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An Orally Administered Multitarget Tyrosine Kinase Inhibitor, SU11248, Is a Novel Potent Inhibitor of Thyroid Oncogenic RET/Papillary Thyroid Cancer Kinases

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Context: The oncogenic RET/PTC tyrosine kinase causes papillary thyroid cancer (PTC). The use of inhibitors specific for RET/PTC may be useful for targeted therapy of PTC.

Objective: The objective of the study was to evaluate the efficacies of the recently developed kinase inhibitors SU11248, SU5416, and SU6668 in inhibition of RET/PTC.

Design: SU11248, SU5416, and SU6668 were synthesized, and their inhibitory potencies were evaluated using an *in vitro* RET/PTC kinase assay. The inhibitory effects of the compounds on RET/PTC were evaluated by quantifying the autophosphorylation of RET/PTC, signal transducer and activator of transcription (STAT)-3 activation, and the morphological reversal of RET/PTC-transformed cells.

Results: An *in vitro* kinase assay revealed that SU5416, SU6668, and SU11248 inhibited phosphorylation of the synthetic tyrosine kinase substrate peptide E4Y by RET/PTC3 in a dose-dependent manner with IC_{50} of approximately 944 nM for SU5416, 562 nM for SU6668, and 224 nM for SU11248. Thus, SU11248 effectively inhibits the kinase activity of RET/PTC3. RET/PTC-mediated Y705 phosphorylation of STAT3 was inhibited by addition of SU11248, and the inhibitory effects of SU11248 on the tyrosine phosphorylation and transcriptional activation of STAT3 were very closely correlated with decreased autophosphorylation of RET/PTC. SU11248 caused a complete morphological reversion of transformed NIH-RET/PTC3 cells and inhibited the growth of TPC-1 cells that have an endogenous RET/PTC1.

Conclusion: SU11248 is a highly effective tyrosine kinase inhibitor of the RET/PTC oncogenic kinase. **(*J Clin Endocrinol Metab* 91: 4070–4076, 2006)**

THYROID CANCER IS a leading cause of death among endocrine malignancies. The major morbidity and mortality of thyroid cancer is related to disease progression despite surgery, radioiodine treatment, and T_4 suppression of TSH (1). The RET/PTC oncogenes are unique genetic alterations that play a role in the pathogenesis of a significant subset of papillary thyroid carcinomas (PTC) (2). Chromosomal rearrangements linking the promoter and N-terminal domains of unrelated genes to the C-terminal fragment of the RET protooncogene result in the ectopic expression of chimeric constitutively active forms of the tyrosine kinase, the RET/PTCs, in thyroid cells (2). Generation of a RET/PTC kinase is believed to be a key event in thyroid carcinogenesis because of its high incidence in occult or microscopic PTC (3). The transforming ability of RET/PTC requires tyrosine kinase activity and the signal transducer and activator of transcription (STAT) proteins have been identified as key substrates of the RET/PTC (4–9). Inhibition of the tyrosine kinase activity could form the basis for target-specific treatment of PTCs that bear the RET/PTC rearrangement.

As knowledge of thyroid cancer biology improves, a number of molecular components, which could be targeted for treatment of thyroid cancers that do not respond to conventional therapies, have been investigated (10). Several compounds that alter pathways involved in thyroid cancer are currently being evaluated in clinical trials, and several of these agents have been tested in thyroid cancer models both *in vitro* and *in vivo* (10–12). SU11248 is a selective, orally administered receptor tyrosine kinase (RTK) inhibitor that targets platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and

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Abbreviations: DMSO, Dimethylsulfoxide; DTT, dithiothreitol; FLK, fetal liver kinase; FLT3, fms-related tyrosine kinase 3; KDR, kinase insert domain-containing receptor; PDGFR, platelet-derived growth factor receptor; PP, pyrazolo-pyrimidine; PTC, papillary thyroid cancer; RTK, receptor tyrosine kinase; SDS, sodium dodecyl sulfate; STAT, signal transducer and activator of transcription; VEGFR, vascular endothelial growth factor receptor.

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fms-related tyrosine kinase 3 (FLT3) with IC_{50} values of 5–50 nM in cellular autophosphorylation assays (13, 14). SU11248 was also designed to target KIT and is predicted to inhibit KIT kinase activity in cells. SU11248 has been shown to possess antitumor activity correlated with inhibition of RTKs expressed in tumor cells (13). For example, SU11248 blocks the activity of wild-type FLT3, as well as activated FLT3 expressed by acute myelogenous leukemia-derived cell lines (14). SU11248 also blocks tumor proliferation and survival by inhibiting RTKs expressed on endothelial or stromal cells (13). For example, SU11248 has been shown to inhibit the VEGFR, fetal liver kinase (FLK)/kinase insert domain-containing receptor (KDR), and PDGFRβ, each of which plays a prominent role in angiogenesis (14, 15).

This study evaluates the activity of the indolinone kinase inhibitor SU11248 against the RET/PTC tyrosine kinase and examines the usefulness of SU11248 in the treatment of RET/PTC-positive papillary thyroid cancers.

Materials and Methods

Compounds

The indolinone derivatives including SU11248, SU5416, and SU6668 were synthesized in research scale amounts and purified (>99.5% purity) by LG Life Science, Ltd. (Seoul, Korea), using previously established synthetic methods (13, 16, 17). The structures of these compounds are shown in Fig. 1A. The compounds were tested both *in vitro* and in cell cultures. Stock solutions (50 mM) were made in 100% dimethylsulfoxide (DMSO). Equivalent DMSO concentrations served as vehicle controls.

Cell culture and antibodies

Media, cell culture reagents, and culture plasticware were purchased from Life Technologies, Inc. (Gaithersburg, MD), Sigma (St. Louis, MO), and Corning, Inc. (Corning, NY), respectively. Antibodies against RET were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-STAT3, anti-p705-STAT3, anti-p42/44-MAPK, anti-phospho-p42/44-MAPK (pT202/Y204), and anti-phosphospecific (pY905)-RET antibodies were purchased from New England Biolabs, Inc. (Danvers, MA). The antiphosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, Inc. (Chicago, IL).

Plasmids

We used the mammalian expression vector pcDNA3-RET/PTC3, as described in our previous report (8), for the construction of the baculovirus RET/PTC3 expression vector. RET/PTC3 coding sequences were amplified from pcDNA3-RET/PTC3, and the fragment was cloned into the pBacPAK9 vector from CLONTECH (Mountain View, CA). The following PCR conditions were used: denaturation at 95 C for 5 min, followed by 30 cycles of denaturation at 94 C for 1 min, annealing at 55 C for 45 sec, and elongation at 72 C for 1 min. The primers used were: 6X-histidine-tagged RET/PTC3 forward, 5′-gctctagaatgcatcatcatcatcatcatatgaataccttc-3′, and RET/PTC3 reverse, 5′-ggggtaccctagtaaatgcatgggaaattcta-3′. The amplified full-length fragment was digested with *Xba*I and *Kpn*I. Verification of clones was confirmed by PCR and automated DNA sequencing using the forward Bac1 primer 5′-AACCATCTCGCAAATAAATA-3′ and the reverse Bac2 primer 5′-ACGCACAGAATCTAGCGCTT-3′.

In vitro kinase assays

Recombinant baculovirus containing a hexa-histidine-tagged RET/PTC3 insert was collected from transfected Spodoptera frugiperda 9 insect cell cultures. After two rounds of infection, the medium was collected and used for large-scale protein purification. Spodoptera frugiperda 9 cell pellets from 2-liter cultures were resuspended in 50 ml ice-cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1% glycerol, 5 mM EGTA, 1% Triton X-100, 20 mM imidazole, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche, Basel, Switzerland)], and stirred on ice for 15 min. The cell lysate was passed through a microfluidizer at 11,000 psi, and clarified supernatant was harvested after centrifugation at 20,000 × *g* (16,000 rpm, JA20; Beckman, Fullerton, CA) for 30 min at 4 C. The supernatant was loaded onto a 5-ml Ni-charged agarose column (Novagen, Darmstadt, Germany) and washed three times with 50 ml wash buffer [50 mM HEPES (pH 7.5) 10% glycerol, 2 mM β-mercaptoethanol, 150 mM NaCl, 5 mM EGTA, 60 mM imidazole]. The His-tagged protein was eluted using a 25-ml linear imidazole gradient from 60 to 400 mM in wash buffer. The protein purity was assessed by SDS-PAGE and Coomassie staining. The eluted fractions were pooled and dialyzed against 2 liters dialysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA] and then stored at −70 C.

Protein tyrosine kinase assays using the purified His-RET/PTC3 were carried out in a reaction buffer containing 20 mM HEPES (pH 7.5), 150

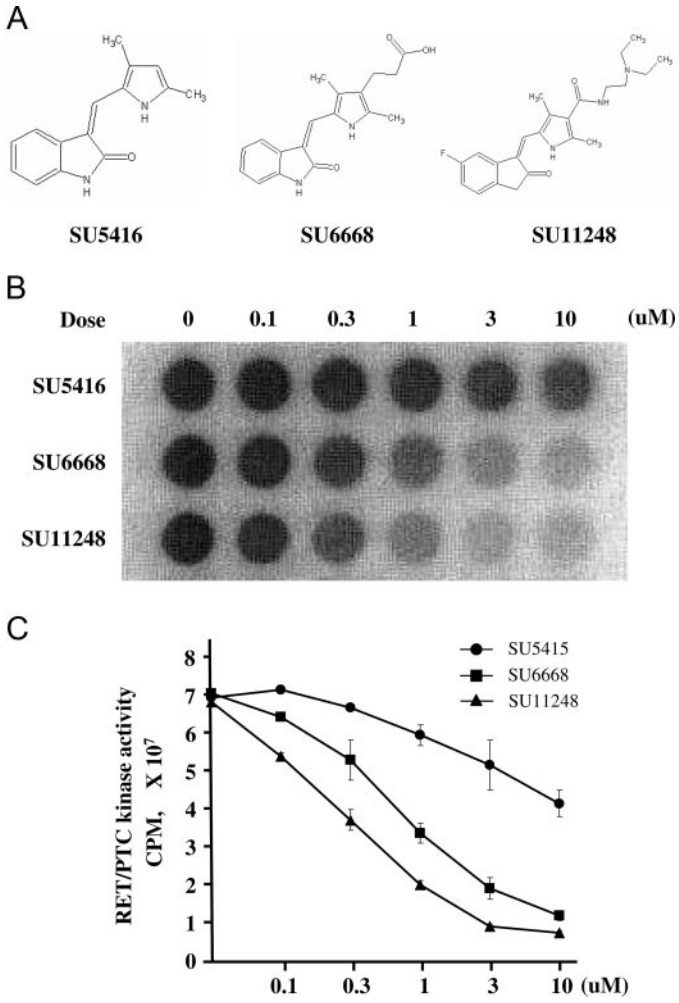

FIG. 1. The indolinone compounds SU5416, SU6668, and SU11248 inhibit the RET/PTC kinase *in vitro*. A, Chemical structures of SU5416, SU6668, and SU11248 are depicted. B, Kinase-active His-RET/PTC3 was produced using a baculovirus expression system. Protein tyrosine kinase assays were conducted using the purified His-RET/PTC3, [γ-P^{32}]ATP, and the E4Y polypeptide as a substrate. The reaction mixtures were transferred onto an Immobilon-polyvinyl difluoride membrane in a 96-well format and the radioactivity quantified using a PhosphorImager (Molecular Dynamics). C, The dose-dependent inhibition of RET/PTC3 kinase from the experiments in B is shown graphically. The IC_{50} was determined from the mean values of more than three repetitions of the assay, using linear regression analysis. CPM, Counts per minute.

mM NaCl, 1% glycerol, 0.1% Triton X-100, 15 mM $MgCl_2$, 15 mM $MnCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4, 5 μM ATP, 0.2 μCi[γ-P^{32}]ATP, 1 μg/ml RET/PTC3, and 69 μg/ml E4Y polypeptide (Sigma) or 54 μg/ml Akt-DD as the kinase substrate. The reaction was carried out in a total reaction volume of 20 μl, with the addition of various concentrations of the inhibitor compounds in 5% DMSO, at 30 C for 10 min and then stopped by the addition of 10% phosphoric acid. The assay solution was transferred onto Immobilon-polyvinyl difluoride membrane (Millipore, Billerica, MA) in a 96-well format, washed four times with 0.5% phosphoric acid, and the amount of radiation adhering to the membrane quantified using a PhosphorImager (Molecular Dynamics, Piscataway, NJ). For each compound, the concentration at which 50% of the total kinase activity was inhibited (IC_{50}) was determined as a mean value calculated from more than three repetitions of the assay using linear regression analysis.

Transfections and reporter assays

NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Paisley, PA). Cells were transfected using the LipofectAMINE method (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Cells were cotransfected with 100 ng Renilla, 100 ng m67 luciferase reporter plasmid, and 500 ng RET/PTC3 plasmid. Briefly, the plasmids were incubated with 6 μl of the LipofectAMINE Plus reagent at room temperature for 15 min, 2 μl of the LipofectAMINE reagent were added, and the mixture was incubated at room temperature. After 15 min, semiconfluent cell cultures were washed twice with 1× PBS and then incubated with DNA-LipofectAMINE Plus complexes at 37 C in a humidified chamber containing 5% CO_2 for 4 h. After transfection, the cells were allowed to recover for 24 h in DMEM with 15% fetal bovine serum. The medium was replaced with fresh medium containing DMSO, SU11248, SU5416, or SU6668 for an additional 12 or 24 h. The cells were then washed with 1× PBS and lysed with 200 μl lysis buffer containing 40 mM tricine (pH 7.8), 50 mM NaCl, 2 mM EDTA, 1 mM $MgSO_4$, 5 mM DTT, and 1% Triton X-100. The fluorescence intensity was measured using a luminometer (Berthold, Bad Wildbad, Germany). The firefly and Renilla luciferase activities were measured with a dual-luciferase reporter assay system (Promega, Madison, WI). Luciferase activity was integrated over a 10-sec period. Firefly luciferase values were standardized to Renilla values.

Immunoblotting analysis

Cells were lysed by adding sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris-HCl (pH 6.8), 6% (wt/vol) SDS, 30% glycerol, 125 mM DTT, 0.03% (wt/vol) bromophenol blue]. Total cell lysates were denatured by boiling for 5 min, resolved on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 5% (wt/vol) milk and 0.1% Tween 20 for 1 h at room temperature. The membranes were incubated with primary antibodies, anti-STAT3, antiphosphospecific (Y705) STAT3, anti-RET/PTC3, or antiphosphospecific (Y431) RET/PTC3, overnight at 4 C. The blots were developed using a horseradish peroxidase-conjugated secondary antibody (Phototope-HRP Western blot detection kit, New England Biolabs).

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability assays were carried out using the MTT dye conversion assay (18) in 96-well plates. After exposure to SU5416 or SU11248, MTT (25 μl of 5 mg/ml MTT in sterile PBS) was added to 100 μl of a cell suspension and allowed to incubate for 2 h at 37 C. The reaction was stopped, and the cells were lysed by the addition of 100 μl lysis buffer consisting of 20% SDS in a water/dimethylformamide (1:1) solution (pH 4.7). Cell lysates were placed at 37 C overnight to allow cell lysis and dye solubilization. The OD was read at 595 nm using a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA). Data are expressed as a percent of vehicle-treated (DMSO) control values. MTT assays were carried out on three independent experiments, each performed in triplicate.

Data analysis

For the experiments examining the effects of SU compounds on the viability of TPC-1 cells, an analysis was performed using SPSS (version 12.0 for Windows; SPSS Inc., Chicago, IL). Paired *t* test and Wilcoxon signed rank test were used to examine differences in inhibition of cellular proliferation based on concentration of SU compounds.

Results

The indolinone compounds SU5416, SU6668, and SU11248 inhibit the RET/PTC kinase in vitro

The indolinone derivatives SU5416, SU6668, and SU11248 are potent antiangiogenic small-molecule inhibitors of RTKs, including those of the VEGFR family (13). These compounds were designed to have broad selectivity for the split kinase family of RTKs as well (14). We measured the effects of SU5416, SU6668, and SU11248 on active RET/PTC3 tyrosine kinase produced using a baculovirus expression system (9). The RET/PTC3 kinase assay (Fig. 1, B and C) revealed that SU5416, SU6668, and SU11248 inhibited phosphorylation of the synthetic E4Y polypeptide tyrosine kinase substrate in a dose-dependent manner with an IC_{50} of approximately 944 nM for SU5416, 562 nM for SU6668, and 224 nM for SU11248 (Fig. 1C). These results indicate that SU11248 is the most active indolinone compound for *in vitro* inhibition of the RET/PTC kinase.

SU11248 inhibits RET/PTC autophosphorylation and RET/PTC-mediated STAT3 activation in vivo

We next investigated the activity of SU11248, SU5416, and SU6668 against RET/PTC activation *in vivo* using transiently transfected NIH3T3 cells. The RET/PTC3 kinase undergoes autophosphorylation on multiple tyrosine residues coincident with dimerization (19). We measured the phosphory-

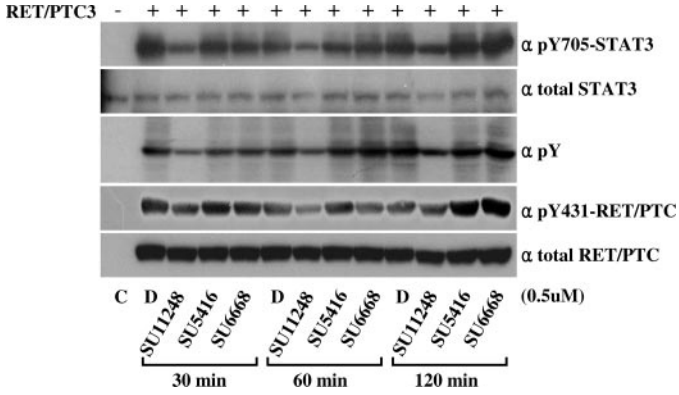

FIG. 2. SU11248 inhibits tyrosine phosphorylation of RET/PTC and STAT3 *in vivo*. NIH3T3 cells cultured in 6-well plates were transfected with RET/PTC3 by the LipofectAMINE method. After transfection, the cells were allowed to recover for 24 h. The medium was then replaced with medium containing DMSO, SU11248, SU5416, or SU6668 for the indicated times. Cells were lysed by boiling in SDS-PAGE sample buffer for 5 min, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were probed with antibodies against STAT3, pY705-STAT3, pY, RET/PTC3, and pY431-RET/PTC3. Total protein expression level (STAT3 and RET/PTC) is not changed by treatment with the drugs, but tyrosine phosphorylation of STAT3 (pY705-STAT3) and RET/PTC (pY431-RET/PTC and pY) is decreased by SU11248 treatment. C, Mock-transfected control; D, DMSO control.

lation of Y431 (the residue homologous to Y905 in wild-type RET) using phosphospecific antibodies (Fig. 2) after treatment of RET/PTC3-transfected NIH3T3 cells with 0.5 μM SU11248, SU5416, or SU6668. The phosphorylation of Y431 was detected in RET/PTC3 transfected cells, but autophosphorylation was effectively inhibited by SU11248, compared with SU5416 and SU6668, in a time-dependent manner (Fig. 2). To determine the tyrosine phosphorylation status of RET/PTC3, we measured the overall tyrosine phosphorylation level of RET/PTC3 using an antiphosphotyrosine antibody. Interestingly, Western analysis revealed a broad range of RET/PTC tyrosine phosphorylation in transfected cells, and the level of RET/PTC phosphorylation was decreased by treatment with SU11248 but not SU5416 or SU6668.

We reported previously that both RET/PTC1 and RET/PTC3 are able to activate STAT3 via phosphorylation of Y705 (7). To determine whether SU11248, SU5416, or SU6668 is able to inhibit RET/PTC-mediated Y705 phosphorylation of STAT3, we measured Y705 phosphorylation after treatment of RET/PTC3-transfected cells with each compound. Y705 phosphorylation was inhibited by addition of SU11248, and the inhibitory effects on tyrosine phosphorylation of STAT3 were very closely correlated with the SU11248-mediated decrease in RET/PTC autophosphorylation (Fig. 2). However, treatment with 0.5 μM SU5416 or SU6668 did not decrease Y705 phosphorylation of STAT3 in RET/PTC3-transfected NIH3T3 cells (Fig. 2).

NIH3T3 cells transfected with RET/PTC3 expression plasmids were exposed to increasing concentrations of SU11248,

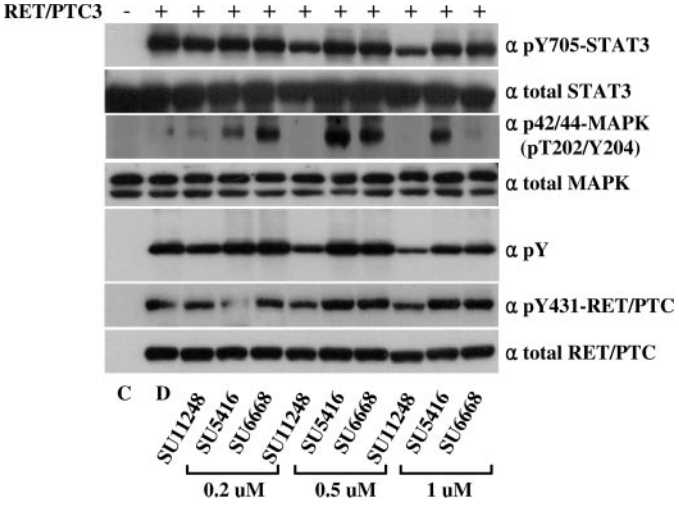

FIG. 3. Dose-dependent inhibition of tyrosine phosphorylation of RET/PTC3 and STAT3. NIH3T3 cells cultured in six-well plates were transfected with RET/PTC3 using the LipofectAMINE method. After transfection, the cells were allowed to recover for 24 h. The medium was replaced with medium containing DMSO, SU11248, SU5416, or SU6668, at the indicated doses, for 30 min. Cells were lysed by boiling in SDS-PAGE sample buffer for 5 min, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were probed with antibodies against STAT3, pY705-STAT3, pY, p42/44-MAPK (pT202/Y204), p42/44-MAPK, RET/PTC3, and pY431-RET/PTC3 antibodies. The drugs did not alter the amount of protein expressed (total STAT3, total MAPK, and total RET/PTC3), but SU11248 did inhibit phosphorylation of STAT3 (pY705-STAT3), p42/44-MAPK [p42/44-MAPK (pT202/Y204)], and RET/PTC3 (pY431-RET/PTC3). C, Mock-transfected control; D, DMSO control.

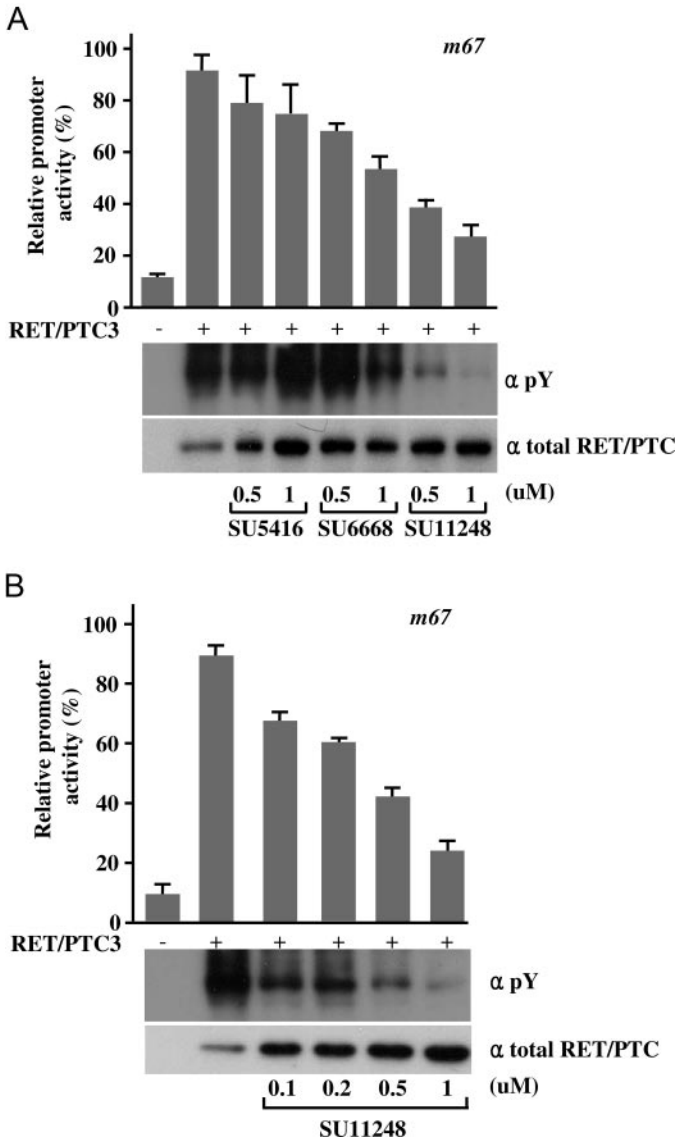

FIG. 4. RET/PTC3-mediated transactivation of STAT3 is suppressed by SU11248. NIH3T3 cells cultured in 12-well plates were cotransfected with RET/PTC3 (500 ng) and m67 luciferase reporter plasmid (100 ng). The medium was replaced 3 h after transfection with medium containing various doses of the test compounds. Cultures were incubated for 18 h, the cells were lysed, and the luciferase activities were measured. The relative luciferase activities of the whole-cell extracts are expressed on the y-axis as mean values ± SD. Experiments were performed in quadruplicate and normalized to Renilla luciferase activity. A, Incubation with 0.5 or 1 μM of each compound shows that transactivation of STAT3 is effectively suppressed by SU11248 treatment but not SU5416 or SU6668 treatment. B, Incubation with increasing concentrations of SU11248 shows that inhibition of transactivation is dose dependent.

SU5416, or SU6668, and the effects on autophosphorylation and substrate phosphorylation were measured. RET/PTC3-transfected NIH3T3 cells expressed significant levels of RET/PTC3 and autophosphorylated the protein (Fig. 3). The Y705 phosphorylation of STAT3, Y431 phosphorylation, and overall tyrosine phosphorylation of RET/PTC3 were inhibited by SU11248 in a dose-dependent manner, whereas

SU5416 or SU6668 had no effect on RET/PTC3 or STAT3 phosphorylation (Fig. 3).

The p42/44 MAPK plays critical roles in cell growth and proliferation and is activated by RET/PTC (20). We assessed phosphorylation levels of p42/44 MAPK in RET/PTC3-transfected cells in the presence of the inhibitors (Fig. 3). RET/PTC3 transfection resulted in an enhanced level of p42/44-MAPK phosphorylation, which was significantly decreased by treatment of cells with 0.5 or 1 μM SU11248 but was only slightly decreased by treatment with 1 μM SU6668.

The activation of STAT3 by RET/PTC3 is involved in cellular transformation and proliferation of RET/PTC3-transformed cells. We measured the transcriptional activity of STAT3 in RET/PTC3 transfected NIH3T3 cells in the presence of SU5416, SU6668, and SU11248. The luciferase reporter construct m67 contains three repeats of the STAT3 binding element and is activated by coexpression of STAT3 and RET/PTC3. A 12-h incubation in the presence of 0.5 or 1 μM SU5416, SU6668, or SU11248 showed various effects on m67 reporter activity, compared with the DMSO control. SU5416 did not show any significant inhibition of m67 reporter activity, and SU6668 showed weak inhibitory effects. In contrast, SU11248 significantly suppressed m67 reporter activity, compared with the vehicle control (Fig. 4A). We measured the dose-dependent effects of SU11248 on RET/PTC3-mediated activation of the STAT3 reporter (Fig. 4B). SU11248 (0.2 μM) effectively suppressed STAT3 transactivation by RET/PTC3, and STAT3 transactivation was further suppressed with increasing doses of SU11248.

SU11248 inhibits the transforming effects of RET/PTC

RET/PTC3 expression induces morphological transformation and serum-independent proliferation of NIH3T3 fibroblasts. We treated RET/PTC transfected NIH3T3 cells with 0.5 μM SU11248 or SU5416 for 24 h and investigated the morphological changes induced by the drugs. SU11248 caused a complete morphological reversion of NIH-RET/PTC3 cells, whereas neither DMSO nor SU5416 had any effect (Fig. 5). As a negative control, we used ras-transformed stable cell lines because ras acts downstream of most receptors, including RET (20). Neither K-ras- nor H-ras-transformed Rat2 cells were affected by SU11248. These data suggest that

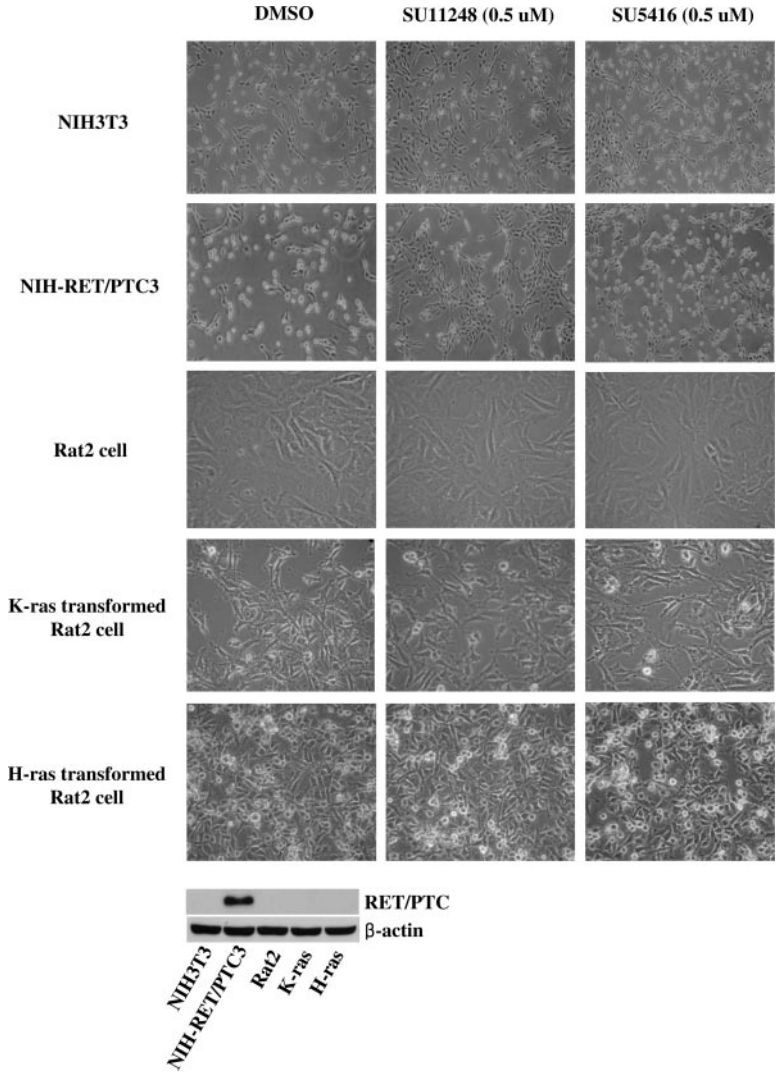

FIG. 5. SU11248 restores normal cell morphology in NIH3T3 cells transformed with RET/PTC3. The NIH3T3 cells transfected with RET/PTC3 and the ras-transformed stable cell lines were treated for 24 h with DMSO, 0.5 μM SU11248, or 0.5 μM SU5416. Cells were photographed using a phase-contrast light microscope (×200). The control and SU5416-treated RET/PTC3 cells show a transformed morphology, but the SU11248-treated RET/PTC3 cells display a normal morphology.

the effect of SU11248 is specific for RET/PTC, rather than inhibiting ras downstream signaling pathways.

SU12248 inhibits proliferation of TPC-1 cells

In NIH-RET/PTC cells, SU11248 treatment was accompanied by a remarkable reduction in RET/PTC autophosphorylation levels (Fig. 2). To investigate this effect further, we measured the proliferation of TPC-1 cells harboring an endogenous RET/PTC1 rearrangement in the presence of SU5416, SU6668, or SU11248 in cultures treated for 1–3 d with various doses (0.02–1 μM) of SU5416, SU6668, or SU11248 (Fig. 6). Whereas SU5416 and SU6668 did not significantly affect the growth of TPC-1 cells, SU11248 treatment resulted in dose-dependent suppression of TPC-1 proliferation.

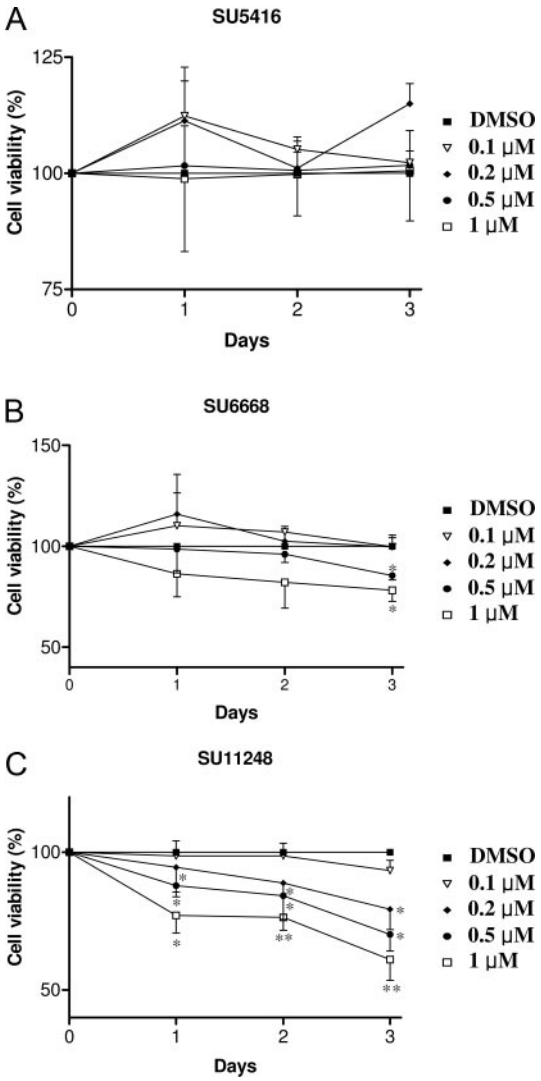

FIG. 6. SU11248 causes growth inhibition of TPC-1 cells. Cell viability assays were carried out using the MTT dye conversion assay in 96-well cell culture plates after exposure to SU5416, SU6668, or SU11248. Data are expressed as a percentage of the DMSO control. MTT assays were carried out in three independent experiments each performed in triplicate. Cell viability was reduced in SU11248-treated TPC-1 cells, compared with DMSO-, SU5416-, or SU6668-treated cultures. As a negative control, K-ras- and H-ras-transformed cells were used. Neither Rat2-K-Ras nor Rat2-H-Ras cells were affected by SU11248 or SU5416. **, $P < 0.01$ compared with DMSO; *, $P < 0.05$, compared with DMSO.

Discussion

SU11248, SU5416, and SU6668 are derivatives of indolinone and have a wide spectrum of inhibitory actions on tyrosine kinases (21). The compounds were developed as inhibitors of the ATP binding site of tyrosine kinases and have entered preclinical and clinical trials (22, 23). Administered orally, SU11248 is a small organic molecule with antitumor and antiangiogenic activity that acts through selectively targeting PDGFR, VEGFR, KIT, and FLT3 (13). By inhibiting the activity of these RTKs, SU11248 directly targets tumor cell proliferation and survival in cancers in which these receptors are involved. SU11248 has been demonstrated to inhibit both FLK/KDR and PDGFRβ phosphorylation *in vivo* (13) and has exhibited antitumor activity in a large number of preclinical xenograft tumor models, including leukemia expressing FLT3-activating mutations (14).

The kinase domain of RET/PTC shows 50% overall homology to the kinase domains of KIT, FLT3, PDGFR, and VEGFR. Most tyrosine kinase inhibitors that bind to ATP-binding pockets showed significant inhibitory activities on multiple classes of tyrosine kinases, which show relatively low homology in the kinase domain. We have shown that SU11248 inhibits the RET/PTC3 kinase with an IC_{50} of 224 nM *in vitro*. This IC_{50} is very similar to the IC_{50} (250 nM) for inhibition of the wild-type fms-related tyrosine kinase/Flk2/Stk-2 (FLT3). FLT3 belongs to the type III split-kinase domain family of RTKs and is primarily expressed on immature hematopoietic progenitors but is also found on some mature myeloid and lymphoid cells. Two classes of mutations activating FLT3 have been identified in acute myelogenous leukemia patients: internal tandem duplication mutations in the juxtamembrane region expressed in 25–30% of acute myelogenous leukemia patients and point mutations in the activation loop of the kinase domain found in approximately 7% of patients (22). SU11248 inhibits phosphorylation of FLT3 and causes regression of sc FLT3 tumors (14).

The 2-indolinone derivative cpd1 has been described as a RET/PTC inhibitor (23); but with an IC_{50} for RET/PTC1 of approximately 30 μM, it appears to be a relatively weak inhibitor, compared with SU11248. The pyrazolo-pyrimidine (PP)-1 and the anilinoquinazoline CZD6474 are potent inhibitors of RET/PTC both *in vitro* and *in vivo* (11, 24). PP1 and ZD6474 inhibited RET-derived oncoproteins with IC_{50} values of 80 and 100 nM, respectively (11, 24). Furthermore, RET/PTC3-transformed cells treated with PP1 and ZD6474 lost proliferative autonomy and showed morphological reversion. Both compounds prevented the growth of two human PTC cell lines that carry spontaneous RET/PTC1 rearrangements and blocked anchorage-independent growth and tumorigenicity of NIH3T3 fibroblasts expressing the RET/PTC3 oncogene transplanted into nude mice. These findings have suggested that PP1 and ZD6474 could be used to treat RET-associated cancers.

The current study shows that SU11248 is a potent (IC_{50} 224 nM) inhibitor of RET/PTC oncoproteins. SU11248-mediated block of RET/PTC inhibited RET/PTC signaling, decreased

RET/PTC autophosphorylation and STAT3 activation, and blocked the transforming capacity of RET/PTC. Furthermore, SU11248 exerted a powerful growth-inhibitory effect on a thyroid carcinoma cell line (TPC-1) that spontaneously harbors a RET/PTC rearrangement. Because these carcinoma cells do not express detectable levels of KDR and SU5416 and SU6668 did not inhibit proliferation, our data suggest that the effects of SU11248 are mediated by RET/PTC1 inhibition, and the possibility that they are mediated by KDR inhibition can be excluded. SU11248 has several possible advantages over previously developed compounds in the treatment of RET/PTC-associated PTC, including its antiangiogenic effects, low toxicity, and the possibility of oral administration. Furthermore, a recent phase I trial reported that potentially active target plasma concentrations between 50 and 100 ng/ml (250 nM) can be achieved with moderate interpatient variability and a long half-life compatible with a single daily dosing (25). Our IC_{50} value for SU11248 was 224 nM, so this dose is projected to be an effective concentration *in vivo*.

SU11248 (sunitinib) is currently undergoing phase III clinical trials for treatment of renal cell carcinoma, lung cancer, and gastrointestinal stromal tumors (25, 26), and our current evidence demonstrating SU11248 inhibition of RET/PTC *in vitro* provides substantive data indicating the benefit of initiating *in vivo* studies with animal models of PTC and subsequent clinical trials.

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