The antitumor effects of sunitinib (formerly SU11248) against a variety of human hematologic malignancies: enhancement of growth inhibition via inhibition of mammalian target of rapamycin signaling

Takayuki Ikezoe,[1] Chie Nishioka,[1] Taizo Tasaka,[2] Yang Yang,[1] Naoki Komatsu,[1] Kazuto Togitani,[1] H. Phillip Koeffler,[3] and Hirokuni Taguchi[1]

[1]Department of Hematology and Respiratory Medicine, Kochi University, Nankoku, Kochi, Japan; [2]Department of Internal Medicine, Kagawa University, Kita-gun, Kagawa, Japan; and [3]Department of Hematology and Oncology, Cedars-Sinai Medical Center, University of California at Los Angeles School of Medicine, Los Angeles, California

Abstract

We studied antitumor effects of receptor tyrosine kinase inhibitor sunitinib (formerly SU11248) against a variety of hematologic malignancies including the following leukemias: eosinophilic (EOL-1), acute myeloid (THP-1, U937, Kasumi-1), biphenotypic (MV4-11), acute lymphoblastic (NALL-1, Jurkat, BALL-2, PALL-1, PALL-2), blast crisis of chronic myeloid (KU812, Kcl-22, K562), and adult T-cell (MT-1, MT-2, MT-4), as well as non-Hodgkin's lymphoma (KS-1, Dauji, Akata) and multiple myeloma (U266). Thymidine uptake studies showed that sunitinib was active against EOL-1, MV4-11, and Kasumi-1 cells, which possessed activating mutations of the *PDGFRα*, *FLT-3*, and *c-KIT* genes, respectively, with IC_{50}s of <30 nmol/L. In addition, sunitinib inhibited the proliferation of freshly isolated leukemia cells from patients possessing mutations in *FLT3* gene. Annexin V staining showed that sunitinib induced apoptosis of these cells. Sunitinib inhibited phosphorylation of FLT3 and PDGFRα in conjunction with blockade of mammalian target of rapamycin signaling in MV4-11 and EOL-1 cells, respectively. Interestingly, rapamycin analogue RAD001 enhanced the ability of sunitinib to inhibit the proliferation of leukemia cells and down-regulate levels of mammalian target of rapamycin effectors p70 S6 kinase and eukaryotic initiation factor 4E–binding protein 1 in these cells. Taken together, sunitinib may be useful for treatment of individuals with leukemias possessing activation mutation of tyrosine kinase, and the combination of sunitinib and RAD001 represents a promising novel treatment strategy. [Mol Cancer Ther 2006;5(10):2522–30]

Introduction

Class III and V receptor tyrosine kinases (RTK), including fms-like tyrosine kinase 3 (FLT3), c-KIT, platelet-derived growth factor receptor (PDGFR), and endothelial growth factor receptors (VEGFR), respectively, consist of five immunoglobulin-like domains in their extracellular regions and a juxtamembrane domain, a kinase domain interrupted by a kinase insertion domain, and a COOH-terminal domain in intracellular regions (1). On ligand binding, RTK activates its downstream effectors including protein kinase B/Akt, signal transducers and activators of transcription (STAT), and extracellular signal-regulated kinases (ERK)-1/2, leading to cell proliferation, differentiation, and/or survival (2, 3). Recent studies found that activating mutations of RTKs frequently occurred in acute myeloid leukemia (AML): internal tandem duplications (ITD) of the juxtamembrane domain of *FLT3* (*FLT3-ITD*) has been found in 20% to 30% of the cases of *de novo* AML (4). Approximately 7% of cases of AML possessed D835 mutation, a point mutation in the activation loop of the second kinase domain of *FLT3* (4). These mutations constitutively activate this receptor kinase without ligand binding, resulting in the activation of downstream prosurvival signals, and are associated with elevated blast counts, increased relapse rate, and poor overall survival (5–8). Mutations of *c-KIT* and *PDGFR* are also associated with subsets of AML (9–12) as well as gastrointestinal stromal tumors (GIST; refs. 13, 14). Thus, RTKs represent promising molecular targets for treatment of AML.

The serine/threonine kinase mammalian target of rapamycin (mTOR) is activated by phosphatidylinositol 3-kinase/Akt signaling and regulates cell proliferation, in part, by ribosomal protein translation and the initiation of cap-dependent translation (15, 16). mTOR phosphorylates p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E–binding protein 1 (4E-BP-1) and increases the translation of mRNAs with long, highly structured 5′-untranslated regions, such as cyclin D1 and c-Myc, which regulate cell cycle transition from G_1 to S phase (15, 16). The mTOR

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Requests for reprints: Takayuki Ikezoe, Department of Hematology and Respiratory Medicine, Kochi University, Nankoku, Kochi 783-8505, Japan. Phone: 81-88-880-2345; Fax: 81-88-880-2348. E-mail: ikezoet@med.kochi-u.ac.jp

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Table 1. Inhibition of the proliferation of malignant hematopoietic cells by SU11248

Cell line	Gene alterations	IC_{50} (μmol/L)
Eosinophilic leukemia		
EOL-1	FIP1L1/PDGFRα	0.004
Biphenotypic leukemia		
MV4-11	FLT3-ITD	0.025
Myeloid leukemia		
Kasumi-1	$Asn^{822}Lys$ in c-Kit	0.03
U937		Not achieved
THP-1		3.5
Kcl-22	Bcr/Abl	7.5
K562	Bcr/Abl	3.5
KU812	Bcr/Abl	2.5
Lymphoblastic leukemia		
PALL-1	Bcr/Abl	2.7
PALL-2	Bcr/Abl	6
NALL-1		5
Multiple myeloma		
U266		8
Pleural effusion lymphoma		
KS-1		4
BCBL-1		Not achieved
Burkitt's lymphoma		
Akata		5
Daudi		10
Adult T-cell leukemia		
MT-1		Not achieved
MT-2		Not achieved
MT-4		4

NOTE: IC_{50} of SU11248 was calculated from dose-response curves.

inhibitors, rapamycin or its analogue RAD001, have been shown to be active against many types of solid tumors, as well as subsets of leukemia, and are now being used in clinical trials (17).

Sunitinib (formerly SU11248) is a novel, orally available, multitargeted receptor tyrosine kinase inhibitor with selectivity against FLT3, c-KIT, PDGFR, and VEGFRs (18). Sunitinib caused regression of various established tumor xenografts derived from human colon, lung, and breast cancers (19). In addition, we have found that sunitinib induced growth inhibition and apoptosis of GIST-T1 cells that possessed the activating mutation in exon 11 of *c-KIT*, in association with blockade of c-KIT (20). Recently, sunitinib has been approved by the U.S. Food and Drug Administration for the treatment of GIST and advanced renal cell carcinoma (21). Furthermore, sunitinib has been shown to be active against AML cells with FLT3-ITD *in vitro* and *in vivo* (22), and the synergistic growth inhibition of these cells occurred when sunitinib was combined with the conventional anticancer agent cytarabine or daunorubicin (23). A phase 1 clinical study showed the efficacy and tolerability of this compound in treatment of individuals with AML with mutation of *FLT3*, although remissions were partial and the duration of remissions was short (24).

The present study explored the effects of sunitinib on a wide variety of hematologic malignancies and found that it was active in leukemia cells with activating mutations in RTK. In addition, rapamycin analogue RAD001 potentiated the antiproliferative activity of sunitinib in these cells.

Materials and Methods

Reagents

Sunitinib and the rapamycin analogue RAD001 were provided by Pfizer (Kalamazoo, MI) and Novartis (Basel, Switzerland), respectively, and were dissolved in 100% DMSO (Burdick & Jackson, Muskegon, MI) to a stock concentration of 10^{-2} mol/L and stored at −80°C.

Table 2. Effect of SU11248 on freshly isolated acute leukemia cells

Patient no.	Age/sex	FAB	WBC ($\times 10^9$)	%Blast	Genetic abnormalities	FLT3 mutations	IC_{50} (μmol/L)	Disease status
1	80/M	M_1	7,900	56		—	1	Initial presentation
2	80/M	M_1	10,600	25	−7, −13, −16, −12, −21, add(8)(q24)	—	0.8	Initial presentation
3	59/M	M_1	1,300	43	-5, −7, −8, −13, −14, −16, add(17)(q23)	—	Not achieved	Initial presentation
4	72/F	M_2	46,900	79		ITD	<0.01	Initial presentation
5	80/M	M_1	5,800	88	−3, −7, −8, −17, −18, −21, −22, add(18)(p11)	—	0.1	Relapse
6	32/M	L_2	77,100	54	t(9;22)(q34;q11), add(8)(q24), add(7)(q34)	—	1	Relapse
7	55/M	M_2	84,700	70		ITD	<0.01	Relapse
8	45/M	M_1	6,100	16	t(6;9)(p23;q34), +8, +13	ITD	<0.01	Relapse
9	44/F	M_4	7,100	11		—	0.2	Initial presentation
10	69/M	M_4	28,100	96	+1, der(1;12)(q10;q10)	—	Not achieved	Relapse
11	64/F	M_1	7,000	47		—	Not achieved	Initial presentation
12	74/M	M_1	5,200	86	del(16)(q?)	—	Not achieved	Initial presentation
13	77/F	M_1	1,900	14		—	0.9	Initial presentation
14	72/F	M_2	18,900	94	t(8;21)(q22;q22)	ITD	<0.01	Relapse
15	50/F	M_3	6,300	89	t(15;17)(q22;q12)	D835	0.01	Initial presentation
16	68/M	M_1	87,700	96		ITD	<0.01	Initial presentation

NOTE: The freshly isolated leukemia cells were cultured in the presence of various concentrations of SU11248 (0.01–1 μmol/L). After 2 days, the proliferation of cells was measured by [^{3}H]thymidine uptake. The IC_{50} of SU11248 was calculated from the dose-response curves.

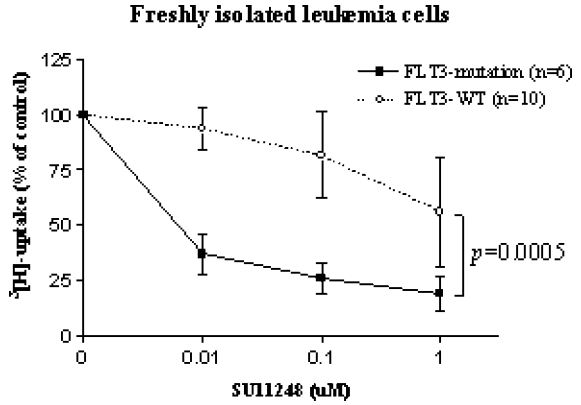

Figure 1. Effect of sunitinib on freshly isolated leukemia cells either with or without *FLT3* mutations. [^{3}H]Thymidine uptake study. Freshly isolated leukemia cells (1×10^6/mL) were plated in 96-well plates and cultured with various concentrations of sunitinib (0.01 – 1 μmol/L). After 2 d, proliferation was measured by [^{3}H]thymidine uptake. The experiments were done in triplicate. Cytotoxic effects of sunitinib on leukemia cells with (n = 6) or without (n = 10) *FLT3* mutation were compared. Statistical significance between two groups was determined by Student's t test.

Cells

FLT3-ITD-expressing MV4-11, Bcr/Abl-expressing KU812 and K562, and FIP1L1/PDGFRα-expressing EOL-1 leukemia cells were obtained from American Type Culture Collection (Manassas, VA). Kasumi-1 cells, which possess Asn^{822}Lys c-Kit mutation, were a kind gift from Dr. H. Asou (Hiroshima University, Hiroshima, Japan; ref. 25). The Bcr/Abl-expressing PALL-1 and PALL-2 lymphoblastic cells and Kcl-22 cells, which were established from chronic myeloid leukemia in blast crisis, were described elsewhere (26, 27). Leukemia cells from patients were freshly isolated as previously described (28).

FLT3 Genotyping

FLT3-ITD mutations were detected with the use of site-specific primers to exons 14 and 15 to amplify a 324-bp fragment in the wild-type *FLT3* sequence. ITD mutations were detected by the appearance of a larger band in gel electrophoresis as previously described (24). To detect *FLT3-D835* mutations, a 263-bp fragment was amplified by PCR. If the *Eco*RV-digested product produced two bands (148 and 115 bp), wild-type *FLT3* sequences were present as described (8). The presence of an uncut 263 fragment following digestion indicated loss of the *Eco*RV site and a D835 mutation.

Thymidine Uptake Studies

DNA synthesis was measured by tritiated thymidine uptake [^{3}H]TdR (Perkin-Elmer, Boston, MA). Cells (5×10^5/mL) were cultured with various concentrations of sunitinib or RAD001, either alone or in combination, for

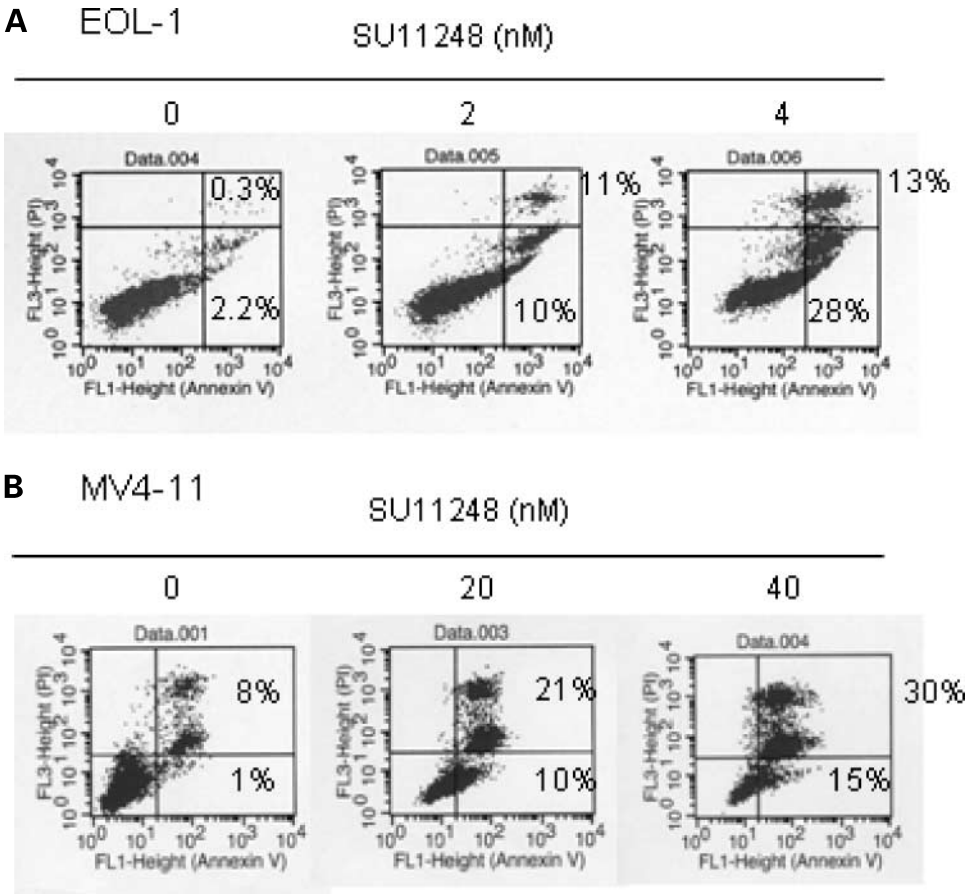

Figure 2. Annexin V binding. Cells (5×10^5/mL) were plated in 24-well plates and cultured with various concentrations of sunitinib. After 2 d, cells were harvested and Annexin V binding and propidium iodide staining were analyzed by FACScan. *Bottom left quadrants,* viable cells; *bottom right quadrants,* early apoptotic cells. *Top right quadrants,* nonviable, late apoptotic/necrotic cells. Results represent the mean of triplicate plates.

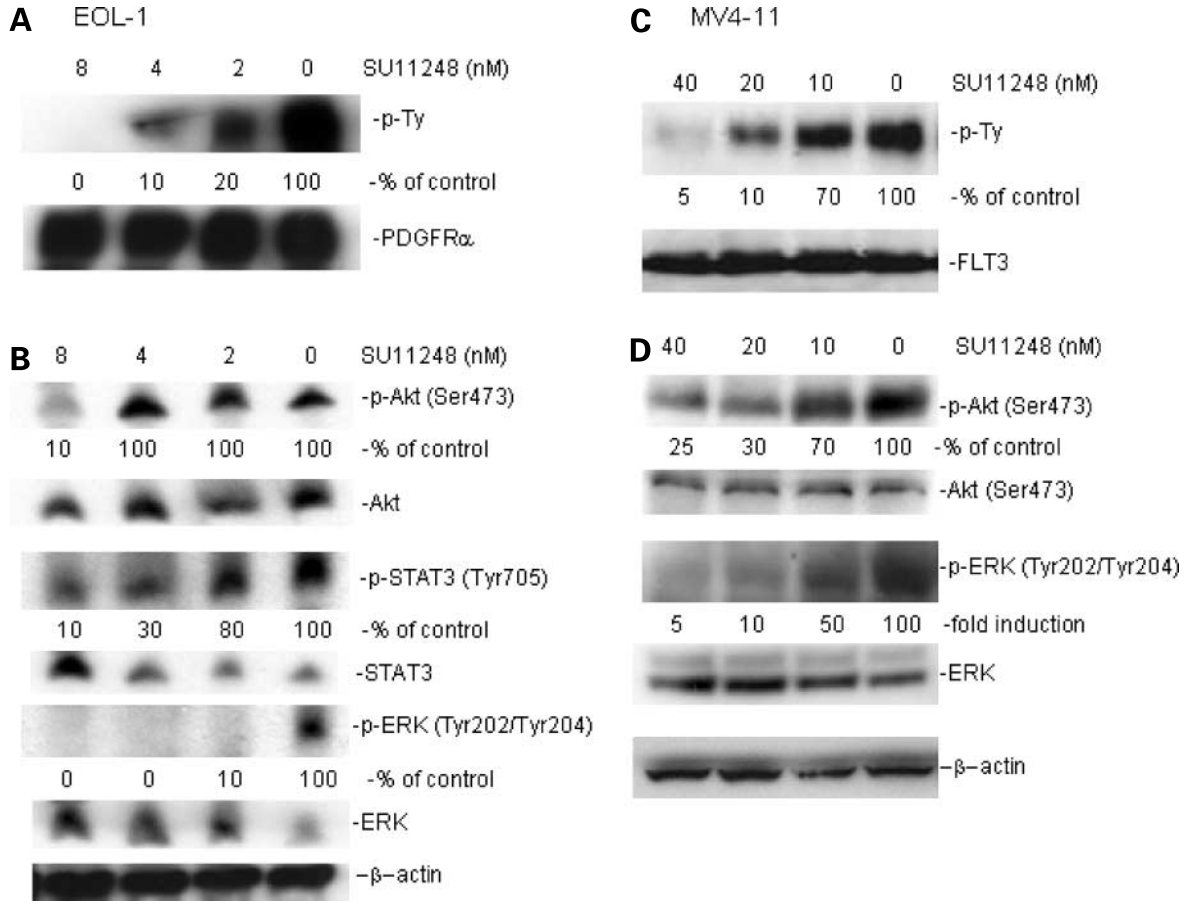

Figure 3. Sunitinib inhibits autophosphorylation of PDGFRα and MV4-11 and their downstream effectors in leukemia cells. Coimmunoprecipitation. EOL-1 (**A**) and MV4-11 cells (**C**) were cultured with various concentrations of sunitinib. After 1 h, cells were harvested and proteins were extracted. The PDGFRα (**A**) and FLT3 (**C**) proteins were immunoprecipitated and subjected to Western blot analyses. The membrane was probed sequentially with an anti-phosphotyrosine antibody (*top*) and an anti-PDGFRα (**A**) or anti-FLT3 (**C**; *bottom*) antibody. *p-Ty,* phosphotyrosine. Western blot analysis. EOL-1 (**B**) and MV4-11 cells (**D**) were cultured in the presence of sunitinib. After 1 h, cells were harvested and proteins were extracted and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with antibodies to p-Akt (Ser^{473}), p-STAT3 (Tyr^{705}), p-STAT5 (Tyr^{694}), p-ERK (Tyr^{202}/Tyr^{204}), Akt, STAT3, STAT5, ERK, and β-actin, and band intensities were measured by densitometry.

2 days in 96-well plates. Cells were pulsed with [^{3}H]TdR [0.5 μCi (0.185 MBq)/well] during the last 6 hours of a 48-hour culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted by with the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). All experiments were done in triplicate and repeated at least thrice.

Apoptosis Assays

The ability of sunitinib to induce apoptosis of leukemia cells was measured with Annexin V-FITC apoptosis detection kit according to the instruction of the manufacturer (PharMingen, Inc., San Diego, CA).

Phosphorylation Analysis of PDGFRα and FLT3

Lysates from MV4-11 or EOL-1 cells were prepared as previously described (20) and were immunoprecipitated with anti-FLT3 antibody (C-18, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or PDGFRα (C-20; Santa Cruz Biotechnology) and protein G-Sepharose (Pierce, Rockford, IL). The precipitated samples were subjected to Western blot analysis as previously described (18). The membrane was sequentially probed with anti-phosphotyrosine (Cell Signaling, Beverly, MA) and anti-FLT3 (Santa Cruz Biotechnology) or anti-PDGFRα (Santa Cruz Biotechnology) antibodies.

Immunoblotting

Immunoblotting was done as previously described (28). Anti-p-Akt (Ser^{473}) (Cell Signaling), Akt (Cell Signaling), anti-p-p70S6K (Thr^{389}) (Cell Signaling), anti-p70S6K (Santa Cruz Biotechnology), anti-p-4E-BP-1 (Thr^{70}) (Cell Signaling), anti-4E-BP-1 (Cell Signaling), anti-p-ERK (Tyr^{202}/Tyr^{204}) (Cell Signaling), anti-ERK (Cell Signaling), anti-p-STAT5 (Tyr^{694}) (Cell Signaling), anti-STAT5 (Abcam, Cambridge, United Kingdom), anti-p-STAT3 (Tyr^{705}) (Cell Signaling), anti-STAT3 (Cell Signaling), and anti-β-actin (Santa Cruz Biotechnology) antibodies were used. The band intensities were measured by densitometry.

Data Analysis

The combination index of sunitinib and RAD001 in freshly isolated leukemia cells was calculated using the

median effect method of Chou and Talalay (ref. 29; Calcusyn Software, Biosoft, Cambridge, United Kingdom). Combination index < 1 indicates synergy, combination index = 1 indicates an additive effect, and combination index > 1 indicates antagonism between the two agents.

Statistical Analysis

Statistical analysis was done to assess the difference between two groups under multiple conditions by one-way ANOVA followed by Bonferroni's multiple comparison tests using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

Results

Sunitinib Induced Growth Arrest of a Variety of Hematologic Malignant Cells

To explore the antitumor effects of sunitinib on hematologic malignancies, we cultured a variety of hematologic malignant cells in the presence of sunitinib (0.001–10 μmol/L) for 2 days. Sunitinib profoundly inhibited the proliferation of FIP1L1/PDGFRα-expressing EOL-1, FLT3-ITD-expressing MV4-11, and Kasumi-1 cells, which possess gain-of-function mutation in the *c-KIT* ($Asn^{822}Lys$), with IC_{50}s of 4, 25, and 30 nmol/L, respectively, on the second day of culture, as measured by thymidine uptake (Table 1). In addition, sunitinib was active against various types of hematologic malignant cells, including Bcr/Abl-expressing Kcl-22, K562, KU812, PALL-1, and PALL-2 cells with IC_{50}s ranging from 2.5 to 7.5 μmol/L (Table 1). On the other hand, myelomonocytic leukemia U937, primary effusion lymphoma BCBL-1, and human T-cell lymphotrophic virus-1–infected MT-1 and MT-2 cells were resistant to sunitinib (Table 1).

Sunitinib Induced Growth Arrest of Freshly Isolated Leukemia Cells

We next explored whether sunitinib affected the survival of freshly isolated leukemia cells. We cultured cells in the presence of various concentrations of sunitinib (0.01–1

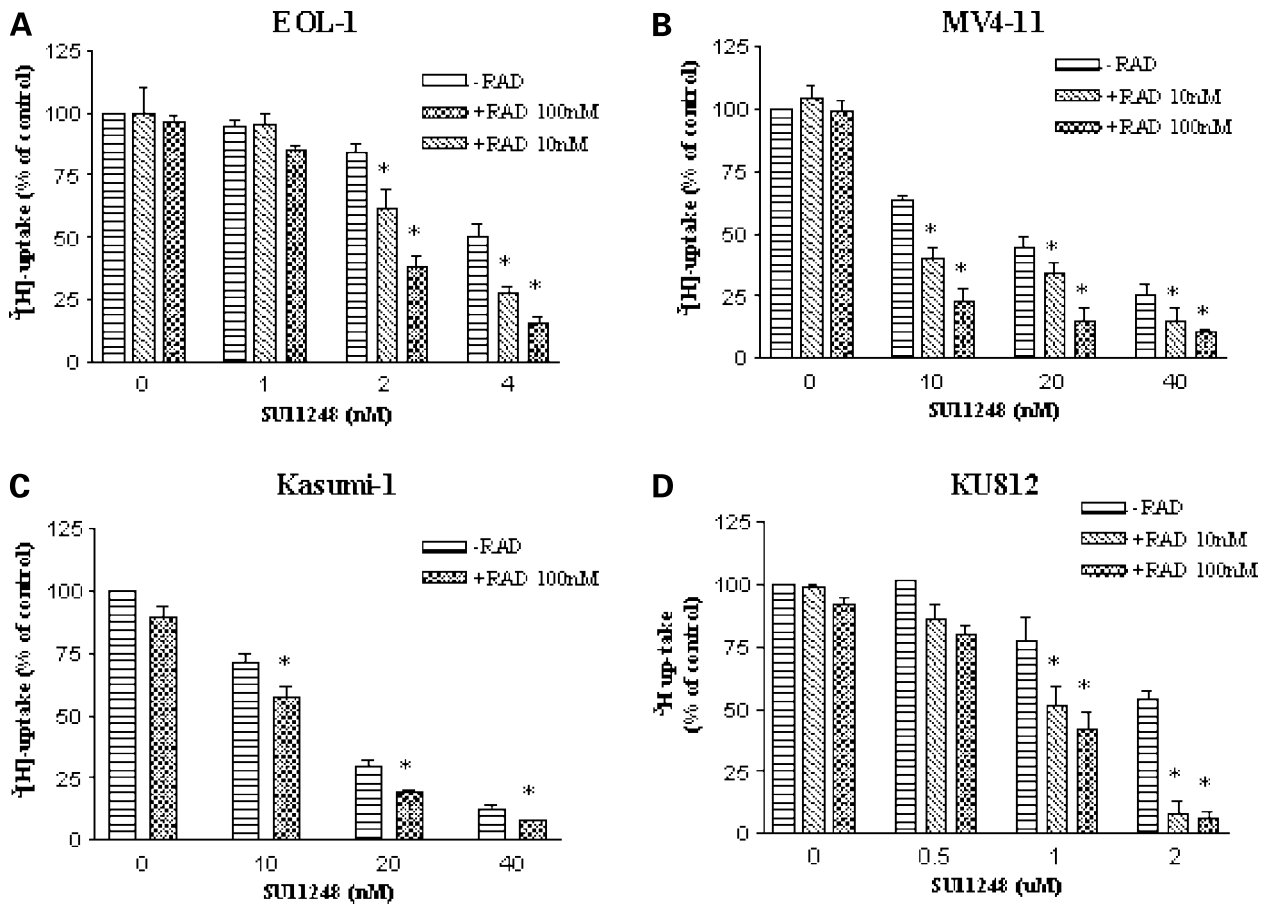

Figure 4. Combination of sunitinib and RAD001 induces enhanced growth inhibition of leukemia cells. EOL-1 (**A**), MV4-11 (**B**), Kasumi-1 (**C**), KU812 (**D**), K562 (**E**), PALL-2 (**F**), and freshly isolated leukemia cells from case #3 (**G**), case #6 (**H**), and case #14 (**J**) were cultured with either sunitinib or RAD001, alone or in combination. After 2 d, proliferation was measured by [^{3}H]thymidine uptake. Statistical significance was determined by one-way ANOVA followed by Bonferroni's multiple comparison tests. *Columns,* mean of three experiments done in triplicate; *bars,* SD. *, $P < 0.01$. *RAD,* RAD001. **J,** combination index of sunitinib and RAD001 in freshly isolated leukemia cells from case #14 was calculated using the median effect method. Combination index < 1 indicates synergy; combination index = 1 indicates an additive effect; and combination index > 1 indicates antagonism between the two agents.

μmol/L). After 2 days, the proliferation was measured by thymidine uptake. *FLT3* genotyping by PCR showed that 5 of 16 (31%) samples expressed *FLT3-ITD* (figure not shown) and 1 of 16 (6%) possessed an *FLT3-D835* mutation (figure not shown; Table 2). Sunitinib profoundly inhibited the proliferation of freshly isolated leukemia cells with *FLT3* mutations: 0.01 or 1 μmol/L sunitinib inhibited the proliferation of mutant *FLT3*-expressing cells (n = 6) by either 63 ± 8% or 80 ± 8%, respectively. On the other hand, the same concentrations of sunitinib (0.01 or 1 μmol/L) inhibited the proliferation of leukemia cells having a wild-type *FLT3* (n = 10) by 6 ± 9% or 44 ± 23%, respectively (P = 0.0005; Fig. 1). Sunitinib (1 μmol/L, 48 hours) inhibited the proliferation of leukemia cells by >50% in 6 of 10 (60%) cases without mutations of *FLT3*, including one case of acute lymphoblastic leukemia (L2) with Philadelphia chromosome who relapsed after conventional chemotherapy with imatinib (Table 2). The cytogenetic analysis found

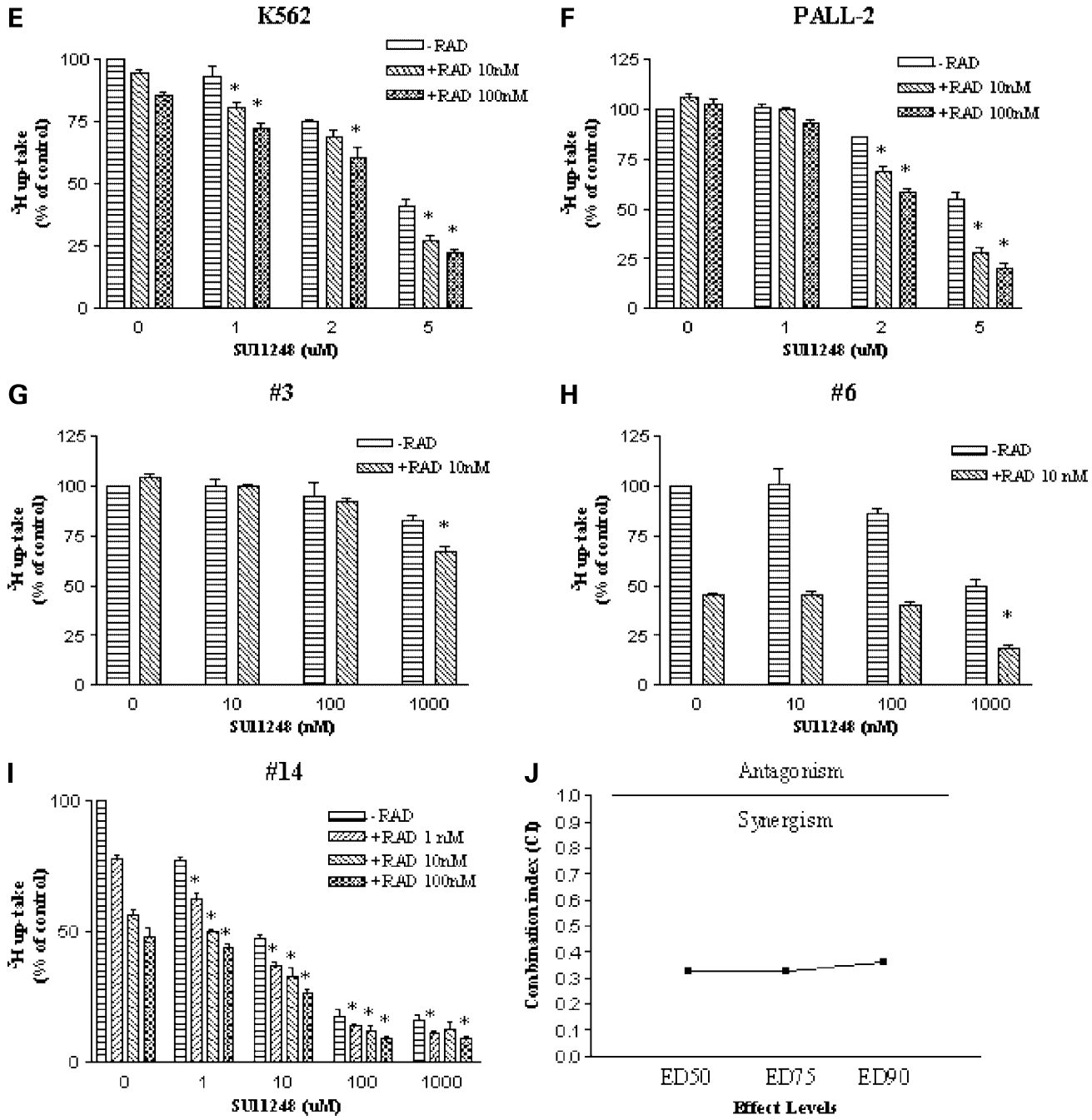

Figure 4 ***Continued.***

t(9;22; q34;q11) as a single abnormality when the patient was diagnosed; however, the complicated abnormalities were found at relapse including add(8; q24), add(7; q34), add(3; p21), as well as add(19; p13). These aberrant chromosomal abnormalities might contribute to imatinib resistance. Our sequence analysis found no mutations in the imatinib-binding site of *c-Abl* (data not shown).

Sunitinib Induced Apoptosis of Leukemia Cells

The ability of sunitinib to induce apoptosis was measured by Annexin V staining. Exposure of EOL-1 cells to sunitinib (2 or 4 nmol/L, 48 hours) induced either 21% or 41% of these cells to become Annexin V staining positive, and <5% of control cells were positive for Annexin V (Fig. 2A; $P < 0.05$). Similarly, sunitinib (20 or 40 nmol/L, 48 hours) induced apoptosis of MV4-11 cells in a dose-dependent manner (Fig. 2B). Profound induction of apoptosis developed in Kasumi-1 and Bcr/Abl-expressing KU812, K562, PALL-1, and PALL-2 cells after their exposure to high concentration of sunitinib (2 μmol/L) for 48 hours (data not shown).

Sunitinib Inhibited Autophosphorylation of PDGFRα and FLT3 and Their Downstream Effectors in EOL-1 and MV4-11 Cells

Exposure of EOL-1 cells to sunitinib (2–8 nmol/L) for 1 hour blocked autophosphorylation of PDGFRα in a dose-dependent manner (Fig. 3A) in conjunction with inactivation of its downstream effectors Akt, STAT3, and ERK, as measured by detection of the phosphorylated forms of these proteins by Western blot analysis (Fig. 3B). Similarly, sunitinib (10–40 nmol/L, 1 hour) inhibited autophosphorylation of FLT3 in MV4-11 cells in conjunction with down-regulation of p-ERK and p-Akt in these cells (Fig. 3C and D).

RAD001 Enhanced the Ability of Sunitinib in Leukemia Cells

We explored whether rapamycin analogue RAD001, the inhibitor of mTOR that locates downstream of Akt, potentiated the ability of sunitinib to inhibit the growth of leukemia cells. RAD001 significantly enhanced the ability of sunitinib; 2 or 4 nmol/L sunitinib inhibited the proliferation of EOL-1 cells by 26 ± 3% or 50 ± 4%, respectively, on the second day of culture (Fig. 4A). The effect of RAD001 (100 nmol/L) on growth of EOL-1 cells was negligible. Exposure of these cells to RAD001 (100 nmol/L) and sunitinib (2 or 4 nmol/L) inhibited the proliferation of these cells by 62 ± 6% and 84 ± 3%, respectively ($P < 0.01$; Fig. 4A). Similarly, RAD001 enhanced the ability of sunitinib to inhibit the growth of MV4-11, Kasumi, KU812, K562, and PALL-2 cells (Fig. 4B–F). We also explored the effect of RAD001 and sunitinib on freshly isolated leukemia cells. RAD001 (10 nmol/L, 48 hours) failed to inhibit the proliferation of leukemia cells from case #3. Sunitinib (1,000 nmol/L, 48 hours) inhibited their proliferation by only 17 ± 3%; however, when these cells were exposed to combination of sunitinib and RAD001 at the same concentration, their growth was inhibited by 33 ± 4% ($P < 0.01$; Fig. 4G). RAD001 also sensitized freshly isolated leukemia cells (cases #6 and #14) to growth inhibition mediated by sunitinib (Fig. 4G and H). Combination index values of RAD001 and sunitinib in case #14 were <1 (IC_{50}, 0.3; IC_{75}, 0.3; IC_{90}, 0.4; Fig. 4I), indicating that combination of these compounds produced the synergistic growth inhibition of freshly isolated leukemia cells, as measured by the median effect method of Chou and Talalay (29).

Sunitinib and RAD001 Down-Regulated Levels of p-p70S6K and/or p-4E-BP-1 in Leukemia Cells

To explore whether the sunitinib- and RAD001-induced growth inhibition of leukemia cells was correlated with impaired mTOR signaling, we measured levels of the phosphorylated forms of mTOR targets, p70S6K and 4E-BP-1, after exposure of these cells to sunitinib and/or RAD001. Sunitinib (4 nmol/L, 1 hour) and RAD001 (100 nmol/L, 1 hour) down-regulated levels of p-p70S6K in EOL-1 cells by ~60% and 80%, respectively (Fig. 5A). When these cells were exposed to the combination of both at the same concentration, levels of p-p70S6K decreased by 85% (Fig. 5A). Sunitinib (8 nmol/L, 1 hour) and RAD001 (100 nmol/L, 1 hour) alone down-regulated levels of p-4E-BP-1 by 40%. Combination of both decreased phosphorylation of 4E-BP-1 by 60% in these cells (Fig. 5A). Similarly, sunitinib and RAD001 decreased levels of p-p70S6K and p-4E-BP-1 in MV4-11, KU812, and PALL-2 cells (Fig. 5B, and data not shown). Combination of both further decreased levels of p-p70S6K and p-4E-BP-1 in these cells (Fig. 5B, and data not shown).

Discussion

RTKs, including PDGFRα, FLT3, and c-KIT, are emerging as promising molecular targets of AML. Sunitinib profoundly inhibited the proliferation of EOL-1, MV4-11, and Kasumi-1 cells, which express activating RTK mutations in *PDGFRα*, *FLT3*, and *c-KIT*, respectively. In addition, sunitinib was more potent against freshly isolated leukemia cells with *FLT3* mutations compared with those with wild-type *FLT3* (Fig. 1), which was in line with the results of a phase 1 clinical study with sunitinib for individuals with AML (25); p.o. administration of sunitinib (50 or 75 mg) for 4 weeks followed by a 2-week rest period caused remission in all patients ($n = 4$) with mutations of *FLT3*. On the other hand, remission was induced in only 2 of 7 (29%) patients with wild-type *FLT3*. In this clinical study, 8 of 13 (62%) patients experienced treatment-related adverse events including grade 4 fatigue and hypertension, which was dose limiting. In addition, two patients experienced fatal bleeding in the lung and cerebrum, respectively. Mean duration of remissions induced by treatment with sunitinib was merely 3 months. Together with these observations, addition of other compound(s) to sunitinib should be considered.

The mTOR inhibitor rapamycin appeared as one of the attractive candidates in the adjuvant setting with protein kinase inhibitor. Previous studies showed that rapamycin enhanced the activity of imatinib and PKC412, an RTK inhibitor, in Bcr/Abl- or FLT3-ITD-expressing leukemia cells, respectively (30, 31). In this study, we found that the rapamycin analogue RAD001, which was shown to be

active against AML cells *in vitro* (32), enhanced the antiproliferative activity of sunitinib in EOL-1, MV4-11, Kasumi-1, as well as Bcr/Abl-expressing lymphoblastic leukemia cells (Fig. 4A–F). In addition, RAD001 sensitized freshly isolated leukemia cells, which were relatively resistant to sunitinib (case #3; Fig. 5G). Interestingly, Bcr/Abl-expressing acute lymphoblastic leukemia cells from case #6, which relapsed after conventional chemotherapy with imatinib, responded to both sunitinib (1 μmol/L, 48 hours) and RAD001 (10 nmol/L, 48 hours). When these cells were exposed to combination of both analogues,

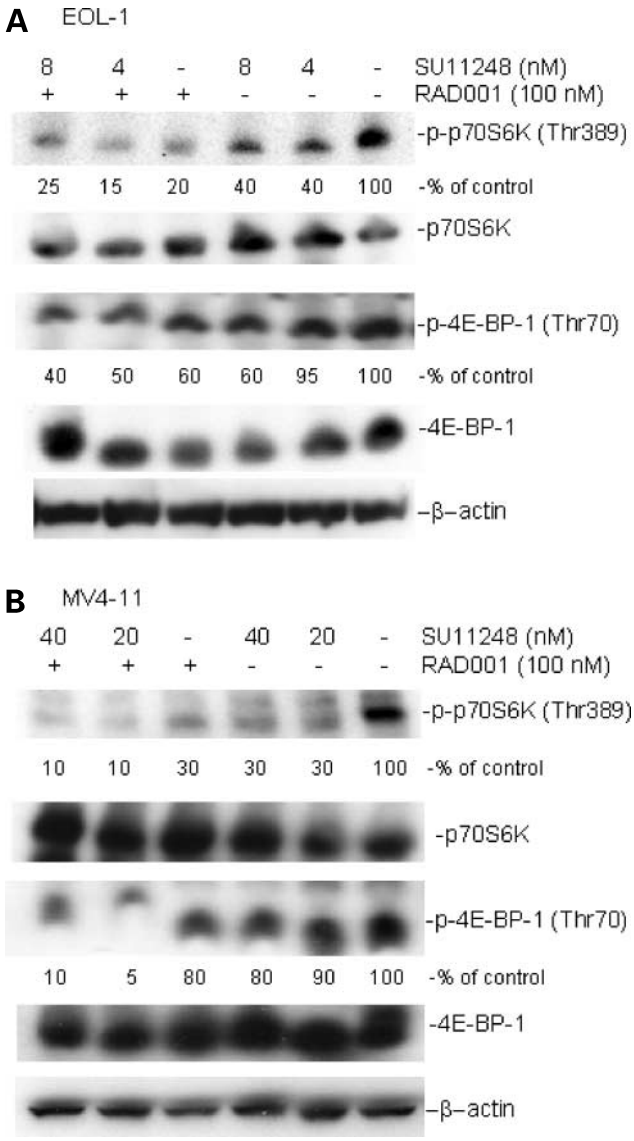

Figure 5. Sunitinib and RAD001 block mTOR signaling. EOL-1 (**A**) and MV4-11 (**B**) cells were cultured with either sunitinib or RAD001, alone or in combination. After 1 h, cells were harvested and proteins were extracted and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with antibodies against p-p70S6K (Thr^{389}), p-4E-BP-1 (Thr^{70}), p70S6K, and 4E-BP-1, and band intensities were measured by densitometry.

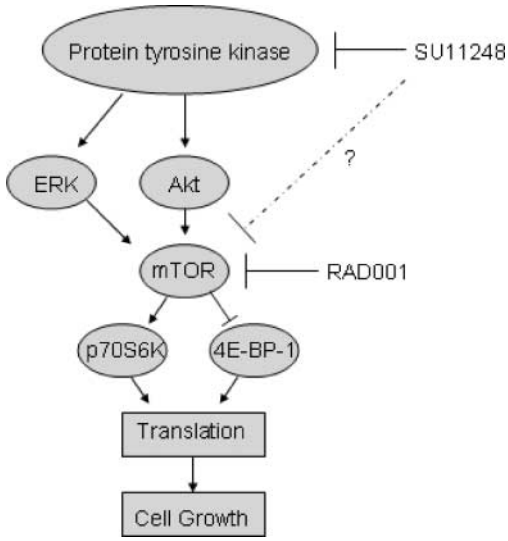

Figure 6. Hypothesis of how sunitinib and RAD001 block proliferative signals mediated by activated protein tyrosine kinase/Akt/mTOR pathway.

growth inhibition was significantly enhanced compared with either compound alone (Fig. 5H).

Sunitinib inhibited autophosphorylation of PDGFRα and FLT3 in EOL-1 and MV4-11 cells, respectively, and blocked their downstream effectors Akt, STAT, and ERK (Fig. 3). Furthermore, sunitinib down-regulated levels of phosphorylated forms of the mTOR effectors p70S6K and 4E-BP-1 in these cells (Fig. 6). As we expected, RAD001 dephosphorylated p70S6K and 4E-BP-1 in leukemia cells; when RAD001 was combined with sunitinib, levels of the phosphorylated forms of these proteins further decreased compared with either compound alone (Fig. 5). mTOR may be a common target of both the RTK inhibitor and rapamycin. The enhanced blockade of this serine/threonine kinase signaling by combination of these compounds could result in the augmented growth inhibition of leukemia cells (Fig. 6).

We have recently explored the medicinal action of sunitinib and rapamycin in GIST-T1 cells. Both sunitinib and rapamycin blocked mTOR signaling and their combination caused the enhanced growth inhibition of GIST-T1 cells (22), which was consistent with the results shown in this study using leukemia cells.

Taken together, sunitinib may be useful for the treatment of individuals with leukemias possessing activating mutations in RTK; and the combination of sunitinib and RAD001 represents a promising novel treatment strategy.

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Takayuki Ikezoe, Chie Nishioka, Taizo Tasaka, et al.

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