

Tyrosine kinase inhibitors target cancer stem cells in renal cell cancer

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Received September 20, 2015; Accepted October 31, 2015

DOI: 10.3892/or.2015.4514

Abstract. This study was designed to analyze the impact of multi-targeted tyrosine kinase inhibitors on the cancer stem cell subpopulation in renal cell cancer. The second objective was to evaluate the effect of tumor growth inhibition related to a tumor niche factor - oxygen deprivation - as hypoxia develops along with the anti-angiogenic activity of tyrosine kinase inhibitors in renal tumors. Cells were treated with tyrosine kinase inhibitors, sunitinib, sorafenib and axitinib, in 2D and 3D culture conditions. Cell proliferation along with drug toxicity were evaluated. It was shown that the proliferation rate of cancer stem cells was decreased by the tyrosine kinase inhibitors. The efficacy of the growth inhibition was limited by hypoxic conditions and 3D intratumoral cell-cell interactions. We conclude that understanding the complex molecular interaction feedback loops between differentiated cancer cells, cancer stem cells and the tumor microenvironment in 3D culture should aid the identification of novel treatment targets and to evaluate the efficacy of renal cancer therapies. Cell-cell interaction may represent a critical microenvironmental factor regulating cancer stem cell self-renewal potential, enhancing the stem cell phenotype and limiting drug toxicity. At the same time the role of hypoxia in renal cancer stem cell biology is also significant.

Introduction

The clinical and biological anticancer effects produced by tyrosine kinase inhibitors (TKIs) in renal cell cancer (RCC) are the result of their inhibitory activities on a variety of cell receptors on cancer cells, endothelial cells, pericytes, and stromal cells (1). The targeted effects of TKIs are dependent on the inhibition of downstream mediators upregulated in response to molecular abnormalities (i.e., VHL, c-MET) in

RCC. Specifically, in clear cell renal cell cancer (ccRCC), the mutations and/or epigenetic silencing of the VHL gene promote subsequent overexpression of growth factors, including the vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor (PDGF), and multiple other hypoxia-regulated genes (EPO, NOS, GLUT-1, CA IX), all of which are co-responsible for tumor angiogenesis and cell proliferation (2,3). A group of targeted agents selective against RCC has been developed and introduced in the clinic over the last decade (4,5). More recently, cancer stem cells (CSCs) or tumor-initiating cells have come into focus as potential treatment targets in solid tumors (6,7) and RCCs (8,9).

Sunitinib (SU11248) is a multi-targeted kinase inhibitor of VEGFR-1, -2 and -3, PDGFR- β , mast/stem cell growth factor receptors (SCFR, c-Kit) and FMS-like tyrosine kinase 3 (FLT3) (10). Sunitinib inhibits cancer growth primarily through an anti-angiogenic mechanism, by halting endothelial cell proliferation and motility (11). The elucidated mechanisms of action of sunitinib may also include targeting CSCs. The first study to show that sunitinib targets CSCs was conducted in pancreatic cancer. In this study, CD24⁺CD44⁺ESA⁺ triple-positive pancreatic CSCs were shown to be responsive to this TKI in combination with liposome-coated doxorubicin (12). In prostate cancer, in a PC3 cell-based model, sunitinib was shown to reduce the number of ALDH⁺ cancer stem-like cells, and to sensitize these cells to the radiation-mediated loss of clonogenicity (13). In xenograft RCC models, sunitinib has been shown to generate resistance to its own therapeutic mechanism due to the induction of hypoxia in perinecrotic areas. Moreover, CD133/CXCR4 co-expressing cells, also considered CSCs, were found in these areas at higher numbers. Under hypoxia, the tumorigenic potential of CD133/CXCR4⁺ cells increased, and their sensitivity to sunitinib decreased (14).

Sorafenib (BAY 43-9006) was discovered based on its ability to inhibit kinases, including C-RAF and B-RAF (wild-type and V600E mutant), and subsequent MEK and ERK phosphorylation. It also targets VEGFR-2 and VEGFR-3, PDGFR- β , FLT3, and c-Kit (15). The complete mechanisms of action include a reduction in angiogenesis and cancer cell proliferation. However, the exact mechanisms remain undefined. In the case of hepatocellular carcinoma (HCC), it was demonstrated that label-retaining cancer cells (LRCCs), a subpopulation of CSCs, were significantly resistant to sorafenib. In addition, the proportion of LRCCs in the HCC cell lines was increased in the total culture after sorafenib treatment (16).

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Key words: renal cell cancer, cancer stem cells, tyrosine kinase inhibitors, sunitinib, axitinib

Moreover, CD44/CD133⁺ and CD44/ALDH1A1⁺ HCC cells (CSCs) were shown to increase in number after treatment with sorafenib (17,18). After treatment with sorafenib, starting at a concentration of 1 μ M and increased by 10% every 2 weeks until reaching the maximum tolerated dose (4–7 μ M), the number of CD44/CD133⁺ stem cells increased in the HCC cell lines (17). However, another HCC subpopulation, CD133⁺/CD166⁺/ALDH⁺ CSCs, was determined to be sensitive to sorafenib (19). On the contrary, HCC CSC-defined by the expression of the Nanog gene also exhibited resistance to sorafenib (20).

It was later determined that it is the combination of two drugs that enables sorafenib to target the HCC CSCs. Sorafenib, in combination with FH535 (an inhibitor of Wnt/ β -catenin signaling and dual antagonist of PPAR γ / δ activity), inhibited the proliferation of liver CSCs (CD133, CD44, CD24, and aldehyde dehydrogenase 1-positive) (21). A similar effect was noted for the combination of sorafenib and gedatolisib (PKI-587), a highly potent dual inhibitor of PI3K α , PI3K γ and mTOR (22). Sorafenib was also shown to effectively target pancreatic CSCs (CD24⁺/CD44⁺ and ALDH⁺) when administered with sulforaphane, a phytochemical belonging to the family of isothiocyanates (23). In the case of breast cancer, sorafenib has been shown to be effective against breast cancer CD44⁺CD24⁺ stem cells in combination with radiation (24). Contrary to its low activity in HCC, sorafenib was shown to be effective *in vitro* against CSCs in glioblastomas (tumor-initiating cells) in primary cell cultures by inhibiting the PI3K/Akt and MAPK pathways. In this case, sorafenib significantly induced apoptosis by downregulating the survival factor myeloid cell leukemia 1 (Mcl-1) (25), which was further potentiated by metformin (26). The combination of metformin and sorafenib was also shown to be significantly toxic to radioiodine refractory anaplastic thyroid carcinoma CSCs (27). In addition, it was shown to be effective against prostate cancer stem-like cells isolated from holoclones of the PC3 cell line (28). Finally, RCC CXCR4⁺ cells, which express stem cell-associated transcription factors (*NANOG*, *OCT3/4*, and *SOX2*) at elevated levels, were reported to be more resistant to sorafenib (and sunitinib) than the parental cells in adherent cultures (9).

Axitinib (AG-013736) is a potent small-molecule inhibitor of multiple tyrosine kinases, including VEGFR-1, -2 and -3 and PDGFR β . Therefore, axitinib inhibits endothelial cell survival, new tube formation, and nitric oxide (NO), protein kinase B (PKB, Akt), and extracellular signal-regulated kinase (ERK) signaling in endothelial cells (29). It was shown that AG-013736 alone, or in combination with radiotherapy treatment, induces functional normalization of the tumor vasculature (30). In glioblastomas, in an S1-M1-80 cell line xenograft model, axitinib was shown to target a side population (SP), referred to by the authors as cancer stem-like cells. It was shown to inhibit the transporter activity of the adenosine triphosphate (ATP)-binding cassette subfamily G member 2 (ABCG2), reversing ABCG2-mediated drug resistance. In this model, axitinib (every 4 days \times 9, p.o., 25 mg/kg) enhanced the cytotoxicity of topotecan and mitoxantrone against an SP (31). Subsequently, it was also shown that axitinib exerts direct cytotoxic activity against patient-derived glioblastoma CSCs (32) and potentiates myxoma virus-based treatment directed against

brain tumor-initiating cells (33). In another study based on the PC3 cell line, holoclone-derived cancer stem-like cells (called PC3/2G7) in a prostate cancer model were shown to be sensitive to axitinib (28). Later, it was shown that 1 μ M axitinib *in vitro* increased the toxic effects of non-small cell lung cancer (NSCLC) cell irradiation respectability when applied to spheres derived from CD24/CD44⁺ NSCLC CSCs (34).

In terms of the tumor microenvironment-dependent activity of TKIs, it was first suggested that sunitinib (and possibly other VEGF inhibitory compounds) increased the population of CSCs in the tumor by generating intratumoral hypoxia. Xenograft-based breast cancer studies revealed that hypoxia-driven cancer stem or progenitor cell enrichment resulted from hypoxia-inducible factor 1 α (HIF1 α) signaling. Therefore, it was concluded that an increase in the number of hypoxia-driven CSCs limits the effectiveness of anti-angiogenic agents as a result of CSC drug resistance (35). It was multitargeted VEGFR inhibition (with sunitinib), not VEGF sequestration (with bevacizumab), that rapidly created a vascular gradient, inducing tumor hypoxia, promoting the aggressive mesenchymal phenotype, and increasing the cancer stem cell number (36). In a mouse based study, oral sunitinib administered at 80 mg/kg/2 days for 4 weeks significantly reduced the tumor volume and angiogenesis, but increased the number of CSCs in the tumors (37). We hypothesized that TKIs suppress tumor angiogenesis and tumor growth and progression via inhibition of the paracrine and autocrine effects of VEGF; however, TKI-induced tumor hypoxia may promote the CSC phenotype. Since the data on TKI activity against renal cell CSCs is not available, we aimed to verify whether renal CSCs are targeted by TKIs in an RCC model. We aimed to verify the influence under both normoxic and hypoxic conditions, which would represent conditions prior to and post-TKI exposure.

Materials and methods

Renal cell cancer-cancer stem cell isolation. Individual donor samples were selected for the study, and selected donations were used for analysis. Primary tumor tissue was obtained. The tumor samples obtained after surgery were placed immediately in tumor transportation media and shipped at 4–8°C for processing. Tissues were washed with 1X PBS solution, divided into two halves and aseptically cut into 0.5-mm sections and cultured in 6-well tissue culture plates pre-coated with extracellular matrix. First, the control half was processed in regular media with serum and was referred to as the parental cell line, while CSCs were selected in Kidney Cancer Stem Cell Complete Growth Medium (Celprogen, San Pedro, CA, USA) (38,39). All cell cultures remained viable and maintained their native architecture for at least 14 days. After 14 days of culture, the cells were characterized for stem cell phenotype.

As previously described, cancer, kidney cancer and CSC surface markers, transcription factors and epithelial-mesenchymal transition (EMT) markers (linked to the induction of a stem-cell like phenotype) were quantified by qPCR or immunocytochemistry per cell culture. These markers included: human kidney injury molecule-1 (hKIM-1), renal cell carcinoma marker (RCC-Ma), chromophobe

renal cell carcinoma (chRCC), calveolin-1 (CAV-1), carbonic anhydrase IX (CA9/CAIX), vascular endothelial growth factor (VEGF), chemokine (C-X-C motif) ligand 16 (CXCL16), A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10, CD156c), programmed death-ligand 1 (PD-L1; also known as cluster of differentiation 274, CD274 or B7 homolog 1, B7-H1), Ki-67, survivin, P53, glucose transporter 1 (GLUT-1), galactosyl transferase II (GalT-II), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cancer antigen 19 (CA19-9), cancer antigen 72-4 (CA72-4), carcinoembryonic antigen (CEA), α -fetoprotein (AFP), β -2-microglobulin (B2M), octamer-binding transcription factor 4 (OCT4), sex-determining region Y-box 2 (SOX-2), stage-specific embryonic antigen-3 and -4 (SSEA3/4), aldehyde dehydrogenase (ALDH), alkaline phosphatase (ALP), tissue-non-specific alkaline phosphatase (TRA1-81 and TRA1-61), telomerase, CD9, CD10, CD24, CD34, CD40, CD44, CD105 (endoglin), CD133 (prominin-1), CXCR-4, SHH (sonic hedgehog), epithelial cell adhesion molecule (EpCAM), epithelial-specific antigen (ESA), CBX7, SNAIL, SLUG, TWIST, Ki-67, E-cadherin, β -catenin, nestin and vimentin (38,40,41).

SCID nude mice were subcutaneously injected with 1,000 CSCs in the hind limbs. Subcutaneous tumors developed. Cells were capable of generating tumors in mice within 20 days. The mice were also evaluated for metastases. Cells tumorigenic at the above mentioned injection concentration were selected for further experiments. Stem cell marker expression was re-confirmed in the developed tumors (38,41,42).

Cells positive for Sox 2, Oct4, SSEA3/4, ALDH, ALP, telomerase, calveolin-1, CD133 and CD44, that were also capable of clonal self-renewal and were tumorigenic with <1,000 cells injected as described above, were used for further investigation. Cells were characterized and stable for markers for up to seven passages. Markers were significantly expressed at a higher level compared with the expression levels in normal kidney tissues and primary renal cancer cells (38,40,41).

Cell culture. Human kidney cancer stem cells (HKCSCs, RCC-CSCs; Celprogen, Torrance, CA, USA) were cultured in Human Kidney Cancer Stem Cell media as per the manufacturer's protocol requirements. 786-0 (CRL-1932), a primary tumor-derived VHL mutated cell line, was obtained from the American Type Culture Collection (ATCC) Global Bioresource Center (Manassas, VA, USA). The SK-RT-42 metastatic bone-derived cell line was established in the laboratory of Dr Lloyd Old, from patients undergoing nephrectomy at Memorial Hospital, Memorial Sloan Kettering Cancer Center (MSKCC) and was obtained from the Core Facility, MSKCC (New York, NY, USA) (43). The 786-0 and SK-RT-42 cell lines were cultured in RPMI-1640 medium with 10% FBS (Biochrom GmbH, Cambridge, UK) and GlutaMAX (Life Technologies, Carlsbad, CA, USA). RCC-CSCs were differentiated with RPMI-FBS under culture conditions described above. Cells were cultured under normoxic (21% O₂) and hypoxic (1% O₂) conditions. Human Kidney Cancer Stem Cell Extracellular Matrix was used to increase RCC-CSC viability (Celprogen).

3D cell culture. In the 3D approach, CSCs were grown in media as above but as aggregates and in hanging drop culture

forming spheroids as previously described (44,45), with modification of 100 cells/10 μ l drop. After formation of the spheroids (72 h), sunitinib was added in a volume of 5 μ l to each drop in order to obtain 0, 1, 2, 4, 8, and 15 μ M concentrations. Spheres were photographed under a Nikon TMS-F phase contrast microscope after 24, 48 and 72 h. The size of the colonies was analyzed using ImageJ software as previously published (46,47).

Assessment of drug toxicity. The cells were treated with TKIs, sunitinib malate, axitinib (Sigma-Aldrich, St. Louis, MO, USA) or sorafenib (Cayman Chemical Co., Ann Arbor, MI, USA) at different concentrations (0, 1, 2, 4, 6, 8, 10, 12, 15 and 20 μ M) with DMSO <0.5% (control). Subsequently IC₅₀ values of the TKIs were evaluated after 24, 48 and 72 h. The cells were also treated with TKIs under normoxic (21% O₂) or hypoxic (1% O₂) conditions. HKCSCs were seeded in 96-well 2D plates and cultured under standard conditions (37°C, 5% CO₂). After 24 h, sunitinib was added and the plates were moved to a normoxic or hypoxic incubator. Subsequently, inhibition of proliferation was quantified after 24, 48 and 72 h. Alamar blue (resazurin) (Life Technologies) assay was performed as per the manufacturer's protocol to quantitatively measure cell viability and cytotoxicity (48) which were read by microplate reader Multiskan GO and analyzed using ScanIt™ software package (Thermo Scientific, Waltham, MA USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Life Technologies) was used to assess cell viability under TKI treatment as previously published (49,50).

Results

Tyrosine kinase inhibitors target renal cell cancer-cancer stem cells. RCC-CSCs were found to be slow proliferators in comparison to stable (differentiated) renal cell cancer cells, and all TKIs (sunitinib, sorafenib, axitinib) were found to directly influence RCC-CSCs. Under TKI treatment, the proliferation rate of the RCC-CSCs was decreased (Figs. 1 and 2). The inhibition of proliferation was dose-dependent, with a significant RCC-CSC toxic minimal dose of 2 μ M TKI (Fig. 1); however, this inhibition was evident after 22 h of drug treatment at high sunitinib doses (>6 μ M) (Fig. 1A), and also significant at a low dose (2 μ M) (Fig. 1B) after 72 h of culture. The metabolic activity of the cells (seeded at least at 1x10⁴ cells/ml) growing under TKIs appeared to slow down by day 3, suggesting that the surfaces were advancing toward confluency. At a lower seeding number (starting at 500/well), the cell culture was extended for 6 days of treatment in order to prolong the observation of the exponential/linear growth phase, focusing on the effect of TKIs on the cells at their point of maximum growth (Figs. 1B and 3). The inhibition of the proliferation by sunitinib was shown to be dose-dependent (Figs. 1 and 3A), and it was determined (for RCC-CSCs) that the half-maximal inhibitory concentration (IC₅₀) for sunitinib was at the 10 μ M level (Fig. 2). The activity of axitinib against RCC-CSCs was lower than that for sunitinib. In regards to the regular ccRCC 786-0 cell line, the inhibition of cell proliferation was significant on day 6 of the axitinib treatment, while the cell growth of the RCC-CSCs was not inhibited using the same concentration of the drug (Figs. 3C vs. E and 4). A similar

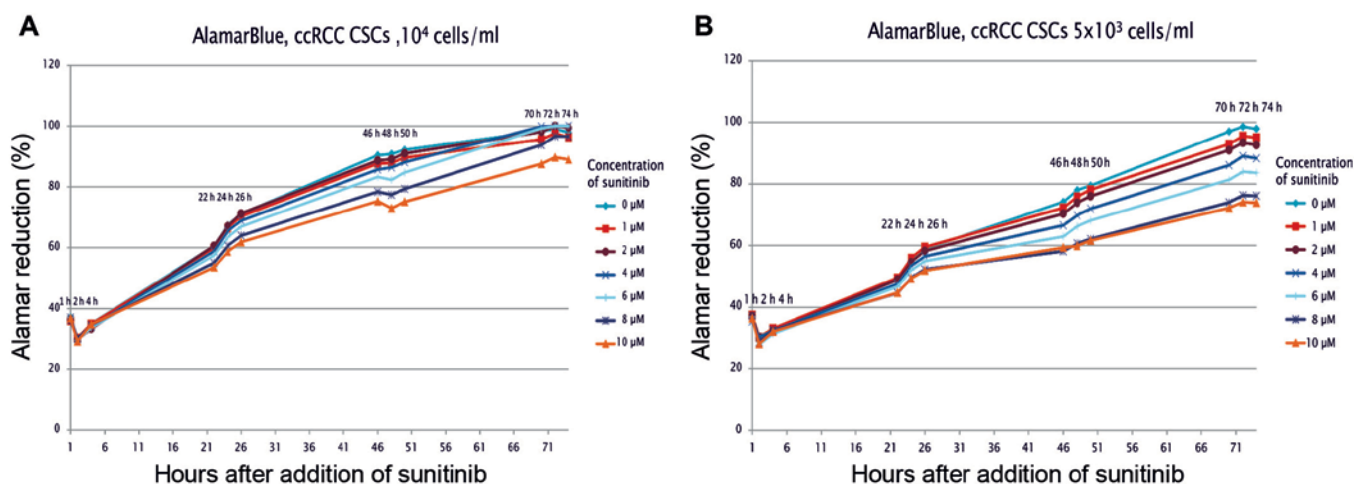


Figure 1. Sunitinib induces renal cell cancer-cancer stem cell (ccRCC-CSC) toxicity at a higher (A) and lower (B) cell seeding density.

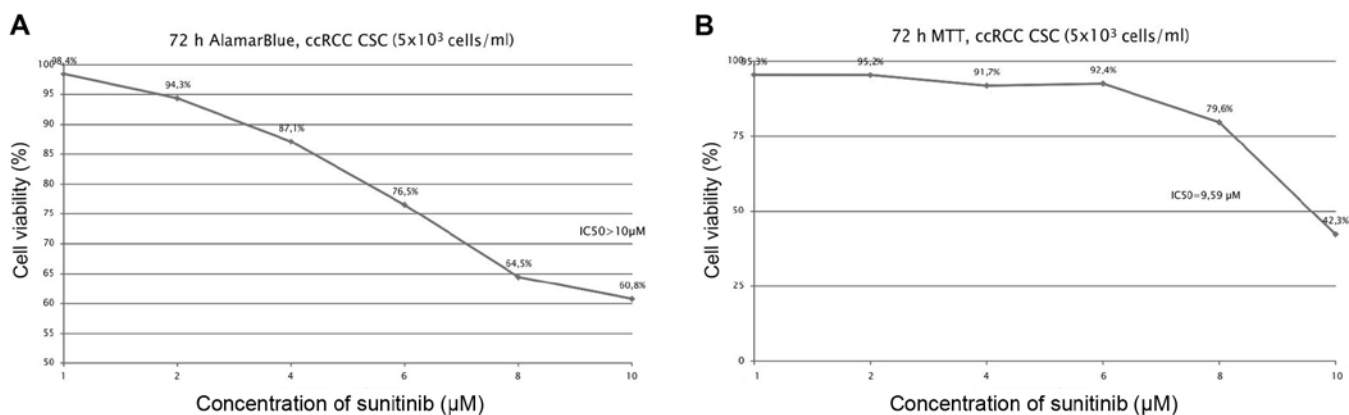


Figure 2. The half maximal inhibitory concentration (IC_{50}) of sunitinib against renal cell cancer-cancer stem cells (ccRCC-CSCs) as determined using AlamarBlue (A) and MTT (B) methods.

trend was confirmed for the SK-RC-42 metastatic cell line (data not shown).

Anti-proliferative activity of tyrosine kinase inhibitors is altered by hypoxia. The proliferation of renal cell cancer cells representing differentiated cancer cells (786-0 cells and post-RCC-CSCs), as well as RCC-CSCs/HKSCs are influenced by oxygen tension in the environment. Normoxic (21% O_2) conditions promoted the growth of the renal cancer cell lines 786-O and SM-KT-42 under no-TKI treatment (Fig. 5). Moreover, when differentiated by the overload of growth factors (FBS), RCC-CSCs also proliferated at a significantly higher rate under high oxygen availability. Differentiated post-RCC-CSCs showed a high proliferation rate, similar to that of the 786-0 cell line, and significantly higher than in RCC-CSCs in stem cell promoting conditions (Fig. 6). At the same time, RCC-CSCs under hypoxia changed their morphology and developed less invadopodia (Fig. 7D) (51), which is also typical when RCC-CSCs are cultured in ECM-rich conditions (Fig. 7A). Under a hypoxic condition, the activity of sunitinib (Fig. 8) and other TKIs was limited with the highest activity of sorafenib (Fig. 4B), and the influence of low oxygen tension was visible after 72 h in a hypoxic culture. During the

first 24 h, cells in normoxia and hypoxia proliferate at similar rates, but the reduction in sunitinib toxicity and concurrent induction of RCC-CSC proliferation under hypoxia were time-dependent (48 h; data not shown), which increased with time at low cell seeding and were visible after 6 days of culture (Fig. 3B).

The anti-proliferative activity of sunitinib decreased under a hypoxic condition, which may occur due to tumor cell-cell interactions between cancer cells, and has been investigated in tumorspheres. Additionally, the anti-proliferative activity of sunitinib also decreased in the 3D environment, which further increased the hypoxic condition, likely to occur in tumors (Fig. 9). The size of the spheres that developed in hypoxia under sunitinib treatment was larger than those developing during normoxia (Fig. 10), which further confirms the pro-proliferatory effect of hypoxia on RCC-CSCs.

Discussion

Recent achievements in the development of multi-targeted molecular inhibitors has necessitated a better understanding of the activity against individual targets with regard to their efficacy. Sunitinib, sorafenib, and axitinib are the most recently

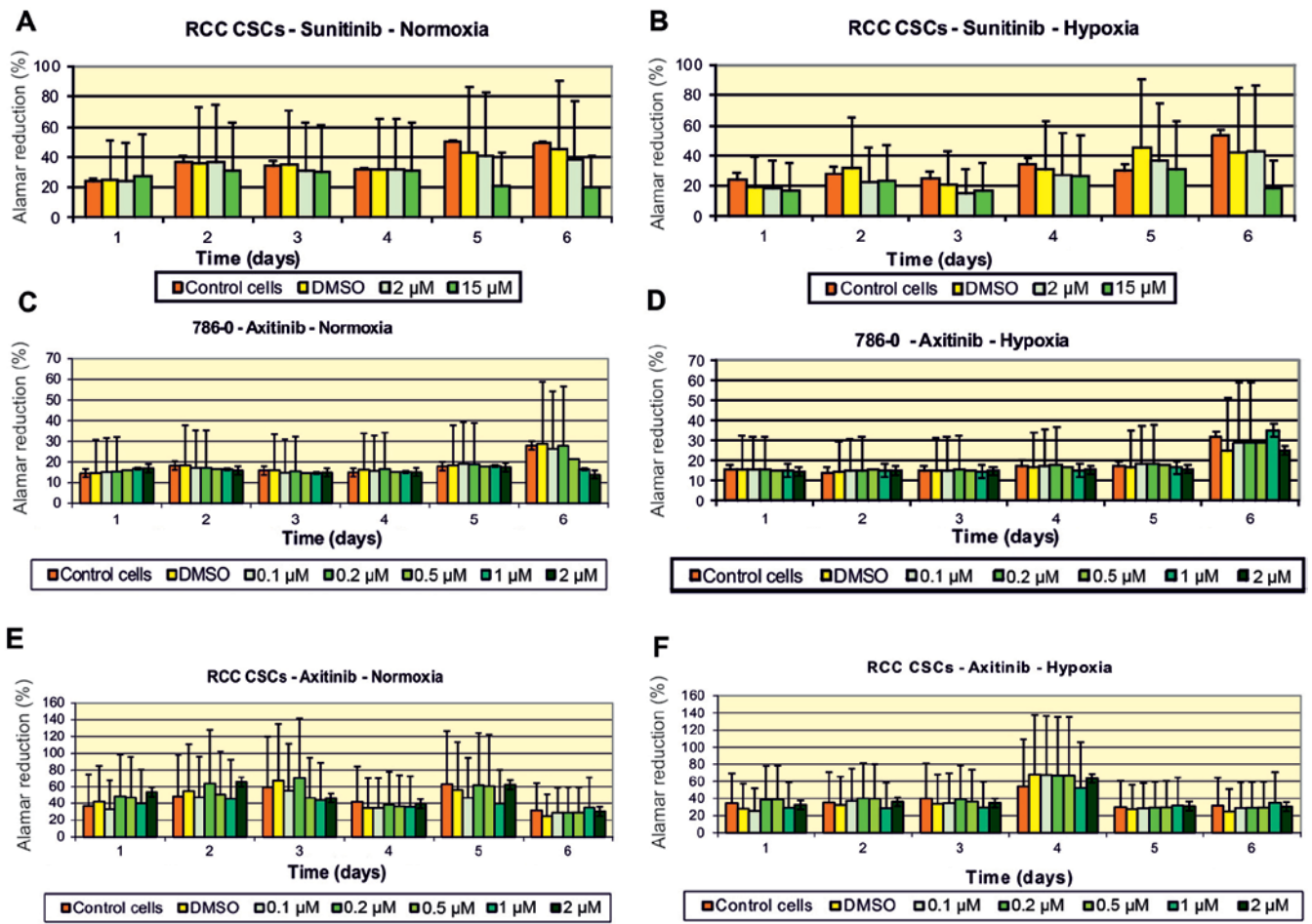


Figure 3. Sunitinib (A and B) and axitinib (C-F) induce a toxic effect on renal cell cancer 786-0 cells (C and D) and renal cell cancer-cancer stem cells (RCC-CSCs) (A, B, E and F) under hypoxia (B, D and F) and normoxia (A, C and E).

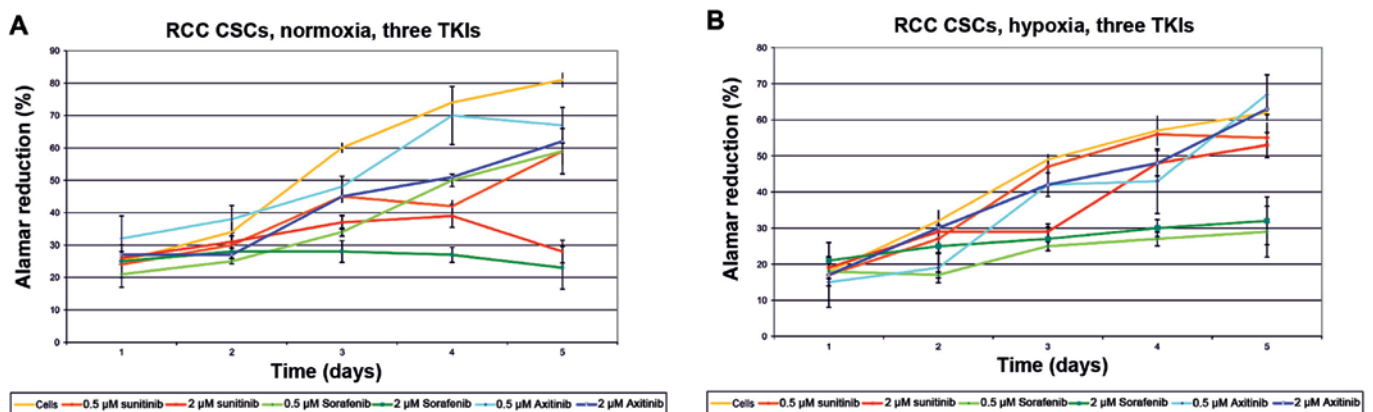


Figure 4. Relative activity of sunitinib, sorafenib and axitinib against renal cell cancer-cancer stem cells (RCC-CSCs) under normoxia (A) and hypoxia (B). TKIs, tyrosine kinase inhibitors.

identified and extensively investigated anti-angiogenic drugs (52); nevertheless, it must be kept in mind that, at a cellular level, it has been shown that TKIs target not only endothelial cells, but also RCC cancer cells, pericytes and renal stromal cells (1). The first pathological condition that was shown to be sunitinib-sensitive was acute myelogenous leukemia (AML), in which sunitinib markedly inhibited cellular proliferation, including AML-stem cell proliferation, in a dose-dependent

manner with an IC_{50} of 10-50 nM (53,54). In this study, we report the activity of TKIs against RCC-CSCs, which has not been confirmed previously.

It is known that the anti-proliferative activity is dependent on the presence of constitutively active receptor tyrosine kinase (RTK) targets. Sunitinib binds 73 kinases in addition to its main target, VEGFR-2, while sorafenib binds 40 additional kinases. However, axitinib is the most selective, with

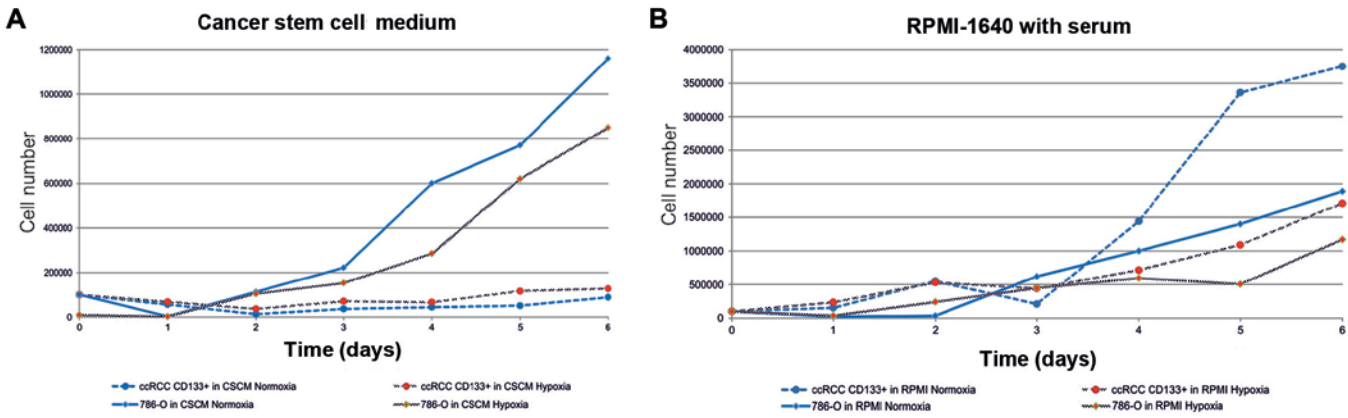


Figure 5. Proliferation of renal cell cancer-cancer stem cells and renal cancer 786-0 cells under hypoxia and normoxia under stem cell-promoting (A) and differentiating (B) conditions.

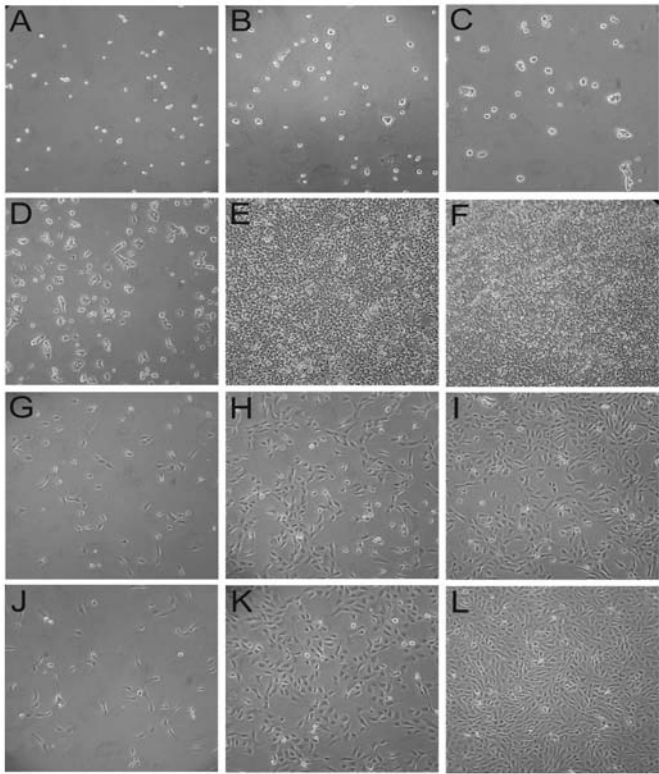


Figure 6. Proliferation of renal cell cancer-cancer stem cells (RCC-CSCs) and renal cancer cells under stem cell-promoting and differentiating conditions. RCC-CSCs in cancer stem cell medium after 24 (A), 48 (B), and 96 h (C) of culture. ccRCC CSC differentiation in RPMI-1640/FBS after 24 (D), 48 (E) and 96 h (F). 786-O cells in cancer stem cell medium after 24 (G), 48 (H) and 96 h (I) of culture; 786-O cells in RPMI-1640/FBS after 24 (J), 48 (K) and 96 h (L) of culture.

a limited number of targets (55). The activity of sorafenib in our CSC research appeared to be the highest under the harsh conditions of hypoxia. In addition, the high activity against a generally drug-resistant target (CSCs) remains in accordance with a wide spectrum of kinases that are inhibited by sorafenib, and therefore, a wider panel of anti-stem cell proliferation inhibitors and hypoxia induced genes. It is known that sorafenib targets kinases in addition to VEGFR, which are in order of increasing IC_{50} value: platelet-derived growth

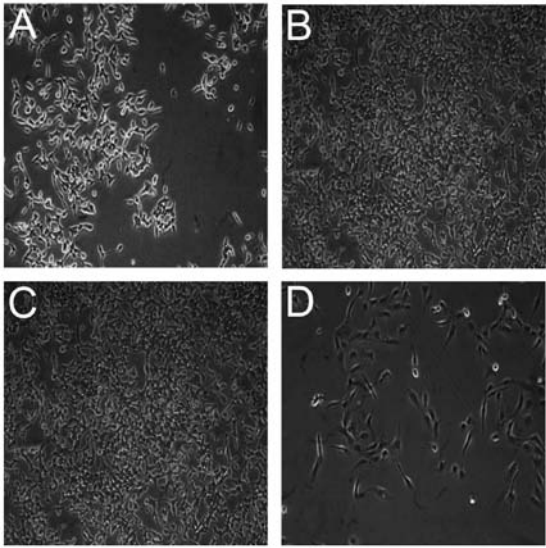


Figure 7. Morphology of renal cell cancer-cancer stem cells in ECM-rich culture (A) and under no-ECM coating (B) conditions in normoxia (B and C) and hypoxia (D).

factor receptor α (PDGFR α) \rightarrow discoidin domain receptor tyrosine kinase 2 (DDR2) \rightarrow rearranged during transfection (RET) \rightarrow homeodomain-interacting protein kinase 4 (HIPK4) \rightarrow fms-like tyrosine kinase 4 (FLT4, also known as VEGFR3) \rightarrow FLT1 (also known as VEGFR1) \rightarrow kinase insert domain receptor (KDR, also known as VEGFR2) \rightarrow PDGFR β \rightarrow RAF1 and FLT3. The IC_{50} values of sorafenib for these 10 kinases were no greater than 100-fold those against its top target. At the same time, sunitinib had 30 kinase targets within similar IC_{50} ranges, suggesting that sorafenib might be more selective against the VEGFR family (Fig. 4) (56).

In vitro, sunitinib inhibits the growth of cancer cell lines driven by VEGF, SCF and PDGF, and also induces apoptosis of vein endothelial cells (57); however, a full understanding of the targets and mechanism of action of sunitinib, as well as the other TKIs in ccRCC treatment, remains incomplete. To complement this activity data, we showed direct inhibition of the ccRCC-CSC growth in a dose-dependent manner (Figs. 1, 2 and 4). At the same time, axitinib that was

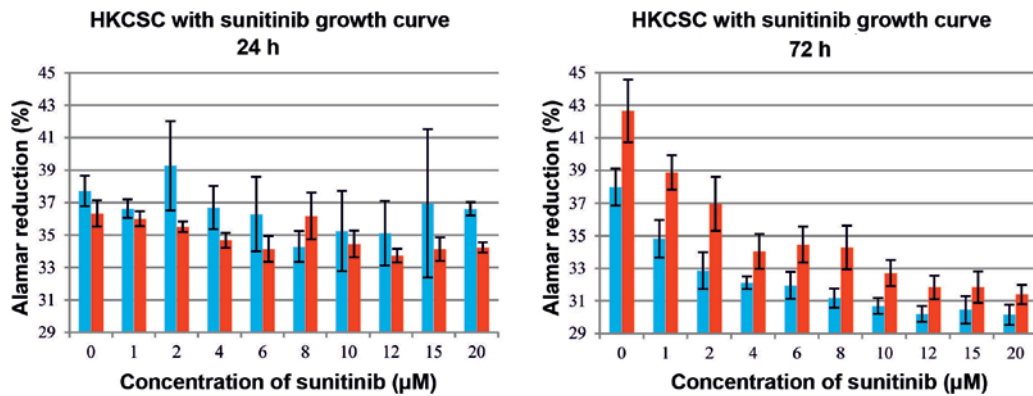


Figure 8. Proliferation of renal cell cancer-cancer stem cells (HKCSCs) under hypoxia (red) and normoxia (blue) after 24 (A) and 72 h (B) of sunitinib treatment.

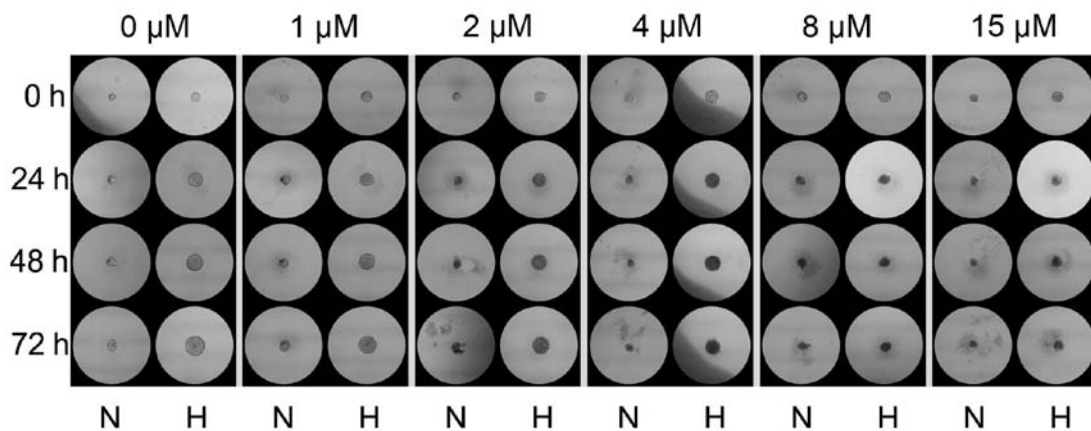


Figure 9. Inhibition of 3D renal cell cancer-cancer stem cell spheroid formation under hypoxia (H) and normoxia (N) after 24, 48 and 72 h of sunitinib treatment.

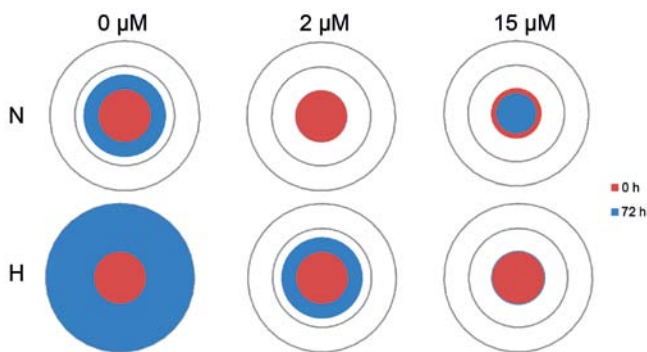


Figure 10. Mean size of spheroids formed by renal cell cancer-cancer stem cells under normoxia (blue) or hypoxia (red) in comparison to maximal spheroid size developed (grey line) - developed without and under sunitinib treatment (0, 2 and 15 μM).

found to have lower activity against RCC-CSCs (Fig. 3) was shown to have an 8- to 25-fold higher IC_{50} against PDGFR- β , KIT, and PDGFR- α (1.6-2.0 nmol/l) and a significantly lower activity against FGFR-1, Flt-3 and RET (1 μmol/l) (29). This specificity may be contradictory to the phenotype of RCC-CSCs and their proliferation triggering pathways.

Our results with regard to the direct activity of TKIs against RCC cancer cells are confirmed by other *in vitro* and animal studies in which sunitinib targeted the tumor cells themselves, since these cells express one or more target RTKs, including the human kidney cancer 786-O cell line which we used as the control (58). In 786-O cells, the PDGFR β is highly constitutively activated and VEGFR-2 expression is upregulated (58); therefore, it is a target of TKIs. Similar gene expression deregulation has been reported in RCC tumors in clinical reports; for example, PDGFR β and VEGFR-2 were reported to be overexpressed in RCCs, relative to normal renal tissues (11). Therefore, we suggest that the multi-targeted inhibition of tyrosine kinase by sunitinib/sorafenib/axitinib contributes to its anti-proliferative effects against ccRCC-CSCs, and may contribute to its clinical efficacy in RCC (Figs. 4, 8 and 10).

The concentrations that are being described as inhibiting RCC-CSCs (Figs. 2, 3 and 7) are in the range that is found within RCC tumors in patients. For example, the intra-tumoral concentrations of sunitinib in mice and human patients are 10.9 ± 0.5 and 9.5 ± 2.4 μmol/l, respectively, whereas the serum measured concentrations are 10-fold lower, and described as 1.0 ± 0.1 and 0.3 ± 0.1 μmol/l, respectively (59). The serum concentration of sunitinib was similar to that in other healthy organs, including the skin, where the concentration was measured at a level of 0.1 to 0.4 μM (60). The high concentra-

tion of TKIs in RCC tumors has been investigated, and was determined to result in multiple codependent mechanisms. The TKIs extravasate into the tumor's extracellular matrix (ECM), while the decrease in the tumor interstitial fluid pressure that arises as a result of anti-angiogenesis slows the leakage of TKIs from the tumor, leading to longer TKI retention in the tumor. At the same time, as the blood flow to the tumor decreases in response to anti-angiogenesis, the blood flow out of the tumor may also decrease. In particular, axitinib was shown to slow the drug efflux from tumors (61); as a result, sunitinib inhibits the tumor cell growth at clinically relevant concentrations *in vitro*, with IC_{50} values of at least 1.4 to 2.3 μM (59). In addition, in RCC cell lines, the anti-proliferative effects of sorafenib are both concentration- and time-dependent, as is the case in our RCC-CSCs. The calculated IC_{50} of sorafenib was in the range of 7.5-10 μM , depending on the RCC cell line, and sorafenib-induced RCC cell apoptosis was reported after 48-72 h of treatment (62).

In terms of the RCC cell targeted activity of TKIs, it was shown that the short-term (24 h) application of sunitinib in renal cell carcinoma Caki-1 and KTC-26 cell lines induced cell growth inhibition, which was halted in the M and G₂ phases. The signs of anti-RCC cell toxicity became apparent when the cells were exposed to 10 μM of sunitinib; additionally, sorafenib caused a distinct downregulation of the tumor cell number at a dosage of $\geq 5 \mu M$ (63). However, when RCC cells were exposed to 1 μM sunitinib for 8 weeks (equivalent to a 1.3 treatment cycle) cdk1 and cdk2 were overexpressed, p27 was downregulated, and Akt, Rictor, and Raptor were activated (63). It was previously shown that the wild-type pVHL expressing CAKI-1 and 786-0-VHL (VHL-transfected) cells were ~2-fold more resistant to the anti-proliferative effects of sorafenib (2.5-20 μM) under hypoxic conditions, when compared with the mutant pVHL expressing CAKI-2 and 786-0 cells. Such a difference was not reported under normoxia (62). The phenomenon of TKI activity dependence on tumor niche oxygen availability is also true for RCC-CSCs (Figs. 3, 6 and 7). In order to explain this phenomenon in RCCs, the gene expression in the 786-0-VHL cells (wt VHL transfected) vs. the 786-0-neo cells (VHL mutant) were investigated. As many as 40 genes, mostly related to the inhibition of apoptosis (e.g., BAG1-bcl2-associated athanogene) and angiogenesis (e.g., PDGF β), were shown to be >5-fold overexpressed under hypoxia in the RCC model. These genes, including BAG-1 and PDGF β , were downregulated >2-fold under hypoxia with sorafenib treatment in both cell types. It was finally concluded that wt-VHL ccRCC under hypoxic (1% O₂) conditions promoted the overexpression of anti-apoptotic and pro-angiogenic genes that attenuated the anti-proliferative effects of sorafenib (62). In our study, hypoxia limited the efficacy of the TKIs in both the 3D model and RCC-CSC proliferation and influenced their morphology, which further confirmed the significant role of oxygen in tumor development (Figs. 7, 8 and 10).

Anti-angiogenic agents generate intratumoral hypoxia, modulating the metastatic process and stimulating cancer stem cells (CSCs) (37). Currently, determining the most effective application of TKIs clinically in RCC treatment is imperative to investigate the mechanisms underlying their efficacy. It remains to be determined whether the *in vivo* efficacy of compounds such as sunitinib, sorafenib or axitinib can be

explained in terms of its inhibition of one (or a combination) of its targets in various tumor cells. As an example of this approach, the primary goal of our study was to describe the action of these TKIs *in vitro*, but in a clinically relevant model or RCC-CSCs. The findings presented here not only broaden our understanding of the role of hypoxia in RCC-CSC biology, but may have significant clinical implications, since angiogenesis has been a long-standing therapeutic target in ccRCC.

Acknowledgements

This project was supported by the Foundation for Polish Science Team project TEAM/2010-6/8. Scribendi proof-reading service (Scribendi Inc. Chatham, ON, Canada) was covered by A.M.C.

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