SUPPLEMENTARY INFORMATION

Synergistic anti-leukemic activity of imatinib in combination with a small molecule Grb2 SH2 domain binding antagonist

LETTER TO THE EDITOR

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Supplementary Table 1. Combination indices of imatinib and TB03*

Imatinib:TB03 ratio	ED_{50}	ED_{75}	ED_{90}
1:100	1.867	1.201	0.774
1:50	0.939	0.665	0.472
1:25	0.391	0.313	0.251
1:12.5	0.202	0.171	0.144
1:6.25	0.089	0.082	0.076

*$r > 0.978$

Supplementary Table 2. MicroRNAs affected most by treatment with imatinib, TB03, or both*.

Imatinib 0.25 μM		TB03 20 μM		Imatinib 0.25 μM + TB03 20 μM	
upregulated					
hsa-miR-548c-A	16.7	hsa-miR-661-P	3.8	hsa-miR-646-P	27.4
hsa-miR-801-A	7.7	hsa-miR-644-P	3.7	hsa-miR-548c-A	26.6
hsa-miR-92b-P	7.2	hsa-miR-550-1-P	3.1	hsa-miR-548b-P	15.1
hsa-miR-548b-P	7.1	hsa-miR-484-A	1.5	hsa-miR-128a-P	14.4
hsa-miR-602-P	6.4	hsa-miR-30c-1-A	1.3	hsa-miR-801-A	10.2
hsa-miR-30c-1-A	1.4	hsa-miR-34b-P	1.2	hsa-miR-644-P	7.1
hsa-miR-34b-P	1.2			hsa-miR-346-A	6.3
hsa-miR-484-A	1.1			hsa-miR-548d1-P	6.0
				hsa-miR-579-P	5.5
				hsa-miR-34b-P	5.0
				hsa-miR-484-A	3.2
				hsa-miR-30c-1-A	1.4
downregulated					
hsa-miR-376a2-A	0.09	hsa-miR-516-1-P	0.17	hsa-miR-616-A	0.05
hsa-miR-545-A	0.09	hsa-miR-376a2-P	0.23	hsa-miR-376a-3p-A	0.08
hsa_miR_147 left	0.10	hsa-miR-182-5pA	0.30	hsa-miR-9-A	0.11
hsa-miR-21No1	0.12	hsa-miR-633-P	0.31	hsa-miR-429-A	0.12
let-7d-A	0.13	hsa-miR-181b-1-P	0.32	hsa-miR-125b1-A	0.14

* Values are fold change in microRNA expression by K562 cells treated with imatinib (0.25 μM), TB03 (20 μM), or imatinib (0.25 μM) plus TB03 (20 μM) relative to vehicle-treated control cells, from most affected (top) to least affected (bottom).

Color code:

	Unique to treatment group
	Common to all treatment groups
	TB03 alone and imatinib + TB03 groups only
	Imatinib alone and imatinib + TB03 groups only

Supplementary Figure Legends

Supplementary Figure 1. Chemical structure of TB03.

Supplementary Figure 2. Inhibition of leukemia stem cell population. K562 cells were treated with the indicated concentrations of imatinib, TB03 alone, or imatinib in combination with TB03 for 48 h in 6-well plates. The cells were then collected and 10^6 viable cells per group were used for ALDH assay according to the manufacture's instruction. This consisted of using 5 μl ALDH substrate for testing sample and 5 μl ALDH substrate plus 5 μl ALDH inhibitor as negative control, followed by flow cytometry analyses. Data from one representative experiment of three independent experiments is shown.

Supplementary Figure 3. MicroRNA expression induced by imatinib, TB03 or imatinib + TB03 treatments. Expression levels of miR-34b, miR-30c, and miR-484 were detected in total RNA samples from K562 cells treated with vehicle, imatinib, TB03, or imatinib in combination with TB03 using an individual miroRNA TaqMan assay, TaqMan microRNA reverse transcription kit, 2×PCR Master Mix. Analyses employed 10 ng total RNA, following the manufactory's instruction on an Applied Biosystems 7900HT Fast Real-Time PCR System. Gene expression was presented by using $2^{-\Delta Ct}$ method with RNU48 for normalization (*$p<0.05$, **$p<0.01$, $n=3$).

Supplementary Materials and Methods

Compounds

TB03 was prepared as previously described[1] and dissolved in PBS as a 1 mg/ml stock solution. Imatinib (imatinib mesylate) was purchased from LC Laboratories (Woburn, MA) and dissolved in DMSO, diluted in PBS as a 10 mM stock solution.

Cell culture

The CML-derived cell line K562 was purchased from ATCC (Manassas, VA). Cells were grown in RPMI1640 containing 10% FBS, 2 mM L-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell proliferation assays

K562 cells were seeded in 96-well plates at a density of 5,000 cells per well (n = 3 per group) with varying concentrations of TB03, imatinib, or TB03 in combination with imatinib, for 48 h at 37°C, 5% CO_2. Cell Counting Kit-8 (CCK-8) reagent (Dojindo Inc., Gaithersburg, MD) was then added to the plate followed by incubation at 37°C for 4 h. Absorbance at 450 nm was recorded using a microplate reader (Molecular Devices, Sunnyvale, CA). Optical density readings from the wells treated with vehicle alone were regarded as 100% growth. IC_{50} values were computed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA) with a nonlinear curve fit function. Possible drug combination synergy was computed using CalcuSyn 2.1 (Biosoft, Cambridge, UK), with a combination index of <1 as synergism and >1 as antagonism.[2]

Apoptosis, cell division, and cell cycle assays

K562 cells treated with vehicle, TB03, imatinib alone or TB03 plus imatinib were stained with Annexin V-FITC-PI, CellTrace Violet, or Vybrant DyeCycle™ Green (Life Technologies, Carlsbad, CA) followed by flow cytometry analysis according to

the manufacturer's instructions. Cell division analysis was performed using the Cell Proliferation mode of the Flowjo program (Tree Star, Inc. Ashland, OR). Cell cycle analysis was conducted using the ModFit LT program (Verity Software House, Augusta, ME).

ALDH assay

The ALDEFLUOR kit (Stem Cell Technologies, Durham, NC, USA) was used to analyze the population of leukemia stem cells as indicated by high-level ALDH enzymatic activity. Vehicle- or drug-treated K562 cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 μM per 1×10^6 cells) and incubated over 40 min at 37°C. As negative control for each sample of cells, an aliquot was treated with 50 mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. The number of cells stained with DEAB was used as a gate to obtain the percentage of ALDH positive (ALDH+) cells by flow cytometry.

Immunoprecipitation and immunoblotting

K562 cells (1×10^7) were treated with varying concentrations of imatinib, TB03, or a combination of imatinib and TB03 for different time intervals. The cells were then harvested and washed twice with PBS on ice. To prepare detergent cell extracts, cells were lysed in ice cold buffer containing 1% Triton X-100, 50 mM HEPES, pH 7.4, 10% glycerol, 137 mM NaCl, 10 mM NaF, 100 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 2 mM EDTA, 10 μg/ml leupeptin, and 1 mM PMSF; lysates were cleared by centrifugation. Protein quantitation of the supernatant was performed using the BCA method (Pierce Inc., Rockford, IL). For immunoprecipitation, cleared cell extracts containing 200–500 μg of total protein were incubated at 4°C with Grb2 antibody (Santa Cruz, Santa Cruz, CA) for 2 h and subsequently mixed with 25 μl of 50% protein A-Sepharose slurry and kept mixing with a rotating device for 1.5 h. Immunocomplexes were sedimented by centrifugation and rinsed three times in the same buffer at 4°C, followed by mixing with an equal volume of 2x Laemmli loading buffer and boiling. For direct immunoblotting, 50 μg aliquots of the cleared extract were mixed with Laemmli loading buffer, boiled, separated by SDS-PAGE, and

transferred to a nitrocellulose membrane.[3,4] Phospho-Bcr-Abl (Cell Signaling, Danvers, MA), tyrosine phosphorylated proteins (Millipore, MA), Grb2, and GAPDH (protein loading control, Sigma, St. Louis, MO) were identified using specific antibodies and visualized by enhanced chemiluminescence detection (ECL, GE Healthcare, Piscataway, NJ). The levels of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) secreted by K562 cells (1×10^7) treated with imatinib (0.25 μM), TB03 (20 μM), or a combination of imatinib and TB03 for 48 h was analyzed in conditioned media using the Proteome Profiler™ Human Angiogenesis Array (R&D Systems, Inc. Minneapolis, MN) as specified by the manufacturer; spots on the array were quantitated by densitometry using Image J software.[5] Statistically significant differences were determined by student's t-test.

MicroRNA expression profiling and qPCR

High-quality total RNA (10 μg) was extracted and purified from K562 cells treated for 48 h with vehicle, imatinib alone, TB03 alone, or imatinib in combination with TB03. RNA samples were subjected to genome-wide microRNA expression profiling using a 10K microRNA expression array developed by the Microarray Core Facility of the Ohio State University Comprehensive Cancer Center.[6,7] Validation of the expression of individual microRNAs was performed using individual microRNA TaqMan assays, TaqMan microRNA reverse transcription kit, and 2x PCR Master Mix (Life technologies. Carlsband, CA), by initiating with 10 ng total RNA, according to the manufacturer's instructions for the Applied Biosystems 7900HT Fast Real-Time PCR System. Gene expression was analyzed using $2^{-\Delta Ct}$ method[8] with RNU48 for normalization.

Supplementary References

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