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

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Online Supplementary Design and Methods

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

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation with lymphocyte cell separation medium (Cedarlane Laboratories, Hornby, Ontario, Canada) and cultured in RPMI 1640 (Lonza, Walkersville, MD, USA) containing 10% fetal bovine serum, L-glutamine and penicillin/streptomycin (GIBCO BRL, Grand Island, NY, USA). Cell lines were cultured in RPMI 1640 (MOLM, NB4 and HL60), ISCOVE (MV4-11) or α-MEM (OCI-AML3), all from Lonza, supplemented with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin (GIBCO). OCI-AML3, NB4 and HL60 cells are $FLT3^{wild\text{-}type}$ while MOLM and MV4-11 are characterized by the presence of FLT3-ITD ($FLT3^{mutated}$).

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

Cell cycle profile was analyzed by flow cytometry after 5-bromodeoxyuridine (BrdU) incorporation.[1] Briefly, leukemic cells were incubated with 50 μM BrdU (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 1 h. After incubation with the anti-BrdU antibody (BD Biosciences Pharmingen, San Diego, CA, USA), binding to BrdU incorporated into neosynthesized DNA, the complex was detected by fluorescein isothiocyanate-conjugated secondary antibody (Immunotech, Marseille, France). Cells were then stained with propidium iodide (PI, 50 μg/mL) and analyzed by flow cytometry as previously described.[2]

Apoptotic and autophagic cells were initially distinct from viable cells by using physical parameters of forward and side light scatter-height (FSC-H and SSC-H) at flow cytometry examination. In order to quantify the degree of apoptosis, leukemic cells were stained by annexin V/7-amino-actinomycin D (7-AAD) using the annexin V apoptosis detection kit I (BD Biosciences Pharmingen) following the manufacturer's instructions. In order to quantify autophagy, leukemic cells were stained for 1 h at 37°C with monodansylcadaverine (MDC, 50 μM, Sigma-Aldrich) and then analyzed by flow cytometry. In some experiments, leukemic cells were pre-treated for 5 h with the pharmacological inhibitor of autophagy 3-methyladenine (3MA, 2.5 mM, Sigma-Aldrich).

Antibody arrays, western blot and immunoprecipitation analyses

For antibody arrays, 300 μg of cellular extracts were incubated with the human phospho-kinase array kit (Proteome Profiler™; R&D Systems, Abingdon, UK) following the manufacturer's instructions. 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.

For western blot and preparation of total cell lysates, cells were lysed as previously described[3,4] and proteins were determined using the BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). Samples were supplemented with loading buffer (250 mM Tris pH 6.8, 2% sodium dodecylsulfate, 10% glycerin, 4% β-mercaptoethanol, 1% bromophenol blue) and boiled for 2 min. Equal amounts of protein for each sample were migrated in sodium dodecylsulfate-polyacrylamide gels and blotted onto nitrocellulose filters. The following antibodies were used: monoclonal anti-Mcl-1 (S-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-p53 (DO-1, Santa Cruz), polyclonal anti-Bak (Millipore, Billerica, MA, USA), monoclonal anti-phospho-Thr202/Tyr204 p44/42 MAPK (ERK1/2) and anti-ERK1/2 (both from Promega, Madison, WI, USA), monoclonal anti-LC3 and anti-tubulin (both from Sigma-Aldrich). After incubation with anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich), specific reactions were revealed with the ECL Lightning detection kit (Perkin Elmer, Boston, MA, USA). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

To evaluate the active Bak conformation specifically, cells were lysed in buffer containing 1% CHAPS, 150 mM NaCl, 150nM KCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, pH 7.4, anti-proteases, and protease inhibitors. Subsequently, 300 μg of protein lysates were pre-cleared with Protein A/Protein G Sepharose 4 Fast Flow mixed media (GE Healthcare, Chalfont St. Giles, UK). Next, the pre-cleared lysates were subjected to immunoprecipitation with 2 μg of

anti-Bak monoclonal antibody-1 (Calbiochem, La Jolla, CA, USA), recognizing only Bak protein that has undergone conformational changes and, in the negative controls, isotypic control Ig antibodies. Immunoprecipitates were then subjected to western blot analysis followed by detection with polyclonal anti-Bak (Millipore), as described previously.[5]

RNA analyses

Total RNA was extracted from cells using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The quality of the total RNA preparation was verified by agarose gel and, when necessary, further purification was performed with the RNeasy cleanup system (Qiagen) to remove chromatin DNA. Total RNA was transcribed into cDNA, using the QuantiTect Reverse Transcription kit (Qiagen). p21, MDM2, Bax and Bak gene expression was analyzed using the SYBR Green real-time polymerase chain reaction (PCR) detection method with SABiosciences RT2 Real-Time™ Gene expression assays, which include specific validated primer sets and PCR master mixes (SABiosciences, Frederick, MD, USA). All samples were run in triplicate using the real time thermal analyzer rotor-gene™ 6000 (Corbett, Cambridge, UK). Expression values were normalized to a housekeeping gene, *POLR2A*, amplified in the same sample.

References

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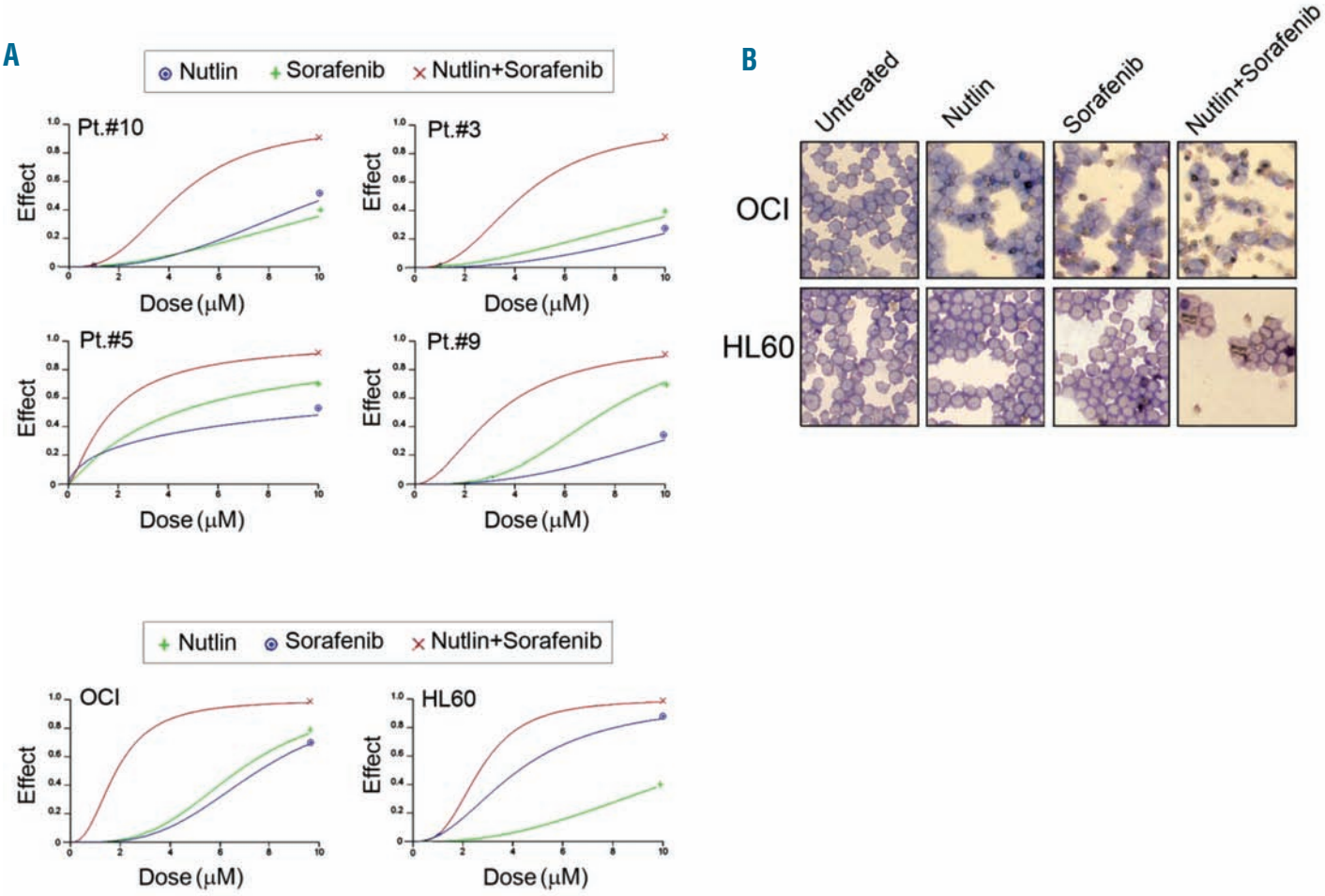
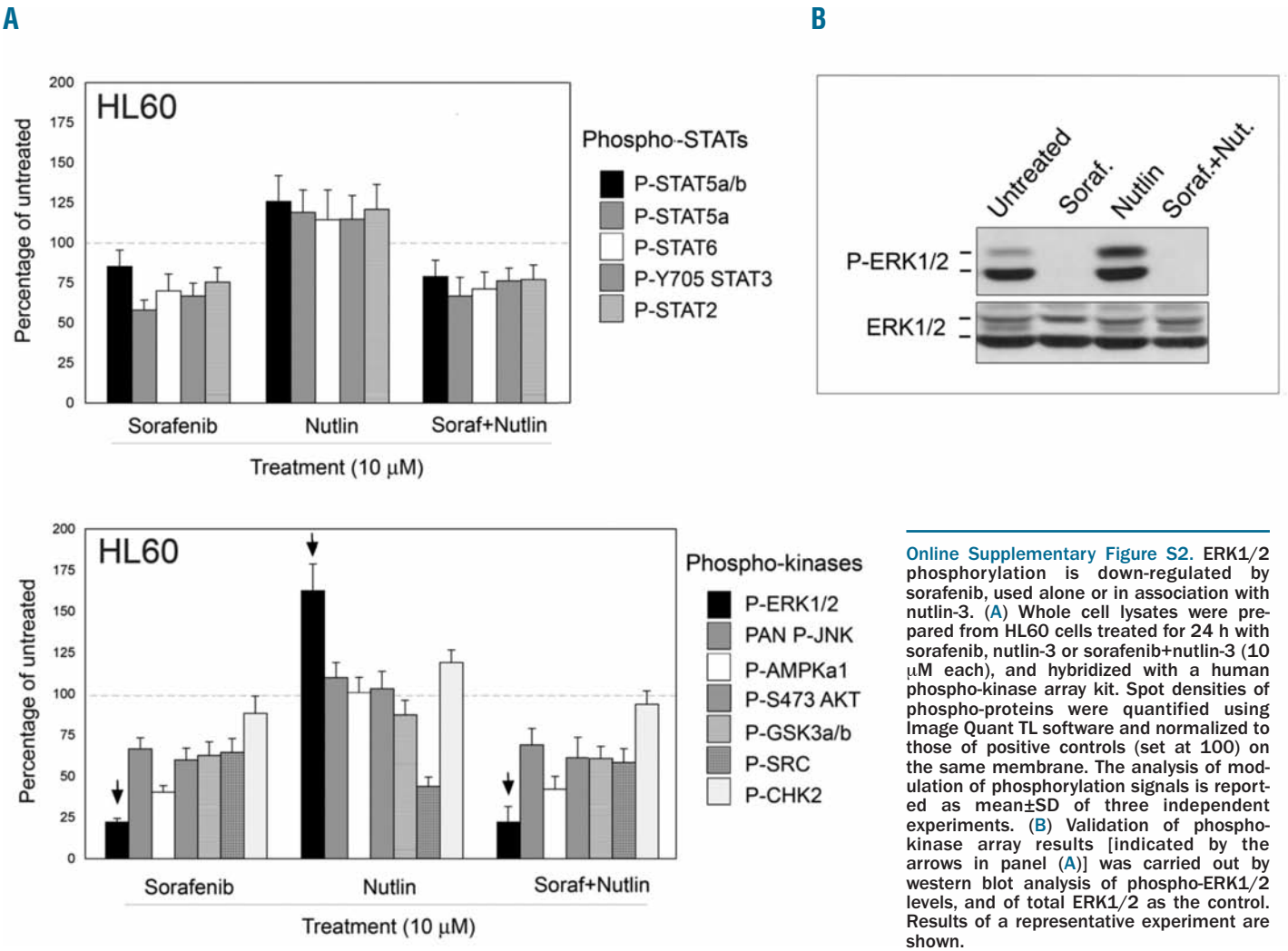
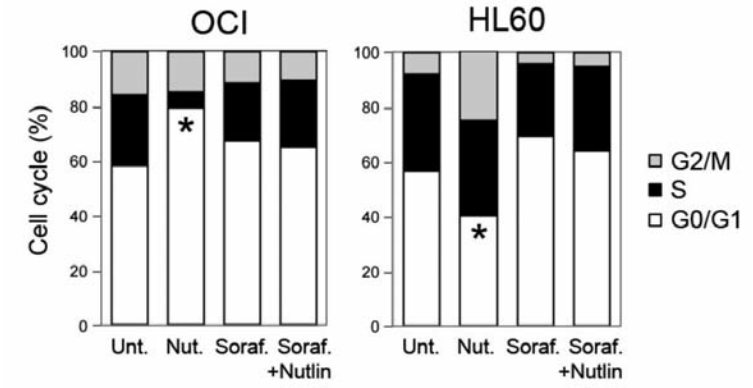


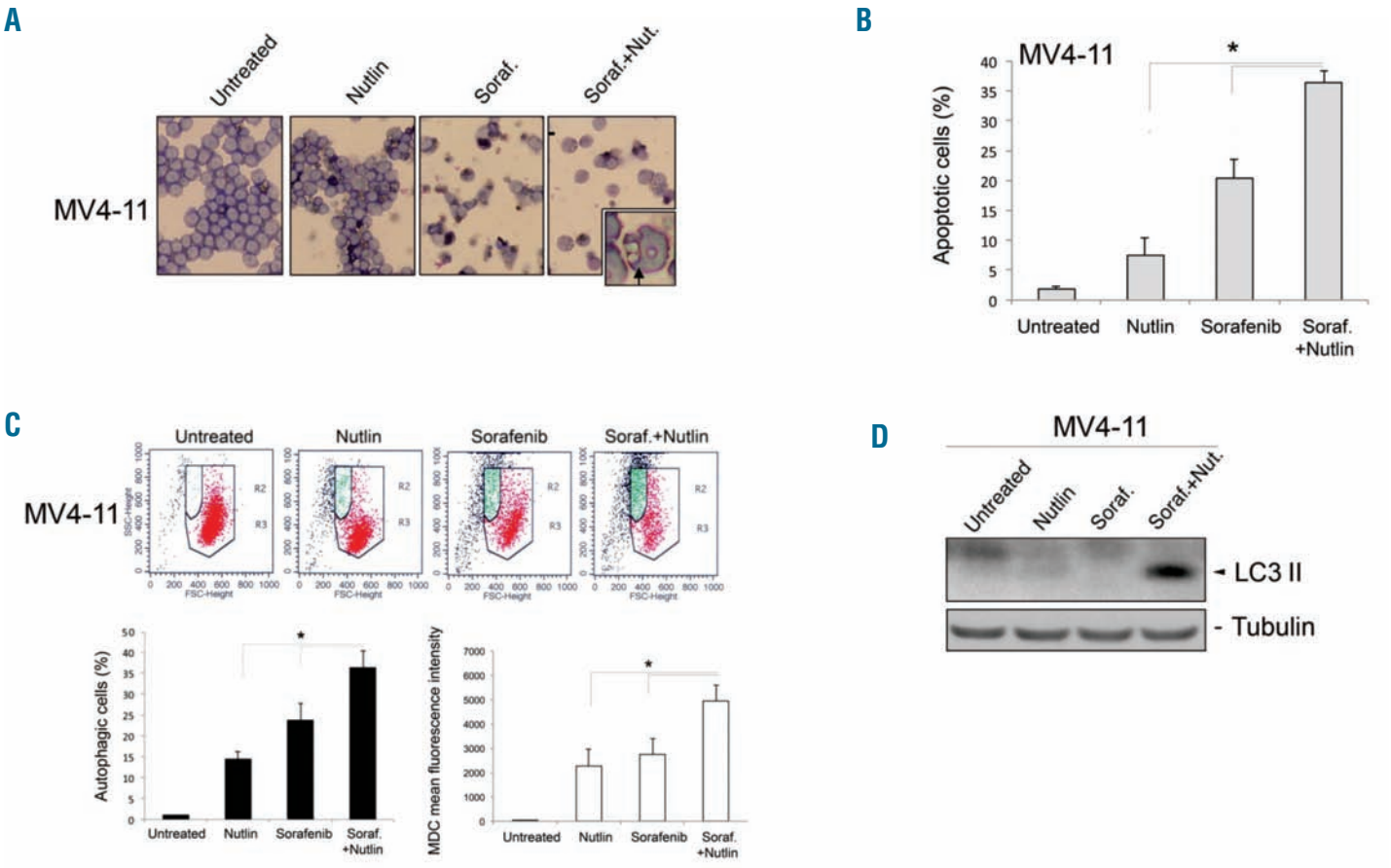
Online Supplementary Figure S1. Synergistic cytotoxicity by the sorafenib+nutlin-3 combination in AML leukemic cells. Primary AML leukemic cells from patients and myeloid cell lines were exposed for 48 h to serial doses of nutlin-3 and sorafenib, used either alone or in combination, at a fixed ratio. (A) Dose-effect plots, to determine drug efficacy, are shown for representative primary AML patients' samples and AML cell lines. The decrease of cell viability, labeled "effect" on the Y-axis, was determined in assays done at least twice in duplicate. (B) Light microscopy images of May-Grünwald-stained cell cultures exposed to nutlin-3 and sorafenib used either alone or in combination (10 μM each). Original magnification, 20X. Representative results are shown.



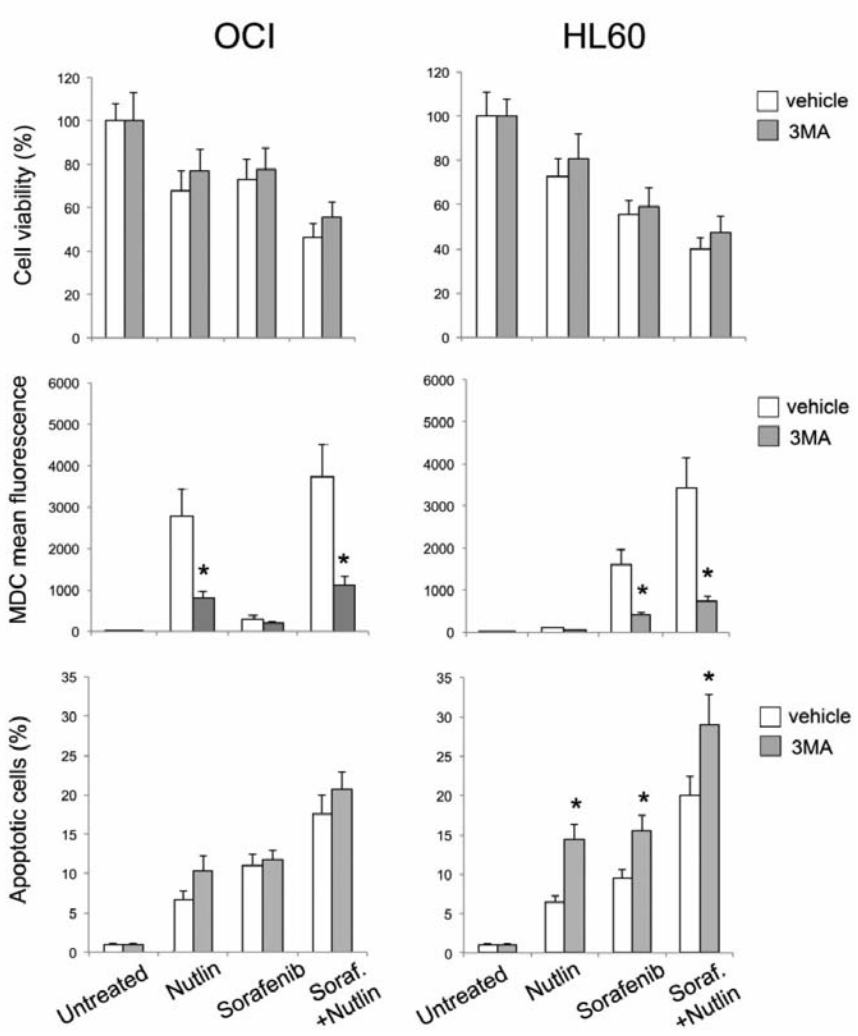
Online Supplementary Figure S2. ERK1/2 phosphorylation is down-regulated by sorafenib, used alone or in association with nutlin-3. (A) Whole cell lysates were prepared from HL60 cells treated for 24 h with sorafenib, nutlin-3 or sorafenib+nutlin-3 (10 μM each), and hybridized with a human phospho-kinase 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 is reported as mean±SD of three independent experiments. (B) Validation of phospho-kinase array results [indicated by the arrows in panel (A)] was carried out by western blot analysis of phospho-ERK1/2 levels, and of total ERK1/2 as the control. Results of a representative experiment are shown.



Online Supplementary Figure S3. Nutlin-3 selectively induces cell cycle block in G1 and G2/M in $p53^{wild\text{-}type}$ OCI-AML3 and $p53^{deleted}$ HL60, respectively. After 24 h of treatment with sorafenib, nutlin-3 or sorafenib+nutlin-3 (10 μM each), the distribution of OCI-AML3 and HL60 cells in the different phases of the cell cycle was calculated from the flow cytometry dot plots after BrdU/PI staining, and expressed as percentage of the total population. Asterisks indicate $P<0.05$ compared to Untreated (Unt.). Results of a representative experiment are shown.



Online Supplementary Figure S4. The sorafenib+nutlin-3 combination promotes both apoptosis and autophagy in FLT3^{mutated} MV4-11 cells. After exposure to nutlin-3 or sorafenib, used either alone or in combination (1 μM each), MV4-11 cells were analyzed by light microscopy (A) and flow cytometry for apoptosis (B) and autophagy (C). (B) The percentage of apoptotic cells was determined by flow-cytometry after annexin-V/7-ADD staining. Results are expressed as means±SD of three independent experiments. The asterisk indicates $P<0.05$. (C) 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. (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 $P<0.05$. (D) Equal amounts of cell lysates were analyzed for LC3II cleavage by western blot. Tubulin staining is shown as the loading control. Representative examples of western blot results are shown.



Online Supplementary Figure S5. 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). Leukemic cells were pre-incubated with 3MA or control vehicle before treatments. The activity of 3MA was assessed by analyzing percentage of cell viability (upper panels), quantification of MDC staining (medium panels), and percentage of apoptosis (lower panels) determined by flow-cytometry after annexin-V/7-ADD staining. Panels report mean±SD of three independent experiments. Asterisks indicate $P<0.05$ compared to vehicle.

Online Supplementary Table S1. Clinical and laboratory features of AML patients.

Patient #	Age	Sex	FAB	WBC count ($x10^9$/L)	% Blasts	FLT3/ITD
1	71	F	M2	26.0	40	+
2	76	F	M0/M1	5.0	40	-
3	76	F	M4/M5	56.0	40	-
4	57	M	M4	55.0	50	n.a.
5	50	F	M2	50.0	86	-
6	43	F	M2	12.5	70	+
7	71	M	M0	6.0	67	-
8	61	M	M5	49.0	41	-
9	65	F	from MDS	266.0	58	-
10	86	M	M1	50.0	35	n.a.
11	61	F	M2	48.0	26	-
12	59	F	M2	33.0	60	-
13	21	F	M5	156.0	99	+

FAB: French-American-British classification; n.a., not available; MDS: myelodysplastic syndrome.

Online Supplementary Table S2. Combination index values for effects of sorafenib+nutlin-3 on viability of AML patients' cells and AML cell lines.

Cells	ED50	ED75	ED90	Average CI*
Patient #1	0.74	0.65	0.68	0.69
Patient #2	0.51	0.59	0.67	0.59
Patient #3	0.58	0.52	0.47	0.52
Patient #4	0.30	0.34	0.38	0.34
Patient #5	0.55	0.39	0.29	0.41
Patient #6	0.80	0.45	0.25	0.50
Patient #7	nd	nd	nd	nd
Patient #8	0.89	0.86	0.90	0.88
Patient #9	0.67	0.82	1	0.83
Patient #10	0.74	0.69	0.65	0.69
Patient #11	1.06	0.29	0.09	0.48
Patient #12	0.43	0.35	0.29	0.36
Patient #13	0.53	0.73	0.96	0.74
HL60	0.75	0.77	0.82	0.78
NB4	0.95	0.83	0.77	0.85
OCI	0.71	0.33	0.16	0.40
MV4-11	0.07	0.11	0.17	0.12
MOLM	0.63	0.55	0.56	0.56

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