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The Kinase Inhibitors Sunitinib and Sorafenib Differentially Affect NK Cell Antitumor Reactivity In Vitro1

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Sunitinib and Sorafenib are protein kinase inhibitors (PKI) approved for treatment of patients with advanced renal cell cancer (RCC). However, long-term remissions of advanced RCC have only been observed after IL-2 treatment, which underlines the importance of antitumor immune responses in RCC patients. Because PKI, besides affecting tumor cells, also may inhibit signaling in immune effector cells, we determined how Sunitinib and Sorafenib influence antitumor immunity. We found that cytotoxicity and cytokine production of resting and IL-2-activated PBMC are inhibited by pharmacological concentrations of Sorafenib but not Sunitinib. Analysis of granule-mobilization within PBMC revealed that this was due to impaired reactivity of NK cells, which substantially contribute to antitumor immunity by directly killing target cells and shaping adaptive immune responses by secreting cytokines like IFN-γ. Analyses with resting and IL-2-activated NK cells revealed that both PKI concentration dependently inhibit cytotoxicity and IFN-γ production of NK cells in response to tumor targets. This was due to impaired PI3K and ERK phosphorylation which directly controls NK cell reactivity. However, while Sorafenib inhibited NK cell effector functions and signaling at levels achieved upon recommended dosing, pharmacological concentrations of Sunitinib had no effect, and this was observed upon stimulation of NK cell reactivity by tumor target cells and upon IL-2 treatment. In light of the important role of NK cells in antitumor immunity, and because multiple approaches presently aim to combine PKI treatment with immunotherapeutic strategies, our data demonstrate that choice and dosing of the most suitable PKI in cancer treatment requires careful consideration.

A

dvanced renal cell carcinoma (RCC)5 is a lethal urologic cancer due to its resistance to cytotoxic chemotherapy. Until recently, the only effective treatment for advanced RCC were immunotherapeutic approaches with IL-2 or IFN-α resulting in response rates up to 15% and a limited improvement of overall survival (1). RCC is associated with genetic mutations leading to the activation of several protein kinases (PK), among them vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), and intracellular kinases like PI3K or MAPK (2). The advances in understanding deregulated pathways in RCC provided the rationale to evaluate the protein kinase inhibitors (PKI) Sunitinib (Sutent, SU11248) and Sorafenib ( Nexavar, BAY43–9006) in patients with advanced RCC. Sunitinib inhibits, among others, the VEGFR-1, -2, and -3, the PDGFR-α and -β, the Fms-like tyrosine kinase 3 (FLT3), and the stem cell factor receptor (c-kit) (3). In a randomized phase III trial, Sunitinib treatment resulted in a significantly higher response rate (47 vs 12%) and a significant improvement in progression-free survival compared with IFN-α (11 vs 5 mo) (4). Sorafenib was initially developed as MAPK kinase kinase (RAF) inhibitor but also inhibits signaling via VEGFR-2 and -3, PDGFR-β, c-kit, and FLT3 (5). Compared with placebo, Sorafenib doubled the median duration of progression free survival in patients with RCC after failure of cytokine treatment (5.5 vs 2.8 mo) (6). These results led to the approval of Sunitinib and Sorafenib for the treatment of metastasized (advanced) RCC (3, 5).

Despite the improved treatment results obtained with PKI, i.v. high-dose IL-2 therapy remains up to now the only systemic treatment under which long term remissions have been observed in selected patients (7). This sole chance of cure strongly emphasizes the importance of an effective antitumor immune response for disease control in RCC patients. Of note, IL-2 does not appear to have any direct effects on tumor cells, but, upon treatment of advanced RCC, has been described to mediate its effects through stimulation of host-immune effector cells (8). Importantly, IL-2 is a potent stimulus for NK cells as central components of the innate immunity (9). NK cells can control both local tumor growth and metastasis due to their ability to exert direct cellular cytotoxicity without prior sensitization and to secrete immunostimulatory cytokines like IFN-γ (10–12). The latter participates in cancer elimination by inhibiting cellular proliferation and angiogenesis, promoting apoptosis, and stimulating the adaptive immune system and is instrumental for enhancing Ag processing and presentation (10, 13, 14). Thus, NK cell activity also influences the efficacy of immunotherapeutic approaches like vaccination strategies with dendritic cells (DC), peptides, or RNA aiming to enhance antitumor reactivity of T cells. Multiple attempts currently aim to stimulate NK cell

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4 Abbreviations used in this paper: RCC, renal cell carcinoma; PK, protein kinases; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; PKI, protein kinase inhibitor; FLT3, Fms-like tyrosine kinase 3; RAF, MAPK kinase kinase; DC, dendritic cell; PI, propidium iodine; XTT, 2,3-bis [methoxyl-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide.

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reactivity in the immunotherapy of cancer (15). Of note, PKI may, besides affecting tumor cells, also inhibit signaling events responsible for activation of immune effector cells. Because the multikinase inhibitors Sorafenib and Sunitinib target partially different PK, and clinical application of the two compounds in recommended dosing causes largely differing plasma levels in patients, differential effects on NK cell antitumor immunity may be the consequence (16–18). In this study, we compared the effects of these two compounds on NK cell antitumor reactivity and the underlying intracellular signaling events.

Materials and Methods

Cell lines, reagents, and Abs

The NK cell line NK-92 was purchased from DSMZ. The human cell lines A498, ACHN, CAKI-2, and MZ1774 (renal), K562, RPMI866, and Raji (leukemia), as well as Sunitinib and Sorafenib were obtained internally at Eberhard Karls University. Parental and FLT3-ITD-transfected Baf3 cells were generated and cultured as previously described (19). PD98059 was obtained from Tocris Bioscience. All PKI were dissolved in DMSO (Sigma-Aldrich). FACS Abs were from BD Pharmingen. rIL-2 was from ImmunTox. All other reagents were obtained from Carl Roth.

Preparation of NK cells

NK cells were isolated from peripheral blood by negative selection using the NK Cell Isolation Kit II and MACS columns (Miltenyi Biotec). Polyclonal NK cells were generated by incubation of nonplastic-adherent PBMC with irradiated RPMI866 feeder cells over 10 days as previously described (20). Experiments were performed when purity was above 90% as determined by flow cytometry.

Degranulation assay

PBMC from healthy donors were cocultured with K562 cells at an E:T ratio of 1:1 for 3 h. Subsequently, cells were stained with anti-CD107a-FITC or isotype control. To identify the different cytotoxic lymphocyte subpopulations, PBMC were counterstained with anti-CD56-FITC, anti-CD8-FITC, anti-y8-TCR-FITC, and/or anti-CD3-PeCy5. Analysis was performed on a FACScan (BD Biosciences).

Cytotoxicity assay

Cytotoxicity was determined by standard 4-h chromium release assays as previously described (20). PBMC from healthy donors or NK cells were cocultured with the different cell lines. Where indicated, Sorafenib, Sunitinib, or DMSO as vehicle control were added to effector cells 30 min before the assays. In all experiments spontaneous release was below 15% of maximum release.

Determination of IFN-γ

IFN-γ concentrations in cell culture supernatants were analyzed using OptEIA sets from BD Pharmingen according to the manufacturer’s instructions.

Determination of apoptosis

Baf3 cells were cultured in the presence of Sunitinib and Sorafenib for 48 h. Apoptosis was assessed using a FITC-labeled Annexin V/propidium iodide (PI) assay (Immunotech) as previously described (19).

Proliferation assay

Parental and FLT3-ITD-transfected Baf3 cells (20,000/well) were cultured in 96-well plates in the presence or absence of Sunitinib and Sorafenib. Proliferation was measured at 72 h using a 2,3-bis [4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT)-based assay (Roche Molecular Biochemicals) as previously described (19).

Detection of ERK phosphorylation

NK-92 cells or NK cells from healthy donors were incubated for 30 min in the presence of the indicated concentrations of Sunitinib, Sorafenib, PD98059, or DMSO as vehicle control and then exposed to formalin-fixed Raji cells or 100 U/ml IL-2. Subsequently, cells were lysed and Western blot analysis was performed as previously described (21). The active forms of ERK1/2 and p38 were detected using phosphospecific Abs, and equal loading was assessed by reblotting with an Ab against whole ERK1/2 protein (Phospho-p44/42 MAPK (Thr202/Tyr204), Phospho-P38 p85 (Tyr458)/p55 (Tyr199), and p44/42 MAPK Ab, Cell Signaling Technology).

Statistical analyses

Statistical analyses of results of chromium release assays and ELISA were performed by one-way ANOVA. Statistical analysis of results of degranulation assays was performed by Wilcoxon-signed Rank Test. Significant differences are indicated by *, p < 0.05 and **, p < 0.01.

Results

Antitumor reactivity of PBMC is impaired by pharmacological levels of Sorafenib but not Sunitinib

To analyze the effects of the PKI Sorafenib and Sunitinib on tumor cell lysis by cytotoxic lymphocytes, we isolated PBMC from healthy donors and determined cytotoxicity in the presence or absence of pharmacological concentrations of the two PKI corresponding to plasma levels achieved in patients by recommended dosing (17, 18). Treatment with Sorafenib (10 μg/ml) caused a substantial and statistically significant reduction of PBMC-mediated cytotoxicity against the RCC cells A498, ACHN, CAKI-2, and MZ1774 (up to 95% reduction; E:T ratio 90:1). In contrast, pharmacological concentrations of Sunitinib (100 ng/ml) did not alter RCC cell lysis (Fig. 1A, upper panels). To determine whether inhibition of cytotoxicity by Sorafenib also occurred in the presence of the activating cytokine IL-2, PBMC were treated with IL-2 (100 U/ml) for 24 h. Subsequently, the effect of PKI treatment on cytotoxicity was determined. Although target cell lysis was in general slightly higher following IL-2 pretreatment, presence of Sorafenib but not Sunitinib again substantially inhibited cytotoxicity in cultures with A498, ACHN, CAKI-2, and MZ1774 RCC cells (Fig. 1A, lower panels). Next, we analyzed whether Sorafenib and/or Sunitinib altered IFN-γ production of PBMC and cultured the RCC cell lines with resting or IL-2-activated PKI alone or in the presence of the PKI. Presence of tumor cells potently enhanced IFN-γ production of PBMC with higher cytokine levels detectable in cultures with IL-2-activated effector cells. In line with the results obtained regarding cytotoxicity, 10 μg/ml Sorafenib significantly reduced the detectable levels of IFN-γ in the cultures with both resting (Fig. 1B, upper panels) and IL-2-activated (Fig. 1B, lower panels) PBMC with all renal cancer cells, while 100 ng/ml Sunitinib had no effect. Thus, Sorafenib inhibits cellular cytokine and IFN-γ production of both resting and cytokine-activated immune effector cells.

To analyze whether a combination of Sorafenib and Sunitinib had a synergistic effect on immune effector functions, we cultured resting and IL-2-activated PBMC with the classical target cell line K562 in the absence or presence of Sorafenib (10 μg/ml), Sunitinib (100 ng/ml), a combination of both, or DMSO as vehicle control. As observed with the renal cancer cells, cytotoxicity (Fig. 1C) and IFN-γ production (Fig. 1D) were reduced by the presence of Sorafenib but not Sunitinib. No further reduction of cytotoxicity and cytokine production was observed upon combined treatment with both compounds.

To exclude that the observed effects were due to a differential influence of the compounds on the target cells, we performed chromium release assays with untreated and PKI-treated A498, ACHN, CAKI-2, MZ1774, and K562 cells in the absence of PBMC. No direct cytotoxic effect of Sorafenib and Sunitinib on the target cell lines was observed after 4 h of incubation, which corresponds to the time required for analysis of NK cell cytotoxicity (Fig. 2A). Next we analyzed whether pretreatment of the target cells with Sunitinib or Sorafenib influenced subsequent immune effector functions in cultures with PBMC. The RCC cell lines A498, ACHN, CAKI-2, and MZ1774 as well as K562 cells were cultured in the presence or absence of Sorafenib (10 μg/ml), Sunitinib...
(100 ng/ml), or DMSO for 4 h. Subsequently, PBMC-mediated cytotoxicity in the absence of the compounds was analyzed by standard chromium release assays. No relevant differences in the lysis of target cells following pretreatment with the PKI were observed (Fig. 2B). To ascertain that both compounds were active at pharmacological concentrations, we studied how Sunitinib and Sorafenib affect viability and proliferation of FLT3-mutated cells. FACS analysis using Annexin-V/PI revealed that treatment with both Sorafenib and Sunitinib at low pharmacological levels (1 μg/ml Sorafenib, 50 ng/ml Sunitinib) comparably induced apoptosis in (100 ng/ml), or DMSO for 4 h. Subsequently, PBMC-mediated cytotoxicity in the absence of the compounds was analyzed by standard chromium release assays. No relevant differences in the lysis of target cells following pretreatment with the PKI were observed (Fig. 2B). To ascertain that both compounds were active at pharmacological concentrations, we studied how Sunitinib and Sorafenib affect viability and proliferation of FLT3-mutated cells. FACS analysis using Annexin-V/PI revealed that treatment with both Sorafenib and Sunitinib at low pharmacological levels (1 μg/ml Sorafenib, 50 ng/ml Sunitinib) comparably induced apoptosis in
FIGURE 2. Pharmacological levels of Sorafenib and Sunitinib do not differ with regard to direct effects on target cells. A. The indicated tumor cells were treated with 10 μg/ml Sorafenib, 100 ng/ml Sunitinib, or DMSO as vehicle control. After 4 h, chromium release was determined. B. The indicated tumor cells were treated with 10 μg/ml Sorafenib, 100 ng/ml Sunitinib, or DMSO as vehicle control for 4 h. Subsequently, cells were washed and tumor cell lysis by PBMC in the absence of the compounds was determined by chromium release assays. C. Baf3 cells (parental and FLT3-ITD-transfected) were incubated in the absence or presence of Sunitinib (50 ng/ml), Sorafenib (1 μg/ml), or DMSO as vehicle control for 48 h before FACS analysis with Annexin-V-FITC/PI. D. Baf3 cells (parental and FLT3-ITD-transfected) were incubated in the absence or presence of Sunitinib (50 ng/ml), Sorafenib (1 μg/ml), or DMSO as vehicle control for 48 h. Cellular proliferation was assessed with an XTT-based assay. Data shown represent means of triplicates with SD of one representative experiment from a total of three.

FLT3-ITD transfected Baf3 cells (Fig. 2C). Furthermore, treatment with either compound reduced cellular proliferation as revealed by XTT proliferation assays and determination of cell counts in the cultures (Fig. 2D and data not shown). Of note, no effect of the compounds at the used low pharmacological levels on the parental Baf3 cells was observed, which confirmed the specific inhibitory capacity of both PKI. Together, these results demonstrate that our findings obtained in cocultures of PBMC with tumor cells were not due to different effects of the compounds on the target cells and confirm that both compounds are active at the used concentrations.

Pharmacological levels of Sorafenib but not Sunitinib impair granule mobilization of NK cells among PBMC

To determine which effector cell population mediating antitumor reactivity among the PBMC was affected by Sorafenib treatment, we performed degranulation assays in cocultures of PBMC and K562 cells. Counterstaining for CD3, CD8, CD56, and γδ-TCR to discern the different cytotoxic lymphocyte subpopulations was used. In the absence of target cells, no relevant CD107a surface expression on the parental Baf3 cells was observed, which confirmed the specific inhibitory capacity of both PKI. Together, these results demonstrate that our findings obtained in cocultures of PBMC with tumor cells were not due to different effects of the compounds on the target cells and confirm that both compounds are active at the used concentrations.

PKI treatment concentration-dependently inhibits NK cell reactivity

Next, we aimed to ascertain that the observed effects were caused by a direct impact on NK cells and not due to a potential modulation of other immune effector cells among PBMC. In addition, we studied the effect of different pharmacological concentrations of the compounds on NK cell reactivity. To this end, we cocultured tumor cells with polyclonal NK cells in the presence of low, medium, and high concentrations of Sorafenib (1 μg/ml, 5 μg/ml, and 10 μg/ml) and Sunitinib (50 ng/ml, 100 ng/ml, and 200 ng/ml), which have been reported to be achieved in patients upon treatment with the two PKI (17, 18). A clear concentration-dependent reduction of NK cell cytotoxicity against the RCC cell lines A498, ACHN, CAKI-2, and MZI774 was observed upon Sorafenib treatment with a slight inhibitory effect already at concentrations as low as 1 μg/ml (Fig. 4A, upper panels). In contrast, Sunitinib, even at a concentration of 200 ng/ml corresponding to plasma peak levels in patients, had no effect (Fig. 4A, lower panels). In addition, Sorafenib concentration-dependently impaired IFN-γ production of NK cells in cocultures with the RCC cell lines, and a substantial inhibition was observed at 1 μg/ml in most cases. No relevant inhibitory effect was observed with Sunitinib in the used pharmacological concentrations (Fig. 4B). Again we wanted to ascertain
that the reactivity of both resting and activated NK cells was influenced. Thus, we cultured NK cells in the presence or absence of 100 U/ml IL-2 for 24 h before analysis of cytotoxicity and IFN-γ production in response to target cells. As expected, cytotoxicity and IFN-γ production of activated NK cells in response to K562 target cells were enhanced after IL-2 treatment compared with NK cells cultured in medium alone. However, with both resting and activated NK cells, treatment with plasma peak concentrations of Sorafenib (10 μg/ml) but not of Sunitinib (200 ng/ml) significantly inhibited NK cell reactivity (Fig. 4, C and D).

To further characterize and compare the effects of Sorafenib and Sunitinib on NK cell reactivity, we analyzed cytotoxicity and IFN-γ production in the presence of a broader range of concentrations of either PKI (0.1 to 10 μg/ml). Sorafenib concentration-dependently inhibited cellular cytotoxicity starting at 1 μg/ml, while IFN-γ production was already reduced at concentrations as low as 0.1 μg/ml (Fig. 4, E and F). Of note, unphysiologically high levels of Sunitinib also inhibited cellular cytotoxicity and IFN-γ production. However, this was only observed in doses starting at 1 μg/ml which is far above the levels reached in patients upon recommended Sunitinib dosing (Fig. 4, E and F).

Pharmacological levels of Sorafenib but not Sunitinib differentially affect NK cell signaling

Although a variety of different intracellular signaling pathways are involved in the modulation of NK cell reactivity, ERK1/2 have been shown to be central for NK cell cytotoxicity and cytokine release (23–25). To study whether the PKI mediated inhibition of ERK phosphorylation, we first used the NK cell line NK-92, which has been shown to be well suitable to study intracellular signaling pathways in response to tumor target cells (21, 26). As expected, the presence of Raji tumor cells induced ERK1/2 phosphorylation in NK-92 cells. Addition of 50 μM of the specific MEK inhibitor PD98059 and of Sorafenib at high pharmacological levels (10 μg/ml) nearly abrogated ERK1/2 phosphorylation. No inhibitory effects of Sunitinib at high levels (200 ng/ml) or DMSO as vehicle control were observed (Fig. 5A). These findings were also confirmed using polyclonal NK cells from healthy donors. Again, PD98059 and Sorafenib but not Sunitinib substantially reduced the detectable levels of ERK1/2 phosphorylation (Fig. 5B, upper panels). To determine whether inhibition of ERK1/2 phosphorylation by Sorafenib was due to an influence on proximal or distal signaling events in NK cells, we next analyzed the phosphorylation of PI3K, which is a key upstream regulator of NK cell reactivity (23, 24). Presence of tumor cells substantially induced PI3K phosphorylation in primary NK cells. Pharmacological levels of Sorafenib markedly reduced PI3K phosphorylation, indicating that Sorafenib already impairs proximal NK cell signaling and thus prevents the activation of the signal network that controls NK cell cytotoxicity. No effects were observed in the presence of Sunitinib at concentrations corresponding to peak levels achieved in patients or DMSO as vehicle control (Fig. 5B, lower panels). Of note, no further reduction of the detectable levels of phosphorylated PI3K (Fig. 5B, upper panels) or phosphorylated ERK1/2 (Fig. 5B, lower panels) was observed upon combined treatment of NK cells with both Sorafenib and Sunitinib as compared with the effect of Sorafenib alone (Fig. 5B). However, our previous analyses of NK cell effector functions had revealed that unphysiologically high concentrations of Sunitinib also inhibit NK cell reactivity. To study how equally high concentrations of Sunitinib and Sorafenib affect tumor cell-induced phosphorylation of PI3K and ERK1/2 in NK cells we used 5 μg/ml of either PKI in cultures of NK cells and RAJI target cells. Again, presence of tumor cells induced PI3K and ERK1/2 phosphorylation in NK cells, and presence of Sunitinib at 5 μg/ml markedly reduced both PI3K and ERK1/2 phosphorylation. Presence of Sunitinib, at the unphysiologically high concentration of 5 μg/ml, also reduced phosphorylation of PI3K and
ERK1/2, which explains our results observed in functional assays (Fig. 5C).

To characterize the effect of the PKI on primary NK cells, which, in patients, would therapeutically be stimulated by a potential IL-2 treatment, we established an assay system using polyclonal NK cells and IL-2 as activating stimulus. A strong induction of phosphorylated ERK1/2 was observed in NK cells already after 5 min of IL-2 treatment, which lasted for about 2 h (Fig. 5D). Next, we stimulated the polyclonal NK cells with IL-2 in the presence or absence of 10 μg/ml Sorafenib, 200 ng/ml Sunitinib, 50 μM...
PD98059, or DMSO as vehicle control. Again, Sunitinib had no effect, while Sorafenib, alike PD98059, markedly reduced IL-2-induced ERK1/2 phosphorylation. No synergistic effect with regard to the inhibition of ERK1/2 phosphorylation was observed when NK cells were exposed to a combination of both Sunitinib and Sorafenib (Fig. 5E). To characterize the effects of the PKI on IL-2-induced ERK phosphorylation more precisely we stimulated NK cells with IL-2 in the presence of varying concentrations of Sorafenib and Sunitinib ranging from 0.1 to 10 μg/ml (Fig. 5F, upper panels). Although Sunitinib, in concentrations below 1 μg/ml, did not affect ERK1/2 phosphorylation, a concentration-dependent inhibition was observed at concentrations above 1 μg/ml (Fig. 5F, lower panels). To ascertain that our results were not influenced by potential effects of the prolonged culture during the process of generation of the polyclonal NK cells we also performed analyses with freshly isolated NK cells from healthy donors. In line with our results obtained with polyclonal NK cells, Sorafenib but not Sunitinib clearly reduced the activation-induced levels of phosphorylated ERK1/2 protein (Fig. 5G).

**FIGURE 5.** Sorafenib and Sunitinib concentration dependently affect NK cell signaling. A, NK-92 cells were incubated for 30 min with or without 10 μg/ml Sorafenib, 200 ng/ml Sunitinib, 50 μM PD98059, or DMSO and exposed to fixed Raji cells for 10 min. Subsequently, cell lysates were analyzed for phosphorylated (pERK) and whole (panERK) ERK1/2 protein. B, Polyclonal NK cells were incubated for 30 min with or without 10 μg/ml Sorafenib, 100 ng/ml Sunitinib, a combination of both PKI, 50 μM PD98059, or DMSO and exposed to fixed Raji cells for 10 min. Subsequently, cell lysates were analyzed for phosphorylated PI3K (pPI3K) and ERK (pERK) and whole (panERK) ERK1/2 protein. C, Polyclonal NK cells were incubated for 30 min with or without 5 μg/ml Sorafenib, 5 μg/ml Sunitinib, or DMSO and exposed to fixed Raji cells for 10 min. Subsequently, cell lysates were analyzed as described in B. D, Polyclonal NK cells were exposed to IL-2 for the indicated times. Subsequently, cell lysates were analyzed as described in A. E, Polyclonal NK cells were incubated for 30 min with or without 10 μg/ml Sorafenib, 100 ng/ml Sunitinib, a combination of both PKI, 50 μM PD98059, or DMSO and exposed to IL-2 for 5 min. Subsequently, cell lysates were analyzed as described in A. F, Polyclonal NK cells were incubated for 30 min with the indicated concentrations of Sorafenib (upper panels) and Sunitinib (lower panels) and exposed to IL-2 for 5 min. Subsequently, cell lysates were analyzed as described in A. G, Freshly isolated NK cells were incubated for 30 min with or without 10 μg/ml Sorafenib, 200 ng/ml Sunitinib, or DMSO and exposed to IL-2 for 5 min. Subsequently, cell lysates were analyzed as described in A. One representative experiment from a total of three is shown.

**Discussion**

For a long time, immunotherapy using cytokines was the only approved systemic treatment for RCC, which is considered a highly immunogenic tumor. The more recent findings regarding the pathophysiological role of PK like VEGFR and PDGFR in RCC led to the development and successful application of so-called targeted therapies in the treatment of advanced RCC, among
those the PKI Sorafenib and Sunitinib (2). However, patients with metastasized RCC will not be cured by PKI treatment (1, 4, 6). Combinations of targeted therapies with immunotherapeutic approaches are an attractive means to exploit both the largely improved response rates of targeted agents like PKI and the potential for long term remissions of immunotherapy. Accordingly, respective combinations have been and are currently investigated in various studies (for an overview see e.g., http://clinicaltrials.gov/ct2/results?cond=%22Kidney+Cancer%22). When developing strategies combining PKI with approaches aiming to enhance antitumor immunity, one needs to consider that PKI may potently affect antitumor immunity by inhibiting signaling pathways necessary for activation and reactivity of immune effector cells. It has already been demonstrated that the PKI Imatinib,Nilotinib, and Dasatinib, which are approved for the treatment of chronic myeloid leukemia, impair reactivity of different immune effector cells like DC, T cells, and NK cells (27–31). We report in this study that pharmacological concentrations of Sorafenib, achieved upon therapeutic application in patients, dramatically impair cytotoxicity and IFN-γ production of resting and IL-2 activated PBMC. In contrast, Sunitinib, at levels reached in patients upon recommended dosing, does not affect PBMC reactivity despite inhibiting a broader spectrum of kinases than Sorafenib (16). Of note, immune effector functions were not further inhibited by a combination of both PKI, FLT3-dependent BaF3 cells, but not the parental cells comparatively underwent apoptosis and inhibition of proliferation upon treatment with pharmacological concentrations of both Sorafenib and Sunitinib. Compared with Sorafenib, an ~20-fold lower concentration of Sunitinib thus mediates similar inhibitory capacity regarding FLT3 signaling. These data are in line with available studies showing a 25-fold higher affinity of Sunitinib toward FLT3 compared with Sorafenib in kinase assays (16). Degranulation analysis revealed that the reduction of PBMC-mediated cytotoxicity and cytokine production by Sorafenib was due to an inhibitory effect on NK cells and not on other lymphocyte subpopulations. As impaired granule mobilization was observed with NK cells from over ten different donors, the Sorafenib-mediated inhibition of NK cell reactivity is not largely influenced by donor variability but rather a general effect of the compound. Furthermore, the inhibitory effect of pharmacological concentrations of Sorafenib on NK cells was observed both in resting and IL-2-activated state. The observation that presence of IL-2 only slightly enhanced cytotoxicity of the effector cells may be attributed to our allogenic setting, in which IL-2 is only one among many factors within the balance of activating and inhibitory receptors contributing to NK cell reactivity. It should be noted that a concentration-dependent inhibition of NK cell reactivity was observed using unphysiologically high concentrations of Sunitinib starting at 1 μg/ml. The differential effect of the pharmacological levels of the two PKI on NK cells as components of the innate immunity is of great interest because NK cells play an important role in antitumor immunity due to their ability to directly lyse target cells and to produce cytokines like IFN-γ, which shape subsequent adaptive immune responses (10, 32, 33). NK cell reactivity is regulated by a balance of activating and inhibitory receptors as well as soluble mediators like cytokines (11, 34–36). Depending on the engagement of particular receptors, activation of various phosphatases and kinases in NK cells results in the phosphorylation of different intracellular signaling proteins (11, 23, 35, 37). Despite the variety of receptors and intracellular proteins involved in the regulation of NK cell reactivity, activation of PI3K leading to the phosphorylation of ERK was shown to be critical for NK cell cytotoxicity by dictating lytic granule mobilization (21, 24, 26, 37, 38). Compared with the mechanisms guiding cellular cytotoxicity, many different intracellular signaling pathways seem to modulate NK cell IFN-γ release (25, 38–41). Our analyses of the signaling events in healthy NK cells following activation with tumor targets provide an explanation for the differential effects of the two PKI on NK cell effector functions: Pharmacological concentrations of Sorafenib reduced phosphorylation of PI3K and ERK1/2, while this was not observed with Sunitinib. As in the analyses of NK cell effector functions, unphysiologically high concentrations of Sunitinib also impaired PI3K and ERK1/2 phosphorylation in NK cells. This indicates that both PKI may affect proximal signaling events in NK cells depending on the used concentrations. However, our data clearly indicate that, at concentrations reached in patients, only Sorafenib but not Sunitinib impairs NK cell antitumor reactivity, and the higher drug levels upon recommended dosing of Sorafenib compared with Sunitinib cause inhibition of NK cell reactivity. Sorafenib may, in addition to inhibiting proximal signaling events, also inhibit RAF-dependent distal signaling during NK cell activation (5, 38). However, this inhibitory effect on RAF seems not to play a major role in the modulation of NK cell reactivity in our setting, because pharmacological levels of Sorafenib already impair proximal, tumor cell-induced PI3K activation.

Therapeutic application of IL-2, a cytokine which possesses no direct antitumor activity but potently activates host immune effector cells including NK cells can result in regression of metastatic lesions in RCC patients (7, 8). Of note, even in the era of targeted therapies, immunotherapy with IL-2 remains the only systemic treatment able to induce long term remissions in the metastatic situation, albeit only a minority of patients achieves such a favorable response (1, 7). Because we found that Sorafenib, in contrast to Sunitinib, inhibited IL-2-induced ERK1/2 phosphorylation at pharmacological concentrations, it seems likely that Sorafenib, upon recommended dosing, also inhibits the immunostimulatory effect of cytokine treatment in RCC patients. In line with our results regarding NK cells, Sorafenib has recently been shown to impair the immunostimulatory properties of DC in vitro and in vivo (42). As the immunoinhibitory effects of pharmacological levels of Sorafenib are neither limited to NK cells nor to the immunotherapy with cytokines or to RCC, our results have at least theoretically implications for combinations of multitargeted PKI with immunotherapies other than cytokines including peptide or RNA-vaccines both in RCC and in other cancers. In the clinical setting, Sunitinib has shown superior efficacy in treatment-naive metastasized RCC patients with a significant improvement in progression free survival compared with IFN-α (11 vs 5 mo) (4). Interestingly, a randomized phase II trial comparing Sorafenib to IFN-α in treatment-naive patients has not shown any benefit for the PKI (5.7 vs 5.6 mo) (43). Furthermore, cross-trial comparison suggests that Sunitinib achieves tumor shrinkage to a higher extend than Sorafenib. Because our data and the findings of Hipp and coworkers clearly indicate that, in therapeutic doses, Sorafenib but not Sunitinib impairs antitumor immunity it is tempting to speculate that this difference might, at least in part, be due to the distinct immunosuppressive capacity of Sorafenib (42).

The use of human PBMC/NK cells as used in our study serves well to mimic the situation in tumor patients and, in contrast to analyses in mouse models, excludes that results are skewed by species-dependent differences between mice and men. This is of great importance because available data point to differences between rodents and humans regarding the effects of immunotherapeutic approaches, which underlines the need to design preclinical studies that can better predict the risks and benefits of immunotherapeutic strategies in humans (44). When combining multitargeted PKI with immunotherapy, investigators should keep in mind the possibility of unexpected interferences. Our data indicate that Sunitinib, but not Sorafenib is exquisitely suitable for combination with immunotherapeutic approaches aiming to enhance NK cell reactivity. In light of the important role of NK cells in...
antitumor immunity and for future combinatorial immunotherapeutic approaches, choice and dosing of the most suitable PKI for a given cancer patient thus requires careful consideration. In the future it will be critical to define potential differences in immunosuppressive side effects of different compounds among rapidly growing assortment of multitargeted PKI to design promising new combinatorial treatment strategies that include immunotherapeutic strategies.

 Disclosures

The authors have no financial conflict of interest.

 References