(54) **Title**: PDGFR-α AS RESPONSE MARKER FOR PM00104 TREATMENT

(57) **Abstract**: The present invention relates to use of PM00104 in the treatment of cancer in patients having tumors with certain molecular markers profile, in particular, having low PDGFR-α expression levels. The invention also relates to methods for providing personalized PM00104 chemotherapy to cancer patients based on said tumor molecular markers.

PDGFR-α AS RESPONSE MARKER FOR PM00104 TREATMENT

FIELD OF THE INVENTION

5   The present invention relates to the treatment of cancer by PM00104 and more specifically to the treatment of cancer in patients having tumors with certain molecular markers profile, in particular, having low PDGFR-α expression levels. The invention also relates to methods for providing personalized PM00104 chemotherapy to cancer patients based on said

10   tumor molecular markers.

BACKGROUND OF THE INVENTION

      Cancer develops when cells in a part of the body begin to grow out of

15   control. Although there are many kinds of cancer, they all arise from out-of-control growth of abnormal cells. Cancer cells can invade nearby tissues and can spread through the bloodstream and lymphatic system to other parts of the body. In addition, cancer is invasive and tends to infiltrate the surrounding tissues and give rise to metastases. It can spread directly into

20   surrounding tissues and also may be spread through the lymphatic and circulatory systems to other parts of the body.

      Many treatments are available for cancer, including surgery and radiation for localised disease, and chemotherapy. However, the efficacy of

25   available treatments for many cancer types is limited, and new, improved forms of treatment showing clinical benefits are needed. This is especially true for those patients presenting with advanced and/or metastatic disease and for patients relapsing with progressive disease after having been previously treated with established therapies which become ineffective or

30   intolerable due to acquisition of resistance or to limitations in administration of the therapies due to associated toxicities.

      Since the 1950s, significant advances have been made in the chemotherapeutic management of cancer. Unfortunately, more than 50%

2

of all cancer patients either do not respond to initial therapy or experience relapse after an initial response to treatment and ultimately die from progressive metastatic disease. Thus, the ongoing commitment to the design and discovery of new anticancer agents is critically important.

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The ideal antitumor drug would kill cancer cells selectively, with a wide index relative to its toxicity towards non-cancer cells, and would also retain its efficacy against cancer cells, even after prolonged exposure to the drug. Unfortunately, none of the current chemotherapies with known

10 agents posses an ideal profile. Most posses very narrow therapeutic indexes and, in addition, cancerous cells exposed to slightly sublethal concentrations of a chemotherapeutic agent may develop resistance to such an agent, and quite often cross-resistance to several other antitumor agents.

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Conventional medical approaches to diagnosis and treatment of cancer disease are based on clinical data alone, or made in conjunction with a diagnostic test. Such traditional practices often lead to therapeutic choices that are not optimal for the efficacy of the prescribed drug therapy

20 or to minimise the likelihood of side effects for an individual subject. Based on the investigation of gene polymorphisms, gene expression levels and gene mutations, pharmacogenomic studies are aimed to identify predictive biomarkers that can help to define subpopulations of patients who will, or will not, benefit from a particular therapy. Progress in pharmacogenomics

25 and pharmacogenetics, which establish correlations between responses to specific tumor drugs and the genetic profile of individual patients and/or their tumors is foundational for the development of new personalized medicines for the treatment of cancer.

30 PM00104 (Zalypsis®) is a synthetic alkaloid related to jorumycin and renieramycins, and also to safracin and saframycin compounds. Jorumycin is a natural compound isolated from the skin and from the mucus of the Pacific nudibranch *Jorunna funebris* (Fontana A., *et al.* Tetrahedron (2000), 56, 7305-8). In addition, the family of renieramycins is

3

disclosed as being isolated from sponges and tunicates (James M.F. *et al.* J. Am. Chem. Soc. (1982), 104, 265-269; Oku N., *et al.* Journal Natural Products (2003), 66, 1136-9). Safracin and saframycin compounds are disclosed in Manzanares I., *et al.* (Curr. Med. Chem. Anti-Cancer Agents

5   (2001), 1, 257-276), as well as in WO 00/18233 and WO 01/87894.

PM00104 has the following chemical structure:
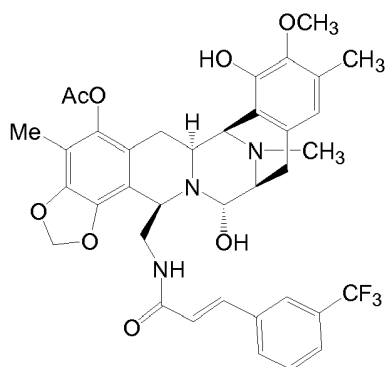
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For further details on PM00104 see WO 01/87894. Additionally, the reader is referred to WO 2007/052076 and WO 2008/135792 which are incorporated herein by specific reference, for pharmaceutical compositions and administration dosages and schedules of PM00104.

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PM00104 is currently in Phase II clinical development for the treatment of solid tumors and haematological malignancies. It previously demonstrated significant *in vitro* activity against solid and non-solid tumor cell lines as well as significant *in vivo* activity in several xenografted human

20   cell lines in mice, such as breast and prostate. The reader is referred to WO 01/87894, WO 2009/140675, and WO 2009/138509, which are incorporated herein by specific reference, for further details on PM00104 biological activity both as single agent and in combination with other agents. Preliminary insights into the mechanism of action of PM00104

25   suggested that the agent has DNA binding properties, induces cell cycle arrest and transcriptional inhibition (Leal *et al.* Biochemical pharmacology (2009), 78, 162-170). Moreover, experimental data revealed that DNA

4

damage repair machinery was essential to overcome PM00104 induced DNA lesions which are mainly due to double strand breaks (Herrero et al., American Association for Cancer Research Annual Meeting, 2007 April 14-18, Abstract nr 5733).

Since cancer is a leading cause of death in animals and humans, several efforts have been and are still being undertaken in order to obtain a safe and effective therapy to be administered to patients suffering from a cancer. Accordingly, an object of the present invention is to provide anticancer therapies that are useful in the treatment of cancer and to provide methods useful to predict and asses tumor drug response in individual subjects and/or allowing to choose the correct treatment regime for each patient.

SUMMARY OF THE INVENTION

In a first aspect, the invention relates to a method for predicting the clinical response of a cancer patient to PM00104 chemotherapy or for designing an individual chemotherapy for a human patient suffering from cancer comprising

(a) determining the expression level of PDGFR-α in a biological sample of the patient before PM00104 chemotherapy, and

(b) comparing the expression level of PDGFR-α in the biological sample with a reference value

wherein an expression level of PDGFR-α equal to or lower than the reference value indicates an increased likelihood of showing a positive response to the treatment with PM00104 or that the patient is to be treated with a PM00104-based chemotherapy.

In a further aspect, the invention relates to a screening method for selecting a human patient suffering from cancer for a treatment with PM00104, comprising the steps of

(a) determining the expression level of PDGFR-α in a biological sample of the patient before PM00104 chemotherapy,

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(b) comparing the amount of expression of PDGFR-α in the biological sample with a reference value, and

(c) selecting a chemotherapy treatment based on PM00104 when said PDGFR-α expression level is equal to or lower than the reference value.

In yet another aspect, the invention relates to PM00104 for use in the treatment of cancer in a patient wherein a biological sample of said patient has a PDGFR-α expression level lower than a reference value.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Panel of low passaged human sarcoma cell lines. Tumor origin, phenotypic characteristics and doubling time.

Figure 2. mRNA expression levels of p16^{INK4a}, p15^{INK4b}, p14^{ARF}, p21^{CIP1}, p27^{KIP1}, MSH-2, MLH-1, Apaf-1, p73, E-cadherin, β-catenin, APC, PTEN and P85 genes in the panel of sarcoma cell lines determined by RT-PCR. β-actin was used as a control.

Figure 3. Protein levels of cyclin D1, CDK4, MDM2, p53 and P27^{Kip1} in the panel of sarcoma cell lines determined by Western-Blot. α-tubulin was used as a control.

Figure 4. Protein levels of pp60^{src}, PDGFR-α, p-C-kit and PTEN in the panel of sarcoma cell lines determined by Western-Blot. α-tubulin was used as a control.

Figure 5. Characterisation of the constitutive activation of the PI3K route. Cells were cultured in two different serum concentrations 10% (+) and 0.5% (-).

6

Figure 6. A. mRNA and protein expression levels of the panel of sarcoma cell lines are shown as discrete values. **B.** p14^{ARF} gene mRNA expression levels are shown together with the score (0 or 1) given to the different expression levels. **C.** PTEN protein expression levels are shown together with their respective score (0, 0.5, 1, 2 or 3).

Figure 7. Correlation between the cytotoxicity (IC$_{50}$) values obtained for the assayed drugs and the biomarkers' expression in the panel of cell lines. A positive coefficient indicates that an increase in marker expression is associated with a decrease in IC$_{50}$ values (i.e. increased sensitivity to the cytotoxic drug), whilst a negative coefficient indicates that an increase in the marker value is associated with an increase in IC$_{50}$ values (i.e. resistance to the drug). Only statistically significant results are shown.

Figure 8. Expression levels of PDGFR-α and phosphorylation status of C-kit determined by Western-Blot in the low passaged cell lines and the correlation with its sensitivity to PM00104.

Figure 9. A. Kaplan-Meier like graph showing the proportion of cell lines having an IC$_{50}$ above the X-axis value according to the cell lines marker profile: -/- (low levels or absence of PDGFR-α and low levels or absence of phosphorylated C-kit), PDGFR/- (high levels of PDGFR-α and low levels or absence of phosphorylated C-kit) and PDGFR/p-C-kit (high levels of PDGFR-α and high levels of phosphorylated C-kit). **B.** Graph showing the variation range of IC$_{50}$ values for cell lines with the following marker profiles: -/- (low levels or absence of PDGFR-α and low levels or absence of phosphorylated C-kit), PDGFR/- (high levels of PDGFR-α and low levels or absence of phosphorylated C-kit) and PDGFR/p-C-kit (high levels of PDGFR-α and high levels of phosphorylated C-kit).

Figure 10. PDGFR-α protein levels relative to α-tubulin expression. Expression of PDGFR-α was analyzed by Western Blot, quantified by

7

densitometry and normalised with those of α-tubulin. Tumor volume (mm³) after drug treatment is shown above each bar.

Figure 11. Characterization by Western Blot analysis of the PDGFR-α, EGFR and p-EGFR expression levels in the cell lines used for the *in vivo* assay. The human epithelial tumor cell lines were grown in the presence of low (0.5%) or standard (10%) percentage of serum.

Figure 12. *In vivo* response of a panel of xenografted human epithelial tumor cell lines to PM00104. C, control (placebo) treatment; Z, Zalypsis® (PM00104) treatment. A reduction higher than 60% of tumor burden with respect that of the control, was considered a positive response.

DETAILED DESCRIPTION

METHOD FOR PREDICTING THE CLINICAL RESPONSE OF A CANCER PATIENT TO PM00104 CHEMOTHERAPY

The authors of the present invention have found that PDGFR-α expression levels correlate with the sensitivity to PM00104 both in in vitro and in vivo assays. These results show that PDGFR-α expression can play an important role in predicting differential chemotherapy sensitivity in patients to the treatment with PM00104.

As shown in Example 4, in *in vitro* studies PDGFR-α protein expression levels were found to correlate with the sensitivity of human low passaged sarcoma cells to PM00104. In particular, when analyzing the correlation of the sensitivity of a panel of low passage human sarcoma cell lines to PM00104 with different molecular tumor markers, it was found that high basal expression levels of PDGFR-α protein were associated with increased resistance to the drug. The only exceptions were the cell lines CNIO-BF and CNIO BI which despite expressing low levels of the marker were quite resistant to the drug. However, it was found that these cell lines

8

constitutively expressed phosphorylated C-kit receptor. Multivariate Cox models analysis confirmed that PM00104 resistance correlated with high levels of PDGFR-α and that the combined presence of constitutively phosphorylated C-kit increased the statistical significance of the correlation. Interestingly, *in vivo* studies in xenograft models (shown in Example 5) confirmed that PDGFR-α was also a suitable marker of *in vivo* response to PM00104. PDGFR-α protein expression levels were found to correlate with the *in vivo* sensitivity of human epithelial tumor cells to PM00104, namely, cells with low levels of PDGFR-α were more sensitive to the drug than those with high levels.

Thus, in a first aspect, the invention relates to a method for predicting the clinical response of a cancer patient to PM00104 chemotherapy comprising
    (a) determining the expression level of PDGFR-α in a biological sample
        of the patient before PM00104 chemotherapy, and
    (b) comparing the expression level of PDGFR-α in the biological sample
        with a reference value
wherein an expression level of PDGFR-α equal to or lower than the reference value indicates an increased likelihood of showing a positive response to the treatment with PM00104.

The term "treating", as used herein, unless otherwise indicated, means reversing, alleviating, inhibiting the progress of, attenuating the symptoms or pathological basis of the disease, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition. The term "treatment", as used herein, unless otherwise indicated, refers to the act of treating as "treating" is defined immediately above.

The term "PM00104 chemotherapy", as used herein, refers to the treatment of a patient with PM00104 thereof either as a single agent or in combination with any other treatment for cancer such as radiation for localised disease and drugs that destroy cancer cells (chemotherapy).

9

Chemotherapy plays a significant part in cancer treatment, as it is required for the treatment of advanced cancers with distant metastasis and often helpful for tumor reduction before surgery (neoadjuvant therapy). It is also used following surgery or radiation (adjuvant therapy) to destroy any

5   remaining cancer cells or prevent recurrence of the cancer. Many anti-cancer drugs have been developed based on various modes of action and can be used in combination with PM00104: alkylating agents that act directly on the DNA (such as cisplatin, carboplatin, oxaliplatin, cyclophosphamide, ifosfamide); antimetabolites that interfere with DNA

10   and RNA synthesis (such as 5-fluorouracil, capecitabine, 6-mercaptopurine, methotrexate, gemcitabine, cytarabine (ara-C), fludarabine); anthracyclines that interfere with enzymes involved in DNA replication (such as daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone); microtubule disrupters (taxanes such as paclitaxel and

15   docetaxel or Vinca alkaloids such as vinblastine, vincristine, and vinorelbine); topoisomerase inhibitors (such as etoposide, topotecan and irinotecan); hormone therapy (such as tamoxifen, flutamide) and recently introduced targeted therapy (such as the inhibitors of EGFR cetuximab, gefitinib or the protein tyrosine kinase inhibitor imatinib), are the most

20   frequently used.

To provide a more concise description, some of the quantitative expressions given herein are not qualified with the term "about". It is understood that, whether the term "about" is used explicitly or not, every

25   quantity given herein is meant to refer to the actual given value, and it is also meant to refer to the approximation to such given value that would reasonably be inferred based on the ordinary skill in the art, including equivalents and approximations due to the experimental and/or measurement conditions for such given value. In addition, unless

30   otherwise stated, where values are given as ranges, the upper and lower values are specifically contemplated as preferred values.

This method of the invention is suitable for the prediction of the clinical outcome of a patient. The expression "clinical outcome" or "clinical

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response", as used herein, relates to the determination of any parameter that can be useful in determining the evolution of a patient. As will be understood by those skilled in the art, the prediction of the clinical response to PM00104 chemotherapy, although preferred to be, need not be correct for 100% of the subjects to be diagnosed or evaluated. The term, however, requires that a statistically significant portion of subjects can be identified as having an increased probability of having a positive response. Whether a subject is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test, etc. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%. The p-values are, preferably, 0.2, 0.1 or 0.05. The determination of the clinical outcome can be done by using any endpoint measurements used in oncology and known to the skilled practitioner. Useful endpoint parameters to describe the evolution of a disease include objective response, tumor control, progression free survival, progression free survival for longer than 6 months and median survival.

"Objective response", as used in the present invention, describes the proportion of treated people in whom a complete or partial response is observed.

"Tumor control" relates to the proportion of treated people in whom complete response, partial response, minor response or stable disease ≥ 6 months is observed.

"Progression free survival" or PFS, as used herein, is defined as the time from start of treatment to the first measurement of cancer growth.

11

"Six-month progression free survival or PFS6" rate, as used herein, relates to the percentage of people wherein free of progression in the first six months after the initiation of the therapy.

5   "Median survival" time, as used herein, is defined as the time from either diagnosis or treatment at which half of the patients with a given disease are found to be, or expected to be, still alive. This term is also called median overall survival and median survival.

10   Step (a) of the method of the invention requires the determination of the expression level of PDGFR-α in a biological sample of the patient before PM00104 treatment.

The term "patient" refers to an animal, preferably a mammal
15   including a non-primate (e.g. a cow, pig, horse, cat, dog, rat, or mouse) and a primate (e.g. a monkey or a human). In a preferred embodiment, the patient is a human patient. In a preferred embodiment, the cancer patient is a human cancer patient.

20   The term "PDGFR-α", as used herein, refers to a member of the PDGF receptor family which can be activated by different PDGF homo- or heterodimers such as PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD, said activation leading to cell migration, proliferation and survival. Platelet-derived growth factors (PDGFs) and their tyrosine kinase
25   receptors (PDGFRs) have been implicated in the pathogenesis of a number of tumor types and play an important role in angiogenesis. A number of PDGFR antagonists have been described to be useful in the treatment of cancer, such as imatinib or sorafenib. Interestingly, many of the PDGFR antagonists that have been developed are not specific for PDGF and act on
30   a number of tyrosine kinase receptors (Board, R. and Jayson G.C., Drug Resistance Updates (2005), 75-83). PDGFs are a growth factor family, wherein each member contains one of four different polypeptide chains: PDGF-A, PDGF-B, PDGF-C and PDGF-D. The polypeptide chains are linked with an amino acid disulfide bond forming homo- or heterodimers, of

12

which five have so far been described: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. These factors exert their cellular effects through PDGF-α and PDGF-β protein tyrosine kinase receptors.

5   The skilled person will appreciate that the determination of the expression level of PDGFR-α can be carried out by determination of the PDGFR-α mRNA levels or of the PDGFR-α protein levels.

        If the determination is carried out by measuring the PDGFR-α mRNA
10   levels, any suitable technique can be used including, without limitation, RT-PCR, SAGE or DNA microarrays. Typically, expression levels of a given mRNA are determined by reverse transcription polymerase chain reaction (RT-PCR). Preferably, the determination is carried out by quantitative (q-) RT-PCR, using a 5'-nuclease assay such as TaqMan®. The detection can be
15   carried out in individual samples or in tissue microarrays.

        In order to normalize the values of mRNA expression among the different samples, it is possible to compare the expression levels of the mRNA of interest in the test samples with the expression of the RNA
20   mixture derived from multiple cell lines. Said RNA mixture can be a commercial one, such as the universal human reference RNA (Stratagene) or a preparation made by pooling RNA preparations from all the samples to be analyzed. In a preferred embodiment, the quantification of gene expression is done by the comparative Ct method using a control RNA.
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        An "endogenous control RNA" as used herein, relates to a mRNA whose expression levels do not change or change only in limited amounts in tumor cells with respect to non-tumorigenic cells. Preferably, the "endogenous control RNA" is mRNA derived from housekeeping genes and
30   which code for proteins which are constitutively expressed and carry out essential cellular functions. More than one endogenous control RNA may be used. Preferred housekeeping genes for use in the present invention include β-2-microglobulin, ubiquitin, 18-S ribosomal protein, cyclophilin,

13

GAPDH, α-tubulin and β-actin. In a preferred embodiment, the control RNA is β-actin mRNA.

The term "biological sample", as used herein, generally refers to a
5   sample obtained from a biological subject, including a sample of a biological tissue or fluid origin, obtained, reached, or collected in vivo or in situ. Exemplary biological samples include but are not limited to cell lysate, a cell culture, a cell line, a tissue, an oral tissue, a gastrointestinal tissue, an organ, an organelle, a biological fluid, a blood sample, a serum sample,
10 a urine sample, and a skin sample. The present method can be applied to any type of biological samples from a patient, such as a biopsy sample, tissue, cell or fluid (serum, saliva, semen, sputum, cerebral spinal fluid (CSF), tears, mucus, sweat, milk, brain extracts and the like). In a preferred embodiment the sample from the patient is a tumor tissue
15 sample. In a further preferred embodiment, a portion of normal tissue from the patient from which the tumor is obtained it is also examined.

In performing the methods of the present invention, tumor cells are preferably isolated from the patient. Tumors or portions thereof are
20 surgically resected from the patient or obtained by routine biopsy. RNA isolated from frozen or fresh samples is extracted from the cells by any of the methods typical in the art, for example, Sambrook, Fischer and Maniatis, Molecular Cloning, a laboratory manual, (2nd ed.), Cold Spring Harbor Laboratory Press, New York, (1989). Preferably, care is taken to
25 avoid degradation of the RNA during the extraction process.

Preferably, the expression level is determined using mRNA obtained from a formalin-fixed, paraffin-embedded tissue sample. Other tissue samples are envisaged, such as fresh tissue from a biopsy or blood
30 samples depending on their availability. Fixed and paraffin-embedded tissue samples are preferred because they are broadly used storable or archival tissue samples in the field of oncology. mRNA may be isolated from an archival pathological sample or biopsy sample which is first deparaffinized. An exemplary deparaffinization method involves washing

14

the paraffinized sample with an organic solvent, such as xylene. Deparaffinized samples can be rehydrated with an aqueous solution of a lower alcohol. Suitable lower alcohols, for example include, methanol, ethanol, propanols, and butanols. Deparaffinized samples may be rehydrated with successive washes with lower alcoholic solutions of decreasing concentration, for example. Alternatively, the sample is simultaneously deparaffinized and rehydrated. The sample is then lysed and RNA is extracted from the sample.

Alternatively, the expression levels of PDGFR-α can be determined by measuring the PDGFR-α protein levels in a sample from a patient before PM00104 treatment. This determination can be carried out by immunological techniques such as ELISA, Western Blot, immunofluorescence, immunoprecipitation or immunohistochemistry. Western blot is based on the detection of proteins previously resolved by gel electrophoreses under denaturing conditions and immobilized on a membrane, generally nitrocellulose by the incubation with an antibody specific and a developing system (e.g. chemoluminiscent). The analysis by immunofluorescence requires the use of an antibody specific for the target protein for the analysis of the expression and subcellular localization by microscopy. Generally, the cells under study are previously fixed with paraformaldehyde and permeabilised with a non-ionic detergent. ELISA is based on the use of antigens or antibodies labelled with enzymes so that the conjugates formed between the target antigen and the labelled antibody results in the formation of enzymatically-active complexes. Since one of the components (the antigen or the labelled antibody) are immobilised on a support, the antibody-antigen complexes are immobilised on the support and thus, it can be detected by the addition of a substrate which is converted by the enzyme to a product which is detectable by, e.g. spectrophotometry or fluorometry. This technique does not allow the exact localisation of the target protein or the determination of its molecular weight but allows a very specific and highly sensitive detection of the target protein in a variety of biological samples (serum, plasma, tissue homogenates, nuclear supernatants, ascites and the like).

In a preferred embodiment, the determination of PDGFR-α protein levels is carried out by Western Blot, as described in Example 1. Scoring of the expression results may be performed, for example, by assigning a score according to the protein expression after visual analysis as specified in Example 2. Figure 6C shows an example of semi-quantitative determination of the protein expression of the PTEN protein, wherein scoring values of 0, 1, 2 or 3 were assigned after visual determination of the PTEN protein expression levels.

In another embodiment, the determination of PDGFR-α protein is carried out by immunohistochemistry (IHC) analysis using thin sections of the biological sample immobilised on coated slides. The sections, when derived from a paraffinised tissue sample, are deparaffinised and treated so as to retrieve the antigen. The detection can be carried out in individual samples or in tissue microarrays.

Any antibody or reagent known to specifically bind with high affinity to the target protein can be used for detecting the amount of target protein. It is preferred nevertheless the use of an antibody, for example polyclonal sera, hybridoma supernatants or monoclonal antibodies, antibody fragments, Fv, Fab, Fab' y F(ab')2, ScFv, diabodies, triabodies, tetrabodies and humanised antibodies. Preferably, a monoclonal antibody is used. Said antibody or reagent specifically binding to the target protein will be labelled with a suitable marker, such as a fluorescent, chemoluminiscent, isotope marker, etc.

Once the expression level of PDGFR-α in the sample under study is determined, step (b) of the method of the invention comprises comparing the amount of expression of PDGFR-α in the biological sample with a reference value.

In the context of the invention, the term "reference value" "is used to denote a laboratory value used as a reference for values/data obtained by

16

laboratory examinations of patients or samples collected from patients. The term "reference value" can be an absolute value; a relative value; a value that has an upper and/or lower limit; a range of values; an average value; a median value; a mean value; or a value as compared to a particular control or baseline value. Suitable reference values for use in the present invention include the expression levels of PDGFR-α in a tumoral or non-tumoral tissue (protein or mRNA) which may be obtained from a different subject or from the same subject. The tumoral tissue will preferably be from patients suffering the same type of cancer.

In another preferred embodiment, the reference value is the median value of the expression of PDGFR-α (mRNA or protein) measured in a collection of biological samples. The collection of samples from which the reference level is derived will preferably be constituted from patients suffering from the same type of cancer. The collection may comprise samples from three, four, five, ten, 15, 20, 30, 40, 50 or more individuals, preferably, a statistically representative number of samples. In any case it can contain a different number of samples.

In the particular case that the expression levels of PDGFR-α are determined by measuring the levels of PDGFR-α mRNA, this is more conveniently carried out by the comparative Ct method using a housekeeping gene as an endogenous control and commercial RNA controls as calibrators or comparator. This provides the fold-change in expression in the test sample with respect to the control sample normalized to the amount of the endogenous control. Final results, are determined according to the formula $2^{-(\Delta Ct \; sample - \Delta Ct \; calibrator)}$, where ΔC_T values of the calibrator and sample are determined by subtracting the C_T value of the target gene from the value of the housekeeping gene. Suitable housekeeping genes that can be used as calibrators in the method of the invention include β-actin, GAPDH, β2-microglobuline, cyclophilin and the like.

17

The expression levels of PDGFR-α mRNA levels can also be done using discrete values and classifying the sample as "high" or "low" expressing sample. This is typically done by comparison to reproducible standards which correspond to the median value of expression levels of
5   PDGFR-α mRNA measured in a collection of tumor tissue in biopsy samples from cancer patients, previous to their treatment with PM00104. Once this median value is established, the level of this marker expressed in tumor tissues from patients can be compared with this median value, and thus be assigned a level of "low", when PDGFR-α mRNA expression levels
10 are lower than the median value of expression levels of PDGFR-α mRNA, or "high", when PDGFR-α mRNA expression levels are equal to or higher than the median value of expression levels of PDGFR-α mRNA.

In the particular case that the expression levels of PDGFR-α are
15 determined by measuring the levels of PDGFR-α protein, the determination is carried out using one or more reference proteins. Reference proteins are generally derived from housekeeping genes, are constitutively expressed and carry out essential cellular functions. Preferred housekeeping proteins for use in the present invention include β-2-microglobulin, ubiquitin, 18-S
20 ribosomal protein, cyclophilin, GAPDH, α-tubulin and β-actin. In a preferred embodiment, the reference protein is α-tubulin.

Similarly as with the situation when the levels of PDGFR-α mRNA are determined, the samples can be classified in two or more groups
25 according to its PDGFR-α protein expression level. For example, two groups "low level" and "high level" can be defined. In a preferred embodiment, said reference protein is a housekeeping protein. In a particular embodiment, said reference protein is alpha-tubulin (α-tubulin) and the patient is classified in the "low level" group when PDGFR-α levels are equal or lower
30 than 50% of those of α-tubulin and the patient is classified in the "high level" group when his PDGFR-α levels are higher than 50% of those of the α-tubulin protein. According to the results shown in Figures 8 and 9, a patient classified in the "low level" group will have an increased likelihood

18

of showing a positive response after treatment with PM00104. If desired, one or more "intermediate levels" can be defined.

The determined value of the reference protein expression level will depend on the reference protein to be assayed and on the methodology used to perform the protein determination. Protein determination can be carried out by any suitable methodology, such as for example by Western Blot analysis as described in Example 1 or other protein determination methodologies defined herein.

Alternatively, PDGRF-α protein expression levels can be determined as discrete values. As shown in Figure 6, the protein expression levels of different biomarkers were determined by Western Blot analysis in the panel of low passaged human sarcoma cell lines and discrete values where given to protein expression levels. PDGFR-α protein expression levels were recorded as negative expression (0), low expression (0.5), moderate expression (1), or high expression (2). According to the correlation results between the PDGFR-α expression levels and the tumor cell lines sensitivity to PM00104, which are shown in Figure 9, those cell lines with a negative expression (0) or low expression (0.5) of PDGFR-α protein have shown a higher sensitivity to PM00104.

Once the expression level of PDGFR-α in the sample isolated from the patient has been compared with the reference value, the method of the invention allows the identification of patients likely to show a positive response to the treatment with PM00104 based as those showing expression levels of PDGFR-α which are equal or lower than the reference value.

The PDGFR-α expression level is considered to be low when the levels in a sample from the subject under study are decreased with respect to the reference value by at least 5%, by at least 10%, by at least 15%, by at least 20%, by at least 25%, by at least 30%, by at least 35%, by at least

19

40%, by at least 45%, by at least 50%, by at least 55%, by at least 60%, by at least 65%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 100%, by at least 110%, by at least 120%, by at least 130%, by at least 140% by at least 150%, or

5   more.

The authors of the present invention have also observed that an increased accuracy of the prediction of the clinical outcome can be achieved by also measuring the phosphorylation status of C-kit and, in

10   particular, the presence of constitutively phosphorylated C-kit results in a correlation with increased statistitical significance. Moreover, the authors of the present invention have also observed that cell lines showing resistance to the treatment with PM00104 and which do not show increased levels of PDGFR-α show increased levels of Epidermal Growth

15   Factor Receptor (EGFR), suggesting that there is certain degree of cooperation between PDGFR-α and EGFR in the resistance to PM00104.

Thus, in a particular embodiment, the method for predicting the clinical response of a cancer patient to PM00104 chemotherapy corresponds to a

20   method further comprising the steps of

   (a) determining the activity of a receptor tyrosine kinase in the biological sample, and

   (b) comparing the activity levels in the biological sample with a reference value

25   wherein (i) an expression level of PDGFR-α equal to or lower than the reference value and (ii) an activity level of the receptor tyrosine kinase lower than the reference value indicates an increased likelihood of showing a positive response to the treatment with PM00104.

30   The term "receptor tyrosine kinase" or "RTK", as described herein, refers to any member of said family of receptors that regulates major cellular events, such as cell proliferation, differentiation, cell migration, adhesion and apoptosis. They are transmembrane proteins with enzymatic

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activity in the cytoplasmic part, catalyzing the transfer of the γ-phosphate of ATP to tyrosine residues in protein substrates. The RTK family includes a plethora of growth factor receptors, such as platelet-derived growth factor receptor (PDGF-R), epidermal growth factor receptor (EGF-R), the insulin

5   growth factor receptor (IGF-R) and the receptor of stem cell factor C-kit. Aberrant tyrosine kinase activity and/or tyrosine kinase overactivity is associated with the development and progress of various human cancers and hyperproliferative diseases. Hence, several strategies for inhibition of aberrant tyrosine kinase activity, such as antisense oligonucleotides,

10   antigenic stimulation and small molecular inhibitors have been developed (Roussidis et al., In vivo (2002), 16(6), 459-469; Roussidis et al., Current Medicinal Chemistry (2007), 14, 735-743; and Pytel et al., Anticancer Agents Med. Chem. (2009), 9(1),66-76).

15   In a further preferred embodiment of the predicting method of the invention, the receptor tyrosine kinase which activity is determined is C-kit. The C-kit proto-oncogene encodes C-kit receptor, a transmembrane glycoprotein with a tyrosine kinase activity in the intracellular domain. It belongs to the same subclass as the receptors for PDGF and colony

20   stimulating factor-1. Its natural ligand, SCF, is a hematopoetic growth factor, which activates the receptor promoting dimerization and autophosphorylation at specific tyrosine residues. C-kit signals major cellular functions, such as cell survival, proliferation, differentiation, adhesion, and chemotaxis. It also induces apoptosis and enhances the

25   invasive potential activating multiple signal transduction pathways, such as the MAP kinase cascade, PI3K, PLC and STAT3 (Roussidis et al., Current Medicinal Chemistry (2007), 14, 735-743).

The determination of the activity of C-kit can be carried out by any

30   method known to the skilled person and includes an enzymatic assay for determination of C-kit tyrosine kinase activity such as the assay described in Majumder et al. (Mol. Cell Biol. (1988), 8, 4896-4903) or by determining the degree of C-kit phosphorylation wherein absence or low level of phosphorylation of C-kit is indicative of decreased receptor tyrosine kinase

21

activity, i.e. the phosphorylation status of C-kit acts as a surrogate of C-kit activity.

The term "phosphorylation", as used herein, refers to the post-translational covalent addition of a phosphate group to the side chain in an amino acid within a peptide chain. Typically, protein phosphorylation is mediated by a protein kinase. Regulation of proteins by phosphorylation is one of the most common modes of regulation of protein function, and is often termed "phosphoregulation". In almost all cases of phosphoregulation, the protein switches between a phosphorylated and an unphosphorylated form, and one of these two forms is the active form, while the other one is inactive. Phosphorylated C-kit is the active form of the C-kit receptor.

While protein phosphorylation occurs in serine, threonine and tyrosine residues in higher eukaryotes, the method preferably involves the determination of phosphorylation at tyrosine residues. Typically, tyrosine phosphorylation can be determined by using any method known to the skilled person including ELISA, Western Blot using anti-phosphotyrosine specific antibodies, mass spectrometry or immunohistochemistry. In a preferred embodiment, phosphorylation can be determined by Western blot using phosphoamino acid-specific antibody and, more specifically, anti-phosphotyrosine antibodies on samples previously immunoprecipitated with anti-C-kit-specific antibodies.

The methods of the invention are suitable for predicting the clinical outcome of patients suffering from a wide variety of cancer types. By way of a non-limiting example, the invention allows to predict the clinical response to chemotherapy with PM00104 of patients suffering from varied types of cancer, including, without limitation, lung cancer, sarcoma, malignant melanoma, pleural mesothelioma, bladder carcinoma, prostate cancer, pancreas carcinoma, gastric carcinoma, ovarian cancer, hepatoma, breast cancer, colorectal cancer, kidney cancer, esophageal cancer, suprarenal cancer, parotid gland cancer, head & neck carcinoma, cervix

22

cancer, endometrial cancer, liver cancer, mesothelioma, multiple myeloma, leukaemia, and lymphoma.

METHOD FOR DESIGNING AN INDIVIDUAL CHEMOTHERAPY FOR A
5   PATIENT SUFFERING FROM CANCER

As mentioned before, it has been found that chemotherapy based on PM00104 is particularly effective in patients having low expression levels of PDGFR-α (protein or mRNA). Thus, PDGFR-α can be used as a marker for
10   the selection of cancer patients to be efficaciously treated with PM00104.

Accordingly, in another aspect, the invention provides a method for designing an individual chemotherapy for a human patient suffering from cancer comprising
15   a)   determining the expression level of PDGFR-α in a
       biological sample of the patient before PM00104 chemotherapy, and
              b)   comparing the expression level of PDGFR-α in the
       biological sample with a reference value
       wherein an expression level of PDGFR-α equal to or lower than the
20   reference value indicates that the patient is to be treated with a PM00104-
       based chemotherapy.

It will be understood that the different elements of these methods of the invention, such as determination of PDGFR-α mRNA or protein levels,
25   biological sample, determination of the expression value of mRNA, the comparison with a reference value and the determination of said reference value and the like are carried out essentially as described before.

The method for designing an individual chemotherapy for a patient
30   suffering from cancer according to the invention results in the selection of a given therapy for a patient which has been analysed for the expression levels of PDGFR-α. Accordingly, for those patients having an expression level of PDGFR-α equal to or lower than the reference value, the therapy of

23

choice will be a therapy based on the treatment with a PM00104-based chemotherapy. The particulars of the different types of PM00104-based chemotherapy have been described in detail in the context of the method for predicting the clinical outcome according to the invention.

In a preferred embodiment, the method for designing an individual chemotherapy for a patient suffering from cancer involves the determination of the PDGFR-α (mRNA or protein). In another preferred embodiment, the reference value used for determination of whether the expression levels are high or low corresponds to the expression levels of a control sample (or from a pool of samples obtained from a series of individuals essentially as described above). In another embodiment, the method for designing an individual chemotherapy for a patient suffering from cancer involves the determination of the activity of a receptor tyrosine kinase in the biological sample wherein an activity level of the receptor tyrosine kinase (RTK) lower than the reference value indicates that the patient is to be treated with a PM00104-based chemotherapy. In a still more preferred embodiment, the RTK is C-kit. Preferably, the activity of C-kit is measured by determining the degree of phosphorylation of C-kit, being said phosphorylation determined in the tyrosine residues in C-kit. Since phosphorylation in tyrosine residues in C-kit is associated with increased activity, an increased phosphorylation is indicative of increased activity and thus, the method involves the selection of PM00104 chemotherapy in patients having an expression level of PDGFR-α equal to or lower than the reference value and an phosphorylation level in C-kit lower than the reference value.

In a preferred embodiment, the patient is classified in one of two or more groups according to his PDGFR-α protein expression level. For example, two groups "low level" and "high level" can be defined. In a preferred embodiment, said reference protein is a housekeeping protein. In a particular embodiment, said reference protein is alpha-tubulin (α-tubulin) and the patient is classified in the "low level" group when PDGFR-α levels

are equal or lower than 50% of those of α-tubulin and the patient is classified in the "high level" group when his PDGFR-α levels are higher than 50% of those of the α-tubulin protein. According to the results shown in Figures 8 and 9, a chemotherapy treatment based on PM00104 will be

5   selected when the patient classified in the "low level" group.

Moreover, the authors of the present invention have also observed that the personalized chemotherapy based on PM00104 can also be decided on the basis of the determination of PDGFR-α protein expression

10   levels in combination with the phosphorylation status of C-kit.

Thus, in a preferred embodiment, the *in vitro* method for designing an individual chemotherapy for a human patient suffering from cancer based on the levels of PDGFR-α protein further comprises the

15   determination of the phosphorylation status of C-kit, wherein (i) a level of PDGFR-α protein equal or lower than those of the reference protein and (ii) low levels or absence of phosphorylated C-kit will determine the selection of a chemotherapy treatment based on PM00104.

20   In another preferred embodiment of the *in vitro* method, the determination of the expression levels of PDGFR-α protein is followed by recording the results of the determination as negative expression (0), low expression (0.5), moderate expression (1), or high expression (2). Accordingly, (i) a PDGFR-α protein expression level of (0) or (0.5) and (ii)

25   low levels or absence of phosphorylated C-kit will determine the selection of a chemotherapy treatment based on PM00104.

METHOD FOR SELECTING A PATIENT SUFFERING FROM CANCER FOR A TREATMENT WITH PM00104

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In a further aspect, the invention relates to a screening method for selecting a human patient suffering from cancer for a treatment with PM00104, comprising the steps of

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(a) determining the expression level of PDGFR-α in a biological sample of the patient before PM00104 chemotherapy,

(b) comparing the amount of expression of PDGFR-α in the biological sample with a reference value, and

5 (c) selecting a chemotherapy treatment based on PM00104 when said PDGFR-α expression level is equal to or lower than the reference value.

It will be understood that the different elements of these methods of 10 the invention, such as determination of PDGFR-α mRNA or protein levels, biological sample, determination of the expression value of mRNA, the comparison with a reference value and the determination of said reference value and the like are carried out essentially as described before.

15   The method for selecting a human patient suffering from cancer for a treatment based on PM00104 according to the invention results in the selection of a given patient as a candidate for the therapy based on PM00104 based on the presence of equal or lower levels PDGFR-α in a sample from said patient when compared to a reference sample. The 20 particulars of the different types of PM00104-based chemotherapy have been described in detail in the context of the method for predicting the clinical outcome according to the invention.

In a preferred embodiment, the method for selecting a patient 25 suffering from cancer for PM00104-based chemotherapy involves the determination of the PDGFR-α mRNA or protein levels. In another preferred embodiment, the reference value used for determination of whether the expression levels are high or low corresponds to the expression levels of a control sample or from a pool of samples obtained from a series of 30 individuals essentially as described above. In another embodiment, the method for selecting a patient suffering from cancer for PM00104-based chemotherapy involves the determination of the activity of a receptor tyrosine kinase in the biological sample wherein an activity level of the

receptor tyrosine kinase (RTK) lower than the reference value indicates that the patient is to be treated with a PM00104-based chemotherapy. In a still more preferred embodiment, the RTK is C-kit. Preferably, the activity of C-kit is measured by determining the degree of phosphorylation of C-kit,

5　being said phosphorylation determined in the tyrosine residues in C-kit. Since phosphorylation in tyrosine residues in C-kit is associated with increased activity, an increased phosphorylation is indicative of increased activity and thus, the method involves the selection of PM00104 chemotherapy in patients having an expression level of PDGFR-α equal to

10　or lower than the reference value and an phosphorylation level in C-kit lower than the reference value.

In a preferred embodiment, the patient is classified in one of two or more groups according to his PDGFR-α protein expression level. For

15　example, two groups "low level" and "high level" can be defined. In a preferred embodiment, said reference protein is a housekeeping protein. In a particular embodiment, said reference protein is alpha-tubulin (α-tubulin) and the patient is classified in the "low level" group when PDGFR-α levels are equal or lower than 50% of those of α-tubulin and the patient is

20　classified in the "high level" group when his PDGFR-α levels are higher than 50% of those of the α-tubulin protein. According to the results shown in Figures 8 and 9, a patient classified in the "low level" group will be selected for a treatment with PM00104.

25　In yet another aspect, the invention relates to a screening method for selecting a human patient suffering from cancer for a treatment with PM00104, comprising the steps:

　　　　a)　determining at least the expression level of PDGFR-α protein in a biological sample of the patient before the PM00104 chemotherapy,

30　　　　b)　recording the results of the determination in step (a) as negative expression (0), low expression (0.5), moderate expression (1), or high expression (2), and

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c)   selecting said patient classified in the (0) and (0.5) groups for a chemotherapy treatment based on PM00104.

Moreover, the authors of the present invention have also observed that the screening method for selecting a human patient suffering from cancer for a treatment with PM00104 can also be decided on the basis of the determination of PDGFR-α protein expression levels in combination with the phosphorylation status of C-kit. Thus, in a preferred embodiment, the screening method for selecting a human patient suffering from cancer for a treatment with PM00104 based on the levels of PDGFR-α protein further comprises the determination of the phosphorylation status of C-kit, wherein (i) a level of PDGFR-α protein equal or lower than those of the reference protein and (ii) low levels or absence of phosphorylated C-kit will determine the selection of said patient for a chemotherapy treatment based on PM00104.

In a further preferred embodiment of the screening method for selecting a cancer patient, the determination of the expression levels of PDGFR-α protein is followed by recording the results of the determination as negative expression (0), low expression (0.5), moderate expression (1), or high expression (2). Accordingly, (i) a PDGFR-α protein expression level of (0) or (0.5) and (ii) low levels or absence of phosphorylated C-kit will determine the selection of said patient for a chemotherapy treatment based on PM00104.

The methods of the invention for selecting a patient for chemotherapy based on PM00104 or for designing an individual chemotherapy based on PM00104 for a patient can be applied to patients suffering from varied types of cancer, including, without limitation, lung cancer, sarcoma, malignant melanoma, pleural mesothelioma, bladder carcinoma, prostate cancer, pancreas carcinoma, gastric carcinoma, ovarian cancer, hepatoma, breast cancer, colorectal cancer, kidney cancer, esophageal cancer, suprarenal cancer, parotid gland cancer, head & neck

28

carcinoma, cervix cancer, endometrial cancer, liver cancer, mesothelioma, multiple myeloma, leukaemia, and lymphoma.

As already described for the methods of the invention for predicting the clinical response of a patient suffering from cancer to the treatment with PM00104, the methods of the invention for selecting a patient for chemotherapy based on PM00104 or for designing an individual chemotherapy based on PM00104 for a patient can also be carried out in any type of sample from the patient, such as a biopsy sample, tissue, cell or fluid (serum, saliva, semen, sputum, cerebral spinal fluid (CSF), tears, mucus, sweat, milk, brain extracts and the like). In a preferred embodiment the sample from the patient is a tumor tissue sample. In a further preferred embodiment, a portion of normal tissue from the patient from which the tumor is obtained it is also examined.

THERAPEUTIC METHODS

The authors of the present invention have found that, surprisingly, the use of PM00104 in human cancer patients having certain expression levels of PDGFR-α, as PDGFR-α protein or as PDGFR-α mRNA, can lead to an increased antitumor efficacy in humans.

Thus, in another aspect, the invention is directed to PM00104 for the treatment of cancer in human patients having low levels of PDGFR-α expression. Alternatively, the invention relates to the use of PM00104 for the manufacture of a medicament for the treatment of cancer in a patient having low levels of PDGFR-α expression. Alternatively, the invention relates to a method for the treatment of cancer in human patients having low levels of PDGFR-α expression comprising the administration of PM00104.

The expression "low levels of PDGFR-α expression" refers to the levels of expression in a biological sample isolated from a patient when

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compared with a reference sample. Suitable biological samples for the determination of the PDGFR-α expression and suitable reference values have been described in detail with respect of the methods for selecting an individualised chemotherapy of the invention. The determination of

5   PDGFR-α expression can be carried by determination of the PDGFR-α mRNA levels or of the PDGFR-α protein levels.

The values for "low" or "high" levels of PDGFR-α expression are determined by comparison with the expression levels of at least a reference

10 protein in the sample. In a more preferred embodiment, said reference protein is a housekeeping protein. In a particular embodiment, said reference protein is alpha-tubulin (α-tubulin), and accordingly, "low" levels of PDGFR-α expression correspond to those when PDGFR-α expression levels are equal or lower than 50% of those of the α-tubulin protein and

15 "high" levels of PDGFR-α expression correspond to those when PDGFR-α expression levels are higher than 50% of those of the α-tubulin protein. If desired, one or more "intermediate levels" can be defined.

Alternatively, PDGRF-α protein expression levels can be determined

20 as discrete values. Accordingly, in a further aspect, the invention relates to a use of PM00104 in the treatment of a cancer patient comprising

a) determining at least the expression level of PDGFR-α protein in a biological sample of the patient before the PM00104 chemotherapy,

25 b) recording the results of the determination in step a) as negative expression (0), low expression (0.5), moderate expression (1), or high expression (2), and

c) treating with PM00104 said patient having negative (0) or low expression levels (0.5) of PDGFR-α protein.

30

Even more preferably, the patients to be treated with PM00104 are those having low levels of PDGFR-α mRNA or PDGFR-α protein and presenting C-kit in a non-phosphorylated status.

The invention also provides methods of treatment based on the levels of PDGFR-α, as PDGFR-α protein or as PDGFR-α mRNA. Thus, in another aspect, the invention is directed to a method for treating a human cancer
5   patient with PM00104, wherein said patient has low levels of PDGFR-α expression, in terms of mRNA expression or in terms of protein expression. Preferably, the patients also present C-kit in a non-phosphorylated status.

For these uses and methods of treatment mRNA and protein
10 expression levels are determined as described above for other methods of the invention.

As mentioned above, PM00104 is a synthetic alkaloid related to the marine compounds jorumycin and renieramycins, and also to safracin and
15 saframycin compounds, having the following structure:
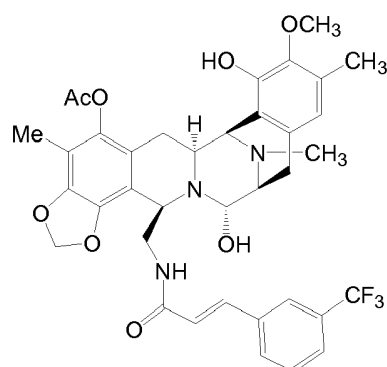
The term "PM00104" is intended here to cover any pharmaceutically
20 acceptable salt, solvate, hydrate, prodrug, or any other compound which, upon administration to the patient is capable of providing (directly or indirectly) the compound as described herein. The preparation of salts, solvates, hydrates, and prodrugs can be carried out by methods known in the art.
25

Pharmaceutically acceptable salts can be synthesized from the parent compound, which contains a basic or acidic moiety, by conventional

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chemical methods. Generally, such salts are, for example, prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent or in a mixture of the two. Generally, nonaqueous media

5   like ether, ethyl acetate, ethanol, isopropanol or acetonitrile are preferred. Examples of the acid addition salts include mineral acid addition salts such as, for example, hydrochloride, hydrobromide, hydroiodide, sulphate, nitrate, phosphate, and organic acid addition salts such as, for example, acetate, trifluoroacetate, maleate, fumarate, citrate, oxalate, succinate,

10 tartrate, malate, mandelate, methanesulphonate and p-toluenesulphonate. Examples of the alkali addition salts include inorganic salts such as, for example, sodium, potassium, calcium and ammonium salts, and organic alkali salts such as, for example, ethylenediamine, ethanolamine, N,N-dialkylenethanolamine, triethanolamine and basic aminoacids salts.

15

Any compound that is a prodrug of PM00104 is within the scope and spirit of the invention. The term "prodrug" is used in its broadest sense and encompasses those derivatives that are converted in vivo to PM00104. The prodrug can hydrolyze, oxidize, or otherwise react under biological

20 conditions to provide PM00104. Examples of prodrugs include, but are not limited to, derivatives and metabolites of PM00104 that include biohydrolyzable moieties such as biohydrolyzable amides, biohydrolyzable esters, biohydrolyzable carbamates, biohydrolyzable carbonates, biohydrolyzable ureides, and biohydrolyzable phosphate analogues.

25 Prodrugs can typically be prepared using well-known methods, such as those described by Burger "Medicinal Chemistry and Drug Discovery 6th ed. (Donald J. Abraham ed., 2001, Wiley) and "Design and Applications of Prodrugs" (H. Bundgaard ed., 1985, Harwood Academic Publishers).

30 In addition, any drug referred to herein may be in crystalline form either as free compound or as solvates (e.g. hydrates) and it is intended that both forms are within the scope of the present invention. Methods of solvation are generally known within the art.

32

PM00104 for use in accordance of the present invention may be prepared following the synthetic process disclosed in WO 01/87894, which is incorporated herein by reference.

5   Pharmaceutical compositions of PM00104 that can be used include solutions, suspensions, emulsions, lyophilised compositions, etc., with suitable excipients for intravenous administration. Preferably, PM00104 may be supplied and stored as a sterile lyophilized product, comprising PM00104 and excipients in a formulation adequate for therapeutic use. In

10 particular a formulation comprising sucrose and a phosphate salt buffered to an adequate pH is preferred. Further guidance on PM00104 formulations is given in WO 2007/052076 which is incorporated herein by reference in its entirety.

15   Administration of PM00104 or of pharmaceutical compositions comprising the compound is preferably by intravenous infusion. Infusion times of up to 72 hours can be used, more preferably between 1 and 24 hours, with either about 1, about 3 or about 24 hours most preferred. Short infusion times which allow treatment to be carried out without an

20 overnight stay in hospital are especially desirable. However, infusion may be around 24 hours or even longer if required.

      Preferably, the administration PM00104 is performed in cycles. In a preferred administration method an intravenous infusion of PM00104 is

25 given to the patients typically the first day of each cycle and then the patients are allowed to recover for the remainder of the cycle. The preferred duration of each cycle is typically of 3 or 4 weeks; multiple cycles can be given as needed. Alternatively, in another preferred administration method the drug is administered in a weekly basis, with a recovery period wherein

30 the drug is not administered to the patient. Dose delays and/or dose reductions and schedule adjustments are performed as needed depending on individual patient condition and tolerance to treatments. For further guidance on PM00104 administration and dosages, see for example WO 2008/135792 which is incorporated herein by specific reference.

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In addition, representative schedules and dosages are for example:

a)   about 3.0 mg/m² body surface area, administered as 1-hour intravenous infusion every three weeks;

b)   about 2.8 mg/m² body surface area, administered as 3-hour intravenous infusion every three weeks;

c)   about 4.0 mg/m² body surface area, administered as 24-hour intravenous infusion every three weeks; and

d)   about 2.0 mg/m² body surface area, administered as 1-hour weekly intravenous infusion for three consecutive weeks in a four-week cycle treatment schedule.

Although guidance for the dosage is given above, the correct dosage of the compound may change according to the particular formulation, the mode of application, and the particular situs, patient and tumor being treated. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the patient, drug combinations, reaction sensitivities and severity of the disease shall be taken into account. Dose delays and/or dose reductions and schedule adjustments are performed as needed depending on individual patient condition and tolerance of treatments.

Depending on the type of tumor and the development stage of the disease, anticancer effects of the methods of treatment of the present invention include, but are not limited to, inhibition of tumor growth, tumor growth delay, regression of tumor, shrinkage of tumor, increased time to regrowth of tumor on cessation of treatment, slowing of disease progression, and prevention of metastasis. It is expected that when a method of treatment of the present invention is administered to a patient in need of such treatment, said method of treatment will produce an effect, as measured by, for example, the extent of the anticancer effect, the response rate, the time to disease progression, or the survival rate. In particular, the methods of treatment of the invention are suited for human

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patients, especially those who are relapsing or refractory to previous chemotherapy. First line therapy is also envisaged.

The following examples further illustrate the invention. They should
5   not be interpreted as a limitation of the scope of the invention.

EXAMPLES

10   **Example 1: Molecular characterisation of a low passaged sarcoma cell lines panel.**

A panel of low-passaged tumor cell lines is of high value to gain knowledge on the molecular basis of sensitivity/resistance to a given
15   antitumoral compound. In particular, keeping the cell lines low passaged avoid undesired alterations occurring as a consequence of genomic instability of the tumor. A panel of low passaged tumor cell lines was generated from chemonaive sarcoma patients as described in Moneo et al. (Journal of Cellular Biochemistry (2007), 100, 399-348) and in Moneo et al.
20   (Mol. Cancer Ther. (2007), 6(4), 1310-1316). Molecular characterisation of the cell lines in the panel (Figure 1) was performed by determining the expression levels (mRNA or protein) of several biomarkers which often are found altered in cancer disease.

25   mRNA analysis (RT-PCR)
The expression levels of 14 genes which mRNA levels are often altered in human tumors were determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). In particular, the genes under study were $p16^{INK4a}$, $p15^{INK4b}$, $p14^{ARF}$, $p21^{CIP1}$, $p27^{KIP1}$, MSH-2, MLH-1, Apaf-1,
30   p73, E-cadherin, β-catenin, APC, PTEN and P85. β-actin was used as a control.

Total RNA was collected using the TRI-REAGENT (Molecular Research Center, Inc). RT was performed with 1 μg of Rnasy (Promega)

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following the manufacturer's protocol. The primers used to amplify regions are shown in Table 1 below.

<u>Table 1</u>

5

Genes	Oligos		Fragment size
PTEN	Oligo +: CAGAAAGACTTGAAGGCGTAT Oligo - : GTAACGGCTGAGGGAACTC	Tm 60 Tm 60	620 pb
P85	Oligo +: AA GAA CAG TGC CAG ACC CAA Oligo -:ACT CGT TCA ACT TCT TTT GCC	Tm60 Tm 60	310 pb
Apaf-1	Oligo +:AGG ACA TCA AGA CAT CCT AC Oligo -:CAC ACA GGA CTG TCC TTA CA	Tm 58 Tm 58	310 pb
p16	Oligo +: CAACGCACCGAATAGTTACG Oligo - : TACCGTGCGACATCGCGAT	Tm 60 Tm 60	270 pb
p15	Oligo + : ATGCGCGAGGAGAACAAGGGC Oligo - : GGGCGGCTGGGGAACCTGG	Tm 68 Tm 68	440 pb
p21	Oligo +: CATGTCAGAACCGGCTGGGGA Oligo -: CTTCCTCTGCTGTCCCCTGCA	Tm 68 Tm 68	320 pb
p27	Oligo + : ATGTCAAACGTGCGAGTGTC Oligo -: TTACGTTTGACGTCTTCTGAG	Tm 60 Tm 60	541 pb
p14ARF	Oligo +: GAGTGGCGCTGCTCACCTC Oligo - TACCGTGCGACATCGCGAT	Tm 60 Tm 60	510 pb
E-cadherin	Oligo +: TCCCATCAGCTGCCCAGAA Oligo - : ATGACTCCTGTGTTCCTGTTA	Tm 60 Tm 60	500 pb
p73	Oligo +: TTTAACAGGATTGGGGTGTCC Oligo -: CGTGAACTCCTCCTTGATGG	Tm62 Tm 62	410 pb
APC	Oligo + : GAATAGCCAGAATTCAGCAAATCG Oligo -: GAAAGTTTCATTAGAACACACACAG	Tm 68 Tm 68	720 pb
β-cathenin	Oligo +: AGCTGATTTGATGGAGTTGGA Oligo - : GCTACTTGTTCTTGAGTGAAG	Tm 60 Tm 60	240 pb
MSH2	Oligo +:AGAGATTGAATTTAGTGGAAGC Ooligo -: ACAATAGCTTATCAATATTACCTT	Tm 60 Tm 60	1777 pb
MLH1	Oligo +: GCATCTAGACGTTTCCTTGG Oligo -: GAGGAATTGGAGCCCAGGA	Tm 60 Tm 60	367 pb
β-actin	Oligo +: AGGCCAACCGCGAGAAGATGAC Oligo-: GAAGTCCAGGGCGACGTAGCA	Tm 70 Tm 68	330 pb

cDNA was subjected to PCR in a Biometra T3 termocycler. PCR conditions were as follows: 3 minutes at 95°C, 30 amplification cycles (1 minute at 95°C, 1 minute at 65°C or 55°C (according to the primers Tm)

36

and 1 minute at 72°C) with a final extension of 10 minutes at 72°C. PCR products were analyzed by electrophoresis on a 1 % agarose gel. The size of the PCR amplification fragments is also shown in Table 1. Results are shown in Figure 2.

5

Protein analysis

Other relevant genes in cancer disease do not show alterations at the transcription level but are affected by alteration of the protein expression or the presence of post-translational modifications. Protein expression

10   levels of the following molecular markers were determined by Western blot analysis: cyclin D1, Cyclin-dependent kinase 4 (CDK4), MDM2, p53, P27[Kip1], pp60src, Platelet-derived growth factor receptor (PDGFR-α), PTEN, AKT and phosphorylated AKT (p-AKT). The determination of phosphorylated C-kit (p-C-kit) levels was carried out by Western Blot after

15   immunoprecipitation.

-   Western Blot assay

To prepare the whole-cell extract, cells were washed once in cold phosphate-buffered saline (PBS) and suspended in 1 ml lysis-buffer (50

20   mM Tris-HCl pH 7.5, 1% NP-40, 10% glycerol, 150 mM NaCl, 2 mM complete protease inhibitor cocktail (Roche, Switzerland)). The protein content of the lysates was determined by the modified method of Bradford (Fazekas de St Groth et al. Biochim Biophys Acta (1963), 14, 71, 377-91). Samples were diluted in a sample buffer (Tris-HCl 62.5 mN pH 6.8 (Sigma),

25   glycerol 10% (Sigma), SDS 1% (Merck), 2-mercaptoethanol 5% (Sigma), bromophenol blue 0.0025% (Sigma)) and were denatured for 5 minutes at 95°C. Proteins were separated on 7.5% SDS-PAGE gels in a electrophoresis buffer (5x: TrisHCl 0.13M (Sigma), glycine 0.95M (Sigma), SDS 0.5%). Then, proteins were transferred onto Immobilon-P membrane (Millipore) using

30   Mini trans-Blot Electrophoretic transfer Cell (Biorad). Transfer was carried out according to the manufacturer's protocol. Finally, the membranes were blocked by being maintained 4 hours under agitation with a blocking buffer containing Tris-buffered saline (TBS) buffer, 0.1% Tween 20 (Sigma)

and 1% Bovine serum albumin (BSA) (Sigma) and immunostained by using the antibodies shown in Table 2 below:

Table 2

Biomarker	Primary Antibody	Secondary Antibody
p53	Anti-p53 FL393 (polyclonal; Santa Cruz)	Anti-rabbit IgG (Calbiochem)
MDM2	Anti-MDM2 (monoclonal; Santa Cruz)	Anti-mouse IgG (Promega)
CDK4	Anti-CDK4 (polyclonal; Santa Cruz)	Anti-rabbit IgG (Calbiochem)
cyclin D1	Anti- cyclin D1 (monoclonal; Dako)	Anti-mouse IgG (Promega)
p27	Anti-p27 (monoclonal; Transduction laboratories)	Anti-mouse IgG (Promega)
PTEN	Anti-PTEN (monoclonal; Upstate biotechnology)	Anti-mouse IgG (Promega)
pp60 src	Anti-pp60 src (monoclonal; Upstate biotechnology)	Anti-mouse IgG (Promega)
PDGF-α	Anti-PDGF-α c-20 (polyclonal; Santa Cruz)	Anti-rabbit IgG (Calbiochem)
C-kit	Anti-C-kit c-19 (polyclonal; Santa Cruz)	Anti-rabbit IgG (Calbiochem)
AKT	Anti –AKT 9272 (polyclonal; Cell Signalling)	Anti-rabbit IgG (Calbiochem)
AKT-P	Anti-AKT-P Ser 473 9271 (polyclonal; Cell Signalling)	Anti-rabbit IgG (Calbiochem)

The protein containing membranes were incubated overnight at 4°C in agitation with the appropriate dilution of primary antibody in blocking buffer. Membranes were washed (three times, 5 minutes) with TBS containing 0.1% Tween 20, and then incubated for 30 minutes at room temperature with the peroxidase conjugated secondary antibody in blocking buffer. Proteins were visualized using the Enhanced Chemiluminiscence (ECL) detection system (Amersham Pharmacia Biotech) and Kodak-X.Omat LS autoradiography film (Kodak).

- Immunoprecipitation

Phosphorylated C-kit levels were determined by Western Blot after immunoprecipitation. Protein lysates were obtained as described for Western Blot analysis. An extract volume containing 150 μg of protein was used. 5 μl of the C-kit primary antibody (Santa Cruz) were added to the

38

lysates to form antigen-antibody complexes. The complexes precipitate by reaction with an immunoadsorbent solid phase, in particular, a sepharose-protein A complex (Sigma). Mild agitation was maintained for 3 hours. Further 25 µl of sepharose-protein A were added and agitation was
5   maintained for an additional hour. Then, the immunoprecipitated was washed 4 times with a washing buffer (20 mM Hepes pH 7.5 (Sigma), 150 mM NaCl (Panreac), 0.1% Triton X-100 (Sigma), 10% glycerine (Sigma), 10 mM $Na_4P_2O_7$ (Sigma)). Finally, quantification of the phosphorilation status of C-kit was determined by carrying out an electrophoresis in a 7.5% SDS-
10   PAGE gel followed by immunodetection using an anti-p-tyrosine (Santa Cruz) as the primary antibody, as described in the above section.

Protein expression levels of cyclin D1, CDK4, MDM2, p53 and P27[Kip1] are shown in Figure 3 and those of pp60[src], PDGFR-α, p-C-kit and PTEN
15   are shown in Figure 4.

Results of Western Blot analysis of AKT and phosphorylated AKT (p-AKT) expression levels are shown in Figure 5. It was known in the art (Kauffmann-Zeh et al. Nature (1997),385, 544-548) that cells grown in a
20   low serum medium have a reduced expression of p-AKT but when the PTEN/PI3K route is altered phosphorylation levels are maintained. Accordingly, AKT and p-AKT levels were determined after culturing cells during 24 h in 0.5% and 10% serum containing medium. It was observed that in 14 of the assayed cell lines the phosphorylation level was
25   maintained which could be indicative of a constitutive activation of the PTEN/PI3K route.

Example 2: Pharmacological characterisation of the low passaged sarcoma cell lines.

30
The sensitivity and resistance profiles of the low passaged tumor cell lines of Table 1 to twelve well-known antitumor drugs were studied. In particular, the drugs assayed in the sarcoma cell lines of the panel were: gemcitabine (Lilly), cisplatin (Sigma), oxaliplatin (Sigma), camptothecin

39

(Sigma), doxorubicin (Sigma), paclitaxel (Sigma), vinblastine (Sigma), flavopiridol (Sigma), UCN-01 (Sigma), imatinib (LC Laboratories), rapamycin (Bio Mol) and LY-294002 (Sigma).

5   Cytotoxicity assay

Firstly, cytotoxicity of the compounds against each of the cell lines was determined by the tetrazolium colorimetric assay (MTT assay) described by Denizot and Lang (J. Immunol. Methods (1986), 89, 271-277) and Mosmann (J. Immunol. Methods (1983), 65, 55-63). The compounds
10   were tested on 96-well trays. Cells growing in a flask were harvested just before they became confluent, counted using a haemocytometer (Coulter) and diluted down with media adjusting the concentration to the required number of cells per 0.2 ml (volume for each well). Details on the culture media used for each of the cell lines of the panel are provided in Moneo et
15   al. (Journal of Cellular Biochemistry (2007), 100, 399-348). Cells were then seeded in 96-well trays at a density between 1000 and 4000 cells/well, depending on the cell size. Cells were left to plate down and grow for 24 hours at 37°C, 5% CO_2 before initiating the cytotoxicity assays. Drugs were diluted with dimethyl sulfoxide (DMSO) (Sigma) to get them into solution.
20   The final concentration of DMSO in the tissue culture media did not exceed 0.5%. The appropriate volume of the drug solution (usually 2 μl) was added automatically (Beckman FX 96 tip) to the medium to make it up to the final concentration for each drug. The drug concentrations assayed were as follows: imatinib and LY-294002 from 0.002 to 100 μM; UCN-01 from
25   0.00016 to 10 μM; rapamycin, flavopiridol, vinblastine, paclitaxel and campthotecin from 0.002 to 100 nM; gemcitabine and doxorubicin from 0.005 to 300 nM; cisplatin from 0.005 to 300 μM; and oxaliplatin from 0.00084 to 50 μM. Each concentration was assayed in triplicate. Two sets of control wells were left on each plate, containing either medium or
30   medium with the same concentration of DMSO as the sample being assayed. A third control (seeding control) was used to determine the number of cells starting the culture. Cells were exposed to the drugs for 96 hours. Then, 50 μl of a freshly prepared solution of MTT (Sigma) in culture

40

medium were added to each well at a final concentration of 1mg/ml. Plaques with MTT were incubated for 5 hours at 37°C. Afterwards, medium was discarded and 50 µl of DMSO were added to each well and surviving cells were quantified by measuring absorbance using a Victor 3 multilabel reader. Finally, IC$_{50}$ values (the concentration of drug at which absorbance was reduced 50% with regards to the DMSO control sample) were determined. The IC$_{50}$ values were calculated by adjusting the results to a sigmoid curve using the GraphPad Prism 3.0 software (GraphPad Software, Inc.).

Cytotoxicity results (IC$_{50}$ values) are shown in Tables 3 and 4 below.

Table 3

CELL LINES	Gemcitabine (nM)	Cisplatin (µM)	Oxaliplatin (µM)	Camptothecin (nM)	Doxorubicin (nM)	Paclitaxel (nM)
CNIO AW	18.5	>300	4.02	30.8	45	7.44
CNIO AX	8.9	>100	3.37	18.9	44	16.4
SW872	11.93	>300	>50	14.2	>300	5.76
1455	8.43	>100	2.86	29.3	>300	2.7
CNIO AA	5.3	>100	3.03	17.7	21.5	1.67
CNIO AY	65.6	>100	0.5	>50	44	6.3
CNIO AZ	4.4	>300	>50	>50	14	>100
CNIO BC	34	>300	>50	24.5	>300	>100
CNIO BB	>100	0.35	>50	>50	232	>100
A673	7.2	>100	>50	>50	50	2.8
CNIO BJ	>300	>300	0.7	>50	>300	5.6
CNIO BF	12.6	>300	16.97	38.3	15	18.8
CNIO BP	7.5	>100	2.45	27.43	>300	21.5
SAOS-2	23	>100	21.28	100	>300	2.19
CNIO BG	5.78	>100	1.5	13.54	22	5
CNIO BM	>300	>300	>50	>100	>300	>100
CNIO BN	>300	>100	1.45	>100	>300	>100
CNIO CE	>300	>100	0.8	>50	>300	>100
CNIO BI	10.9	>300	2.9	>100	50	3.1

Table 4

CELL LINES	Vinblastine (nM)	Flavopiridol (nM)	UCN-01 (µM)	Imatinib (µM)	Rapamycin (nM)	LY-294002 (µM)
CNIO AW	13.73	29.08	0.09	>100	0.64	15.66
CNIO AX	>100	17.85	0.11	>100	0.16	6.8
SW872	>100	9.6	0.052	39.4	0.7	7.46
1455	6.3	6.75	0.016	30.33	0.22	5.62
CNIO AA	6.3	6	0.158	61.58	0.5	6.99
CNIO AY	13.6	4.1	0.05	>100	0.12	19.14
CNIO AZ	10.87	5	0.13	>100	18.3	6.84
CNIO BC	25.16	15.85	0.05	>100	4.89	>100
CNIO BB	>100	23.7	0.041	>100	1.27	>100
A673	7.9	12	0.87	>100	>100	6.45
CNIO BJ	19.06	6.8	0.078	28.49	>100	>100
CNIO BF	>100	33.1	0.1	>100	0.3	8.76
CNIO BP	4.83	26	0.124	37.04	2.68	12.13
SAOS-2	5.56	3.8	0.031	>100	1.24	75.72
CNIO BG	6.58	24.5	0.05	31.57	>100	6.28
CNIO BM	>100	>100	>10	>100	>100	27
CNIO BN	>100	2.85	0.024	54.01	0.52	2.17
CNIO CE	>100	17.5	0.05	>100	>100	5.8
CNIO BI	13.09	22.5	0.42	21.31	0.5	22.19

Identification of biomarkers of sensitivity/resistance

The cytotoxicity data obtained with these well-known antitumor drugs together with the molecular characterisation of the sarcoma cell lines of the panel was analysed. A bioinformatics algorithm was used to determine genes which correlate with the pharmacologic activity of the compound. Particularly, a univariable Cox method was used to analyse the relationship between IC_{50} values and biomarkers. It is to note that the data of the p21[CIP1], p27[KIP1], MSH2, MLH1, PTEN, P85, β-Catenin, Apaf-1 and APC genes were not considered because it was found that they had constant values. Similarly, response to cisplatin was similar in all cell lines except one and thus, it was decided not to consider the data of this compound. Accordingly, 16 gene markers and 11 compounds were ultimately used for the calculations.

Discrete values are needed to perform the analysis by correlation analysis software. Accordingly, the presence or absence of mRNA

42

expression for the different markers was scored as 1 or 0, meaning presence and absence, respectively. The assignation of the score values to the mRNA expression levels was performed after visual analysis of the intensity of RT-PCR products in the agarose gels. Similarly, scoring values

5   of 0, 0.5, 1, 2 or 3 were assigned after visual determination of the protein expression levels after Western Blot analysis, according to the observed intensity. Score data of mRNA and protein expression levels is shown in Figure 6.

10   Fifteen Cox models were obtained for each compound, one for each marker with the exception of p16[INK4a] and p14[ARF] which had shown identical patterns. In addition, the t-test P value was also used to evaluate the statistical significance of the correlation. The results of the statistical analysis giving the correlation between the cytotoxicity values obtained for

15   the assayed drugs and the biomarkers' expression in the panel of cell lines are shown in Figure 7.

     It was found that flavopiridol showed a correlation with CDK4 levels, namely, those cell lines with high levels of CDK4 were more sensitive to the

20   treatment with flavopiridol. This relationship was prior described by Lu et al., (Lu et al. Cancer Chemother. Pharmacol. (2000) 46, 293-304). In connection with paclitaxel, results are coincident with those published by other authors proposing that cells with non-functional p53 are more sensitive to paclitaxel (Kielb et al. J. Urol. (2001), 166, 482-487; and

25   Kurschat and Mauch, Clin. Exp. Dermatol. (2000), 25, 482-489). Furthermore, the obtained data showed a correlation between an increase of the PDGFR-α and resistance to paclitaxel. This relation was prior described by Isonishi et al. (Oncol. Rep. (2007), 18, 195-201) which described that a pre-treatment with PDGF increases the *in vitro* resistance

30   of cells to paclitaxel. Similarly, Kim et al. (Science (2004), 266, 2011-2015) disclosed that treatment of cell lines with PDGF inhibitor molecules (e.g. STI571) increased paclitaxel sensitivity. Finally, a statistically significant relationship was found between the phosphorylated form of p60Scr and

43

resistance to oxaliplatin. This relation was prior suggested by Griffiths et al. (J. Biol. Chem. (2004), 279, 46113-46121).

The above results show that the sensitivity/resistance profile of the
5   low passaged tumor cell lines panel to several well-known antitumor drugs was consistent with those prior described in the literature. Therefore, the predictive value of this panel together with the bioinformatics algorithms used was confirmed.

10 **Example 3: Cytotoxicity of PM00104 against the low passaged sarcoma cell lines.**

With the aim of identifying new markers of sensitivity and resistance to PM00104 we analysed the cytotoxicity (IC_{50} values) of the antineoplastic
15 agent on the human tumor cell lines of Figure 1. The cytotoxicity assay was performed as described in Example 2. Drug concentrations from 0.002 to 100 nM and from 0.002 to 100 mM were assayed. After 96-hour exposure, cells were washed twice with phosphate buffered saline (PBS) (Sigma) before being fixed with 10% glutaraldehyde (Panreac). Cells were
20 washed twice and fixed with crystal violet (Merck) 0.5% during 30 minutes. Then washed extensively, solubilized with 15% acetic acid (Panreac) and absorbance was measured at 595 nm. Cytotoxicity values (IC_{50}) were calculated as an average of three independent experiments done in triplicate. Response to PM00104 varied from < 1 nM to > 1 mM, as it can
25 be seen in Table 5 below.

Table 5

Cell line	Origin	PM00104 (nM)
CNIO AW	Liposarcoma	0.16
CNIO BP	Osteosarcoma	0.29
CNIO AX	Liposarcoma	0.32
1455	Liposarcoma	0.34
A673	Ewing's Sarcoma	0.42
CNIO AA	Leiomyosarcoma	0.5
SW872	Liposarcoma	0.87
SAOS-2	Osteosarcoma	0.94
CNIO BJ	Osteosarcoma	103
CNIO BF	Osteosarcoma	501

Cell line	Origin	PM00104 (nM)
CNIO BC	MPNST	664
CNIO AZ	Fibrous tumor of the pleura	870
CNIO BB	MPNST	1008
CNIO BG	Myxoid fibrosarcoma	1200
CNIO CE	Rhabdomyosarcoma	2401
CNIO AY	Leiomyosarcoma	2580
CNIO BM	Hibernoma	3473
CNIO BN	Fibrohistiocytoma	4750
CNIO BI	GIST	4933

Example 4: Identification of biomarkers of sensitivity/resistance to PM00104.

PDGFR-α protein levels and c-Kit phosphorylation status were previously determined by Western Blot as described in Example 1. These protein expression results were correlated with the IC_{50} values obtained after exposure to PM00104 as described in Example 3, showing that high basal expression levels of PDGFR-α protein were associated with increased resistance to the drug (Figure 8). Furthermore, relative expression levels of PDGFR-α protein expressed as percentage of the reference protein levels (i.e. α-tubulin) for the assayed low passaged human tumor cell lines was determined. It was observed that those cells which were found to be sensitive to PM00104 had expression levels of PDGFR-α equal or lower than 50% of those of the α-tubulin protein. The only exceptions were the cell lines CNIO-BF and CNIO-BI which despite expressing low levels of the marker were quite resistant to the drug. However, as shown in Figure 8 these cell lines constitutively expressed phosphorylated c-Kit receptor.

Furthermore, the previously validated bioinformatics algorithm was used to correlate the gene markers profile of the tumor cell lines in the panel with their sensitivity/resistance to PM00104. Accordingly, we carried out a univariate Cox model analysis to determine the relationship between IC_{50} cytotoxicity values and the tumor cell lines biomarkers profile. Based on the outcome of the Cox model analysis, it was evaluated how relevant

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each of the markers was for IC_{50} by using, for instance, the p-value for the likelihood-ratio test of the Cox model. Multivariate Cox models were also used to evaluate whether a better signature was obtained by the combination of several markers.

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By applying these statistical analyses to all the markers, the likelihood value for each marker for the sensitivity to one specific treatment was obtained. Statistically relevant values ($p<0.05$) were only obtained with regards to PDGFR-α. The correlation index between PDGFR-α as an individual marker and the IC_{50} values obtained by univariate Cox models was -1.45. A negative coefficient means that increases in the marker are associated with increases in IC_{50} (i.e., a biomarker of resistance). Accordingly, PM00104 sensitivity correlated with low levels of PDGFR-α protein. In addition, the combined presence of constitutively phosphorylated C-kit increased the statistical significance of the correlation. Results are shown in Figure 9A and 9B.

Example 5: Evaluation of *In vivo* response to PM00104 in human xenograft models

20

As shown in Example 4, we found that high levels of PDGFR-α expression determined resistance to the treatment with PM00104. In order to validate PDGFR-α expression as a marker predictive of the sensitivity to the treatment with PM00104 in the *in vivo* setting, we generated xenografts from different cell lines expressing high or low levels of PDGFR-α. It was found that the low passaged sarcoma cells of the predictive panel did not grow exponentially when xenografted in immunosuppressed mice. Thus, the following commercial cell lines were used for the *in vivo* assay:

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- A2780 (ECACC 93112519), ovary adenocarcinoma;
- HepG2 (ATCC HB-8065), hepatocellular carcinoma;
- HGC-27 (CLS), gastric carcinoma;
- MDA-MB-231 (ATCC HTB-26), breast adenocarcinoma;

- UMUC-3 (ATCC CRL-1749), bladder carcinoma;

- SW1990 (ATCC CRL-2172), pancreatic adenocarcinoma;

- SKOV-3 (ATCC HTB-77), ovary adenocarcinoma;

- MX-1 (CLS), breast carcinoma; and

5   - Calu-6 (ATCC HTB-59), lung carcinoma.

Cells were maintained in the appropriate culture medium (RPMI-1640 (Sigma) for A2780, MX-1 and SW1990; MEME (Sigma) for HepG2, Calu-6 and UMUC-3; IMDM (Sigma) for HGC-27; DMEM (Sigma) for MDA-

10   MB-231; McCoy's (Sigma) for SKOV-3) supplemented with 10% FBS, 2 mM L-Glutamine (Gibco/Invitrogen) and 100 units/mL of penicillin-streptomycin (Gibco/Invitrogen) at 37°C and 5% CO_2.

First, we determined by Western-Blot analysis the PDGFR-α

15   expression levels in these cells (as above described) using an Anti-PDGFR-α (c-20) polyclonal antibody (Santa Cruz) as primary antibody. Results are shown in Figure 10. Tumor cell lines HGC-27, SW1990, Calu-6 and A2780 showed high levels of PDGFR-α, whilst UMUC-3, SKOV-3, MX-1, MDA-MB-231 and HepG2 showed low levels of PDGFR-α.

20

Four- to six-week-old athymic nu/nu mice (Harlan Sprague Dawley, Madison, WI, USA) were subcutaneously xenografted into their right flank with (0.5–1) x 10^7 cells in 0.2 ml of a mixture (50:50; v:v) of Matrigel basement membrane matrix (Beckton Dickinson) and serum-free medium.

25   When tumors reached approximately 150 mm³, mice were randomly assigned into treatment or control groups. PM00104 was intravenously administered either in 3 consecutive weekly doses of 0.9 mg/kg/day (HepG2, SKOV-3, Calu-6, A2780, MDA-MB-231) or in 2 cycles of 5 consecutive daily doses of 0.3 mg/kg/day (SW1990, HGC-27, UMUC-3,

30   MX-1). Control animals received an equal volume of vehicle (500 mg Sucrose + 34 mg Potassium Phosphate + Phosphoric acid q.s. pH 3.8-4.4). Caliper measurements of the tumor diameters were done twice weekly and tumor volumes calculated according to the following formula: (a x b^2)/2,

47

where *a* and *b* were the longest and shortest diameters, respectively. Differences in tumor volumes between treated and control groups were evaluated using the unpaired t-test. Statistical significance was defined as p < 0.05. Statistical analyses were performed by LabCat1 v8.0 SP1 (Innovative Programming Associates, Inc. NJ, USA). All animal studies were conducted under approval from an IACUC in an AAALAC accredited animal facility.

An observed reduction of the tumor burden with respect to the control untreated tumor which was higher than 60% was considered a positive response. Only the xenografts of 4 cell lines did not show a positive outcome after the treatment with PM00104: HGC-27, SW1990, SKOV-3, Calu-6 and A2780, as shown in Figure 12. Interestingly, the non-respondent xenografts were obtained with cells presenting high levels of PDGFR-α protein, with the exception of SKOV-3. Therefore, a relationship between low levels of PDGFR-α and sensitivity to PM00104 was confirmed in the *in vivo* model.

Further analysis of marker expression was performed in the panel of human epithelial tumor cell lines. In particular, Epidermal Growth Factor Receptor (EGFR) and phosphorylated EGFR (p-EGFR) expression levels were determined by Western Blot using Anti-EGFR polyclonal antibody (Cell Signaling Technology) and Anti-phospho-EGFR-Y1068 polyclonal antibody (Invitrogen) as primary antibodies, respectively. It was found that SKOV-3 was the only cell line expressing high levels of constitutively active EGF receptor (Figure 11). This data indicates that there is certain degree of cooperation between PDGFR-α and EGFR in the resistance to PM00104 in the *in vivo* setting.

48

CLAIMS

1. A method for predicting the clinical response of a cancer patient to
    PM00104 chemotherapy or for designing an individual chemotherapy
    for a human patient suffering from cancer comprising
        (a) determining the expression level of PDGFR-α in a biological
            sample of the patient before PM00104 chemotherapy, and
        (b) comparing the expression level of PDGFR-α in the biological
            sample with a reference value
    wherein an expression level of PDGFR-α equal to or lower than the
    reference value indicates an increased likelihood of showing a positive
    response to the treatment with PM00104 or that the patient is to be
    treated with a PM00104-based chemotherapy.

2. A method as defined in claim 1 wherein the reference value is selected
    from the group of (i) the expression level of PDGFR-α in a non-tumor
    tissue and (ii) the median value of the PDGFR-α expression level in a
    collection of biological samples.

3. A method as defined in claims 1 or 2 wherein the expression level of
    PDGFR-α is determined by determination of the PDGFR-α mRNA
    levels or the PDGFR-α protein levels.

4. A method as defined in claim 3 wherein the determination of the
    PDGFR-α protein levels is carried out by Western blot or by
    immunohistochemistry.

5. A method according to any of claims 1 to 4 further comprising the
    steps of
        (a) determining the activity of a receptor tyrosine kinase in the
            biological sample, and
        (b) comparing the activity levels in the biological sample with a
            reference value

wherein (i) an expression level of PDGFR-α equal to or lower than the reference value and (ii) an activity level of the receptor tyrosine kinase lower than the reference value indicates an increased likelihood of showing a positive response to the treatment with PM00104 or that the patient is to be treated with a PM00104-based chemotherapy.

6.   A method as defined in claim 5 wherein the receptor tyrosine kinase is C-kit.

7.   A method as defined in claim 6 wherein the activity level of the C-kit is determined by measuring the phosphorylation level of said receptor, wherein high level of phosphorylation results in an increased C-kit activity.

8.   A screening method for selecting a human patient suffering from cancer for a treatment with PM00104, comprising the steps of

(a) determining the expression level of PDGFR-α in a biological sample of the patient before PM00104 chemotherapy,

(b) comparing the amount of expression of PDGFR-α in the biological sample with a reference value, and

(c) selecting a chemotherapy treatment based on PM00104 when said PDGFR-α expression level is equal to or lower than the reference value.

9.   A method as defined in claim 8 wherein the reference value is selected from the group of (i) the expression level of PDGFR-α in a non-tumor tissue and (ii) the median value of the PDGFR-α expression level in a collection of biological samples.

10.   A method as defined in claims 8 or 9 wherein the expression level of PDGFR-α is determined by determination of the PDGFR-α mRNA levels or the PDGFR-α protein levels.

11. A method as defined in claim 10 wherein the determination of the PDGFR-α protein levels is carried out by Western blot or by immunohistochemistry.

5   12. A method according to any of claims 8 to 11 further comprising the steps of

(a) determining the activity of a receptor tyrosine kinase in the biological sample, and

(b) comparing the activity levels in the biological sample with a

10   reference value

wherein (i) an expression level of PDGFR-α equal to or lower than the reference value and (ii) an activity level of the receptor tyrosine kinase lower than a reference value determines selecting a chemotherapy treatment based on PM00104.

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13. A method as defined in claim 12 wherein the receptor tyrosine kinase is C-kit.

14. A method as defined in claim 13 wherein the activity level of C-kit is

20   determined by measuring the phosphorylation level of said receptor, wherein low level of phosphorylation results in a decreased C-kit activity.

15. PM00104 for use in the treatment of cancer in a patient wherein a

25   biological sample of said patient has a PDGFR-α expression level lower than a reference value.

16. PM00104 as defined in claim 15 wherein the PDGFR-α expression level is determined by measuring PDGFR-α mRNA expression level or

30   PDGFR-α protein expression level.

51

17. PM00104 according to any of claims 15 or 16 wherein a biological sample of said patient further presents a decreased activity of a receptor tyrosine kinase with respect to a reference value.

5   18. PM00104 according to claim 17 wherein the receptor tyrosine kinase is C-kit.

19. PM00104 according to claim 18 wherein the activity level of the C-kit is determined by measuring the phosphorylation level of said receptor, wherein high level of phosphorylation results in an increased C-kit activity.

Cell line	Origin	Phenotype	Duplication time (h)
CNIO AW	Liposarcoma	Fibroblastic	48h
CNIO AX	Liposarcoma	Epithelial	48h
SW872	Liposarcoma	Fibroblastic	24h
CNIO BK	Liposarcoma	Fibroblastic	96h
1455	Liposarcoma	Fibroblastic	48h
CNIO AA	Leiomyosarcoma	Epithelial	36h
CNIO AY	Leiomyosarcoma	Fibroblastic	48h
CNIO AZ	fibrous tumor of the pleura	Fibroblastic	96h
CNIO BC	MPNST	Epithelial	96h
CNIO BB	MPNST	Fibroblastic	72h
A673	Ewing's Sarcoma	Fibroblastic	24h
CNIO BJ	Osteosarcoma	Epithelial	96h
CNIO BF	Osteosarcoma	Epithelial	24h
CNIO BP	Osteosarcoma	Epithelial	48h
SAOS-2	Osteosarcoma	Epithelial	48h
CNIO BG	myxoid fibrosarcoma	Fibroblastic	24h
CNIO BM	Hibernoma	Fibroblastic	96h
CNIO BN	Fibrohistiocytoma	Fibroblastic	72h
CNIO CE	Rhabdomyosarcoma	Fibroblastic	48h
CNIO BI	GIST	Epithelial	24h

FIGURE 1

FIGURE 2A

3/14

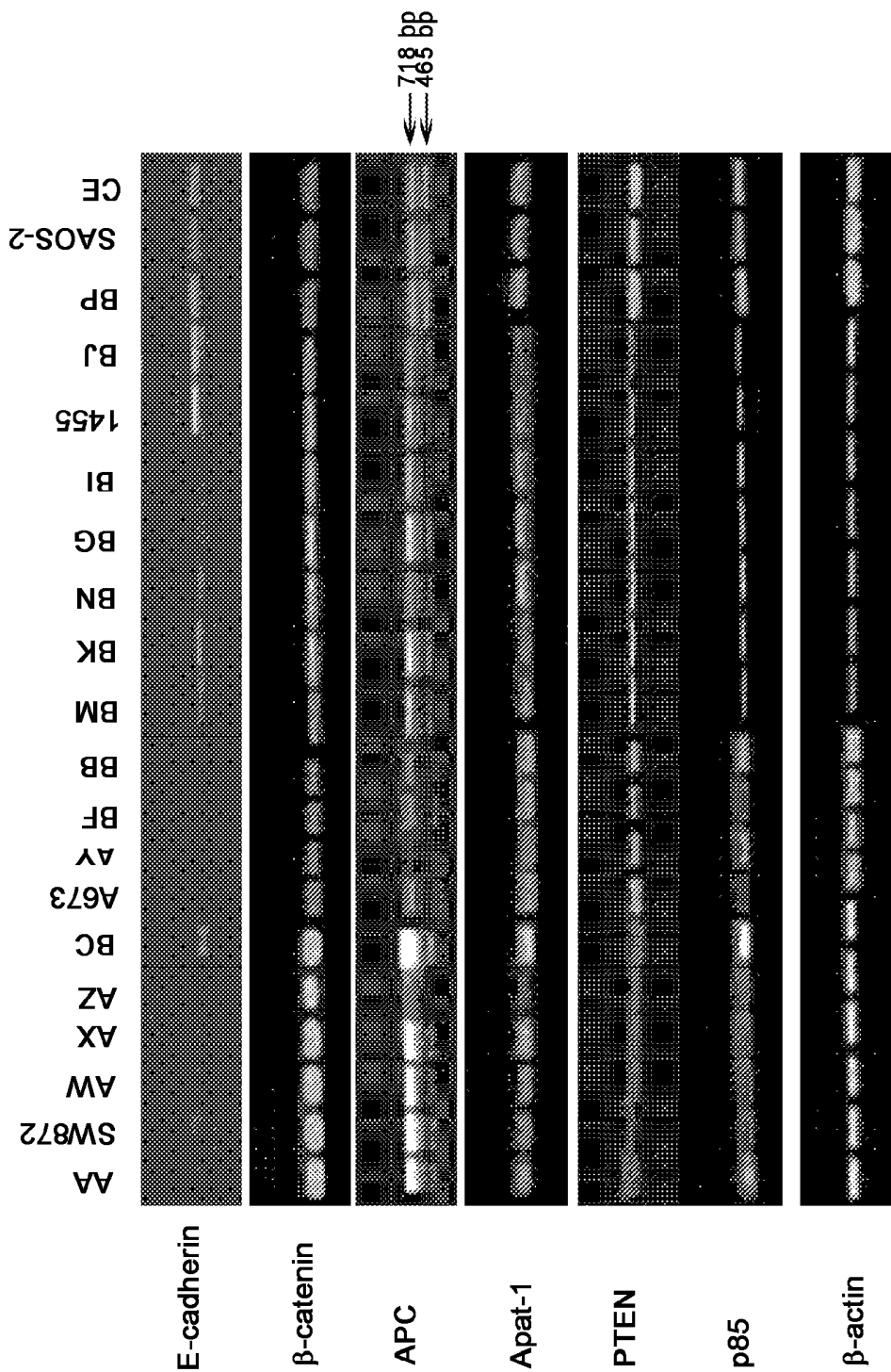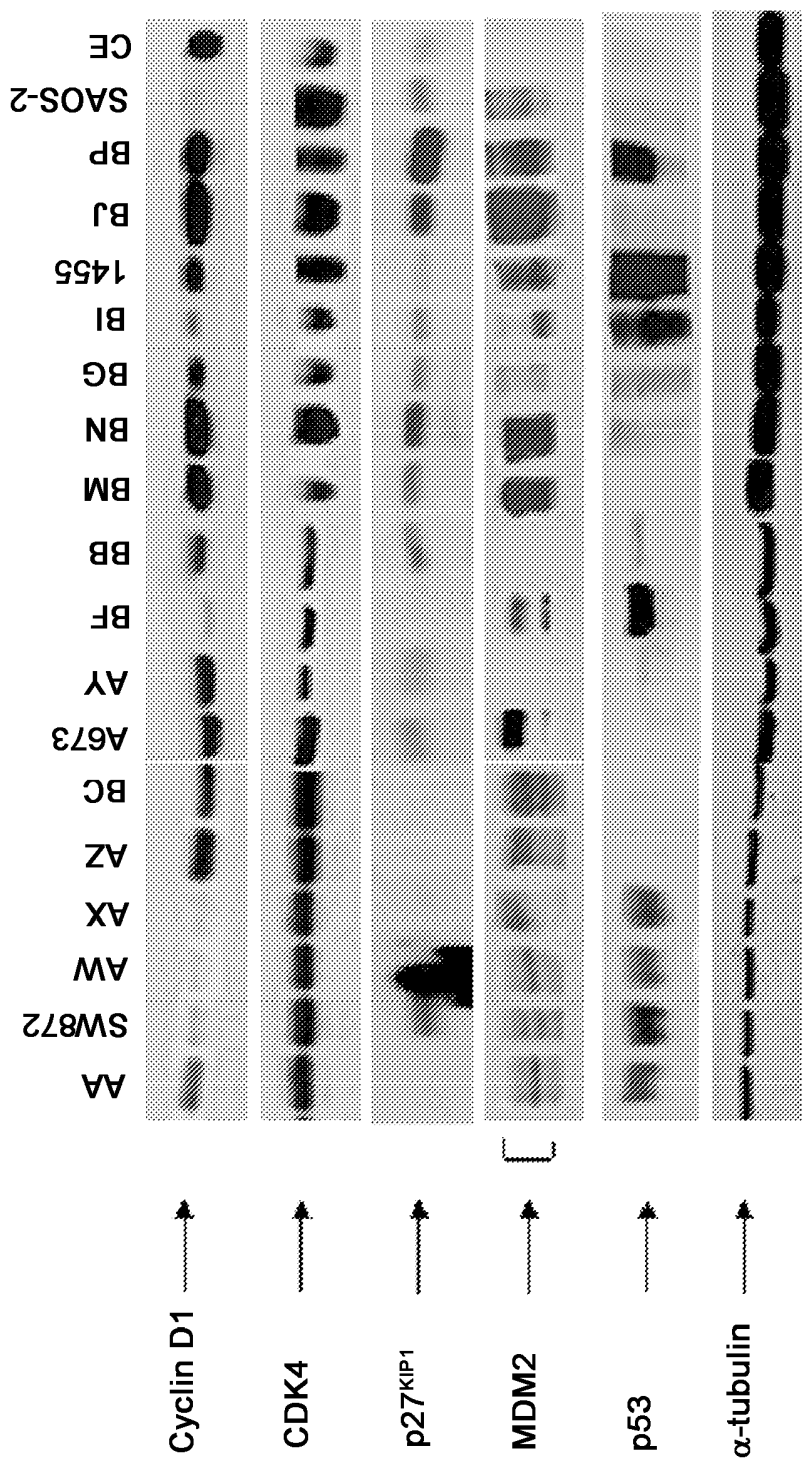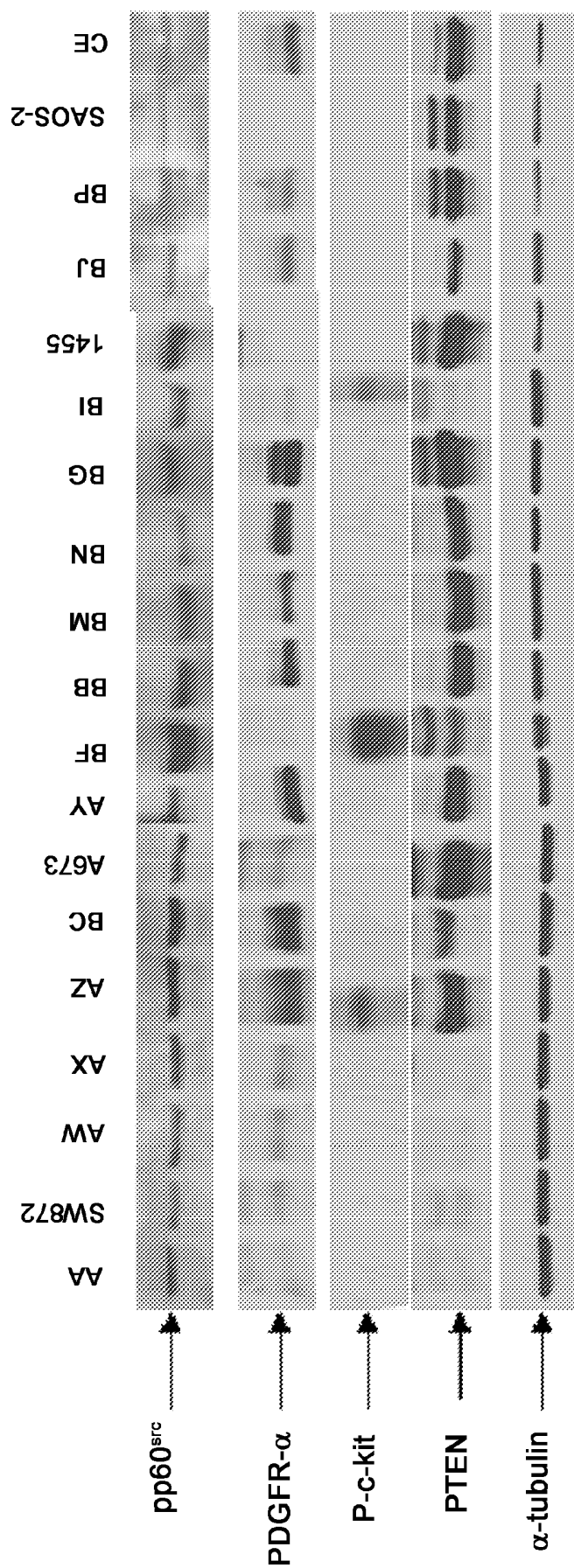

FIGURE 2B

FIGURE 3

FIGURE 4
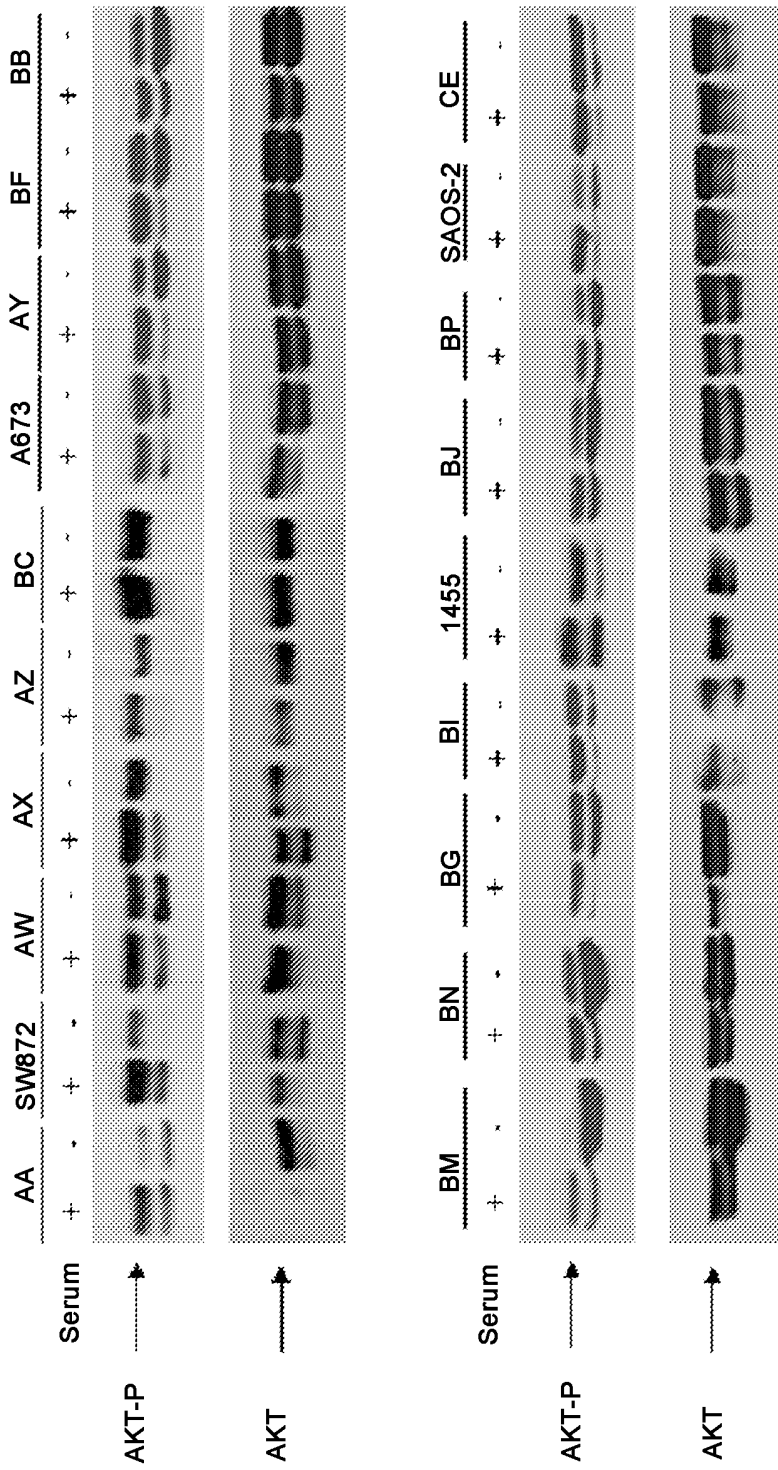
FIGURE 5

	CNIO AW	CNIO AX	SW872	1455	CNIO AA	CNIO AY	CNIO AZ	CNIO BC	CNIO BB	A673	CNIO BJ	CNIO BF	CNIO BP	SAOS-2	CNIO BG	CNIO BM	CNIO BN	CNIO CE	CNIO BI
p21^{CIP}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
p27^{KIP1}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
p16^{INK4a}	0	0	1	1	0	1	1	0	1	C	1	0	1	1	1	1	1	1	0
p15^{INK4b}	0	0	1	1	0	1	1	1	1	C	1	0	1	1	1	1	1	1	0
p14^{ARF}	0	0	1	1	0	1	1	0	1	C	1	0	1	1	1	1	1	1	0
MSH2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MLH1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Apaf-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
p73	1	1	0	0	1	0	0	1	0	1	0	1	0	0	C	0	0	0	1
PTEN	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
p85	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E-cadherin	0	0	1	1	0	0	0	1	0	C	1	0	1	1	C	1	1	1	0
B-catenin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
APC	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
p53	1	1	1	1	1	0	0	0	0	C	0	1	1	1	1	0	0	0	1
MDM2	0	0	0	0	0	0	0	1	0	1	1	0	0	0	C	1	1	0	0
CDK4	0	0	0	0	0	0	0	1	0	1	1	0	0	1	C	0	1	0	0
Ciclin D1	0	0	0	0,5	0	1	1	1	1	1	1	0	1	0	0,5	1	1	1	0
p27^{KIP1}	1	1	2	0,5	0,5	0,5	1	2	1	2	1	0,5	2	1	0,5	1	1	1	1
pp60^{SRC}	1	1	1	2	1	1	2	2	2	1	1	2	1	1	2	2	1	1	1
PDGFR-α	0,5	0,5	0,5	0	0	1	1	1	1	0,5	0,5	0	0,5	0	1	1	1	1	0,5
P-C-KIT	0	0	0	0	0	0	1	0	0	C	0	1	0	0	C	0	0	0	1
PTEN	0	0	0	3	0	2	3	1	2	ε	1	1	2	2	ε	2	2	2	0
AKT-P	1	1	0	1	0	0	1	1	1	1	1	1	1	0	1	0	1	1	1

FIGURE 6A

RT-PCR
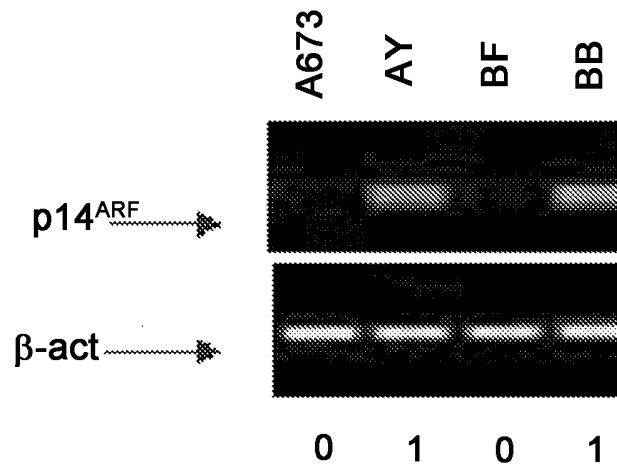
FIGURE 6B

WESTERN BLOT
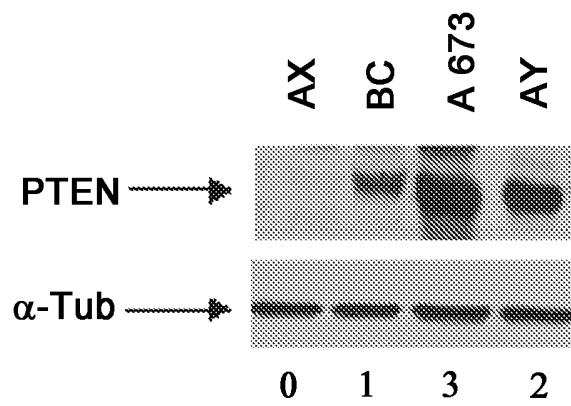
FIGURE 6C

	p15^INK4B	p16^INK4A	p14^ARF	p73	E-caderin	p53	MDM2	CDK4	Cyclin D1	p27^KIP1	p60^SRC-P	PDGFR-α	C-KIT-P	PTEN	P-value
Doxorubicin	-1,91	-1,36	-1,36	1,36	-27,36		-1,57	-1,57					1,77		>0,05
Gemcitabine	-1,27	-1,03	-1,03	1,03	-1,02	2,12			-1,08	-1,29				-2,56	>0,05
Oxaliplatin												-1,1			>0,1
Camptothecin						2,55			-1,19	-1,82					>0,05
UCN-01															>0,05
Flavopiridol							1,45	1,45							>0,05
Paclitaxel						1,73			-1,33		-2,66				>0,05
Imatinib															>0,05
Rapamycin						1,2	-1,34		-1,54						>0,05
Vinblastine									2,25						>0,05
LY294002														-2,07	>0,05

Statistical value

Positive coefficient   Negative coefficient

IC50   Biomarker   IC50   Biomarker
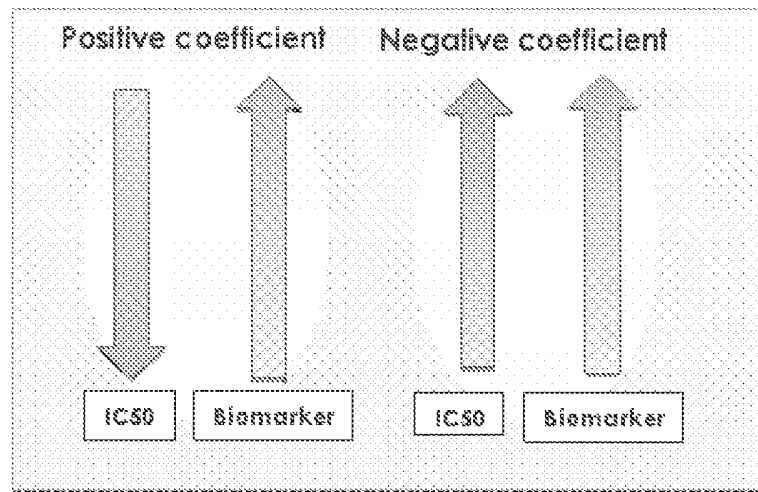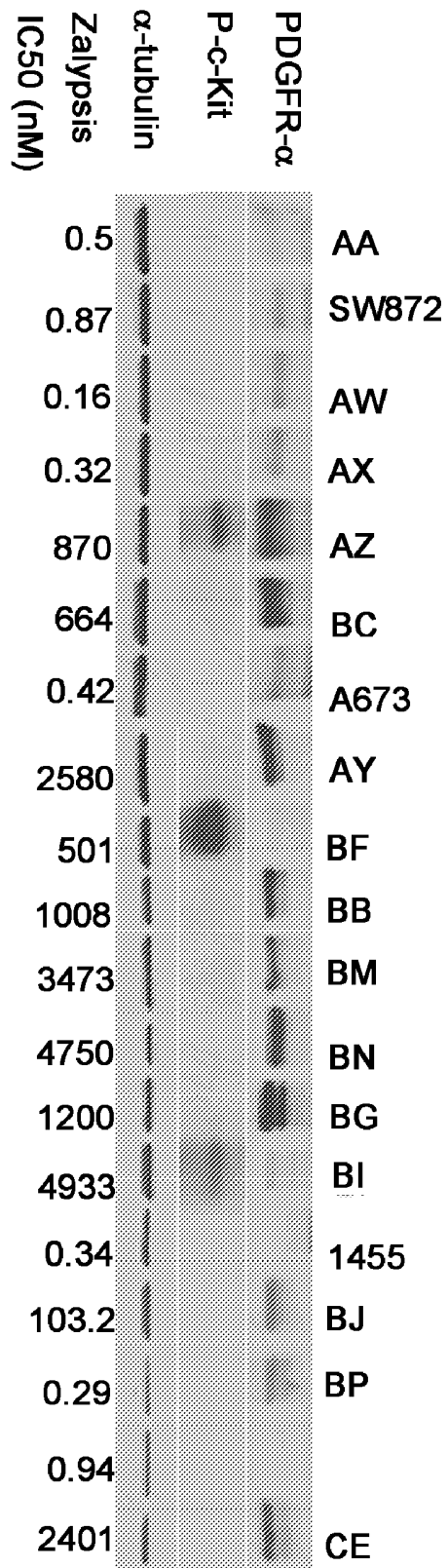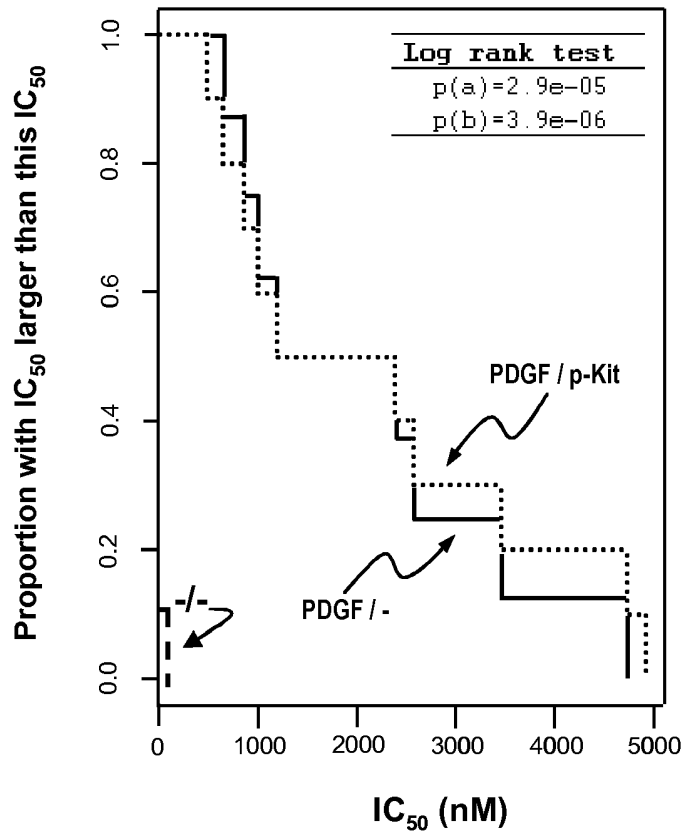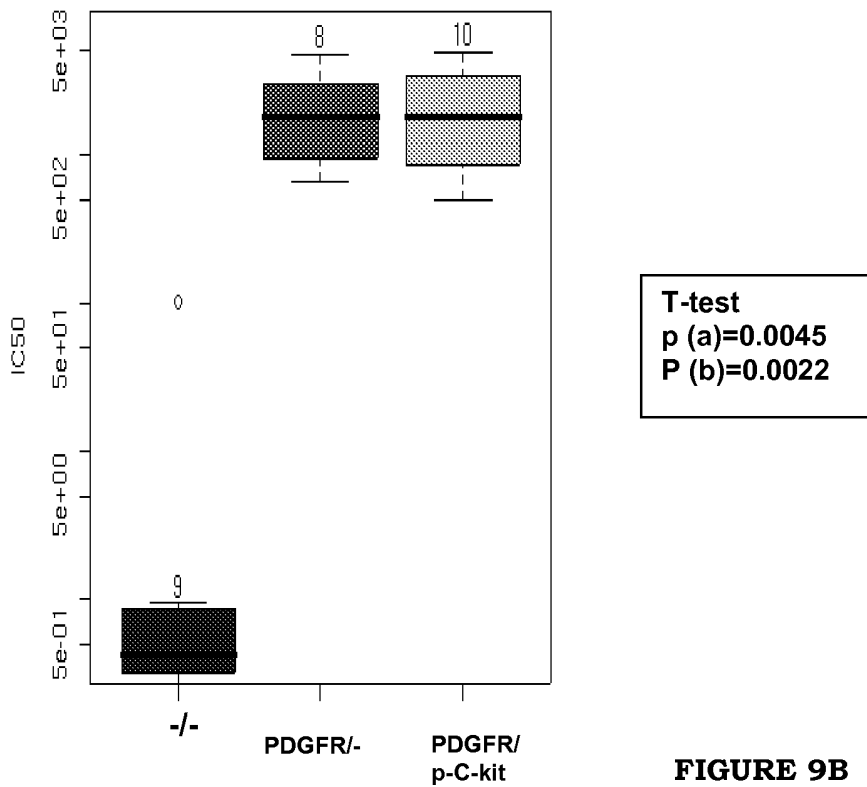
FIGURE 7
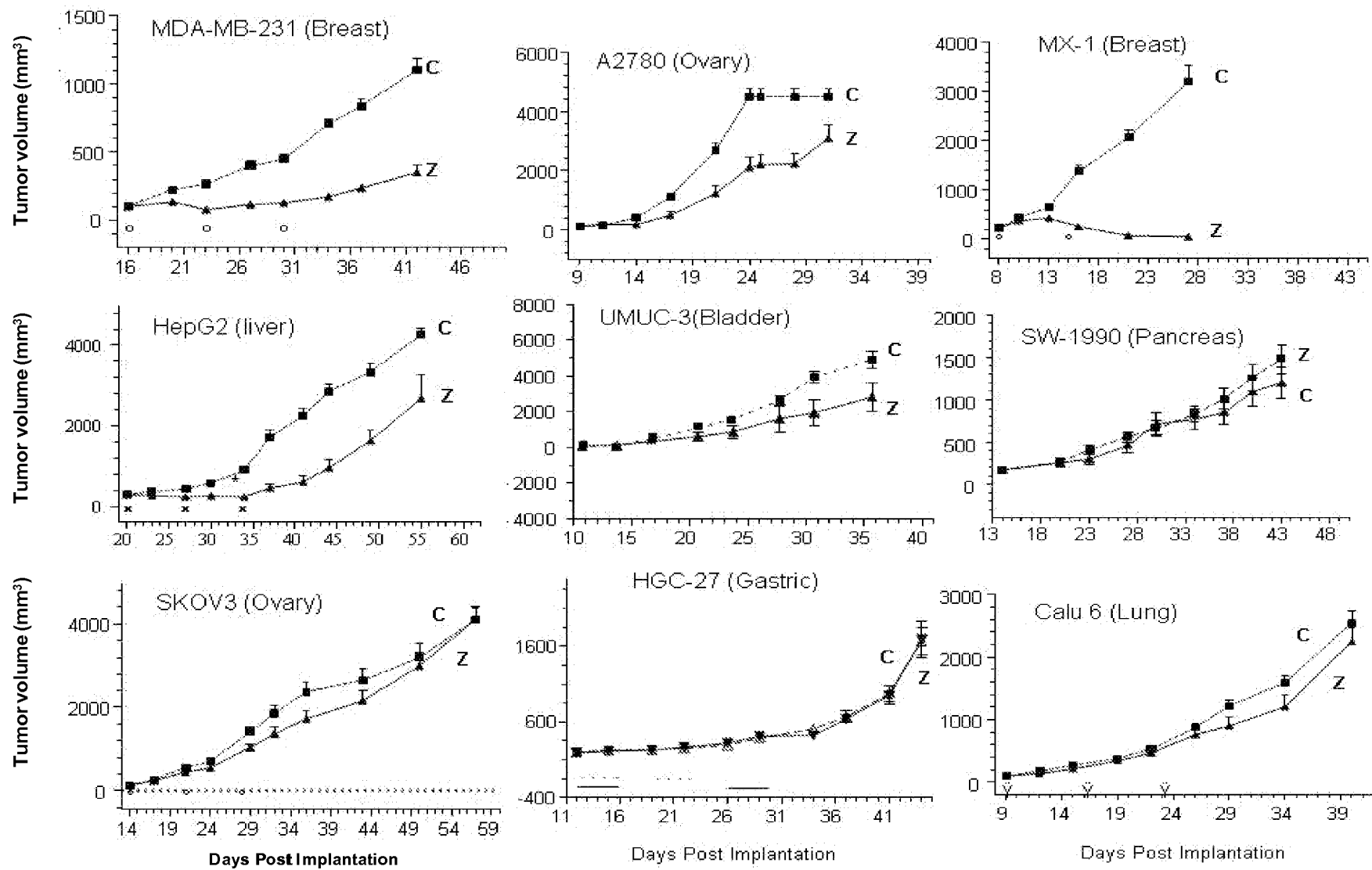
FIGURE 8

FIGURE 9A

FIGURE 9B
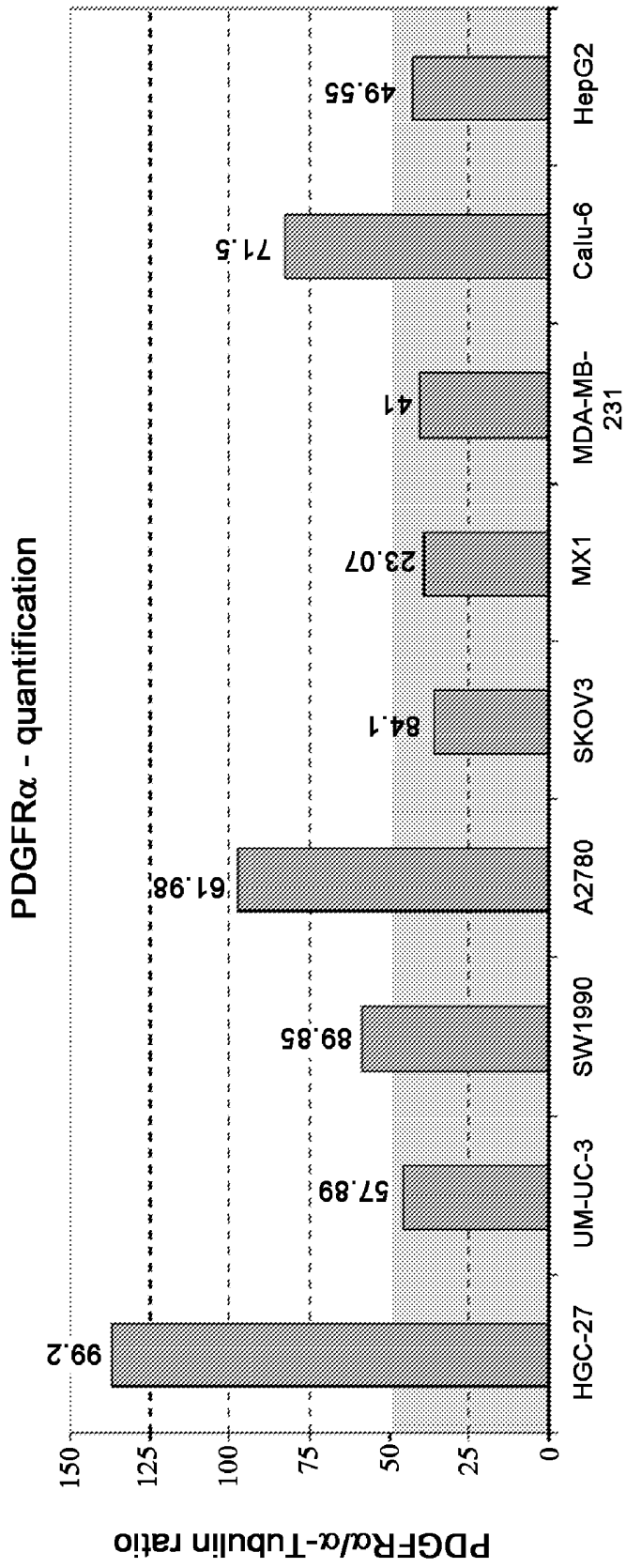
FIGURE 10
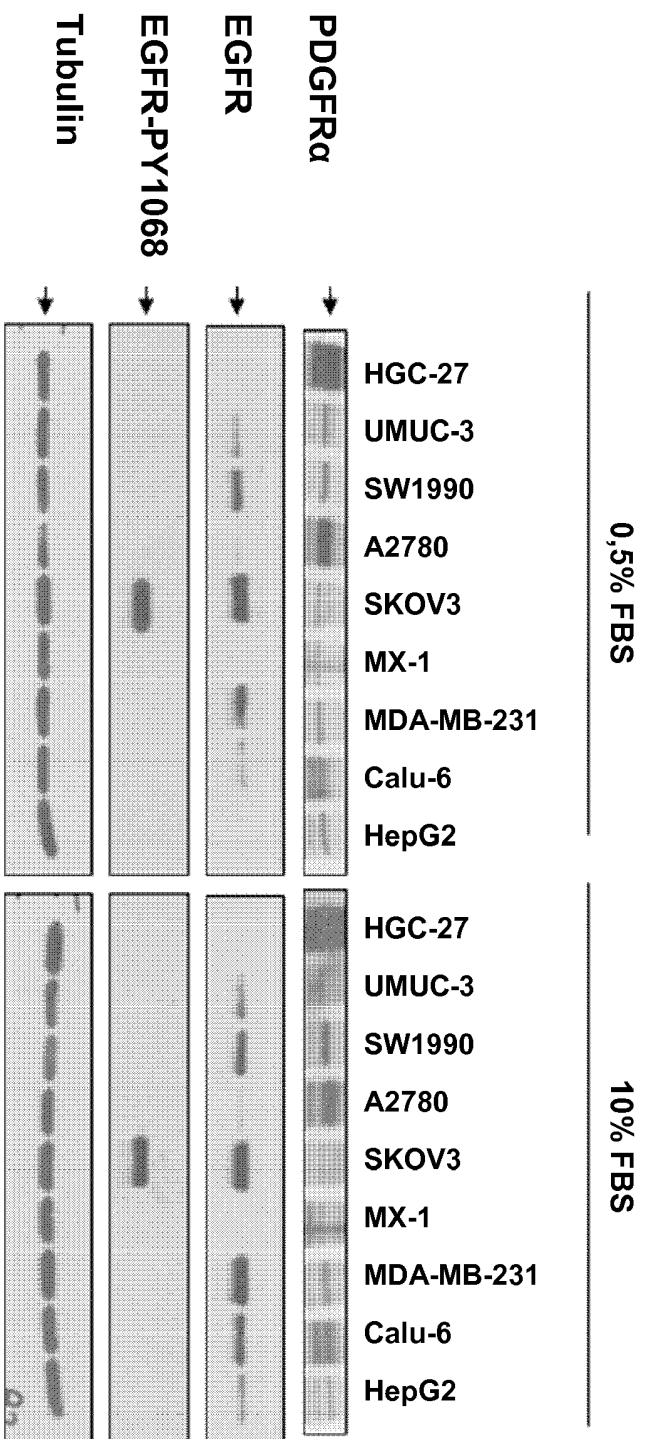
FIGURE 11

FIGURE 12

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV.   C12Q1/68   A61K31/4995
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q   A61K

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 practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHARUPANT KORNVIKA ET AL: "Microarray-based transcriptional profiling of renieramycin M and jorunnamycin C, isolated from Thai marine organisms.", MARINE DRUGS 2009 LNKD- PUBMED:20098592, vol. 7, no. 4, 2009, pages 483-494, XP002613943, ISSN: 1660-3397 the whole document -/--	1-19

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

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2010	30/12/2010

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

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DUAN ZHENFENG ET AL: "ZNF93 increases resistance to ET-743 (Trabectedin; Yondelis) and PM00104 (Zalypsis) in human cancer cell lines.", PLOS ONE 2009 LNKD- PUBMED:19742314, vol. 4, no. 9, 2009, page E6967, XP002613944, ISSN: 1932-6203 the whole document	1-19
Y	DUAN ZHENFENG ET AL: "Diverse cross-resistance phenotype to ET-743 and PM00104 in multi-drug resistant cell lines.", CANCER CHEMOTHERAPY AND PHARMACOLOGY MAY 2009 LNKD- PUBMED:18828019, vol. 63, no. 6, May 2009 (2009-05), pages 1121-1129, XP002613945, ISSN: 1432-0843 the whole document	1-19
Y	WO 2007/052076 A2 (PHARMA MAR SA [ES]; RUFFLES GRAHAM KEITH [GB]; CALVO SALVE PILAR [ES];) 10 May 2007 (2007-05-10) cited in the application the whole document	1-19
Y	WO 2008/135792 A1 (PHARMA MAR SA [ES]; WILLIAMS GARETH [GB]; DE BONO JOHANN [GB]; PAZ-ARE) 13 November 2008 (2008-11-13) cited in the application the whole document	1-19
Y	WO 2009/114836 A1 (GENOMIC HEALTH INC [US]; NSABP FOUNDATION INC [US]; BAKER JOFFRE B [US) 17 September 2009 (2009-09-17) claim 14	1-19
Y	WO 2008/109423 A1 (UNIV TEXAS [US]; COLMAN HOWARD [US]; ZHANG LI [US] UNIV TEXAS [US]; AL) 12 September 2008 (2008-09-12) claims 3,4	1-19
Y	WO 2008/006517 A2 (BAYER HEALTHCARE AG [DE]; GEHRMANN MATHIAS [DE]) 17 January 2008 (2008-01-17) table 4	1-19

Box No. I	Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐   on paper

   ☒   in electronic form

   b. (time)

   ☒   in the international application as filed

   ☐   together with the international application in electronic form

   ☐   subsequently to this Authority for the purpose of search

2. ☐   In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
WO 2007052076	A2		10-05-2007	AU	2006310260	A1	10-05-2007
				CA	2625096	A1	10-05-2007
				CN	101300011	A	05-11-2008
				EP	1968592	A2	17-09-2008
				JP	2009513618	T	02-04-2009
				KR	20080064959	A	10-07-2008
				US	2009076016	A1	19-03-2009
				ZA	200803420	A	28-10-2009
WO 2008135792	A1		13-11-2008	NONE			
WO 2009114836	A1		17-09-2009	US	2009305277	A1	10-12-2009
WO 2008109423	A1		12-09-2008	CA	2679943	A1	12-09-2008
				EP	2132336	A1	16-12-2009
				US	2010167939	A1	01-07-2010
WO 2008006517	A2		17-01-2008	EP	2041307	A2	01-04-2009
				US	2009239223	A1	24-09-2009