

# The Multi-tyrosine Kinase Inhibitor TKI258, Alone or in Combination with RAD001, Is Effective for Treatment of Human Leukemia with *BCR-ABL* Translocation *In Vitro*

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**Abstract.** *Background/Aim:* *BCR-ABL*-positive (*BCR-ABL*<sup>+</sup>) leukemia is very difficult to treat although much improvement has been achieved due to the clinical application of imatinib and the second-generation tyrosine kinase inhibitors (TKIs). This study aimed to evaluate for the first time the treatment value of the multiple tyrosine kinase inhibitor TKI258 in *BCR-ABL*<sup>+</sup> leukemia. *Materials and Methods:* Proliferation of different *BCR-ABL*<sup>+</sup> leukemic cells was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; cell apoptosis with Annexin V/propidium iodide (PI) and flow cytometry. Gene expression at the protein level was determined by western blotting. *Results:* This drug showed treatment efficacy in naïve and imatinib-resistant *BCR-ABL*<sup>+</sup> leukemia cells, particularly in cells harboring T315I-mutated *BCR-ABL*, for which no effective inhibitor is available to date. By combination with the mTOR inhibitor RAD001, a synergistic effect on cell proliferation was observed in these cell lines. *Conclusion:* TKI258 may become a potent therapeutic agent, either alone or in combination with RAD001, for treatment of *BCR-ABL*<sup>+</sup> leukemia.

*BCR-ABL* translocation is the most common cytogenetic abnormality found in patients diagnosed with acute lymphoblastic leukemia (ALL) (3-5% of childhood ALL, 20-30% of adult ALL) and chronic myelogenous leukemia. The resulting *BCR-ABL* fusion protein is a constitutively-activated tyrosine kinase with oncogenic potentials and is the primary therapeutic target for tyrosine kinase inhibitors (TKIs). Imatinib, the first TKI approved a decade ago,

combined with chemotherapy represents the current standard-of-treatment for patients with newly-diagnosed disease. However, up to one third of patients require alternative therapy because of drug intolerance, suboptimal response, primary and secondary resistance or progression to advanced disease (1). The new generation of *BCR-ABL* inhibitors nilotinib and dasatinib, have been shown to be more potent than imatinib and have demonstrated activity against most clinically relevant imatinib-resistant *BCR-ABL* mutants; however, none of them is effective against T315I-mutated *BCR-ABL* (2). Therefore, *BCR-ABL* with T315I point-mutation, which is seen in 4-15% of resistant disease, remains a significant clinical issue because it is insensitive to all currently-available agents (1, 2).

As we have learned from imatinib resistance, use of TKIs as a single-agent may lead to the quick emergence of drug-resistance. New multi-tyrosine kinase inhibitors (TKIs), which simultaneously target multiple aberrantly-regulated signal transduction pathways, may therefore represent a promising therapeutic strategy to enhance the treatment efficacy and overcome drug resistance.

An alternative approach to overcome *BCR-ABL* resistance is the use of other compounds that do not target directly the *BCR-ABL* protein; instead, these molecules act on several downstream pathways which are regulated by or linked in different ways to *BCR-ABL* that lead to the malignant transformation of the cells.

The phosphatidylinositol 3-kinases (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) pathway is one of these pathways. PI3K and its downstream targets, including AKT and mTOR, stimulate anti-apoptotic and leukaemogenic signals, making this pathway an interesting target for leukemia therapy. Its activation starts from the phosphorylation of the *BCR-ABL* Tyr177, forming a complex with growth factor receptor-bound protein 2 (GRB2) and binding the adapter protein GAB2. Consequently, the latter is phosphorylated and binds to PI3K (3). Kim and colleagues demonstrated that the activation of

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Table I. Growth-inhibitory profile of TKI258.

Cell line	Characteristics	IC <sub>50</sub> (Imatinib, μM)	IC <sub>50</sub> (TKI258, μM)
SupB15	Human B-ALL ( <i>BCR-ABL</i> <sup>+</sup> )	0.224	0.449
SupB15-R	B-ALL, Imatinib resistant derivative of SupB15	Not reached	0.558
BaF3-pSRα	Murine pro-B cells (transfected with pSRα control plasmid)	Not reached	0.668
BaF3-p210 <sup>Bcr-Abl</sup>	Murine pro-B cells (transfected with wild-type p210 <sup>Bcr-Abl</sup> )	4.142	0.692
BaF3-p210 <sup>Bcr-Abl</sup> -T315I	Murine pro-B cells (transfected with p210 <sup>Bcr-Abl</sup> with T315I mutation), extremely imatinib-resistant	Not reached	2.626

Different human ALL cells and transfected murine BaF3 cells were seeded into 96-well plates at a density of 5×10<sup>3</sup> cells per well and were exposed to different concentrations of TKI258 or imatinib in growth medium. After a 5-day incubation, cell proliferation was determined by the MTT assay. The concentrations necessary to result in a 50% decrease in the number of various cells (IC<sub>50</sub>) was calculated by the CalsuSyn software.

the PI3K/mTOR pathway by BCR-ABL contributes to the increased production of reactive oxygen species (ROS), linking this pathway to mechanisms implicated in genomic instability and imatinib resistance (4).

Consequently, inhibitors of the PI3K/AKT pathway reduce BCR-ABL-mediated transformation *in vitro*. A recent study by Kharas *et al.* demonstrated that BCR-ABL transformation of PI3K/AKT-deficient cells was severely impaired. The few surviving clones showed reduced fitness *in vitro*, increased sensitivity to imatinib and failure to cause leukaemia *in vivo*. The same authors also demonstrated that the mTOR signalling is a vital pathway that remains in cells lacking PI3K activity, indicating that the combined targeting of PI3K, mTOR and BCR-ABL would provide an attractive therapeutic strategy in *BCR-ABL*<sup>+</sup> leukaemia (5). These results suggest that the mTOR signaling pathway has become an important therapeutic target for *BCR-ABL*<sup>+</sup> leukemias.

TKI258 (dovitinib) is a new drug developed by Novartis that inhibits multiple receptor tyrosine kinases (RTKs) involved in tumor growth as well as tumor angiogenesis of solid and hematological cancers. TKI258 has a great potency as an inhibitor of class III/IV/V RTKs associated with the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), stem cell factor (c-KIT), Flt3 and colony-stimulating factor-1 (CSF-1) (7) with IC<sub>50</sub> values of approximately 10 nM. Due to its super activity and oral availability, TKI258 is now in phase III development in renal cell carcinoma, in phase II development in advanced breast cancer, relapsed multiple myeloma and urothelial cancer.

Based on the tyrosine kinase profiles that are inhibited by TKI258 and the published data available so far, we expected that targeting multiple tyrosine kinases with TKI258 would be more appealing in *BCR-ABL*<sup>+</sup> leukemias. In addition, we tested in this study the possibility to intensify the treatment effect of TKI258 by combining it with the mTOR inhibitor RAD001 to evaluate if this combination had the potential to become a new treatment regimen for this leukemia entity.

## Materials and Methods

**Cell lines, reagents and cell culture.** Human childhood *BCR-ABL*<sup>+</sup> ALL cell lines SupB15 and its imatinib resistant derivative SupB15-R were kindly provided by Dr. M. Ruthardt of the Department of Hematology, Goethe University, Frankfurt am Main, Germany. SupB15-R was generated by cultivating the SupB15 cells with increased concentration of imatinib for a long time and maintained in 1 μM imatinib-containing culture media. Murine pro-B cell line BaF3 cells transfected with mammalian expression vector pSRα (BaF3-SRα), wild type p210<sup>Bcr-Abl</sup> oncoproteins (BaF3-p210<sup>Bcr-Abl</sup>) or highly imatinib-resistant T315I mutated p210<sup>Bcr-Abl</sup> (BaF3-p210<sup>Bcr-Abl</sup>-T315I) were kindly provided by Dr. P. La Rosée of the Department of Hematology and Oncology, University Hospital of Jena, Germany (8). These cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (PS) and 500μg/ml G418 except for BaF3-SRα cells which additionally required 15% mouse myelomonocytic leukemia WEHI-3 cells-conditioned medium as a source of Interleukin-3 (IL-3). Cells in logarithmic growth phase were used for experiments. The characteristic of cell lines used in this study are summarized in Table I.

TKI258 and everolimus (RAD001) were kindly provided by Novartis Institutes for Biomedical Research (Basel, Switzerland). A 10mM stock solution for RAD001 and 1mM for TKI258 were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Munich, Germany), stored at -20°C and diluted with fresh culture medium immediately before use.

**Determination of cell proliferation by 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.** Different leukemic cells were seeded into a 96-well plate at a density of 5×10<sup>3</sup> cells per well and exposed to different concentrations of TKI258, with or without RAD001, in culture medium. After incubation for indicated time points, cell proliferation was carried-out, as previously described (9).

**Apoptosis analysis.** Cell apoptosis was detected by determining phosphatidylserine expression on the cell surface with the Annexin V kit (BD Biosciences, Heidelberg, Germany) by using flow cytometry according to the instructions of the manufacturers.

**Western blot analysis.** Protein concentrations of whole-cell lysates were measured using a BCA protein assay kit (Pierce, Bonn, Germany). Western blot analysis was performed as described

previously (9). Antibodies against STAT5, p-4EBP1 (Th37/46), p-AKT (T308) were from Cell Signaling Technology (New England Biolabs, Frankfurt am Main, Germany); Antibodies against BCR and  $\beta$ -Actin were from Santa Cruz (Heidelberg, Germany).

*Statistical analysis.* Numerical data were reported as mean $\pm$ SD, unless otherwise specified. All data represent the results of at least three independent experiments. Synergistic, additive effects on cell proliferation by the combination of TKI258 and RAD001 were assessed using the Chou-Talalay method (10) and CalcuSyn software (Biosoft, Ferguson, MO, USA), as described previously (9).

## Results

*The multi-tyrosine kinase inhibitor TKI258 inhibited cell proliferation of cell lines with BCR-ABL translocation.* *BCR-ABL*<sup>+</sup> ALL cell line SupB-15, imatinib-resistant SupB15-R cell line, murine BaF3 cells transfected with control vector (BaF3-pSR $\alpha$ ), wild type p210<sup>Bcr-Abl</sup> (BaF3-p210<sup>Bcr-Abl</sup>) or p210<sup>Bcr-Abl</sup> harbouring T315I-mutation (BaF3-p210<sup>Bcr-Abl</sup>-T315I) were exposed to different concentrations of TKI258 and imatinib (Table I). Among these cell lines, TKI258 inhibited proliferation of the childhood *BCR-ABL*<sup>+</sup> ALL cell line SupB15 and its imatinib resistant counterpart cell line with almost equal efficiency (IC<sub>50</sub> values at about 0.5  $\mu$ M for both of the two cell lines) whereas imatinib was only able to block the proliferation of the naïve SupB15 cells (Table I). In order to test whether TKI258 was also able to inhibit *BCR-ABL*<sup>+</sup> leukemic cells with T315I mutation for which no effective agents are yet available, murine Ba/F3 pro-B cell lines transfected with either wild-type or T315I-mutated *BCR-ABL* fusion genes were employed. Interestingly, TKI258 inhibited the proliferation of the BaF3-p210<sup>Bcr-Abl</sup>-T315I cells although to a lesser extent compared to the cells with wild-type *BCR-ABL* oncoproteins (IC<sub>50</sub> value of 2.63  $\mu$ M for BaF3-p210<sup>Bcr-Abl</sup>-T315I cells *versus* 0.69  $\mu$ M for BaF3-p210<sup>Bcr-Abl</sup> cells). This result indicated that this agent has the potential to be a new therapy for leukemia with T315I mutation, which is hard to treat. In contrast to TKI258, imatinib could only slightly suppress the growth of the transfected cells with wild-type p210<sup>Bcr-Abl</sup> oncoproteins (IC<sub>50</sub> 4.14  $\mu$ M) but not, as expected, the cells with T315I mutation.

*TKI258 inhibited cell proliferation by inducing apoptosis.* To investigate whether the decreased cell proliferation was caused by cell death, cell apoptosis was detected by measuring the Annexin V-FITC/PI-staining with flow cytometry. In correlation with the results from the cell proliferation assay, TKI258 induced apoptosis to the same degree in the *BCR-ABL*<sup>+</sup> ALL cell line SupB15 and its imatinib-resistant subclone, as demonstrated by 77.75% apoptotic cells of SupB15 *vs.* 84.99% for SupB15-R when cells were treated with 2  $\mu$ M TKI258, indicating that the imatinib-resistant ALL cells were not cross-resistant to TKI258 (Figure 1A). Importantly, TKI258 also effectively

induced cell apoptosis dose-dependently in *Bcr-Abl*<sup>+</sup> BaF3 cell line harbouring T315I-mutation as measured with AnnexinV-FITC/PI-staining (Figure 1B). However, these cells were more resistant to TKI258 treatment with 42.13% apoptotic cells caused by 2  $\mu$ M TKI258.

*Combination of TKI258 and mTOR inhibitor RAD001 at low concentrations resulted in synergistic effect on cell proliferation.* We believe that simultaneously targeting this pathway will enhance the cytotoxic effect of TKI258 and overcome drug resistance. To evaluate this hypothesis in *BCR-ABL*<sup>+</sup> leukemias, we investigated the combination effect of TKI258 and mTOR inhibitor RAD001 with the *Bcr-Abl*<sup>+</sup> BaF3 cell line harbouring T315I-mutation, because leukemia cells with this mutation are insensitive to all currently available agents (11). Although some new agents are in development, which holds promise for activity in T315I-mutated disease, their clinical effect remains a question. Figure 2 shows that the cell proliferation rate was reduced to 20% of control for BaF3-p210<sup>Bcr-Abl</sup>-T315I cells when 0.5  $\mu$ M of TKI258 was combined with 10 nM of RAD001. For reaching the same inhibitory effects as with the combination treatment regimen, about 3- to 4-fold higher concentrations of TKI258-alone were required.

Synergistic inhibitory effects of TKI258 and RAD001 on cell proliferation of BaF3- p210<sup>Bcr-Abl</sup>-T315I cells was further analyzed with the CalcuSyn software based on the experimental data and demonstrated as dose-effect curve (Figure 2B) and combination index values (data not shown).

*Inhibition of oncoproteins by TKI258 or TKI258 in combination with RAD001 in BCR-ABL<sup>+</sup> leukemia.* Most interestingly, TKI258 was also able to directly inhibit the *BCR-ABL* kinase with the T315I mutation in BaF3-T315I cells and its downstream signaling such as STAT5, AKT and mTOR (with 4EBP1 as the indicator) as assessed by western blot (Figure 3). These inhibitions were further enhanced by addition of RAD001 to the treatment regimen. Therefore, the inhibitory effects of TKI258 and RAD001, in combination, on *BCR-ABL* kinase and its downstream signaling may account for the synergistic effects of this combination on growth and apoptosis, as described above.

## Discussion

In clinical practice, resistance to imatinib becomes a serious problem and has driven the development of the second generation of *BCR-ABL* inhibitors such as dasatinib and nilotinib. These new inhibitors are treatment options after imatinib failure. However, quick emergence of secondary resistances against these inhibitors limits their long-term efficacies. Based on our results, TKI258 may also hold promise as an alternative drug to treat *BCR-ABL*<sup>+</sup> ALL

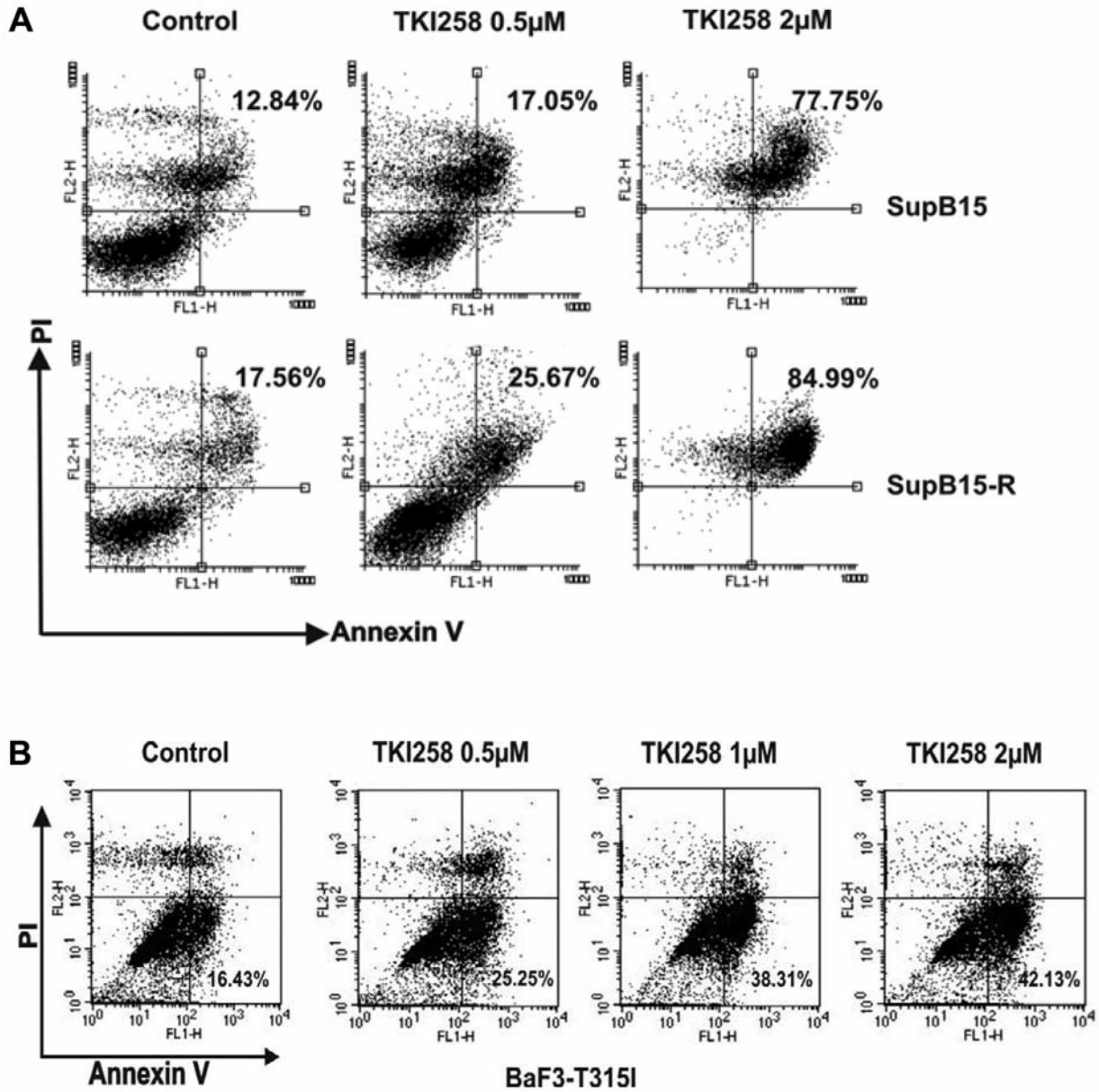


Figure 1. TKI258 inhibited cell proliferation of ALL cells by inducing apoptosis. A-B: Cells were cultured in the presence of TKI258 at the indicated concentrations for 48 h, respectively. Cell apoptosis was measured with Annexin V/PI-staining and flow cytometry as described in Materials and Methods. (A): *BCR-ABL*<sup>+</sup> ALL cell line SupB15 and its imatinib-resistant counterpart SupB15-R; (B): BaF3-p210<sup>Bcr-Abl</sup>-T315I (Murine pro-B cell line BaF3 cells transfected with mammalian expression vector imatinib-resistant T315I mutated p210<sup>Bcr-Abl</sup>).

following imatinib resistance: TKI258 demonstrated effects on growth inhibition and apoptosis in childhood *BCR-ABL*<sup>+</sup> ALL cell line SupB15 as well as the imatinib-resistant counterpart cell line SupB15-R with almost equal efficiency, suggesting that cells which are resistant to imatinib are not cross-resistant to TKI258, conferring this agent the potential to be integrated into the treatment regimen for childhood *BCR-ABL*<sup>+</sup> ALL. Even more interestingly, TKI258 effectively inhibited the cell

proliferation and induced apoptosis in BaF3-p210<sup>Bcr-Abl</sup>-T315I cells although its effect was less than that in BaF3-p210<sup>Bcr-Abl</sup> cells. Since there is no effective drug to treat leukemic cells bearing *BCR-ABL* with T315I-mutation which frequently occurs in patients causing their clinical resistance to almost all *BCR-ABL* inhibitors available up-to-date, this result is of much clinical importance and indicates that the drug TKI258 holds promise to treat this clinically difficult issue. In order to

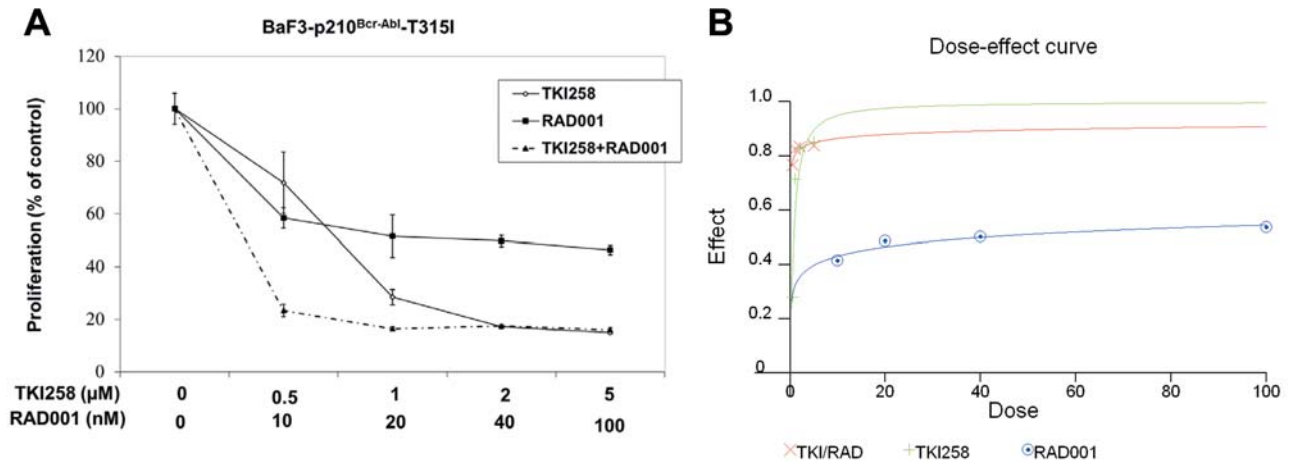


Figure 2. Combination effect of TKI258 and mTOR inhibitor RAD001 at low concentrations on cell proliferation of *BCR-ABL*<sup>+</sup> cell line BaF3-p210<sup>Bcr-Abl</sup>-T3151. A: Cells were seeded into 96-well plates at a density of  $5 \times 10^3$  cells per well and were exposed to different concentrations of TKI258 alone, RAD001 alone and TKI258 in combination with RAD001, respectively. After a 3-day incubation, cell proliferation was determined by the MTT assay. Data were analyzed with CalcuSyn software; (B): dose-effect curve of TKI258 alone, RAD001 alone or the combination of both as analyzed with CalcuSyn software.

further enhance the inhibitory effect of TKI258 on BaF3-p210<sup>Bcr-Abl</sup>-T3151 cells, we tried to combine TKI258 with mTOR inhibitor RAD001, since PI3K/AKT/mTOR is one of the major downstream pathways of *BCR-ABL* and contributes to the survival of the leukemic cells (12, 13). Additional inhibition of mTOR signaling by RAD001 resulted in synergistic growth inhibition and significant apoptosis of BaF3-T3151 cells. In the presence of RAD001, less than 0.5 μM TKI258 was required to reach 50% proliferation inhibition of BaF3-p210<sup>Bcr-Abl</sup>-T3151 cells whereas more than 2 μM TKI258 was necessary when it were used as a single agent. Therefore, combination of TKI258 and mTOR inhibitor is probably a new therapeutic concept for leukemia with *BCR-ABL* T3151 mutation and deserves further study.

To investigate the action mechanism of TKI258 in BaF3-p210<sup>Bcr-Abl</sup>-T3151 cells, we performed Western blot analysis to assess the possible effects of TKI258 on *BCR-ABL* kinase and its downstream signaling. TKI258 effectively inhibited the expression of *BCR-ABL* and blocked its downstream AKT/mTOR and STATs signaling as judged by reduction of phosphorylated AKT and STAT5, respectively. Addition of RAD001 led to additional reduction of phosphorylated 4EBP1 without further alteration of STAT5 suggesting an enhanced suppression of mTOR signaling. This enhancement accounts possibly for the observed synergy between TKI258 and RAD001. However, we believe that inhibition of the mTOR signaling may not be the only reason for the observed synergy since the additional inhibition of 4EBP1 by combination of TKI258 and RAD001 was only moderate. Involvement of additional signaling in the above described synergy remains to be investigated.

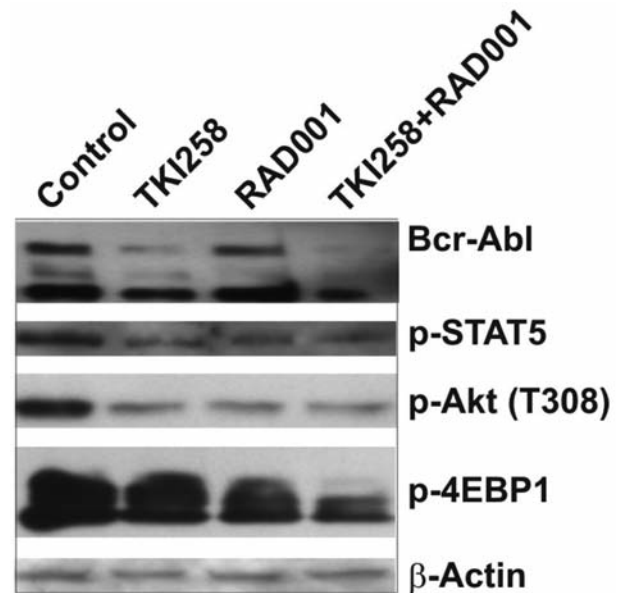


Figure 3. Inhibition of oncoproteins by TKI258 or TKI258 in combination with RAD001 in *BCR-ABL*<sup>+</sup> leukemia. BaF3-p210<sup>Bcr-Abl</sup>-T3151 cells were cultured in the presence of TKI258 (2 μM), RAD001 (10 nM) or the combination of the two drugs for 24 h, respectively. Cells were lysed, *BCR-ABL*, STAT5 and p-AKT (T308) were determined by western Blot.

In conclusion, our data demonstrated that TKI258 induces growth arrest and apoptosis of *BCR-ABL*<sup>+</sup> leukemia. TKI258 is also active against imatinib-resistant *BCR-ABL*<sup>+</sup> ALL and BaF3-p210<sup>Bcr-Abl</sup>-T3151 cells with ectopic expression of the *BCR-ABL* mutant, *BCR-ABL*-T3151, which confers clinical

resistance to almost all available clinical TKIs. Of more clinical importance, addition of the clinically-approved RAD001 synergistically enhances the cytotoxic effects of TKI258 in *BCR-ABL*<sup>+</sup> leukemic cell lines. Overall, our data suggest that TKI258, either alone or with mTOR inhibitors, could represent a novel targeted therapy for this leukemia entity.

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