Clinical Cancer Research

Dasatinib Plus Nutlin-3 Shows Synergistic Antileukemic Activity in Both p53$^{wild\text{-}type}$ and p53^{mutated} B Chronic Lymphocytic Leukemias by Inhibiting the Akt Pathway

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Abstract

Purpose: To analyze the effect of the combination of Dasatinib, a multikinase inhibitor, plus Nutlin-3, a nongenotoxic activator of the p53 pathway, in primary B chronic lymphocytic leukemia (B-CLL) patient samples and B leukemic cell line models.

Experimental Design: The induction of cytotoxicity was evaluated in both primary B-CLL cell samples ($n = 20$) and in p53$^{wild\text{-}type}$ (EHEB, JVM-2) and p53$^{deleted/mutated}$ (MEC-2, BJAB) B leukemic cell lines. The role of Akt in modulating leukemic cell survival/apoptosis in response to Dasatinib or Dasatinib + Nutlin-3 was documented by functional experiments carried out using specific pharmacological inhibitors and by overexpression of membrane-targeted constitutively active form of Akt.

Results: The combination of Dasatinib + Nutlin-3 exhibited a synergistic cytotoxicity in the majority (19 out of 20) of B-CLL samples, including patients carrying 17p- ($n = 4$), and in both p53$^{wild\text{-}type}$ and p53$^{deleted/mutated}$ B leukemic cell lines. At the molecular level, Dasatinib significantly counteracted the Nutlin-3-mediated induction of the p53 transcriptional targets *MDM2* and *p21* observed in p53$^{wild\text{-}type}$ leukemic cells. Conversely, Nutlin-3 did not interfere with the ability of Dasatinib to decrease the phosphorylation levels of ERK1/2, p38/MAPK, and Akt in both p53$^{wild\text{-}type}$ and p53$^{deleted/mutated}$ B leukemic cell lines. A critical role of Akt downregulation in mediating the antileukemic activity of Dasatinib and Dasatinib + Nutlin-3 was demonstrated in experiments carried out by specifically modulating the Akt pathway.

Conclusions: These findings suggest that Dasatinib + Nutlin-3 might represent an innovative therapeutic combination for both p53$^{wild\text{-}type}$ and p53$^{deleted/mutated}$ B-CLL. *Clin Cancer Res; 17(4); 762–70. ©2010 AACR.*

Introduction

The prolonged survival of B chronic lymphocytic leukemia (B-CLL) cells has been linked to deregulated expression and/or activity of related nonreceptor tyrosine kinases (1) and members of the Src family kinases (SFK; ref. 2), which are basic components of the cell signaling machinery and are involved in pathways regulating growth, survival, motility, and adhesion. Additional studies have underlined a primary role of the Akt pathway in promoting B-CLL survival (3). In this context, the oral, small-molecule multikinase inhibitor Dasatinib has demonstrated toxicity on B-CLL cells at concentrations of 1–10 μM (4, 5). In contrast to chlorambucil- and fludarabine-resistance of B-CLL, which correlated with lack of integrity of the p53 pathway (4, 6), the cytotoxic activity of Dasatinib does not seem to be mediated by p53. It is particularly noteworthy that the few studies performed to evaluate the *in vitro* activity of Dasatinib on primary B-CLL cells have shown that Dasatinib is potentially active also in B-CLL samples obtained from patients with a worse prognosis (4, 5).

Because it is still uncertain whether Dasatinib alone can induce B-CLL cytotoxicity at clinically relevant concentrations (4), the combination of Dasatinib with innovative therapeutic compounds will likely represent an important therapeutic strategy. In this respect, small molecule inhibitors of the p53–MDM2 interaction, the

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Translational Relevance

The authors have evaluated the effect of the combination of Dasatinib + Nutlin-3 in primary B chronic lymphocytic leukemia (B-CLL) patient cells and in a panel of B leukemic cell lines with different p53 status. These experiments demonstrated that the Dasatinib + Nutlin-3 combination induced synergistic cytotoxicity in both $p53^{wild\text{-}type}$ and $p53^{deleted/mutated}$ leukemic cells, and allowed to dissect the primary role of Akt downregulation in mediating the response to either Dasatinib alone or Dasatinib + Nutlin-3. Given the relatively low toxicity of these drugs in initial human testing, the results of this study provide insight for the potential clinical evaluation of Dasatinib + Nutlin-3 in B-CLL.

Nutlins, able of rising the levels of p53 protein and, subsequently, of promoting the induction of cell cycle arrest and apoptosis in a variety of tumor cells, have been suggested for innovative therapeutic purposes (7). Interestingly, at variance to solid tumors, mutations, and/or deletions of p53 in hematological malignancies have been detected in less than 20% of patients at diagnosis (8), mostly in patients with 17p monosomy, which is often associated to *TP53* mutation of the second allele (9). Of note, recent studies have demonstrated that Nutlin-3, used alone or in combination with chemotherapeutic drugs, effectively increases the degree of apoptosis in hematological malignancies (10–13), and in particular in B-cell malignancies (6, 14–18).

On these bases, the aim of this study was to investigate the effect of the combination of Dasatinib plus Nutlin-3 in primary B-CLL cells obtained from patients ($n = 20$) characterized by different prognostic markers as well as in B leukemic cell models with different p53 status ($p53^{wild\text{-}type}$ and $p53^{deleted/mutated}$).

Methods

Primary B-CLL patient samples and B leukemic cell lines

For experiments with primary cells, peripheral blood samples were collected in heparin-coated tubes from 20 B-CLL patients (Supplementary Table 1) following informed consent, in accordance with the Declaration of Helsinki and in agreement with institutional guidelines (University-Hospital of Ferrara). The main clinical parameters of the patients were abstracted from clinical records. B-CLL samples were also characterized by CD38 surface expression, interphase FISH, ZAP-70 protein levels, and p53 status. All patients had been without prior therapy at least for 3 weeks before blood collection. Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation with lymphocyte cell separation medium (Cedarlane Laboratories). T lymphocytes, NK lymphocytes, granulocytes, and monocytes were negatively depleted from peripheral blood leucocytes (PBL) with immunomagnetic microbeads (MACS microbeads, Miltenyi Biotech), with a purity >95% of resulting $CD19^+$ B-CLL population, as assessed by flow cytometry. Patient cells were cultured in RPMI containing 10% FBS, L-glutamine and penicillin/streptomycin (Gibco).

The $p53^{wild\text{-}type}$ (EHEB, JVM-2) and $p53^{deleted/mutated}$ (MEC-2, BJAB) B leukemic lines (19, 20) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig). In MEC-2 cells, p53 is mutated with a deletion of 40aa at the N-term, whereas in BJAB p53 mutation is aa substitution His193Arg. EHEB, JVM-2 and BJAB cells were routinely cultured in RPMI-1640 medium (Gibco BRL) whereas MEC-2 cells were cultured in ISCOVE medium (Euroclone), containing 10% FBS, L-glutamine and penicillin/streptomycin (Gibco).

Culture treatments

Dasatinib and Nutlin-3 were purchased from Selleck Chemicals and from Cayman Chemical, respectively. For *in vitro* treatments with Dasatinib and Nutlin-3, used either alone or in combination, cells were seeded at a density of 1×10^6 cells/mL. In selected experiments, before treatment with Dasatinib or Nutlin-3, cells were preincubated (at 37°C for 1 hour) with the following cell-permeable pharmacological inhibitors: LY294002 (inhibitor of PI3-kinase/Akt), PD98059 (inhibitor of the ERK pathway), and SB203580 (inhibitor of the p38/MAPK pathway), all purchased from Calbiochem. The optimal concentrations of pharmacological inhibitors were determined in preliminary dose–response experiments. In other experiments, cells were transfected with pEGFP-C1 or pCMV empty vectors or with a construct expressing a membrane-targeted constitutively active form of Akt (pMyr-Akt). For transfection, cells (1×10^6) were resuspended into 0.1 mL of Nucleofector™ solution of human nucleofector kit V (Amaxa). Two micrograms of plasmid DNA were mixed with the 0.1 mL of cell suspension, transferred into a 2.0 mm electroporation cuvette, and nucleofected using an Amaxa Nucleofector II apparatus, following the manufacturer guidelines. After transfection, cells were immediately transferred into complete medium and cultured at 37°C. Optimal transfection efficiency, estimated in each experiment by scoring the number of GFP-positive cells by flow cytometry analysis, was in the range 35% to 50% in the B-cell lines used in this study.

Assessment of cell viability and apoptosis

At different time points after treatment, cell viability was examined by Trypan blue dye exclusion and MTT (3-(4,5-dimethilthiazol-2yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Roche Diagnostics Corporation) for data confirmation. Induction of apoptosis was quantified by Annexin V-FITC/propidium iodide (PI) staining

(Immunotech) followed by analysis using a FACScan flow cytometer (Becton-Dickinson). To avoid nonspecific fluorescence from dead cells, live cells were gated tightly using forward and side scatter, as described (21).

The caspase activity was measured in cell extracts by using the colorimetric Caspase-3/CPP32 assay (Biovision). To determine Caspase-3/CPP32 protease activity, assays were performed in triplicates in 96-well flat-bottom plates by incubating 100 μg of cell proteins/sample with the specific labeled substrate DEVD-*p*NA according to the manufacturer's instructions. After 1 hour of incubation, the *p*NA light emission was quantified by using a spectrophotometer plate reader at 405 nm and expressed in OD (Optical Density). Increase in OD was linear over time and extract concentration.

Western blot analyses and antibody arrays

For Western blot analyses, cells were lysed as previously described (22). Protein determination was performed by using the BCA Protein Assay (Thermo Scientific). Samples were supplemented with loading buffer (250 mM Tris pH 6.8, 2% SDS, 10% Glycerin, 4% β-mercaptoethanol, 1% bromophenol blue) and boiled for 2 min. Equal amounts of protein for each sample were migrated in SDS-polyacrylamide gels and blotted onto nitrocellulose filters, as previously described (22). The following Abs were used: anti-p53 (DO-1), anti-MDM2 (SMP14), anti-p21 (C-19) were purchased from Santa Cruz Biotechnology; anti-Akt (55) from BD Biosciences Pharmingen; anti-Phospho Thr180/Tyr182 p38MAPK and anti-p38 from Cell Signaling Technology; anti-Phospho Thr202/Tyr204 p44/42 MAPK (ERK1/2) and anti-ERK1/2 from Promega; anti-Phospho Ser473 Akt (SK703) and anti-ZAP70 (2F3.2) from Millipore; and anti-tubulin from Sigma-Aldrich. After incubation with anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary Abs (Sigma), specific reactions were revealed with the ECL Lightning detection kit (Perkin Elmer). For antibody arrays, 300 μg of cellular extracts were incubated with the Human Phospho-Kinase Array Kit (Proteome Profiler; R&D Systems) following manufacturer's instructions.

Densitometry values for Western blot and antibody array experiments were estimated by the ImageQuant TL software (GE Healthcare) and were expressed as arbitrary units (a.u.). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film. In Western blot, the expression of ZAP-70 was considered low/absent when the densitometric values were not significantly over the film background. In antibody arrays, the average signal of the pair of duplicate spots, representing each phosphorylated kinase protein, was calculated after subtraction of background values (pixel density) from negative control spots and normalization to average values from positive control spots.

Immunofluorescence confocal microscopy

For analysis of p53 nuclear traslocation, cytospin cell preparations were fixed in paraformaldheyde 4%, washed with PBS, permeabilized with 0.3% Triton X-100, and nonspecific binding of primary antibody was blocked with PBS/BSA 1% for 1 hour, as previously reported (23). Cells preparations underwent indirect immunofluorescence staining with anti-p53 monoclonal Ab (DO-1, Santa Cruz Biotechnology). Then, cells preparations underwent DNA staining with the dye TOPRO-3 Iodide (1 μM, Molecular Probes, Life Technologies) and were examined using 60× plan apoVC objective on an inverted Swept Field Nikon (Nikon Corporation) microscope.

Statistical analysis and assessment of the effect of combination treatment

The results were evaluated by using analysis of variance with subsequent comparisons by Student's *t*-test and with the Mann–Whitney rank-sum test. Statistical significance was defined as $P < 0.05$. To investigate the effect of Dasatinib plus Nutlin-3 combination, leukemic cells were then treated with serial 2-fold dilutions (from 10 to 0.1 μM) of Dasatinib or Nutlin-3, individually or in combination using a constant ratio (Dasatinib:Nutlin-3) 1:1. Results were analyzed with the method of Chou and Talalay (24) to determine whether combined treatment yields greater effects than expected from summation alone. A combination index (CI) of 1 indicates an additive effect, whereas a CI below 1 indicates synergism.

Results

The Dasatinib + Nutlin-3 combination promotes synergistic cytotoxicity in primary CLL cells as well as in $p53^{wild\text{-}type}$ and $p53^{deleted/mutated}$ leukemic cell lines

In the first group of experiments, we have investigated the induction of cytotoxicity by Dasatinib and Nutlin-3, used either alone or in combination, in B-CLL cell samples obtained from patients ($n = 20$) with different prognostic characteristics (Supplementary Table 1). The rationale for using such combination was that Dasatinib and Nutlin-3 are thought to target distinct intracellular pathways controlling the survival/proliferation of leukemic cells (4, 5, 25). To evaluate whether the combined effect of Dasatinib plus Nutlin-3 was additive or synergistic, primary B-CLL cell samples were treated with serial concentrations of Dasatinib and Nutlin-3 at a constant Dasatinib:Nutlin-3 ratio for data analysis by the method of Chou and Talalay (ref. 24; Fig. 1). After 48 hours of treatment, the Dasatinib + Nutlin-3 combination induced a synergistic (average CI < 1) cytotoxic activity in 19 out of 20 primary B-CLL samples (Table 1). Importantly, the synergistic cytotoxicity of Dasatinib + Nutlin-3 was observed even in 4 patient samples with chromosome 17p deletion (Supplementary Table 1, Table 1, and Fig. 1), suggesting that functional loss of p53 does not seem to preclude the efficacy of the drug combination.

To further explore the potential therapeutic activity of Dasatinib + Nutlin-3 in relationship with the p53 status, next experiments were carried out by using B leukemic cell lines characterized for being $p53^{wild\text{-}type}$ (EHEB and

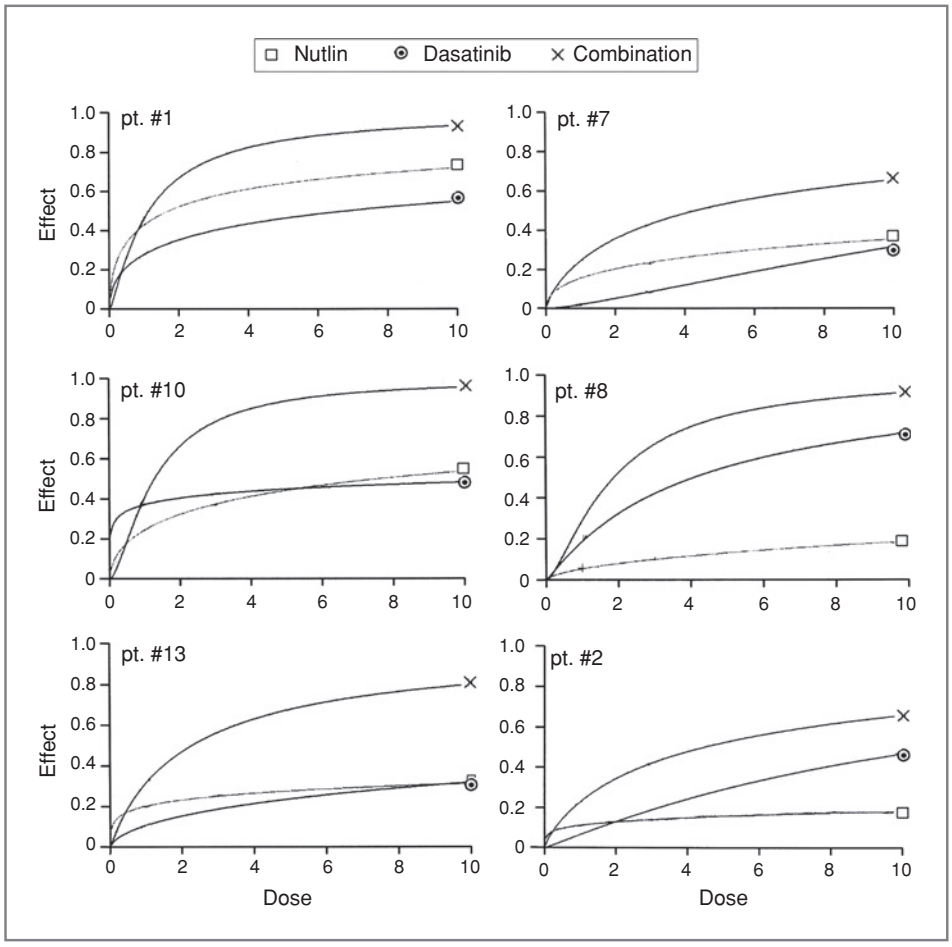

Figure 1. Cytotoxicity by Dasatinib and Nutlin-3 used alone or in combination in B-CLL patient leukemic cells. B-CLL patient leukemic cells were exposed to serial doses of Dasatinib or Nutlin-3 used either alone or in combination, with a fixed ratio, for 48 hours. Dose-effect plots, to determine drug efficacy, are shown for representative B-CLL samples, including 3 patients carrying 17p- (Pt. #7, Pt. #8, and Pt. #10). The decrease of cell viability, labeled "effect" on the *Y*-axis, was determined in assays done at least twice in duplicate.

JVM-2), or $p53^{deleted/mutated}$ (MEC-2 and BJAB). As expected (25), $p53^{deleted/mutated}$ cell lines showed a poor susceptibility to Nutlin-3 alone, but the combined treatment of Dasatinib + Nutlin-3 significantly ($P < 0.05$) reduced cell viability (Fig. 2A) and promoted apoptosis (Fig. 2B), with respect to the treatments with the single compounds, in both $p53^{wild\text{-}type}$ and $p53^{deleted/mutated}$ leukemic cell lines. Indeed, at 48 hours of treatment, synergy of Dasatinib plus Nutlin-3 combination treatment (average CI < 1) was observed not only in $p53^{wild\text{-}type}$ but notably also in $p53^{deleted/mutated}$ cell lines (Table 2).

Dasatinib interferes with the p53 transcriptional activity induced by Nutlin-3

As expected (25), exposure to Nutlin-3 induced rapid p53 accumulation in $p53^{wild\text{-}type}$ (EHEB and JVM-2), but not in $p53^{deleted/mutated}$ (MEC-2 and BJAB), leukemic cells, as documented by indirect immunofluorescence (data not shown) and Western blot analyses performed on total cell lysates (Fig. 3A). On the other hand, Dasatinib marginally affected both the basal levels of p53 and the accumulation of p53 induced by Nutlin-3 in the $p53^{wild\text{-}type}$ cells (Fig. 3A). Interestingly, however, levels of both MDM2 and p21 proteins, 2 of the best characterized transcriptional targets of p53 (25), were significantly ($P < 0.05$) decreased in cells treated with Dasatinib + Nutlin-3 with respect to cells treated with Nutlin-3 alone (Fig. 3B).

Critical role of P-Akt downregulation in mediating the cytotoxicity of Dasatinib and Dasatinib + Nutlin-3

To further explore the intracellular pathways mediating the synergistic cytotoxicity induced by Dasatinib + Nutlin-3, we next performed antibody phospho-kinase arrays to compare the effect of the treatment with each single agent versus the treatment with the combination both in the $p53^{wild\text{-}type}$ EHEB and in the $p53^{mutated}$ BJAB leukemic cells. After 16 hours of treatment, when the levels of culture cytotoxicity were still low, the most consistent modulations observed in response to Dasatinib and Dasatinib + Nutlin-3 both in $p53^{wild\text{-}type}$ EHEB and in $p53^{mutated}$ BJAB cells were observed on the ERK1/2, p38/MAPK, and Akt phosphorylation levels. Indeed, although Nutlin-3 had no effects on these pathways, Dasatinib alone as well as Dasatinib + Nutlin-3 markedly inhibited the basal phosphorylation of ERK1/2, p38/MAPK, and Akt (Fig. 4A). Of interest, the combination of Dasatinib + Nutlin-3 selectively enhanced ($P < 0.05$) the inhibition of Akt

Table 1. Combination index values for effects of Dasatinib and Nutlin-3 on patient B-CLL cell viability, evaluated after 48 hours of treatment

B-CLL patient no.	ED50	ED75	ED90	Average CI*
1	0.83	0.25	0.08	0.39
2	0.86	0.46	0.26	0.53
3	0.32	0.51	0.83	0.55
4	0.85	0.75	0.66	0.75
5	0.36	0.49	0.91	0.59
6	>1	>1	>1	>1
7	0.36	0.49	0.82	0.56
8	0.48	0.35	0.26	0.36
9	1.60	0.41	0.13	0.71
10	0.43	0.81	1.53	0.92
11	0.24	0.31	0.46	0.34
12	0.12	0.25	2.1	0.82
13	0.24	0.002	1.17e−5	<0.1
14	0.33	0.27	0.28	0.29
15	0.30	0.70	1.62	0.87
16	0.26	0.05	0.01	0.11
17	0.30	0.32	0.37	0.33
18	0.79	0.82	1.00	0.87
19	0.50	0.55	1.23	0.76
20	0.27	0.27	0.28	0.27

NOTE: ED indicates effect dose.
*The average combination index (CI) values were calculated from ED50, ED75, and ED90.

phosphorylation with respect to Dasatinib alone, as validated by Western blot analysis (Fig. 4A).

Because it has been recently shown that Dasatinib-mediated inhibition of Src kinases emanates via Syk and PLC-γ to the phosphatidylinositol (PI) 3-kinase/Akt axis (5, 26) and Akt has been shown to play a primary role in the survival of B-CLL cells (4), in the attempt to verify the effective role played by the inhibition of the Akt pathway in mediating the antileukemic activity of Dasatinib, we have comparatively analyzed the effect of Dasatinib with that of cell-permeable pharmacologic inhibitors specific for different pathways: LY294002 (inhibitor of PI3K-Akt pathway), PD98059 (inhibitor of the ERK pathway), and SB20380 (inhibitor of the p38/MAPK pathway). For this purpose, cells were preincubated with Dasatinib or with each single pharmacological inhibitor, to selectively inhibit the phosphorylation of Akt, ERK1/2, or p38/MAPK, before exposure to Nutlin-3. As shown in Fig. 4B, although LY294002, PD98059, and SB20380 did not significantly affect p53 accumulation induced by Nutlin-3, pretreatment with LY294002 significantly reduced the induction of MDM2 with an effect comparable to that of Dasatinib. Moreover, although PD98059 and SB20380 did not reduce cell viability of leukemic cells and did not affect the response to Nutlin-3, LY294002 exhibited an effect comparable to that of Dasatinib also on cell viability (Fig. 4C). Indeed, LY294002 significantly ($P < 0.05$) reduced cell viability and enhanced the cytotoxicity of Nutlin-3 both

Table 2. Combination index values for effects of Dasatinib and Nutlin-3 on cell viability, evaluated after 48 hours of treatment

Cell line	ED50	ED75	ED90	Average CI*
EHEB	0.53	0.55	0.61	0.56
JVM-2	0.84	0.97	1.14	0.98
MEC-2	0.75	0.66	0.58	0.66
BJAB	0.55	0.35	0.24	0.38

NOTE: ED indicates effect dose.
*The average combination index (CI) values were calculated from ED50, ED75, and ED90.

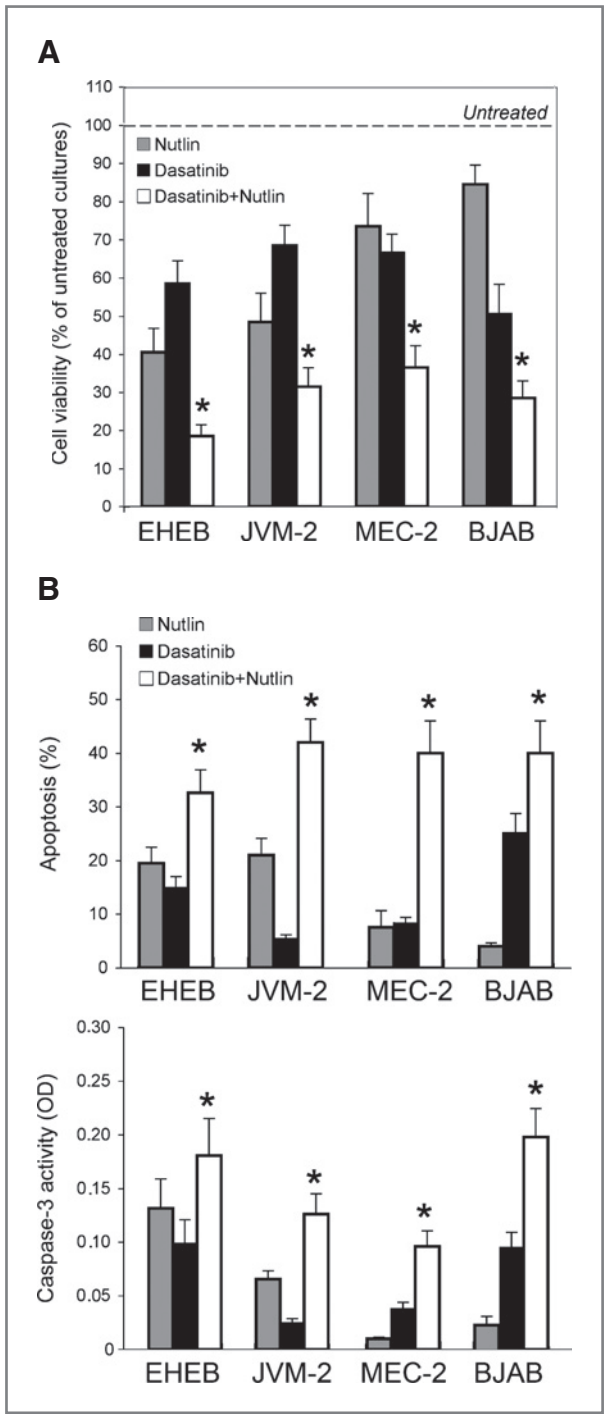

Figure 2. Cytotoxicity by Dasatinib and Nutlin-3 used alone or in combination in B leukemic cell line models. The $p53^{wild\text{-}type}$ (EHEB, JVM-2) and $p53^{deleted/mutated}$ (MEC-2, BJAB) B leukemic lines were exposed for 48 hours to Dasatinib (10 μM) and Nutlin-3 (10 μM), used either alone or in combination, as indicated. A, cell viability was calculated as percentage with respect to the control vehicle cultures (set to 100% for each cell line). B, induction of apoptosis was calculated as percentage of Annexin V^+/PI^+ cells and caspase activation was evaluated on cell lysates by using DEVD-*p*NA substrate. In A to B, data are reported as mean ± SD of results from at least 3 independent experiments, performed in duplicate. *$P < 0.05$ with respect to cultures treated with either Dasatinib or Nutlin-3 alone.

in $p53^{wild\text{-}type}$ and in $p53^{mutated}$ leukemic cells (Fig. 4C). Conversely, overexpression of a membrane-targeted constitutively active form of Akt (pMyr-Akt) upon cell transfection in both $p53^{wild\text{-}type}$ EHEB and $p53^{mutated}$ BJAB, efficiently ($P < 0.05$) counteracted the cytotoxicity induced by Dasatinib and Dasatinib + Nutlin-3 (Fig. 4D). Overall, these experiments support a critical role of the Akt pathway in modulating both the antileukemic activity of Dasatinib and the sinergy of Dasatinib + Nutlin-3.

Discussion

Among the second generation of protein kinase inhibitors, Dasatinib, currently used in clinical trials for the treatment of chronic myeloid leukemia (CML; ref. 27) is a novel multikinase inhibitor, which displays broader spectrum of activity, higher efcacy and lower toxicity compared to Imatinib. Several studies have already proposed a potential cytotoxic activity of Dasatinib for B-CLL treatment and recent works have documented an antiproliferative effect of Dasatinib on B-CLL cells *in vitro* (4, 5, 28, 29). However, although these studies have suggested a potential use of Dasatinib in combination with the most commonly used chemotherapeutic agents chlorambucil and fludarabine, this is the first study evaluating the potential effect of Dasatinib in combination with Nutlin-3, a nongenotoxic activator of the p53 pathway.

The rationale for using this drug combination was that the mechanism of action of Dasatinib differs from Nutlin-3, because Dasatinib recognized molecular targets are represented by several kinases (1–3), which have been reported to be overexpressed in B-CLL cells. On the other hand, Nutlin-3 disrupts the interactions between p53 and MDM2 and promotes a rapid and potent accumulation of p53 selectively in $p53^{wild\text{-}type}$ cells. Moreover, recent results of phases I and II clinical trials have shown that Dasatinib alone might be benecial only in a subset of previously treated B-CLL patients (29), suggesting that therapeutic combinations will likely be more effective than Dasatinib alone. In this respect, the results of our study confirm and expand recent data demonstrating a synergism between the first-generation tyrosine kinase inhibitor Imatinib and Nutlin-3 in Bcr-Abl-positive leukemias in a p53-dependent manner (30, 31). In fact, we could demonstrate that Dasatinib synergized with Nutlin-3 not only in p53 proficient, but also in p53 deficient leukemic B cell lines, suggesting a potential broader antileukemic activity as compared to Imatinib plus Nutlin-3. Importantly, the therapeutic combination Dasatinib + Nutlin-3 was effective also in primary B-CLL patient cell samples with bad prognosis. Indeed, to our surprise, a synergistic cytotoxic activity was documented also in primary B-CLL cells carrying 17p-.

At the molecular level, Dasatinib counteracted the Nutlin-3 mediated p53 transcriptional activity in $p53^{wild\text{-}type}$ leukemic cells. This effect was not observed

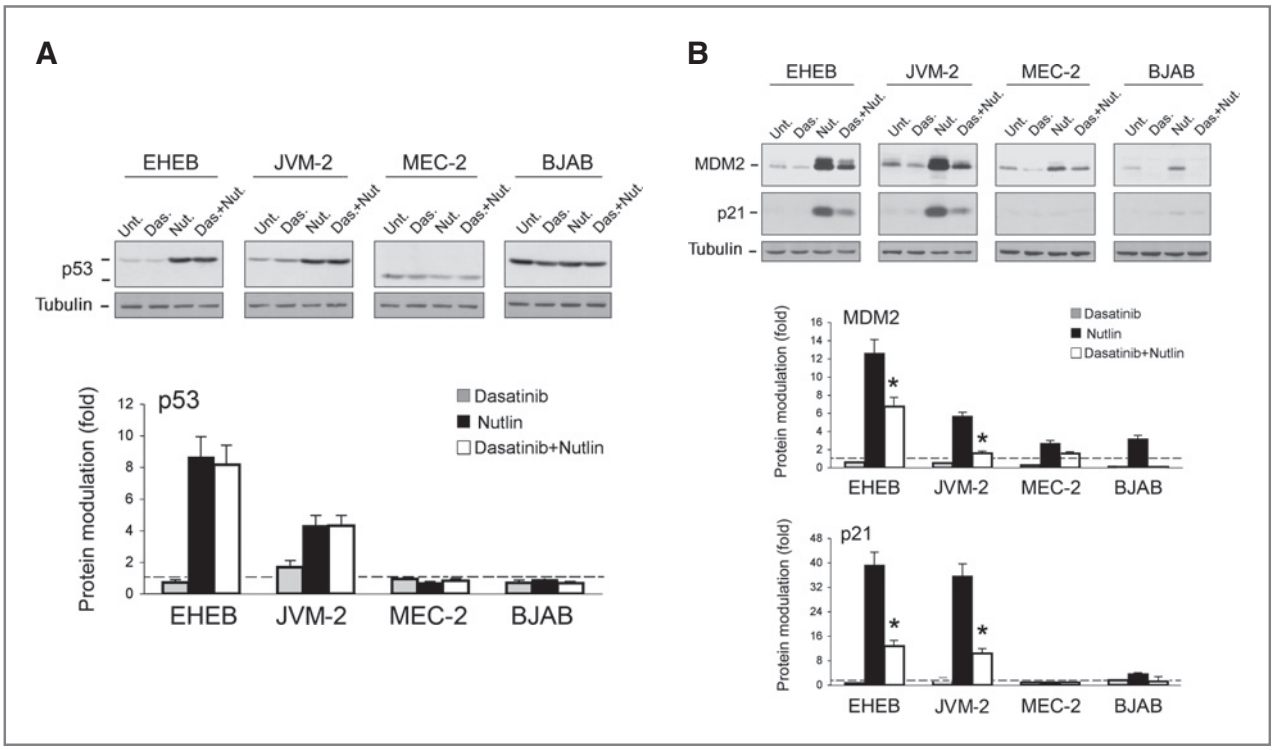

Figure 3. Dasatinib interferes with the p53 transcriptional activity induced by Nutlin-3. Leukemic cell lines were exposed for 24 hours to Dasatinib (10 μM) and Nutlin-3 (10 μM), used either alone or in combination, as indicated. Levels of p53 (A), MDM2 and p21 (B) were assessed by Western blot analysis of total cell lysates. Representative examples of Western blot results are shown. Tubulin staining is shown as loading control; after densitometric analyses, p53 as well as MDM2 and p21 protein levels are expressed as folds of protein modulation, by the indicated treatments, with respect to the control untreated cultures set to 1 (hatched line). *$P < 0.05$ with respect to the Nutlin-3-treated cultures.

in $p53^{deleted/mutated}$ cells and therefore could not account for the synergistic cytotoxicity observed in $p53^{deleted/mutated}$ leukemic cells. On the contrary, Nutlin-3 selectively enhanced the downregulation of Akt phosphorylation induced by Dasatinib in both $p53^{wild\text{-}type}$ and $p53^{deleted/mutated}$ leukemic cells, suggesting that downregulation of Akt played a major role in mediating the combined effect of Dasatinib + Nutlin-3. Consistently with this hypothesis, the experiments carried out with selective pharmacological inhibitors showed an interesting parallelism between the effect of Dasatinib alone or in combination with Nutlin-3 and LY294002, an inhibitor of the Akt pathway. Finally, the key role of Akt inhibition in mediating the cytotoxic activity of Dasatinib, used alone or in association with Nutlin-3, was confirmed by overexpression studies of a membrane-targeted constitutively active form of Akt.

Consistently with our present findings, a previous study has shown that treatment with Dasatinib for 24 hours resulted in a dose-dependent reduction of p21 basal expression levels in primary B-CLL cells (29). In addition, we found that also MDM2, another major transcriptional target of p53, which represents the most important antagonist of p53, was significantly down-regulated in cultures treated with Dasatinib + Nutlin-3 with respect to cultures treated with Nutlin-3 alone. The downregulation of MDM2 and p21 accumulation in the presence of Dasatinib might be due to the inhibition of p53 translocation at the nuclear level, as well as to the inhibition of the Akt pathway, which plays an important role in stabilizing MDM2 (32) and p21 (33) proteins.

In conclusion, this study suggests that the combined treatment of Dasatinib plus Nutlin-3 might offer a novel therapeutic strategy not only for $p53^{wild\text{-}type}$ B-CLL patients that represent >80% of all leukemias at diagnosis (8), but also for $p53^{deleted/mutated}$ leukemias that have the worst prognosis (9) and urgently need innovative therapeutic approaches.

Disclosure of Potential Conflicts of Interest

Authors declare no potential conflicts of interest.

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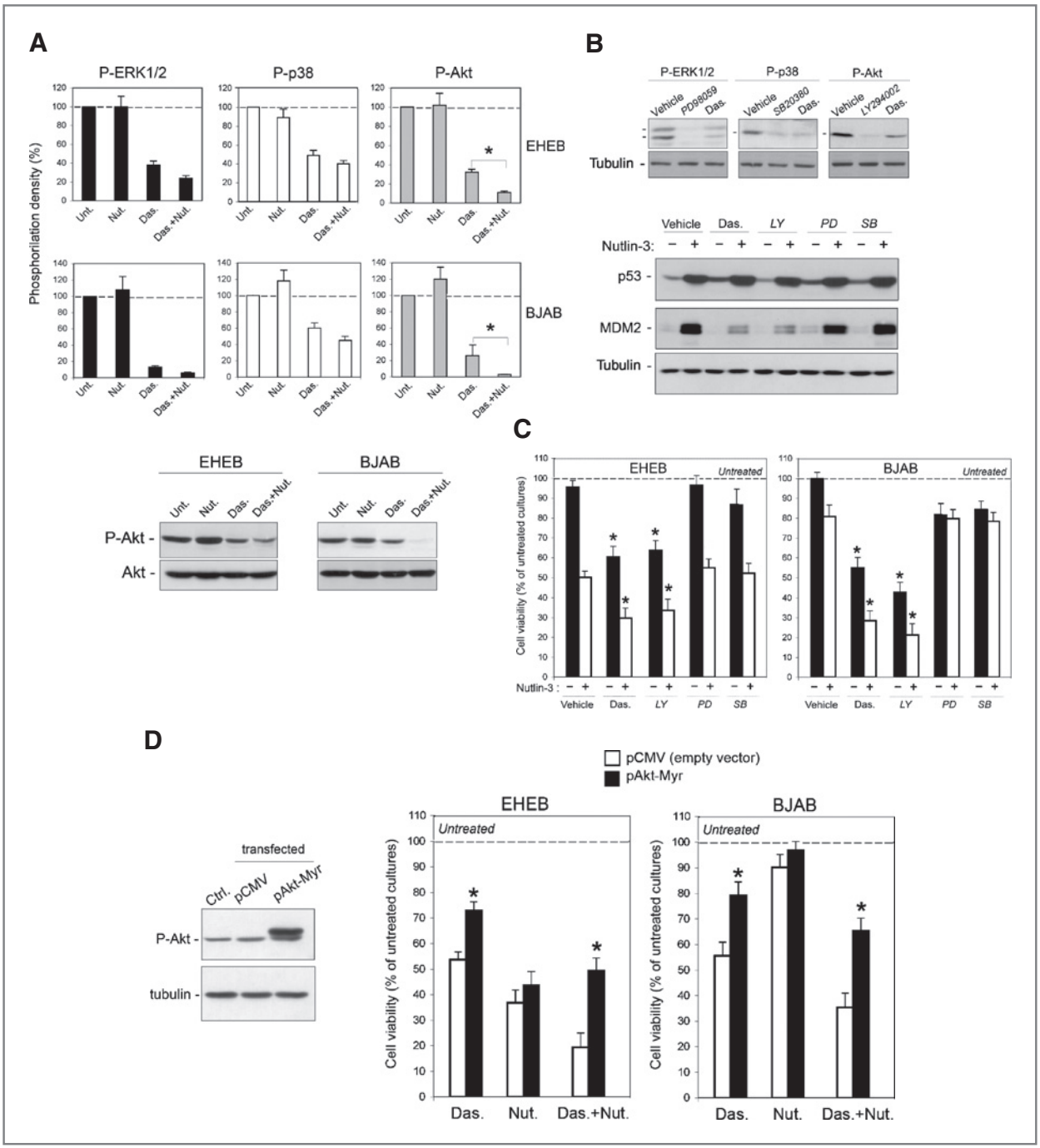

Figure 4. Role of Akt in Dasatinib cytotoxicity and in Dasatinib+Nutlin-3 synergy. A, whole cell lysates were prepared from EHEB and BJAB cell lines treated (for 16 hours) as indicated, and hybridized with a human Phospho-Kinase array kit. Spot densities of phospho-proteins were quantified using Image Quant TL software and normalized to those of positive controls (set at 100) on the same membrane. The analysis of modulation of phosphorylation signals for P-ERK1/2, P-p38, and P-Akt are reported (*$P < 0.05$). Validation of phospho-kinase array results was carried out by Western blot analysis of P-Akt levels. B–C, leukemic cell lines were preincubated with Dasatinib (10 μM), LY294002 (*LY*, 40 μM), PD98059 (*PD*, 40 μM), SB203580 (*SB*, 10 μM), or control vehicle, before treatment with Nutlin-3 for 24 hours (10 μM). In B, the activity of each pharmacological inhibitor was assessed by analyzing the level of phosphorylated ERK1/2, p38, and P-Akt in cell lysates. Moreover, the potential effects on Nutlin-3 induced p53 activation were evaluated by analyzing the levels of p53 and MDM2 by Western blot analysis of cell lysates. Representative examples of Western blot results out of 3 independent experiments are shown; tubulin staining is shown as loading control. D, cells were transfected either with an empty control vector (pCMV) or with a constitutively active form of Akt (pMyr-Akt), as documented by Western blot analysis, before treatment with Dasatinib, Nutlin-3, or Dasatinib plus Nutlin-3 (Ctrl.: control not-transfected cultures). In C and D, upon 48 hours of the indicated treatments, cell viability was calculated as percentage with respect to the control cultures (set to 100% for each cell line). Data are reported as mean ± SD of results from experiments performed in triplicates. In experiments with pharmacological inhibitors ± Nutlin-3, *$P < 0.05$ with respect to cultures treated with either vehicle (black bars) or Nutlin-3 ± vehicle (white bars). In transfection experiments, *$P < 0.05$ with respect to cultures transfected with the control empty vector.

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