The sorafenib plus nutlin-3 combination promotes synergistic cytotoxicity in acute myeloid leukemic cells irrespectively of FLT3 and p53 status

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ABSTRACT

Background

Both the multi-kinase inhibitor sorafenib and the small molecule inhibitor of the MDM2/p53 interaction, nutlin-3, used alone, have shown promising anti-leukemic activity in acute myeloid leukemia cells. Thus, in this study we investigated the effect of the combination of sorafenib plus nutlin-3 in acute myeloid leukemia.

Design and Methods

Primary acute myeloid leukemia blasts (n=13) and FLT3$^{wild\text{-}type}$/p53$^{wild\text{-}type}$ (OCI-AML3), FLT3^{mutated}/p53$^{wild\text{-}type}$ (MOLM), FLT3^{mutated}/p53^{mutated} (MV4-11), FLT3$^{wild\text{-}type}$/p53^{deleted} (HL60) or FLT3$^{wild\text{-}type}$/p53^{mutated} (NB4) acute myeloid cell lines were exposed to sorafenib, used alone or in association with nutlin-3 at a 1:1 ratio, in a range of clinically achievable concentrations (1-10 μM). Induction of apoptosis and autophagy was evaluated by transmission electron microscopy and by specific flow cytometry analyses. The levels of Mcl-1, p53 and Bak proteins were analyzed by western blotting. Knock-down of Bax and Bak gene expression was performed in transfection experiments with specific short interfering RNA.

Results

The sorafenib+nutlin-3 drug combination exhibits synergistic cytotoxicity in primary acute myeloid leukemia blasts and in acute myeloid leukemia cell lines with maximal cytotoxicity in FLT3^{mutated} MV4-11 and MOLM, followed by the FLT3$^{wild\text{-}type}$ OCI-AML3, HL60 and NB4 cell lines. The cytotoxic activity of sorafenib+nutlin-3 was characterized by an increase of both apoptosis and autophagy. Moreover, Bax and Bak showed prominent roles in mediating the decrease of cell viability in response to the drug combination in p53$^{wild\text{-}type}$ OCI-AML3 and p53^{deleted} HL-60 cells, respectively, as demonstrated in transfection experiments performed with specific short interfering RNA.

Conclusions

Our data demonstrate that acute myeloid leukemia cells show a variable but overall good susceptibility to the innovative therapeutic combination of sorafenib+nutlin-3, which differentially involves the pro-apoptotic Bcl-2 family members Bax and Bak in p53$^{wild\text{-}type}$ and p53^{deleted} cells.

Key words: sorafenib, nutlin-3, acute myeloid leukemia, p53, apoptosis, autophagy.

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The online version of this article has a Supplementary Appendix.

Introduction

The second-generation protein kinase inhibitors constitute a new class of pharmacological compounds with the potential to target acute myeloid leukemia (AML) cells.[1] Sorafenib (Nexavar) was found to be particularly active in six of seven FMS-like tyrosine kinase-3-internal tandem duplication (FLT3)-ITD-positive patients included in a phase 1 clinical trial performed on 16 AML patients,[2] as well as in FLT3-ITD positive cell lines.[3] In addition, complete molecular remission has been reported in a subset of AML patients treated with sorafenib,[4] while a long-term response was observed in refractory and relapsed disease before and after allogeneic stem cell transplantation.[5] At the molecular level, sorafenib shows the ability to inhibit several critical tyrosine kinases, besides FLT3-ITD, such as vascular endothelial growth factor receptor (VEGFR-2), c-Kit, Ret, as well as the serine threonine kinase RAF1, an upstream activator of the ERK1/ERK2 pathway.[2-5]

In spite of this promising clinical evidence, recent studies suggest that after a favorable initial outcome, subsequent non-responsiveness occurs.[6] It is, therefore, unlikely that sorafenib will display maximal efficacy as monotherapy and combined therapies have recently been proposed.[7] In this context, we sought to investigate the anti-leukemic activity of this multi-kinase inhibitor in combination with nutlin-3, a small molecule inhibitor of the MDM2/p53 interaction.[8] The rationale for testing this association relies on solid data demonstrating that mutations and/or deletions of p53 in hematologic malignancies are present in less than 20% of patients at diagnosis.[9] In addition, recent studies have demonstrated that nutlin-3, used alone or in combination with chemotherapeutic drugs, effectively increases the degree of cytotoxicity in AML.[10-12] Finally, a reciprocal interplay between MDM2 and FLT3 in AML cells has been proposed.[13]

Thus, we investigated the potential therapeutic efficacy of sorafenib in association with nutlin-3 in primary AML blasts as well as in a panel of myeloid leukemic cells with different FLT3 ($FLT3^{wild\text{-}type}$ and $FLT3^{mutated}$) and p53 ($p53^{wild\text{-}type}$, $p53^{deleted}$ or $p53^{mutated}$) status.

Design and Methods

Primary samples from patients with acute myeloid leukemia and leukemic cell lines

For experiments with primary cells, peripheral blood samples from 13 AML patients, diagnosed according to French-American-British (FAB) criteria, were collected into heparin-coated tubes following informed consent, in accordance with the Declaration of Helsinki and in agreement with institutional guidelines after Ethics Committee approval (Udine University-Hospital and Cremona Hospital). The main clinical parameters of the patients were abstracted from clinical records and are reported in *Online Supplementary Table S1*.

The $p53^{wild\text{-}type}$ (OCI-AML3 and MOLM), $p53^{mutated}$ (NB4 and MV4-11) and $p53^{deleted}$ (HL60) leukemic cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) or the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DSMZ; Braunschweig, Germany).

Further details on primary AML samples and leukemic cell lines cultures are described in the *Online Supplementary Design and Methods*.

Culture treatments and flow cytometric assessment of cell cycle, apoptosis and autophagy

Leukemic cells were treated with sorafenib (range 1-10 μM, Custom Selleck Chemicals, Houston, TX, USA) and nutlin-3 (range 1-10 μM, Cayman Chemicals, Ann Arbor, MI, USA), used alone or in combination. At different time points after treatment, cell viability was examined by trypan blue dye exclusion, as previously described.[14,15] Details of the cytometric analysis of cell cycle,[9,11] apoptosis and autophagy are provided in the *Online Supplementary Design and Methods.*

Transmission electron microscopy

For transmission electron microscopy (TEM), cells were centrifuged at 2000 rpm for 5 min and the pellet fixed with 2.5% glutaraldehyde/0.1 M phosphate-buffered saline (pH 7.3) overnight at 4°C. After several rinses with phosphate-buffered saline, the cells were post-fixed in 1% osmium tetroxide at 4°C for 3 h and then rinsed thoroughly with distilled water, dehydrated by graded ethanol, and freeze-dried. The specimens were sputter-coated with platinum and examined with a TEM H-800 (Hitachi) for the presence of putative autophagic features. The apoptotic and autophagic cells were quantified by counting a total of 500 cells, scoring several fields (magnification 1000X) for each culture condition.

Antibody arrays, western blot and immunoprecipitation analyses

Details on antibody arrays, western blot[16,17] and immunoprecipitation analyses[18] are provided in the *Online Supplementary Design and Methods*.

Transfection experiments

Leukemic cells ($1.2x10^6$ cells/0.1 mL) were mixed with either 1 μg of control enhanced green fluorescence protein (EGFP) plasmid or 2 μg of small interfering RNA (siRNA) cocktails, transferred to a 2.0-mm electroporation cuvette, and nucleofected with the nucleofector kit V (Amaxa, Cologne, Germany) using a nucleofector device (Amaxa Nucleofector II apparatus). After electroporation, cells were immediately transferred to a complete medium supplemented with 10% fetal calf serum and cultured at 37°C until analysis. Transfection efficiency was monitored in each experiment by scoring the percentage of EGFP-positive cells by flow cytometry analysis. For specific Bax and Bak gene knock-down, siRNA were designed and manufactured by Ambion Inc. (Woodward, Austin, TX, USA) according to the current guidelines for effective gene knock-down by this method. A cocktail of three different negative control siRNA, each comprising a 19 bp-scrambled sequence with 3' dT overhangs (Ambion's Silencer negative control siRNA), was used to demonstrate that the transfection did not induce non-specific effects on gene expression. The Ambion's Silencer negative control siRNA sequences have no significant homology to any known gene sequences from humans and they have been previously tested to ensure that they do not have non-specific effects on gene expression (Ambion).

RNA analyses

Details on RNA analyses are provided in the *Online Supplementary Design and Methods*.

Statistical analysis and assessment of the effect of combination treatment

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 $P<0.05$. In order to investigate the effect of the sorafenib+nutlin-3

combination, leukemic cells were treated with serial doses (range 1-10 μM) of sorafenib or nutlin-3, individually or in combination using a constant 1:1 ratio (sorafenib:nutlin-3). Results were analyzed with the method of Chou and Talalay[19] to determine whether combined treatment yielded greater effects than expected from summation alone: a combination index (CI) of 1 indicates an additive effect, while a CI below 1 indicates synergism. For this purpose cell viability data were analyzed with CalcuSyn software and reported either as dose-effect curves drawn directly by the CalcuSyn software (*Online Supplementary Table S1A*) or as CI values (*Online Supplementary Table S2*).

Results

The sorafenib+nutlin-3 combination exhibits synergistic cytotoxicity in both primary acute myeloid leukemia blasts and myeloid cell lines with different FLT3 and p53 status

In the first group of experiments, we investigated the cytotoxic effect of the combination of sorafenib+nutlin-3. Both primary AML cells from patients (*Online Supplementary Table S1*) and AML cell lines were exposed to serial concentrations (1-3-10 μM) of sorafenib and nutlin-3, used either alone or in combination at a constant 1:1 sorafenib:nutlin-3 ratio, and analyzed for cell viability by trypan blue dye exclusion 24 and 48 h after treatment. Synergy (average CI<1) of the sorafenib+nutlin-3 combination was documented in all primary AML samples from patients, including those cells bearing FLT3-ITD (*Online Supplementary Table S2* and *Online Supplementary Figure S1A,B*). Since the percentage of blasts was around 40% in several patients, we cannot exclude that the toxicity of the drug combination also affected non-leukemic peripheral blood mononuclear cells. However, it is noteworthy that sorafenib+nutlin-3 promoted synergistic cytotoxicity in all AML leukemic cell lines investigated (*Online Supplementary Table S2*), with $FLT3^{mutated}/p53^{mutated}$ (MV4-11) and $FLT3^{mutated}/p53^{wild\text{-}type}$ (MOLM) leukemic cells being the most sensitive to the drug combination, followed by $FLT3^{wild\text{-}type}/p53^{wild\text{-}type}$ (OCI-AML3), $FLT3^{wild\text{-}type}/p53^{deleted}$ (HL60) and $FLT3^{wild\text{-}type}/p53^{mutated}$ (NB4) (Figure 1). In this respect, it is noteworthy that treatment for 48 h with a low concentration of sorafenib+nutlin-3 (1 μM each) killed all MV4-11 cells and reduced the viability of MOLM cells by about 80% (Figure 1).

The sorafenib+nutlin-3 combination promotes both apoptosis and autophagy in $p53^{wild\text{-}type}$ and $p53^{deleted/mutated}$ leukemic cells

In order to appreciate the morphological and molecular aspects of the synergistic cytotoxicity of sorafenib+nutlin-3 better, we performed most of the following experiments on $FLT3^{wild\text{-}type}$ OCI-AML3 and HL60 cells, considering that in these cell lines the toxicity of the single agents (sorafenib or nutlin-3), as well as of the sorafenib+nutlin-3 combination, was not as substantial as observed in the $FLT3^{mutated}$ MOLM and MV4-11 cell lines (Figure 1). The concentrations (3-10 μM) of nutlin-3 and sorafenib used in the experiments performed on OCI-AML3 and HL60 cells were chosen on the basis of previous clinical studies demonstrating that when given twice daily at 400 mg, the maximum plasma concentrations of sorafenib reach 9.9 μM after 6 h,[20] 9.7 μM after 6 days[21] and 8.5 μM after 28 days.[22] When used at maximal concentration (10 μM) sorafenib almost completely abrogated the phosphorylation levels of ERK1/2 but not of other kinases, such as JNK and Akt (*Online Supplementary Figure S2A,B*). In addition, sorafenib variably down-regulated the phosphorylation levels of STAT transcription factor family members, while nutlin-3 had minor effects on these pathways and paradoxically increased the phosphorylation levels of ERK1/2 (*Online Supplementary Figure S2A*). Of note, the effect of sorafenib+nutlin-3 on the potential signaling mediators analyzed was not significantly different from the effects of sorafenib alone and even the suppressive effects of sorafenib on ERK1/2 phosphorylation prevailed over the induction by nutlin-3 (*Online Supplementary Figure S2A,B*). With respect to the drug concentrations used, it is also noteworthy that while our study was under consideration, another study showed a synergistic cytotoxic effect of sorafenib+nutlin-3 in renal cell carcinoma cells, using high concentrations of both sorafenib (up to 50 μM) and nutlin-3 (up to 20 μM).[23]

Before characterizing the aspects of cell death induced by the sorafenib+nutlin-3 combination, we investigated the potential anti-proliferative effects. As shown in *Online Supplementary Figure S3*, while nutlin-3 alone induces a prevalent accumulation in G_1 and G_2/M phases of the cell cycle in $p53^{wild\text{-}type}$ OCI-AML3 and $p53^{deleted}$ HL60 cells, respectively, as previously demonstrated by us and by other groups,[10-12] sorafenib had minor effects on the leukemic cell cycle, whether used alone or in combination with nutlin-3. In the next experiments, the morphological aspects of cell death were characterized by TEM (Figure 2) on cell cultures harvested after 24 h of drug treatment, to avoid the excessive cell death observed at 48 h (Figure 1). After treatment with sorafenib and nutlin-3, leukemic cells showed mixed aspects of apoptosis, such as nuclear shrinkage, chromatin condensation, and membrane blebbing, and of autophagy, such as membrane-bound vesicles occupying the major cytoplasmic space, which frequently contained electron-dense material of cytoplasmic fragments and organelles (Figure 2A,B). Higher magnification of images clearly revealed multiple or double membrane-bound autophagosomes with fragmented cellular organelles and debris inside (Figure 2B). By scoring independent fields, the most evident morphological aspect characterizing the dying leukemic cells treated with sorafenib+nutlin-3 with respect to cells treated with single drugs was the appearance of a significantly higher (P<0.05) percentage of cells showing features of apoptosis and autophagy (Figure 2C).

Consistently with the morphological data, analyses of leukemic cell cultures by flow cytometry confirmed the induction of both apoptosis and autophagy upon treatment with sorafenib+nutlin-3. In particular, the staining of leukemic cells with annexin V/7-amino-actinomycin D revealed a significant (P<0.05) increase in the percentage of apoptotic cells following treatment with the sorafenib+nutlin-3 combination, with respect to following treatment with either sorafenib or nutlin-3 used alone, in both $p53^{wild\text{-}type}$ OCI-AML3 and $p53^{deleted}$ HL60 leukemic cultures (Figure 3A). On the other hand, evidence of autophagy was first documented by analysis of the physical parameters FSC-H/SSC-H by flow cytometry (Figure 3B). Autophagic cells were represented by a distinct population of cells that were smaller and denser with respect to viable cells and that were observed in a significantly

higher percentages after treatment with sorafenib+nutlin-3 than after treatment with the single compounds (Figure 3B). The occurrence of autophagy was confirmed by flow cytometry analysis after MDC staining of both p53$^{wild\text{-}type}$ OCI-AML3 and p53^{deleted} HL60 cells (Figure 3C), as well as by western blot analysis of autophagosome-associated generation of endogenous LC3-II (Figure 3D). It is noteworthy that similar results were confirmed by exposing FTL-3^{mutated} MV4-11 leukemic cells to low concentrations of sorafenib+nutlin-3 (1 μM each), as shown in *Online Supplementary Figure S4A-D*. In addition, cell viability of OCI-AML3 and HL60 cultures was analyzed after pre-treatment with a specific autophagy inhibitor[24] (3MA) before adding the sorafenib+nutlin-3 combination. As shown in *Online Supplementary Figure S5*, in the presence of 3MA a small increase of cell viability was observed upon drug treatment. Of note, pre-treatment with 3MA down-modulated autophagy and induced a concomitant increase of apoptosis. Taken together, these data indicate that both apoptosis and autophagy, which are intimately intertwined processes, are involved in the cytotoxic response of leukemic cells to sorafenib+nutlin-3.

Molecular interplay between sorafenib and nutlin-3 in p53$^{wild\text{-}type}$ leukemic cells

It has been previously shown that Mcl-1 degradation is an early event not only following induction of apoptosis, but also under conditions in which Mcl-1 levels regulate activation of autophagy.[25] In particular, we have recently shown that Mcl-1 shows a marked decline in myeloid leukemic cells as early as 2 h after treatment with sorafenib.[26] Thus, in order to investigate the mechanism underlying the synergistic cytotoxicity of sorafenib+nutlin-3 in AML cells, we next investigated changes in the levels of Mcl-1 and p53, two known targets of sorafenib and nutlin-3, respectively. Consistently with previous studies in both leukemic cells[26] and solid tumor models,[27] sorafenib alone showed the ability to down-regulate Mcl-1 in lysates obtained from primary AML blasts as well as OCI-AML3 and HL60 cells (Figure 4A). Although nutlin-3 alone showed either no effects or a paradoxical induction of Mcl-1 (Figure 4A), in keeping with its ability to induce ERK1/2 phosphorylation (*Online Supplementary Figure S2A,B*), the sorafenib+nutlin-3 combination promoted potent down-regulation or a complete shut-off of Mcl-1 protein (Figure 4A), indicating that the suppressive activity of sorafenib was prominent with respect to the induction of Mcl-1 by nutlin-3. On the other hand, nutlin-3 alone selectively induced p53 accumulation in p53$^{wild\text{-}type}$ OCI-AML3 but not in p53^{deleted} HL60 cells (Figure 4B), while sorafenib alone had no effects on p53. Of note, the association of sorafenib+nutlin-3 did not interfere with the accumulation of p53 protein induced by nutlin-3 in either primary AML blasts or p53$^{wild\text{-}type}$ OCI-AML3 cells (Figure 4B). With respect to the p53 transcriptional activity, however, it is noteworthy that sorafenib+nutlin-3 induced signifi-

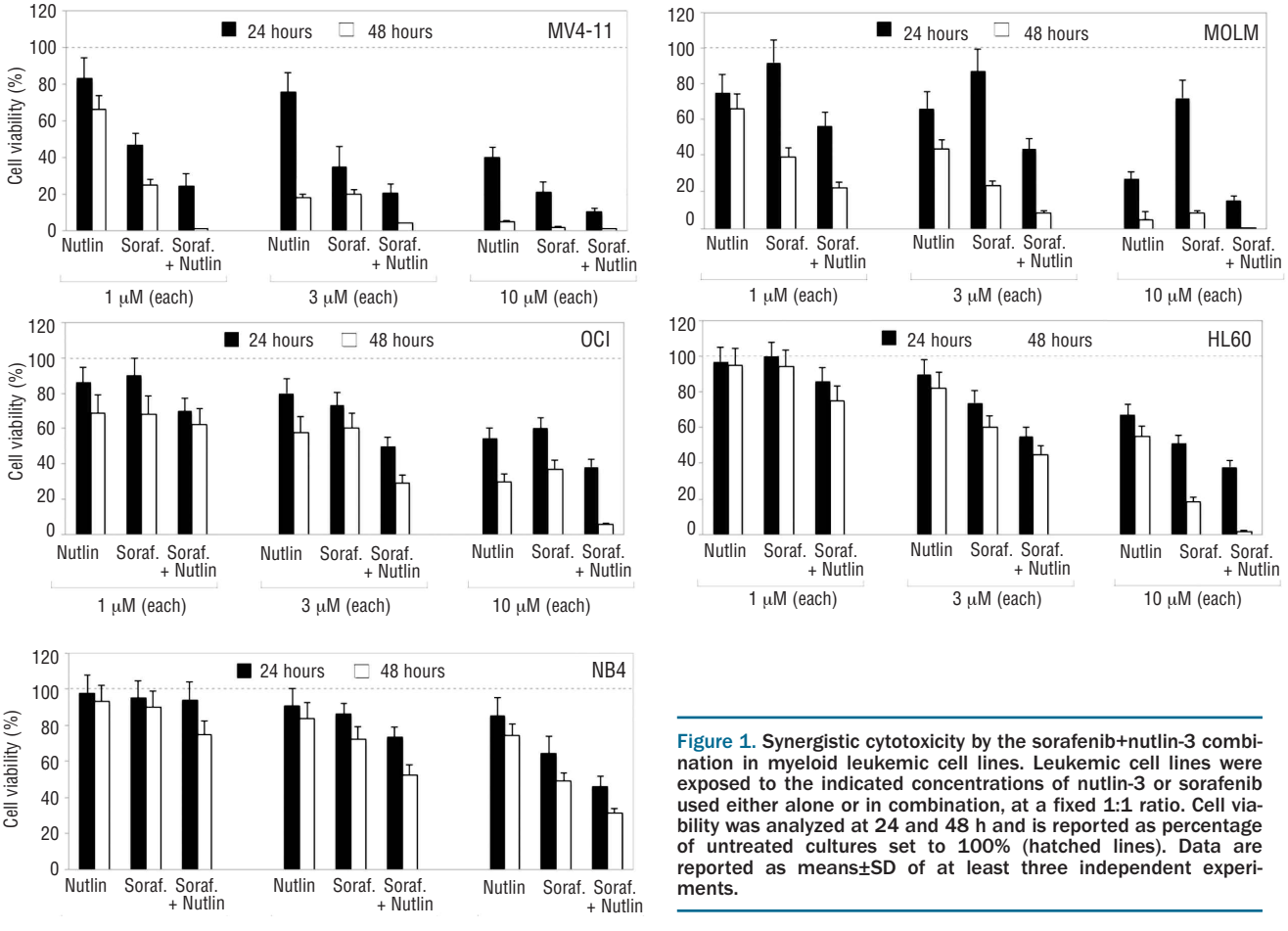

Figure 1. Synergistic cytotoxicity by the sorafenib+nutlin-3 combination in myeloid leukemic cell lines. Leukemic cell lines were exposed to the indicated concentrations of nutlin-3 or sorafenib used either alone or in combination, at a fixed 1:1 ratio. Cell viability was analyzed at 24 and 48 h and is reported as percentage of untreated cultures set to 100% (hatched lines). Data are reported as means±SD of at least three independent experiments.

cantly (P<0.05) higher levels of Bax and MDM2 than did nutlin-3 alone in OCI-AML3 cells but not in HL60 cells (Figure 4C). In order to start to address the molecular mechanism mediating the cytotoxic activity of sorafenib+nutlin-3, it is worth noting that a previous study showed that the autophagic response is converted to apoptosis by the concomitant activation of Bax.[25] Thus, in subsequent experiments OCI-AML3 and HL60 cells were transfected with Bax siRNA and then treated with sorafenib+nutlin-3 (Figure 4D). Down-regulation of Bax efficiently (P<0.05) counteracted the cytotoxicity of sorafenib+nutlin-3 in OCI-AML3 but not in HL60 cells (Figure 4D), suggesting that Bax plays a prominent role in mediating the sorafenib+nutlin-3 cytotoxicity in $p53^{wild\text{-}type}$ cells.

Molecular interplay between sorafenib and nutlin-3 in $p53^{deleted}$ leukemic cells

The ability of sorafenib to potentiate the p53 transcriptional activity of Bax may account for the synergistic cytotoxicity of sorafenib+nutlin-3 in $p53^{wild\text{-}type}$ cells, while it does not explain why this drug combination promotes cytotoxicity also in $p53^{deleted}$ HL60 cells. In this respect, it is noteworthy that the pro-apoptotic Bcl-2 family member Bak interacts with Mcl-1 under normal conditions and is released to be activated upon cell death stimuli.[18] As shown in Figure 5A, sorafenib+nutlin-3 selectively increased the levels of both total and the conformationally active form of Bak in $p53^{deleted}$ HL60 but not in $p53^{wild\text{-}type}$ OCI-AML3 cells. To elucidate the functional role of Bak activation in mediating the cytotoxic activity of sorafenib+nutlin-3, leukemic cells were transfected with Bak siRNA before exposure to sorafenib+nutlin-3 (Figure 5B). Down-regulation of Bak significantly (P<0.05) counteracted cytotoxicity of the drug combination in HL60 cells whereas it had little effect in OCI-AML3 cells (Figure 5B), suggesting that sorafenib+nutlin-3-induced cytotoxicity in $p53^{deleted}$ HL60 cells is Bak-dependent.

Discussion

We have demonstrated for the first time that the sorafenib+nutlin-3 combination exhibits synergistic cytotoxicity in primary AML blasts as well as in a panel of myeloid cell lines. In all the cell lines and primary AML blasts investigated, we found that the cytotoxicity of sorafenib+nutlin-3 showed aspects of both autophagy and apoptosis, which are intimately intertwined processes. The greatest susceptibility to the drug combination was observed in the $FLT3^{mutated}$ MV4-11 and MOLM cell lines, a finding particularly relevant since it has been shown that

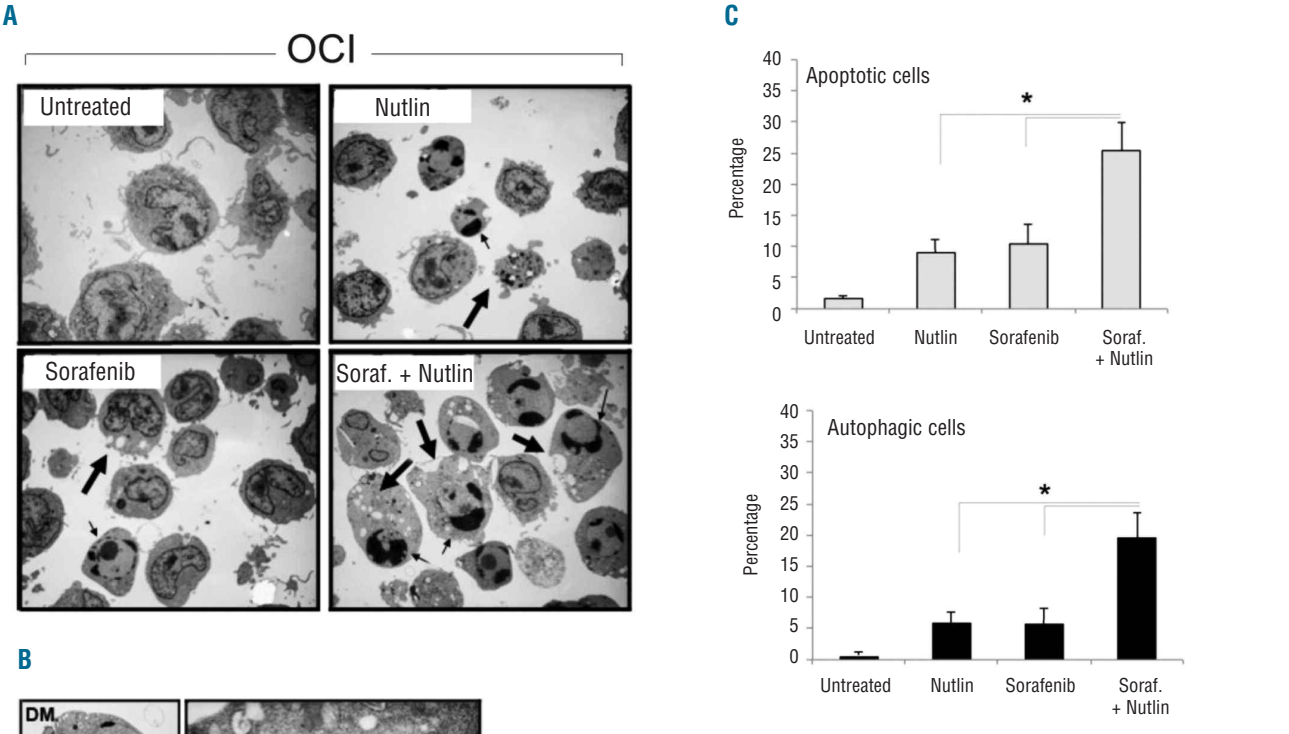

Figure 2. Sorafenib+nutlin-3 promote both apoptosis and autophagy in myeloid leukemic cells. OCI cells were exposed for 24 h to nutlin-3 and sorafenib used either alone or in combination (10 μM each) before analysis by transmission electron microscopy. (A) Representative images of marked morphological modifications, including autophagocytic vacuolization (thick black arrows) and apoptotic chromatin condensation (thin black arrows), frequently observed in cell cultures treated with sorafenib+nutlin-3 (magnification, 2.500X). (B) High-magnification images of autophagosomes (A), an autophagolysosome (AL) and a double membrane vesicle (DM) (a: magnification, 5,000X; b: magnification, 30,000X). (C) Cells with morphological aspects of apoptosis and autophagy were quantified by counting a total of 500 cells (magnification 1,000X) for each culture condition. Data are expressed as means±SD of 11 independent fields. *P<0.05.

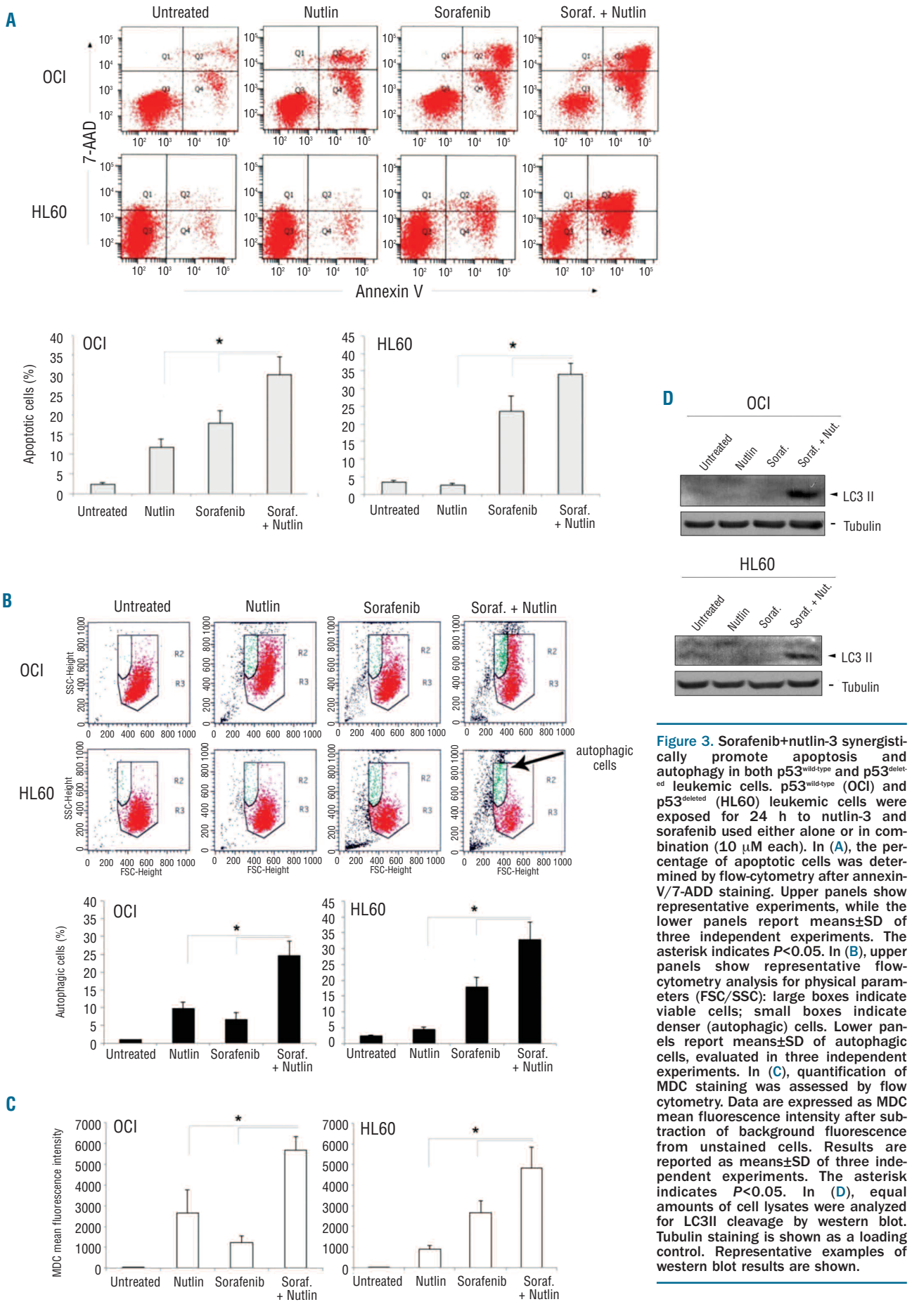

Figure 3. Sorafenib+nutlin-3 synergistically promote apoptosis and autophagy in both $p53^{wild\text{-}type}$ and $p53^{deleted}$ leukemic cells. $p53^{wild\text{-}type}$ (OCI) and $p53^{deleted}$ (HL60) leukemic cells were exposed for 24 h to nutlin-3 and sorafenib used either alone or in combination (10 μM each). In (A), the percentage of apoptotic cells was determined by flow-cytometry after annexin-V/7-ADD staining. Upper panels show representative experiments, while the lower panels report means±SD of three independent experiments. The asterisk indicates $P<0.05$. In (B), upper panels show representative flow-cytometry analysis for physical parameters (FSC/SSC): large boxes indicate viable cells; small boxes indicate denser (autophagic) cells. Lower panels report means±SD of autophagic cells, evaluated in three independent experiments. In (C), quantification of MDC staining was assessed by flow cytometry. Data are expressed as MDC mean fluorescence intensity after subtraction of background fluorescence from unstained cells. Results are reported as means±SD of three independent experiments. The asterisk indicates $P<0.05$. In (D), equal amounts of cell lysates were analyzed for LC3II cleavage by western blot. Tubulin staining is shown as a loading control. Representative examples of western blot results are shown.

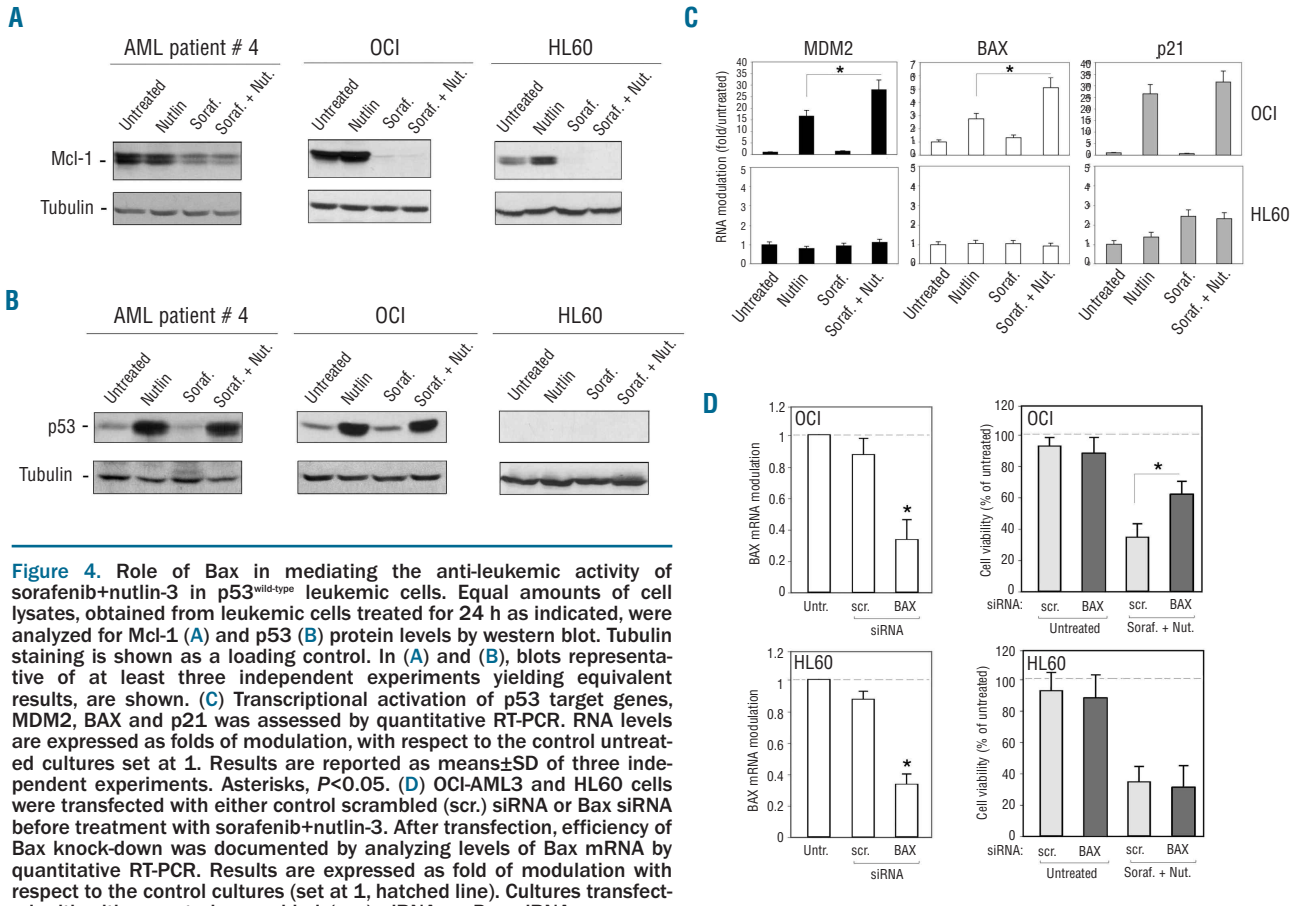

Figure 4. Role of Bax in mediating the anti-leukemic activity of sorafenib+nutlin-3 in p53$^{wild\text{-}type}$ leukemic cells. Equal amounts of cell lysates, obtained from leukemic cells treated for 24 h as indicated, were analyzed for Mcl-1 (A) and p53 (B) protein levels by western blot. Tubulin staining is shown as a loading control. In (A) and (B), blots representative of at least three independent experiments yielding equivalent results, are shown. (C) Transcriptional activation of p53 target genes, MDM2, BAX and p21 was assessed by quantitative RT-PCR. RNA levels are expressed as folds of modulation, with respect to the control untreated cultures set at 1. Results are reported as means±SD of three independent experiments. Asterisks, $P<0.05$. (D) OCI-AML3 and HL60 cells were transfected with either control scrambled (scr.) siRNA or Bax siRNA before treatment with sorafenib+nutlin-3. After transfection, efficiency of Bax knock-down was documented by analyzing levels of Bax mRNA by quantitative RT-PCR. Results are expressed as fold of modulation with respect to the control cultures (set at 1, hatched line). Cultures transfected with either control scrambled (scr.) siRNA or Bax siRNA were analyzed for cell viability upon exposure to sorafenib+nutlin-3. Results are expressed as percentage of viable cells with respect to the control cultures (set at 100%, hatched line). Data are reported as means±SD of results from three independent experiments. Asterisks, $P<0.05$.

FLT3-ITD can up-regulate Mcl-1 and inactivate p53$^{wild\text{-}type}$ through hyper-phosphorylation to promote survival of stem cells in AML.[28-30] Although most of the mechanistic experiments were generated in FLT3$^{wild\text{-}type}$ OCI-AML3 and HL60 cells, further investigation of mechanistic studies in FLT3^{mutated} AML cells are warranted.

A large body of data has shown that there are two main types of programmed cell death: type I, also called apoptosis, refers specifically to a genetically controlled process involving transcription of specific proteins, such as the Bcl-2 pro-apoptotic family members and leading eventually to a cell's demise.[31,32] Type II programmed cell death, also called autophagy, is a non-selective process in which cytoplasm and organelles are (apparently) randomly assorted into the autophagosome, where they are degraded.[33-35] The levels of autophagy in cells treated with a combination of sorafenib+nutlin-3 rapidly increased, as judged by increased processing of LC3 to LC3II, morphological tests and flow cytometry assays. Since sorafenib alone,[36] similarly to other stress inducers,[37,38] can induce either a protective form of autophagy or a toxic form of autophagy, which appears to be based on the stimulus and the tumor cell type being examined, the ability of the small molecule inhibitor 3MA to increase the number of apoptotic cells suggests that the sorafenib+nutlin-3 combination induces a protective form of autophagy in AML cell models.

At the molecular level, it is noteworthy that while nutlin-3 was unable to down-regulate Mcl-1 and rather promoted an increase of Mcl-1 levels in both p53$^{wild\text{-}type}$ OCI-AML3 and p53^{deleted} HL60 cells, sorafenib potently down-regulated Mcl-1 both when used alone and when used in association with nutlin-3 in all leukemic cell lines investigated. In addition, the combined treatment selectively enhanced the transcriptional activation of the p53 target pro-apoptotic gene Bax in p53$^{wild\text{-}type}$ AML leukemic cells with respect to nutlin-3 alone. This is an important finding since, in contrast to solid tumors, more than 80% of the AML cases at diagnosis comprise wild-type p53,[5] but, on the other hand, the p53 inhibitor MDM2 is usually strongly expressed in AML, contributing to blocking the effect of p53.[6] The role of Bax up-regulation in mediating the synergistic cytotoxicity of sorafenib+nutlin-3 in p53$^{wild\text{-}type}$ leukemic cells, but not in p53^{deleted} cells, was underscored in knock-down experiments performed with siRNA for Bax.

Another important finding of our study was that the synergistic cytotoxicity of sorafenib+nutlin-3 was documented also in p53^{deleted} HL60 cells, clearly suggesting that nutlin-3 also exerts p53-independent effects. In this respect, we have demonstrated that sorafenib+nutlin-3 act synergistically in promoting the up-regulation of Bak in

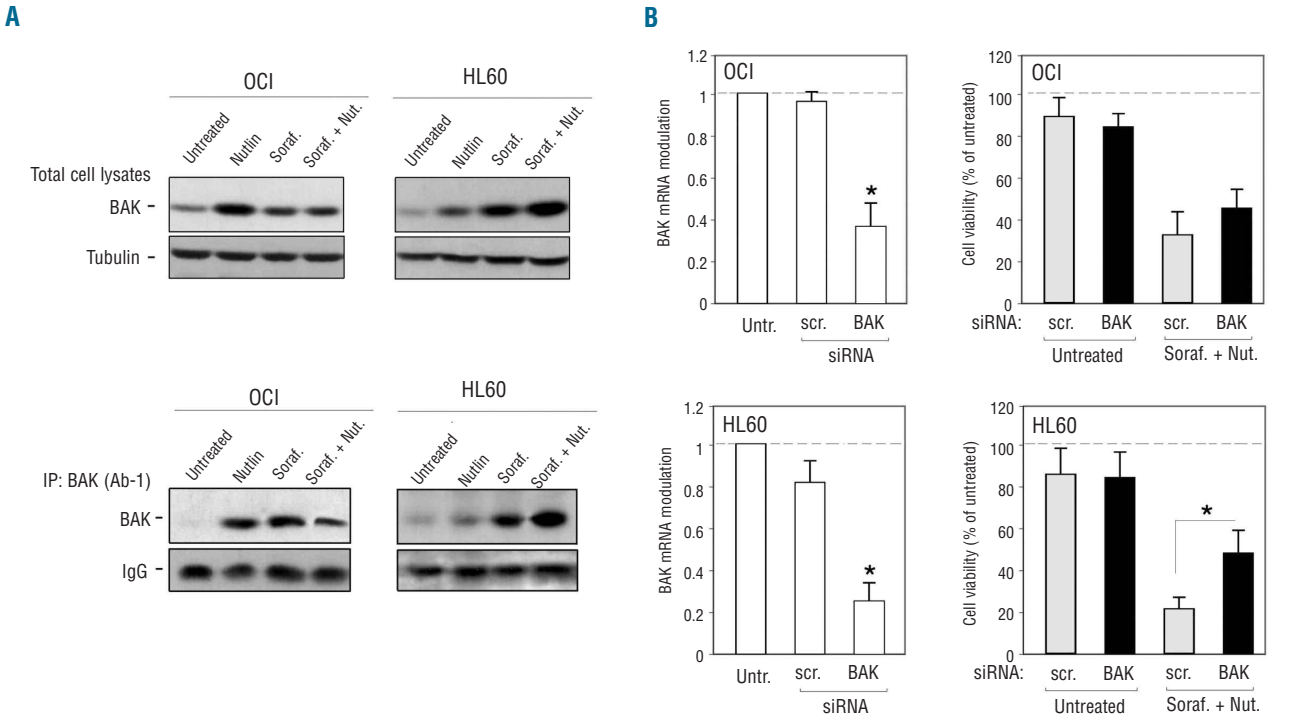

Figure 5. Role of Bak in mediating the anti-leukemic activity of sorafenib+nutlin-3 in p53^{deleted} leukemic cells. (A) Equal amounts of cell lysates, obtained from OCI-AML3 and HL60 leukemic cells, treated for 24 h as indicated, were analyzed for Bak protein by western blot peformed on total cell lysates or after immunoprecipitation (IP) of conformationally active Bak. Blots representative of three independent experiments, yielding equivalent results, are shown. (B) OCI-AML3 and HL60 cells were transfected with either control scrambled (scr.) siRNA or Bak siRNA before treatment with sorafenib+nutlin-3. After transfection, the efficiency of Bak knockdown was documented by analyzing levels of Bak mRNA by quantitative RT-PCR. Results are expressed as fold of modulation with respect to the control cultures (set at 1, hatched line). Cultures transfected with either control scrambled (scr.) siRNA or Bak siRNA were analyzed for cell viability upon exposure to sorafenib+nutlin-3. Results are expressed as percentage of viable cells with respect to the control cultures (set at 100%, hatched line). Data are reported as means±SD of results from three independent experiments. Asterisks, $P<0.05$.

HL60 cells and the role of Bak in mediating the cytotoxicity of sorafenib+nutlin-3 in this cell line was suggested by knock-down experiments performed with siRNA specific for Bak. The ability of nutlin-3 to up-regulate Bak in synergism with sorafenib may be due to the existence of a molecular mimicry between MDM2 and the anti-apoptotic family members Bcl-2 and Bcl-Xl,[39,40] which suggests that nutlin-3 could bind with comparable efficacy to MDM2 and to Bcl-2 and Bcl-Xl and free Bak from sequestration.

In conclusion, we have established for the first time that the sorafenib+nutlin-3 combination is particularly active in FLT3^{mutated} leukemic cells but also in FLT3$^{wild/type}$/p53$^{wild\text{-}type}$ and in FLT3$^{wild/type}$/p53^{deleted} leukemic cells, opening new therapeutic perspectives for the combined use of sorafenib+nutlin-3 in the majority of AML cases.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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