like the CHOICES trial, the combination of HCQ with a targeted therapy, or in combination with cytotoxic chemotherapy or metabolic stress inducers, for example in multiple myeloma, where HCQ is used in combination with the proteasome inhibitor bortezomib (velcade).[90] But the overall hypothesis in all these trials is that autophagy is a survival mechanism of therapeutic resistance and blocking autophagy might increase the effect of the anticancer treatment. Although CQ has convincingly been shown to block autophagy *in vitro*, it still remains to be shown that drug/radiation-induced autophagy can be blocked using 400-800 mg daily dosing of HCQ in human tumours *in vivo*. Therefore, developing rigorous biomarkers in order to monitor autophagy *in vivo* will be critical.

**Conclusion:** In many ways CML represents a model disease and a paradigm for cancer stem cells, oncogene addiction and targeted therapies. With the introduction of TKI for the treatment of CML we have embarked on a journey aiming to cure the disease with orally administered, non-toxic agents. The major current impediment to cure for patients in CP resides in the cancer stem cell population that is neither oncogene addicted nor sensitive to TKI. The recent discovery that TKI induce the process of autophagy, which in turns protects CML stem cells, has presented the field with an exciting opportunity to kill one bird, CML, with two stones, TKI and autophagy inhibitors, with potential for cure.

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## Authorship

G.V.H. M.K and T.L.H wrote the paper and contributed to revisions. T.L.H. checked the final version of the manuscript.
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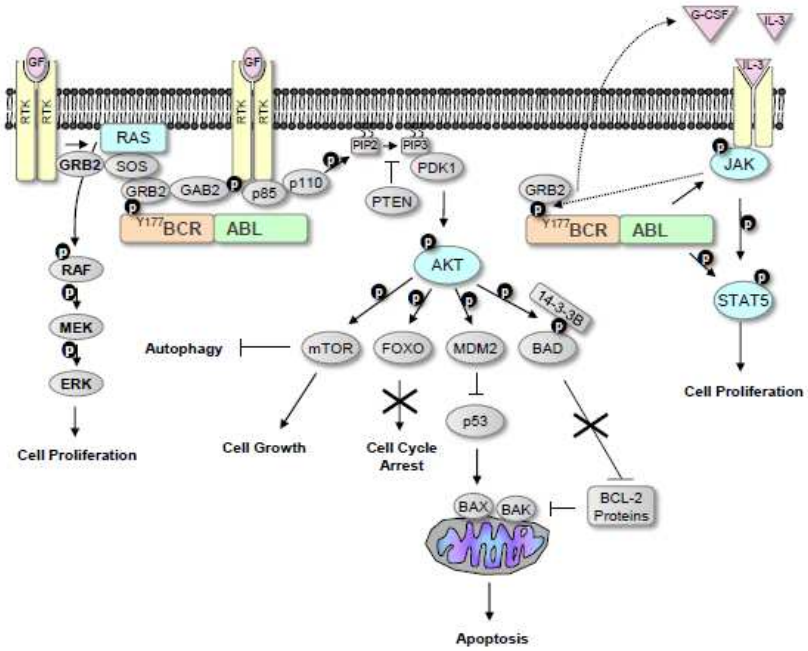
**Figure legends**

**Figure 1: BCR-ABL signaling mimics growth factor activation**
Growth factors are known to activate receptor tyrosine kinases (RTKs) leading to activation of the RAS and PI3-K/AKT pathways. These pathways are commonly activated in human cancers. Phosphorylation of tyrosine 177 within the BCR part of BCR-ABL leads similarly to activation of these pathways by interacting with the SH2 domain in GRB2. GRB2 activates RAS by interacting with SOS and it also activates PI3-K by mediating phosphorylation of GAB2. Constitutive activation of PI3-K leads to conversion of phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3). This can be inhibited by PTEN, a phosphatase that is downregulated in many cancers. PIP3 provides a platform for the recruitment of the serine/threonine kinases AKT and 3-phosphoinositide-dependent protein kinase-1 (PDK1), leading to phosphorylation and activation of AKT. Activated AKT phosphorylates many downstream targets that affect proliferation and survival, including the FOXO transcription factors and the pro-apoptotic protein BAD. AKT mediated phosphorylation of FOXO inhibits its nuclear entry and therefore suppresses its activity, leading to increased cell proliferation. The pro-apoptotic activity of BAD is also suppressed following AKT mediated phosphorylation. Phosphorylation of BAD prevents it binding to and inhibiting the function of anti-apoptotic BCL-2 proteins which inhibit the pro-apoptotic proteins BAK and BAX. AKT activation leads to downregulation of the tumour suppressor p53 by increasing the levels of the negative regulator of p53, MDM2. AKT also phosphorylates the serine/threonine kinase mTOR. Activated mTOR promotes protein translation and inhibits autophagy. BCR-ABL also leads to the activation of the JAK/STAT pathway, a pathway that is frequently activated in myeloproliferative diseases. JAK is a non-receptor tyrosine kinase and is normally activated by growth factors. BCR-ABL has been shown to induce IL-3 and G-CSF production that could lead to activation of this pathway. Active JAK phosphorylates the transcription factor STAT5 and BCR-ABL has also been shown to directly phosphorylate STAT5, leading to increased proliferation.

**Figure 2: Molecular mechanism of autophagy**
Autophagy initiation starts with activation of the ULK1/2 kinase complex which also includes ATG13 and FIP200. mTOR suppresses the activity of this complex by phosphorylating ULK1 and ATG13 on "negative" sites. This complex then becomes active following AMPK activation and mTOR inhibition, for example during starvation or treatment with rapamycin. Phosphorylated and now active ULK1 promotes phosphorylation of ATG13 and FIP200 and dissociates from mTOR. The PI3-K-III VPS34 is critical for further autophagosome formation. VPS34 forms a complex with VPS15, UVRAG, AMBRA1 and BECLIN1. This complex can be inhibited by the anti-apoptotic protein BCL-2 which can interact with BECLIN1 through the BH3 domain in BECLIN1. Autophagosome completion is mediated by the ATG8 (LC3)-PE and ATG12/ATG5 conjugation systems. These systems perform the lipid modification of LC3-I leading to LC3-II-PE binding to the autophagosomal membrane (see text for more details). The conversion of LC3-I to LC3-II is commonly used to monitor autophagy by various assays. Completed autophagosomes contain materials such as proteins and organelles that can be digested following autophagosome fusion with lysosomes. Lysosomes have low pH and abundance of pH sensitive enzymes that can break down waste materials and cellular debris. The autolysosome degradation can therefore remove unwanted materials such as damaged proteins in addition to providing the cell with new building blocks for cellular maintenance. The mTOR inhibitor rapamycin can be used to promote autophagy induction. VPS34 inhibitors, such as 3-MA and Wortmannin can be used to inhibit early stages of autophagy. Bafilomycin $A_1$, CQ, HCQ and $NH_4Cl$, which inhibit the fusion of autophagosomes and lysosomes, can be used to inhibit autophagy at later stages.

**Figure 1**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RTK
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SOS
GAB2
p85
p110
PIP2
PIP3
PDK1
PTEN
JAK
Y177 BCR
ABL
AKT
RAF
MEK
ERK
Cell Proliferation
Autophagy
mTOR
FOXO
MDM2
BAD
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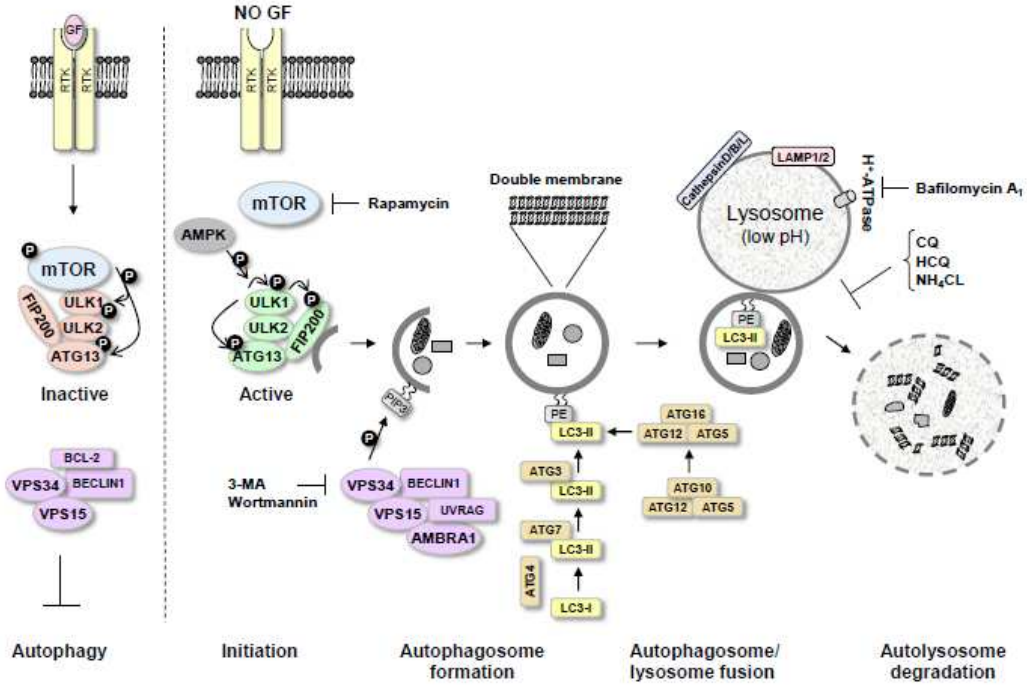
STAT5
Cell Growth
Cell Cycle Arrest
p53
BAX
BAK
BCL-2 Proteins
Apoptosis

**Figure 2**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VPS34
BECLIN1
VPS15
mTOR
Rapamycin
AMPK
ULK1
ULK2
FIP200
ATG13
Active
3-MA
Wortmannin
PIP3
VPS34
BECLIN1
VPS15
UVRAG
AMBRA1
Double membrane
PE
LC3-II
ATG3
LC3-II
ATG7
LC3-II
ATG4
LC3-I
ATG16
ATG12
ATG5
ATG10
ATG12
ATG5
CathepsinD/B/L
LAMP1/2
Lysosome
(low pH)
H+-ATPase
Bafilomycin A1
CQ
HCQ
NH4CL
PE
LC3-II
Autophagy
Initiation
Autophagosome formation
Autophagosome/ lysosome fusion
Autolysosome degradation

**Table 1: Autophagy induction in CML cells following various drug treatments**

| Drug | Main drug target | Cell type | Drug affect | Autophagy inhibitor | Affect of drug & autophagy inhibitor | Reference |
|---|---|---|---|---|---|---|
| Crotoxin | Not known | Cell line (K562) | Apoptosis and autophagy induction | 3-MA $NH_4Cl$ | Increased cell death | [80] |
| SAHA | Histone deacetylases | Cell lines, primary CML MNC | Apoptosis and autophagy induction | 3-MA CQ | Increased cell death | [81-82] |
| Bafetinib | BCR-ABL | Cell line (K562) | Apoptosis and autophagy induction | CQ 3-MA | Increased cell death | [83] |
| Imatinib | BCR-ABL | Cell line (K562) | Apoptosis and autophagy induction | CQ | Increased cell death | [84] |
| Imatinib, Dasatinib | BCR-ABL | Cell lines, primary CD34+ cells, including CD34+38- | Apoptosis and autophagy induction | CQ Bafilomycin $A_1$ | Increased cell death | [49-50] |
| Imatinib | BCR-ABL | K562 | Apoptosis and autophagy induction | 3-MA | Increased cell death | [85] |
| OSI-027 | mTOR | Cell line (K562) | Apoptosis and autophagy induction | CQ | Increased cell death | [88] |
| Resveratrol | Not known AMPK? | Cell lines, primary CD34+ cells | Apoptosis and autophagy induction | Bafilomycin $A_1$ | Decreased cell death | [89] |

MNC = Mononuclear cell.