Sunitinib induces apoptosis in pheochromocytoma tumor cells by inhibiting VEGFR2/Akt/mTOR/S6K1 pathways through modulation of Bcl-2 and BAD

Yuria Saito,‡ Yuki Tanaka,‡ Yui Aita, Kiy-aki Ishii, Tatsuhiko Ikeda, Kazumasa Isobe, Yasushi Kawakami, Hitoshi Shimano, Hisato Hara, and Kazuhiro Takekoshi

1Department of Laboratory Medicine, 2Department of Endocrine Surgery, and 3Department of Internal Medicine (Endocrinology and Metabolism), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

Submitted 24 January 2011; accepted in final form 29 August 2011

Sunitinib induces apoptosis in pheochromocytoma tumor cells by inhibiting VEGFR2/Akt/mTOR/S6K1 pathways through modulation of Bcl-2 and BAD. Am J Physiol Endocrinol Metab 302: E615–E625, 2012. First published August 30, 2011; doi:10.1152/ajpendo.00035.2011.—Sunitinib is an oral multitargeted receptor tyrosine kinase inhibitor with antiangiogenic and antitumor activity that mainly targets vascular endothelial growth factor receptors (VEGFRs). Very recently, sunitinib has been shown to be an active agent for the treatment of malignant pheochromocytomas. However, it is unclear whether sunitinib acts only through an antiangiogenic mechanism or whether it may also directly target tumor cells. Sunitinib markedly induced apoptosis of PC12 cells in a dose-dependent and time-dependent manner. Furthermore, in support of these findings, we found that sunitinib induced a reduction in the expression of the antiapoptotic molecule Bcl-2 as well as dephosphorylation of the proapoptotic molecule BAD, which results in the activation of BAD in these cells. Consistent with these apoptotic effects, our results showed that sunitinib inhibited phosphorylation of Akt and mTOR and was followed by a reduction of S6K1, which is a well-known target of mTOR. Knockdown of VEGFR-2 attenuated the sunitinib-induced effects, including apoptosis and inhibition of signaling pathways such as the phosphorylation of Akt as well as mTOR, and Bcl-2, which confirmed that these effects could be mediated by VEGFR-2. In addition, silencing of S6K1 induced apoptosis accompanied by a decrease in the phosphorylation of BAD and Bcl-2, similar to that observed with sunitinib treatment. Thus, these results together suggest that sunitinib initially exerts its apoptotic effect through the inhibition of VEGFR-2, which, when followed by reduction of its downstream effectors, including Akt/mTOR/S6K1, may lead to inhibition of the antiapoptotic molecule Bcl-2 and activation of the proapoptotic molecule BAD in PC12 cells. However, PC12 cells do not precisely reflect the pathogenesis of malignant pheochromocytomas. Therefore, we confirmed the key findings by replicating these experiments in human neuroblastoma SK-N-SH cells.

PC 12 cells; SK-N-SH cells; proliferation; cyclin; hypoxia-inducible factor

SUNITINIB IS AN ORAL MULTITARGETED RECEPTOR tyrosine kinase inhibitor with antiangiogenic and antitumor activity that targets platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptors (VEGFRs), the stem cell growth factor receptor KIT, and fms-related tyrosine kinase-3 (FLT3) (13). It has been well established that sunitinib suppresses tumor angiogenesis through VEGFR and PDGFR, which are expressed on endothelial cells and are known to play critical roles in angiogenesis (9, 13, 20). Sunitinib is also reported to have direct antiproliferative and/or apoptotic effects on tumor cells that express target kinases. Indeed, sunitinib has been shown to directly inhibit the survival and proliferation of a variety of cancer cells, including small-cell lung carcinoma, gastrointestinal stromal tumors, acute myelogenous leukemia, chronic myelogenous monocytic leukemia, and renal cell carcinoma (RCC) (2, 12, 22, 23, 34, 41, 46, 49).

Advanced-stage malignant pheochromocytomas are resistant to conventional therapies such as radiation therapy and chemotherapy. Three recent reports suggest that sunitinib may be an active agent in the treatment of malignant pheochromocytomas (17, 26, 27). It is well established that pheochromocytomas often display hypervascularity. Indeed, significant VEGF expression has been shown in most tumors by immunohistochemical staining (8, 28). Furthermore, the VEGF expression and increased microvessel density evidenced by CD31 staining has been associated with an aggressive malignant phenotype (15, 43). In a study comparing hereditary and sporadic paragangliomas and pheochromocytomas, Gimenez-Roqueplo et al. demonstrated prominent expression of VEGF and hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in both tumor cells and endothelial cells lining tumor blood vessels (15). Interestingly, the expression levels of VEGF, HIF-1α, and HIF-2α were increased to a greater degree in hereditary tumors than in sporadic tumors, which suggests that angiogenesis has an important role in these inherited syndromes. On the basis of all these data, it is reasonable to consider the inhibition of angiogenesis as a therapeutic target in these tumors, probably through a mechanism of action similar to that seen in other hypoxia-inducible tumors such as RCC (26, 34). However, the antitumor effects of sunitinib in the treatment of malignant pheochromocytoma have not been clearly established. In particular, it is unclear whether sunitinib acts only through an antiangiogenic mechanism or whether it may also directly target tumor cells.

The phosphoinositide 3-kinase/Akt cell survival pathway is activated in many cancers, and increased phosphorylation of Akt at Ser473, which is linked to its activation, is observed in human pheochromocytoma samples (5, 14). The mammalian target of rapamycin (mTOR) is divided into two multiprotein complexes, named mTORC1 and mTORC2. In particular, mTORC1 is a well-known positive regulator of cell cycle progression and cellular proliferation. mTOR is regulated by an upstream effector via multiple phosphorylation sites, including Ser2448. Activation of mTOR induces phosphorylation of ribosomal protein S6 kinase (S6K1) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1),...
which results in activation of both ribosome biogenesis and cap-dependent mRNA translation (19, 39). Indeed, high levels of S6K1 are reported to be associated with proliferation of the adrenal medulla in both mouse and human pheochromocytomas (37). It is noteworthy that mTOR has been reported to be an upstream regulator of HIF-1α (21, 33).

The BCL-2 family of proteins regulates cell death. BAD is a “BH3 domain-only” proapoptotic member of this family. In the presence of survival factors, cells phosphorylate BAD on two serine residues (Ser112 and Ser136) that are embedded within 14-3-3 consensus binding sites. Phosphorylated BAD appears to be the inactive form that is sequestered in the cytosol and bound to 14-3-3, which thus frees BCL-XL or BCL-2 to promote survival. In contrast, nonphosphorylated activated BAD heterodimerizes with BCL-XL or BCL-2 to promote cell death (1, 10, 11).

To confirm the direct effects of sunitinib, other than angiogenesis, on tumor cells, we examined a number of sunitinib responses (i.e., apoptosis, proliferation, and the molecules involved in the underlying mechanisms of action, including the Akt/mTOR/S6K1 family and the BCL family) in the rat pheochromocytoma cell line PC12. We have, for the first time, introduced the hypothesis that the antitumor effect of sunitinib observed in malignant pheochromocytomas seems to be associated with direct effects on tumor cells. It is known that PC12 cells do not accurately reflect the pathogenesis of malignant cells. Therefore, we confirmed the effects of sunitinib in human neuroblastoma SK-N-SH cells.

**Fig. 1.** Effects of sunitinib on apoptosis in rat pheochromocytoma PC12 cells. PC12 cells were treated with sunitinib as described and apoptosis was assessed by 3 different methods as follows. A: TUNEL assay. 1) Representative images of TUNEL staining (red) are shown. These data were reproducible in 3 independent experiments. 2) Histogram showing the average of number of TUNEL-positive cells (red) per total cells. Significantly increased numbers of TUNEL-positive cells (red) were seen in the 1,000 nM sunitinib treatment group (*P < 0.05). B: cleavage of caspase. Cleaved caspase was analyzed by immunoblotting of total cell lysates after a 24-h sunitinib treatment. Representative data are shown, and these data were reproducible in 3 independent experiments. C: cytoplasmic histone-associated DNA fragments. Cytoplasmic histone-associated DNA fragments were determined by ELISA; details are described in MATERIALS AND METHODS. PC12 cells were treated with sunitinib at indicated the concentrations for 24 h (1, C-I) or sunitinib at 1,000 nM for 12 or 24 h (1, C-II). The right 2 columns in C–I show the effect of vascular endothelial growth factor receptor 2 (VEGFR-2) knockdown on sunitinib-induced apoptosis and the effect of S6 kinase (S6K1) knockdown on apoptosis in PC12 cells. The data shown represent the mean ± SE of 3 independent experiments (1 experiment was performed with 2 samples). *P < 0.05 vs. basal value; #significantly different (P < 0.05) from the value induced by sunitinib (1,000 nM) alone.
MATERIALS AND METHODS

Reagents. Unless otherwise noted, all reagents were purchased from Wako Seiyaku (Tokyo, Japan). Sunitinib was a gift from Pfizer Inc. (NY).

Cell culture. The PC12 cell line (RCB009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). Cells were grown in 75-cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Gaithersburg, MD) containing 10% inactivated horse serum (GIBCO-BRL) and 10% fetal bovine serum (GIBCO-BRL) in a humidified atmosphere of 5% CO₂:95% O₂ at 37°C. The culture medium was changed three times per week. Cells were removed from the flasks for subculture and for plating into assay dishes using a Ca²⁺/Mg²⁺-free solution: 172 mM NaCl, 5.4 mM KCl, 1 mM NaH₂PO₄, and 5.6 mM glucose, at pH 7.4. After about 2 min in this solution, the cells were detached by tapping the side of the flask. The cells (1 × 10⁶) were plated into 35-mm polystyrene dishes and cultured with 2 ml of DMEM for 2 days under similar conditions to those described above. The cells were then used for experiments in a serum-starved condition (4).

Human neuroblastoma SK-N-SH cells were obtained from the RIKEN Cell Bank (Ibaraki, Japan). Under serum conditions, the cells were maintained at 37°C and 5% CO₂ in minimal essential medium with Eagle’s salts containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.4% minimal essential medium vitamins, 0.4% minimal essential medium nonessential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% normal fetal bovine serum. Under serum-free conditions, the cells were maintained as in serum conditions except that the media lacked nonessential amino acids and fetal bovine serum (36).

Immunoblotting. Immunoblotting was carried out as described previously (4, 23). Anti-p Akt (Ser⁴⁷³) (Cell Signaling), Akt (Cell Signaling), anti-p-70 S6K (Thr³⁸⁹) (Cell Signaling), anti-p-mTOR Ser²⁴⁸ (Cell Signaling), anti-p70 S6K (Cell Signaling), anti-p-4E-BP1 (Thr⁷⁰) (Cell Signaling), anti-4E-BP1 (Cell Signaling), anti-Bcl-2 (Santa Cruz Biotechnology), anti-Bcl-xL (Cell Signaling), and anti-HIF-1α (Cell Signaling) antibodies were used. Antibodies against α-tubulin, cyclin B1, cyclin D, and p27 were obtained from Santa Cruz Biotechnology. Cleaved caspase was obtained from Cell Signaling Technology. The band intensities were measured by densitometry.

Apoptosis assay. The TUNEL method was performed by commercially available TMR-red kit (Roche, Mannheim, Germany) in accordance with the manufacturer’s instructions.

Apoptosis was assessed by Cell Death Detection ELISA PLUS kit (Roche) (23), used in accordance with the manufacturer’s instructions (24). In brief, each well of 96-well plates was seeded with 5,000 cells in culture medium with 1% FBS. The following day, the cells were treated with indicated concentrations of sunitinib for a 12- or 24-h period. After treatment, all cells, including both floating and attached cells, were collected, and the apoptotic cells were detected by photometric enzyme immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes).

Proliferation assay. MTT ASSAY. Cells were plated in 96-well culture plates at a density of 10,000 cells/well. At the indicated periods medium was removed and 100 µl of DMEM containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Cells were then incubated at 37°C for 45 min in a CO₂ incubator. Finally, the MTT-containing medium was removed, and purple formazan crystals were dissolved by adding 100 µl of dimethyl sulfoxide to each well. Absorbance was measured in a microplate reader at 570 nm with a reference filter at 655 nm (24).

BRDU ASSAY. Cell incorporation of BrdU was measured by enzyme-linked immunoabsorbent assay (ELISA) with a Cell Proliferation Biotrak ELISA system (version; GE Healthcare, Piscataway, NJ), according to the manufacturer’s instructions. In brief, cells were incubated in control (DMSO) or 1,000 nM of sunitinib for 24 h. Then, cells were fixed, cellular DNA was partially digested by nuclease treatment, and incorporated BrdU was detected with anti-BrdU monoclonal antibodies (mAb) conjugated with peroxidase. The absorbance was measured by a microtiter plate reader at 450 nm and was directly correlated to the level of BrdU incorporation into cellular DNA (4).

Small interfering RNA transfection. RNA interference was performed as previously described (24). In brief, PC12 cells were seeded in six-well plates and 24 h later were transfected with small interfering
(si)RNA (iGENE, Tokyo, Japan) targeting either VEGFR-2 or S6K1 using lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. At the same time, nontargeting siRNA (iGENE, Tokyo, Japan) was transfected as a negative control. The knockdown gene was confirmed by Western blotting 24 h after transfection.

**Microarray analysis.** Microarray analyses were performed as previously described (24). In brief, we processed total cellular RNA extracts according to the protocol recommended by Takara. We then hybridized 5 g of labeled cRNA from each sample to the rat genome Agilent Expression Array (Takara) representing ~43,000 genes.

**Statistical analysis.** All data are expressed as means ± SE. The significance of differences in the data was determined by an analysis of Student’s t-test. P values <0.05 were considered significant.

**RESULTS**

**Effect of sunitinib on apoptosis in PC12 cells.** We determined whether sunitinib has apoptotic effects on PC12 cells. Initially, we performed a TUNEL assay to confirm these findings. Prior to sunitinib treatment (at the end of the incubation period), the presence of FBS in the medium did not significantly affect cell death (Fig. IA, histogram). Although minimal numbers of TUNEL-positive cells (red) were detected in vehicle (DMSO)-treated control cells, in sunitinib-treated (1,000 nM, 24 h) cells, we observed extensive apoptosis of PC12 cells (Fig. IA, top). To further confirm that the cell death induced by sunitinib treatment was indeed apoptosis, immunoblotting analyses were performed to detect cleavage of caspase (inactive caspase). Sunitinib treatment (1,000 nM, 24 h) increased the level of cleaved caspase (Fig. IB). Finally, we further confirmed these two findings by assessing oligonucleosomal DNA fragmentation. PC12 cells were treated with either vehicle (DMSO) or increasing concentrations of sunitinib for 12 and 24 h. As shown in Fig. IC, sunitinib markedly induced apoptosis of PC12 cells in a dose- and time-dependent manner.

**Effect of sunitinib on phosphorylation of akt at Ser473 in PC12 cells.** Receptor tyrosine kinases (RTKs) transduce proliferation, migration, and survival signals from the extracellular environment through a series of downstream signal transduction pathways. Akt is one of the major downstream effectors of RTK signaling (13). Thus, we examined the effect of sunitinib on the phosphorylation of Akt at Ser473 in PC12 cells. As shown Fig. 2A, sunitinib at 100 nM significantly inhibited Akt phosphorylation. Furthermore, this inhibitory effect was already evident 15 min after the onset of stimulation (Fig. 2B).

**Effect of sunitinib on antiapoptotic and proapoptotic proteins in PC12 cells.** The expression of the antiapoptotic proteins Bcl-2 and Bcl-XL was investigated in PC12 tumor cells after sunitinib treatment. As shown in Fig. 3A, sunitinib significantly reduced Bcl-2 expression, indicating that it may induce its apoptotic effect through inhibition of antiapoptotic protein expression. However, sunitinib did not affect the expression of Bcl-XL (data not shown). Furthermore, as shown in Fig. 3B, phosphorylation of the proapoptotic protein BAD at Ser136 was markedly diminished, implying that sunitinib contributes to the activation of this proapoptotic factor.

**Fig. 3.** Effects of sunitinib on antiapoptotic and proapoptotic proteins in PC12 cells. PC12 cells were treated with sunitinib at 1,000 nM for 12 h. Cell lysates were subjected to SDS-PAGE and immunoblotted with (A) anti-phosphospecific BAD (Ser136) or anti-total BAD antibody, and (B) Bcl-2 antibody. Representative data are shown; these data were reproducible in 3 independent experiments. Details are described in MATERIALS AND METHODS. Values in the histogram represent the mean ± SE of the densitometric measurements of the parameter indicated. Under basal conditions, this ratio was set to 100%. *P < 0.05 vs. basal.
Effect of sunitinib on PC12 cell proliferation and expression of cell cycle regulatory proteins. To evaluate the effects of sunitinib on PC12 tumor cell growth, cells were treated with either vehicle (DMSO) or sunitinib for 24 h. Cell proliferation was determined by performing an MTT. As shown in Fig. 4A, 1–100 nM sunitinib did not affect cell proliferation; however, 1,000 nM of sunitinib significantly reduced cell proliferation by \( \sim 30\% \). This reduction in cell proliferation was confirmed using the bromodeoxyuridine (BrdU) assay (Fig. 4B). To further investigate whether sunitinib affected the expression of cell cycle regulatory proteins, immunoblotting analyses were performed to measure the positive regulators of the cell cycle cyclin B1 and cyclin D, and the negative cell cycle regulator p27. Figure 4C shows that cyclin B was downregulated by sunitinib, whereas p27 was upregulated by sunitinib. These results are consistent with sunitinib-induced inhibition of cell proliferation in these cells.

Effect of sunitinib on mTOR and its downstream targets S6K1 and 4E-BP1 in PC12 cells. As shown Fig. 2, A and B, sunitinib-induced inhibition of Akt phosphorylation at Ser473 was an early response in PC12 cells. To examine whether inhibition of Akt phosphorylation influences its downstream targets, mTOR phosphorylation was investigated after a 24-h treatment with sunitinib. We found that mTOR phosphorylation at Ser2448 was inhibited by 1,000 nM sunitinib (Fig. 5A). Next, we examined the effect of sunitinib on downstream targets of mTORC1, such as S6K1 and 4E-BP1. As shown in Fig. 5B, 1,000 nM sunitinib significantly inhibited phosphorylation of S6K1 at Thr389, which is known to be highly correlated with S6K1 activation. In contrast, sunitinib did not significantly affect 4E-BP1 phosphorylation at Thr70 (data not shown).

Effect of sunitinib on HIF-\( \alpha \) and its transcriptional target genes in PC12 cells. Sunitinib is believed to mediate its antitumor effects by suppressing HIF-\( \alpha \) and its transcriptional targets. Furthermore, it is of note that mTOR has been demonstrated to be an upstream regulator of HIF-1\( \alpha \) expression (21, 33). Thus, we examined the effect of sunitinib on HIF-\( \alpha \) expression. Unexpectedly, we found that sunitinib had no effect on either the transcript or protein levels of HIF-1\( \alpha \) or HIF-2\( \alpha \) in PC12 cells (Fig. 5C). Consistent with the lack of an effect on HIF-1\( \alpha \) expression, the results of microarray-based profiling studies showed that sunitinib had no significant effect on several parameters, including GLUT1 and VEGF, which are known HIF-1\( \alpha \) transcriptional target genes in PC12 cells in vivo (data not shown). We also confirmed that sunitinib had no significant effect on GLUT1 and VEGF mRNA expression by using real-time PCR analysis (data not shown).

Although HIF-1\( \alpha \) is primarily involved in glucose metabolism, HIF-2\( \alpha \) is uniquely involved in tumor growth and cell cycle progression through its interaction with c-Myc (7). Similar to the expression results shown here for HIF-1\( \alpha \), microarray-based profiling studies showed that sunitinib also did not have a significant effect on several parameters, such as c-Myc (data not shown).

Effect of VEGF receptor 2 knockdown on sunitinib-induced apoptosis and related molecules in PC12 cells. It is well known that sunitinib exerts its effects via RTKs, mainly VEGF receptor 2 (VEGFR-2) (13, 26). As shown in Fig. 6A, VEGFR-2 protein was expressed in PC12 cells; this finding was consistent with that of a previous study (6). To further
confirm whether sunitinib exerts its effects through VEGFR-2, we examined the effect of VEGFR-2 knockdown on several molecules, such as Akt, mTOR, and Bcl-2. As shown in Fig. 6A, VEGFR-2 protein expression was significantly lower in cells transfected with VEGFR-2 siRNA than in the negative control siRNA. As shown in the right column of Fig. 1, C–I, knockdown of VEGFR-2 attenuated sunitinib-induced apoptosis, suggesting that VEGFR-2 could be involved in the direct cytotoxic effects of sunitinib in pheochromocytoma cells. Also, silencing of VEGFR-2 attenuated sunitinib-induced effects such as the inhibition of Akt and mTOR phosphorylation (Fig. 6B and C) and Bcl-2 protein expression (Fig. 6D) compared with that in the negative control siRNA. This showed that these sunitinib-induced effects could be mediated, at least in part, by VEGFR-2.

Effect of S6K1 knockdown on apoptosis and related molecules in PC12 cells. To further examine whether inhibition of S6K1 is sufficient to induce apoptosis, we examined the effect of S6K1 knockdown on apoptosis and apoptosis-related molecules, such as BAD and Bcl-2. Similar to what was observed with sunitinib treatment, silencing of S6K1 induced both apoptosis (Fig. 1, C–I, right) and defects in BAD phosphorylation and Bcl-2 (Fig. 7). Therefore, it is likely that S6K1 inhibition induced by sunitinib is sufficient to induce apoptosis in PC12 cells.

Effect of sunitinib in human neuroblastoma SK-N-SH cells. It is known that PC12 cells do not accurately reflect the pathogenesis of malignant cells. Also, recent findings imply that the two neural crest-derived tumors pheochromocytomas and neuroblastomas have a common pathogenesis, including genetic background, such as mutation of KIF1Bbeta and SDHB (44, 45). Therefore, we confirmed the key findings of this study, such as apoptosis and proliferation, in a second cell line, human neuroblastoma SK-N-SH cells. Similar to what was observed in PC12 cells (Fig. 8), in SK-N-SH cells treated

Fig. 5. Effect of sunitinib on mammalian target of rapamycin (mTOR) and its downstream target S6K1 in PC12 cells. PC12 cells were treated with sunitinib at 1,000 nM for 12 h. Cell lysates were subjected to SDS-PAGE and immunoblotted with (A) anti-phospho-specific mTOR (Ser2448) or anti-total mTOR antibody, (B) anti-phospho-specific S6K1 (Thr389) or anti-total S6K1 antibody, and (C) anti-hypoxia-inducible factor (HIF)-1α or anti-HIF-2α antibody. Representative data are shown; these data were reproducible in 3 independent experiments. Details are described in MATERIALS AND METHODS. Values shown in the histogram represent the mean ± SE of densitometric measurements of the parameter indicated. Under basal conditions, the ratio was set to 100%. *P < 0.05 vs. basal value.
Fig. 6. Effect of VEGFR-2 knockdown in PC12 cells. A: effect of VEGFR-2 knockdown on its protein expression in PC12 cells. Cell lysates from VEGFR-2 siRNA (siVEGFR-2)-transfected cells or control siRNA (siCON)-transfected cells were subjected to SDS-PAGE and immunoblotted with a VEGFR-2-specific antibody. Effect of sunitinib (at 1,000 nM for 24 h) on either siVEGFR-2-transfected or siCON-transfected cells. Cell lysates were subjected to SDS-PAGE and immunoblotted (B) with phospho-Akt antibody, (C) phospho-mTOR antibody, or (D) Bcl-2-specific antibody. Representative data are shown; these data were reproducible in 3 independent experiments. Details are described in MATERIALS AND METHODS. Values shown in the histogram are the mean ± SE of the densitometric measurement of parameter indicated. Under basal conditions, this ratio was set to 100%. *P < 0.05 vs. basal value. (Note: effect of VEGFR-2 knockdown on sunitinib-induced apoptosis in PC12 cells is depicted in the right column of Fig. 1, C–I.)
with 1,000 nM sunitinib for 24 h, induction of apoptosis and inhibition of proliferation was observed. In addition, concomitant inactivation of phospho-Akt (Ser473) and phospho-mTOR (Ser2448) was also seen.

**DISCUSSION**

In the present study, we have demonstrated for the first time that sunitinib has several antitumor effects in pheochromocytoma PC12 cells, which indicates that sunitinib may exert its antitumor effects, at least in part, directly through tumor cells rather than through angiogenesis.

Initially, using several different methods, we demonstrated that sunitinib markedly induced apoptosis and inhibited proliferation in PC12 cells, (Figs. 1 and 4). Although inhibition of proliferation was observed at relatively high levels, the apoptotic effect was seen at clinically relevant concentrations (125–250 nM) (13, 34). Thus, it is possible that the apoptotic effect induced by sunitinib in tumor cells may also occur during treatment of pheochromocytoma.

Our observation of this apoptotic effect was consistent with the finding that sunitinib inhibited phosphorylation of Akt (Ser473) as well as mTOR (Ser2448) and S6K1 (Thr389). Growing evidence suggests that S6K1 inactivates BAD through phosphorylation at Ser136, ultimately leading to its interaction with 14-3-3 proteins and its degradation (18, 37). In contrast, the precise relationship between S6K1 and antiapoptotic members of the Bcl family, including Bcl-2, remains unclear. Recently, it was reported that a mouse model with both S6K1 and S6K2 knocked out (S6K1;S6K2−/−/−) showed a defect in BAD phosphorylation and expression of the antiapoptotic factor Bcl-2 (37), which is consistent with our present results. Indeed, these authors claimed that S6K might regulate the expression of Bcl-2, possibly acting through multiple pathways, leading to the equilibrium between intracellular proapoptotic and antiapoptotic factors (37).

Consistent with this hypothesis, we demonstrated that silencing of S6K1 induced apoptosis (Fig. 1, C–I, right) as well as defects in the phosphorylation of BAD and the antiapoptotic factor Bcl-2 in PC12 cells (Fig. 7, B and C), similar to the sunitinib-induced effects. Therefore, it is likely that inhibition of S6K1 alone is sufficient to induce apoptosis. However, whether select S6K1-regulated Bcl-2 family members might be
sufficient to induce apoptosis remains unknown. Hence, the possibility that another S6K1 regulating mechanism may trigger mitochondrial outer membrane permeabilization cannot be excluded. Indeed, a recent study in cardiac myocytes described an alternative pathway by which S6K1 affects mitochondrial ROS release, which acts on the permeability of the transition pore complex through inhibition of glycogen synthase kinase-3β (29).

Very recently, it was reported that S6K1 plays a pivotal role in regulating proliferation of the adrenal medulla in mouse. It was also shown that the human adrenal gland primarily expresses S6K1. Indeed, high levels of S6K1 are reported to be associated with human pheochromocytomas (37). Combined with our findings, it is reasonable to speculate that S6K1 may play a key role in signaling downstream of mTOR in the adrenal gland. In addition, it also suggests that targeting of S6K1 kinase may represent a potential avenue for treatment of pheochromocytomas.

Why sunitinib did not significantly affect the phosphorylation of 4E-BP1 at Thr70 also remains unknown (data not shown). Nevertheless, the most plausible explanation for this phenomenon may be the difference in the roles of 4E-BP1 in each specific cell. Recently, a difference in the regulatory mechanism between S6K1 and 4E-BP1 has been shown. Indeed, 4E-BP1 is subject to a more complicated regulatory mechanism than S6K1. This point should also be taken into account when considering the mechanism described above (25, 47, 48).

Another interesting point in the present study is that sunitinib did not affect expression of either HIF-1α or HIF-2α (Fig. 5C). Despite some overlapping effects, it has been reported that HIF-1α and HIF-2α regulate distinct genes (7, 32). For example, HIF-1α is primarily involved in glucose metabolism, including upregulation of glycolytic enzymes and downregulation of electron transport chain activity (7). In contrast, HIF-2α is uniquely involved in tumor growth and cell cycle progression through interaction with c-Myc (7). We confirmed that sunitinib had no significant effect on these target genes by using microarray-based profiling studies and real-time PCR analysis (data not shown). Therefore, under our experimental conditions, the growth inhibitory effects of sunitinib did not appear to be mediated through the suppression of HIF-1α or HIF-2α and their target genes.

Our findings are supported by the fact that sunitinib also downregulated the phosphorylated forms of mTOR as well as its effectors, namely, S6K1 and 4E-BP1, in leukemia cells, indicating that mTOR may be a target of RTK inhibitors (22). Taken together, our results indicate that sunitinib inhibits the PI3K/Akt/mTOR/S6K1 pathway but not HIF in pheochromocytoma cells.
It is essential to determine the specific kinase that sunitinib inhibits and that is responsible for the antitumor effects of sunitinib in PC12 cells. In the present study, we demonstrated that knockdown of VEGFR-2 attenuated sunitinib-induced apoptosis and inhibition of signaling pathways, such as Akt, mTOR phosphorylation and Bcl-2, confirming that these sunitinib-induced effects could be mediated by VEGFR-2 (right column of Figs. 1, C–I, right, and 6, B–D). Thus, sunitinib possibly exerts its apoptotic effect initially through the inhibition of VEGFR-2, which, when followed by its downstream effectors such as Akt/mTOR/S6K1, may lead to inhibition of the antiapoptotic molecule Bcl-2 and activation of proapoptotic molecule BAD in PC12 cells.

This suggests that multitargeted approaches, including sunitinib, are likely to have more antitumor activities than approaches targeting a single pathway (2, 13). Although silencing of VEGFR-2 alone markedly attenuated sunitinib-induced apoptosis and related pathways, this result cannot rule out the possibility that other RTKs could have important roles in Akt/mTOR/S6K1 signaling. Further studies (i.e., evaluating the expression of multiple RTKs) will be needed to clarify this.

Growing evidence suggests that the PI3K/Akt/mTOR/S6K1 pathway may play an important role in the pathogenesis of neuroendocrine tumors (16, 30, 35, 39, 41). Those findings, together with those of our present study, indicate that sunitinib exerts its antitumor effect, at least in part, through inhibition of the PI3K/Akt/mTOR/S6K1 pathway in the treatment of human malignant pheochromocytoma.

It is known that PC12 cells, although well established and extensively used, do not accurately reflect the pathogenesis of malignant pheochromocytomas. Also, similar to pheochromocytomas, neuroblastosomas are neural crest-derived tumors and reported to have a common genetic background, such as mutation of KIF1Bbeta and SDHB (44, 45). Despite SK-N-SH cells being a human derived neuroblastoma cell line, we confirmed that sunitinib exerted the same effects, with regard to apoptosis, proliferation, and Akt and mTOR, in these cells (Fig. 8).

Further development of these studies may make it possible to predict the response to and to enhance the safety of these molecular target therapies in the future.

GRANTS

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, (no. 21591168) (K. Takekoshi).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


44. Thoreen CC, Sabatini DM. Rapamycin inhibits mTORC1, but not completely. _Autophagy_ 5: 725–726, 2009.