Activation of the RAS/RAF/ERK Signaling Pathway Contributes to Resistance to Sunitinib in Thyroid Carcinoma Cell Lines

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Context: Sunitinib is currently being evaluated in advanced human thyroid carcinomas, based on the rationale that the vascular endothelial growth factor and platelet-derived growth factor receptors and the RET/PTC rearrangement are valuable targets for the treatment of this malignancy. However, criteria for selecting thyroid tumors that may benefit from sunitinib are lacking.

Design: The effect of activating somatic mutations in the KRAS and BRAF genes on the responsiveness to sunitinib was evaluated in a panel of thyroid cancer cell lines harboring wild-type KRAS and BRAF genes, the RET/PTC1 rearrangement, the G12R KRAS, or the V600E BRAF mutation.

Results: Sunitinib was found to selectively inhibit cell proliferation, induce cell accumulation in the G0-G1 phase, and inhibit the phosphorylation of ERK1/2 in both KRAS/BRAF wild-type thyroid cancer cells and in tumor cells harboring the RET/PTC rearrangement, whereas it was completely ineffective in KRAS- or BRAF-mutated thyroid carcinoma cells. This differential antitumor activity of sunitinib did not correlate with the expression profile of the vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor-alpha, and cKIT genes. Of note, the constitutive activation of RAS/RAF/ERK signaling in KRAS/BRAF wild-type cells by transfection of the R12 HRAS or V600E BRAF mutants or stimulation with epithelial growth factor resulted in the loss of responsiveness to sunitinib, whereas pharmacological inhibition of MAPK kinase activity resulted in the resensitization of KRAS- or BRAF-mutated cells to the multikinase inhibitor.

Conclusions: The constitutive activation of the RAS/RAF/ERK pathway may favor resistance to sunitinib in thyroid carcinoma cells. (J Clin Endocrinol Metab 97: 0000–0000, 2012)
tive criteria to help select tumors which may benefit from these treatments.

In recent years, a variety of molecular-targeted agents that inhibit tyrosine kinases (TK) related to tumor growth and/or angiogenesis have been developed, and some of them (i.e. axitinib, motesanib, sorafenib, sunitinib, and pazopanib) have been evaluated in advanced TC in humans with promising results (3, 4). The rationale behind these studies is provided by the evidence that most papillary TC are driven in part by single activating somatic mutations of the BRAF and RAS oncogenes, and/or by translocations producing RET/PTC oncogenes (5).

Sunitinib is a multitargeted agent, with antiangiogenic and antitumor properties, acting as a selective inhibitor of vascular endothelial growth factor receptors (VEGFR) 1, 2, and 3, platelet-derived growth factor receptor (PDGFR)-α, cKIT, and RET/PTC subtypes 1 and 3. Sunitinib is currently used as standard treatment for clear-cell renal carcinoma and gastrointestinal stromal tumor and is under investigation in several other human malignancies (6). The interest in sunitinib for the treatment of TC arises from its activity against tumor cells bearing the RET/PTC rearrangement (7). Several preclinical studies have evaluated sunitinib in RET/PTC TC cell lines demonstrating, indeed, that this agent is able to inhibit cell proliferation and cause cell cycle arrest by inhibiting the RET/PTC kinase (8, 9). Interestingly, sunitinib appears to target the cytosolic MAPK kinase (MEK)/ERK and stress-activated protein kinase/c-Jun N-terminal kinase pathways in RET/PTC cell lines, suggesting that blocking these pathways is at least part of the mechanism by which sunitinib exerts its direct antiproliferative activity (9). Sunitinib has been tested in phase II clinical trials in metastatic iodine-refractory TC, showing promising results (10, 11). However, under sunitinib therapy, the majority of TC showed disease stability, whereas few of them exhibited major tumor regression (10, 11), suggesting that the clinical activity of sunitinib may be restricted to a specific subgroup of human thyroid malignancies (4). In fact, given the indolent nature of most TC, a report highlighting tumor stability may be of limited value. Thus, taken all together, these observations suggest that criteria for identifying tumors that may benefit from sunitinib therapy are lacking.

Because of several factors (including 1) only 20% of human papillary TC harbor the RET/PTC rearrangement (12), 2) the molecular characterization of human thyroid cancers revealed a great heterogeneity with genotypes showing driving mutations in other oncogenes (12), and 3) little information is available on the antiproliferative activity of sunitinib in non-RET/PTC TC cell lines), we sought to evaluate sunitinib activity in a panel of TC cell lines at different degrees of cell differentiation and characterized by different molecular genotypes, in an attempt to find the molecular determinants responsible for sensitivity/resistance.

### Materials and Methods

#### Chemicals, cell cultures, and constructs

Reagents were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise specified. The MEK inhibitor PD98059 was purchased from Cell Signaling Technology (Milan, Italy). The following unique human TC cell lines were used: papillary TPC-1; follicular ML-1; poorly differentiated WRO; and anaplastic CAL-62, FRO, and BHT-101 cells (13). TPC-1 cells were kindly provided by Professor Pontecorvi (Endocrinology Unit, Catholic University, Rome, Italy), whereas the other cell lines were purchased from DSMZ (Braunschweig, Germany). Cells were cultured in DMEM containing 10% (vol/vol) fetal bovine serum (20% for BHT-101), 1.5 mM glutamine, and 100 U/ml penicillin and streptomycin. Cell lines were routinely monitored by microscopic morphology.

Sunitinib was kindly provided by Pfizer (New York, NY) and dissolved in dimethylsulfoxide at 10 mM. Sunitinib or the same dimethylsulfoxide volume was added to the cultures at the concentrations specified in Results. To evaluate the growth rate, cells were cultured in six-well plates in the presence and absence of the reported concentrations of sunitinib, harvested after 4 d, and counted in a Bürker chamber (three countings per sample). Incubation with drugs was carried out continuously, and drug-containing fresh medium was changed at 48-h intervals. Cell viability was evaluated using the dimethylthiazoliphenyltetrazoliumbromide (MTT) dye assay as previously described (14). Results represent the average of three experiments. Apoptosis was measured by cytofluorimetric evaluation of 7-aminoactinomycin D incorporation in nonpermeabilized cells.

The R12 HRAS construct was a kind gift of Dr. Alfonso Bellacosa (Fox Chase Cancer Center, Philadelphia, PA), and the wild-type BRAF and BRAF V600E constructs were kindly provided by Professor Franca Esposito (University of Naples Federico II, Naples, Italy). All the constructs were cloned in pcDNA3.1 vector (Invitrogen, San Giuliano Milanese, Italy). Transient transfection of DNA plasmids was performed with Polyfect Transfection reagent (QIAGEN, Milan, Italy), according to the manufacturer’s protocol.

#### Cell cycle analysis

Cells were grown in the presence and the absence of 0.5 and 1 μM sunitinib for 48 h, incubated in the same medium supplemented with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 15 min, and harvested. Cell pellets were resuspended in a solution containing 10 N HCl for 30 min at room temperature to obtain DNA denaturation and subsequently incubated in the presence of anti-BrdU monoclonal antibodies (Becton Dickinson and Co., Milan, Italy) for 1 h at room temperature in the dark. After washing with PBS, cells were incubated with 1 μg/ml propidium iodide (PI) for 20 min and evaluated using the EPICS XL flow cytometer (Beckman Coulter, Cassina De’ Pecchi-Milan, Italy). In specific experiments, cells were transiently cotransfected with pcDNA3.1 (scramble), R12 HRAS, wild-type BRAF or V600E BRAF constructs, and pEGFP-F (CLONTECH, Mountain View, CA) and 24 h later treated with sunitinib. The transfection vector
pEFGP-F, which encodes for a farnesylated enhanced green fluorescent protein, was used as a reporter vector both to monitor transfection efficiency and as a cotransfection marker. Cell cycle was evaluated by PI labeling in green fluorescent protein–positive cells. Experiments were performed at least three times using three replicates for each drug concentration.

**Immunoblot analysis**

Immunoblot analysis was performed as previously reported (15). Specific proteins were detected by using a rabbit polyclonal antiphosphorylated ERK1/2 (Upstate Cell Signaling Technology), a mouse monoclonal antiphosphorylated ERK1/2 (Thr202/Tyr204-E10; Cell Signaling Technology), a rabbit polyclonal anti-ERK1/2 (Calbiochem, Rome, Italy), a mouse monoclonal anti-BRAF (F-7, sc-5284; Santa Cruz Biotechnology, Segrate, Italy), a rabbit polyclonal anti-HER2-3-phosphate dehydrogenase (GAPDH; sc-47724; Santa Cruz Biotechnology), and a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-47724; Santa Cruz Biotechnology) antibodies.

**RNA extraction and real-time RT-PCR analysis**

Total RNA from cell pellets was extracted using the TRIzol reagent (Invitrogen). For the first-strand synthesis of cDNA, 3 μg of RNA was used in a 20-μl reaction mixture using a cDNA SuperScript II (Invitrogen). For real-time PCR analysis, 1 μl of cDNA sample was amplified using the Platinum SYBR Green qPCR Supermix UDG (Invitrogen) in an iCycler iQ real-time detection system (Bio-Rad Laboratories GmbH, Segrate, Italy). The following primers were used: VEGFR1, forward, 5′-CAAGTGGCCAGAGGACCGGAGTT-3′ reverse, 5′-GATGTATCTTTACCATCTGTGG-3′ (PCR product 417 bp); VEGFR2, forward, 5′-TAAGGGCATGGGATTTCTTG-3′ reverse, 5′-AGGAAACAGGTGGTACGTGGACCTGGC-3′ (PCR product 562 bp); PDGFRα, forward, 5′-TTTGACCGTTCCCCGAGGCCA-3′ reverse, 5′-TGCGGCGCACATTGCTA-3′ (PCR product 60 bp); and GAPDH, forward, 5′-CAAGGCAGAAGGGAAGAGACGG-3′ reverse, 5′-GCATGCCCAACTTGATTTTGTACGGTCCCCGAGGCCA-3′ (PCR product 90 bp). Primers were designed to be intron spanning. Reaction conditions were 50 C for 2 min, 95 C for 2 min, followed by 45 cycles of 15 sec at 95 C, 30 sec at 60 C (VEGFR2, 58 C; PDGFRα, 62 C), and 30 sec at 72 C. GAPDH was chosen as an internal control. Primers specific for VEGFR3 and cKIT mRNA were purchased from QIAGEN (catalog no. QT00063637 and QT01679993) and used according to the manufacturer’s protocol.

**Mutational analysis**

DNA was extracted from TC cells using the QIAamp DNA minikit (QIAGEN), according to the manufacturer’s protocol. Assays for samples for KRAS and BRAF mutations were performed by using, respectively, the anti-epithelial growth factor receptor (EGFR) monoclonal antibodies response-KRAS status and the anti-EGFR monoclonal antibodies response-BRAF status kits (Diatech Pharmacogenetics, Ancona, Italy), according to the manufacturer’s instructions. Codons 12, 13, 61, and 146 (exon 2) of the KRAS gene and codons 464, 466, and 469 (exon 11), and 600 (exon 15) of the BRAF gene were amplified by PCR reactions on the Rotor-Gene Q 6000 (QIAGEN), single-stranded DNA templates were prepared using the PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden) and, lastly, pyrosequencing analysis was performed on the PyroMark Q96 ID (Biotage, Uppsala, Sweden).

**Statistical analysis**

The paired Student’s t test was used to establish the statistical significance between different levels of growth rate and cell cycle distribution. Statistically significant values (P < 0.05) are reported in the figure legends.

**Results**

Sunitinib antiproliferative activity is selective for specific TC cell lines

To explore the antiproliferative activity of sunitinib in TC cells, a panel of six TC cell lines at distinct degrees of cell differentiation was evaluated for growth rate (Fig. 1A) and cell viability by MTT incorporation (Fig. 1B) in response to increasing concentrations of sunitinib. Thus, in addition to papillary TPC-1 cells harboring the RET/PTC translocation...
(16), follicular ML-1, poorly differentiated WRO, and anaplastic FRO, BHT-101, and CAL-62 cell lines were evaluated. Sunitinib induced a similar inhibition of cell proliferation in TPC-1, ML-1, and WRO cells, whereas it was ineffective in CAL-62, FRO, and BHT-101 cells (Fig. 1, A and B). Of note, this sunitinib-dependent differential antiproliferative activity was not dependent on a selective induction of apoptosis, as demonstrated by cytofluorimetric evaluation of 7-aminoactinomycin D incorporation in nonpermeabilized cells (data not shown) but correlated with the accumulation of cells in the G0-G1 phase of the cell cycle and the parallel inhibition of the S phase. Consistently, sunitinib failed to induce similar effects in FRO, BHT-101, and CAL-62 cells (Fig. 1C).

Because it is well established that the direct antitumor activity of sunitinib depends mostly on its ability to inhibit ERK signaling (17), the ability of the drug to inhibit ERK phosphorylation was evaluated in TC cell lines (Fig. 2).

The exposure of ML-1, TPC-1, and WRO cells to 0.5 \( \mu \)M sunitinib for 4 h resulted in a significant inhibition of ERK phosphorylation, whereas the same drug concentration failed to inhibit ERK signaling in FRO, CAL-62, and BHT-101 cells.

The resistance to sunitinib correlates with the mutational status of KRAS and BRAF genes in TC cell lines

A major aim of this study is to find molecular determinants of sunitinib sensitivity/resistance in TC cell lines. Thus,
the panel of TC cells was evaluated, by real-time PCR, for the expression profile of VEGFR1, VEGFR2, and VEGFR3, PDGFRα, and cKIT, all well-established targets of sunitinib (17). Of note, the expression of none of these genes correlated with the antiproliferative activity of sunitinib. Indeed, VEGFR1 and VEGFR3, as well as cKIT mRNA levels were below the detection limit of a quantitative RT-PCR assay (data not shown). The expression of the VEGFR2 gene was up-regulated in FRO and CAL-62 cells that are nonresponsive to sunitinib, whereas it was poorly expressed in both sunitinib-insensitive BHT-101 cells and the three sunitinib-sensitive TC cell lines (Fig. 3A). Furthermore, PDGFRα was up-regulated in both sunitinib-sensitive WRO and sunitinib-insensitive BHT-101 cells (Fig. 3A).

Because the inhibition of the RAS/RAF/ERK signaling downstream of the TK receptors is a critical step for the direct antitumor activity of sunitinib (17) and taking into account that mutations of KRAS or BRAF genes have been proven to drive oncogenic events in thyroid carcinogenesis (12), we further evaluated the relationship between the mutational status of BRAF and KRAS genes and the responsiveness to sunitinib. As reported in Fig. 3B, sunitinib-resistant CAL-62 cells were shown to be heterozygous for the KRAS G12R mutation, whereas FRO and BHT-101 cell lines were revealed to harbor the BRAF V600E heterozygous mutation. By contrast, all sunitinib-sensitive TC cell lines exhibited wild-type KRAS and BRAF genotypes.

The activation of RAS/RAF/ERK pathway favors the resistance to sunitinib in TC cells

To establish whether the activation of the RAS/RAF/ERK signaling pathway may be responsible for resistance...
to sunitinib in TC cells, we evaluated sunitinib activity on the constitutive activation of the ERK pathway in KRAS/ BRAF wild-type WRO cells. This was achieved by transfecting a construct encoding for the HRAS R12 mutant, which is known to induce fibroblast transformation and the constitutive activation of ERK signaling (18), and a construct encoding for the BRAF V600E mutant, which is the most frequent BRAF mutation in human papillary TC and known to be responsible for the constitutive activation of ERK phosphorylation (19, 20). In these experimental conditions, sunitinib failed to inhibit ERK phosphorylation (Fig. 4A) and the S phase with accumulation of cells in the G0-G1 phase (Fig. 4B) in both WRO HRAS R12 and BRAF V600E transfectants. Furthermore, because the increased expression of alternative growth factors with proangiogenic potential and capable of directly stimulating tumor cells has been proposed as an escape mechanism from anti-vascular endothelial growth factor therapies (21), we hypothesized that epithelial growth factor (EGF), which is known to be involved in thyroid tumor progression (22), may favor the appearance of sunitinib resistance in TC cells. Thus, cell proliferation and ERK phosphorylation was evaluated in sunitinib-treated KRAS/BRAF wild-type WRO cells upon stimulation with high concentrations of EGF. Indeed, the EGF-dependent activation of ERK pathway reduced the ability of sunitinib to inhibit both ERK phosphorylation (Fig. 4C, upper panel) and the rate of cell proliferation (Fig. 4C, lower panel).

In a parallel experiment, we sought to inhibit ERK signaling in KRAS- or BRAF-mutated TC cells to reestablish sensitivity to sunitinib. This objective was achieved by pretreating CAL-62, BHT-101, and FRO cells with the MEK inhibitor, PD98059, before exposure to sunitinib (Fig. 5). Of note, inhibition of the ERK signaling pathway resulted in the partial reestablishment of sunitinib inhibitory activity on cell proliferation (Fig. 5A), cell cycle (Fig. 5B), and ERK phosphorylation (Fig. 5C).

Discussion

The mechanism of action of the multitargeted TK inhibitor, sunitinib, depends on both its antiangiogenic activity toward endothelial cells and its direct antiproliferative activity against tumor cells (17). The present study was specifically designed to evaluate the mechanisms responsible for sunitinib’s direct antiproliferative activity in TC cell

![Image](85x128 to 505x398)
The results suggest that sunitinib does the following: 1) is equally active in both RET/PTC and non-RET/PTC cell lines, 2) induces arrest of the cell cycle in the G0-G1 phase with parallel inhibition of ERK signaling, and 3) exerts antiproliferative activity, likely independently of the expression of its molecular targets. Of note, sunitinib showed inefficacy in TC cells bearing activating somatic mutations of \textit{KRAS} or \textit{BRAF} oncogenes, which are known to be responsible for the constitutive activation of ERK signaling. Consistently, the constitutive activation of ERK signaling by the transfection of HRAS or BRAF constitutive active mutants resulted in the loss of sunitinib antiproliferative activity (Fig. 6). Although this is, to our knowledge, the first demonstration that oncogenic mutations of \textit{KRAS} or \textit{BRAF} genes are predictive factors of sunitinib inefficacy \textit{in vitro}, similar results have been reported for KRAS-activating mutations and for both the anti-EGFR monoclonal antibodies in human colorectal carcinomas (24) and the EGFR TK inhibitors in non-small cell lung carcinomas (25).

The second finding highlighted by this study is the observation that the stimulation of thyroid cancer cells with high concentrations of EGF results in loss of sensitivity to sunitinib and in its inability to inhibit ERK phosphorylation. Indeed, it has been previously suggested that, during anti-vascular endothelial growth factor therapy, the up-regulation of alternative growth factors may be responsible for secondary drug resistance. This is due either to the recruitment of alternative angiogenic circuits or to a direct tumor stimulating activity (21). Thus, it is likely that the ligand-dependent activation of TK receptors that are not specific targets of sunitinib may favor the activation of ERK signaling, and this in turn may be responsible for the loss of sunitinib activity, despite its still effective inhibitory activity on receptor TKs.

These results highlight the relevance of the inhibition of ERK signaling in the antiproliferative activity of sunitinib in TC cells. Indeed, the RAS/RAF/ERK pathway is a highly conserved system, responsible for transducing proliferative and survival signals from TK receptors to the nuclear transcriptional machinery (23). Thus, our results suggest that the inhibitory activity of sunitinib on multiple TK receptors is responsible for the inactivation of RAS/RAF/ERK signaling, this representing a major mechanism of its direct antitumor effect. Furthermore, the constitutive activation of this signaling pathway downstream of the TK receptors, due to either activating mutations of KRAS or BRAF oncogenes or the transfection of HRAS or BRAF constitutive active mutants, results in the loss of sunitinib antiproliferative activity (Fig. 6). Although this is, to our knowledge, the first demonstration that oncogenic mutations of \textit{KRAS} or \textit{BRAF} genes are predictive factors of sunitinib inefficacy \textit{in vitro}, similar results have been reported for KRAS-activating mutations and for both the anti-EGFR monoclonal antibodies in human colorectal carcinomas (24) and the EGFR TK inhibitors in non-small cell lung carcinomas (25).
dependent phenotype or the escape from the antitumor activity of chemotherapeutics and TK-specific agents.

These observations may provide useful insights for the development of novel molecular-targeted therapies in human TC. In the last few years, several clinical trials evaluated novel agents for the treatment of radioiodine refractory progressive thyroid cancer (3, 4), all of them with antiangiogenic and antitumoral activity. Because most of these studies demonstrated promising but conflicting results (3, 4), it is likely that a selective approach to the use of these agents, based on the molecular profiling of thyroid tumors, may provide clearer evidence. In such a light, predictive criteria to drive the selection of the appropriate inhibitor are needed in human TC. This issue is even more critical in the light of recent studies demonstrating that mutations of BRAF and KRAS oncogenes and the RET/PTC translocations are the most common driving events responsible for papillary thyroid carcinogenesis (28, 29). Indeed, the RET/PTC translocation and the BRAF V600E mutation have been demonstrated in, respectively, about 20 and 30–40% of human papillary TC (12), whereas activating mutation of RAS gene represents the predominant molecular alteration of poorly differentiated thyroid cancers (30). Furthermore, it has been suggested that papillary TC bearing the BRAF V600E mutation are characterized by a worse prognosis, due to increased nodal metastases, high risk to multicentricity and extrathyroidal invasion, and reduced responsiveness to radioiodine therapy (31). Thus, in the light of this molecular heterogeneity, a differential sensitivity toward agents that inhibit different targets is expected. In such a perspective, based on the observation that sunitinib is active irrespective of the RET/PTC translocation but fails to inhibit TC cells bearing BRAF or KRAS mutations, we suggest that activating mutations of both these oncogenes need to be prospectively evaluated as predictive biomarkers in thyroid cancer clinical trials. In fact, even though KRAS- or BRAF-mutated human TC may still respond to sunitinib due to its antiangiogenic activity, it is intriguing to speculate that the constitutive activation of ERK signaling may result in insensitivity to sunitinib in human TC. Indeed, this hypothesis would be consistent with recent observations suggesting that sunitinib fails to inhibit BRAF-mutated TC cells (32) and would explain the sometimes conflicting results provided by sunitinib in clinical trials (10, 11). Indeed, although sorafenib, which is known to inhibit BRAF, has been suggested to be more effective in BRAF-mutated papillary TC (33), other agents, such as the recently developed BRAF inhibitors (34), may provide interesting clinical activity in BRAF- or KRAS-mutated thyroid cancers. Thus, future studies will aim to answer the question as to whether KRAS or BRAF wild-type tumors are the only subgroup of thyroid cancers responsive to sunitinib therapy, BRAF- or KRAS-mutated TC likely being more responsive to agents that target ERK signaling downstream of the TK receptors.

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