

Lysosomal Sequestration of Sunitinib: A Novel Mechanism of Drug Resistance

Kristy J. Gotink¹, Henk J. Broxterman¹, Mariette Labots¹, Richard R. de Haas¹, Henk Dekker¹, Richard J. Honeywell¹, Michelle A. Rudek⁵, Laurens V. Beerepoot⁴, René J. Musters², Gerrit Jansen³, Arjan W. Griffioen¹, Yehuda G. Assaraf^{1,6}, Roberto Pili⁷, Godefridus J. Peters¹, and Henk M.W. Verheul¹

Abstract

Purpose: Resistance to antiangiogenic tyrosine kinase inhibitors such as sunitinib is an important clinical problem, but its underlying mechanisms are largely unknown. We analyzed tumor sunitinib levels in mice and patients and studied sensitivity and resistance mechanisms to sunitinib.

Experimental Design: Intratumoral and plasma sunitinib concentrations in mice and patients were determined. Sunitinib exposure on tumor cell proliferation was examined. Resistant tumor cells were derived by continuous exposure and studied for alterations in intracellular sunitinib accumulation and activity.

Results: Intratumoral concentrations of sunitinib in mice and patients were 10.9 ± 0.5 and 9.5 ± 2.4 $\mu\text{mol/L}$, respectively, whereas plasma concentrations were 10-fold lower, 1.0 ± 0.1 and 0.3 ± 0.1 $\mu\text{mol/L}$, respectively. Sunitinib inhibited tumor cell growth at clinically relevant concentrations *in vitro*, with IC_{50} values of 1.4 to 2.3 $\mu\text{mol/L}$. Continuous exposure to sunitinib resulted in resistance of 786-O renal and HT-29 colon cancer cells. Fluorescent microscopy revealed intracellular sunitinib distribution to acidic lysosomes, which were significantly higher expressed in resistant cells. A 1.7- to 2.5-fold higher sunitinib concentration in resistant cells was measured because of increased lysosomal sequestration. Despite the higher intracellular sunitinib accumulation, levels of the key signaling p-Akt and p-ERK 1/2 were unaffected and comparable with untreated parental cells, indicating reduced effectiveness of sunitinib.

Conclusion: We report that sunitinib inhibits tumor cell proliferation at clinically relevant concentrations and found lysosomal sequestration to be a novel mechanism of sunitinib resistance. This finding warrants clinical evaluation whether targeting lysosomal function will overcome sunitinib resistance. *Clin Cancer Res*; 17(23): 7337–46. ©2011 AACR.

Introduction

Resistance to antiangiogenic tyrosine kinase inhibitors (TKI) is a major clinical problem and mechanistic insight into the possible underlying mechanisms of resistance is limited (1). Sunitinib is an antiangiogenic TKI which prolongs progression-free and overall survival of patients with advanced renal cell carcinoma (RCC; refs. 2, 3) and of

patients with gastrointestinal stroma cell tumors refractory to imatinib treatment (4). Recently, sunitinib has shown activity in patients with advanced pancreatic neuroendocrine tumors as well (5). Several distinct resistance mechanisms to sunitinib and other antiangiogenic TKIs have been proposed on the basis of preclinical studies including induction of alternative growth factor signaling as well as epithelial to mesenchymal transformation (EMT; 6–9), but these cannot fully explain the clinical observations of resistance. Acquired mutations in target kinases play an important role in TKI resistance such as to imatinib in chronic myeloid leukemia and to B-RAF inhibitors in melanoma, but altered pharmacokinetics and pharmacodynamics should be evaluated as potential resistance mechanisms as well (10). While sunitinib has been developed as an antiangiogenic agent, intended to primarily target endothelial and perivascular cells through its high-affinity binding to VEGF receptor (VEGFR) 2 and platelet-derived growth factor receptor (PDGFR), it inhibits many other kinases (22). In fact, Karaman and colleagues (23) studied the affinity of TKIs to a panel of 317 kinases and found that sunitinib has a low selectivity for specific tyrosine kinases.

Authors' Affiliations: Departments of ¹Medical Oncology, ²Physiology, and ³Rheumatology, VU University Medical Center, Amsterdam; ⁴Department of Internal Medicine, St. Elisabeth Hospital, Tilburg, the Netherlands; ⁵The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, Maryland; ⁶The Fred Wyszowski Cancer Research Laboratory, Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel; and ⁷Genitourinary Program, Roswell Park Cancer Institute, Buffalo, New York

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Author: Henk M.W. Verheul, Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, Rm 3A46, Amsterdam 1081 HV, the Netherlands. Phone: 31-20-444-4321; Fax: 31-20-444-4079; E-mail: h.verheul@vumc.nl

doi: 10.1158/1078-0432.CCR-11-1667

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Translational Relevance

In this study, we obtained more insight in the clinical antitumor activity of and resistance to sunitinib. On the basis of intratumoral concentrations and *in vitro* experiments, we envision that clinical resistance to sunitinib is transient and rechallenging with sunitinib after an alternative treatment regimen may be beneficial for patients. In addition, on the basis of our results, we hypothesize that treatment with clinical available drugs interfering with lysosomal function, such as chloroquine, may potentiate the antitumor activity of sunitinib. Whether this mechanism of resistance mediated by acidic lysosomes will also play a role in other (antiangiogenic) tyrosine kinase inhibitors warrants further examination.

On the basis of this potential broader profile of kinase inhibition, we investigated whether an alternative mechanism of action may play a role in the antitumor activity of sunitinib rather than solely its antiangiogenic activity. We determined intratumoral concentrations of sunitinib and studied these clinically relevant concentrations on tumor cells and endothelial cells *in vitro*. In addition, we studied whether continuous sunitinib exposure would induce tumor cell resistance. We found that sunitinib resistance of tumor cells is mediated by lysosomal sequestration. This sunitinib resistance mechanism is transient as shown by recovery after drug-free culture and can be modulated by interference with lysosomal function. Therefore, this new resistance mechanism warrants further clinical translation.

Materials and Methods

Cell culture

The tumor cell lines 786-O, HT-29, DLD-1, HCT116, and MCF-7 were cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% FBS. The 786-O and HT-29 cell lines were authenticated by the American Tissue Culture Collection. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords. Endothelial colony-forming cells (ECFC) were isolated from the mononuclear cell fraction that was obtained from cord blood. To induce resistance, 786-O and HT-29 cancer cell lines were continuously exposed for more than 12 months to gradually increasing concentrations of sunitinib up to 6 (786-O) or 12 $\mu\text{mol/L}$ (HT-29). Cells were maintained in a humidified incubator containing 5% CO_2 at 37°C. Sunitinib was provided by Pfizer Global Pharmaceuticals and was prepared as 20 mmol/L stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at -20°C .

Sunitinib measurements in murine samples, human samples, and *in vitro*

BALB/c female mice were injected with 5×10^5 Renca RCC tumor cells and treated with sunitinib (40 mg/kg/d)

and sacrificed after 1 month. Experiments were approved by the Institutional Care and Use Committee at the Johns Hopkins Medical Institutions and in accordance with the NIH Guide for the care and use of laboratory animals.

Three patients diagnosed with advanced malignancies [malignant solitary fibrous tumor, colorectal cancer (CRC), and leiomyosarcoma] were treated with sunitinib in context of a clinical trial at a dose of 37.5 to 50 mg/d for at least 4 weeks. Tumors of these patients were biopsied and in parallel plasma samples were collected. The use of these samples was in accordance with the local ethical guidelines at VU University Medical Center. For the determination of sunitinib in tissue (tumor or skin), an amount of about 10 mg of snap frozen tissue was cut, put into a vial and weighed. A total of 200 μL of water was added, the sample was snap-frozen and freeze-dried overnight. For extraction, 200 μL of ice-cold 83% acetonitrile (ACN) was added and left on ice for 1 hour. After centrifugation, 50 μL of supernatant was transferred for subsequent liquid chromatography—tandem mass spectrometry (LC/MS-MS) analysis as reported previously for plasma and cell pellet homogenates (13). The data are expressed in micromole to allow comparison of tissue concentrations with the IC_{50} values in cell culture and are based on the conversion of 1 g tissue to 1 mL liquid (14).

Proliferation and clonogenic assays

For proliferation and clonogenic assays, cells were seeded and allowed to attach for 24 hours. After these 24 hours, sunitinib was added at different concentrations. For proliferation assays, a $t = 0$ measurement was carried out at the same time. Cell proliferation was studied 96 hours after sunitinib treatment. $T = 0$ and $t = 96$ hours measurements are carried out using MTT or by cell counts. Cell proliferation was calculated using the following formula:

$$\% \text{ of proliferation} = [(96 \text{ hours measurement of treated cells} - 0 \text{ hours measurement}) / (96 \text{ hours measurement of untreated cells} - 0 \text{ hours measurement})] \times 100\%$$

Subtracting the measurement at the beginning of treatment ($t = 0$ measurement) might result in negative value, representing cell killing.

For clonogenic assays (15), medium was refreshed after 72 hours of sunitinib treatment. After 10 days in drug-free medium, colonies were fixed, stained with 10% Giemsa and counted. Proliferation and clonogenic assays were carried out in triplicate and repeated a minimum of 3 times independently. IC_{50} values of the parental and resistant cell lines were estimated in parallel in 4 independent experiments, by direct reading from the proliferation curve. Results were normalized to DMSO controls.

Western blot analysis

Cells were treated as indicated. The cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce) supplemented with protease and phosphatase inhibitor cocktails (Pierce). Protein concentrations were determined by Micro BCA protein assay (Pierce). Samples containing 50 μg protein underwent electrophoresis on 8% to 12% SDS

polyacrylamide gels and were subsequently transferred to PVDF membranes. Proteins were detected using the following antibodies (with catalogue numbers in parentheses): Akt (9272), phospho-Akt (on Ser473; 9271), ERK 1/2 (9102), phospho-ERK 1/2 (on Thr202 and Tyr204; 9101; Cell Signaling Technology), LAMP-1 (sc-20011), LAMP-2 (sc-18822; Santa Cruz Biotechnology), β -actin (A5441; Sigma-Aldrich). After incubation with IRDye (infrared dye)-labeled secondary antibodies (LI-COR Biosciences), membranes were scanned and analyzed with the Odyssey Infrared Imaging System and accompanying software program (LI-COR Biosciences; ref. 16).

Subcellular colocalization studies

Cells were incubated with sunitinib, LysoTracker Red DND-99 (Invitrogen) or Mitotracker Red FM (Invitrogen), Hoechst 33342 (Invitrogen) and bafilomycin A1 (LC laboratories) or ammonium chloride (NH_4Cl ; Sigma-Aldrich) as indicated. Viable cells were imaged in real time with a Zeiss Axiovert 200 Marianas inverted microscope (ZEISS) equipped with a motorized stage (stepper-motor z-axis increments, 0.1 μm), multiple fluorescence (FITC filter for sunitinib, Cy3 filter for LysoTracker or Mitotracker and DAPI filter for Hoechst nuclear stain), and a Cooke Sensicam cooled charge-coupled device camera (Cooke; 1,280 by 1,024 pixels) with true 16-bit capability at 63 \times oil immersion objective. The acquisition protocols included three-dimensional optical sections in real time. Image acquisition and analysis was carried out under full software control (SlideBook 5.0.0.18; Intelligent Imaging Innovations). Three-dimensional optical sections were deconvoluted using the same software. Representative images from more than 3 independent experiments are shown.

Statistical analysis

Data are expressed as means \pm SEM. When appropriate, results are shown as normalized data (percentage of DMSO controls). Statistical analyses were carried out using Student *t* test. A *P* value less than 0.05 was considered to be statistically significant. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Results

Intratumoral sunitinib concentrations are significantly higher than plasma concentrations

After 4 weeks of sunitinib treatment at a dose of 40 mg/kg/d, intratumoral sunitinib concentrations in the murine Renca RCC model were 10-fold higher than the corresponding steady-state plasma concentrations [mean \pm SEM (range): 10.9 \pm 0.5 (9.95–11.8) $\mu\text{mol/L}$ vs. 1.0 \pm 0.1 (0.84–1.2) $\mu\text{mol/L}$ sunitinib; *n* = 3, respectively; *P* < 0.001; Fig. 1A]. The intratumoral sunitinib concentrations in micromoles correspond to, in micrograms sunitinib per gram tissue: 4.33 \pm 0.21 (3.96–4.69) $\mu\text{g/g}$. In normal skin tissue of these mice, sunitinib concentrations were comparable with intratumoral concentrations [mean (range): 7.4 (6.6–8.3) $\mu\text{mol/L}$, or in $\mu\text{g/g}$: 3.0 (2.6–3.3); *n* = 2). Subsequently, tumor biopsies from 3 patients undergoing

sunitinib treatment were obtained. In line with the murine data, intratumoral concentrations in patients were 30-fold higher than plasma concentrations. Intratumoral concentrations of sunitinib in patients were 9.5 \pm 2.4 (5.1–13.4) $\mu\text{mol/L}$, whereas their plasma concentrations were 0.3 \pm 0.1 [(0.22–0.34) $\mu\text{mol/L}$; *n* = 3; *P* < 0.05; Fig. 1A]. The intratumoral sunitinib concentrations in $\mu\text{mol/L}$ correspond to, in micrograms sunitinib per gram tissue: 3.79 \pm 1.67 (2.02–5.32) $\mu\text{g/g}$.

Sunitinib directly inhibits tumor cells at intratumoral concentrations

In a panel of RCC, CRC, and breast cancer cell lines, sunitinib inhibited the proliferation at clinically achievable intratumoral drug concentrations, with inhibitory concentrations of 50% (IC_{50}) ranging between 1.4 and 2.3 $\mu\text{mol/L}$ (Fig. 1B). These results were confirmed in a clonogenic assay in a subset of cancer cell lines (Fig. 1C). In addition, sunitinib inhibited the proliferation of HUVECs and ECFCs grown in complete medium at similar concentrations (Fig. 1D). Western blot analysis of tumor cell lysates revealed that sunitinib reduced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and Akt, which are 2 downstream signaling proteins of sunitinib targets (17, 18; Fig. 1E). No inhibition of Akt phosphorylation was observed in 786-O cells in accordance with a mutated PTEN status in this cell line causing constitutive activation of the Akt pathway (19).

Prolonged sunitinib exposure results in tumor cell resistance *in vitro*

786-O and HT-29 cells were cultured for more than 12 months at gradually increasing sunitinib concentrations. This prolonged drug exposure resulted in tumor cell resistance, which is reflected by a continuation of growth at sunitinib concentrations above 6 $\mu\text{mol/L}$, whereas at these concentrations, cell death is induced in the parental 786-O and HT-29 cells (Fig. 2A). IC_{50} values were increased in the sunitinib-resistant cells compared with the parental cells from 1.2 to 3.1 $\mu\text{mol/L}$ in 786-O cells (*P* < 0.001) and from 1.9 to 3.5 $\mu\text{mol/L}$ in HT-29 cells (*P* < 0.05; Fig. 2B). In addition, resistant cells had a reduced growth rate (Fig. 2C) and a reduced clonogenic capacity (HT-29; Fig. 2D). Morphologic examination by light microscopy and immunofluorescent staining with β -actin and Hoechst revealed that a subpopulation of the resistant cells was more flattened, had enlarged nuclei, and/or were multinuclear compared with their parental cells (Fig. 2E). No signs of increased senescence characteristics were found, as mean cell volume and expression of pH-dependent β -galactosidase activity of the resistant cell population was comparable with parental cells (Supplementary Fig. S1A and S1B).

Sunitinib resistance is transient

To determine whether *in vitro* resistance is transient or irreversible, sunitinib-resistant 786-O and HT-29 cells were cultured in drug-free medium for a period of up to 12 weeks. Sunitinib sensitivity was gradually restored and these

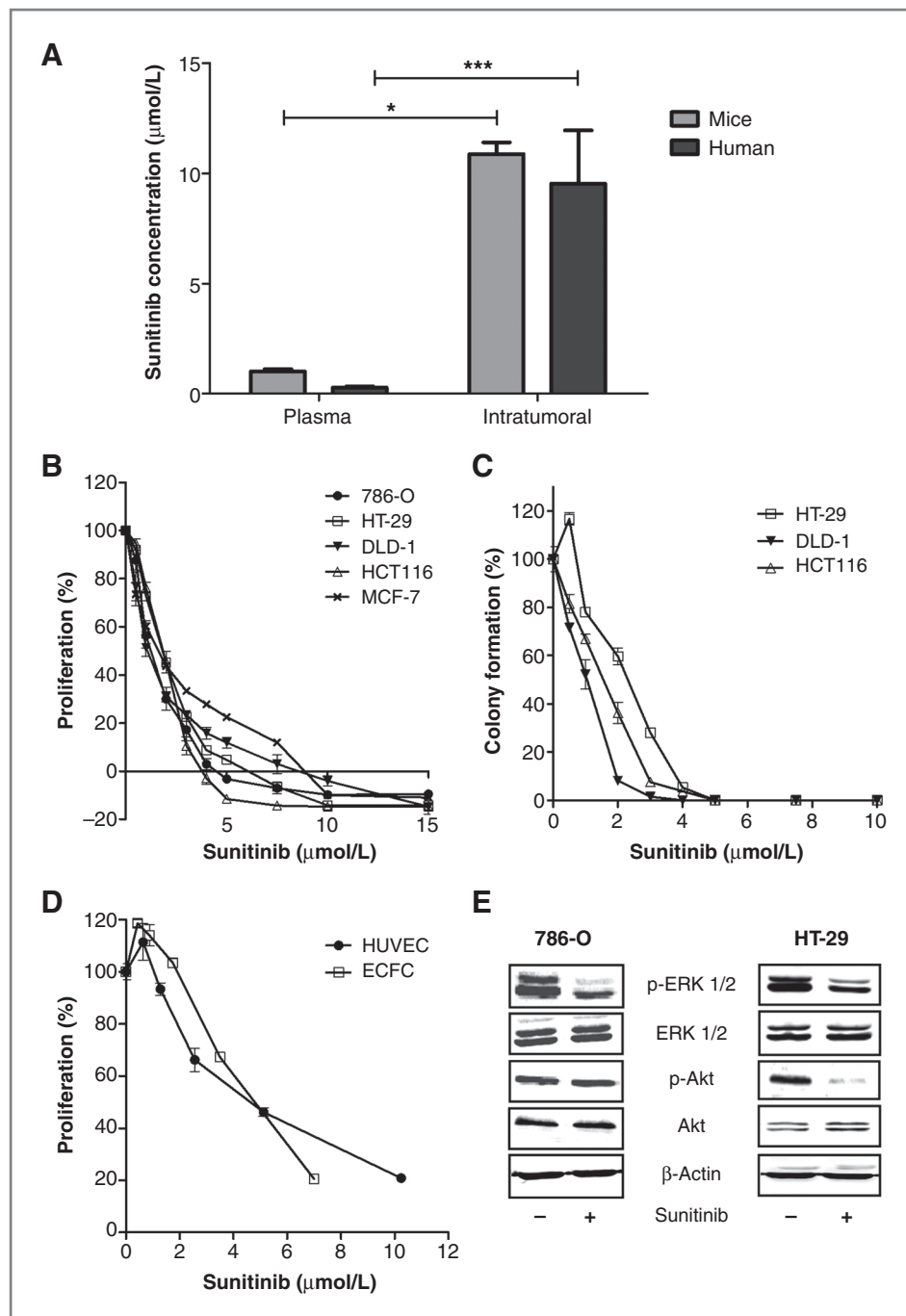


Figure 1. High intratumoral concentrations of sunitinib inhibit tumor cells directly. **A**, intratumoral and plasma concentrations of sunitinib in mice with RCC tumors ($n = 3$) and patients with advanced malignancies ($n = 3$), treated with sunitinib at a dose of 40 mg/kg/d for 1 month (mice) or 37.5 to 50 mg/d for at least 4 weeks (patients). **B**, proliferation (MTT assay) of tumor cell lines incubated with increasing sunitinib concentrations. Sunitinib inhibited proliferation of tumor cells at clinically relevant intratumoral concentrations. **C**, sunitinib inhibition of colony formation of tumor cell lines. **D**, proliferation of endothelial cells incubated with increasing concentrations of sunitinib while grown in complete medium. HUVECs were cultured in M199 medium supplemented with 10% FBS and 10% human serum, whereas ECFCs were cultured in endothelial basal medium (EBM-2) supplemented with 10% FBS, VEGF, epidermal growth factor (EGF), insulin-like growth factor (IGF), and FGF. **E**, Western blot analysis of (phosphorylated) ERK 1/2 and Akt in 786-O and HT-29 cell lines treated with 2 $\mu\text{mol/L}$ sunitinib for 1 hour, compared with DMSO controls. Results are shown as means \pm SEM. *, $P < 0.05$; ***, $P < 0.001$.

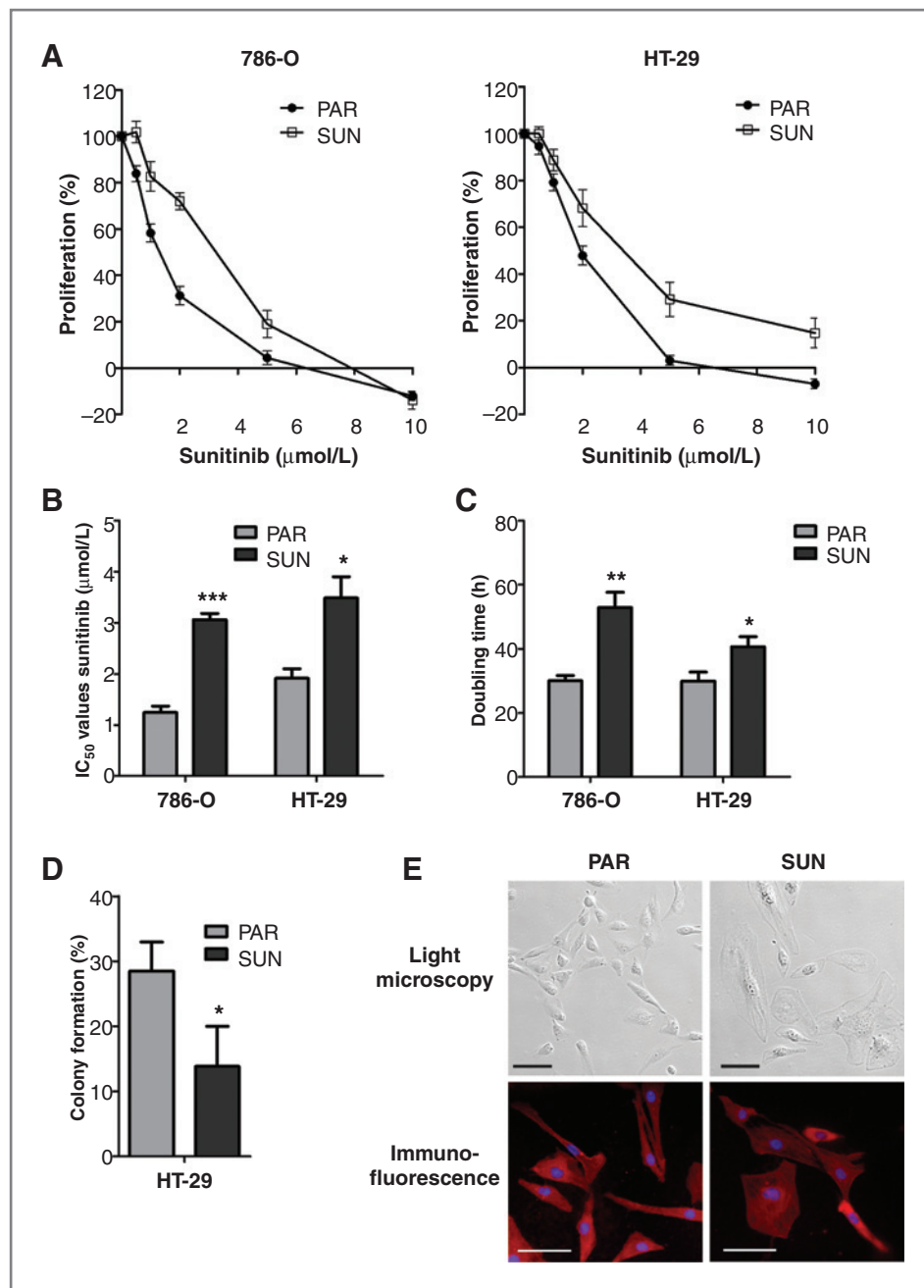
revertant 786-O and HT-29 cells reached the original drug sensitivity after 12 weeks (Fig. 3A). Concomitantly, their growth rate and cell morphology restored to that of their parental cells (Fig. 3B).

Sunitinib is sequestered in lysosomes

A classical mechanism of drug resistance includes increased drug efflux, resulting in decreased intracellular drug concentration in the resistant cells (10). To investigate whether a decreased intracellular drug concentration was

involved in the sunitinib-resistant tumor cells, we analyzed the intracellular sunitinib accumulation. In contrast to expectations from classical drug resistance studies, pharmacologic analysis revealed that the intracellular sunitinib concentrations were significantly higher in the resistant tumor cells than in the parental cells incubated with 5 $\mu\text{mol/L}$ sunitinib for 24 hours. Intracellular concentrations in the resistant 786-O and HT-29 cells were 5.04 ± 0.11 and 4.25 ± 0.86 mmol/L sunitinib (mean \pm SEM), respectively (Fig. 4A). These concentrations in the

Figure 2. Acquired resistance to sunitinib in tumor cells. To induce resistance, 786-O and HT-29 cancer cell lines were continuously exposed for more than 12 months to gradually increasing concentrations of sunitinib up to 6 (786-O) or 12 $\mu\text{mol/L}$ (HT-29). A, proliferation assay showing reduced drug sensitivity of sunitinib-resistant (SUN) cancer cells compared with their parental (PAR) cells. B, IC_{50} values of sunitinib were increased in the resistant 786-O and HT-29 cells, compared with their parental cells. C, sunitinib-resistant 786-O and HT-29 cells had a reduced cell growth (higher doubling time), compared with their parental cells. D, sunitinib-resistant HT-29 cells had a reduced clonogenic capacity, compared with parental cells. E, morphologic changes in sunitinib-resistant 786-O cells. For immunofluorescent staining, cells were incubated with β -actin antibody, followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody and Hoechst. Scale bars are 100 μm . Results are shown as means \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



resistant cells are 1.7-fold ($P < 0.05$) and 2.5-fold ($P < 0.05$) higher than the parental 786-O and HT-29 cells, in which intracellular concentrations of 2.93 ± 0.42 and 1.68 ± 0.29 mmol/L sunitinib were measured, respectively (Fig. 4A). These intracellular concentrations in the resistant cells are up to 1,000-fold the sunitinib concentration in the conditioned medium. The intracellular concentration of sunitinib calculated as the amount of drug per gram of cellular protein are 23.8 ± 0.4 and 13.0 ± 2.6 $\text{ng sunitinib}/\mu\text{g protein}$ in the resistant 786-O and HT-29 cell, respectively, and to 12.2 ± 1.3 and 5.9 ± 0.8

$\text{ng sunitinib}/\mu\text{g protein}$ in the parental 786-O and HT-29 cell, respectively.

To further define the mechanism of sunitinib resistance in the tumor cells, we analyzed the subcellular distribution of sunitinib, to investigate whether sunitinib accumulates in specific subcellular compartments. Taking advantage of the autofluorescent properties of sunitinib, fluorescence microscopy revealed that sunitinib is specifically sequestered in subcellular compartments. This sequestration was significantly higher in resistant cells than in the parental cells. Consistent with the physicochemical properties of

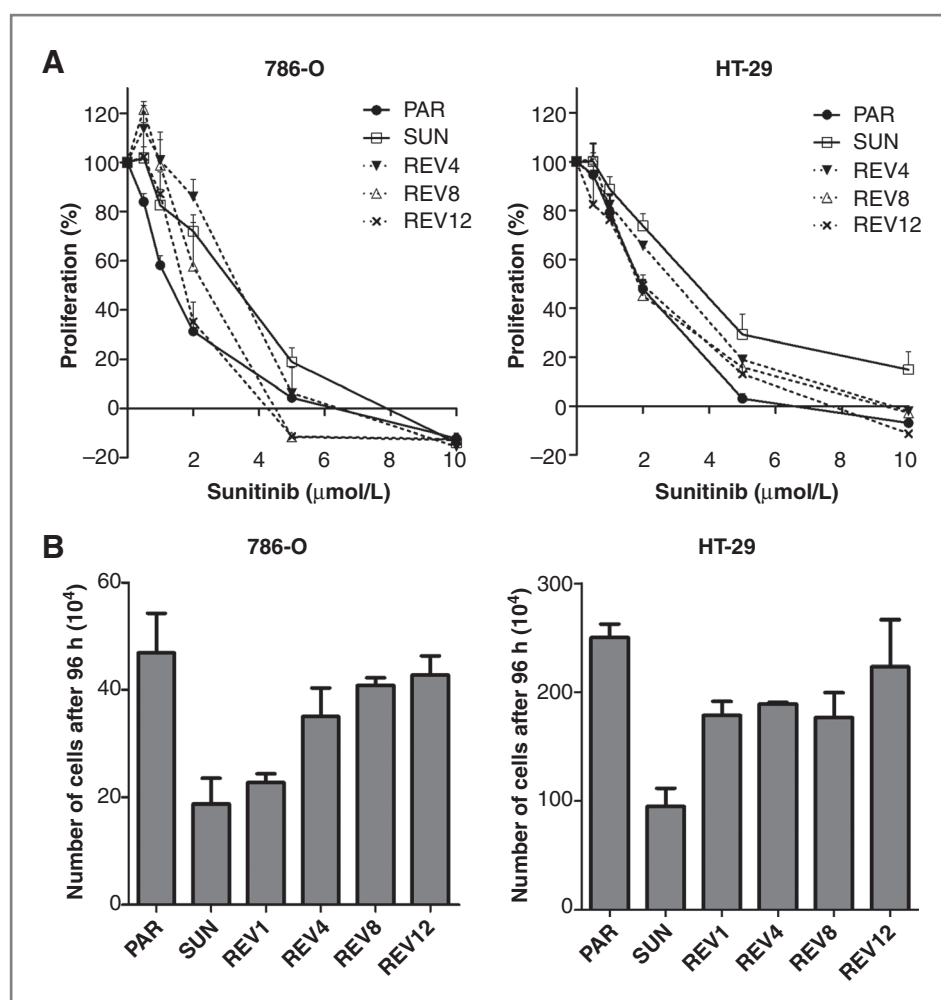


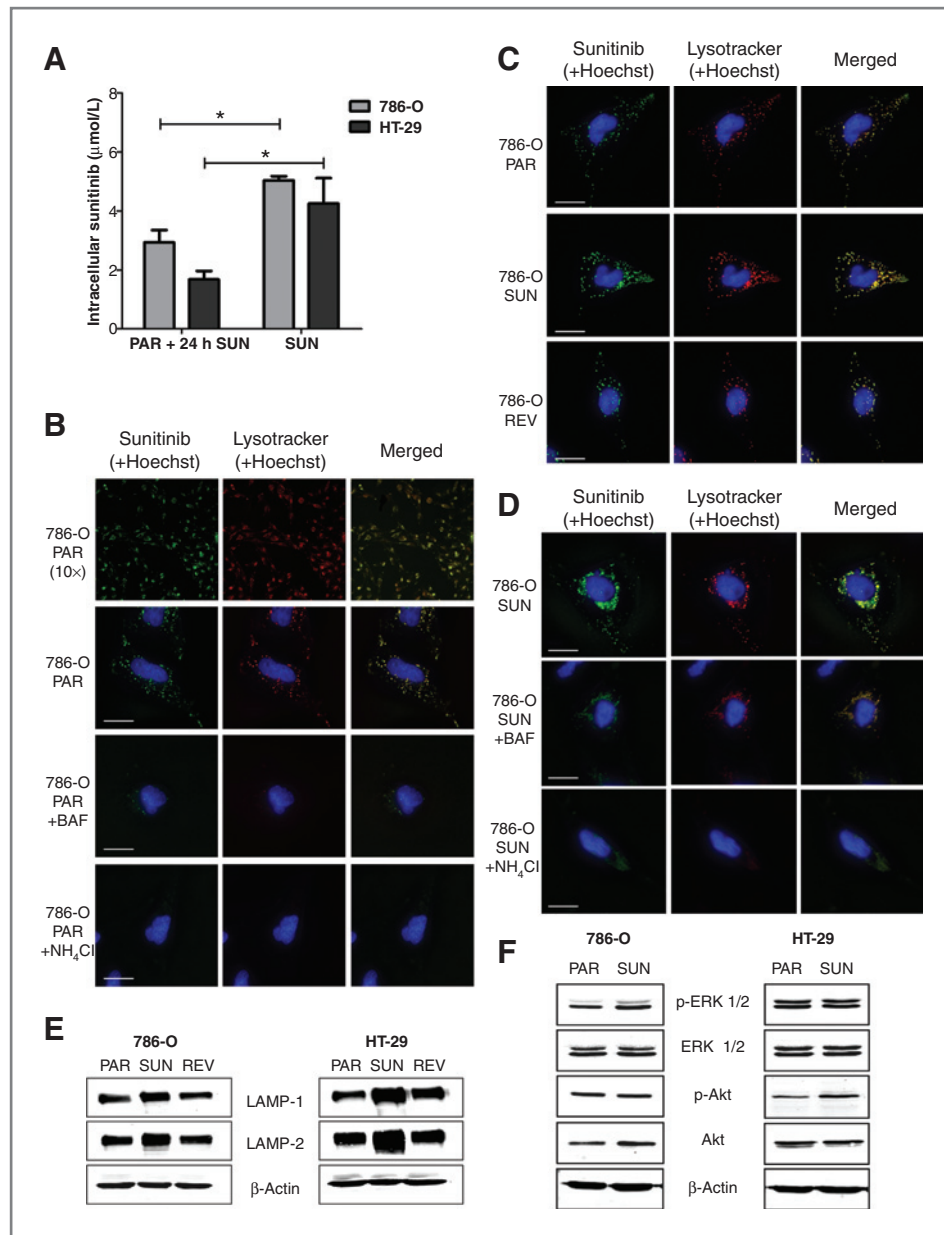
Figure 3. Transient resistance to sunitinib *in vitro*. To recover cells from continuous sunitinib exposure, 786-O- and HT-29-resistant cells were cultured in sunitinib-free medium for a period of up to 12 weeks. A, sensitivity of revertant cells after 4, 8, and 12 weeks of drug-free culture conditions. Cells recovering from continuous sunitinib exposure gradually regained sensitivity to the level of parental cells. B, growth rate of revertant cells after 1, 4, 8, and 12 weeks of drug-free culture conditions. PAR, parental cells; SUN, sunitinib-resistant cells; REV, revertant cells after 1 week (REV1); 4 weeks (REV4), 8 weeks (REV8), and 12 weeks (REV12) in drug-free medium. Results are shown as means \pm SEM.

sunitinib, which is a hydrophobic ($\log P = 5.2$) weak base (pK_a , acid dissociation constant, = 8.95), it appeared that sunitinib predominantly colocalized with lysosomal staining upon treatment of 786-O and HT-29 cells in combination with a viable lysosomal-specific probe (Lysotracker Red DND-99; 786-O: Fig. 4B, first and second rows in second column; HT-29: data not shown). Lysosomal accumulation of sunitinib was also present in HUVECs (Supplementary Fig. S2). We studied whether the subcellular localization of sunitinib can be disturbed by interfering with the function of acidic lysosomes. 786-O cells were coincubated with bafilomycin A1, a specific inhibitor of vacuolar type H^+ -ATPase (V-ATPase), which abolishes acidification of lysosomes (20), and ammonium chloride, a lysosomotropic weak base, which rapidly increases lysosomal pH (21). These incubations resulted in a significantly reduced sequestration of sunitinib within lysosomes (Fig. 4B, third and fourth rows in second column), confirming acidic lysosomal localization of sunitinib. Alternative sequestration in mitochondria was ruled out by the absence of colocalization of sunitinib with Mitotracker Red FM staining (Supplementary Fig. S3).

Lysosomal sequestration is involved in sunitinib resistance

Comparison of parental and sunitinib-resistant 786-O and HT-29 cells indicated that the lysosomal capacity of resistant cells was substantially increased (Fig. 4C, middle). In addition, Fig. 4D shows that incubation of resistant 786-O cells with bafilomycin or ammonium chloride largely decreased the lysosomal fluorescence. Western blot analysis indeed showed increased expression of the major lysosome-associated membrane proteins (LAMP) 1 and LAMP-2 (22) in the resistant 786-O and HT-29 cells compared with their parental cells (Fig. 4E). Supportive for the increased sequestration in lysosomes of sunitinib as a mechanism of resistance includes similar phosphorylation levels of ERK 1/2 and Akt in resistant cells compared with untreated parental cells (Fig. 4F), despite the high intracellular sunitinib concentrations. In addition, after 12 weeks of drug-free culture of the sunitinib-resistant cells, these revertant cells had normalized their lysosomal capacity and LAMP expression to the level of the parental cells (Fig. 4C, bottom, and E). During this same period, sunitinib sensitivity was also restored to that of the

Figure 4. Lysosomal sequestration of sunitinib. A, intracellular sunitinib concentration of parental cells, incubated for 24 hours with 5 $\mu\text{mol/L}$ sunitinib, and resistant cells, continuously incubated with sunitinib. B and C, confocal images after incubation with sunitinib (green), 75 nmol/L LysoTracker (red), and 0.5 $\mu\text{g/mL}$ Hoechst (blue) for 60 minutes. Sunitinib and LysoTracker were found to be highly colocalized (yellow). B, first and second rows, 786-O parental cells (PAR), treated with 5 $\mu\text{mol/L}$ sunitinib for 60 minutes, 10 \times and 63 \times magnification, respectively; third row, 786-O PAR, treated with 50 nmol/L bafilomycin A1 (BAF) for 90 minutes, starting 30 minutes before addition of sunitinib, LysoTracker, and Hoechst. Scale bars are 25 μm . C, top, 786-O PAR, treated with 5 $\mu\text{mol/L}$ sunitinib for 60 minutes; middle, 786-O sunitinib-resistant cells (SUN), continuously treated with sunitinib; bottom, 786-O revertant cells (REV), cultured for 12 weeks in sunitinib-free medium, and subsequently retreated as the parental cells. Scale bars are 25 μm . D, top, 786-O sunitinib-resistant cells (SUN), continuously treated with sunitinib; middle, 786-O SUN, treated with 50 nmol/L bafilomycin A1 (BAF) for 90 minutes; bottom, 786-O SUN, treated with 10 mmol/L NH_4Cl for 90 minutes. Scale bars are 25 μm . E, Western blot analysis of LAMP-1 and LAMP-2 of parental, sunitinib-resistant, and revertant 786-O and HT-29 cells. F, Western blot analysis of (phosphorylated) ERK 1/2 and Akt of parental and sunitinib-resistant 786-O and HT-29 cells. Results are shown as means \pm SEM. *, $P < 0.05$.



parental cells (Fig. 3A), supporting the role of lysosomes in sunitinib resistance.

Discussion

On the basis of the pharmacokinetic properties of sunitinib with a high apparent volume of distribution ($>2,000 \text{ L}$), the majority of the drug is distributed into various tissues as reflected by yellow discoloration of the skin in patients, whereas only a relatively small fraction of the drug remains in the circulation. To gain quantitative insight into the distribution of sunitinib in normal tissues and tumors, we determined sunitinib concentrations in skin and tumor tissues of a murine RCC model and in tumor biopsies from

patients during sunitinib treatment. We found that intratumoral sunitinib concentrations in mice and patients were at least 10-fold higher than plasma concentrations. These plasma concentrations in mice and patients were similar to previously published observations (13, 23). Together, (pre) clinical intratumoral concentrations indicated that sunitinib plasma pharmacokinetics are unlikely to provide adequate insight into the antitumor mechanisms operative at the tumor tissue level.

While sunitinib has been developed as an antiangiogenic agent, intended to primarily target endothelial and perivascular cells through its high affinity binding to VEGFR2 and PDGFR, it inhibits many other kinases (11). Karaman and colleagues (12) studied the affinity of TKIs to a panel of 317

kinases and found that sunitinib has a low selectivity for specific tyrosine kinases. Given the fact that high intratumoral sunitinib concentrations in patients may not only inhibit angiogenesis but may also target tumor cells directly, we studied the activity of sunitinib *in vitro*. We found that sunitinib inhibits tumor and endothelial cells at intratumoral concentrations achieved in tumors of patients, explaining clinical observations that sunitinib treatment not only results in tumor stabilization due to its antiangiogenic activity, but also can cause partial or complete clinical tumor responses.

Previous reports indicated that sunitinib antitumor activity could not be mediated by direct antitumor activity, first because no activity of sunitinib at assumed relevant plasma concentrations on tumor cells *in vitro* was seen and second because endothelial cells were several fold more sensitive to sunitinib treatment than tumor cells in previous reports (24). By gaining more insight into the pharmacokinetics of sunitinib, we here show that plasma concentrations do not truly reflect physiologic intratumoral pharmacokinetics of sunitinib due to its high volume of tissue distribution and by our finding that sunitinib concentrations are 10-fold higher in (tumor) tissue than plasma levels. Second, while tumor cell proliferation experiments are mostly carried out with a fixed concentration of bovine serum (5%–20% of the growth medium), for endothelial cells experimental conditions are being varied extensively. Mostly for specific proliferation assays, endothelial cells are being starved and stimulated at low serum level (0.1%–1%) with the addition of a specific growth factor, such as VEGF or basic fibroblast growth factor (bFGF; refs. 24, 25). Although these specific conditions are important to study specific growth factor pathway signaling modifications, vascular cells in the *in vivo* situation or in patients are not only being stimulated by one specific growth factor. Vascular cells *in vivo* are stimulated by multiple growth factors and proteins, due to tumor release of several growth factors, extracellular matrix proteins and platelet releasates, similar to the proteins present in serum (26, 27). Therefore, in our experimental set up we determined the sensitivity of endothelial cells *in vitro* under full growth stimulating conditions for endothelial cells and found under these conditions similar sunitinib sensitivity of endothelial cells as for tumor cells. These results further support our findings that sunitinib may not only inhibit angiogenesis but has a direct antitumor effect *in vivo* and in patients as well.

Furthermore, we found that sunitinib inhibits key signaling pathways in our cancer cell lines similar to what was previously reported by Yang and colleagues (28). While possible primary targets of the multitargeted agent sunitinib on tumor cells are multiple and remain to be studied, we reasoned that p-Akt and p-ERK would be a rational first choice as read-out of sunitinib activity, because they are 2 major downstream proteins of several known targets of sunitinib. In addition, they are major downstream junctions in many signaling pathways controlling cancer cell proliferation and/or apoptosis. Indeed, we found decreased p-Akt and p-ERK in the sensitive, but not the continuously

exposed cells. This inhibitory action on signaling pathways is in support of the activity profile of sunitinib at higher doses due to its affinity to other kinases as previously reported by Karaman and colleagues (12). In preliminary studies using a peptide-based kinase activity array, we indeed confirmed that multiple kinases are inhibited by sunitinib in tumor cells (data not shown).

One of the major clinical problems with antiangiogenic TKIs including sunitinib is their intrinsic and acquired drug resistance (1, 17, 29). On the basis of preclinical studies, the upregulation of VEGF production as well as other compensatory cytokines and growth factors such as HGF/cMET, IL-8, PlGF, SDF-1, or erythropoietin and epithelial EMT have been suggested to play a role in sunitinib resistance (6–9, 30–32). On the basis of our findings that sunitinib inhibits tumor cell proliferation directly, we investigated the possible role of tumor cells in sunitinib resistance. We found that continuous exposure to sunitinib resulted in resistance of tumor cells by an adaptive tumor cell mechanism identified as an increased intracellular lysosomal sunitinib sequestration. The resistance factors were moderate, but were not due to variation in the MTT assays, which were highly consistent in 4 independent experiments per cell line. Moreover, the continuously exposed cells kept growing at concentrations (5 $\mu\text{mol/L}$ for 786-0 and 10 $\mu\text{mol/L}$ for HT-29) that kill the parental cells. Because this form of adaptive resistance to sunitinib might not be the result of a stable mutation in its target such as often occurs with TKIs in cells addicted to one oncogenic target receptor, we decided to investigate other possibilities. We reasoned that sunitinib might preferentially accumulate in acidic lysosomes, because sunitinib is a hydrophobic ($\log P = 5.2$) weak base ($\text{pK}_a = 8.95$; 4) as is described for some other substances. Because sunitinib is a hydrophobic compound, it can easily cross plasma membranes and other intracellular membranes. In addition, sunitinib is a weak base with a pK_a of 9, implying that decreasing the pH below 9, sunitinib will be increasingly protonated and thereby losing its ability to cross membranes. At a pH of 5, all sunitinib will be protonated. Therefore, upon entering an acidic organelle such as a lysosome, sunitinib becomes protonated and cannot cross membranes anymore. To test this hypothesis we studied co-localization of sunitinib and acidic lysosomes. Indeed, we found clear colocalization of acidic lysosomes by staining of lysosomal-associated membrane proteins and sunitinib due to its auto-fluorescence. No colocalization with other intracellular organs could be detected. In addition, subsequent disturbance of the lysosomal function resulted in sunitinib release from lysosomes. Subsequently, we found that resistant cells are able to sequester sunitinib from their cytoplasmic compartment into acidic lysosomes to a higher extent contributing to a decreased growth inhibitory activity. Despite higher intracellular concentration of sunitinib in resistant cells, intracellular kinase activity was comparable with untreated parent cells. This result supports that sequestration of sunitinib in acidic lysosomes reduces its cytoplasmic availability and provides a novel mechanism of resistance that has not

been previously reported. This mechanism of resistance may play a role for other agents, including TKIs, as well and it may reflect a natural defense mechanism of cells.

Lysosomal sequestration as a mechanism of drug resistance is reversible, as drug-free culture of resistant tumor cells resulted in normalization of their lysosomal capacity and recovery of drug sensitivity. Transient *in vitro* resistance of tumor cells supports clinical resistance data that were recently reported. While resistance to sunitinib is acquired during treatment in almost all patients (29), it is transient in some of them after treatment interruption and subsequent rechallenge (33). The latter is also supported by our recent finding with a tumor-derived cell line from a progressive skin metastasis (9).

Although *in vitro* prolonged primary endothelial cell culture experiments are not easily feasible, our results indicate that continuous clinical exposure to sunitinib may cause an increased lysosomal capacity in various cell types and provide a general mechanism of resistance. Whether this novel resistance mechanism is unique for sunitinib or whether this is a general mechanism of TKI resistance remains to be studied. Other TKIs either have a relatively high pKa (pazopanib and dasatinib) or a high lipophilicity

(gefitinib and nilotinib), whereas sunitinib is both a weak base and hydrophobic. In conclusion, this novel mechanism of resistance warrants further clinical investigation either by re-treatment with sunitinib after prior tumor progression in patients or by concomitant treatment with clinically available drugs that interfere with lysosomal function, such as chloroquine (34).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This study was supported by a research grant of Pfizer Global Pharmaceuticals and by an AEGON Research Fellowship Program. YGA is the recipient of a visiting professor fellowship from the Royal Netherlands Academy of Arts and Sciences (KNAW) and from the Netherlands Organization for Scientific Research (NWO).

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Received June 29, 2011; revised September 21, 2011; accepted September 26, 2011; published OnlineFirst October 6, 2011.

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