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Targeting 14-3-3 sensitizes native and mutant BCR-ABL to inhibition with U0126, rapamycin and Bcl-2 inhibitor GX15-070

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Abstract

Small molecule tyrosine kinase inhibitors, such as imatinib, are effective therapies for BCR-ABL-mediated human leukemias. However, clinical drug resistance occurs, which warrants development of alternative and/or complementary therapeutic strategies to target critical downstream signaling molecules. We recently demonstrated that disrupting 14-3-3/ligand association by a peptide-based 14-3-3 competitive antagonist R18 induces significant apoptosis, partially through reactivation of AKT-inhibited proapoptotic FOXO3a, in FGFR1 fusion-transformed hematopoietic cells. Here, we report that targeting 14-3-3 by R18 effectively induced significant apoptosis in Ba/F3 and K562 cells expressing BCR-ABL, similarly through liberation and reactivation of FOXO3a. Moreover, R18 sensitized BCR-ABL-transformed cells to inhibition with MEK1 inhibitor U0126, Bcl-2 inhibitor GX15-070, or mTOR inhibitor rapamycin. Treatment with these reagents potentiated R18-induced reactivation of proapoptotic FOXO3a with enhanced expression of downstream transcription targets $p27^{kip1}$ and Bim1. Furthermore, R18-induced apoptotic cell death in cells expressing diverse imatinib-resistant BCR-ABL mutants, including T315I. This inhibition was enhanced by R18 in combination with U0126 and rapamycin. Thus, our findings suggest that targeting 14-3-3 may potentiate the effects of conventional therapy for BCR-ABL-associated hematopoietic malignancies, and overcome drug resistance.

Keywords

BCR-ABL; chronic myelogenous leukemia (CML); peptide-based 14-3-3 antagonist R18; BCR-ABL imatinib-resistant mutations

Introduction

Small molecule tyrosine kinase inhibitors, such as imatinib (Gleevec), are effective therapies in the clinical treatment of BCR-ABL-positive chronic myelogenous leukemia (CML) and TEL-PDGFβR associated chronic myelomonocytic leukemia.[1] However, the emergence of molecular resistance to imatinib poses a significant clinical problem in treatment of BCR-ABL-positive CML patients, which is often caused by point mutations in *BCR-ABL* gene.[2,3] Identification of the spectrum of imatinib-resistant BCR-ABL mutations has led to rapid development of new generation of small molecule ABL inhibitors with distinct mechanism of action against imatinib-resistant cell lines. The clinical activity of these agents such as AMN107 and SKI606 is currently evaluated in ongoing Phase I/II clinical trials, while dasatinib, a Src/ABL dual inhibitor, has received FDA approval for clinical treatment of imatinib-resistant CML patients. However, although these agents in general are very active in

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treatment of imatinib-resistant CML, they still fail to inhibit some BCR-ABL imatinib-resistant mutants including T315I, which is among the most common BCR-ABL mutations identified in imatinib-resistant CML patients.[4,5] Therefore, it is of interest to develop alternative and/or complementary therapeutic strategies to target critical signaling molecules of aberrant signaling pathways activated by leukemogenic tyrosine kinases, which may attenuate their transforming potential and overcome drug resistance.

BCR-ABL has been demonstrated to mediate hematopoietic transformation by providing prosurvival and proliferative signaling through activation of PI3K/AKT and Ras/Raf/MAPK pathways.[6-8] We have recently shown that constitutively activated ZNF198-FGFR1 fusion tyrosine kinase, which is associated with human t(8;13)(p11;q12) 8p11 stem cell myeloproliferative disorder,[9] activates the AKT and MAPK pathways in hematopoietic cells, and 14-3-3 proteins integrate prosurvival signals through sequestering the proapoptotic FOXO3a and BAD downstream of AKT and MAPK.[10] Moreover, disrupting 14-3-3/ligand association by a peptide-based 14-3-3 competitive antagonist R18 induces apoptosis by, in part, disrupting the interaction of 14-3-3/FOXO3a but not 14-3-3/BAD in ZNF198-FGFR1-transformed cells.[10]

Here, we report that targeting 14-3-3 by R18 similarly induces significant apoptosis in hematopoietic cells expressing BCR-ABL, through liberation and reactivation of FOXO3a. Moreover, R18 sensitizes BCR-ABL-transformed cells to inhibition with anticancer agents targeting prosurvival signaling effectors in parallel to AKT-inhibited FOXO3a, including MEK1 inhibitor U0126, Bcl-2 inhibitor GX15-070[11] and mTOR inhibitor rapamycin. Targeting 14-3-3 also induces apoptotic cell death in cells expressing diverse imatinib-resistant BCR-ABL mutants, including T315I, and this apoptosis is further enhanced in combination of U0126 and rapamycin treatment.

Materials and methods

DNA constructs and reagents

Native and mutant BCR-ABL cDNA constructs were subcloned into pMSCV-neo or -puro derived Gateway destination vectors as previously described.[12] The plasmids of pREV-TRE-Hyg-YFP-R18 dimer or mutant, pECFP-R18 dimer or mutant were described.[10] GX15-070 was provided by Gemin X Biotechnologies Inc.(Montreal, Quebec, Canada).

Retroviral infections, proliferation and apoptosis assays

Doxycycline (Dox)-inducible R18 dimer or mutant expressing TonBaF cell lines were described.[10] Cell lines inducibly expressing R18 dimer or mutant were transduced by retroviral supernatant carrying pMSCV-puro vectors encoding BCR-ABL, followed by antibiotic selection. MTT assay and apoptosis assays were described.[10]

Immunoprecipitation and western blot

The immunoprecipitation and immunoblotting were performed as described.[10] Applied antibodies included antibodies against BAD, phospho-BAD (S112), β-actin, p44/42 ERK, phospho-p44/42 ERK (Thr202/Tyr204), c-Abl, AKT, phospho-AKT (Cell Signaling Technology Inc., Danvers, MA, USA); antibodies against p27, GFP, 14-3-3β (Santa Cruz Biotechnology, Santa Cruz, CA, USA); antibodies against Bim1 (Affinity Bio Reagents, Golden, CO, USA) and antibodies against phospho-tyrosine (clone 4G10), FOXO3a and phospho-FOXO3a (Thr32) (Upstate, Lake Placid, NY, USA).

Purification of recombinant TAT-YFP-R18 fusion proteins

In brief, the expressed fusion protein was purified by sonication of high expressing BL21(DE3) pLysS cells obtained from 250 ml of culture with IPTG-induction. Cellular lysates were resolved by centrifugation and loaded onto a Ni-NTA column in 20 mM imidazole. After a step of two times washing, the protein was eluted with 250 mM imidazole. TAT fusion proteins were desalted on a PD-10 column into phosphate-buffered saline (PBS) and the purification efficiency was examined by silver staining and western blotting.

Results

Induced expression of the 14-3-3 antagonist, R18, induces apoptosis in hematopoietic cells transformed by BCR-ABL

We generated hematopoietic TonBaF cell lines inducibly expressing a dimeric version of R18 combining two R18 peptides (R18 dimer; Figure 1a), or a mutant form of R18 (R18 mutant, Figure 1a) that contains one R18 peptide with two substitutions D12K and E14K that abolish binding ability to 14-3-3.[10] These cell lines were used to generate TonBaF cell lines that express either R18 dimer or R18 mutant in an inducible manner with stable expression of BCR-ABL (Figure 1b, right). The resulting cell lines were cultured in the presence or absence of Dox, followed by analysis of R18-induced apoptosis, in the absence or presence of interleukin-3 (IL-3). Expression of YFP-R18 dimer induced significant apoptosis in cells stably transformed by BCR-ABL, which was assessed as the fraction of annexin V/YFP double-positive cells in a dose-dependent manner (Figure 1b, left). In contrast, R18 mutant failed to induce significant apoptosis in these cells. Moreover, in the presence of IL-3, R18 dimer induced much less apoptosis in the control parental TonBaF cells (Figure 1b, left), suggesting that the cells stably transformed by BCR-ABL are more sensitive to apoptosis induced by R18 expression.

We have also generated bacterial expression plasmids to express fusion protein TAT-YFP-R18 proteins, containing a N-terminal 6-histidine leader followed by the 11-amino acid-TAT protein transduction domain (YGRKKRRQRRR) and the YFP-tagged R18 dimer or mutant (Figure 1c).[10] As shown in Figure 1c, purified TAT-YFP-R18 dimer and mutant effectively crossed the cell membrane and transduced into human leukemia cell lines, including K562 that express BCR-ABL, as well as HL-60 and Jurkat-T cells. Moreover, treatment with TAT-YFP-R18 dimer effectively induced apoptosis in BCR-ABL-transformed K562 cells, but not in HL-60 and Jurkat-T cells that are not transformed by any constitutively activated tyrosine kinase. Apoptosis was assessed as the fraction of annexin V+/YFP+ cells, compared with cells treated with TAT-YFP-R18 mutant.

Taken together, these data suggest that the constitutively activated BCR-ABL-transformed human leukemia cells are more sensitive to the R18-induced apoptosis, whereas R18 has minimal nonspecific cytotoxicity in human leukemia cells that are not transformed by leukemogenic fusion/mutant tyrosine kinases. We also observed that induced R18 expression disrupted the interaction between 14-3-3 and FOXO3a, but not 14-3-3/BCR-ABL association as well as BCR-ABL kinase activity in Ba/F3 cells (Supplementary Figure 1).

Induced R18 expression enhances apoptosis induced by MEK1 inhibitor U0126 or a Bcl-2 inhibitor GX15-070 in TonBaF cells expressing BCR-ABL

Our data (Supplementary Figure 1) and previous publication[10] suggest a model that targeting 14-3-3 by R18 induces apoptosis in BCR-ABL or FGFR1 fusion transformed cells, partially through liberation and reactivation of FOXO3a, but not BAD, from 14-3-3 binding and inhibition. Thus, we hypothesize that targeting 14-3-3 by R18 may potentiate the inhibition of

BCR-ABL-transformed cells by in parallel targeting the MEK1/ERK pathway or blocking the downstream Bcl-2 prosurvival pathway (Figure 2a).

TonBaF cells stably expressing BCR-ABL and inducibly expressing R18 dimer or mutant (Supplementary Figure 2a) were cultured in the absence of IL-3 and presence of increasing doses of U0126, with or without Dox treatment at increasing concentrations. We observed that although treatment with the MEK1 inhibitor U0126 at 12.5 and 25 μ_M was sufficient to abolish ERK phosphorylation and activation (Supplementary Figure 2b), U0126 as a single agent was insufficient to induce significant apoptosis in BCR-ABL-transformed cells (Figure 2b). In contrast, induced R18 expression enhanced the proapoptotic activity of MEK1 inhibitor U0126 in TonBaF cells transformed by BCR-ABL, compared with R18 mutant. The enhanced apoptosis was assessed as the increased fraction of annexin V/YFP double-positive cells in a dose-dependent manner (Figure 2b), and enhanced cleavage of poly(ADP-ribose) polymerase (PARP) (Supplementary Figure 2c), compared to cells treated with R18 and U0126 alone.

GX15-070 (Gemin X Biotechnologies Inc.) is a small molecule pan-Bcl-2 inhibitor that targets the Bcl-2 family of proteins, which is currently being studied in clinical trials for the treatment of patients with chronic lymphocytic leukemia and solid tumors. We observed that GX15-070 treatment inhibited cell growth of TonBaF cells expressing BCR-ABL in a dose-dependent manner as assessed in an MTT assay, and induced expression of YFP-R18 dimer, but not R18 mutant, significantly enhanced GX15-070 inhibitory effects (Figure 2c, left and middle). The cellular IC_{50} of GX15-070 was decreased more than 4-fold in cells with R18 induction compared with cells not expressing R18 (Figure 2c, right).

R18 enhances the mTOR inhibitor rapamycin-induced apoptotic cell death in BCR-ABL-transformed TonBaF cells

We also hypothesize that simultaneously targeting two separate signaling effectors mTOR and FOXO3a downstream of AKT in parallel may result in enhanced abrogation of the prosurvival PI3K/AKT pathway, and achieve synergistic inhibition of leukemia cells (Figure 3a). We treated the cells with rapamycin at 1 and 4 n_M, which were sufficient to inhibit the phosphorylation and activation of mTOR downstream kinase p70 S6 kinase (Supplementary Figure 2d), but insufficient to induce significant apoptosis in BCR-ABL-transformed cells as a single agent (Figure 3b). However, R18 enhanced the proapoptotic activity of rapamycin in TonBaF cells transformed by BCR-ABL, with enhanced annexin V staining (Figure 3b) and PARP cleavage (Supplementary Figure 2e), compared with treatment with rapamycin alone.

U0126, rapamycin or GX15-070 potentiate R18-induced reactivation of FOXO3a with enhanced expression of FOXO3a transcription targets Bim1 and p27^{kip1}

We next found that combined treatment with R18 and U0126 (Figure 4a), GX15-070 (Figure 4b) or rapamycin (Figure 4c) results in enhanced upregulation of FOXO3a transcriptional targets, such as the cyclin-dependent kinase inhibitor p27^{kip1} and the proapoptotic Bcl-2 family member Bim1, including Bim1 L (long isoform) and Bim1 S (short isoform). p27^{kip1} and Bim1 have been implicated in regulation of cell cycle and apoptosis. These data suggest the potential molecular mechanism by which combined treatment with R18 and U0126, rapamycin or GX15-070 results in enhanced inhibition of BCR-ABL-expressing cells.

Targeting 14-3-3 by R18 alone, and in combination with U0126 or rapamycin induce apoptosis in cells transformed by diverse imatinib-resistant BCR-ABL mutants

We next evaluated the effects of targeting 14-3-3 on overcoming drug resistance. We generated distinct TonBaF-R18 dimer cell lines stably expressing individual clinically identified, imatinib-resistant BCR-ABL mutants, including E255V, M351T, G250E, F317L, T315I, A380T and F486S. Among these mutants, T315I is a potent imatinib-resistant BCR-ABL

mutant, which is also resistant to the second generation ABL tyrosine kinase inhibitors including AMN107.[4] We observed that induction of R18-induced apoptosis in a dose-dependent manner in cells expressing BCR-ABL T315I mutant, and the R18 proapoptotic activity was significantly enhanced in combination of either U0126 or rapamycin (Figures 5a and b, left, respectively). Moreover, U0126 or rapamycin enhanced R18-induced apoptosis in cells expressing diverse BCR-ABL imatinib-resistant mutants (Figures 5a and b, right, respectively).

Discussion

Emerging data have shown that clinical emergence of imatinib-resistant mutations in BCR-ABL confers drug resistance and results in a relapse of the disease. Although new molecules were rapidly developed to tackle the imatinib-resistant mutants of BCR-ABL, few agents have activity against the T315I mutant. Here, we show that targeting 14-3-3 by a peptide-based, competitive pan-14-3-3 inhibitor R18 alone, and in combination with other agents such as U0126 or rapamycin induce apoptosis in cells transformed by native BCR-ABL or diverse imatinib-resistant mutants, including T315I. Our findings suggest that targeting 14-3-3 may represent a helpful strategy to potentiate the effects of conventional therapy for BCR-ABL-associated hematopoietic malignancies, and overcome drug resistance.

14-3-3 interacts with both BCR and c-ABL, as well as BCR-ABL.[13,14] The exact role of 14-3-3-binding in regulation of BCR-ABL is still unknown. 14-3-3 was implied to link specific signaling protein components to BCR-ABL to mediate transformation signals or regulate BCR-ABL activation via protein–protein interaction.[15] Interestingly, R18 as a competitive antagonist of 14-3-3 is insufficient to dissociate BCR-ABL from 14-3-3 binding (Supplementary Figure 1a). Since the phosphorylation levels of BCR-ABL were not altered by expression of R18 (Supplementary Figure 1a), this difference might be due to the relatively high binding affinity of 14-3-3 for BCR-ABL such that R18 is able to compete with FOXO3a for 14-3-3 (Supplementary Figures 1b and c), but insufficient to disrupt 14-3-3/BCR-ABL association. These data suggest that R18 induces apoptosis in BCR-ABL-transformed cells probably in part through liberation and reactivation of FOXO3a that is downstream of AKT, but not by disrupting 14-3-3/BCR-ABL association or attenuating BCR-ABL tyrosine kinase activity.

Moreover, targeting 14-3-3 by R18 alone or in combination with U0126 or rapamycin effectively induced apoptosis in cells expressing a spectrum of imatinib-resistant BCR-ABL mutants, including T315I (Figure 5), suggesting that targeting 14-3-3 may present an alternative solution to the clinical problem of drug-resistance in BCR-ABL-associated hematopoietic malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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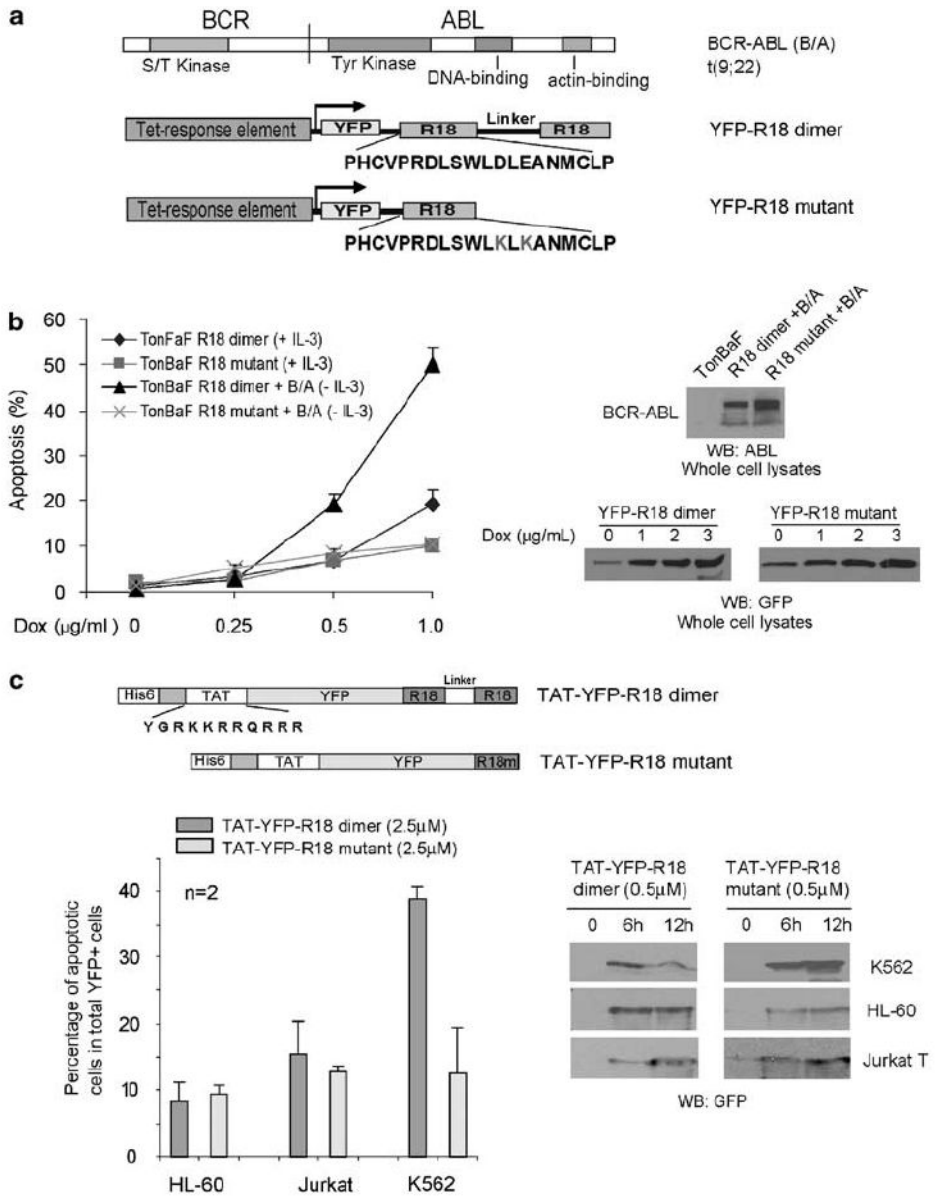

Figure 1.

Inducible expression of a 14-3-3 antagonist R18 induces apoptosis in hematopoietic cells transformed by BCR-ABL. (**a**) Schematic diagram of BCR-ABL, as well as structure and amino-acid sequences of YFP-tagged R18 dimer and mutant. R18 dimer construct contains two R18 motifs separated by a linker of 11 amino acids. (**b**) TonBaF cells transformed by BCR-ABL are more sensitive to R18 dimer-induced apoptosis compared with control TonBaF-R18 dimer cells. Cells were stained with PE-conjugated anti-annexin V reagent and analyzed by FACS for apoptotic population that is characterized as the fraction of annexin V/YFP double-positive cells in total YFP-positive cells (%). (**c**) Upper: schematic diagram of structure of TAT-YFP-R18 dimer and -R18 mutant. Lower: TAT-YFP-R18 dimer transduces into and induces significant apoptosis in human leukemia K562 cells, but not in control HL-60 and Jurkat-T cells.

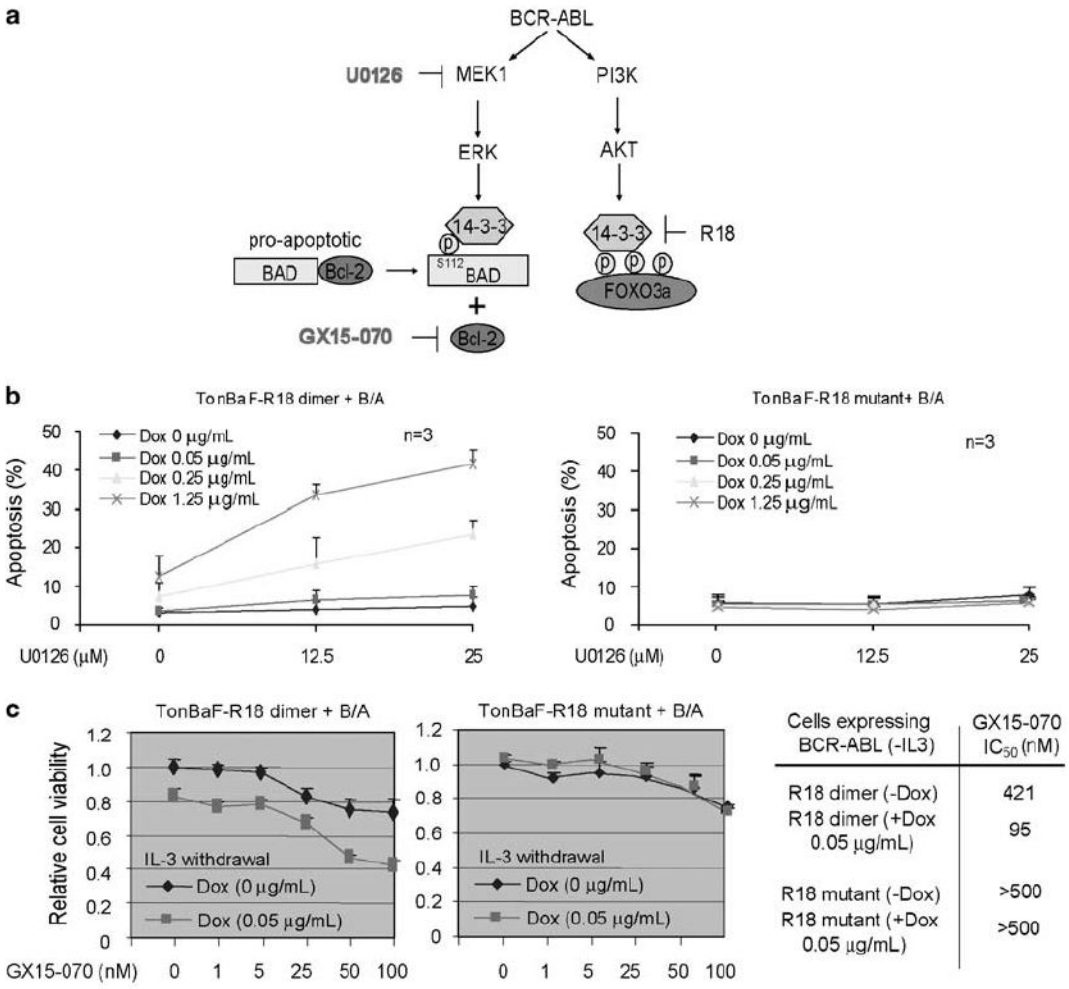

Figure 2.

Targeting 14-3-3 by R18 enhances MEK1 inhibitor U0126 or Bcl-2 inhibitor GX15-070-induced apoptosis in BCR-ABL-transformed TonBaF cells. (**a**) Proposed model of targeting 14-3-3 by R18 to induce apoptosis through reactivation FOXO3a and sensitize BCR-ABL-dependent transformation signaling to inhibition with U0126 or GX15-070. (**b**) Cells were treated with indicated concentrations of doxycycline in the absence or presence of increasing concentrations of U0126, followed by annexin V staining and FACS analysis for apoptotic population. (**c**) The cells were treated with increasing concentrations of GX15-070 in the absence or presence of Dox. The relative cell viability determined by MTT assay was normalized to cells without treatment with both doxycycline and GX15-070. Right: cellular IC_{50} was determined for each cell line treated with GX15-070 in the presence or absence of Dox.

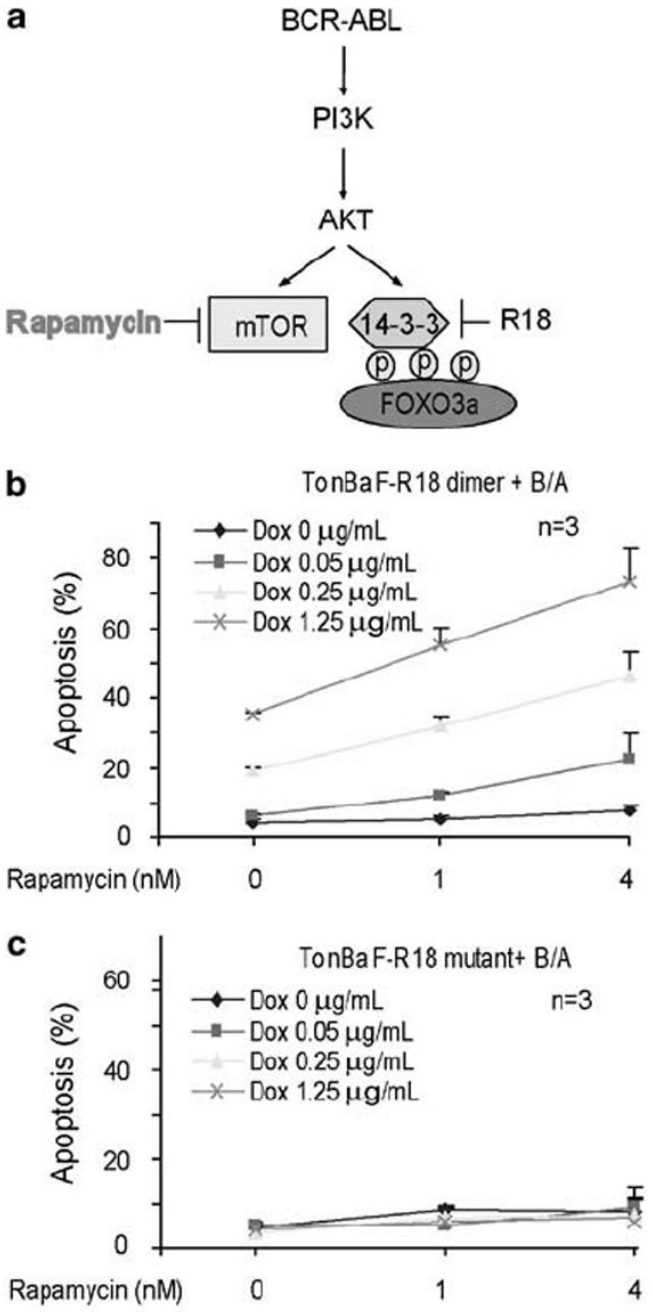

Figure 3.

Targeting 14-3-3 by R18 potentiates mTOR inhibitor rapamycin-induced apoptotic cell death TonBaF cells expressing BCR-ABL. (**a**) Proposed model of simultaneous inhibition of targeting 14-3-3 by R18 and mTOR by rapamycin to induce synergistic apoptosis in BCR-ABL-transformed cells. (**b**) Cells were treated with indicated concentrations of Dox in the absence or presence of increasing concentrations of rapamycin, followed by annexin V staining and FACS analysis for apoptosis.

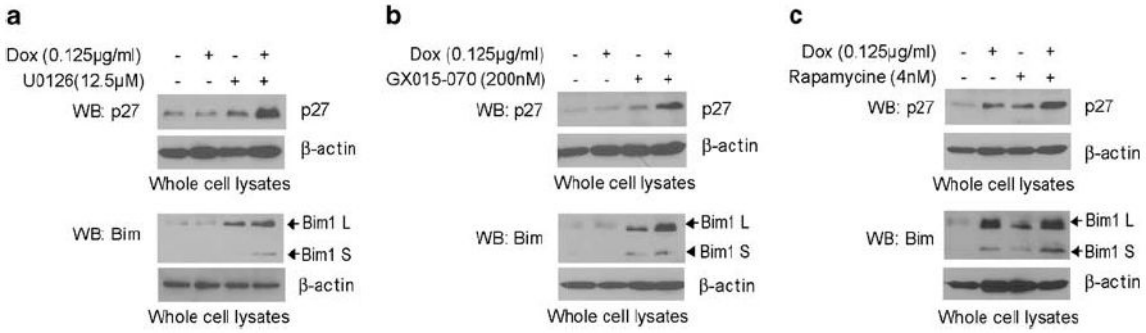

Figure 4.

U0126, GX15-070 or rapamycin potentiate R18-induced reactivation of FOXO3a with enhanced expression of FOXO3a transcription targets Bim1 and $p27^{kip1}$. TonBaF-R18 cells expressing BCR-ABL were treated with (**a**) U0126, (**b**) GX15-070 or (**c**) rapamycin in the presence and absence of Dox at indicated concentrations.

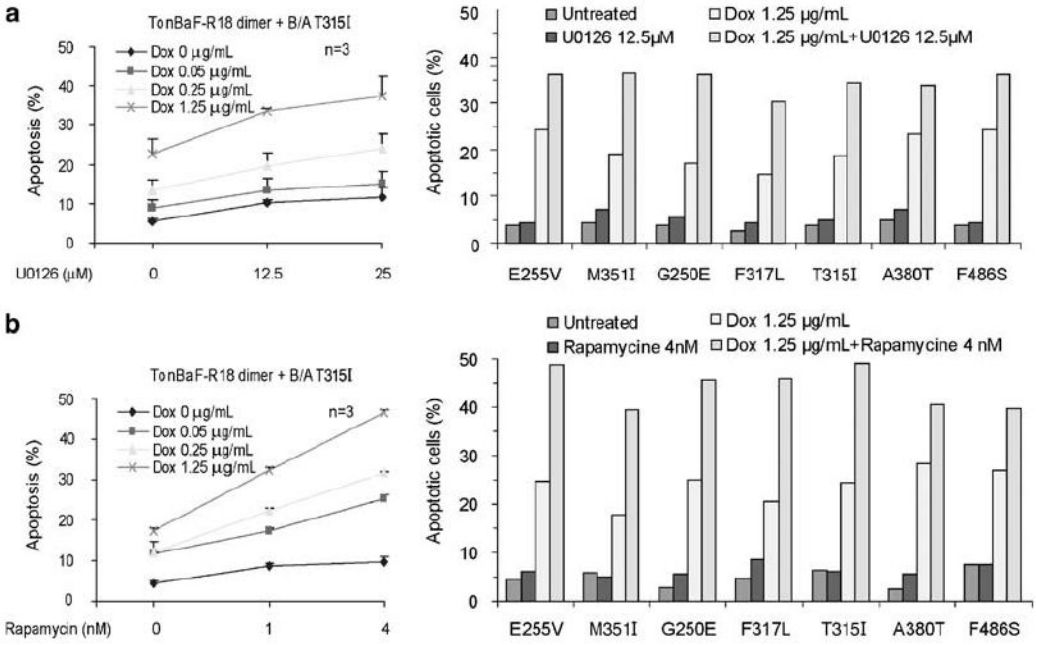

Figure 5.

Combined treatment with R18 expression and U0126 (**a**) or rapamycin (**b**) induces enhanced apoptosis in TonBaF cell lines expressing distinct imatinib-resistant BCR-ABL mutants.