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Apoptosis and autophagy have opposite roles on imatinib-induced K562 leukemia cell senescence

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Imatinib, the anti-Abl tyrosine kinase inhibitor used as first-line therapy in chronic myeloid leukemia (CML), eliminates CML cells mainly by apoptosis and induces autophagy. Analysis of imatinib-treated K562 cells reveals a cell population with cell cycle arrest, p27 increase and senescence-associated beta galactosidase (SA-β-Gal) staining. Preventing apoptosis by caspase inhibition decreases annexin V-positive cells, caspase-3 cleavage and increases the SA-β-Gal-positive cell population. In addition, a concomitant increase of the cell cycle inhibitors p21 and p27 is detected emphasizing the senescent phenotype. Inhibition of apoptosis by targeting Bim expression or overexpression of Bcl2 potentiates senescence. The inhibition of autophagy by silencing the expression of the proteins ATG7 or Beclin-1 prevents the increase of SA-β-Gal staining in response to imatinib plus Z-Vad. In contrast, in apoptotic-deficient cells (Bim expression or overexpression of Bcl2), the inhibition of autophagy did not significantly modify the SA-β-Gal-positive cell population. Surprisingly, targeting autophagy by inhibiting ATG5 is accompanied by a strong SA-β-Gal staining, suggesting a specific inhibitory role on senescence. These results demonstrate that in addition to apoptosis and autophagy, imatinib induced senescence in K562 CML cells. Moreover, apoptosis is limiting the senescent response to imatinib, whereas autophagy seems to have an opposite role.
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Chronic myeloid leukemia (CML) is a clonal proliferation malignancy characterized by a t(9;22) (q34;q11) reciprocal translocation leading to the Philadelphia chromosome (Ph^+) with a fusion gene *BCR-ABL*, the molecular hallmark of CML and Ph^+ acute lymphoblastic leukemia.[1] Targeting the tyrosine kinase (TK) activity of Bcr-Abl has been an attractive therapeutic strategy in CML with the development of new drugs such as tyrosine kinase inhibitors (TKIs). Imatinib mesylate (previously known as Gleevec or STI571) is a Bcr-Abl TKI that competes with the ATP-binding site of Bcr-Abl and stabilizes the protein in its inactive conformation, thereby inhibiting its TK activity.[2] Bcr-Abl is one of the main targets of the TKI imatinib mesylate (Gleevec^R or Glivec^R, previously STI571), which also inhibits c-Kit (the receptor for SCF), the PDGF receptor, Abl and the Abl-related gene.[3] The resulting inhibition of the kinase activity leads to apoptosis of leukemic cells sparing normal hematopoiesis.[2] Several studies suggest that TKIs induce caspase-independent cell death (CICD) among which necrosis or lysosomal permeabilization but also surviving mechanisms like autophagy. Some of these different deaths or survival pathways have a role in the response to imatinib.[4,5] However, other cell deaths still remain to be studied. Although the efficient therapeutic use of TKI-targeting Abl in CML disease has been associated with the apoptosis of the Philadelphia-positive cells, CICD have been reported at least *in vitro* and frequently unmasked by caspase inhibition.[6,7]

Apoptosis is by far the best characterized type of cell death and classified as programmed cell death I. It is defined by morphological features (rounding up of the cell, reduction of cellular and nuclear volume, nuclear fragmentation, plasma membrane blebbing and phosphatidylserine exposure, loss of mitochondrial membrane potential) and caspase activation. For a long time, apoptosis was the only death reported in response to imatinib associated with a controlled mechanism. However, recent studies suggest that necrosis, considered for a long time as an accidental kind of death, would result from accurate mechanisms.[8] Interestingly, there are other ways to die such as when autophagy is overbooked.[9] Macroautophagy (refered as autophagy) has first been demonstrated to be a self-proteolysis system involved in the rescue of the cell to maintain homeostasis.[10] It is a well-organized catabolic mechanism allowing recycling of macromolecules triggered by stress conditions.[11] Autophagy is characterized by double

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Abbreviations: ATF5, activating transcription factor 5; ATG, autophagy-related genes; Bcl-2, B-cell CLL/lymphoma 2; Bim, Bcl-2 interacting mediator; CML, Chronic myeloid leukemia; shRNA, short hairpin RNA; TAF6, Transcription initiation factor TFIID subunit 6; TEM, Transmission Electron Microscopy; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor; Z-Vad-fmk, Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone
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membrane vesicle formation called autophagosome, a massive vacuolization and can become a death pathway in not yet well-defined conditions.[9,12] Even CML cells can be eliminated through a resveratrol-mediated autophagic cell death.[13] There is now mounting evidence that autophagy and apoptosis share several common regulatory elements.[14,15] In contrast, senescence has been associated with age and telomere shortening or stress conditions.[16,17] Senescence is characterized by molecular and morphological cell changes such as an irreversible cell cycle arrest, an increase of cell granulation and size and an increase of lysosome.[18,19] Senescent cells also share biochemical modifications, such as an increase of senescence-associated β-galactosidase activity (SA-β-gal), overexpression of cell cycle inhibitors (p21, p53, p16), specific wnt protein expression and a strong resistance to apoptosis.[20] They have still an active metabolism and lots of attention is now on the secreted proteins during senescence. Indeed, senescence could participate in the quality of cell response to therapeutic treatment but also to eliminate oncogenic-transformed cells.[17,21]

Imatinib has recently been described to be involved in a senescent response of thyroid cancer cells or a quiescent phase of gastrointestinal stromal tumor cells.[22,23] However, these responses have yet to be determined in imatinib-treated CML cells. To answer if imatinib triggers senescence in CML cells, we studied how K562 CML cells died in response to imatinib and the interplay between apoptosis, autophagy and senescence.

Results

Imatinib induced apoptosis, autophagy and a senescent-like phenotype in K562 cells. In an attempt to characterize if imatinib is inducing other cell death than apoptosis, we detect apoptosis, autophagy and senescence in K562 cells. K562 cells were first incubated with 1 μM of imatinib for 24, 48 and 72 h. Then, annexin V binding and SA-β-Gal were measured. In response to imatinib, apoptosis was detected at 24 h and still increased at 72 h (Supplementary Figure 1A). SA-β-Gal was maximal at 48 h and led us to choose 48 h of incubation to detect apoptosis, autophagy and senescence. The apoptotic response of K562 cells upon 48 h imatinib treatment was also confirmed by the detection of the cleaved caspase-3 fragment (Figure 1a). A mean of 35% of annexin V-labeled K562 cells were detected in response to imatinib on eight independent experiments. To detect autophagy, the ATG8-related human protein LC3 (the microtubule-associated protein 1 light chain 3) was detected by western blot. K562 cells were treated with 20 nM of Bafilomycin A1 2 h before the end of the 48-h imatinib incubation to block the autophagic flux through the inhibition of the autophagolysosome formation. Bafilomycin incubation leads to the accumulation of the lipidated form of LC3 (LC3BII) allowing easier detection of the autophagic marker (Supplementary Figures 2A and B). Upon 48-h imatinib incubation, LC3BII accumulates in K562 cells showing an autophagic response (Figure 1b). This was also confirmed by the detection of mcherry-tagged LC3 proteins (Supplementary Figure 2C). Senescence was observed by the detection of SA-β-Gal-positive cells, the increase of cell size and the expression of the cell cycle inhibitors p21 and p27 (Figure 1c and Supplementary Figure 1e). Imatinib induced an increase of SA-β-Gal-positive cells (38% of SA-β-Gal-positive cells were detected in response to imatinib *versus* 14% in untreated cells, Figure 1c). A decrease of the cell cycle inhibitors p21 (3-fold) and an increase of p27 (4.6-fold) were detected in imatinib-treated cells upon 48 h in comparison to untreated cells (Figure 1d).

Inhibition of imatinib-induced apoptosis potentiates cell cycle inhibitor expression and SA-β-Gal-positive cell population. As apoptosis, autophagy and senescence are detected in K562 cells upon 48-h imatinib incubation, we would determine if regulations exist between these responses. Using the broad caspase inhibitor Z-Vad-fmk (Z-Vad), the annexin V-positive cell population was lesser than 8% in response to imatinib plus Z-Vad (Figure 1a). In addition, incubation of K562 cells with imatinib plus Z-Vad induced 70% of SA-β-Gal-positive cells (Figure 1c). This was paralleled by an increase of p21 (12–fold) and p27 (21–fold) expression, suggesting that apoptosis limits the senescent response may be through p21 and p27 downregulation (Figure 1d and Supplementary Figure 1D).

To test this hypothesis, we generate apoptosis-deficient K562 cells by inhibiting the expression of the proapoptotic protein Bim or by overexpressing the anti-apoptotic protein Bcl2 (Figure 2a). The detection of annexin V-positive cells in response to imatinib in the K562 shRNA Bim (KS sh Bim) and K562 Bcl2 (KS Bcl2) show no significant increase in comparison to untreated cells (Figure 2b), confirming that both Bim inhibition and Bcl2 overexpression inhibit imatinib-induced apoptosis. The SA-β-Gal-positive cells were increased in untreated KS sh Bim or KS Bcl2 cells and still increase upon 48-h imatinib incubation (Figure 2c).

Imatinib-induced senescence in K562 cells is linked to autophagy. Because autophagy is limiting the apoptotic response to imatinib, we investigate if it also regulates imatinib-induced senescence. ATG7-deficient K562 cells (KS shATG7) were generated using a short hairpin RNA (shRNA) anti-ATG7 by lentiviral infection of K562 cells as previously described[24] (Figure 3a). These cells show a moderate increase of the autophagic marker LC3B in response to imatinib confirming the inhibition of the autophagy process (compare Figure 3a with Figure 1a and Supplementary Figures 2B and C). ATG7-deficient K562 cells treated with 1 μM imatinib for 48 h have an increase of annexin V binding (Figure 3b). Imatinib induced a similar level of SA-β-Gal in KS shATG7 cells in comparison to K562 cells, suggesting that ATG7 inhibition did not abrogate imatinib-induced senescence, which is associated with a p21 decrease (5-fold) and a p27 increase (4-fold). In contrast, incubation with imatinib plus Z-Vad was not associated with an increase of SA-β-Gal-positive cells but there is still an increase of p21 (3.5-fold) and p27 (11-fold) expression (Figure 3c). Transmission electron microscopy (TEM) analysis of KS shATG7 cells treated with imatinib or imatinib plus Z-Vad show large vacuoles but no autophagosome (Supplementary Figure 3a).

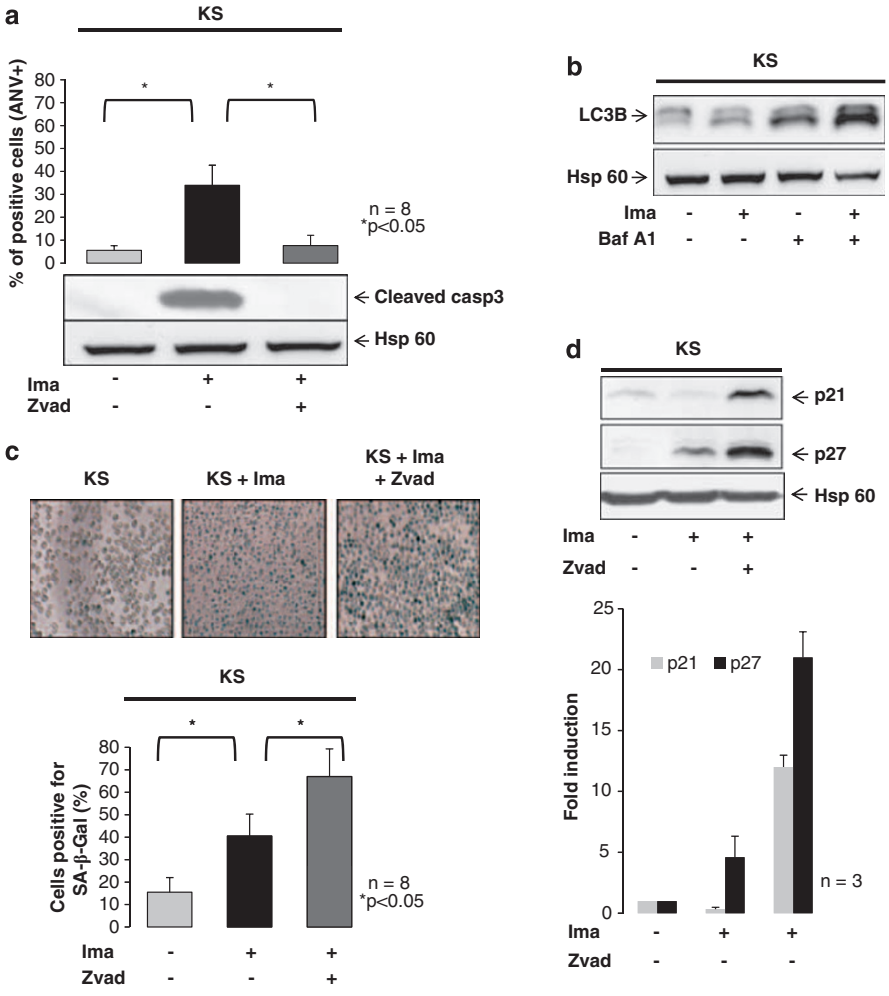

Figure 1 Imatinib-induced senescence of K562 cells is potentiated by caspase inhibition. K562 cells were grown in the presence of vehicle only, imatinib (Ima, 1 μM) or preincubated 30 min with Z-vad (50 μM) and then Ima (1 μM) for 48 h. An aliquot was incubated for 10 min in the presence of annexin-V-FITC. Samples were analyzed by flow cytometry and labeled cells were quantified as described in Materials and Methods. Results from eight experiments are expressed as the percentage of annexin V-labeled cells (**a**). Samples treated as above were used for detection of the cleaved form of caspase 3 by western blot and Hsp60 (as loading control). LC3B proteins were detected by western blot and Hsp60 was used as loading control (**b**). K562 cells treated as above (10^5 cells) was washed in PBS, fixed in PFA (4%) during 15 min and then incubated overnight in a 96-well plate in the presence of X-Gal (1 mg/ml) at 37 °C as described in Materials and Methods. The day after, the cells were washed once in PBS and SA-β-Gal activity was detected by a blue cell staining visualized under an inverted microscope. Pictures were acquired and analyzed using the NIS Nikon software. A representative result is shown in the top of the figure (**c**). SA-β-Gal-positive cells were quantified by counting 10^2 cells on three separate fields for each condition. Results show the mean of eight independent experiments (**c**). The proteins p21, p27 and Hsp60 were detected and the latter was used as a loading control. Results are from one experiment representative of three. The level of expression of p21 and p27 were quantified by densitometry analysis using ImageJ and results were normalized to the untreated condition (**d**)

Imatinib-induced senescence in apoptotic and autophagic-deficient K562 cells. To confirm the limiting role of apoptosis on imatinib-induced senescence, we used tetracycline-inducible shRNA anti-caspase 3 and 9. Although induction of shRNA anti-caspase 3 and 9 decreased their targets, respectively, imatinib induced a decrease of p21 and an increase of p27, which is surprisingly still associated with apoptosis and SA-β-Gal (Supplementary Figure 4), suggesting that these caspases are not critical for K562 cells responses to imatinib.

To investigate the role of autophagy in imatinib-induced senescence, we used the Bim-deficient and Bcl2-overexpressing K562 cells to generate double knock-down Bim/ATG7 cells and Bcl2-overexpressing/ATG7-deficient cells (Figure 4a). Incubation with imatinib for 48 h did not increase the number of annexin V-labeled cells confirming the inhibition of the apoptotic response previously observed (Figure 4b). To test the effect of the inhibition of both apoptosis and autophagy on imatinib-induced senescence, we incubate these cells with imatinib for 48 h. Although the level of the SA-β-Gal-positive cells in untreated K562 Bim/ATG7- and KS Bcl2/sh ATG7-deficient cells was elevated and similar to the single KS sh Bim or KS Bcl2 cells, imatinib still induced an increase of the SA-β-Gal-positive cells (Figure 4c). However, inhibition of both autophagy and apoptosis decreases the senescent response to imatinib similar to the imatinib plus Z-Vad-treated KS shATG7 cells (compare Figures 3c and 4c). To confirm this result, inhibition of Beclin-1 and ATG5 was performed by

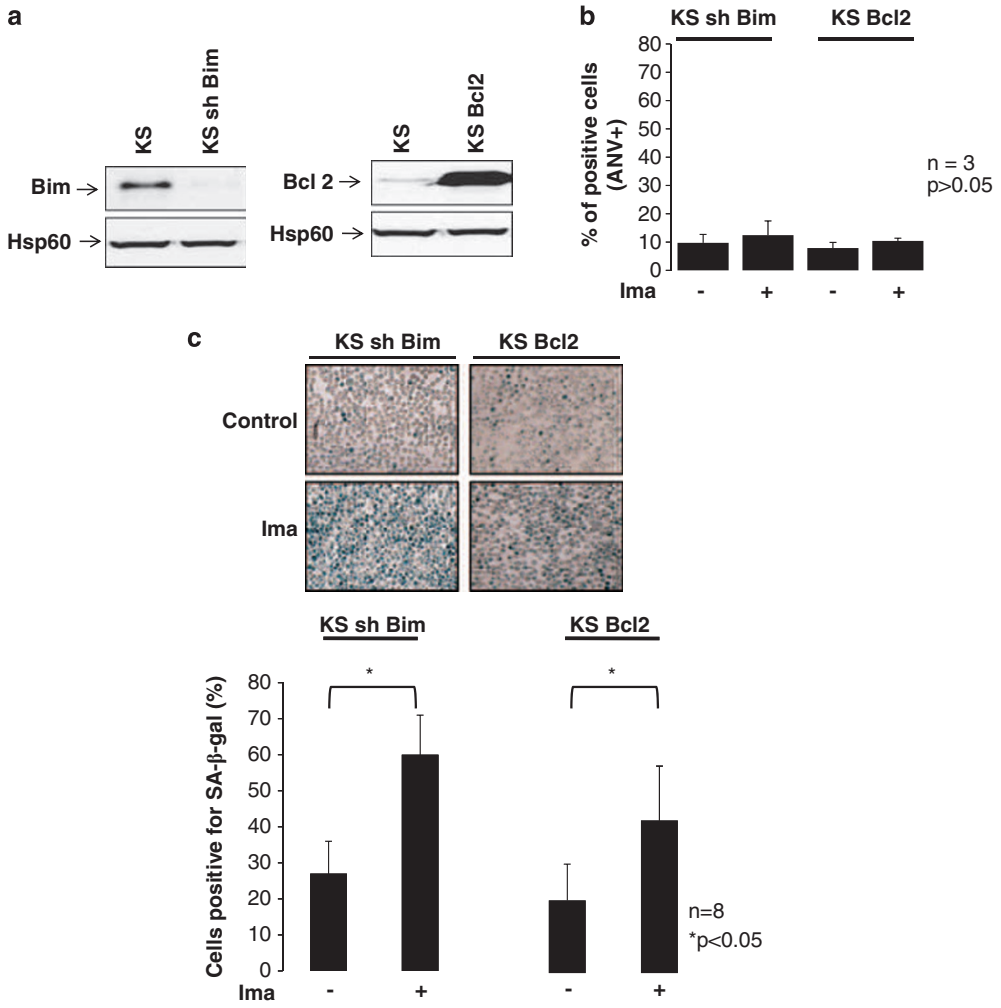

Figure 2 Inhibition of apoptosis increases senescence of K562 cells. K562 cells were infected by 24 h incubation with lentivirus coding for a shRNA anti-Bim or the complementary DNA of Bcl2. Then, the cells were washed twice in PBS and grown for 6 days before sorting using GFP selection. Bim and Bcl2 expression were detected by western blotting (**a**). K562 sh Bim and K562 Bcl2 cells were incubated with vehicle only or imatinib (Ima, 1 μM) for 48 h. Then, an aliquot was incubated for 10 min in the presence of annexin-V-FITC. Samples were analyzed by flow cytometry and labeled cells were quantified as described in Materials and Methods. Results from three experiments are expressed as the percentage of annexin V-labeled cells (**b**). K562 sh Bim and K562 Bcl2 cells were treated as above (10^5 cells) and washed in PBS, fixed in PFA (4%) during 15 min and then incubated overnight in a 96-well plate in the presence of X-Gal (1 mg/ml) at 37 °C as described in Materials and Methods. The day after, the cells were washed once in PBS and SA-β-Gal activity was detected by a blue cell staining visualized under an inverted microscope. Pictures were acquired and analyzed using the NIS Nikon software. A representative result is shown in the top of the figure (**c**). SA-β-Gal-positive cells were quantified by counting 10^2 cells on three separate fields for each condition. Results show the mean of eight independent experiments (**c**)

shRNA (Supplementary Figure 5). In parallel, a shRNA scramble (KS sh-scr) was used as control. KS, Ks sh-scr, Ks sh-Bcn and KS sh-ATG5 cells were incubated with imatinib for 48 h with or without preincubated Z-Vad. Then, the apoptotic and senescent responses were compared with untreated cells. Imatinib induced apoptosis in all cell lines, although KS sh-ATG5 cells respond by a significant higher annexin V-positive cell population (Supplementary Figure 5b). This apoptotic response was strongly decreased by Z-Vad preincubation. Imatinib-induced SA-β-Gal was detected in KS cells and still increased when apoptosis was inhibited by Z-Vad (Supplementary Figures 5c and d). Similar to the KS sh ATG7 cells, imatinib-induced SA-β-Gal was increased in KS sh-Bcn but no more when cells were preincubated with imatinib plus Z-Vad confirming previous results (compare Figure 3c and Supplementary Figure 5c). In contrast, untreated KS sh-ATG5 cells have an increase of the SA-β-Gal-positive cells, which is not modified by imatinib or imatinib plus Z-Vad treatment (Supplementary Figures 5c and d).

Discussion

In this study, we report that imatinib induced, in addition to apoptosis and autophagy, a senescence-like phenotype in K562 CML cells.[4,25] Moreover, the senescent response was potentiated by apoptosis inhibition in an autophagy-dependent way. The critical role of the BH3-only domain-containing protein Bim or Bcl2 in imatinib-induced CML cell apoptosis allows us to generate apoptosis-deficient K562 cells.[26] Indeed, the inhibition of Bim expression or the overexpression

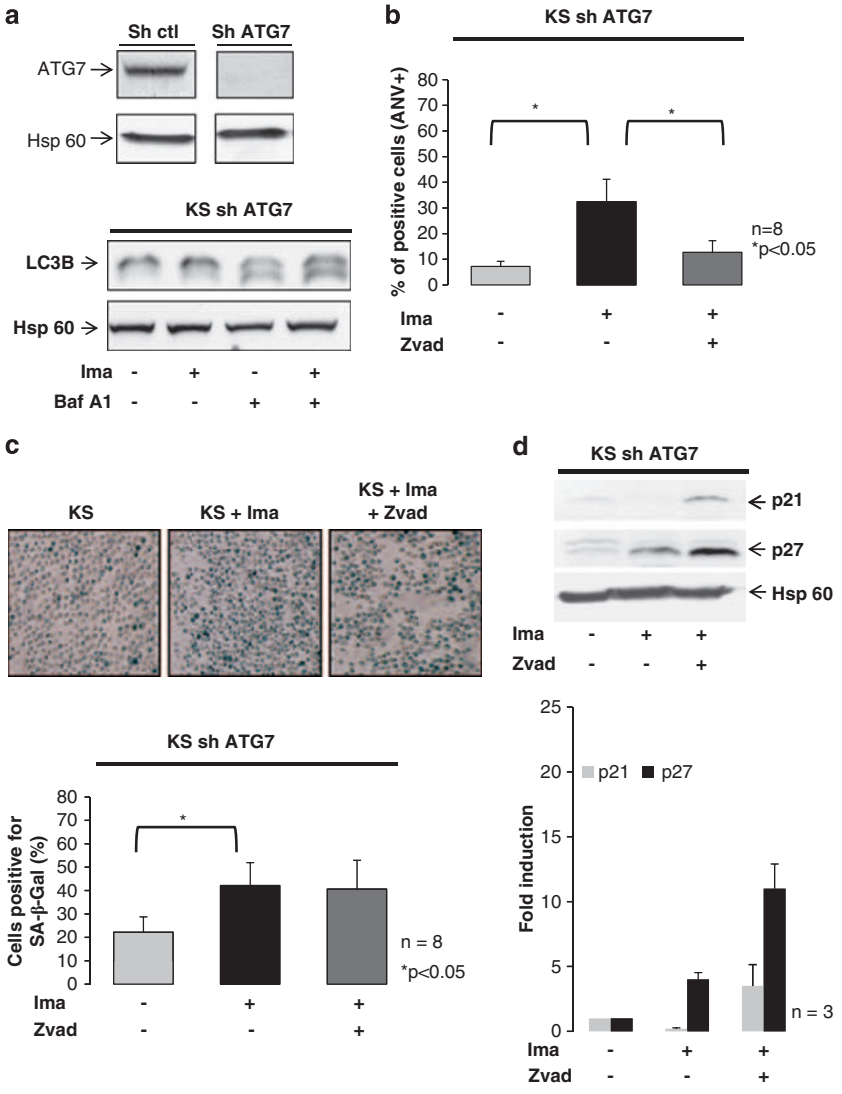

Figure 3 Autophagy-deficient cells have impaired potentiation of senescence. K562 cells were infected by lentivirus coding for a shRNA anti-ATG7 by incubation for 24 h. Then, the cells were washed twice in PBS and grown for 6 days before sorting. Samples were analyzed for ATG7 expression by western blotting and inhibition of autophagy by detecting LC3B proteins by western blot (**a**). K562 shATG7 cells were incubated with vehicle only, imatinib (Ima, 1 μM) or incubated with Z-vad (50 μM) and Ima (1 μM) for 48 h. Then, an aliquot was incubated for 10 min in the presence of annexin-V-FITC. Samples were analyzed by flow cytometry and labeled cells were analyzed as described in Materials and Methods. Results from eight experiments are expressed as the percentage of annexin V-labeled cells (**b**). K562 shATG7 cells treated as above (10^5 cells) was washed in PBS, fixed in PFA (4%) during 15 min and then incubated overnight in a 96-well plate in the presence of X-Gal (1 mg/ml) at 37 °C as described in Materials and Methods. The day after, the cells were washed once in PBS and SA-β-Gal activity was detected by a blue cell staining visualized under an inverted microscope. Pictures were acquired and analyzed using the NIS Nikon software. A representative result is shown in the top of the figure (**c**). SA-β-Gal-positive cells were quantified by counting 10^2 cells on three separate fields for each condition. Results show the mean of eight independent experiments (**c**). The proteins p21, p27 and Hsp60 were detected and the latter was used as a loading control. Results are from one experiment representative of three. The level of expression of p21 and p27 was quantified by densitometry analysis using ImageJ and results were normalized to the untreated condition (**d**)

of Bcl2 blocks apoptosis and increases senescence in response to imatinib, emphasizing previous results. Moreover, these results confirmed that CICD occurred in imatinib-treated CML cells and increased when caspases or apoptosis are inhibited.[6,7] Lavallard *et al.*[7] report CICD in CML cells in response to imatinib. This has not been associated with senescence, although the response to imatinib was correlated with the inhibition of colony formation in clonogenic assay and even more when apoptosis is inhibited, suggesting a cell cycle arrest or cell death. Another CICD has been reported in response to imatinib when apoptosis was prevented by caspases inhibition.[6] In regard to the necrosis-like cell death reported by Okada *et al.*,[6] we investigated the role of the serine protease Omi/HtrA2 in imatinib-induced senescence using the inhibitor ucf-101. At least in our hand, both the increase of p21 and p27 and the SA-β-Gal-positive cell population was not prevented or decreased by blocking Omi/HtrA2, suggesting that the release of this protease is not involved in the senescent response (Drullion, unpublished data). In addition, no significant propidium iodide (PI)-labeled

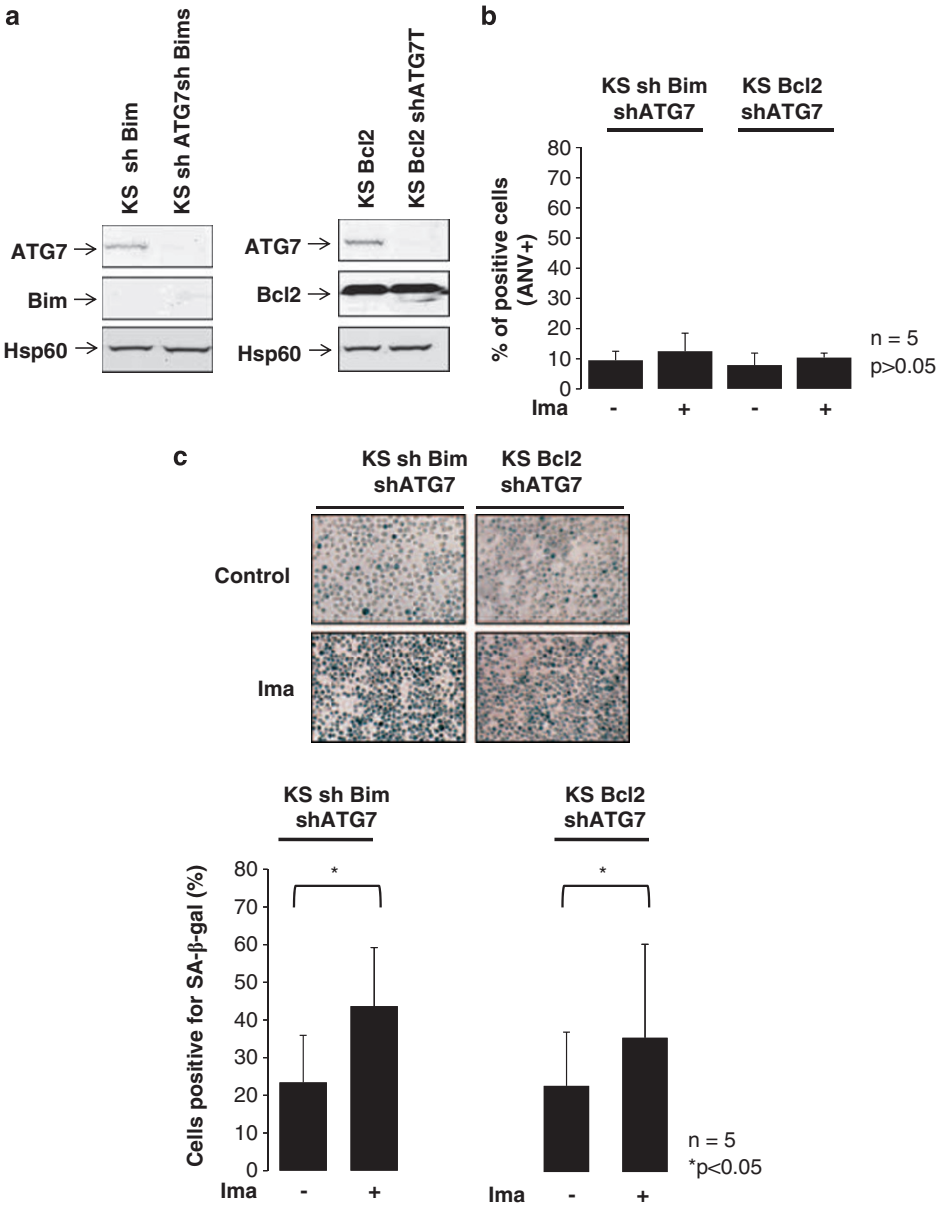

Figure 4 Inhibition of apoptosis and autophagy limits imatinib-induced K562 cell senescence. K562 sh Bim and K562 Bcl2 cells were infected by incubation for 24 h with lentivirus coding for a shRNA anti-ATG7. Then, the cells were washed twice in PBS and grown for 6 days before sorting as described in Materials and Methods. Samples were analyzed for Bim, Bcl2 and ATG7 expression by western blotting (**a**). K562 sh Bim/sh ATG7 and K562 Bcl2/sh ATG7 cells were incubated with vehicle only or imatinib (Ima, 1 μM) for 48 h. Then, an aliquot was incubated for 10 min in the presence of annexin-V-APC. Samples were analyzed by flow cytometry and labeled cells were analyzed as described in Materials and Methods. Results from five experiments are expressed as the % of annexin V-labeled cells (**b**). K562 sh Bim/sh ATG7 and K562 Bcl2/sh ATG7 cells were treated as above (10^5 cells) and washed in PBS, fixed in PFA (4%) during 15 min and then incubated overnight in a 96-well plate in the presence of X-Gal (1 mg/ml) at 37 °C as described in Materials and Methods. The day after, the cells were washed once in PBS and SA-β-Gal activity was detected by a blue cell staining visualized under an inverted microscope. Pictures were acquired and analyzed using the NIS Nikon software. A representative result is shown in the top of the figure (**c**). SA-β-Gal-positive cells were quantified by counting 10^2 cells on three separate fields for each condition. Results show the mean of five independent experiments (**c**)

cells was detected upon 48-h imatinib incubation and this was not increased by inhibition of caspase. Only dual PI/annexin V-labeled cells were detected in our experiments at longer time. This may suggest that K562 cells are more competent to die through senescence than necrosis in response to imatinib; a response more convincing when apoptosis is blocked. Interestingly, it should be noted that another CML cell line, BV173, undergoes more PI labeling than K562 cells upon 48-h imatinib incubation. Indeed, only cells undergoing mitochondrial depolarization were PI labeled, suggesting again that PI labeling may reflect the late stage of the apoptosis.

One major result of this study is imatinib induced, in addition to apoptosis and autophagy, a senescence-like change in K562 cells. It was already reported that imatinib triggers senescence (detected by an increase of p21 expression) in radiation-treated thyroid cancer cells or induces quiescence in GIST cells.[22,23] Furthermore, the senescent response observed was amplified by caspase or apoptosis inhibition.

A similar result was reported in neuroblastoma cells treated by increasing concentration of doxorubicin without caspase activation.[27] This is in accordance with the limiting role of apoptosis on senescence in a caspase-dependent way. This is also highlighted by the increase of p21 expression and SA-β-Gal-positive cells at a concentration of doxorubicin, which did not activate caspase 3. At higher concentration of doxorubicin, leading to caspase-3 activation and p21 degradation, death switches to apoptosis. Conversely, p21 increase cannot be observed until caspases are inhibited emphasizing our result in K562 CML cells. However, we were not able to confirm these results using K562 cells deficient for caspases 3 or 9. Although tetracycline-induced shRNA against caspase 3 or 9 decreased their expression and activation, they did not prevent imatinib-induced apoptosis and senescence. We also cannot rule out that Z-Vad has additional targets. For example, the cysteine-protease calpains have been involved in the proteolysis of specific substrates and may be inhibited to some extent by Z-Vad. Indeed, calpain activation has been associated with senescence in response to DNA damage.[28] However, using E-64-d, a calpain and cathepsin inhibitor, or CA074-Me, a cathepsin B inhibitor, we were unable to prevent p21 decrease in response to imatinib. The cell cycle arrest and the senescent response detected in imatinib-treated K562 cells could be linked to some other caspase substrates, such as the retinoblastoma protein (Rb) involved in E2F binding and proliferation or TAF6, a transcription factor involved in p21 expression even in the absence of p53.[29,30]

Besides the induction of senescence by imatinib, we identified two levels of senescence. The first one occurred in addition to apoptosis in response to imatinib, whereas the last was unmasked when apoptosis is inhibited. This later seems to be dependent on autophagy, as it was significantly decreased in two autophagic-deficient K562 cell lines (KS sh ATG7 and sh-Bcn). Indeed, autophagy has been connected to senescence in physiopathology, but its role is still controversial.[15,31–33] For example, mTor inhibition by resveratrol or rapamycin was reported to increase autophagy and suppress cellular senescence.[34] In our condition, senescence in response to imatinib was neither increased nor modified by rapamycin, an inhibitor of mTor (results not shown). Inhibition of ATG5 expression in K562 cells was accompanied by a strong increase of SA-β-Gal, but not anymore by imatinib or imatinib plus Z-Vad incubation. Although shATG7, sh-Bcn and sh-ATG5 inhibit imatinib-induced autophagy in K562 cells, basal and induced SA-β-Gal were maximal in KS sh-ATG5. We have not yet identified the molecular pathway explaining the discrepancy observed. In addition, the role of ATG5 in the senescent response is still controversial. Indeed, a similar increase of senescence has been reported in fibroblasts in response to ATG5 protein inhibition, whereas it inhibits both autophagy and senescence induced by H_2O_2 in the WI38 fibroblast cells through a p38 mitogen-activated protein kinase and p21 pathway.[35,36] Because apoptosis is limiting autophagy, at least through the proteolysis of autophagy-regulating proteins, potentiating senescence upon inhibition of apoptosis may take place in an autophagy-dependent way. This may explain why Bim-deficient K562 cells (sh Bim) undergo high basal SA-β-Gal-positive cell population, although it is not significantly different from K562 sh Bim/sh ATG7. When apoptosis was blocked by overexpression of Bcl2, the SA-β-Gal-positive cell population was similar.[37,38] A recent report describes that Bcr-Abl inhibits autophagy through an ATF5-mediated regulation of *mTOR* transcription.[39] In this way, this may contribute to the absence of senescence in Bcr-Abl-expressing cells while such oncogene expression should normally induce an oncogene-induced senescence response. Indeed, the inhibition of Bcr-Abl activity by imatinib blocks the BCR-ABL/PI3K/AKT/FOXO4/ATF5/mTOR pathway and consequently may induce autophagy and senescence.

This study reports for the first time that imatinib is able to induce senescence in K562 CML cells and confirms interplay between the different death and survival pathways. Many questions have still to be answered concerning the molecular network interconnecting these responses. However, the possibility to induce senescence in cancer cells is very exciting as it is the first barrier against tumorigenesis.

Materials and Methods

Reagents. RPMI 1640 medium, fetal calf serum, phosphate-buffered saline (PBS), were from Invitrogen (Life Technologies SAS, Saint Aubin, France). Trypan blue and the antibody against LC3 were from Sigma (St. Quentin Fallavier, France). TKIs Imatinib and Nilotinib were kindly provided by Novartis Pharma (Basle, Switzerland). The broad caspase inhibitors Z-VAD-fmk were purchased from Peptanova (Sandhausen, Germany). Cyto-ID autophagy detection kit was from Enzo Life Sciences (Villeurbanne, France) and used in flow cytometry. The following antibodies: caspase 3 and 9, p21, p27 were from Cell Signalling (Danvers, MA, USA), and Hsp60 was from Santa Cruz (Bergheimer, Germany). Annexin-V-FITC and APC were from Beckman Coulter (Villepinte, France).

Cell lines. The human erythroleukemia Bcr-Abl-positive human cell line used in this study: K562 (KS) was from ATCC. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Aliquots were taken at 24 h intervals for assessment of cell viability by Trypan blue exclusion. K562 sh caspase 3 and sh caspase 9 were generated as previously described.[40]

Transmission electron microscopy (TEM). K562 cells were processed for ultramicrotomy according to standard procedures. Cell pellets were fixed for 2 h in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4) and post-fixed for 1 h at 4 °C with 1% osmium tetroxide in the same buffer. The pellets were dehydrated with ethanol and embedded in Epon-Araldite. Thin sections were stained successively with 5% uranyl acetate and 1% lead citrate. TEM observation was performed with a FEI CM120 operated at 120 kV (FEI, Eindhoven, The Netherlands). Images were recorded with a USC1000 slow scan CCD camera (Gatan, CA, USA).

Western blot. Protein lysates were prepared according to Mahon *et al.*[41] Protein concentration was measured by the BCA Protein Assay (Pierce, Rockford, IL, USA) and the lysates were stored at -80 °C. Equal amounts of protein were separated by electrophoresis on an SDS-PAGE 12.5 or 15% and transferred to a PVDF membrane as described[42] (Biorad, Marnes-La-Coquette, France). After blocking, the membrane is incubated with primary antibodies and secondary antibodies. Protein–antibody complexes were detected by an enhanced chemiluminescence immunoblotting ECL (Perkin Elmer, Courtaboeuf, France).

Flow cytometry. Cells (10^5 cells) were incubated for 15 min in 500 μl of PBS with 2 mM Ca^{2+}, 2 μl of Annexin-V-FITC (or APC) and 0.25 μg of PI before flow cytometry analysis on Facscalibur. Ten thousand events are acquired for statistical analysis. Detection of autophagy was performed according to the manufacturer instructions (Enzo life sciences). Briefly, K562 cells (5×10^4) were incubated with dual-detection solution for 30 min in the dark and then diluted with PBS before flow cytometry analysis.

SA-β-galactosidase labeling. Sa-β-galactosidase was detected according to Dimri *et al.*[43] Briefly, cells (10^5 cells) were fixed for 15 min with 3% PFA, then washed once with PBS. Cells were then incubated in a 96-well plate with a mix (1vol/20vol) of solution I containing X-Gal (20 mg/ml X-Gal in dimethylformaldehyde) and solution II (5 mM ferricyanure, 5 mM ferrocyanure, 2 mM $MgCl_2$ 150 mM NaCl, 30 mM Citric acid/phosphate pH = 6) for 24 h at 37 °C. Cells were then washed with PBS and SA-β-gal activity was observed by detection of cells with a blue staining using an inverted Nikon Microscope (Eclipse Ti) and analyzed with the Nikon software NIS (Nikon France Instruments, Champigny, France). For SA-β-gal-positive staining cell quantification, 100 cells were counted on three separate fields and the mean of blue-stained cells was calculated as followed (number of blue cells/(number of total cells)). Results are expressed as percent of SA-β-gal-positive cells.

Apoptosis inhibition by Bim shRNA silencing or Bcl2 overexpression. To inhibit apoptosis, HIV-1 lentivirus-based vectors were used to introduce shRNAs against Bim or the complementary DNA of Bcl2 into cells as previously described.[44] Briefly, shRNAs were cloned in FG12 lentivector, HEK293FT cells were used as packaging cells for virus production. To confirm silencing of Bim or overexpression of Bcl2, infected cells were sorted using GFP-positive cell detection and specific protein expression was detected by western blot.

Autophagy inhibition by RNA silencing. To inhibit autophagy, HIV-1 lentivirus-based vectors were used to introduce shRNAs against ATG7 or Beclin-1 as already reported.[38] Briefly, shRNAs were cloned in FG12 lentivector, HEK293FT cells were used as packaging cells, and virus production was performed as previously described.[24,38] ShRNA anti-ATG5 were purchased from ABgene (Courtabeuf, France) and five different sequences were tested for ATG5 inhibition (target set, pLKO.1). To confirm autophagy inhibition by silencing of ATG7, and Beclin-1, cells were grown in nutrient-deprivated medium (HBSS) in the absence or in the presence of Bafilomycin A, and both LC3B I and II form were detected by western blot. For ATG5-deficient cells, the inhibition of autophagy was confirmed using the Cyto-ID autophagy detection kit in comparison to K562 cells.

Statistical analysis. A Wilcoxon test was used to calculate differences between means; differences were considered significant only when $P \leq 0.05$ and shown by an asterisk.

Conflict of Interest

The authors declare no conflict of interest.

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