

Imatinib Mesylate Induces Cisplatin Hypersensitivity in Bcr-Abl⁺ Cells by Differential Modulation of p53 Transcriptional and Proapoptotic Activity

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Abstract

Imatinib is highly effective in inducing remission in chronic myelogenous leukemia (CML). However, complete eradication of the malignant clone by imatinib is rare. We investigated the efficacy of combining imatinib with cisplatin. Inhibition of Bcr-Abl by imatinib induced a hypersensitive phenotype both in Bcr-Abl⁺ cell lines and in CD34⁺ cells from CML patients. Importantly, cisplatin sensitivity of leukemic cells harboring an inactive Bcr-Abl greatly exceeded that of Bcr-Abl⁻ parental cells. The cisplatin response of Bcr-Abl⁺ cells treated with imatinib was characterized by an impaired G₂-M arrest and by rapid induction of mitochondrial cell death after the first passage through G₂. Imatinib abrogated ATM activation on cisplatin selectively in Bcr-Abl⁺ cells. As a consequence, phosphorylation of p53 on Ser¹⁵ and its activity as a transcription factor was significantly diminished. Furthermore, p53 accumulated predominantly in the cytoplasm in Bcr-Abl⁺ cells treated with imatinib and cisplatin. Silencing of p53 significantly reduced sensitivity to cisplatin in imatinib-treated Bcr-Abl⁺ cells, indicating that p53 retains its proapoptotic activity. Simultaneous downregulation of Bcl-x_L was an additional requirement for cisplatin hypersensitivity, as p53-dependent cell death could be antagonized by exogenous Bcl-x_L. We conclude that imatinib sensitizes Bcr-Abl⁺ cells to cisplatin by simultaneous inhibition of p53 transactivation, induction of p53 accumulation predominantly in the cytoplasm, and reduction of Bcl-x_L. [Cancer Res 2009;69(24):9337–45]

Introduction

Bcr-Abl activates several signaling pathways essential for chronic myelogenous leukemia (CML; refs. 1–6). This oncogene protects cells from DNA damage–induced apoptosis (7–10), thereby inducing genomic instability (11). Bcr-Abl exerts its antiapoptotic functions by inhibiting proapoptotic and inducing antiapoptotic proteins (12). In addition, Bcr-Abl modulates DNA repair (9, 13–15) and extends G₂-M arrest on DNA damage (8, 16) mediated by an enhanced stimulation of ATR-CHK1 (8, 17).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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These pathways are liable to be altered by imatinib in Bcr-Abl⁺ cells. Imatinib functions as a competitive Bcr-Abl inhibitor, leading to reduced proliferation, restoration of checkpoint control, induction of apoptosis, and reversal of genetic instability in Bcr-Abl⁺ cells (10, 11, 18, 19). Imatinib has also proven to be highly effective for CML treatment (20). However, there is mounting evidence that resting CML stem cells are not eliminated by imatinib *in vivo* (21). Therefore, most patients remain Bcr-Abl⁺. Furthermore, therapy with imatinib may select the emergency of resistance and impair genomic stability in Bcr-Abl⁻ cells (22, 23). Moreover, therapy results of imatinib are less impressive in accelerated disease and Philadelphia chromosome–positive (Ph⁺) acute lymphoblastic leukemia (ALL; ref. 20). A possibility to overcome these concerns involves the combination of imatinib with DNA damaging agents. Therefore, a detailed understanding of effects of Bcr-Abl on DNA damage response pathways is warranted.

An approach to understand the mechanisms by which Bcr-Abl modulates DNA damage response has been proposed based on the cellular homologue c-Abl. c-Abl functions as a tightly regulated kinase in the nucleus and cytoplasm. In the nucleus, c-Abl is activated on DNA damage and binds to DNA damage response proteins (24). In this context, it is of importance that c-Abl influences the ATM-p53 axis: c-Abl binds to ATM (25), complexes with p53 and stimulates its tetramerization and DNA binding (26), and phosphorylates Mdm2 and MdmX, thereby neutralizing their inhibitory effects on p53 (27, 28). Bcr-Abl may disrupt some of the c-Abl functions (e.g., by impeding its nuclear localization). However, this has never been documented yet. Furthermore, c-Abl and Bcr-Abl share most of their sequences and therefore may also share most of their binding partners. The fact that Bcr-Abl is localized in the cytoplasm impedes its binding to nuclear partners. However, it has been shown that Bcr-Abl relocalizes to the nucleus following imatinib (29). Bcr-Abl was also found to translocate to the nucleus and bind to ATR in the presence of etoposide, leading to reduced ATR-CHK1 activation (30). However, this finding contrasts other reports showing an enhanced CHK1 activation in Bcr-Abl⁺ cells (8, 17). Therefore, it remains open whether DNA damaging agents are sufficient to mediate Bcr-Abl translocation as an active kinase.

To investigate whether imatinib-mediated inhibition of Bcr-Abl may enhance sensitivity to DNA damage, we combined imatinib with cisplatin. We found that imatinib not only reversed the Bcr-Abl–mediated resistance to cisplatin but also led to a hypersensitive phenotype. This was mediated by a diminished activation of ATM-p53 and reduced Bcl-x_L, leading to an impaired G₂-M arrest and to an enhanced transcriptional-independent proapoptotic function of p53.

Materials and Methods

Cell culture. BaF3 harboring wt-p53 (16) and BaF3BA (expressing p185bcr-abl) were gifts from Justus Duyster (Technische Universität München, Munich, Germany). p185bcr-abl is a human/mouse chimera containing human *bcr* and murine *abl* (31). K562 and BV173 were from the American Type Culture Collection. Cells were cultivated in RPMI 1640 (Biotech) with 10% FCS and glutamine.

CD34⁺ cells were purified using MACS (Miltenyi) and cultured in Stem-Pro-34 (Life Technologies) with 5 ng/mL FLT3 (RDI), 10 ng/mL human interleukin-3 (IL-3; Novartis), and 200 ng/mL granulocyte colony-stimulating factor (G-CSF; Roche). The investigation was approved by the local ethics committee (reference: 035-06-f); informed consent was obtained from patients.

Reagents. Imatinib (Research Chemicals) was used at 3 $\mu\text{mol/L}$ and added 2 h before cisplatin to ensure complete Bcr-Abl inhibition at this time point. We tested different imatinib concentrations in BaF3BA and found complete Bcr-Abl inhibition not before 3 $\mu\text{mol/L}$ (data not shown). We also studied imatinib preincubation up to 24 h and found no differences in cisplatin sensitivity (data not shown). Nutlin (Sigma) was used at 10 $\mu\text{mol/L}$. ABT737 (Research Chemicals) was used at 1 $\mu\text{mol/L}$. KU-55933 and LY294002 were used at 10 and 25 $\mu\text{mol/L}$, respectively (Merck).

Protein and mRNA expression. For separation of cellular lysates, we used a cytoplasmic and nuclear isolation kit (Pierce). For total lysates, the cellular pellet was resuspended in Laemmli, boiled, and sonicated. Western blot was performed using the following antibodies: anti-p21,

anti-Bcl-x_L, and anti-Abl (1:1,000) from Pharmingen; anti-p53, anti-apoptosis-inducing factor (AIF), and anti-(Ser²¹⁶)Cdc25C (1:250) from Santa Cruz Biotechnology; anti-Mdm2 (clone 2A10, 1:10,000); anti-(Ser¹⁵)p53, anti-ATM, anti-(Ser¹⁹⁸¹)ATM, anti-BAX, anti-PUMA, anti-(Tyr¹⁵)Cdc2, anti-phosphotyrosine, and anti-histone H3 (1:1,000) from Cell Signaling; anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000) from Biodesign; and anti- β -actin (1:5,000) from Sigma.

Total RNA was extracted and cDNA was prepared according to standard protocols. Expression analysis was done using equal amounts of cDNA and the following primer pairs: p21, 5'-ATCCTGGTCTGGACTGTCTACCCTTA-3' (sense) and 5'-GTGAGACGCTTACAATCTGAGTGGAG-3' (antisense); Mdm2, 5'-TGCAAGCACCTCACAGATGC-3' (sense) and 5'-ACA-CAATGTGCTGCTGCTTC-3' (antisense); p53, 5'-GCTCCTGCAGTCTGG-GACA-3' (sense) and 5'-ACTGCACAGGGCAGTCTTC-3' (antisense). Other primers were from SABiosciences. The relative amount of synthesized cDNA was determined through PCR (LightCycler, Roche).

Colony assay. Following pulse treatment with/without imatinib and cisplatin, cells were washed, suspended in drug-free semisolid medium, and plated at 5,000 per well (CD34⁺) or 100 per well (BaF3). A liquid overlay was added to the wells containing 200 ng/mL G-CSF and 10 ng/mL IL-3 (CML) or 2 ng/mL murine IL-3 (BaF3). After 14 d (CML) or 10 d (BaF3), colony-forming units (CFU; >50 cells) were evaluated.

Apoptosis. Apoptosis was assessed by FITC-conjugated or Alexa Fluor 568-conjugated Annexin V (Pharmingen) as described (32).

Carboxyfluorescein diacetate succinimidyl ester staining. Cells were stained with 1 $\mu\text{mol/L}$ carboxyfluorescein diacetate succinimidyl ester

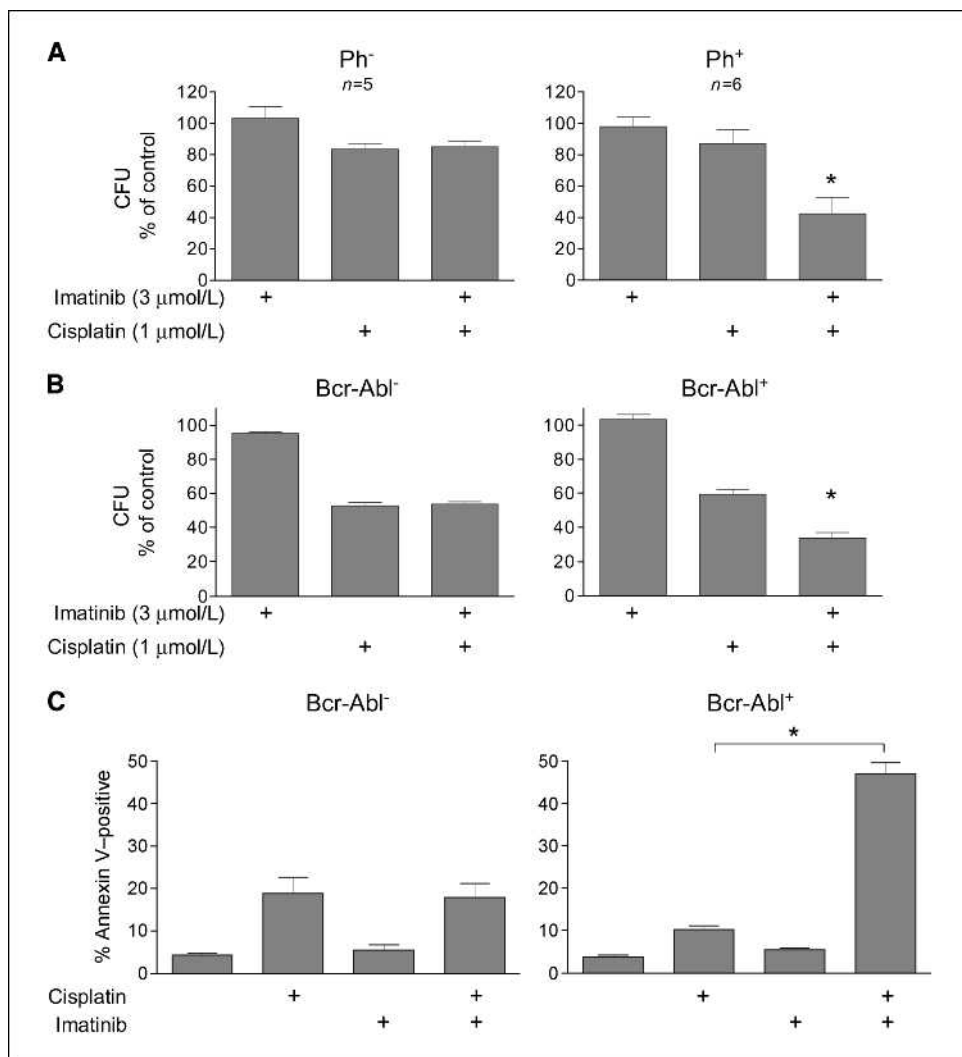


Figure 1. Imatinib sensitizes Bcr-Abl⁺ cells to cisplatin. **A**, granulocyte macrophage CFUs from CD34⁺ cells from five Ph⁻ donors and six CML patients after short-term incubation with/without imatinib (2 h before cisplatin) and 1 $\mu\text{mol/L}$ cisplatin (16 h). Following treatment, cells were plated in drug-free semisolid medium. CFUs were evaluated after 14 d. **B**, long-term survival of Bcr-Abl⁺ and Bcr-Abl⁻ BaF3 cells pulse-treated with/without imatinib (2 h before cisplatin) and 1 $\mu\text{mol/L}$ cisplatin (16 h). After treatment, cells were plated in drug-free semisolid medium. CFUs were evaluated after 10 d. **C**, induction of cell death in Bcr-Abl⁺ and Bcr-Abl⁻ BaF3 cells on imatinib and cisplatin. Cells were pretreated with/without imatinib 2 h before cisplatin (5 $\mu\text{mol/L}$). After 16 h, cells were stained with Annexin V-FITC. Columns, mean of 12 (Bcr-Abl⁺) and 6 (Bcr-Abl⁻) experiments; bars, SE. *, $P < 0.05$.

(CFSE; Molecular Probes) for 30 min. A homogenous subset of CFSE⁺ cells was sorted using a narrow gate.

Cell cycle analyses. Cells were fixed in 70% ethanol, stained with propidium iodide (PI), and analyzed by fluorescence-activated cell sorting (FACS).

Mitochondrial membrane integrity. Cells were incubated with 50 nmol/L tetramethylrhodamine methyl ester (TMRM) or 1.6 μ mol/L calcein AM (Molecular Probes) for 20 min and analyzed by FACS.

Short hairpin RNA experiments. For p53 silencing, we used SureSilencing short hairpin RNA (shRNA) plasmid with green fluorescent protein (GFP; SABiosciences). The following sequences were used: p53, AGAG-TATTTCCACCCTCAAGAT; control, GGAATCTCATTCGATGATAC. Twenty-four hours after electroporation, GFP⁺ cells were sorted and treated as indicated. To evaluate the efficacy of p53 silencing, cells were fixed (An-Der-Grub) and stained with FITC-conjugated anti-p53 (Santa Cruz Biotechnology) and DyLight 649-conjugated anti-FITC (Dianova).

Immunofluorescence. Cells were incubated with MitoTracker CMXRos (Molecular Probes) for 45 min and then fixed in methanol, washed, and blocked in 1% bovine serum albumin/PBS. Antibodies used were anti-AIF and FITC-conjugated anti-p53 (1:100; Santa Cruz Biotechnology), DyLight 648-conjugated anti-FITC (1:100; Dianova), and FITC-conjugated goat anti-mouse (1:20; Dianova). DNA was counterstained with 1 μ mol/L PicoGreen or TO-PRO (1:2,000; Molecular Probes). Samples were examined by confocal microscopy.

Statistics. Data are expressed as SE. Changes in paired samples were analyzed using two-sided paired *t* test. Data points marked with an asterisk were found to be significantly different from controls (*P* < 0.05).

Results

Imatinib enhances sensitivity to cisplatin selectively in Bcr-Abl⁺ cells. We examined effects of cisplatin and imatinib on colony formation of Ph⁺ CML and normal myeloid progenitors. Pulse treatment with imatinib for 18 hours had no significant effect on colony formation of Ph⁺ (range, 89.4–120.9% of control) or Ph⁻ (range, 87.5–128.1% of control) cells (Fig. 1A). Pulse treatment with low-dose cisplatin for 16 hours caused a 12.8 \pm 5.1% or a 16.5 \pm 2.84% decrease in number of colonies developed from Ph⁺ or Ph⁻ progenitors, respectively. Imatinib had no further effect on normal progenitors. In contrast, pulse treatment of Ph⁺ cells with imatinib and cisplatin produced a significant decrease of 57.8 \pm 5.9% in the number of colonies (Fig. 1A).

To address the mechanisms behind this sensitizing effect of imatinib to cisplatin in Ph⁺ CML, we used a cell model based on Bcr-Abl⁻ and Bcr-Abl⁺ BaF3. IL-3 completely prevented imatinib-induced apoptosis in Bcr-Abl⁺ BaF3 cells, allowing us to study effects of imatinib on DNA damage response in c-Abl⁻/Bcr-Abl⁻ cells and c-Abl⁺/Bcr-Abl⁺ cells within the same cell type (32). Pretreatment of Bcr-Abl⁺ BaF3 with imatinib had a comparable effect on clonogenic survival following pulse treatment with low-dose cisplatin as observed in primary CML cells. Again, imatinib had no further effect on cisplatin sensitivity in Bcr-Abl⁻ cells (Fig. 1B).

Active Bcr-Abl protected BaF3 cells from cisplatin-induced cell death (10.2 \pm 0.9% versus 18.8 \pm 3.8% in Bcr-Abl⁻ counterparts). Pretreatment with imatinib not only abolished the protective effect of Bcr-Abl but also led to a pronounced increase in cisplatin-induced apoptosis. This was dependent on Bcr-Abl, as pretreatment of Bcr-Abl⁻ controls with imatinib had no further effect on cisplatin-induced cell death (Fig. 1C).

Importantly, the cisplatin sensitivity of imatinib-treated Bcr-Abl⁺ BaF3 was in the same range as observed for cells derived from testicular germ cell tumors (TGCT), which are hypersensitive to cisplatin (Supplementary Fig. S1; ref. 33).

Hence, imatinib renders Bcr-Abl⁺ cells exquisitely sensitive to cisplatin.

Imatinib shifts cisplatin-mediated DNA damage response from cell cycle arrest to mitochondrial cell death. Bcr-Abl⁺ cells responded to cisplatin with a G₂-M arrest. However, preincubation of these cells with imatinib led to a decreased percentage of cells in G₂-M on cisplatin. This effect was most pronounced at 16 and 24 hours (Fig. 2A, top). To evaluate possible molecular mechanisms of this imatinib-mediated loss of G₂-M arrest, we investigated phosphorylation of Cdc25C and Cdc2 at these time points. The inhibitory phosphorylation of Cdc25C phosphatase was reduced and Cdc2 was activated by dephosphorylation of the inhibitory Tyr¹⁵ residue in cells pretreated with imatinib (Fig. 2A, bottom).

Tracking of cell divisions by CFSE showed that imatinib-mediated inhibition of Bcr-Abl led to rapid onset of mitochondrial outer membrane permeabilization (MOMP) already one cell division after adding cisplatin. This is in contrast to cells treated with cisplatin alone. These cells accomplished the first division and went into G₂-M arrest in the second division without significant induction of MOMP. In the absence of imatinib, a small percentage of cisplatin-treated cells even started the third division without induction of MOMP. The presence of imatinib alone led to a minor retardation of cell proliferation when compared with nontreated controls and did not induce MOMP (Fig. 2B).

Hence, imatinib leads to rapid cell death after one single cell division instead of arresting in G₂-M on cisplatin in Bcr-Abl⁺ cells.

MOMP commonly results in cytochrome *c* release and apoptosome-dependent caspase activation. Indeed, we observed poly(ADP-ribose) polymerase cleavage starting 16 hours after addition of cisplatin (data not shown). However, the cell death observed in Bcr-Abl⁺ cells treated with imatinib and cisplatin was independent of caspases, as the pan-caspase inhibitor zVAD-fmk had no effect (Fig. 2C). In contrast, zVAD-fmk blocked cisplatin-induced apoptosis in TGCT cells (Fig. 2C). This finding not only shows that zVAD-fmk was active but also suggests the existence of different mechanisms in different cell types leading to comparable sensitivities.

Mitochondrial cell death can also proceed independently of caspases via release of factors tethered to the inner mitochondrial membrane such as AIF, which then serve as a cell death trigger in the nucleus (34). Indeed, we observed AIF translocation from mitochondria to nucleus and release of calcein AM (which is trapped in the matrix of healthy mitochondria and released following disruption of the inner mitochondrial membrane) on cisplatin in imatinib-treated Bcr-Abl⁺ cells (Fig. 2D).

These results indicate that in Bcr-Abl⁺ cells treated with imatinib and cisplatin, AIF is released from mitochondria following disruption of mitochondrial membranes.

Cisplatin-induced cell death in imatinib-treated Bcr-Abl⁺ cells depends on p53. BV173 cells showed a higher percentage of apoptosis on cisplatin when pretreated with imatinib, whereas imatinib had no effect on K562 cells (Fig. 3A). Of note, BV173 cells harbor wt-p53, whereas K562 cells do not express functional p53. To examine the role of p53 for the observed hypersensitivity in imatinib-treated Bcr-Abl⁺ cells, we evaluated whether forced p53 accumulation by nutlin may affect cisplatin sensitivity. Indeed, nutlin enhanced cisplatin-induced p53 accumulation and further augmented the sensitivity to cisplatin in imatinib-treated Bcr-Abl⁺ cells (Fig. 3B). To assess the effect of p53 silencing, we transfected BaF3 with p53shRNA coexpressing GFP. Analysis of GFP-positive cells confirmed that *p53* mRNA expression was

reduced by $64.9 \pm 8.7\%$ (Fig. 3C, *left*). In addition, p53 protein induction on cisplatin was attenuated by $\sim 50\%$ in p53shRNA-transfected cells compared with control transfectants (Fig. 3C, *middle*). Notably, shRNA-mediated p53 silencing significantly reduced induction of apoptosis in cells treated with imatinib and cisplatin relative to controls (Fig. 3C, *right*).

These data show that imatinib-mediated sensitization of Bcr-Abl⁺ cells depends at least partially on p53.

Imatinib impairs ATM activation on cisplatin in Bcr-Abl⁺ cells and diminishes DNA damage-induced transcriptional p53 activity. ATM is a key activator of p53 on DNA damage (35). We investigated whether altered ATM activation might contribute to hypersensitivity in imatinib-treated Bcr-Abl⁺ cells. Imatinib reduced ATM autophosphorylation on cisplatin selectively in Bcr-Abl⁺ cells (Fig. 4A). This was mirrored by the observation that in Bcr-Abl⁺ BaF3 (Fig. 4A, *left*) and primary CML cells (Fig. 4A, *right*), but not in Bcr-Abl⁻ cells, imatinib impeded cisplatin-induced phosphorylation of p53 on Ser¹⁵ (Ser¹⁸ in mouse p53, subsequently called Ser¹⁵), which is directly phosphorylated by ATM (36, 37). Importantly, imatinib had only a minor effect on p53 protein stabilization on cisplatin in Bcr-Abl⁺ BaF3 and in CML cells (Fig. 4A).

In BaF3 cells harboring active Bcr-Abl, we found an enhanced induction of *Mdm2* and *p21* mRNA and protein on cisplatin as compared with the Bcr-Abl⁻ counterparts. However, imatinib-mediated inhibition of Bcr-Abl led to a significant reduction of *p21* and *Mdm2* mRNA and protein on cisplatin in Bcr-Abl⁺ cells, whereas no effect on p53 transcriptional activity was observed in Bcr-Abl⁻ cells (Fig. 4B).

We also investigated the regulation of the proapoptotic p53 targets *BAX*, *PUMA*, and *NOXA*. We found a constitutively high expression of these genes in nonstressed Bcr-Abl⁺ and Bcr-Abl⁻ cells, which was not further induced by cisplatin. Furthermore, imatinib had no effect on expression of these genes (Fig. 4C).

Therefore, the extensive cell death observed in Bcr-Abl⁺ cells treated with imatinib and cisplatin seems not to be due to a selective p53-dependent induction of proapoptotic genes.

The impaired capability of p53 to transactivate *p21* and *Mdm2* was not simply due to reduced p53 level, as nutlin-mediated p53 accumulation was not capable to restore the transactivation of *Mdm2* and *p21*. Moreover, imatinib completely impaired p53 phosphorylation even on forced nutlin-induced p53 accumulation, indicating that the abolished p53 transactivation is due to inhibition of

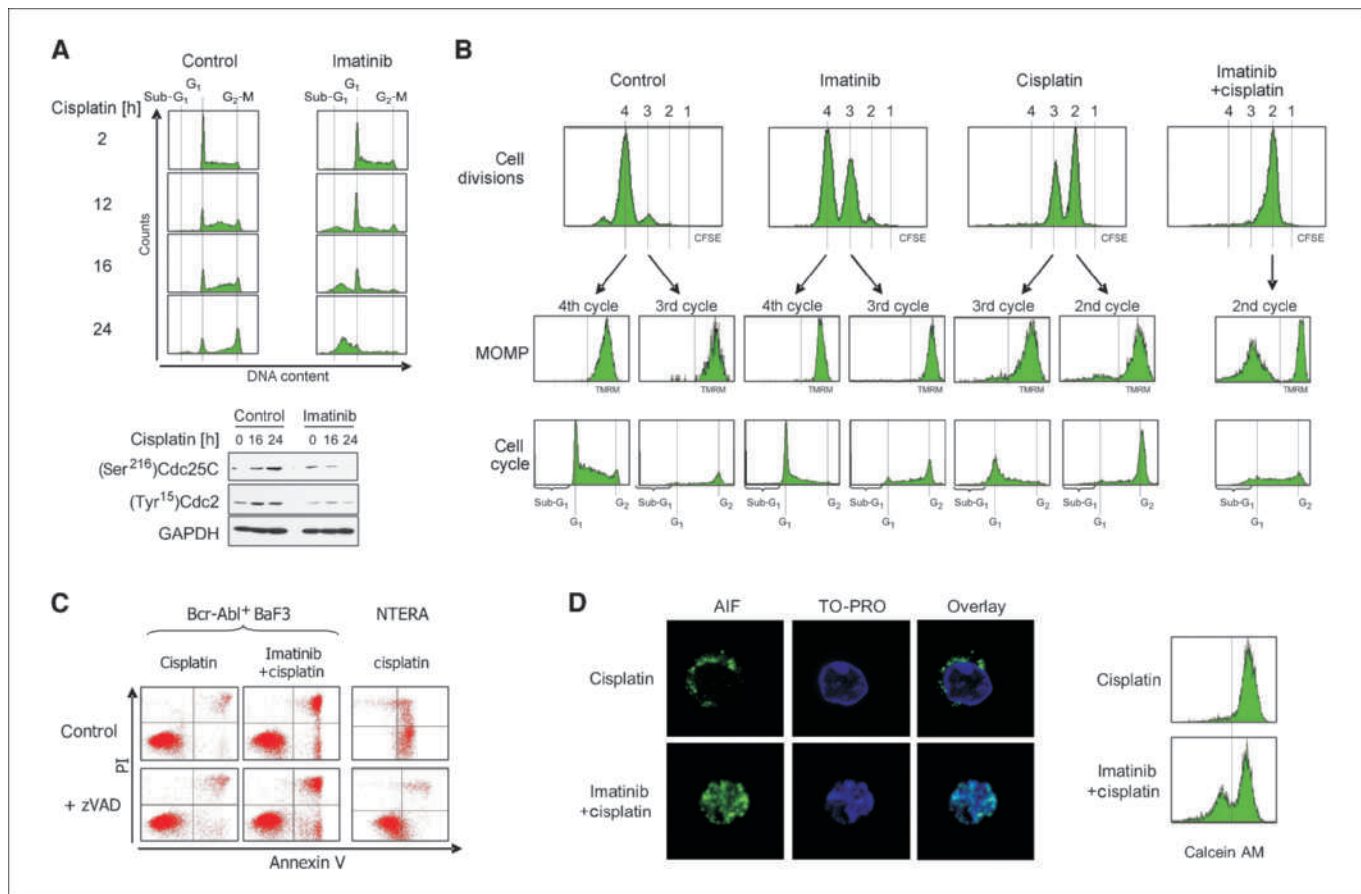


Figure 2. Imatinib abrogates cisplatin-induced G₂-M arrest in Bcr-Abl⁺ BaF3 cells and induces mitochondrial cell death. *A*, imatinib alters cisplatin-induced G₂-M arrest in Bcr-Abl⁺ cells. *Top*, cells were preincubated with/without imatinib, treated with 5 μmol/L cisplatin for indicated times, and stained with PI; *bottom*, cells were incubated with/without imatinib and cisplatin (5 μmol/L). After 16 and 24 h, cells were harvested for Western blot using anti-(Ser²¹⁵)Cdc25C and anti-(Tyr¹⁵)Cdc2. *B*, imatinib alters onset of MOMP and proliferation on cisplatin. BaF3 cells were labeled with CFSE, sorted, and preincubated with/without imatinib and with/without 5 μmol/L cisplatin (24 h). Cells were stained with TMRM or PI, respectively. *C*, cell death on imatinib and cisplatin is caspase independent. One hour before imatinib, BaF3 cells were preincubated with/without zVAD-fmk. Cells were treated with 5 μmol/L cisplatin 2 h after addition of imatinib, harvested after 16 h, and stained with Annexin V-FITC/PI. As controls, we used the TGCT cells NTERA. *D*, cell death is accompanied by AIF translocation and mitochondrial membrane disruption. Cells were preincubated with/without imatinib and treated with 5 μmol/L cisplatin (16 h). Cells were stained with anti-AIF and TO-PRO (blue) or labeled with calcein AM.

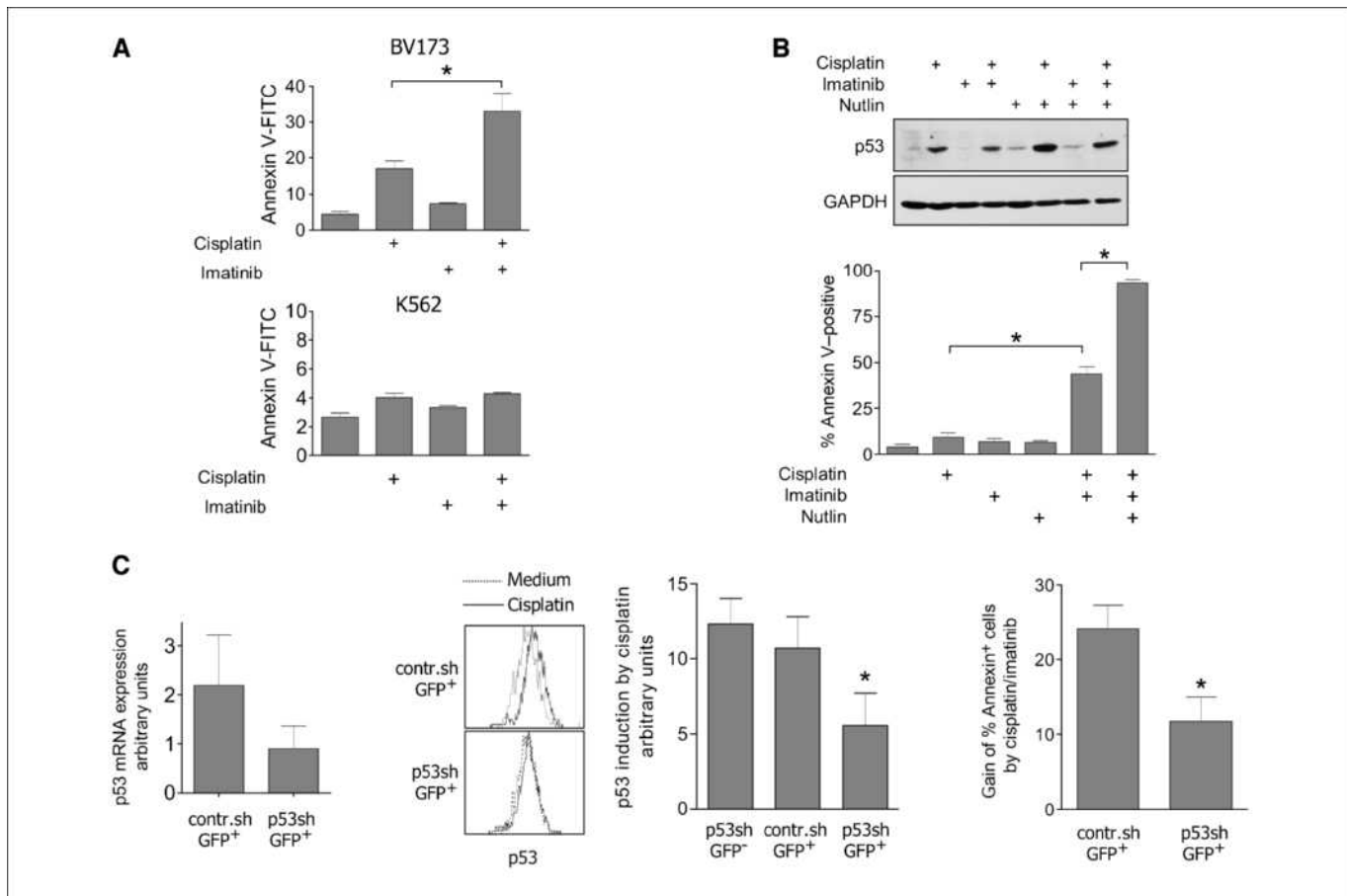


Figure 3. Hypersensitivity depends on functional p53. *A*, imatinib induces hypersensitivity in cells harboring wt-p53. BV173 and K562 cells were treated with/without imatinib and with/without 5 μ M cisplatin (16 h). Cells were stained with Annexin V-FITC. *Columns*, mean of four experiments; *bars*, SE. *B*, nutlin further enhances hypersensitivity. BaF3 cells were preincubated with/without nutlin 1 h before imatinib (2 h before cisplatin) and treated with 5 μ M cisplatin (16 h). Cells were analyzed for p53 expression (*top*) and apoptosis by Annexin V-FITC staining (*bottom*). *Columns*, mean of three experiments; *bars*, SE. *C*, p53 silencing reduces hypersensitivity. Bcr-Abl⁺ BaF3 cells were transfected with p53shRNA/GFP or control-shRNA/GFP. GFP⁺ cells were sorted and treated with imatinib and cisplatin and analyzed for p53 mRNA (*left*), p53 protein accumulation (*middle*), and induction of apoptosis (*right*). *Columns*, means of four experiments; *bars*, SE. *, $P < 0.05$.

ATM-dependent p53 phosphorylation (Supplementary Fig. S2A). To ensure that the impaired p53 transcriptional activity was due to Bcr-Abl inhibition, we performed experiments with imatinib-resistant Bcr-Abl mutants (Bcr-AblT315I). Imatinib had no effect on cisplatin-dependent induction of p21 and Mdm2 in these cells (Supplementary Fig. S2B).

Imatinib leads to predominant localization of p53 in the cytoplasm in Bcr-Abl⁺ cells after cisplatin. p53 regulates apoptosis as a transcription factor of proapoptotic targets (38) but also acts as a proapoptotic protein in the cytoplasm (39). We examined subcellular distribution of cisplatin-induced p53 in BaF3 and CD34⁺ CML cells incubated with or without imatinib. Equal accumulation of p53 in the nucleus and in the cytoplasm was detected in Bcr-Abl⁺ BaF3 and CML cells after cisplatin alone. In contrast, preincubation with imatinib led to a predominant accumulation of p53 in the cytoplasm (Fig. 5A; Supplementary Fig. S3). Interestingly, a considerable fraction of p53 colocalized to mitochondria (Fig. 5A). To confirm this, BaF3 lysates were biochemically fractionated into cytoplasmic and nuclear extracts. In agreement with our immunofluorescence data, p53 accumulation was abundant in the cytoplasm but almost lost in the nucleus in imatinib-treated cells, whereas in the absence of imatinib p53 accumulation was detected in both fractions (Fig. 5B).

Hypersensitivity requires inhibition of ATM-p53 and Bcl-x_L.

The finding that imatinib blocked ATM activation in Bcr-Abl⁺ cells prompted us to ask whether direct ATM inhibition by the phosphoinositide 3-kinase inhibitor LY294002 or by the specific ATM inhibitor KU-55933 (40) could mimic the effect of imatinib on cisplatin sensitivity. Both inhibitors blocked ATM autophosphorylation, p53 Ser¹⁵ phosphorylation, and Mdm2 induction. However, these compounds failed to induce cisplatin hypersensitivity, suggesting that ATM inhibition alone is not sufficient for inducing hypersensitivity (Fig. 6A).

The findings that the hypersensitive phenotype depends on p53, p53 transactivation is impaired, and p53 predominantly accumulates in the cytoplasm implicate a role of p53 as a proapoptotic protein in the cytoplasm in imatinib-treated Bcr-Abl⁺ cells. In the cytoplasm, Bcl-2/Bcl-x_L binds p53 and thereby prevents p53-mediated BAX activation (39). In addition, cells with constitutively active tyrosine kinases have high Bcl-x_L levels (41). We found that Bcl-x_L was significantly reduced in Bcr-Abl⁺ cells on cisplatin and imatinib (Fig. 6B), whereas Bcl-2 remained unchanged (data not shown). Exogenous Bcl-x_L attenuated induction of MOMP and AIF translocation and consequently reduced induction of apoptosis in Bcr-Abl⁺ cells treated with imatinib and cisplatin (Fig. 6C). However, inhibition of the Bcl-x_L-BHX protein-protein interaction

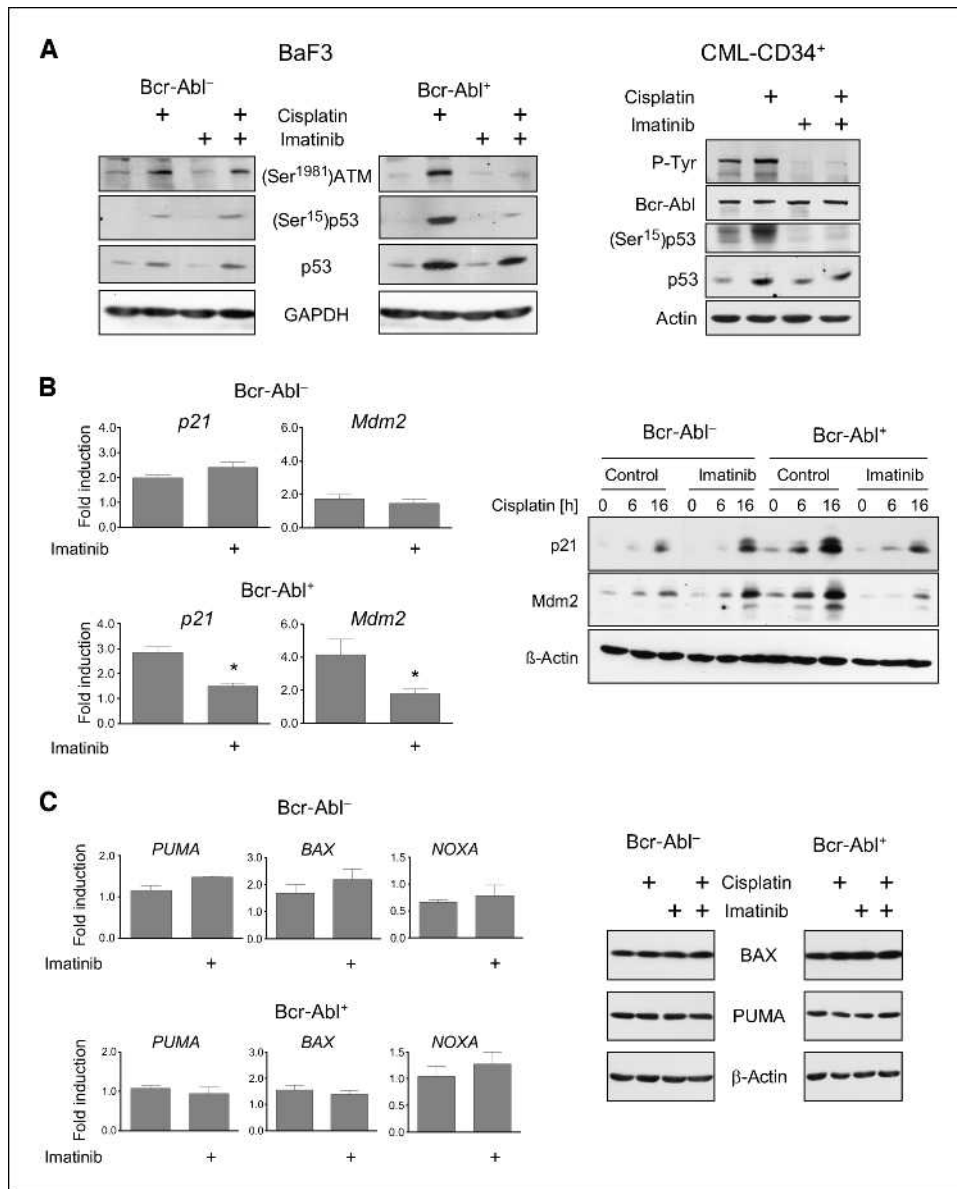


Figure 4. Imatinib impairs cisplatin-induced ATM-p53 activation in Bcr-Abl⁺ cells. **A**, Bcr-Abl⁻ and Bcr-Abl⁺ BaF3 or CML cells were cultivated in the presence/absence of imatinib and 5 μmol/L cisplatin (6 h). Western blot was performed using anti-(Ser¹⁹⁸¹)ATM, anti-(Ser¹⁵)p53, anti-p53, anti-GAPDH, and anti-β-actin. **B**, imatinib reduces cisplatin-induced p21 and Mdm2 in Bcr-Abl⁺ cells. Cells were cultivated in the presence/absence of imatinib and 5 μmol/L cisplatin and harvested after 6 h for RNA and protein extraction and after 16 h for protein extraction. *Left*, RNA was quantified by PCR. *Columns*, mean fold induction on cisplatin from nine (Bcr-Abl⁺) and six (Bcr-Abl⁻) experiments. *Right*, immunoblotting was performed using anti-p21, anti-Mdm2, and anti-β-actin antibodies. **C**, PUMA, BAX, and NOXA are highly expressed and not altered by cisplatin or imatinib. Cells were cultivated in the presence/absence of imatinib and 5 μmol/L cisplatin (6 h), harvested, and lysed. *Left*, RNA levels were quantified by PCR. *Columns*, mean of three experiments (*PUMA* and *NOXA*) and six independent experiments (*BAX*); bars, SE. *Right*, for Western blot, anti-BAX and anti-PUMA antibodies were used.

by ABT737 was not sufficient to mimic the effect of imatinib on sensitization of Bcr-Abl⁺ cells to cisplatin. Interestingly, simultaneous inhibition of both ATM and Bcl-x_L had an additive effect on induction of cell death on cisplatin, reaching almost the sensitizing effect of imatinib (Fig. 6D).

These findings suggest that both downregulation of Bcl-x_L and inhibition of ATM-p53 are necessary for imatinib-induced hypersensitivity in Bcr-Abl⁺ cells.

Discussion

Bcr-Abl activates multiple signaling pathways, leading to enhanced proliferation (42), reduced growth factor dependence (43), G₂-M cell cycle delay (8, 16), enhanced DNA repair (9, 14, 15), and protection against apoptosis stimuli such as cisplatin (9). Here, we show that imatinib-mediated inhibition of Bcr-Abl induces hypersensitivity to cisplatin in Bcr-Abl⁺ cell lines and in primary CML cells. The sensitivity of cells harboring inhibited Bcr-Abl

highly exceeds that of normal Bcr-Abl⁻ cells. This phenotype is caused by impairment of DNA damage response pathways, leading to altered p53 activation accompanied by downregulation of Bcl-x_L. Therefore, imatinib-mediated inhibition of Bcr-Abl not only reverses the malignant phenotype but also represents a strategy to induce "leukemia-specific lethality" after exposure to DNA damaging drugs.

The effect of Bcr-Abl on p53 has been extensively studied. It has been shown that Bcr-Abl elevates constitutive and DNA damage-induced p53 (17, 44). In agreement with these studies, we show that DNA damage-induced p53 accumulation in Bcr-Abl⁺ cells is reduced on imatinib treatment. More importantly, cells harboring imatinib-inactivated Bcr-Abl showed reduced ATM activation on cisplatin, leading to impaired p53 phosphorylation on Ser¹⁵. This depends on Bcr-Abl and inhibition of its kinase activity, as imatinib had no effect on cisplatin-induced p53 phosphorylation in Bcr-Abl⁻ cells or in cells expressing an imatinib-resistant Bcr-Abl. The impaired activation of ATM-p53 in Bcr-Abl⁺ cells pretreated with

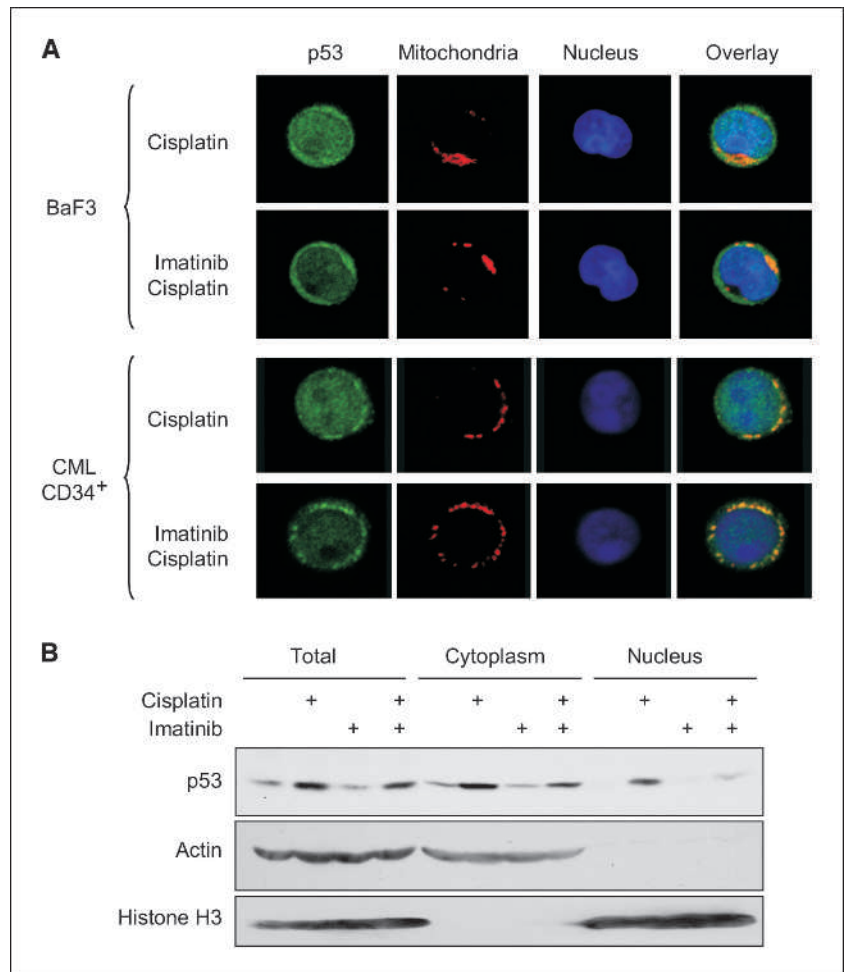
imatinib significantly alters DNA damage response, which is shifted from predominant cell cycle arrest to predominant cell death. We have identified two mechanisms that seem to be responsible for the preferential cell death response in imatinib-treated leukemic cells: (a) accumulation of transcriptionally inactive p53 predominantly in the cytoplasm together with decreased Bcl-x_L and (b) reduced activation of the Cdc25C/Cdc2-dependent checkpoint.

Modification of p53 on Ser¹⁵ by ATM/ATR triggers a cascade of damage-induced post-translational events, leading to p53 stabilization and enhanced transcriptional activity (45, 46). This phosphorylation site is also critical for p53 localization. Loss of DNA damage-induced Ser¹⁵ phosphorylation results in enhanced nuclear export (47). A central role for p53 localization has also been shown for Mdm2: low-level Mdm2 leads to p53 monoubiquitination, allowing its nuclear export (48). Both events may participate in the preferential localization of p53 in the cytoplasm on cisplatin in imatinib-treated Bcr-Abl⁺ cells. The absence of nuclear (Ser¹⁵) p53 is most likely the cause for the diminished p53-dependent transcriptional response: we observed a reduced induction of p21 and Mdm2 in imatinib-treated Bcr-Abl⁺ cells. On the other hand, imatinib-mediated inhibition of Bcr-Abl did not affect BAX, PUMA, and NOXA expression. This is consistent with the finding that these proapoptotic p53 targets are also not induced effectively by cisplatin in Bcr-Abl⁺ and Bcr-Abl⁻ BaF3 cells. The constitutively high levels of BAX and PUMA are likely to contribute to the resulting

imbalance of p53-dependent targets after cisplatin in imatinib-treated Bcr-Abl⁺ cells.

The fact that p53 silencing reduced sensitivity to cisplatin in imatinib-treated Bcr-Abl⁺ cells indicates that despite its impaired transactivation capability, p53 still retains its proapoptotic activity. This proapoptotic function of p53 relies on its presence in the cytoplasm, where p53 is sequestered by Bcl-2/Bcl-x_L and can be liberated by BH3-only proteins to activate BAX and induce MOMP (39, 49). Alternatively, p53 has been proposed to act more directly at the mitochondria by promoting BAK/BAX oligomerization (50, 51). Either way, the total levels of p53 in the cytoplasm relative to proapoptotic BAX and antiapoptotic Bcl-x_L/Bcl-2 seem to be pivotal for the biological outcome. We found that treatment of Bcr-Abl⁺ cells with cisplatin led to p53 accumulation in the cytoplasm and predominant localization at the mitochondria, whereas BAX was highly expressed and not further induced by cisplatin. On the other hand, Bcl-x_L expression was diminished in cells treated with imatinib and cisplatin. We propose that the altered Bcl-x_L/p53-BAX ratio allows p53 to activate BAX at the mitochondria, leading to disruption of mitochondrial membranes and AIF translocation. This is supported by the ability of exogenous Bcl-x_L to rescue imatinib-treated Bcr-Abl⁺ cells from cisplatin-induced MOMP. This may also explain our finding that inhibition of ATM was not sufficient to mimic the effect of imatinib on cisplatin sensitivity in Bcr-Abl⁺ cells. ATM inhibition contributes to the

Figure 5. p53 predominantly accumulates in the cytoplasm following cisplatin in imatinib-treated Bcr-Abl⁺ cells. Cells were preincubated with/without imatinib and treated with 5 μmol/L cisplatin (6 h). **A**, following treatment, cells were incubated with MitoTracker CMXRos (red) and stained for p53 (green) and nuclei (PicoGreen; blue). **B**, following treatment, cells were harvested and cytoplasmic and nuclear extracts were prepared for Western blot using anti-p53, anti-β-actin, and anti-histone H3 antibodies.



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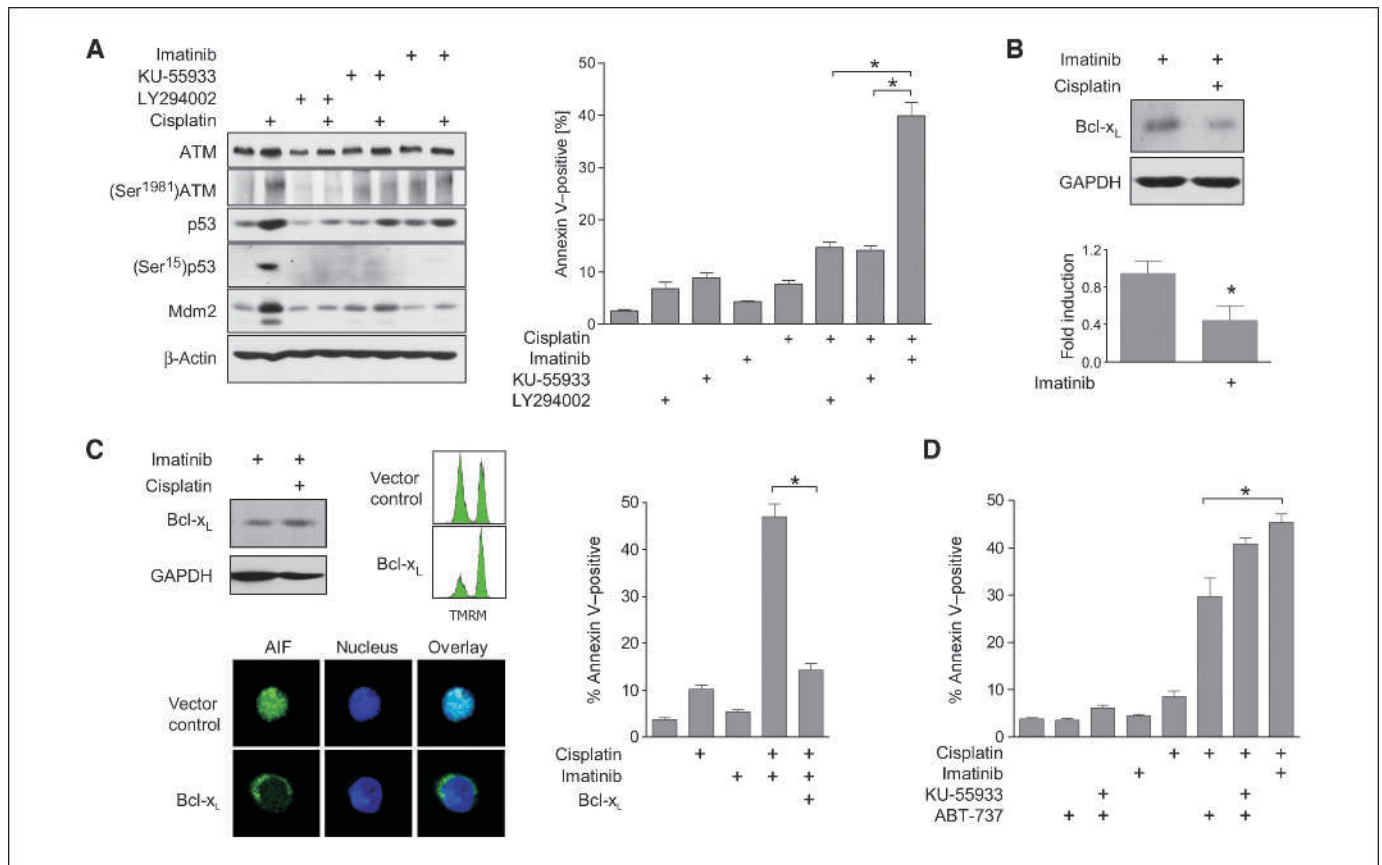


Figure 6. Hypersensitivity requires inhibition of ATM-p53 and Bcl-x_L. **A**, ATM inhibitors were sufficient to reduce p53 phosphorylation and Mdm2 induction but did not induce hypersensitivity. Bcr-Abl⁺ BaF3 cells were preincubated with/without KU-55933 or LY294002 2 h before cisplatin (5 μmol/L). Cells were harvested after 6 h for Western blot using anti-(Ser¹⁹⁸¹)ATM, anti-ATM, anti-p53, anti-(Ser¹⁵)p53, anti-Mdm2, and anti-β-actin (*left*) and after 16 h for Annexin V-FITC staining (*right*). **B**, Bcl-x_L is downregulated in Bcr-Abl⁺ cells on imatinib and cisplatin. Cells were pretreated with imatinib and incubated with/without 5 μmol/L cisplatin (6 h). Western blot was performed with anti-Bcl-x_L and anti-GAPDH. Relative changes were measured by densitometry. *Columns*, mean of three experiments; *bars*, SE. **C**, MOMP, AIF translocation, and induction of apoptosis in cells treated with imatinib and cisplatin are prevented by exogenous Bcl-x_L. *Top left*, cells with exogenous Bcl-x_L were pretreated with imatinib, incubated with/without 5 μmol/L cisplatin (6 h), and harvested for Western blot using anti-Bcl-x_L and anti-GAPDH. Bcr-Abl⁺ cells transfected with control vector or Bcl-x_L were treated with imatinib and cisplatin for 16 h and analyzed for induction of MOMP (by TMRM labeling; *top right*), AIF translocation [by staining with anti-AIF, FITC-goat anti-mouse (*green*), and TO-PRO-3 (*blue*); *bottom*], and apoptosis (by Annexin V; *right*). **D**, Inhibition of Bcl-x_L is not sufficient to mimic the effect of imatinib but has additive effects in combination with ATM inhibition. Bcr-Abl⁺ cells were pretreated with ABT737, imatinib, or ABT737 and KU-55933 2 h before cisplatin (5 μmol/L). After 16 h, cells were stained with Annexin V-FITC. *, *P* < 0.05.

impaired G₂-M arrest via impaired Cdc25C/Cdc2 pathway and may also force p53 accumulation in the cytoplasm. However, in contrast to imatinib, neither LY294002 nor KU-55933 altered Bcl-x_L expression (data not shown). On the other hand, inhibition of the Bcl-x_L BH3 protein-protein interaction by ABT737 also failed to completely mimic the sensitizing effect of imatinib. Our data therefore indicate that the hypersensitive phenotype in imatinib-treated Bcr-Abl⁺ cells requires inactivation of ATM-p53, accumulation of p53 in the cytoplasm, and unleashing of the apoptotic capacity of p53 via Bcl-x_L downregulation.

It remains to be elucidated if Bcr-Abl acts on ATM-p53 by directly interacting with ATM or by yet unknown mechanisms. Conflicting data have been reported on the interaction of active Bcr-Abl with ATM and ATR (9, 31). In addition, the efficacy of nuclear localization of imatinib-inhibited Bcr-Abl in the absence of export inhibitors and presence of DNA damaging agents is unclear and requires further investigation.

Imatinib has revolutionized therapy for Bcr-Abl⁺ leukemias. However, neither CML nor Ph⁺ ALL can be cured by imatinib alone. Our data indicate that imatinib not only reverses the Bcr-Abl-mediated abnormal growth but also causes a leukemia-specific

phenotype, allowing the selective targeting of Bcr-Abl⁺ cells by DNA damaging agents such as cisplatin. Therefore, these observations may contribute to the development of rational strategies combining DNA damaging agents and imatinib to eliminate residual Ph⁺ cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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