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RAD 001 (Everolimus) Prevents mTOR and Akt Late Re-Activation in Response to Imatinib in Chronic Myeloid Leukemia

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ABSTRACT
The mammalian target of rapamycin (mTOR) is one target of BCR-ABL fusion gene of chronic myeloid leukemia (CML). Moreover, it drives a compensatory route to Imatinib mesylate (IM) possibly involved in the progression of leukemic progenitors towards a drug-resistant phenotype. Accordingly, mTOR inhibitors are proposed for combined therapeutic strategies in CML. The major caveat in the use of mTOR inhibitors for cancer therapy comes from the induction of an mTOR-phosphatidylinositol 3 kinase (PI3k) feedback loop driving the retrograde activation of Akt. Here we show that the rapamycin derivative RAD 001 (everolimus, Novartis Institutes for Biomedical Research) inhibits mTOR and, more importantly, revokes mTOR late re-activation in response to IM. RAD 001 interferes with the assembly of both mTOR complexes: mTORC1 and mTORC2. The inhibition of mTORC2 results in the de-phosphorylation of Akt at Ser^{473} in the hydrophobic motif of C-terminal tail required for Akt full activation and precludes Akt re-phosphorylation in response to IM. Moreover, RAD 001-induced inhibition of Akt causes the de-phosphorylation of tuberous sclerosis tumor suppressor protein TSC2 at 14-3-3 binding sites, TSC2 release from 14-3-3 sigma (restoring its inhibitory function on mTORC1) and nuclear import (promoting the nuclear translocation of cyclin-dependent kinase [CDK] inhibitor $p27^{Kip1}$, the stabilization of $p27^{Kip1}$ ligand with CDK2, and the G_0/G_1 arrest). RAD 001 cytotoxicity on cells not expressing the BCR-ABL fusion gene or its p210 protein tyrosine kinase (TK) activity suggests that the inhibition of normal hematopoiesis may represent a drug side effect. J. Cell. Biochem. 109: 320–328, 2010. © 2009 Wiley-Liss, Inc.

KEY WORDS: CHRONIC MYELOID LEUKEMIA; mTOR; mTOR INHIBITORS; RAD 001; Akt

BCR-ABL fusion gene, resulting from reciprocal translocation of chromosomes 9 and 22, is the molecular hallmark and the causative event of chronic myeloid leukemia (CML) [Heisterkamp et al., 1985; Daley et al., 1990]. Its oncogenic potential arises from the constitutive TK activity of p210 kDa protein that promotes leukemic progenitor proliferation and survival through interactions with multiple signal transduction pathways. Accordingly, TK inhibitor Imatinib mesylate (IM) induces long-lasting hematologic and cytogenetic responses in the great majority of CML patients in chronic phase [Druker et al., 2006]. mTOR is one BCR-ABL target. Its activation evokes critical events for CML pathogenesis, including cap-dependent mRNA and protein translation, production of angiogenesis-promoting vascular endothelial growth factor (VEGF), generation of reactive oxygen species (ROS), and suppression of pro-apoptotic signals [Mayerhofer et al., 2002; Ly et al., 2003; Kim et al., 2005; Prabhu et al., 2007; Carayol et al., 2008]. Moreover, mTOR drives a compensatory route to IM possibly involved in the disease progression towards drug resistance [Burchert et al., 2005]. Accordingly, mTOR inhibitor rapamycin enhances IM toxicity in BCR-ABL-expressing cells in vitro and in vivo [Mohi et al., 2004; Sillaber et al., 2008].

mTOR belongs to the family of PI3k-related kinases (PIKKs) including DNA-PK, ataxia telengectasia mutated (ATM), and ataxia telengectasia and RAD-3-related (ATR) proteins. It exists in two distinct complexes. The one referred to as mTORC1 contains Raptor (regulatory associated protein of mTOR, the mammalian homolog of

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yeast KOG1), GβL (a mammalian ortholog of yeast Lst8p), and PRAS40 (proline-rich Akt substrate of 40 kDa). It is activated by nutrients, growth factors/hormones, and energy signals and inhibited by rapamycin. Raptor contributes to mTOR specificity by binding specific substrates via TOR signaling (TOS) motifs and promoting their recruitment to the mTOR kinase domain. In particular, it phosphorylates the ribosomal p70 S6 kinases S6K1 and S6K2, which drive ribosome biogenesis and increase the capacity of the translational machinery for protein synthesis, and the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BP) 1 and 2, that initiate the transcription of a subset of mRNAs important for cell proliferation [Wullschleger et al., 2006]. GβL is required for mTORC1 full activation, while PRAS40 functions as a negative regulator by blocking TOS-mediated substrate access to Raptor [Wang et al., 2007]. mTORC1 activity is further regulated by a complex formed by TSC2 (also referred to as tuberin) and TSC1 (also referred to as hamartin). TSC1 induces the stabilization of TSC2 that acts as a GTPase-activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain) and promotes the conversion of Rheb-GTP to Rheb-GDP [Li et al., 2004]. TSC2 phosphorylation in response to growth signals promotes its dissociation from TSC1 at plasma membranes and association with 14-3-3 scaffolding proteins thereby precluding the complex inhibitory function on mTORC1 [Cai et al., 2006]. The other complex, referred to as mTORC2, contains Rictor (the mammalian homolog of AVO3) in place of Raptor, Sin1, GβL, and PRR5 (PRoline-Rich protein 5). It functions as a phosphoinositide-dependent protein kinase 2 (PDK2) to phosphorylate Akt at Ser^{473} and mediates a rapamycin-insensitive pathway [Jacinto et al., 2004; Sarbassov et al., 2005]. Sin1 is required for mTORC2 complex formation and enzymatic activity while the only PRR5 role identified so far is on the regulated expression and signaling of platelet-derived growth factor receptor β (PDGF β) [Jacinto et al., 2006; Woo et al., 2007].

Moreover, mTOR is one major regulatory component of authophagy, a degradative process resulting in the breakdown of intracellular material within lysosomes [Kondo et al., 2005]. While in certain cellular contexts autophagy can serve as a death mechanism alternative to apoptosis (named type II cell death), it may be involved in cancer cell adaptation to environmental stress signals (such as low oxygen, starvation, or growth factor deprivation) thereby acting as a survival mechanism. Accordingly, high levels of mTOR kinase inhibit autophagy and the rapamycin-induced inhibition of mTOR triggers autophagy [Kondo et al., 2005]. A recent study showed that the autophagic process is rapidly activated by IM in CML cells and its inhibition by chloroquine potentiates IM-induced leukemic cell death in vitro and in vivo [Bellodi et al., 2009].

The aim of our study was to investigate the impact of rapamycin derivative RAD 001 (everolimus) on BCR-ABL-expressing cells. RAD 001 (at the 1 μM concentration required to inhibit leukemic cell proliferation through the repression of protein synthesis) was first studied for its effects on a cell clone (3B) generated from the murine hematopoietic progenitor cell line 32D expressing a temperature-sensitive (ts) BCR-ABL construct (whose p210 protein owns TK activity only at the permissive temperature of 33°C) [Mazzacurati et al., 2004]. RAD 001 inhibited mTOR-activating phosphorylation and, more importantly, revoked mTOR late re-activation in response to IM. RAD 001 interfered with the assembly of both mTORC1 and mTORC2 complex components and prevented mTOR re-association with Raptor, Rictor, and Sin1 occurring after a prolonged exposure to IM (24 h). The drug inhibitory effect on mTORC2 precluded Akt re-phosphorylation at Ser^{473} thereby preventing its late re-activation in response to IM. Such a RAD 001 "high-dose effect" on Akt reduced TSC2 phosphorylation at an Akt site (Ser^{1462}) allowing its release from 14-3-3 sigma (that restores its inhibitory function on mTORC1) and nuclear import (that drives the G_0/G_1 arrest through functional interactions with CDK inhibitor $p27^{Kip1}$) [Liu et al., 2002; Rosner et al., 2007a]. RAD 001 effects on mTOR, Akt, and TSC2 were confirmed in $CD34^+$ hematopoietic progenitors purified from bone marrow samples of CML patients at diagnosis expressing the BCR-ABL gene. RAD 001 cytotoxicity against 32D parental cell line (not expressing the BCR-ABL gene) and ts BCR-ABL-transduced cells kept at 39°C (lacking p210 BCR-ABL TK activity) suggests that the inhibition of normal hematopoiesis may represent one major limit for drug clinical use in the therapy of CML.

MATERIALS AND METHODS

CELLS AND TREATMENTS

A ts BCR-ABL mutant subcloned into a pDG retroviral vector under the control of myeloproliferative sarcoma virus LTR promoter was expressed in the murine myeloid progenitor cell line 32D through electroporation [Mazzacurati et al., 2004]. The temperature dependence of its p210 protein TK activity in clone 3B (generated from single colonies of 32D grown in methylcellulose following electroporation) was preliminarily assessed (data not shown). The ts BCR-ABL-transduced clone 3B was maintained in RPMI medium (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 1% L-glutamine, antibiotics, and 10% WEHI-3 conditioned medium (CM) as source of IL-3 when required in 5% CO_2 and fully humidified atmosphere at either permissive (33°C) or non-permissive (39°C) temperature. Parental 32D cell lines was maintained in RPMI medium additioned with FCS, 10% WEHI-3 CM, and antibiotics at 37°C. Cell sensitivity to IM and RAD 001 (both provided by Novartis Institutes for Biomedical Research, Oncology, Basel, Switzerland) was measured in clonogenic assays (0.9% methylcellulose additioned with 30% FCS). Lethal dose 50 (LD_{50}) was calculated by means of linear regression analysis in three individual experiments. The time-course signal induction in response to drugs was investigated following in vitro exposure to 1 μM IM and RAD 001 either alone or associated.

IMMUNO-MAGNETIC PURIFICATION OF $CD34^+$ CELLS

$CD34^+$ hematopoietic progenitors were isolated from bone marrow of CML patients at diagnosis after informed consent. They were obtained by indirect immuno-magnetic labeling (mini-MACS from Milteny Biotech, Bergish Gladback, Germany) of mononuclear cell fractions. Their content was measured by means of cytometric analysis with a FacScan (Becton Dickinson, Franklin Land, PI). Twenty to 30 $CD34^+$ cells were scored for the presence of BCR-ABL re-arrangement by means of fluorescent in situ hybridization (FISH) using the LSI BCR-ABL ES dual Colour Translocation probe (Vysis, Downers Grove, IL).

PROTEIN ANALYSIS

Western blot and immunoprecipitation (IP)/immunoblotting analyses were performed on whole cell and nuclear lysates according to published methods [Mazzacurati et al., 2004; Mancini et al., 2007]. Anti-mTOR, Sin1, and Rictor antibodies were purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Anti-phospho-mTOR Ser^{2448} antibody was purchased from GeneTex, Inc. (Irvine, CA) and anti-Raptor from Epitomics, Inc. (Burlingame, CA), anti-$p27^{kip1}$, and -TSC2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Akt Ser^{473}, anti-phospho-Akt Thr^{308}, and anti-phospho-TSC2 Thr^{1462} were purchased from Cell Signalling (Danvers, MA). Anti-beta actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Signal intensities in single blots from three repeated experiments were measured by a GS-700 Imagining densitometer (BioRad, Waltham, MA) equipped with a dedicated software (Molecular Analyst, BioRad).

CYTOFLUORIMETRIC ANALYSIS OF CELL-CYCLE DISTRIBUTION

Cytofluorimetric analysis of cell-cycle distribution and cell death was performed by measuring the uptake of propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO) and Annexin V (Hoffmann-La Roche, Basel, Switzerland) according to published methods [Mazzacurati et al., 2004]. Cell fluorescence and PI uptake were measured by means of a FACScan flow cytometer (set on 488 nm excitation and 530 nm bandpass filter wavelength for fluorescein detection or 580 nm for PI detection) and a dedicated software (both from Beckton Dickinson). Statistical significance of differences in cell-cycle distribution and death following drug exposure was determined by means of Student's t-test ($P < 0.05$ was considered significant).

RESULTS

THE mTOR INHIBITOR RAD 001 ENHANCES IM EFFECTS ON PROLIFERATION AND CELL-CYCLE PROGRESSION OF BCR-ABL-EXPRESSING CELLS

The effects of IM and RAD 001 were first investigated in clone 3B kept at the permissive temperature for p210 BCR-ABL TK. IM and RAD 001 induced a dose-dependent reduction of its reproductive integrity, with LD_{50} of 0.39 ± 0.08 and 1.67 ± 0.11 μM, respectively (Fig. 1A). The association of 0.5 μM IM significantly reduced RAD 001 LD_{50} to 0.49 ± 0.07 μM ($P < 0.01$) (Fig. 1A). The findings support that higher doses of mTOR inhibitors (falling in the μM range) are required to inhibit leukemic cell proliferation compared to nM doses that are sufficient for the suppression of mTOR activity [Shor et al., 2008]. We therefore used RAD 001 at 1 μM concentration (the dose needed for the so-called "high-dose effect") alone or in association with 1 μM IM to investigate the drug impact

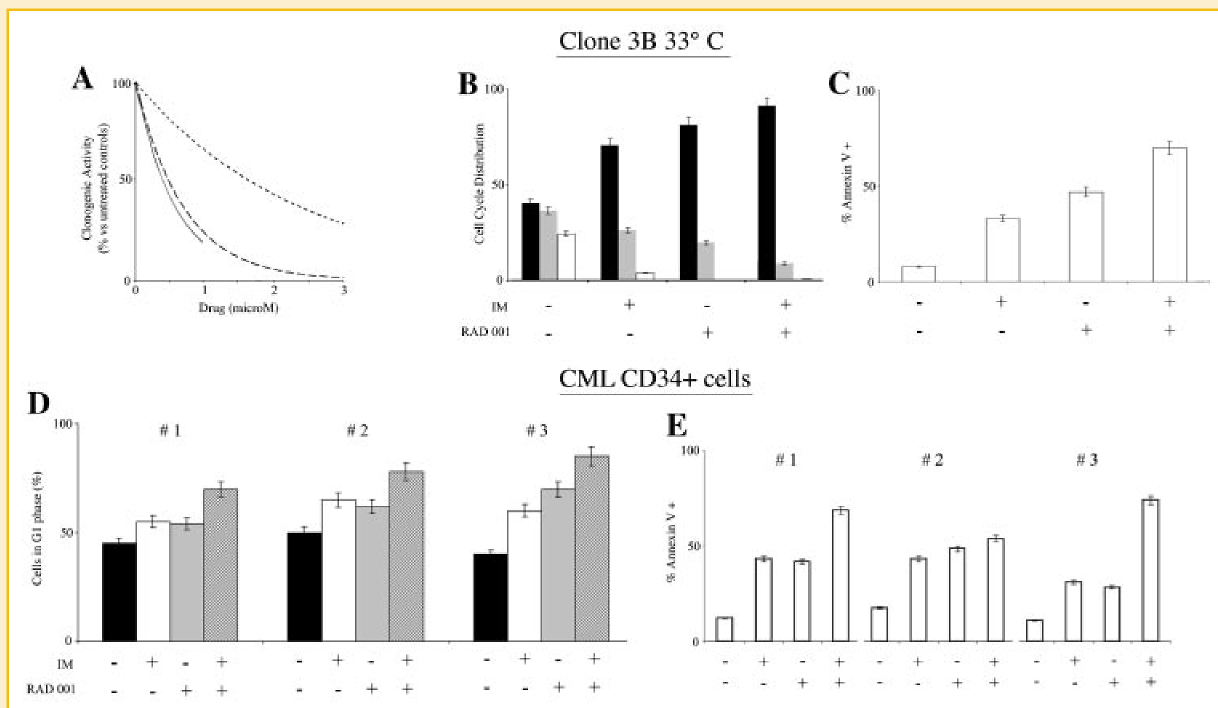

Fig. 1. RAD 001 effects on proliferation, survival, and cell-cycle progression of BCR-ABL-expressing cells. A: Survival curves of clone 3B kept at 33°C exposed to scalar doses of IM (———) and RAD 001 alone (••••••) or associated with 0.05 μM IM (— —). The drug effects on clone 3B reproductive integrity were assayed in clonogenic assays (0.9% methyl cellulose) in three separate experiments. Standard deviations did not exceed 10% (data not shown). B: Changes in cell-cycle distribution of clone 3B kept at 33°C following 24 h exposure to 1 μM IM and RAD 001 alone or associated. Black: G_1 phase; gray: S phase; white: G_2/M phase. Cell-cycle distribution was assessed by means of cytofluorimetric analysis of PI uptake. C: Cell death induction by IM and RAD 001 alone or associated in clone 3B kept at 33°C. Cell death was measured by cytofluorimetric analysis of Annexin V uptake. D: Changes in G_1 size of $CD34^+$ cells from three CML patients at diagnosis in response to IM and RAD 001 alone and associated. E: IM- and RAD 001-induced death in $CD34^+$ cells from three CML patients at diagnosis.

on cell-cycle distribution and survival. RAD 001 induced a significant cell recruitment in G_1 phase ($P < 0.001$), paralleling a significant reduction of cells in S and G_2 phases ($P < 0.05$ or less) and further significantly augmented by IM association ($P < 0.05$) (Fig. 1B). Moreover, RAD 001 promoted the cell commitment to death further significantly upraised by IM association ($P < 0.01$ or less) (Fig. 1C).

RAD 001 impact and additive effects to IM on cell-cycle progression and survival were then investigated in $CD34^+$ hematopoietic progenitors isolated from bone marrow of three CML patients at diagnosis. $CD34^+$ cell final concentration following immuno-magnetic sorting was >95% in all cases. Moreover, in situ fluorescence hybridization (FISH) detected the BCR-ABL fusion gene in more than 80% of $CD34^+$ cells (data not shown). The fraction of $CD34^+$ cells arrested in G_1 or committed to death following in vitro treatment with RAD 001 was significantly higher compared to untreated controls and further increased by IM association ($P < 0.05$ or less) (Fig. 1D,E).

RAD 001 SUPPRESSES mTOR LATE RE-ACTIVATION IN RESPONSE TO IM

In clone 3B kept at 33°C mTOR expression and phosphorylation at Ser^{2448} (which closely parallels mTOR activation by insulin) were significantly reduced by IM at second and fourth hour ($P < 0.01$) but recovered the levels of untreated controls at the 24th hour ($P < 0.1$) (Fig. 2A). As expected, 24 h exposure to 0.1 and 1 μM RAD 001 abrogated mTOR phosphorylation at Ser^{2448} and revoked mTOR late re-phosphorylation in response to IM (Fig. 2A and data not shown).

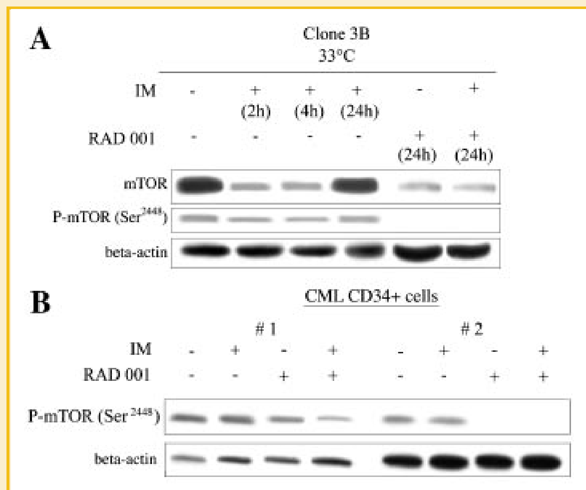

Fig. 2. RAD 001 effects on mTOR expression and activating phosphorylation. A: In clone 3B kept at 33°C mTOR expression and activating phosphorylation at Ser^{2448} were investigated by means of Western blot or IP-immunoblotting technique, respectively, following exposure to 1 μM IM for 2, 4, and 24 h, to 1 μM RAD 001 for 24 h and to the two drug association for 24 h. B: mTOR phosphorylation at Ser^{2448} was investigated in $CD34^+$ cells from CML patients # 1 and # 2 following 24 h exposure to 1 μM IM and RAD 001 alone or associated. Beta actin served as the internal control for protein loading. The results shown here have been confirmed in two other separate experiments.

The inhibitory effects of RAD 001 on mTOR were further investigated in $CD34^+$ cells from two CML patients at diagnosis. In both cases, mTOR phosphorylation at Ser^{2448} after 24 h in vitro exposure to μM IM did not exhibit significant differences compared to untreated control ($P < 0.1$) but was significantly reduced ($P < 0.05$, patient # 1) and completely abrogated (patient # 2) by in vitro exposure to RAD 001 (1 μM for 24 h) (Fig. 2B). RAD 001 and IM association further significantly reduced mTOR phosphorylation at Ser^{2448} in $CD34^+$ cells from patient # 1 ($P < 0.01$) (Fig. 2B).

RAD 001 PROMOTES AKT DE-PHOSPHORYLATION THROUGH THE INHIBITION OF mTORC2 ASSEMBLY

In clone 3B kept at 33°C Raptor and Rictor expression and interaction with mTOR were significantly reduced at second and fourth hour of exposure to 1 μM IM ($P < 0.01$ or less), but partly recovered at the 24th hour (Fig. 3A). Conversely, Sin1 expression and interaction with mTOR were persistently reduced by IM ($P < 0.01$ or less) (Fig. 3A). It is assumed that rapamycin in complex with its intracellular receptor FKBP12 directly binds mTORC1 and weakens the interaction between mTOR and Raptor, but neither binds mTORC2 nor perturbs mTORC2 assembly. As expected, in clone 3B kept at 33°C Raptor expression and interaction with mTOR

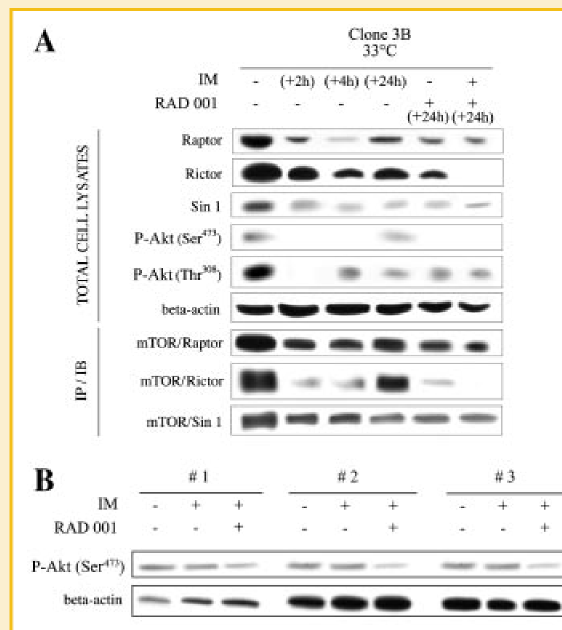

Fig. 3. RAD 001 inhibits the assembly of mTORC1 and mTORC2 components and induces a retrograde signaling resulting in Akt full inactivation. A: In clone 3B kept at 33°C the expression of Raptor, Rictor, and Sin1 was investigated by means of Western blot analysis. mTOR interactions with Raptor, Rictor, and Sin1 were investigated by labeling mTOR IP products with specific anti-Raptor, -Rictor, and -Sin1 antibodies. Akt phosphorylation at relevant residues for its activation (Ser^{473} and Thr^{308}) was investigated by means of IP-immunoblotting technique. The results shown here have been confirmed in two other separate experiments. B: In $CD34^+$ cell from three CML patients at diagnosis the changes in Akt phosphorylation at Ser^{473} following mTOR inhibition by RAD 001 were investigated by means of IP-immunoblotting technique. Beta actin was used as the internal control for protein loading. See Figure 2 legend for drug doses and exposure length.

were significantly reduced following 24 h exposure to 1 μM RAD 001 ($P < 0.01$) (Fig. 3A). Notably, RAD 001 also reduced Rictor and Sin1 expression and their interaction with mTOR ($P < 0.01$ or less). Moreover, RAD 001 significantly reduced the recovery of Raptor expression and interaction with mTOR ($P < 0.01$ and 0.05, respectively) and completely abolished the recovery of Rictor expression and interaction with mTOR after 24 h exposure to IM (Fig. 3A). Sin1 and Rictor are crucial for Akt phosphorylation at Ser^{473} [Navé et al., 1999; Sarbassov et al., 2005]. Accordingly, their persistent reduction in response to RAD 001 resulted in Akt de-phosphorylation at Ser^{473} and, more importantly, prevented AKT re-phosphorylation after 24 h exposure to IM (Fig. 3A). Furthermore, RAD 001 either alone or in association with IM significantly reduced Akt phosphorylation at Thr^{308}, although to a lesser extent than Ser^{473} phosphorylation ($P < 0.01$ or less) (Fig. 3A). The findings are consistent with a study showing that mTORC2 phosphorylation of Akt at Ser^{473} within the C-terminal hydrophobic motif provides a docking site for the recruitment of phosphoinositide-dependent kinase 1 (PDK1) in order to phosphorylate Thr^{308} in the activation loop required for Akt full activation [Frodin et al., 2002].

The effect of 1 μM RAD 001 on Akt activating phosphorylation at Ser^{473} was further investigated in $CD34^+$ progenitors from three CML patients. In all three cases Akt phosphorylation at Ser^{473} was not significantly affected by 24th hour in vitro exposure to IM alone ($P < 0.1$), but significantly reduced by RAD 001 and IM association ($P < 0.05$) (Fig. 3B). Further investigation is required to elucidate why Ser^{473} de-phosphorylation of AKT in response to IM and RAD 001 association is complete in clone 3B and only partial in $CD34^+$ cells expressing p210 BCR-ABL TK.

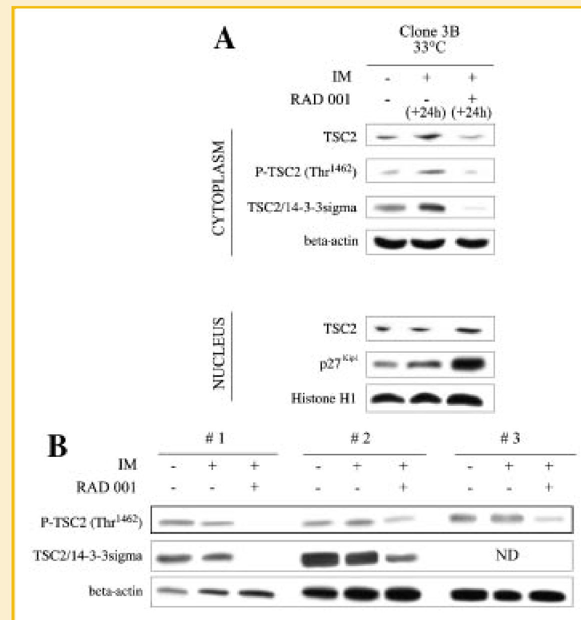

Fig. 4. RAD 001 impact on TSC2 phosphorylation promotes TSC2 release from 14-3-3 sigma and nuclear import. A: TSC2 expression in the cytoplasmatic and nuclear compartments and $p27^{Kip1}$ nuclear expression levels were investigated in clone 3B kept at 33°C by means of Western blot. TSC2 phosphorylation at Thr^{1462} (one Akt site) and interaction with 14-3-3 sigma were investigated by means of IP/immunoblotting technique. The results shown here have been confirmed in two additional experiments. B: TSC2 phosphorylation at Thr^{1462} and interaction with 14-3-3 sigma were investigated in $CD34^+$ cell from three CML patients at diagnosis. Beta actin and histone H1 were used as the internal controls for cytoplasmatic and nuclear protein loading.

TSC2 DE-PHOSPHORYLATION AND RELEASE FROM 14-3-3 CONTRIBUTES TO mTORC1 INHIBITION IN RESPONSE TO RAD 001

TCS2 phosphorylation by AKT creates binding sites for 14-3-3 scaffolding proteins and precludes the formation of TSC1/TSC2 complex thereby precluding its inhibitory function on mTORC1 [Cai et al., 2006]. It may therefore represent an additional route to mTOR compensatory activation in response to IM of CML cells. In clone 3B kept at 33°C Akt late re-phosphorylation at the 24th hour of exposure to 1 μM IM was associated with a significant increase of TSC2 phosphorylation at Thr^{1462} (one of five Akt sites) in the cytoplasmatic compartment ($P < 0.05$) (Fig. 4A). RAD 001 revoked the increment of TSC2 phosphorylation in response to IM ($P < 0.01$) thereby promoting TSC2 release from 14-3-3 sigma ($P < 0.001$) (Fig. 4A). The impact of Akt phosphorylation status on TSC2 expression, whose increment in response to IM was revoked by RAD 001 ($P < 0.001$), may be contingent upon reduced protein stability or sub-cellular repartitioning following de-phosphorylation [Han et al., 2008].

TSC2 phosphorylation status and interaction with 14-3-3 sigma were further investigated in $CD34^+$ progenitors from three CML patients at diagnosis. In all cases TSC2 phosphorylation at Thr^{1462} was not affected by in vitro exposure to IM ($P < 0.1$) but significantly reduced by RAD 001 association in two cases (patients # 2 and # 3; $P < 0.05$ and 0.01, respectively) and completely abrogated in one (patient # 1) (Fig. 4B). TSC2 release from 14-3-3 sigma following its de-phosphorylation was confirmed in two cases (patients # 1 and # 2) (Fig. 4B).

A recent study proved that Akt-induced phosphorylation triggers TSC2 de-localization from the nucleus to the cytoplasm through events at least partly dispensable from 14-3-3 binding [Rosner et al., 2007a]. Accordingly, in clone 3B kept at 33°C TSC2 de-phosphorylation at Thr^{1462} following Akt inactivating de-phosphorylation in response to RAD 001 and IM association induced a significant increment of TSC2 nuclear expression ($P < 0.01$) (Fig. 4A). To elucidate if TSC2 functions were restored by de-phosphorylation we assayed the nuclear expression of $p27^{Kip1}$, the CDK inhibitor that mediates TSC2 negative impact on cell-cycle progression. Previous studies proved, in fact, that TSC2 prevents $p27^{Kip1}$ degradation by SCF^{Skp2} ubiquitin ligase through direct binding and competes with $p27^{Kip1}$ for 14-3-3 binding thereby stabilizing $p27^{Kip1}$ interaction with CDK2 [Rosner and Hengstschläger, 2004; Rosner et al., 2007b]. Indeed, $p27^{Kip1}$ nuclear levels were significantly upraised by IM and further increased by RAD 001 association ($P < 0.05$ and < 0.001, respectively) (Fig. 4A).

RAD 001 EFFECTS ON PROLIFERATION ARE NOT RESTRICTED TO CELLS EXPRESSING p210 BCR-ABL TK

To investigate whether RAD 001 selectivity targets CML progenitors we assayed the drug effects on cells lacking the BCR-ABL re-arranged gene (32D parental cell line) and or the p210 BCR-ABL TK activity (clone 3B kept at the non-permissive temperature of

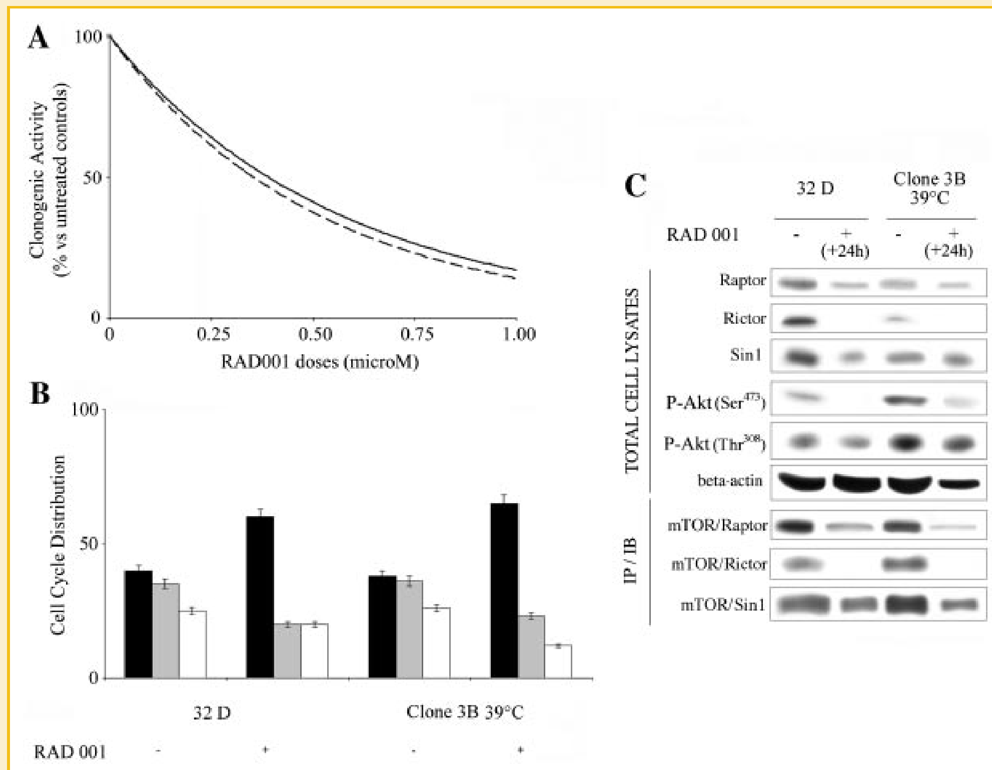

Fig. 5. RAD 001 effects on proliferation and cell-cycle progression of cell not expressing the BCR-ABL gene (parental 32D cell line) or the TK activity of its p210 protein (clone 3B kept at the non-permissive temperature of 39°C). A: Survival curves of parental 32D cell line (continuous line) and clone 3B kept at 39°C (dotted line) exposed to scalar doses of RAD 001. See Figure 1A legend for details. B: Changes in cell-cycle distribution of 32D cell line and clone 3B kept at 39°C following 24 h exposure to 1 μM IM and RAD 001 alone or associated. See Figure 1B legend for details. C: RAD 001 effects on the assembly of mTOR complexes: mTORC1 and mTORC2 and on Akt activating phosphorylations, the signals involved in growth arrest of 32D cell line and clone 3B kept at 39°C. The results shown here have been confirmed in two additional experiments. See Figure 3 legends for technical details.

39°C). Dose–response curves showed that RAD 001 reduces clonogenic activity (with LD_{50} of 0.30 and 0.24 μM, respectively) and induces a G_1 arrest in both cell types ($P < 0.01$ and < 0.005, respectively) (Fig. 5A,B).

The anti-proliferative effects of RAD 001 on the putative normal counterpart of leukemic hematopoiesis proceeded from its impact on mTOR complexes. In 32D parental cell line and clone 3B kept at 39°C the reduction of Raptor, Rictor, and Sin1 expression and interaction with mTOR in response to RAD 001 was similar to that seen in clone 3B kept at 33°C (Fig. 5C). Reduced assembly of mTORC2 components had a significant impact on Akt phosphorylation at Ser^{473} and Thr^{308} (Fig. 5C). Our results are consistent with mTOR participation in the proliferation and survival signaling downstream of IL-3 receptor in hematopoietic progenitor cells [Wieman et al., 2007]. Moreover, they suggest that the inhibition of normal hematopoiesis may represent a side effect of RAD 001 in CML.

DISCUSSION

The TK inhibitor IM is the frontline treatment of CML. However, a subset of patients, particularly those treated in advanced stages, develop IM resistance most frequently due to BCR-ABL point mutations that reduce the drug binding affinity to the fusion protein [Jabbour et al., 2006]. However, BCR-ABL-independent events, including the activation of PI3k/Akt/mTOR axis have been involved in the persistence of leukemic cells eventually preceding the emergence of overt IM resistance [Burchert et al., 2005]. Accordingly, mTOR inhibitors have been proposed for combined therapeutic strategies in CML [Mohi et al., 2004; Sillaber et al., 2008].

The major caveat in the use of rapamycin and its derivatives for cancer therapy comes from the induction of a negative feedback pathway leading to Akt activation as a result of the up-regulation of the IRS-2 adaptor protein and insulin-like growth factor (IGF-1)/IGF-1R signaling [O'Reilly et al., 2006; Wan et al., 2007]. The putative role of mTOR inhibitors in the treatment of hematologic malignancies is still controversial. Akt activation proceeding from IGF-1R signaling suggests caution in their use in acute myeloid leukemias (AML) and multiple myeloma [Shi et al., 2005; Zeng et al., 2007; Tamburini et al., 2008]. Conversely, in CML the cytotoxicity of TK inhibitors is enhanced by rapamycin and RAD 001 [Mayerhofer et al., 2005; Ikezoe et al., 2006]. Notably, rapamycin is also effective in IM-resistant CML patients [Sillaber et al., 2008]. However, the mechanisms underlying the additive effects of TK and mTOR inhibitors in $BCR\text{-}ABL^{+}$ diseases are largely unknown. Here we

demonstrated that in BCR-ABL-expressing cells RAD 001 interferes with both mTOR complexes mTORC1 and mTORC2. In a BCR-ABL-transduced 32D cell clone, (3B) kept under permissive conditions for p210 TK activity (33°C), RAD 001 inhibited mTOR-activating phosphorylation at Ser^{2448}, and more importantly, revoked late mTOR re-phosphorylation in response to IM (Fig. 2A). mTOR inhibition by RAD 001 proceeded from the reduction of Raptor, Rictor, and Sin1 expression and of their interaction with mTOR and from the precluded late re-assembly of mTORC1 and mTORC2 in response to IM (Fig. 3A). Rictor and Sin1 are key components of mTORC2 enzymatic activity [Navé et al., 1999; Sarbassov et al., 2005]. Accordingly, mTOR dissociation from Rictor and Sin1 induced by RAD 001 precluded Akt activating phosphorylation at Ser^{473} and its late recovery in response to IM (Fig. 3A). Moreover, the reduction of Akt phosphorylation at Thr^{308} promoted Akt full inactivation in response to RAD 001 alone or in association with IM [Frodin et al., 2002].

The effects of RAD 001 and IM association on mTOR phosphorylation at Ser^{2448} and Akt phosphorylation at Ser^{473} were confirmed in $CD34^{+}$ progenitors from CML patients at diagnosis (Figs. 2B and 3B). Rapamycin-induced inhibition of mTORC2–Akt axis requires prolonged treatments (24 h or more). It arises from the drug interference with mTORC2 assembly leading to the reduction of intact mTORC2 below the threshold needed to maintain Akt phosphorylation at Ser^{473}, rather than FKBP12-rapamycin binding with preformed mTORC2 as occurs with mTORC1 [Sarbassov et al., 2006]. Accordingly, mTOR inhibitors are now regarded as cell-type-dependent inhibitors of mTORC2 as well as universal inhibitors of mTORC1. Moreover, RAD 001 inhibition of Akt is conditional upon the drug dose and the basal levels of phosphorylated Akt. In this study we used RAD 001 doses ranging from 100 nM to 1 μM and did not detect any significant difference in the impact of such drug doses on mTOR-activating phosphorylation at Ser^{2448} (Fig. 2A and data not shown). Lower RAD 001 doses, in particular the 1 nM dose that significantly increases phosphorylated Akt levels in human myelomonocytic (U937) and T-lymphoid leukemia (Jurkat) cell lines were not tried [Wang et al., 2008]. Notably, Akt hyperphosphorylation has been associated with IM resistance of BCR-ABL-expressing cells [Mancini et al., 2007]. It will be of interest to investigate whether it also drives resistance to mTOR inhibitors.

RAD 001 inhibited mTORC1 assembly and activation, and precluded mTORC1 re-assembly in response to IM through mechanisms encompassing TSC2 post-translational modifications (Fig. 4A). Previous studies proved that Akt-induced phosphorylation of TSC2 creates 14-3-3 binding sites and thereby keeps TSC2 away from TSC1 at the cellular membranes, thereby hindering the TSC1/TSC2 complex inhibitory function on mTORC1 [Liu et al., 2002; Cai et al., 2006]. Moreover, it promotes TSC2 de-localization from nuclear to cytoplasmatic compartments through events most likely at least partly independent from 14-3-3 ligand [Rosner et al., 2007a]. Here we showed that in clone 3B kept at 33°C RAD 001 revokes Akt late re-phosphorylation in response to IM and thereby promotes a significant reduction of TSC2 phosphorylation at Thr^{1462} (one of five Akt sites), release from 14-3-3 sigma and nuclear re-location (Figs. 3A and 4A). The two former events were elicited by RAD 001 also in $CD34^{+}$ cells from CML patients (Figs. 3B and 4B).

$P27^{Kip1}$ is a critical mediator of TSC2 negative impact on cell-cycle progression. Here, we showed that TSC2 de-phosphorylation in response to RAD 001 and IM association causes a massive $p27^{Kip1}$ nuclear import promoting the two drug additive effects on G_1 arrest of BCR-ABL-expressing cells (Figs. 1B,D and 4A,B). P210 BCR-ABL TK abrogates $p27^{Kip1}$ function by inhibiting the gene transcription, enhancing the protein Skp2-mediated degradation and precluding the protein re-location to the nuclear compartment where it binds cyclin/CDK complexes [Sekimoto et al., 2004; Andreu et al., 2005; Hong et al., 2008]. Accordingly, IM raises $p27^{Kip1}$ expression and promotes its nuclear translocation thereby contributing to the BCR-ABL-expressing cell arrest in the G_1 (Figs. 1B and 4A). $P27^{Kip1}$ nuclear re-location in response to RAD 001 may be concurrently promoted by distinct two events: the dissociation of mTORC1 components mTOR and Raptor (that impairs $p27^{Kip1}$ phosphorylation at the 14-3-3 binding residue [Thr^{157}] by the serum/glucocorticoid-regulated kinase 1 [SGK1]) and the interaction with TSC2 following its de-phosphorylation, that promotes the stabilization of $p27^{Kip1}$ and of its ligand with CDK2 [Rosner and Hengstschläger, 2004; Rosner et al., 2007b; Hong et al., 2008].

In conclusion, our results proved RAD 001 and IM additive effects on growth and survival of BCR-ABL-expressing cells arising from the RAD 001-induced block of mTORC2/Akt retrograde signaling pathway and recovery of TSC2 inhibitory function on mTORC1 (Fig. 6). Both events may contribute to prevent the outcome of IM-resistant cell clones during IM therapy in CML [Burchert et al., 2005]. Additional mechanisms, including the remarkable reduction of polysomal mRNA assembly and protein translation, and of VEGF (which acts as an autocrine growth factor for clonal progenitors), may contribute to RAD 001 cytotoxicity against the $BCR\text{-}ABL^{+}$ leukemic clone [Mayerhofer et al., 2002; Shor et al., 2008; Zhang et al., 2008].

Finally, RAD 001 anti-proliferative effects on 32D cells not expressing the BCR-ABL gene or the TK of its p210 protein activity proceeding from its impact on the mTOR–Akt axis suggest that the

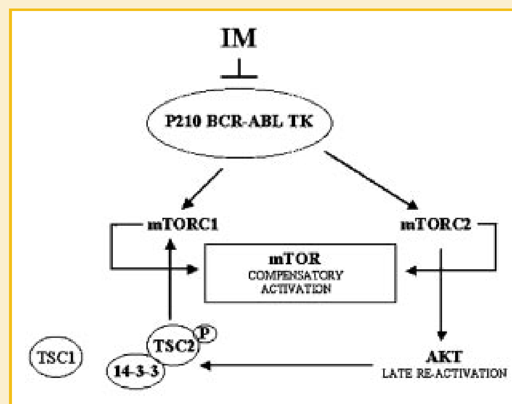

Fig. 6. RAD 001 prevents the compensatory activation of mTOR in response to IM in CML cells through different mechanisms. The drug impact on mTORC2 precludes the late re-activation of Akt that, in turn, induces the phosphorylation of TSC2 at a critical residue for binding to 14-3-3 scaffolding proteins thereby hindering the formation of TSC1/TSC2 complex and its inhibitory function on mTORC1.

drug cytotoxicity may be not restricted to p210 BCR-ABL-expressing cells and that the inhibition of normal hematopoiesis might be a major side effect of RAD 001 in CML (Fig. 5A). However, clinical trials revealed that hematologic abnormalities are uncommon and generally mild events occurring in response to the maximum tolerated dose of RAD 001 malignancies (10 mg/day), leading to mTOR inhibition and AKT down-modulation in relapsed or refractory hematologic [Yee et al., 2006; Tabernero et al., 2008]. Notably, a new perspective in the use of mTOR-targeted therapy for the cure of leukemias has been opened by rapamycin capacity of inhibiting the generation, proliferation, and survival of leukemia-initiating cells generated by the deletion of PTEN (for phosphatase and tensin homolog) without damaging normal hematopoietic stem cells [Yilmaz et al., 2006]. Further investigation is required to explore whether CML stem cells exhibit this unique sensitivity to RAD 001.

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