Nutlin-3 up-regulates the expression of Notch1 in both myeloid and lymphoid leukemic cells, as part of a negative feedback antiapoptotic mechanism

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The small molecule inhibitor of the MDM2/p53 interaction Nutlin-3 significantly up-regulated the steady-state mRNA and protein levels of Notch1 in TP53wild-type (OCI, SKW6.4) but not in TP53deleted (HL-60) or TP53mutated (BJAB) leukemic cell lines. A direct demonstration that NOTCH1 was a transcriptional target of p53 in leukemic cells was obtained in experiments carried out with siRNA for p53. Moreover, inhibition of Notch1 expression using Notch1-specific siRNA significantly increased cytotoxicity in TP53wild-type leukemic cells. Of note, Nutlin-3 up-regulated Notch1 expression also in primary TP53wild-type B-chronic lymphocytic leukemia (B-CLL) cells and the combined use of Nutlin-3 plus pharmacological γ-secretase inhibitors of the Notch signaling showed a synergistic cytotoxicity in both TP53wild-type leukemic cell lines and primary B-CLL cells. A potential drawback of γ-secretase inhibitors was their ability to enhance osteoclastotic maturation of normal circulating preosteoclasts induced by RANKL + M-CSF. Notwithstanding, Nutlin-3 completely suppressed osteoclastogenesis irrespective of the presence of γ-secretase inhibitors. Taken together, these data indicate that the p53-dependent up-regulation of Notch1 in response to Nutlin-3 represents an antiapoptotic feedback mechanism able to restrain the potential therapeutic efficacy of Nutlin-3 in hematologic malignancies. Therefore, therapeutic combinations of Nutlin-3 + γ-secretase inhibitors might potentiate the cytotoxicity of Nutlin-3 in p53wild-type leukemic cells. (Blood. 2009;113:4300-4308)

Introduction

The activation of p53 is tightly regulated by the human homolog of murine double minute 2 (MDM2) gene,1 which is an E3 ubiquitin ligase for p53 and itself and controls p53 half-life mainly via ubiquitin-dependent degradation. In response to a variety of stimuli, such as cellular stress, the p53-MDM2 interaction is disrupted and p53 rapidly accumulates within the cell.2 Potent and selective small molecule inhibitors of the p53-MDM2 interaction, the Nutlins, have been recently reported.2-3 These compounds bind MDM2 in the p53 binding pocket with high selectivity and can release p53 from negative control, leading to effective stabilization of p53 and activation of the p53 pathway.2,3 It has been demonstrated that treatment with the active enantiomer Nutlin-3a results in rising levels of p53 protein and subsequent induction of cell cycle arrest and apoptosis in a variety of tumor cells.1 In contrast to most solid tumors, TP53 is mutated in approximately 10% to 15% of both myeloid and lymphoid leukemias at diagnosis,4 and several recent studies have demonstrated that Nutlin-3 induces ex vivo cytotoxic cell death of most TP53wild-type primary hematologic malignancies, including acute myeloid leukemias, multiple myeloma, B-chronic lymphocytic leukemias (B-CLL), and B-cell lymphomas.5-15

Although it is well established that p53 mediates a variety of cellular functions, such as cell cycle arrest, cellular senescence, and, only as ultimate choice, apoptosis,16,17 some experimental evidence suggests that inhibition of the transcriptional activity of p53 might paradoxically result in an increase of the p53-mediated proapoptotic activity,11 also in B-CLL cells.19 Therefore, the aim of this study was to further investigate the relationship between the transcriptional and cytotoxic activities induced by Nutlin-3 and to elucidate whether Nutlin-3 promotes the transcription of antiapoptotic genes in leukemic cells, which might hamper and/or reduce its potential therapeutic efficacy. In this respect, it has recently emerged that a potential target gene of the p53 pathway is NOTCH1,20 which belongs to an evolutionarily conserved pathway that profoundly impacts mammalian development. Of note, a recent study published while this article was under preparation has demonstrated that circulating B-CLL cells overexpress Notch1 and Notch2 family members with respect to circulating normal B lymphocytes.21 The same study suggested that constitutively activated Notch signaling might be involved in survival and apoptosis resistance of B-CLL cells.21 For the purpose of our present study, it is also of particular interest that Notch family members contribute to the maintenance, renewal, and maturation of normal hematopoietic system,22 and have been implicated in the promotion of different types of cancer, including hematologic malignancies.23,24

On these bases, the aim of the present study was to evaluate the effect of Nutlin-3 treatment on Notch1 expression in both myeloid and lymphoid leukemic cell lines as well as in primary B-CLL cells and the role of Notch signaling in modulating Nutlin-3 cytotoxicity.

Methods

Leukemic cell lines and primary B-CLL cells

The myeloid TP53wild-type HL-60 and TP53wild-type OCI and the lymphoid TP53wild-type SKW6.4 and TP53mutated BJAB leukemic cell lines were either purchased from the American Type Culture Collection (ATCC, Manassas, VA) or prepared in our laboratory from bone marrow aspirates of six newly diagnosed B-CLL patients. The diagnosis of B-CLL was confirmed by histology and immunophenotyping based on the criteria of the Working Group on Chronic Lymphocytic Leukemia.25

The culture medium for SKW6.4 and BJAB cells was Roswell Park Memorial Institute Medium 1640 (Gibco, USA) supplemented with 10% fetal calf serum (Biologica, Italy), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and essential fatty acids (Invitrogen, USA). HL-60 cells were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 1 μg/ml gentamicin. OCI cells were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Biologica, Italy) and 1 μg/ml gentamicin.

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Table 1. Clinical and laboratory features of patients with CLL

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*ZAP-70 expression was determined by Western blot analysis.
†FISH defects were found using a B-CLL FISH panel.

Western blot analyses

Cells were harvested in lysis buffer containing 1% Triton X-100, Pefablock (1 mM), aprotinin (10 μg/mL), pepstatin (1 μg/mL), leupeptin (10 μg/mL), NaF (10 mM), and Na3VO4 (1 mM), as described.29 Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of protein for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters.

RNA analyses

Total RNA was extracted using the RNaseasy Plus Mini Kit (QIAGEN, Hilden, Germany) that also removes chromatin DNA. Total RNA was retrotranscribed into cDNA, using the AccuScript high fidelity 1st strand cDNA synthesis kit (Stratagene, La Jolla, CA). To ascertain the TP53 status, mutation analysis for cDNA of TP53 was performed analyzing 3 overlapping fragments spanning the TP53 coding region of exons II-X, which were amplified by polymerase chain reaction (PCR), purified, and sequenced. The primer sequences were as follows: no. 1, AGT CAC ATG CTC GCG TGC AG (14-53); no. 2, GGA GTA CGT GCG AAT CAC AG (381-386); no. 3, TGC ACC AGC AGC TCC TAC AG (225-244); no. 4. ACA GTC AGA GGC AAC CTC AG (687-668); no. 5, TTC GAC ATA GTG TGG TGC TG (635-654); no. 6, GAA TGT CAG TCT GAG TCA GG (1187-1168). Numbers refer to the human TP53 cDNA sequence.

Evaluation of osteoclastic differentiation

As model system of human osteoclastogenesis, we have used peripheral blood mononuclear cells (PBMCs), obtained from healthy blood donors, as...
previously described. Briefly, adherent PBMCs were cultured in RPMI medium containing 10% FBS and treated with 50 ng/mL human macrophage-colony stimulating factor (M-CSF; PeproTech, London, United Kingdom) alone for 6 days, followed by M-CSF (50 ng/mL) plus RANKL (50 ng/mL; Alexis Biochemicals, Lausen, Switzerland) for additional 12 days, replacing the medium containing fresh cytokines every 3 days. Nutlin-3 and L-685,458, either alone or in combination, were added to the cultures simultaneously with RANKL + M-CSF and at every medium replacement.

At the end of the culture time, the degree of osteoclast differentiation was investigated by staining adherent cells for TRAP, using the leukocyte acid phosphatase kit (387-A; Sigma-Aldrich) according to the manufacturer’s instructions, and for 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), as previously described. After the stainings, cells were photographed under a light (for TRAP; Eclipse TE200 Inverted microscope; Nikon, Tokyo, Japan) or fluorescence (for DAPI; Axioshot 100; Zeiss, Jena, Germany) microscope and scoring of TRAP-positive and multinucleated cells was performed by examination of at least 8 independent fields.

In parallel, the culture supernatants were analyzed for the levels of osteoclast-derived tartrate-resistant acid phosphatase form 5b, a product of osteoclast activity. The assay used (TRACP 5b; Immunodiagnostics systems, Fountain Hills, AZ) detects only the active TRACP molecules, giving an accurate estimation of bone resorption activity. Results were read at an optical density of 450 nm using an Anthos 2010 enzyme-linked immunosorbent assay (ELISA) reader (Anthos Labtec Instruments, Wals Salzburg, Austria). Measurements were done in duplicate and corrected for the dilution factors.

Statistical analysis and assessment of the effect of combination treatment

The results were evaluated using analysis of variance with subsequent comparisons by Student t test and with the Mann-Whitney rank-sum test. Statistical significance was defined as \( P \) value less than .05. To investigate the effect of Nutlin-3 plus L-685,458 combination, leukemic cells were then treated with serial 2-fold dilutions of Nutlin-3 (from 10 to 1.25 \( \mu \)M) or L-685,458 (from 20 to 2.5 \( \mu \)M), individually or in combination using a constant ratio (Nutlin-3/L-685,458) of 1:2, for 24 hours. Dilutions of each compound were performed starting from the maximal concentrations of Nutlin-3 (10 \( \mu \)M) and L-685,458 (20 \( \mu \)M), which avoid specific cytotoxicity, as determined in preliminary experiments. Results were analyzed using the CalcuSyn software program (Biosoft, Cambridge, United Kingdom), which uses the method of Chou and Talalay 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

**Nutlin-3 selectively up-regulates Notch1 in p53\(^{\text{wt}}\)-type leukemic cell lines**

In the first set of experiments, we sought to investigate whether Nutlin-3 was able to affect the steady-state mRNA levels of Notch1 in B lymphoblastoid (SKW6.4 and BJAB) and myeloid (OCI, HL-60) cell lines. Quantitative reverse-transcription (RT–PCR) analysis demonstrated that the exposure for 24 hours to 10 \( \mu \)M Nutlin-3 selectively \( (P < .01) \) up-regulated the mRNA levels of Notch1 in SKW6.4 and OCI but not in BJAB and HL-60 cell lines (Figure 1A). Western blot analysis confirmed that the up-regulation of Notch1 mRNA was accompanied by a significant \( (P < .01) \) accumulation of the transmembrane/cytoplasmic (120-kDa) portion of Notch1 protein in response to Nutlin-3 in SKW6.4 and OCI leukemic cells (Figure 1B). Because the best characterized biologic activity of Nutlin-3 is to disrupt the interaction between MDM2 and p53 proteins and to prevent p53 from ubiquitination, changes in p53 protein were also investigated in the different leukemic cell lines before and after treatment with Nutlin-3 (Figure 1B). The amount of p53 in cell lysates obtained from untreated TP53\(^{\text{wt}}\)-type SKW6.4 and OCI cells markedly increased after exposure to 10 \( \mu \)M Nutlin-3 for 24 hours. On the other hand, HL-60 cells carrying TP53\(^{\text{del}}\)-type or BJAB carrying TP53\(^{\text{mut}}\)-type exhibited absent and high basal levels of p53, respectively (Figure 1B). As expected on the basis of previous findings obtained on a variety of hematologic malignancies, exposure to Nutlin-3 did not modulate p53 levels in either TP53\(^{\text{del}}\)-type HL-60 or TP53\(^{\text{mut}}\)-type BJAB cell lines (Figure 1B).

**Silencing of TP53 counteracts the ability of Nutlin-3 to up-regulate Notch1**

Since it has been shown that p53 might be involved in the up-regulation of Notch1 in human prostate and breast cancer cell

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**Figure 1. Effect of Nutlin-3 on Notch1 mRNA expression levels in leukemic cell lines.** B-lymphoid (BJAB and SKW6.4) and myeloid (HL-60 and OCI) leukemic cell lines were either left untreated or exposed for 24 hours to Nutlin-3 (10 \( \mu \)M). Levels of Notch1 mRNA and protein were analyzed by quantitative RT-PCR (A) and Western blot analysis (B), respectively. (A) For each cell line, after normalization to the level of GAPDH mRNA, results were expressed as fold of Notch1 mRNA induction in Nutlin-3–treated cultures with respect to the control untreated cultures. Data are reported as means plus or minus SD of results from 3 to 5 independent experiments each performed in duplicate. (B) The levels of Notch1 and of p53 proteins were assessed by Western blot analysis in cell lysates. Tubulin staining is shown as a loading control. Representative examples of Western blot results of 5 independent experiments are shown. After densitometric analyses, results were expressed as fold of Notch1 protein induction in Nutlin-3–treated cultures with respect to the control untreated cultures. Data are reported as means ± SD of results from 5 independent experiments. *\( P < .05 \) with respect to untreated cells.
Nutlin-3 was mediated by p53 also in leukemic cells. (Figure 2), strongly suggesting that the induction of Notch1 by p53 activation induced by Nutlin-3 not only of p53 but also of Notch1 protein in cell lysates specifically abrogated the ability of Nutlin-3 to induce the accumulation of p53. Therefore, to investigate whether the inhibition of Notch1 might potentiate the Nutlin-3–mediated apoptosis, we have used siRNAs to attenuate Notch1 expression. Knockdown of Notch1 expression was demonstrated by Western blot, documenting a significant reduction of Notch1 protein both at the basal level as well as in response to Nutlin-3 in both OCI (Figure 3A) and SKW6.4 (Figure 3B) cells, transfected with a cocktail of Notch1-specific siRNAs. On the other hand, the ability of Nutlin-3 to induce the accumulation of p53 in OCI and SKW6.4 cells was not affected by Notch1 siRNAs (Figure 3A,B). Of note, in OCI and SKW6.4 samples in which Notch1 expression was knocked down by transfection with Notch1-specific siRNAs, the cytotoxicity of Nutlin-3 was significantly ($P < .05$) increased with respect to either cells transfected with a scrambled control siRNA or cells not transfected (Figure 3A,B).

In additional experiments, SKW6.4 cells were preincubated with PFTa (25 μM), a pharmacological inhibitor that has been shown to block p53-mediated transcription in B-CLL cells.19 Preincubation with PFTa abrogated Notch1 induction by Nutlin-3 without affecting the levels of p53 (Figure 3C) and resulted in a significant ($P < .05$) increase of Nutlin-3 cytotoxicity. Overall, these experiments strongly suggest that NOTCH1 was a downstream transcriptional target of p53.

Nutlin-3 up-regulates Notch1 also in primary B-CLL cells

To exclude the possibility that the data illustrated above were confined to leukemic cell lines, next experiments were carried out using a series of primary B-CLL samples, obtained from 27 patients all characterized for Rai stage, ZAP70 levels, fluorescent in situ hybridization (FISH) analysis, and TP53 mutational status (Table 1). Nutlin-3 variably up-regulated the steady-state mRNA levels of Notch1 also in the majority of primary B-CLL samples examined (Figure 4A). Of note, all B-CLL samples characterized for having a mutated p53 (samples nos. 1, 16, 25, and 27 of Table 1 and Figure 4A) showed an absent induction of Notch1 mRNA in response to Nutlin-3. Consistently with the data obtained in leukemic cell lines, although TP53wild-type B-CLL samples displayed a significant accumulation of p53 in response to Nutlin-3 (exemplified by samples nos. 3, 10, 19, and 24 of Table 1 and Figure 4B), TP53mutated B-CLL samples displayed high basal levels of p53, which were not modified by Nutlin-3 (exemplified by sample no. 1 of Table 1 and Figure 4B). Moreover, in samples characterized by a high percentage of 17p− deletion at FISH analysis (exemplified by sample no. 11 of Table 1), Nutlin-3 poorly induced p53 accumulation (Figure 4B) and Notch1 mRNA expression (Figure 4A). Taken together, these data strongly suggest that Nutlin-3 up-regulates Notch1 in a p53-dependent manner also in primary B-CLL cells.

Silencing of Notch1 increases the Nutlin-3–mediated cytotoxicity in leukemic cell lines

Notch1 has been shown to play a significant role in promoting leukemic development mainly through inhibition of apoptosis.21-25 Therefore, the data illustrated in the previous paragraphs suggested that the Notch1 up-regulation might represent a regulatory feedback antipoptotic mechanism restraining the cytotoxic effects of p53 activation induced by Nutlin-3. Therefore, to investigate whether the inhibition of Notch1 might potentiate the Nutlin-3-mediated cytotoxicity in leukemic cell lines, next experiments were carried out using a series of primary B-CLL samples, obtained from 27 patients all characterized for Rai stage, ZAP70 levels, fluorescent in situ hybridization (FISH) analysis, and TP53 mutational status (Table 1). Nutlin-3 variably up-regulated the steady-state mRNA levels of Notch1 also in the majority of primary B-CLL samples examined (Figure 4A). Of note, all B-CLL samples characterized for having a mutated p53 (samples nos. 1, 16, 25, and 27 of Table 1 and Figure 4A) showed an absent induction of Notch1 mRNA in response to Nutlin-3. Consistently with the data obtained in leukemic cell lines, although TP53wild-type B-CLL samples displayed a significant accumulation of p53 in response to Nutlin-3 (exemplified by samples nos. 3, 10, 19, and 24 of Table 1 and Figure 4B), TP53mutated B-CLL samples displayed high basal levels of p53, which were not modified by Nutlin-3 (exemplified by sample no. 1 of Table 1 and Figure 4B). Moreover, in samples characterized by a high percentage of 17p− deletion at FISH analysis (exemplified by sample no. 11 of Table 1), Nutlin-3 poorly induced p53 accumulation (Figure 4B) and Notch1 mRNA expression (Figure 4A). Taken together, these data strongly suggest that Nutlin-3 up-regulates Notch1 in a p53-dependent manner also in primary B-CLL cells.

The combination of Nutlin-3 plus γ-secretase inhibitors promotes synergistic cytotoxicity in both leukemic cell lines and primary B-CLL cells

To analyze the potential role of Notch signaling in modulating the survival/apoptotic response of leukemic cells to Nutlin-3, we used a pharmacological approach using the γ-secretase inhibitors DAPT and L-685,458, which block the activation of Notch receptors, and induce phenotypes similar to NOTCH-loss-of-function mutations.33-35 Predetermined optimal concentrations of DAPT and L-685,458 in SKW6.4 and OCI cells induced a variable decrease of leukemic viability and enhanced Nutlin-3 cytotoxicity (Figure 5A).
Among the 2 γ-secretase inhibitors used, L-685,458 exhibited more powerful cytotoxicity both alone and in combination with Nutlin-3.

To better define the nature of the pharmacological interaction between Nutlin-3 and L-685,458 on leukemic cell viability, a fixed ratio of these molecules was analyzed, as exemplified in Figure 5B. Based on the availability of B-CLL samples, the Chou and Talalay median-effect method was used to determine drug efficacy and the nature of the drug interaction for 16 of 27 B-CLL samples, as well as for SKW6.4 and OCI cell lines (Table 2). The interactions were judged to be synergistic, with CI less than 1, for SKW6.4 and OCI cell lines as well as for all TP53 wild-type B-CLL samples (Table 2). Interestingly, the synergistic cytotoxicity was observed also in B-CLL samples carrying unfavorable cytogenetic abnormalities (samples nos. 6 and 8 carrying 11q− and samples nos. 7, 14, and 18 carrying 17p−). Of note, B-CLL sample no. 11 with a CI of 0.98, more additive than synergistic (Table 2), was characterized by a double cytogenetic abnormality (13q−/17p−, Table 1), and a poor accumulation of p53 protein (Figure 4B) as well as absent induction of Notch1 mRNA (Figure 4A) in response to Nutlin-3.

The only TP53 mutated B-CLL included in this analysis was sample no. 1, which showed a CI more than 1 (Table 2).

Nutlin-3 efficiently counteracts the pro-osteoclastic activity of L-685,458

A potential concern in considering the therapeutic use of γ-secretase inhibitors in the antileukemic therapy is the reported ability of Notch1 signaling pathway to suppress osteoclastogenesis. Consistently with these previously published data, we found that L-685,458 significantly (P < .05) increased the number of mature osteoclasts, when added to peripheral blood adherent mononuclear cells simultaneously with M-CSF + RANKL (Figure 6). As previously observed in leukemic cells, exposure to Nutlin-3 induced Notch1 expression in normal
preosteoclasts (data not shown). However, Nutlin-3 either added alone or in combination with L-685,458 completely suppressed the formation of multinucleated (Figure 6) giant osteoclasts in cultures supplemented by M-CSF + RANKL.

Discussion

The Notch receptors and ligands are single-pass transmembrane proteins expressed on the surface of adjacent cells, and, therefore, activation of Notch signaling usually requires cell/cell contact and the cleavage of the amino terminal region of Notch family members by γ-secretase.38 This cleavage releases the intracellular domain of Notch, which translocates to the nucleus and induces the transcription of Notch target genes. Ablative NOTCH activation has been linked to cancer since 1991 when mammalian Notch1 was first identified as part of the translocation t(7;9) in a subset of human T-cell acute lymphoblastic leukemias (T-ALLs).27,38 Recent findings indicate an important role of Notch also in the pathogenesis of human T and B cell-derived lymphomas.38,39 In this context, the interplay between Notch1 and p53 signaling pathways appears rather complex. In fact, previous data obtained in primary keratinocytes40 have demonstrated that a p53-responsive element could be identified in the NOTCH1 promoter, and therefore NOTCH1 gene represents a novel target of p53 with a major role in the process of keratinocyte differentiation. Conversely, a couple of recent studies41,42 have demonstrated that cells expressing the intracellular active domain of human Notch1 are chemoresistant in a wild-type p53-dependent manner. In keeping with an antiapoptotic role of Notch family members, a recent study21 has demonstrated that B-CLL and normal PBL samples. Tubulin staining is shown as loading control.

In this study, we have demonstrated for the first time that the nongenotoxic activator of p53 pathway Nutlin-3 significantly up-regulated Notch1 at both the mRNA and protein levels in both myeloid and lymphoblastoid TP53wild-type cell lines as well as in the majority of primary TP53wild-type B-CLL patient cells. Moreover, down-regulation of Notch1 expression/function using either siRNA specific for Notch1 or 2 different types of γ-secretase inhibitors significantly potentiated the cytotoxic activity of Nutlin-3 toward TP53wild-type leukemic cells. In this respect, it is noteworthy that also p21WAF/CIP1, a major p53 transcriptional target potentially up-regulated by Nutlin-3,15 besides inducing cell cycle arrest also exhibits antiapoptotic activity.43-45 Thus, our present study suggests that, similarly to p21WAF/CIP1, the activation of NOTCH1 by p53 represents a negative feedback loop, able to restrain the induction of apoptosis mediated by p53. On the other hand, since several studies have shown that the basal levels of p53 expression are very low in B-CLL cells,7-10 transcription factors other than p53 likely account for the high basal expression of Notch1 in B-CLL cells.21

Experiments performed with PFT-α, a pharmacologic inhibitor of the transcriptional activity of p53, demonstrated that PFT-α was able to enhance the cytotoxic activity of Nutlin-3, confirming recent data of Steele et al,19 who demonstrated that activation of p53 by Nutlin-3 induced apoptosis of B-CLL mainly through the nontranscriptional association of p53 to the mitochondria. These authors rather showed that the transcriptional activity of p53 counteracts the apoptosis-inducing activity of both Nutlin-3 and chemotherapeutic drugs.19 Since we have demonstrated that PFT-α significantly counteracted the transcriptional induction of NOTCH1, these data further indicate that NOTCH1 is one of the transcriptional targets of p53 with antiapoptotic activity.

The synergistic cytotoxic activity of Nutlin-3 and γ-secretase inhibitors are particularly noteworthy since clinical trials with γ-secretase inhibitors have commenced for refractory T-ALL,
Figure 5. Evaluation of cytotoxicity by Nutlin-3 and γ-secretase inhibitors used alone or in combination in leukemic cells. OCI and SKW6.4 cells were exposed for 24 hours to Nutlin-3 (10 \( \mu \)M), DAPT (20 \( \mu \)M), or L-685,458 (20 \( \mu \)M), used either alone or in combination, as indicated. Cell viability was calculated as percentage with respect to the control (vehicle) cultures. Data are reported as means ± SD of results from 3 independent experiments. * \( P < .05 \) with respect to cultures treated with vehicle + Nutlin-3.

(B) Leukemic cells were exposed to serial doses of Nutlin-3 or L-685,458, used either alone or in combination, with a fixed ratio, for 24 hours. Dose-effect plots to determine drug efficacy are shown for SKW6.4, OCI, representative TP53wttype B-CLL samples, and for one TP53mutated B-CLL (patient no. 1). The decrease of cell viability, labeled “effect” on the y-axis, was determined in assays done at least twice in duplicate.
but it has been shown that γ-secretase inhibitors may be useful for the treatment of hematologic malignancies other than T-ALL.46 Although the mechanism behind the cytotoxic effects of γ-secretase inhibitors remains to be clarified, it has been proposed that Notch1 signaling confers chemoresistance by inhibiting p53 pathway through mTOR-dependent PI3K-Akt/PKB pathway.41

In considering the potential cytotoxic activity of the combination of Nutlin-3 plus γ-secretase inhibitors toward normal tissues, previous studies have shown that Nutlin-3 is much less toxic against normal tissues with respect to neoplastic cells,7 and might even protect normal cells from apoptosis induced by mitotic inhibitors.47 Moreover, although Notch1 mediates cell survival signal in leukemic cells, in vitro and in vivo studies strongly support a role for Notch signaling in the regulation of stem cell renewal and hematopoiesis.48,49 In normal hematopoiesis, proliferation is tightly linked to differentiation in ways that involve cell-cell interaction with stromal elements in the bone marrow stem cell niches, and it has been shown that activation of Notch signaling in hemangioblasts dramatically reduces their survival and proliferative capacity and lowers the levels of hematopoietic stem cell markers CD34 and c-Kit.48 In keeping with a key role of Notch in hematopoiesis, it has also been shown that the down-regulation of Notch1 in osteoclastic precursors enhances osteoclastogenesis,36,37 identifying osteoporosis as a potential complication of therapeutic inhibition of Notch activity in humans. Consistently, we also found that the addition of the γ-secretase inhibitor L-685,458 in vitro significantly increased the number of mature osteoclasts differentiating from peripheral blood adherent mononuclear cells in the presence of M-CSF + RANKL. On the other hand, recently we have demonstrated that Nutlin-3 suppresses osteoclastic maturation.40 In this context, an additional important finding of our study was that although Nutlin-3 potently induced Notch1 accumulation in preosteoclasts, the simultaneous addition of Nutlin-3 + L-685,458 to normal preosteoclasts completely suppressed osteoclastic maturation, driven by M-CSF + RANKL.

In conclusion, we have demonstrated for the first time that Nutlin-3 induces the transcriptional activation of NOTCH1 in TP53+/−/− leukemic cell lines and primary B-CLL cells and that the simultaneous treatment with Nutlin-3 and γ-secretase inhibitors enhances the potential therapeutic efficacy of Nutlin-3. Moreover, the combined use of Nutlin-3 plus γ-secretase inhibitors might be safely used also in hematologic malignancies characterized by hyperactivation of osteoclastogenesis.

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Authorship

Contribution: P.S. conceived and designed the study, provided financial support, assembled, analyzed, and interpreted the data, and wrote the paper; E.M., M.G.d.I., E.R., and F.C. carried out the experiments, and collected, assembled, analyzed, and interpreted data; M.T. and V.G. were responsible for provision of study material, and analyzed and interpreted data; and G.Z. conceived and designed the study, provided financial support, wrote the paper, and was responsible for final approval of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Table 2. Combination index values for effects of Nutlin-3 and γ-secretase inhibitor L-685,458 on cell viability

<table>
<thead>
<tr>
<th>Sample</th>
<th>ED50</th>
<th>ED75</th>
<th>ED90</th>
<th>Average CI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKW6.4</td>
<td>0.22</td>
<td>0.35</td>
<td>0.76</td>
<td>0.44</td>
</tr>
<tr>
<td>OCI</td>
<td>0.74</td>
<td>0.60</td>
<td>0.48</td>
<td>0.61</td>
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<tr>
<td>Patient no. 1</td>
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<td>1.31</td>
<td>2.36</td>
<td>1.47</td>
</tr>
<tr>
<td>Patient no. 2</td>
<td>0.42</td>
<td>0.81</td>
<td>1.59</td>
<td>0.94</td>
</tr>
<tr>
<td>Patient no. 3</td>
<td>0.52</td>
<td>0.50</td>
<td>0.54</td>
<td>0.52</td>
</tr>
<tr>
<td>Patient no. 4</td>
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<td>0.38</td>
<td>0.52</td>
<td>0.42</td>
</tr>
<tr>
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<td>0.62</td>
<td>0.30</td>
</tr>
<tr>
<td>Patient no. 6</td>
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<td>0.63</td>
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<td>0.68</td>
</tr>
<tr>
<td>Patient no. 7</td>
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<td>0.82</td>
<td>1.05</td>
<td>0.84</td>
</tr>
<tr>
<td>Patient no. 8</td>
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<td>0.93</td>
<td>1.61</td>
<td>0.98</td>
</tr>
<tr>
<td>Patient no. 9</td>
<td>0.21</td>
<td>0.45</td>
<td>0.96</td>
<td>0.54</td>
</tr>
<tr>
<td>Patient no. 10</td>
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<td>0.46</td>
<td>0.38</td>
<td>0.47</td>
</tr>
<tr>
<td>Patient no. 11</td>
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<td>0.47</td>
<td>0.53</td>
<td>0.47</td>
</tr>
<tr>
<td>Patient no. 12</td>
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<td>0.95</td>
<td>0.80</td>
</tr>
<tr>
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<td>0.17</td>
<td>0.26</td>
</tr>
<tr>
<td>Patient no. 14</td>
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<td>0.38</td>
<td>0.62</td>
<td>0.41</td>
</tr>
<tr>
<td>Patient no. 15</td>
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<td>0.80</td>
<td>0.51</td>
<td>0.86</td>
</tr>
<tr>
<td>Patient no. 16</td>
<td>0.52</td>
<td>0.60</td>
<td>0.70</td>
<td>0.61</td>
</tr>
</tbody>
</table>

ED indicates effect dose.

*The averaged combination index (CI) values were calculated from ED50, ED75, and ED90.
Nutlin-3 up-regulates the expression of Notch1 in both myeloid and lymphoid leukemic cells, as part of a negative feedback antiapoptotic mechanism

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