The mammalian target of rapamycin inhibitor RAD001 (everolimus) synergizes with chemotherapeutic agents, ionizing radiation and proteasome inhibitors in pre-B acute lymphocytic leukemia

Philip Saunders, Adam Cisterne, Jocelyn Weiss, Kenneth F. Bradstock, and Linda J. Bendall

Westmead Institute for Cancer Research, Westmead Millennium Institute, University of Sydney, Westmead, NSW, Australia

Citation: Saunders P, Cisterne A, Weiss J, Bradstock KF, and Bendall LJ. The mammalian target of rapamycin inhibitor RAD001 (everolimus) synergizes with chemotherapeutic agents, ionizing radiation and proteasome inhibitors in pre-B acute lymphocytic leukemia. Haematologica 2011;96(1):69-77. doi:10.3324/haematol.2010.026997

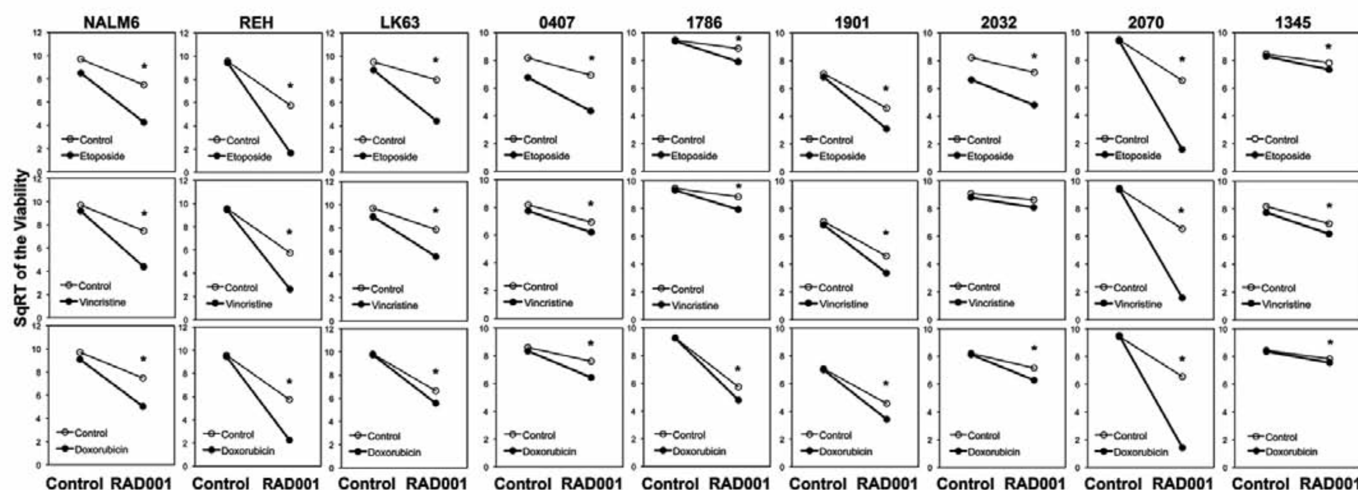
Supplementary Methods

Lentiviral transduction and assessment of cleaved caspase-3

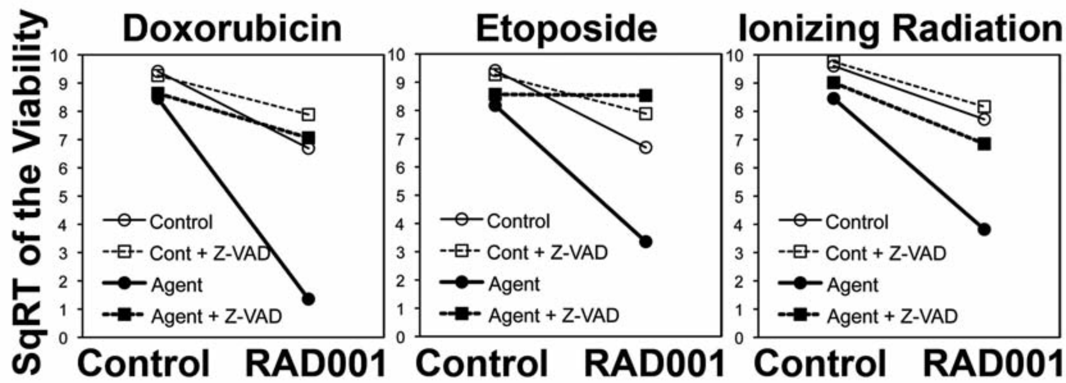
Twenty-four hours before transduction, NALM-6 cells were seeded into T75 flasks at a density of 1×10^6/mL. After 24 h of culture, 1.5×10^6 cells were suspended in 7.5 mL of complete medium. An equal volume of complete medium containing 16 μg/mL polybrene and 400 μL of either luciferase (3.8×10^7 viral particles/mL) or p53 (1.17×10^7 viral particles/mL) shRNA expression vectors was added. After incubation for 48 h, cells were washed in complete medium, seeded at 1×10^6/mL and cultured in complete medium in triplicate for a further 24 h, in the absence or presence of vincristine (5 nM). Cells were harvested, washed in cold phosphate-buffered saline and fixed in 2% paraformaldehyde. Cells were then permeabilized in 70% ethanol for 2 h and washed twice with perm/wash buffer before suspension and incubation in perm/wash buffer containing 10% human AB serum for 1 h. Cells were then washed and resuspended in 100 μL perm/wash buffer and incubated with anti-cleaved caspase-3 for 1 h followed by washing and labeling with an allophycocyanin-conjugated secondary antibody. Cells were resuspended in 500 μL phosphate-buffered saline prior to analysis by flow cytometry. Successfully transduced cells were identified by GFP expression. Viability was assessed on cells within the GFP-positive gate. Cells negative for active caspase-3 expression and considered live at the point of fixation by forward and side scatter characteristics were considered viable.

Viability assay

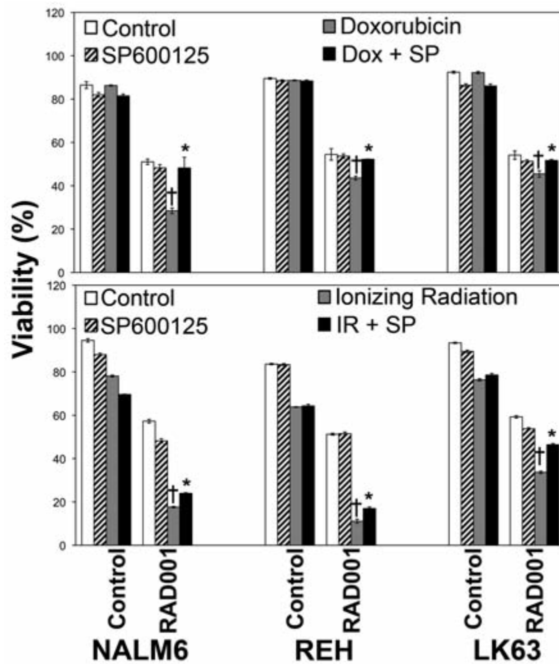
Cells were seeded at a density of 1×10^6/mL and cultured for 24 h unless otherwise stated. Cells were harvested and washed in cold phosphate-buffered saline by centrifugation at 400xg then resuspended in 200 μL annexin V binding buffer. Cells were stained with 5 μL of fluorochrome-conjugated annexin V for 45 min in the dark at room temperature, after which 5 μL of 7-AAD were added and cells analyzed by flow cytometry. Cells negative for annexin V and 7-AAD staining were considered viable.



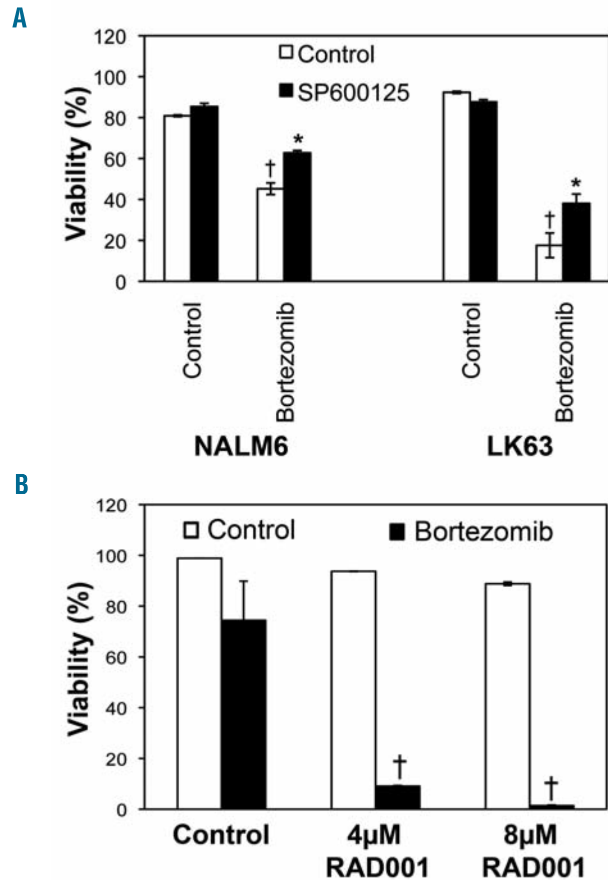
Online Supplementary Figure S1. RAD001 synergizes with cytotoxic agents. Indicated ALL cell lines or patients' samples were cultured in the presence or absence of RAD001 for 24 h with or without exposure to 100 nM etoposide, 0.6 nM vincristine or 10 nM doxorubicin as indicated. RAD001 was used at a dose of 16 μM except for patients' samples 2032, 1345 and 2070 for which 14 μM RAD001 was used. The viability of ALL cells was determined by flow cytometry with cells lacking annexin V and 7-AAD staining considered viable, as described in Figure 1. Viability data shown in Figure 2 were square root transformed. Divergent lines represent agonistic, parallel lines additive, and convergent lines antagonistic relationships. * Indicates a synergistic interaction was detected (*P*<0.05).



Online Supplementary Figure S2. Synergistic cell death is caspase-dependent. NALM6 cells were either untreated or exposed to 10 nM doxorubicin, 100 nM etoposide or 1 Gy of ionizing radiation, 16 μM RAD001 or the combination of RAD001 and cytotoxic agents, in the absence or presence of 20 μM ZVAD-FMK for 24 h. Cells negative for annexin V and 7-AAD staining by flow cytometry were considered viable. Viability data from Figure 3 were square root transformed. Divergent lines represent agonistic, parallel lines additive, and convergent lines antagonistic relationships.



Online Supplementary Figure S3. Synergistic cell death is partially mediated by activation of the JNK pathway. NALM6, REH and LK63 cells were untreated or exposed to 10 nM doxorubicin or 1 Gy (NALM6), 2.5 Gy (REH) or 5 Gy (LK63) of ionizing radiation with or without the addition of 16 μM RAD001 in the absence or presence of 5 μM SP600125 for 24 h. Cells were analyzed by flow cytometry and cells lacking annexin V and 7-AAD staining were considered viable. The mean±SD of triplicate determinations is shown. † Indicates a significant synergistic interaction between a chemotherapeutic agent and RAD001 (P<0.05) * Indicates a significant reversal of the synergistic interaction by SP600125 (P<0.05).



Online Supplementary Figure S4. Proteasome inhibitors induce JNK-dependent killing and synergize with RAD001. (A) NALM6 or LK63 cells were untreated or exposed to 20 nM bortezomib in the absence or presence of 5 μM SP600125 for 24 h. Cells were analyzed by flow cytometry, and those negative for annexin V and 7-AAD staining were considered viable. The mean±SD of triplicate determinations is shown. †Indicates significant killing (P<0.05) and *significant reversal of the cytotoxic action of bortezomib. (B) NALM-6 cells were untreated or exposed to the indicated concentrations of RAD001, 20 nM bortezomib or the combination of RAD001 and the proteasome inhibitor for 24 h. The mean±SD of triplicate determinations is shown. †Indicates synergistic killing by the combination of RAD001 and bortezomib (P<0.05).

Online Supplementary Table S1. Characteristics of the patients with ALL.

ID	Age(y)/ Sex	BM/PB	Blasts (%)	Phenotype	Cytogenetics
0407	45/M	BM	99	$CD34^- CD19^+ CD10^+ CD20^-$	t (1;19)*
1901	5m/F	PB	100	$CD34^+ CD19^+ CD10^- CD20^-$	46 XX, del(12)(p11.2p13)[6]/46, XX[14]
2032	12/M	BM	N/A	$CD19^+ CD10^+$	46 XY add (9)(p24), del(9)(p21), del(13)(q11q21), del(19) t(1;19)(q23;p13)
2070	65/M	BM	N/A	$CD34^+ CD19^+ CD10^+ CD20^+ CD45^+$	45 XY t(9;22) (q34;q11.2) del(9) (p21)
1345	5/F	BM	95	$CD34^- CD19^+ CD10^+ CD20^+$ HLA-DR	45 XX dup (1)(q42 q25), del (3) (q21),-9,del (9)p22,t(18;20)(q21;q13.1)
1786	12/F	BM	90	$CD34^- CD19^+ CD10^+ CD20^+$	No metaphases

*The percentage of blasts at diagnosis is indicated; BM- bone marrow; PB- peripheral blood; N/A- not available. *Complete karyotype not available but t(1:19) detected by polymerase chain reaction.*