Abstract:
The invention relates to methods for treating a patient with VEGFR inhibitor-resistant metastatic renal cell carcinoma (mRCC). More particularly, the invention relates to a drug or a combination of drugs selected from the group consisting of a protea - some inhibitor, an efflux inhibitor or a combination of an efflux inhibitor and a lysosomotropic agent for use in a method for treating mRCC in a patient with an acquired resistance to treatment with a multi-targeted weak base VEGFR tyrosine kinase inhibitor (TKI).
METHODS FOR TREATING A PATIENT WITH VEGFR INHIBITOR-RESISTANT METASTATIC RENAL CELL CARCINOMA

FIELD OF THE INVENTION:
The invention relates to methods for treating a patient with VEGFR inhibitor-resistant metastatic renal cell carcinoma (mRCC).

BACKGROUND OF THE INVENTION:
Metastatic Renal Cell Carcinomas (mRCC) are highly vascularized tumors that are a paradigm for the treatment with anti-angiogenesis drugs particularly with tyrosine kinase inhibitors targeting the Vascular Endothelial Growth Factor (VEGF) pathway. The available drugs increase the time to progression but are not curative and the patients eventually relapse. These inhibitors include notably sunitinib, which since 2006 is considered as the standard first line treatment option for this disease. Sunitinib has been designed to disrupt major signaling pathways (HRAS/RAF1/MAP2K1, 2/MAPK1, 3 and MTOR pathways) that are responsible for the abnormal proliferation of cancer cells and tumor angiogenesis. However, sunitinib has not significantly improved the overall survival of the majority of patients compared to treatment with interferon alpha or interleukin 2 (median time of survival after the diagnosis of about 20 months), the standard treatments used before the development of anti-angiogenesis drugs. Moreover, the fact that mRCC patients gradually become refractory to sunitinib represents an important obstacle to better outcome for patients.

Therefore, it is urgent to better understand the molecular mechanisms associated with resistance to sunitinib to improve the final outcome of the patients. In this context, the fate of tumor cells following chronic exposure to the drug has been analyzed. The selection pressure exerted by chronic exposure has led to the selection of resistant cells, but the mechanisms inducing resistance are unknown.

It was previously reported that sunitinib induced autophagy in bladder cancer cells and that inhibition of autophagy potentiated the anti-proliferative effects of sunitinib. However, in these experiments, cells were exposed to high doses of sunitinib and the cells were not representative of cancers for which sunitinib is the treatment of reference. Moreover, these reports did not investigate the molecular link between sunitinib treatment and autophagy. Lysosomal sequestration of sunitinib may be explained by the fact that it is a hydrophobic weak base (pKa 8.95). Sequestration in lysosomes may prevent access of the
drug to the kinase domain of tyrosine kinase receptors present in the cytoplasm, thus participating in the loss of efficacy of the drug. However, the effect of sunitinib on autophagy has not been described for mRCC cells. Furthermore, the implication of autophagy and lysosome trapping in the mechanisms of resistance has not been addressed.

**SUMMARY OF THE INVENTION:**

In a first aspect, the invention relates to a drug or a combination of drugs selected from the group consisting of a proteasome inhibitor, an efflux inhibitor or a combination of an efflux inhibitor and a lysosomotropic agent for use in a method for treating metastatic renal cell carcinoma (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI).

In a second aspect, the invention relates to a method for predicting the survival time of a patient affected with RCC or mRCC comprising the steps of: (i) determining the expression level of at least one of the proteasome component genes selected from the group consisting of PSMB8, PSMB9, PSMB10 and PSMF1 genes in a primary renal tumor biopsy obtained from said patient; (ii) comparing the level determined at step (i) with a predetermined reference level; and (iii) concluding that the patient has a poor prognosis when the level determined at step (i) is higher than the predetermined reference level.

In a third aspect, the invention relates to a combination comprising a multi-targeted weak base VEGFR TKI and a proteasome inhibitor or a combination comprising a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use in a method for treating RCC or mRCC and/or preventing metastasis in a patient determined as having a poor prognosis according to the method of the invention.

In a fourth aspect, the invention relates to a method for determining whether a patient affected with RCC or mRCC has or is at risk of multi-targeted weak base VEGFR TKI-resistance, comprising a step of determining the expression level of at least one of the proteasome component genes selected from the group consisting of PSMB8, PSMB9, PSMB10 and PSMF1 genes in a primary renal tumor biopsy obtained from said patient; (ii) comparing the level determined at step (i) with a predetermined reference level; and (iii) concluding that the patient has or is at risk of multi-targeted weak base VEGFR TKI-resistance when the level determined at step (i) is higher than the predetermined reference level.
In a fifth aspect, the invention relates to a combination comprising a multi-targeted weak base VEGFR TKI and a proteasome inhibitor or a combination comprising a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use in a method for treating RCC or mRCC and/or preventing metastasis and/or preventing acquired resistance to treatment with a multi-targeted weak base VEGFR TKI in a patient determined as having or being at risk of multi-targeted weak base VEGFR TKI-resistance according to the method of the invention.

**DETAILED DESCRIPTION OF THE INVENTION:**

The invention addresses these needs, as it relates to methods and treatment approaches useful in the treatment of VEGFR inhibitor-resistant metastatic renal cell carcinoma (mRCC).

The inventors have indeed focused their attention on the molecular mechanisms leading to resistance to sunitinib, the first line treatment of mRCC. Because of the anarchic vascularization of tumors the core of mRCC tumors receives sub-optimal concentrations of the drug. To mimic this *in vivo* situation, which is encountered in a neo-adjuvant setting, they exposed sunitinib-sensitive mRCC cells to concentrations of sunitinib below the concentration of the drug that gives 50% inhibition of cell proliferation (IC50). At these concentrations, sunitinib accumulated in lysosomes, which down-regulated the activity of the lysosomal protease cathepsin B and led to incomplete autophagic flux. Amino acid deprivation, which initiates autophagy enhanced sunitinib resistance through the amplification of auto-lysosome formation. Sunitinib stimulated the expression of ABCB1, which participates in the accumulation of the drug in auto-lysosomes and favor its cellular efflux. Inhibition of this transporter by elacridar and the permeabilization of lysosome membranes with Leu-Leu-O-Methyl (LLOM) re-sensitized mRCC cells that were resistant to concentrations of sunitinib superior to the IC50. Proteasome inhibitors also induced the death of resistant cells suggesting that the ubiquitin-proteasome system compensates inhibition of autophagy to maintain a cellular homeostasis.

Based on the present results, they propose a new therapeutic approach combining sunitinib with molecules that prevent lysosomal accumulation or inhibit the proteasome.

Accordingly, a first aspect of the invention relates to a drug or a combination of drugs selected from the group consisting of a proteasome inhibitor, an efflux inhibitor or a
combination of an efflux inhibitor and a lysosomotropic agent for use in a method for treating metastatic renal cell carcinoma (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI).

As used herein, the terms "proteasome inhibitor" or "inhibitor of the ubiquitin-proteasome system" (UPS) refers to compounds which inhibits the activity of the proteasome, more particularly its enzymatic activity. Inhibiting UPS enzymatic activity means reducing the ability of a UPS component to perform its activity.

Such compounds include, but are not limited to, bortezomib (also known as PS-341), MLN 341, carfilzomib, delanzomib (also known as CEP-18770) and MG132.

In one embodiment of the invention, the proteasome inhibitor is bortezomib. Bortezomib is commercially available from Millennium under the trade name Velcade® and may be prepared for example as described in EP788360, EP1312609, EP1627880, US 6066730 and US 6083903 or by processes analogous thereto.

As used herein, the term "efflux inhibitor" refers to compound that inhibits the expression and/or activity of at least one transport protein (e.g., a P-Glycoprotein (P-GP)).

In one embodiment of the invention, the efflux inhibitor is a P-Glycoprotein (ABCB1) inhibitor. In a particular embodiment of the invention, the P-Glycoprotein (ABCB1) inhibitor is selected from the group consisting of elacridar, biricodar, pantoprazole, and tariquidar.

As used herein, the term "lysosomotropic agent" refers to a compound, which diffuses into cellular lysosomes, causes a decrease in the lysosome transmembrane proton gradient, and increases the pH inside the organelle.

In one embodiment of the invention, the lysosomotropic agent is selected from the group consisting of Leu-Leu-OMe (LLME), chloroquine, hydroxychloroquine, 3-methyladenine, quinacrine, mefloquine, monensin and bafilomycin Al.
As used herein, the terms "combination" refers to a "kit-of-parts" in the sense that the combination partners as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners, i.e. simultaneously or at different time points. The parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partners to be administered in the combined preparation can vary. The combination partners can be administered by the same route or by different routes. When the administration is sequential, the first partner may be for instance administered 1, 2, 3, 4, 5, 6, 7, days before the second partner.

As used herein, the term "treatment" refers to an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. Within the context of the invention, the term "treatment" may also mean prolonging survival as compared to expected survival if not receiving treatment.

As used herein, the term "vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI)" refers to a compound which lead to the intracellular inhibition of VEGF signaling pathway by targeting the intracellular kinase domains of the three VEGFRs (VEGF receptor [VEGFR]-1, VEGFR-2, and VEGFR-3).

As used herein, the term "multi-targeted VEGFR TKI" refers to a compound which inhibits several kinase targets in addition to the VEGFRs (e.g. platelet-derived growth factor receptor [PDGFR], stem cell factor receptor [c-kit] and FMS-like tyrosine kinase-3 [Flt3]).

As used herein, the term "weak base" refers to a compound which in an aqueous environment exists in an equilibrium between a neutrally charged basic form which is a proton acceptor (also referred to as a "non-protonated" form) and a positively charged conjugate acid form which is a proton donor (also referred to as a "protonated" form). In one embodiment of the invention, the pKa is higher than a typical physiological pH, for example
in a range from 7-12, and preferably from 8-10, such that the weak base exists predominantly in a positively charged (protonated) form at both a lysosomal pH and at the physiological pH.

In one embodiment of the invention, the multi-targeted weak base VEGFR TKI is selected from the group consisting of sunitinib, axitinib and dovitinib.

In one particular embodiment of the invention, said targeted weak base VEGFR TKI is sunitinib.

As used herein, the term "sunitinib" (also known as SU-1248 and marked as Sutent®) refers to a member of a family of pyrrole substituted 2-indolinone compounds, reported as being receptor tyrosine kinase inhibitors (see, U.S. Pat. Nos. 6,573,293 and 7,211,600). Sunitinib inhibits cellular signaling via a variety of receptors which play a role in tumor angiogenesis and tumor cell proliferation. Hence, the simultaneous inhibition of these receptors promotes reduced tumor vascularization and cancer cell death.

As used herein, the term "drug resistant" refers to a condition which demonstrates acquired resistance. With "acquired resistance" is meant a multifactorial phenomenon occurring in tumor formation and progression that can influence the sensitivity of cancer cells to a drug. Acquired resistance may be due to several mechanisms such as but not limited to; alterations in drug-targets, decreased drug accumulation, alteration of intracellular drug distribution, reduced drug-target interaction, increased detoxification response, cell-cycle deregulation, increased damaged-DNA repair, and reduced apoptotic response. Several of said mechanisms can occur simultaneously and/or may interact with each other.

Various qualitative and/or quantitative methods may be used to determine if a patient has developed or is susceptible to developing a resistance to treatment with a multi-targeted weak base VEGFR TKI such as sunitinib. For example, a patient who showed initial improvement while taking a multi-targeted weak base VEGFR TKI, may display signs that the multi-targeted weak base VEGFR TKI has become less effective or is no longer effective. Symptoms that may be associated with resistance to a multi-targeted weak base VEGFR TKI include, for example, a decline or plateau of the well-being of the patient, an increase in the size of a tumor, arrested or slowed decline in growth of a tumor, and/or the spread of cancerous cells in the body from one location to other organs, tissues or cells.
A decrease in the sensitivity of cancer cells to a multi-targeted weak base VEGFR TKI, an increase in the growth or proliferation of cancer cells, and/or a decrease in cancer cell apoptosis as compared to a control, may also be indicative that the patient has developed or is susceptible to developing a resistance to a multi-targeted weak base VEGFR TKI. It is possible to determine cancer cell sensitivity, growth, proliferation or apoptosis using standard methods as described further herein. For example, cancer cell sensitivity, growth, proliferation or apoptosis may be determined either in situ or in vitro.

In situ measurements may involve, for example, observing the effect of a multi-targeted weak base VEGFR TKI therapy in a patient by examining cancer growth or metastasis. Typically, for mRCC patients, RECIST criteria are analyzed.

As used herein, the term "Response Evaluation Criteria In Solid Tumors (RECIST)" refers to a set of published rules that define when cancer patients improve ("respond"), stay the same ("stable") or worsen ("progression") during treatments. The original criteria were published in February 2000 by an international collaboration including the European Organization for Research and Treatment of Cancer (EORTC), National Cancer Institute (NCI) of the United States and the National Cancer Institute of Canada Clinical Trials Group. RECIST 1.1, published in January 2009, is an update to the original criteria. Usually, the skilled in the art concludes that the disease progresses (and hence that the patient is or is become resistant to a treatment) when at least a 20% increase in the sum of the longest diameter of target lesions, taking as reference the smallest sum longest diameter recorder since the treatment started or the appearance of one or more new lesions) by conventional methods of imaging such as computed tomography (CT).

Within the context of the invention, and according to the RECIST criteria applied to mRCC patients, a patient is considered as resistant when at least a 30% increase of metastases is detected in said patient by \(^{18}\text{F}\)fluoro-2-deoxy-2-d-glucose (FDG) positron emission tomography (PET) imaging (FDG-PET scan).

In one embodiment of the invention, the patient with an acquired resistance is still under multi-targeted weak base VEGFR TKI treatment.
In another aspect, the invention relates to a method for treating metastatic renal cell carcinoma (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI) comprising the followings steps of: a) selecting a patient with mRCC who has developed a resistance to treatment with a VEGFR, and b) administering to said patient an therapeutically effective amount of a drug or a combination of drugs selected from the group consisting of a proteasome inhibitor, an efflux inhibitor or a combination of an efflux inhibitor and a lysosomotropic agent.

By "therapeutically effective amount" is meant an amount sufficient to achieve a concentration of compound which is capable of preventing or slowing down the disease to be treated. Such concentrations can be routinely determined by those of skilled in the art. The amount of the polypeptide actually administered will typically be determined by a physician or a veterinarian, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the patient, the severity of the subject's symptoms, and the like. It will also be appreciated by those of skilled in the art that the dosage may be dependent on the stability of the administered compound.

The compounds of the invention may be administered by any means that achieve the intended purpose. For example, administration may be achieved by a number of different routes including, but not limited to, subcutaneous, intravenous or parenteral, intramuscular, intraperitoneal or oral routes. Parenteral route is particularly preferred.

Dosages to be administered depend on individual needs, on the desired effect and the chosen route of administration. It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment. For example, it is well within the skill of the art to start doses of the compounds at levels lower than those required to
achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the polypeptides may be varied over a wide range from 0.01 to 1.000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 10 mg/kg of body weight per day.

In a second aspect, the invention relates to a method for predicting the survival time of a patient affected with RCC or mRCC comprising the steps of: (i) determining the expression level of at least one of the proteasome component genes selected from the group consisting of PSMB8, PSMB9, PSMB10 and PSMF1 genes in a primary renal tumor biopsy obtained from said patient; (ii) comparing the level determined at step (i) with a predetermined reference level; and (iii) concluding that the patient has a poor prognosis when the level determined at step (i) is higher than the predetermined reference level.

In a third aspect, the invention relates to a method for determining whether a patient affected with RCC or mRCC has or is at risk of multi-targeted weak base VEGFR TKI-resistance, comprising (i) a step of determining the expression level of at least one of the proteasome component genes selected from the group consisting of PSMB8, PSMB9, PSMB10 and PSMF1 genes in a primary renal tumor biopsy obtained from said patient; (ii) comparing the level determined at step (i) with a predetermined reference level; and (iii) concluding that the patient has or is at risk of multi-targeted weak base VEGFR TKI-resistance when the level determined at step (i) is higher than the predetermined reference level.

As used herein, the term "Proteasome subunit beta type-8 (PSMB8) gene" encodes a protein of 204 amino acids, member of the proteasome B-type family, also known as the TIB family, which is a 20S core beta subunit. This term includes naturally occurring PSMB8 and variants thereof. The naturally occurring human PSMB8 protein has an amino acid sequence as shown in UniProt Accession number P28062 and is encoded by the nucleic acid sequence provided in the GenBank database under accession number NM_004159.
As used herein, the term "Proteasome subunit beta type-9 (PSMB9) gene" encodes a protein of 199 amino acids, member of the proteasome B-type family. This term includes naturally occurring PSMB9 and variants thereof. The naturally occurring human PSMB9 protein has an amino acid sequence as shown in UniProt Accession number P28065 and is encoded by the nucleic acid sequence provided in the GenBank database under accession number NM_002800.

As used herein, the term "Proteasome subunit beta type-9 (PSMBIO) gene" encodes a protein of 234 amino acids, member of the proteasome B-type family. This term includes naturally occurring PSMBIO and variants thereof. The naturally occurring human PSMBIO protein has an amino acid sequence as shown in UniProt Accession number P40306 and is encoded by the nucleic acid sequence provided in the GenBank database under accession number NM_002801.

As used herein, the term "Proteasome inhibitor PI31 subunit (PSMFI) gene" encodes a protein of 270 amino acids that inhibits the activation of the proteasome by the 11S and 19S regulators. This term includes naturally occurring PSMFI and variants thereof. The naturally occurring human PSMFI protein has an amino acid sequence as shown in UniProt Accession number Q92530 and is encoded by the nucleic acid sequence provided in the GenBank database under accession number NM_006814.

As used herein, the term "predetermined reference level" refers to the expression levels of PSMB8, PSMB9, PSMBIO and PSMFI genes in biological samples obtained from the general population or from a selected population of patients. For example, the selected population may be comprised of apparently RCC patients who had any sign or symptoms indicating the presence of a multi-targeted weak base VEGFR TKI-resistance. A predetermined reference level may be determined, for example, by determining the expression level of PSMB9, PSMBIO and PSMFI nucleic acids or encoded polypeptides, in a corresponding biological sample obtained from one or more control patient(s) (e.g., no resistance or known not to be resistant). When such a predetermined reference level is used, a higher or increased levels determined in a biological sample (i.e. a primary renal tumor biopsy obtained from the patient to be tested) is indicative for example that said patient has or is at risk of having has or is at risk of multi-targeted weak base VEGFR TKI-resistance. The
predetermined reference level may be established based upon comparative measurements between patients classified with no resistance and patients with established resistance.

The predetermined reference value can be a threshold value or a range. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. For example, retrospective measurement of the expression level of the gene(s) of interest in properly banked historical subject samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the expression level of the gene(s) of interest in a group of reference, one can use algorithmic analysis for the statistic treatment of the expression levels determined in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests.

As used herein, a "higher" or "increased" level refers to a expression level in a biological sample (i.e. a primary renal tumor biopsy obtained from the patient) which is at least 20% higher, in an embodiment at least 30% higher, in a further embodiment at least 40% higher; in a further embodiment at least 50% higher, in a further embodiment at least 100% higher (i.e. 2-fold), in a further embodiment at least 200% higher (i.e. 3-fold), in a further embodiment at least 300% higher (i.e. 4-fold), relative to the predetermined reference level.

Methods for determining the expression level of the biomarker of the invention:

Determination of the expression level of PSMB8, PSMB9, PSMB10 and/or PSMF1 genes may be performed by a variety of techniques. Generally, the expression level as determined is a relative expression level. For example, the determination comprises contacting the biological sample with selective reagents such as probes, primers or ligands, and thereby detecting the presence, or measuring the amount, of polypeptide or nucleic acids of interest originally in said biological sample. Contacting may be performed in any suitable device, such as a plate, micro titer dish, test tube, well, glass, column, and so forth. In specific
embodiments, the contacting is performed on a substrate coated with the reagent, such as a nucleic acid array or a specific ligand array. The substrate may be a solid or semi-solid substrate such as any suitable support comprising glass, plastic, nylon, paper, metal, polymers and the like. The substrate may be of various forms and sizes, such as a slide, a membrane, a bead, a column, a gel, etc. The contacting may be made under any condition suitable for a detectable complex, such as a nucleic acid hybrid or an antibody-antigen complex, to be formed between the reagent and the nucleic acids or polypeptides of the biological sample.

As used herein, the term "detecting" includes qualitative and/or quantitative detection (i.e. detecting and/or measuring the expression level) with or without reference to a control or a predetermined value. As used herein, "detecting" means determining if PSMB8, PSMB9, PSMBIO and/or PSMFI is present or not in a biological sample and "measuring" means determining the amount of PSMB8, PSMB9, PSMBIO and/or PSMFI in a biological sample. Typically the expression level may be determined for example by RT-PCR or immunohistochemistry (IHC) performed on a primary renal tumor biopsy.

In a particular embodiment, the expression level of PSMB8, PSMB9, PSMBIO and/or PSMFI genes may be assessed by determining the quantity of mRNA.

Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the biological samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e.g., Northern blot analysis) and/or amplification (e.g., RT-PCR). Quantitative or semi-quantitative RT-PCR is preferred. Real-time quantitative or semi-quantitative RT-PCR is particularly advantageous.

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical.
In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization. A wide variety of appropriate indicators are known in the art including, fluorescent, radioactive, enzymatic or other ligands (e.g., avidin/biotin).

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are "specific" to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature Tm, e.g., 50% formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above-mentioned methods may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A particular kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

Accordingly, the invention also relates to a kit for performing the above-mentioned methods, wherein said kit comprises means for determining the expression level of the PSMB8, PSMB9, PSMB10 and/or PSMF1 genes in a biological sample of interest.

In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from a primary renal tumor biopsy and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

In another particular embodiment, the expression level of PSMB8, PSMB9, PSMB10 and/or PSMF1 genes may be assessed by determining the quantity of proteins encoded by said genes.

Such methods comprise a step of contacting the biological sample with a binding partner capable of selectively interacting with a marker protein present in the biological sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal. Monoclonal antibodies directed against PSMB8, PSMB9, PSMB10
and/or PSMF1 are well known from the skilled man in the art such as the antibodies commercialized by

The presence of PSMB8, PSMB9, PSMB10 and/or PSMF1 proteins may be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labelled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; Immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith. Labels are known in the art that generally provide (either directly or indirectly) a signal (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE)).

Typically, an immunohistochemistry (IHC) method may be used on a primary renal tumor biopsy. IHC specifically provides a method of detecting a target protein in a biological sample or tissue specimen in situ. The overall cellular integrity of the biological sample is maintained in IHC, thus allowing detection of both the presence and location of the target of interest. Typically a biological sample is fixed with formalin, embedded in paraffin and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labelling or secondary antibody-based or hapten-based labelling. Examples of known IHC systems include, for example, EnVision™ (DakoCytomation), Powervision® (Immunovision, Springdale, AZ), the NBA™ kit (Zymed Laboratories Inc., South San Francisco, CA), HistoFine® (Nichirei Corp, Tokyo, Japan).

In another aspect, the invention relates to a combination comprising a multi-targeted weak base VEGFR TKI and a proteasome inhibitor or a combination comprising a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use in a method for treating RCC or mRCC and/or preventing metastasis in a patient determined as having a poor prognosis according to a method of the invention as above-defined.

In one embodiment of the invention, the combination further comprises a lysosomotropic agent as previously described.
In still another aspect, the invention relates to a combination comprising a multi-targeted weak base VEGFR TKI and a proteasome inhibitor or a combination comprising a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use in a method for treating RCC or mRCC and/or preventing metastasis and/or preventing acquired resistance to treatment with a multi-targeted weak base VEGFR TKI in a patient determined as having or being at risk of multi-targeted weak base VEGFR TKI-resistance according to a method of the invention as above-defined.

In one embodiment of the invention, the combination further comprises a lysosomotropic agent as previously described.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

**FIGURES:**

**Figure 1:** A sunitinib concentration below the IC50 slowed down cell proliferation but did not induce cell death. (A) General scheme illustrating the different concentrations to which RCC cells may be exposed to in a tumor. (B) The proliferative capacity of 786-0 cells in the absence (Ct) or presence of increasing concentrations of sunitinib (sun) was evaluated by counting the cells at the indicated times. Data are the mean fold increase ± SD. The fold increase of untreated cells was taken as the reference value for statistics. Statistical significances of the results compared to untreated cells are indicated; *p<0.05; **p <0.01; ***p < 0.001. (C) Clonal growth of 786-0 cells in the absence (Ct) or presence of sunitinib (sun) (2.5 or 10 µM/L). (D) The proportion of cells in each phase of the cell cycle was determined by DNA labeling with propidium iodide followed by FACS analysis. (E) Determination of viable cells in the absence (Ct) or presence of 2.5 µM/L (2.5) or 10 µM/L (10) of sunitinib (sun).

**Figure 2:** A combination of a lysosome destabilizing agent and an inhibitor of ABC transporters reverted sunitinib resistance of 786-OR cells. Determination of the percentage of dead 786-OS and 786-OR cells after incubation for 24 hours with the indicated
combinations of drugs (sunitinib (sun) 2.5 µM/L; LLOM (L) 1 µM/L; elacridar (E) 5 µM/L). * p < 0.05; *** p < 0.001.

**Figure 3:** Primary tumor cells derived from a patient who progressed on sunitinib were sensitive to sunitinib when in the presence of elacridar or LLOM. Determination of the percentage of viable/dead TFE3 cells after incubation for 24 hours with the indicated combinations of drugs (sunitinib (sun) 2.5 µM/L; LLOM (L) 0.2 µM/L; elacridar (E) 1 µM/L). * p < 0.05; *** p < 0.001.

**Figure 4:** Proteasome inhibitors induced the death of cells resistant to sunitinib. The proteasome inhibitors MG132 (mg, 10 µM/L) or bortezomib (borte, 5 µM/L) alone or in combination with sunitinib (sun, 2.5 µM/L) decreased the viability of 786-OS and 786-OR cells after incubation for 24 hours. **p < 0.01; *** p < 0.001, NS (non significant).

**Figure 5:** Description of the different phases of events justifying a combinatorial approach for treatment. (A) Maximal resistance (+++) is mediated by lysosomal trapping of sunitinib (yellow circles in lysosomes). The presence of auto-lysosomes (green circles) is indicative of incomplete autophagy. The proteasome degrades misfolded proteins and participates in recycling of amino acids. (B) The lysosome destabilizing agent Leu-Leu-O-Methyl (LLOM) prevents the trapping of sunitinib in lysosomes so the drug is localizes to the cytoplasm but is "taken in charge" by the ABCG1, which transport the drug out of the cell thus leading to intermediate resistance (++). (C) Maximal sensitivity to sunitinib can be obtained by destabilization of lysosomes with LLOM combined with inhibitors of ABC transporters (Elacridar) or with proteasome inhibitors (MG132 (mg) or bortezomib (borte)).

**Figure 6:** LLOM, elacridar, bafloymycin or a combination of these different drugs alone or with sunitinib induced a higher mortality in 786-OS cells than in 786-OR cells. (A) Effect of increasing concentrations of elacridar (E) on cell viability after incubation for 24 hours. * p < 0.05; ** p < 0.01; *** p < 0.001. (B) Effect of increasing concentrations of LLOM (L) on cell viability after incubation for 24 hours. ** p < 0.01. (C) Effect of increasing concentrations of elacridar (E) and a sub-optimal concentration of LLOM (L, 1 µM/L see part B of the figure) on cell viability after incubation for 24 hours. ** p < 0.01; *** p < 0.001. (D) Determination of the percentage of viable 786-OS and 786-OR cells after incubation for
24 hours with the indicated combinations of sunitinib (sun 2.5 µmol/L), bafilomycin (B 50 nmol/L), elacridar (E 5 µmol/L). **p < 0.01; ***p < 0.001.

**Figure 7:** The combination of a lysosomal destabilizing agent and an inhibitor of ABC transporters reverts sunitinib resistance of RCC10 cells. Determination of the percentage viable of RCC10s and RCC10R cells after incubation for 24 hours with the indicated combinations of drugs sunitinib (sun 2.5 mmol/L), LLOM (L 1 mmol/L), elacridar (E 5 mmol/L). *p < 0.05; **p < 0.01.

**Figure 8:** Proteasome inhibitors induced the death of cells resistant to sunitinib. The proteasome inhibitors MG132 (mg, 10 µmol/L) or bortezomib (bort, 5 µmol/L) alone or in combination with sunitinib (sun, 2.5 µmol/L for RCC10 cells and 5 µmol/L for CC cells) decreased the viability of RCC10s, RCC10R (A) and CC cells (B) after incubation for 24 hours. *p < 0.05; **p < 0.01; ***p < 0.001, N.S (non significant).

**Figure 9:** Analysis of cbioportal databases highlighted the prognostic value of a cluster of proteasome associated genes. (A) Heatmaps of three microarrays (GSE 14494, 11151 and 22541) for the relative expression of PSMB8, PSMB9, PSMBIO and PSMFl). N = normal tissue; C = carcinoma; P = primary tumor; M = metastasis. (B) Kaplan-Meier analysis of overall survival of patients with RCC at cbioportal. Overall survival was calculated from patient subgroups with mRNA levels for PSMB8, PSMB9, PSMBIO and PSMFl that were 1.4 less or greater than the median value. Statistical significance (p value) is indicated. (C) Kaplan-Meier analysis of disease free survival or overall survival of patients with non metastatic (M0) or metastatic (M+) RCC. Statistical significance (p value) is indicated.

**EXAMPLE:** Resistance to sunitinib in renal clear cell carcinoma results from sequestration in lysosomes and inhibition of the autophagic flux.

**Material & Methods**

**Materials:** Sunitinib and bortezomib came from residual materials given to patients (Centre Antoine Lacassagne, Nice, France) and prepared as 2.5 mmol/L and 6.5 mmol/L stock solutions in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at -20°C. Chloroquine, elacridar and LLOM were purchased from Sigma Aldrich. MG132 was
purchased from Calbiochem. Anti-LC3 antibody (5F10) was obtained from Nanotools. Anti-LAMP1 (H4A3) and anti-EEAl (N-19) were from Santa-Cruz, anti SQSTM1 was from BD Bioscience, CTSB (Ab-1) was purchased from Merck, anti LAMP2 was from Abeam (H4B4), anti-ARD1 antibodies were produced and characterized in our laboratory 50, anti-actin (1-19) was from Santa-Cruz, anti-phospho AKT1 at Ser473 (9271S), anti-AKT1 (9272), anti-phospho MAPK1/3 at Thr85/Tyr87 and Thr202/Tyr204 (4370) and anti-MAPK1/3 (137F5) antibodies were all obtained from Cell Signaling Technology. Hank's Balanced Salt Solution (HBSS) was from Life technology.

**Cell culture:** Human 786-0 cells were purchased from the American Tissue Culture Collection. RCC10 cells were a kind gift from W.H. Kaelin (Dana-Farber Cancer Institute, Boston, MA) and were used in one of our published studies 51. RCC cells were grown in DMEM supplemented with 7% FCS at 37°C in a humidified atmosphere containing 5% CO2. For HBSS experiments, cells were pre-incubated in HBSS for 30 minutes before sunitinib treatment for 24 hours for the determination of cell viability. For clonogenic assays, cells were incubated for seven days in fresh medium after the same procedure. Resistant cells were obtained by chronic exposure to increasing concentrations of sunitinib up to 8 μmol/L. An INVIVO2 200 workstation (Ruskin Technology Biotrace International Pic) set at 1% oxygen, 94% nitrogen and 5% carbon dioxide was used for hypoxic conditions.

**Growth curves and cell viability:** Cells were seeded in six-well dishes and transiently treated with sunitinib the following day. Cells were next detached from days 2 to 6 and counted with a Coulter counter (Beckman) in duplicate to assess cell proliferation. Cell viability and cell death was assessed using the ADAM-MC apparatus (NanoEnTek) based on fluorescent propidium iodide staining according to the manufacturer's instructions.

**Colony formation assay:** RCC cells (500 cells per condition) were treated or not with sunitinib. Colonies were detected after 10 days of culture. Cells were then washed, fixed at room temperature for 20 minutes with 3% paraformaldehyde and colored by crystal violet.

**Kinetics of cell migration:** Cell migration in real time was monitored by using the xCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument equipped with a CIM-plate 16 (Roche, Indianapolis, IN).
Each well of the plate is composed of upper and lower chambers separated by a microporous membrane. Migration was measured as the relative impedance change (cell index) across micro-electronic sensors integrated into the bottom side of the membrane. $10^4$ cells were added in triplicate to the upper chambers. Migration/invasion was monitored every min for 48 hours. For quantification, the cell index at the indicated time points was averaged from three independent measurements.

**Immunoblotting:** Cells treated with sunitinib and/or exposed to pharmacological inhibitors, were lysed in buffer containing 3% SDS, 10% glycerol, 0.825mM Na$_2$HP04. Samples (30 µg) were separated by SDS-PAGE, transferred onto a PVDF membrane (Immobilon, Millipore, France) and then exposed to the appropriate antibodies: anti-LC3, anti-p62, anti-LAMPl, anti-ARDl, anti-cathepsin B or anti-actin. Proteins were visualized with the ECL system using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies.

**Subcellular co-localization studies:** Cells were incubated with sunitinib and Lysotracker Red DND-99 (Invitrogen) or LYSOSENSOR Green DND-153 (Invitrogen). Viable cells were imaged in real time with EVOS Cell Imaging Systems (Life Technologies).

**Immunofluorescence:** RCC cells seeded on glass coverslips (150 000 cells for 24 hours or 60 000 cells for 48 hours) in 6-well dishes were treated or not with sunitinib. 24 or 48 hours after, cells were then washed, fixed at room temperature for 20 minutes with 3% paraformaldehyde and permeabilized with phosphate-buffered saline containing 0.2% Triton X-100 for 2 minutes before being exposed to anti-LC3, anti-p62, anti-LAMPl, anti-LAMP2 or anti-EEAl for 1h at room temperature. Cells were washed three times with PBS, and then incubated for 1 h at room temperature with 1:1000 dilution anti-mouse or anti-rabbit Alexa Fluor 488- or Alexa Fluor 594-labeled secondary antibody (Invitrogen) and mounted using Mountant Permafluor (ThermoScientific). Fluorescence images were examined and collected under a DeltaVision Microscopy Imaging Systems.

**Sub-cellular fractionation:** Sub-cellular fractionation was performed using proteo-extract sub-cellular proteome extraction kit according to the manufacturer's instructions (Calbiochem).
Flow Cytometry - Cell cycle distribution: Cells were trypsinized, washed and re-suspended in cold 70% ethanol overnight. After 2 washes with PBS, cells were re-suspended in propidium iodide (40 µg/ml) containing ribonuclease A (10 µg/ml) for 15 min at room temperature and were analyzed using a fluorescence-activated cell sorter (BD healthcare FACSCALIBUR, analyzer).

Transmission and scanning electron microscopy: RCC cells pellets were collected, fixed with 1.6% glutaraldehyde, post-fixed in 1% OsO₄, dehydrated in a series of alcohol, and embedded in epoxy resin. Thin sections were contrasted with uranyl acetate and lead citrate. Preparations were observed either with a Philips CM12 electron microscope operating at 80 kV (FEI) or with a Jeol 1400 mounted with CCD cameras (Morada, Olympus SIS). For scanning electron microscopy, cells were fixed on poly-L-lysine coated plastic slides, dehydrated and treated with HMDS (Hexamethyldisilazane). Preparations were coated with 3 nm gold-palladium and observed with a JEOL 6700F scanning electron microscope.

CTSB activity: RCC cells treated with sunitinib for 24 h were lysed for 30 min at 4°C in lysis buffer (400 mmol/L Na Phosphate pH 6, 150 mmol/L NaCl, 4 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 1% Triton X-100) and lysates were cleared at 10000g for 15 min at 4°C. Each assay (in quadruplicate) was performed with 50 µg of protein prepared from control or sunitinib treated cells. Briefly, cellular extracts were incubated in a 96-well plate, with 60µM of Z-RR-AMC (7-amino-4-methylcoumarin) as substrate for various times at 37°C. The CTSB activity was measured by following the emission at 460 nm (excitation at 390 nm) in the presence or absence of 1 µM of CA-074Me (an inhibitor of CTSB activity). Enzyme activities were expressed in arbitrary units per mg of protein.

Statistical analysis: Statistical significance and p values were determined by the two-tailed Student's t-test.

Gene expression microarray analysis: Four publicly available microarray datasets were analyzed to determine the relationship between proteasome related genes mRNA levels, patient diagnosis, and outcome (Fig. S12, Table S1). Transcriptomic and clinical data from 519 Renal Clear Cell Carcinoma were downloaded through the cBio Cancer Genomics Portal (www.cbioportal.org, TCGA Provisional; RNA-Seq V2). Three published datasets
(GSE14494, GSE1 1151 and GSE22541; n=70 and 54) queried using the InSilico DB platform (https://insilicodb.com/). Heatmap visualization was performed using GENE-E (http://www.broadinstitute.org/) and Boxplots were created using the BoxPlotR tool (http://boxplot.tyerslab.com/).

**Results**

**mRCC cells showed reduced proliferation in the presence of concentrations of sunitinib below the IC50 (sub-optimal concentration):**

Because of the abnormal vascularization of tumors, the core of primary mRCC or metastases is not exposed to optimal concentrations of the drug (Fig. 1A). We first determined the in vitro concentrations that resulted in progressive adaptation to sunitinib and final selection of resistant cells. The plasma concentrations of patients or mice exposed to sunitinib was low (0.1-1 µmol/L range) compared to the intra-tumor amount, which was ten times higher (10 µmol/L range). Whereas the IC50 of endothelial cells for sunitinib was approximately 0.1 µmol/L, the IC50 of mRCC cells was approximately 5 µmol/L. In the presence of a concentration of sunitinib below the IC50 (2.5 µmol/L), mRCC cells (Fig. 1B, C) have a reduced proliferation rate, which was linked to prolonged S and G2/M phases of the cell cycle (Fig. ID). A sub-optimal concentration of sunitinib (2.5 µmol/L) did not affect cell viability (Fig. ID, E) whereas exposure to a higher concentration resulted in cell death, as measured by cell counting or a clonogenic assay.

**Sunitinib accumulated in lysosomes:**

To decipher the adaptation to sub-optimal concentrations of sunitinib, the rest of the experiments were conducted at the concentration of 2.5 µmol/L.

Phase contrast microscopy highlighted a modification of the cell shape and the appearance of a yellowish color inside the cells after incubation with sunitinib for two days. The intracellular localization of sunitinib was confirmed by visualization of its auto-fluorescence. Sunitinib auto-fluorescence co-localized with a specific lysosomal staining (Lysosome-Associated Membrane Protein 1 (LAMP1)) confirming that sunitinib accumulated in acidic lysosomal structures. Accumulation in lysosomes was also observed in two independent cell lines (RCC10 and A498) and two RCC primary cell lines (CC and TFE3) that we previously described. However, sunitinib did not accumulate in early endosomes (no co-localization with the early endosome antigen 1 (EEAI)). This result suggests accumulation
of sunitinib in intracellular compartments with no major consequences to cell viability. This characteristic defines sunitinib as a lysomotropic agent. FACS analysis showed that sunitinib accumulated in lysosomes in a time-dependent manner and that there was an increase in the lysosomal mass, which coincided with increased expression of LAMP1. Such accumulation of sunitinib was not dependent on the oxygen concentration since sunitinib sequestration was equivalent in normoxia or hypoxia. At the concentration of 2.5 µM/L of sunitinib, hypoxia did not modify the cell viability. Intracellular accumulation of sunitinib was also observed in experimental RCC in mice.

Sunitinib neutralized the pH of lysosomes and inhibited CTSB/cathepsin B:

Sunitinib is a weak base (pKa 8.95), which accumulates in lysosomes where it is protonated by a pH-partitioning process. Once ionized, sunitinib becomes membrane impermeable with the impossibility of diffusing out of the organelle, which results in lysosome trapping. Accumulation continues as long as the low pH is maintained by the vacuolar proton pump (V-type H+ATPase) but ultimately results in buffering of the acidic pH of lysosomes. The lysosensor DND-153 fluorescence (pKa 7.5) was intense in control conditions (this dye fluoresces in an acidic environment) but almost disappeared in the presence of sunitinib suggesting that the acidic pH of lysosomes has been neutralized. This correlated with decreased expression and activity of one of the major lysosome-associated proteases CTSB.

Sub-optimal concentrations of sunitinib initiated incomplete autophagic flux:

In physiological situations, autophagy is responsible for the degradation of dysfunctional organelles and proteins and allows cell survival during nutrient deprivation. So, autophagy is important in maintaining cell homeostasis, but if exacerbated, it can lead to cell death. Sunitinib treatment resulted in an increase in the lysosomal pH and inhibition of the lysosomal protease activity. Hence, we investigated the consequences of these modifications on autophagy. Autophagy is characterized by the accumulation of lipided forms of LC3 (LC3-II) and the degradation of SQSTM1/p62 protein. Sunitinib treatment induced accumulation of LC3-II, which indicated either initiation or blockade of autophagy (in 786-0 cells and in two additional cell lines (RCC10 and A498) and the above mentioned primary cells (CC and TFE3)). However, sustained expression of SQSTM1 strongly suggested incomplete processing. To confirm incomplete autphagic flux, we evaluated the LC3-II level in response to sunitinib in the presence or absence of chloroquine, a
lysomotropic agent that increases the lysosomal pH. A higher fold change compared to control conditions reflected induction of autophagy whereas no modification or a lower fold change in the presence of chloroquine was indicative of inhibition of autophagy. The fold change in LC3-II in sunitinib treated cells after chloroquine treatment was equivalent compared to cells treated with sunitinib alone, indicating an inhibition of the autophagy flux. Furthermore, the merged localization of sunitinib, LC3 and LAMP2 indicated accumulation of non-functional auto-lysosomes, which was visualized by electronic microscopy.

Amino acid deprivation enhanced resistance to sunitinib:

It is well known that cell starvation stimulates autophagy, which helps cells to resist this unfavorable environment. Autophagy is also used by tumor cells to survive when they have consumed all the nutrients easily accessible from the blood stream. Hence, the auto-lysosomes generated through this process may engulf sunitinib thereby amplifying resistance to the drug by preventing drug access to its targets. To test this hypothesis, we cultured 786-OS cells in a saline medium (HBSS) deprived of amino acids, which initiates autophagosome and lysosome formation. We questioned if this increased the capacity of the cells to store sunitinib in acidic intracellular compartments. Phase contrast microscopy clearly showed an enhanced accumulation of yellow granules when cells are cultured in HBSS medium. Quantification by FACS confirmed this qualitative observation. A high concentration (10 \( \mu \text{mol/L} \)) of sunitinib induces cell death, but at a lower concentrations (2.5 \( \mu \text{mol/L} \)), it slows proliferation without inducing cell death, as expected. However, if the cells were first cultured in HBSS medium, cell proliferation was minimally affected by 2.5 \( \mu \text{mol/L} \) sunitinib and cell death was substantially decreased, even at a high concentration of the drug (10 \( \mu \text{mol/L} \)). These results were confirmed with two other independent cell lines (RCC10 and A498 cells).

Sunitinib resistant cells showed exacerbated, incomplete autophagy and a more aggressive phenotype:

We hypothesized that sequestration of sunitinib in lysosomes and the subsequent inhibition of the autophagy flux participated in sunitinib resistance. To test this hypothesis, we generated sunitinib-resistant cells by chronic exposure of cells to the drug (786-OR and RCCIOR). Incomplete autophagy in these cells was attested by accumulation of LC3-II and sustained expression of SQSTM1/p62. Primary sunitinib-resistant cells were also derived from a RCC removed surgically from a patient, as we previously described (TFE3 cells).
The 786-OR and RCC10R cells survive and proliferate in the presence of a high concentration of the drug, which is sufficient to induce parental cell death. TFE3 cells are highly resistant to sunitinib in vitro even at high concentrations of the drug (IC50 10 μmol/L). The ability to accumulate sunitinib inside 786-OR was increased compared to parental cells. As shown previously by Gotink et al., resistance (maintained several weeks) was not genetically acquired since it could be reverted by culturing the cells in the absence of the drug for a few passages (not shown). Electron microscopy showed that 786-OR cells accumulate bigger vacuolar structures, identified above as auto-lysosomes, compared to 786-OS cells when incubated in the presence of 2.5 μmol/L of sunitinib, a finding in favor of an exacerbated incomplete autophagy.

Previous reports have shown that treatment of tumor-bearing mice with sunitinib resulted in the selection of more aggressive cells. This has been observed in vivo, but we hypothesized that such selection did not involve cells from the tumor microenvironment but implicated an intrinsic phenotypic adaptation of tumor cells. We observed that 786-OR cells acquired greater anchorage independency illustrated by the formation of bigger colonies in soft agar (786-OR and RCC10R). In addition, the 786-OR cells showed an increase in their ability to migrate compared to 786-OS cells. These results suggest that incomplete autophagy correlated with the acquisition of the more aggressive phenotype of 786-OR cells. We tested different markers implicated in epithelial/mesenchymal transition (EMT) including N-cadherin, vimentin and the transcription factors slug and snail, but no significant changes were observed between the sensitive and resistant cells.

**Inhibition of the ATP binding cassette (ABC) transporter ABCGL/P-glycoprotein and lysosomal permeabilization enhanced the efficacy of sunitinib:**

We hypothesized that sequestration of sunitinib in lysosomes is involved in resistance to sunitinib. If so, destabilization of lysosomes with LLOM may result in increased cell death in the presence of a sub-optimal concentration of sunitinib since the drug inhibits the kinase activity of target tyrosine kinase receptors located in the cytoplasm. However, we observed that LLOM reduced accumulation of sunitinib inside the cells. Moreover, in the presence of LLOM, the amount of sunitinib in the culture medium was increased. These results strongly suggest that if sunitinib is not sequestered in lysosomal compartment, it is actively exported outside the cells.

Inhibition of the ABC transporters improved sunitinib accumulation in the brain suggesting that these transporters participate in the efflux of the drug. These transporters
are present at the lysosomal membrane and could participate in sunitinib accumulation in this cellular compartment \textsuperscript{15}. Moreover, sunitinib is a substrate of ABCG1 \textsuperscript{16}. Hence, we hypothesized that ABC transporters could be present on the plasma and/or lysosomal membranes to mediate accumulation in specific cell compartments and/or to participate in sunitinib efflux out of the cells. Sunitinib treatment of 786-OS stimulated ABCG1 expression and increased accumulation in 786-OR cells. ABCG1 expression was also increased in A498, TFE3 and CC cells. Whereas elacridar, an inhibitor of ABC transporters, induced slightly 786-OS cell death at a low concentration (1 μM) (Fig. 6A), it potentiated sunitinib activity on 786-OS cells (Fig. 2). Elacridar did not significantly mediate 786-OR cell death when alone (Fig. 6A) but potentiated sunitinib activity (Fig. 2). The lysomotropic agent LLOM had little effect on cell death at a low concentration (1 μM). Higher concentrations are needed to induce cell death probably through the release of cathepsins and induction of lysosome membrane permeabilization leading finally to apoptosis \textsuperscript{17} (Fig. 6B). A low concentration of LLOM increased the efficacy of sunitinib in 786-OS cells (Fig. 2), which strongly suggested that preventing drug sequestration in the lysosomes allowed a better accessibility to its targets.

Finally, the triple combination of sunitinib, LLOM and elacridar resulted in 100 % cell death of 786-OS and 786-OR cells (Fig. 2). Of note, a mix of elacridar plus LLOM had a strong detrimental effect on 786-OS cells even at low concentrations, but had little effect on 786-OR cell viability (Fig. 6C). Although less potent, bafilomycin, an inhibitor of the V-ATPase pump, which is responsible for the maintenance of the low pH of the lysosomes, exerted a comparable effect to LLOM (Fig. 6D). Hence, the triple combination sunitinib/bafilomycin/elacridar was less potent than the sunitinib/LLOM/elacridar mix. Equivalent results were obtained with an independent cell line (RCC10, Fig. 7). These results suggest that blockade of sunitinib trapping in the lysosomes is an efficient way to increase the potency of the drug and prevent resistance.

Elacridar and LLOM alone or in combination had no effect on TFE3 cell viability. Elacridar was more active in the presence of a concentration of 2.5 μM sunitinib, (55% cell death) but this was not the case for the LLOM/sunitinib combination. As for 786-OR cells, massive TFE3 cell death was obtained with the triple combination (Fig. 3).

**Proteasome inhibitors induced the death of sunitinib-resistant cells:**

Autophagy and the ubiquitin-proteasome system are two linked mechanisms leading to the degradation of abnormal proteins and the recycling of amino acids. These two...
mechanisms compensate for each other when one is inhibited. Hence, we speculate that incomplete autophagy resulted in enhanced proteasomal activity. As a consequence, sunitinib and inhibitors of the proteasome may have additional effects on cell death. We observed that MG132, a proteasome inhibitor, or bortezomib, which is approved for the treatment of multiple myeloma, induced the death of sensitive and resistant cells. However, proteasome inhibitors combined with sunitinib induced a higher level of mortality of 786-OR cells than that induced by the individual compounds (Fig. 4). A higher level of mortality was also observed for RCCIOS, RCCIOR and CC cells when proteasome inhibitors, and especially bortezomib, was combined with sunitinib (Fig. 8).

**DISCUSSION:**

Most of the current research in the field of anti-angiogenesis drugs has focused on the adaptation of the endothelial cells to these drugs, which target VEGFA or its receptors. However, it has been shown that other members of the VEGF family especially VEGFC are induced after exposure to anti-VEGFA antibodies. Research has also concentrated on modifications to the genetic program of tumor cells exposed to anti-angiogenesis drugs, which lead to the expression of redundant pro-angiogenesis factors or the ability to migrate. Different reports have described the acquisition of a MET-dependent aggressive phenotype associated with sunitinib treatment, in particular in animal models. However, it is difficult to address this modification in patients since sunitinib is administered mainly after radical nephrectomy to challenge metastatic sites and metastatic cells are generally not sampled for ethical purposes. Dissemination of mRCC cells via the lymphatic system has also been reported but again in animal models. The role of the tumor microenvironment in the adaptation to treatments has also been addressed. Although all the aspects that lead to acquired resistance are of major importance, to become resistant the cells require chronic exposure to the drugs. So it would take several months before genetically modified tumor cells emerge that survive anti-angiogenesis treatment.

The ability of cells to compartmentalize the drug in sub-cellular organelles to avoid accessibility to its target has not been examined in detail. Several reports have shown that certain weakly basic compounds (i.e. daunorubicin, doxorubicin) with pKa values near neutrality are selectively sequestered into lysosomes of multi-drug resistant cell lines. Alternatively, other weakly basic compounds, also with pKa near neutrality, specifically accumulate within mitochondria (i.e. rhodamine 123). Here, it has been observed lysosomal sequestration of sunitinib but no accumulation in mitochondria, as previously shown.
Lysosomal trapping reduces the activity of sunitinib, since its targets, the kinase domain of tyrosine kinase receptors are located in the cytoplasm. This mechanism has been described in chronic myeloid leukemia for which agents that destabilize lysosomes revert resistance to imatinib 17. Among the different drugs that have obtained FDA/EMA approval for the treatment of mRCC, in addition to sunitinib, axitinib and dovitinib can be protonated at physiological pH and subsequently trapped in the lysosomes. Hence, resistance mechanisms equivalent to that described herein for sunitinib may be the cause of reduced efficacy of these drugs. Pazopanib, another ATP mimetic approved for the treatment of mRCC is the only drug that cannot be protonated and trapped in the lysosomes, hence not concerned by this mechanism of resistance. However, sunitinib and pazopanib show the same overall survival 31, but pazopanib is preferred by physicians and patients mainly for its better quality of life 32. Several reports have previously shown that drugs that are lysosomotropic shared certain physicochemical properties, possessing a ClogP>2 and a basic pKa between 6.5 and 11. predictably influenced their intracellular localization 33. Sunitinib enters perfectly into this category with a ClogP= 5.2 and a basic pKa= 8.95. The inventors also observed that the amine group of sunitinib, added to improve the solubility of the drug is responsible for the high pKa value. Thus, the synthesis of an analog of sunitinib devoid of this amine group may prevent its accumulation in lysosomes.

Sequestration of chemotherapeutic agents in lysosomes is largely due to their lysosomotropic properties but sequestration within lysosomes may also be dependent on the ABC transporter activity 15 34. The inventors observed an increase in the expression of the P-gp transporter after sunitinib treatment, but the regulatory mechanism implicated is unknown. Preliminary experiments suggest that the initiation of autophagy induces activating transcription factor 4/ATF4 expression. ATF4 is a major transcription factor implicated in the adaptation to nutrient stress of tumor cells 35. Moreover, ATF4 has also been implicated in resistance to cisplatin and cells overexpressing ATF4 showed multidrug resistance36. Hence, ATF4 may be the driver of a transcriptional program leading to expression of ABCG1, as previously shown 37 38.

Moreover, accumulation of ABCG1 may be due to a lack of its degradation. Several membrane proteins, including receptors and transporters, recycle to the plasma membrane through the recycling endosomal system. Some cargo proteins sort cell membranes and discarded proteins into internal luminal vesicles of multi-vesicular bodies (early endosomes), and mature multi-vesicular bodies (late endosomes) that can fuse with lysosomes for proteolysis by lysosomal enzymes. In the context of the present study, the lysosomal
degradation pathway is impaired because of the modification of the lysosomal pH and could explain the decrease in ABCG1 degradation and its subsequent accumulation. Similar consequences were observed with chloroquine treatment, which resulted in Notch 1 accumulation due to a decrease in the activity of lysosomes.$^{39}$

Lysosomal sequestration is rapid, occurring as soon as the drug is in contact with the target cells, and does not modify the genetic program. Recent studies have also demonstrated that numerous cancer cells have defective acidification of their lysosomes. Hence, lysomotropic agents would be in contact with their targets in the cytoplasm of cancer cells devoid of lysosome trapping.$^{40}$ This elegant approach would limit toxicity to normal cell and would concentrate the cytotoxic/cytostatic effects on tumor cells. However, they showed that the acidification of the lysosomes of mRCC cells was not defective. They observed that the TFE3 cells were resistant to a high concentration of sunitinib (IC50 = 10 μmol/L). However, they did not observe an increase in the number of lysosomes (lysotracker labeling) in TFE3 cells compared to 786-0 cells. The pH of lysosomes dictates the predicted degree of lysosomal accumulation of sunitinib; the greater the lysosome-to-cytosol pH gradient the greater the extent of lysosomal sequestration. As long as the pH gradient is maintained, significant accumulation of the drug is possible. The proton pump, the vacuolar (V)-ATPase which is located on the lysosomal membrane, maintains acidification of lysosomes. Tumor cells with drug resistance exhibit an increase in (V)-ATPase activity, which may explain the resistance to sunitinib of TFE3 cells.$^{41,42}$

To prevent lysosomal trapping and avoid export of the drug out of the cells, they used lysosomal destabilizing agents and inhibitors of ABCG1. This combination was very efficient in promoting cell death of cancer cell lines and cancer cells derived from a patient who progressed on sunitinib. The recapitulative schema they propose to prevent sunitinib resistance is shown in Fig. 5. Lysosome stabilizing agents are far from entering into the clinic, because of major toxic effects whereas clinical assays using inhibitors of ABC transporters are ongoing.$^{43}$ Moreover, they found that proteasome inhibitors induced strong tumor cell death especially on cells resistant to sunitinib. As sunitinib and proteasome inhibitors have independent targets, the toxic effects should be manageable. In silico analysis of online-available microarrays highlighted a cluster of proteasome associated genes that are over-expressed in primary and mRCC but also in paired pulmonary metastasis (Fig. 9A).$^{44,45}$ The proteins encoded by these genes comprised a subset of the proteasome beta sub-units that affect the generation of peptides to promote efficient antigen recognition (PSMB8/Proteasome subunit beta type-8, PSMB9/Proteasome subunit beta type-9, PSMB10/
Proteasome subunit beta type-10) and a cellular regulator of proteasome formation and of proteasome-mediated antigen processing (PSMFl/Proteasome inhibitor PI31 subunit) 47. A slight increase in expression (1.4 (PSMB8, 9, 10) to 2 fold (PSMFI) above the median) of each gene was associated with a decrease in overall survival (OS) with significant p values (p = 0.035 for PSMB8; 0.0006 for PSMB9; 0.018 for PSMB10; 0.036 for PSMFl) as revealed by data analysis at cBioportal 48-49. Moreover, over-expression of the genes of the cluster were indicative of both disease free survival (p = 0.0008) and overall survival is much more decreased for patients that over-expressed the different genes of the cluster (p = 0.0002). Over-expression of the genes of the cluster was also indicative of disease free survival for non metastatic patients (p = 0.007) and of overall survival for metastatic patients (0.006) (Fig. 9B). This in silico analysis clearly showed the prognostic significance of specific proteasome-associated genes. It corroborated our "in cellule*" analysis for the relevance of association of sunitinib and proteasome inhibitors.

Taken together, the present study highlighted a primary mechanism of resistance to the major anti-angiogenic compound used in the treatment of mRCC. Having deciphered this mechanism, the inventors can now propose relevant therapeutic combinations that deserve testing in preclinical models but also putatively in phase I clinical trials.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.


3. Grepin R, Ambrosetti D, Marsaud A, Gastaud L, Amiel J, Pedeutour F, Pages G. The relevance of testing the efficacy of anti-angiogenesis treatments on cells derived from


and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib coadministration. Int J Cancer 2012; 130:223-33.


CLAIMS:

1. A drug or a combination of drugs selected from the group consisting of a proteasome inhibitor, an efflux inhibitor or a combination of an efflux inhibitor and a lysosomotropic agent for use in a method for treating metastatic renal cell carcinoma (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI).

2. The drug or the combination of drugs for use according to claim 1, wherein the multi-targeted weak base VEGFR TKI is characterized by a pKa in a range from 7 to 14.

3. The drug or the combination of drugs for use according to claim 1 or 2, wherein the multi-targeted weak base VEGFR TKI is selected from the group consisting of sunitinib, axitinib and dovitinib.

4. The drug or the combination of drugs for use according to any one claims 1 to 3, wherein the proteasome inhibitor is selected from the group consisting of bortezomib, carfilzomib and delanzomib.

5. The drug or the combination of drugs for use according to any one claims 1 to 4, wherein the efflux inhibitor is P-Glycoprotein (ABCB1) inhibitor selected from the group consisting of elacridar, biricodar, pantoprazole and tariquidar.

6. The drug or the combination of drugs for use according to any one claims 1 to 5, wherein the lysosomotropic agent is selected from the group consisting of chloroquine, mefloquine, monensin and bafilomycin Al.

7. The drug or the combination of drugs for use according to any claims 1 to 6, wherein the patient with an acquired resistance is still under multi-targeted weak base VEGFR TKI treatment.

8. A method for predicting the survival time of a patient affected with RCC or mRCC comprising the steps of: (i) determining the expression level of at least one of the proteasome component genes selected from the group consisting of PSMB8, PSMB9,
PSMB10 and PSMF1 genes in a primary renal tumor biopsy obtained from said patient; (ii) comparing the level determined at step (i) with a predetermined reference level; and (iii) concluding that the patient has a poor prognosis when the level determined at step (i) is higher than the predetermined reference level.

9. A combination comprising a multi-targeted weak base VEGFR TKI and a proteasome inhibitor or a combination comprising a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use in a method for treating RCC or mRCC and/or preventing metastasis in a patient determined as having a poor prognosis according to the method of claim 8.

10. The combination of a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use according to claim 9, further comprising a lysosomotropic agent.

11. A method for determining whether a patient affected with RCC or mRCC has or is at risk of multi-targeted weak base VEGFR TKI-resistance, comprising (i) a step of determining the expression level of at least one of the proteasome component genes selected from the group consisting of PSMB8, PSMB9, PSMB10 and PSMF1 genes in a primary renal tumor biopsy obtained from said patient; (ii) comparing the level determined at step (i) with a predetermined reference level; and (iii) concluding that the patient has or is at risk of multi-targeted weak base VEGFR TKI-resistance when the level determined at step (i) is higher than the predetermined reference level.

12. A combination comprising a multi-targeted weak base VEGFR TKI and a proteasome inhibitor or a combination comprising a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use in a method for treating RCC or mRCC and/or preventing metastasis and/or preventing acquired resistance to treatment with a multi-targeted weak base VEGFR TKI in a patient determined as having or being at risk of multi-targeted weak base VEGFR TKI-resistance according to the method of claim 11.

13. The combination of a multi-targeted weak base VEGFR TKI and an efflux inhibitor for use according to claim 12, further comprising a lysosomotropic agent.
Figure 1
Figure 2

Figure 3
Figure 4

RESISTANCE +++
RESISTANCE ++
NO RESISTANCE → CELL DEATH

Figure 5
Figure 6
Figure 7
Figure 8
Figure 9A
Figures 9B & 9C
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K31/4965 A61K45/06 A61K31/5377 A61K31/4418 A61K31/473

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

**Date of the actual completion of the international search**

19 September 2016

**Date of mailing of the international search report**

23/09/2016

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer
Ansal do, M
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>Hiromi Sato et al.: &quot;Abstract 983: A dual inhibitor of MDR-1 and ABCG2, elacridar, enhances cytotoxic effects of sunitinib on RCC cell lines.&quot;</td>
<td>1-3, 5-7, 9, 12, 13</td>
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<td>Retrieved from the Internet: URL: <a href="http://cancerres.aacrjournals.org/content/73/8/Suppl/983">http://cancerres.aacrjournals.org/content/73/8/Suppl/983</a></td>
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<td></td>
<td>[retrieved on 2015-10-20] abstract</td>
<td></td>
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<tr>
<td>Y</td>
<td>MEDI0NI J et al.: &quot;Salvage Therapy with Bevacizumab-Sunitinib Alone for Metastatic RCC Cell Carcinoma: A Case Series&quot;, EUROPEAN UROLOGY, ELSEVIER BV, NL, vol. 56, no. 1, 1 July 2009 (2009-07-01), pages 207-211, XP026174083</td>
<td>1-4, 7, 9, 10, 12, 13</td>
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<tr>
<td></td>
<td>[retrieved on 2009-01-13] abstract</td>
<td></td>
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<tr>
<td>Y</td>
<td>G. V. KONDAGUNTA: &quot;Phase II Trial of Bortezomib for Patients with Advanced RCC Cell Carcinoma&quot;, JOURNAL OF CLINICAL ONCOLOGY, vol. 22, no. 18, 9 August 2004 (2004-08-09), pages 3720-3725, XP055244973</td>
<td>1-4, 7, 9, 10, 12, 13</td>
</tr>
<tr>
<td></td>
<td>[retrieved on 2009-01-13] abstract</td>
<td></td>
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<tr>
<td>Y</td>
<td>J. AN et al.: &quot;Epidermal growth factor receptor inhibition sensitizes renal cell carcinoma cells to the cytotoxic effects of bortezomib&quot;, MOLECULAR CANCER THERAPEUTICS, vol. 6, no. 1, 1 January 2007 (2007-01-01), pages 61-69, XP055244978,</td>
<td>1-4, 7, 9, 10, 12, 13</td>
</tr>
<tr>
<td></td>
<td>[retrieved on 2009-01-13] abstract</td>
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</table>
INTERNATIONAL SEARCH REPORT

Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6, 8, 11 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
   1-5, 7, 9, 10, 12, 13

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.
This International Search Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3, 5, 7, 9, 10, 12, 13 (all partially)
   an efflux inhibitor for use in a method for treating metastatic renal cell cancer (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI).
   ---

2. Claims: 4 (completely) ; 1-3, 7, 9, 10, 12, 13 (partially)
   proteasome inhibitor for use in a method for treating metastatic renal cell cancer (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI).
   ---

3. Claims: 6 (completely) ; 1-3, 5, 7 (partially)
   combination of an efflux inhibitor and a lysosomotropic agent for use in a method for treating metastatic renal cell cancer (mRCC) in a patient with an acquired resistance to treatment with a multi-targeted weak base vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor (TKI).
   ---

4. Claim: 8
   A method for predicting the survival time of a patient affected with RCC or mRCC
   ---

5. Claim: 11
   A method for determining whether a patient affected with RCC or mRCC has or is at risk of multi-targeted weak base VEGFR TKI-resistance
   ---
Conti nuati on of Box II .2

Claims Nos.: 6, 8, 11

No additional fees paid for inventions 3-5.

The applicant’s attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on a matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the applicant proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.