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(54) **Title:** METHODS FOR DETERMINING THE SENSITIVITY OR RESISTANCE OF CANCER CELLS TO AT LEAST ONE ANTICANCER DRUG AND/OR THERAPEUTICALLY ACTIVE MOLECULE

(57) **Abstract:** The invention relates to methods for determination of sensitivity or resistance of at least one tumor cell to an anticancer drug, a therapeutically active molecule capable of inhibiting growth or inducing death of said tumor cell and/or capable of restoring sensitivity of drug-resistant cancer cells to an anticancer drug and to a combination of the anticancer drug and therapeutically active molecule.

Methods for determining the sensitivity or resistance of cancer cells to at least one anticancer drug and/or therapeutically active molecule

## DESCRIPTION

### 5 FIELD OF THE INVENTION

The present invention relates to methods to determine the sensitivity or resistance of at least one cancer cell to at least one anticancer drug, to at least one molecule with therapeutic activity that is capable of inhibiting growth (proliferation and/or metabolism) of a tumor cell and/or inducing death, and/or capable of restoring in  
10 resistant tumor cells the sensitivity to an anticancer drug and to at least one combination of said at least one anticancer drug and said at least one molecule with therapeutic activity.

### STATE OF THE ART

15 Currently, the major limitation of anticancer treatments is due to poor sensitivity of many tumors, especially solid tumors, to the cytotoxic action of various chemotherapeutic agents, and the subsequent resistance.

For example colon cancer is the third most common form of cancer in both men and women and the fourth leading cause of death worldwide (de Gramont, 2011a),  
20 with 608,000 deaths per year worldwide.

Surgery is the first and most important therapeutic intervention. Subsequent evaluation takes into account the possibility to perform medical therapy in order to avoid relapses (recurrence). Medical therapy is indicated for treating the patient to avoid recurrence of disease after surgery.

25 Patients with stage A disease according to Dukes (B1 according to the Astler-Coller classification) cannot be candidates for surgery, which is indicated for patients with stage C disease. For stage B disease (stage II according to the TNM classification) (B2-3 according to Astler-Coller classification) there are no shared indications since the study of cohorts has shown the same incidence of recurrence  
30 with or without anticancer therapy.

For stage III colon carcinoma, since the early 90's the post-surgical treatment of choice is chemotherapy based on 5-fluorouracil (5FU), usually in combination with

leucovorin. After completion of chemotherapy, 58.9% of patients subjected to such treatment is disease-free at 5 years.

From the middle of the past decade, oxaliplatin (OxPt) has been added to the standard 5-FU therapy, raising to 66.4% the percentage of disease-free patients at 5 years after completion of chemotherapy (de Gramont, 2011a). However, it is evident that chemotherapy is not yet effective in a very high percentage of patients, due to the onset of drug resistance. To date the range of effective drugs is very limited (de Gramont 2011b) although combination chemotherapy protocols have been developed (such as 5-FU in combination with OxPt, irinotecan, capecitabine, tegafur or biologic agents such as bevacizumab and cetuximab) in order to overcome this drawback.

One of the main reasons for the drug resistance developed by tumor cells is their inability to execute the drug-induced death process.

Tumor cells may display very high resistance levels. For instance, first-line therapy based on 5FU determines an effective response only in 10-15% of patients with advanced stage colon carcinoma. Combination of 5FU with other chemotherapy drugs (such as oxaliplatin or irinotecan) leads to an increase in response up to 40-50%, a value not yet completely satisfactory for effective therapeutic action against cancer pathologies (Longley, 2003).

To date many genes have been identified that are involved in the phenomenon of tumor cell resistance to antineoplastic drugs, and others can be still identified.

US 8,232,085 B2 identified 49 genes that, when individually silenced, restore drug-sensitivity in resistant cells. Gene silencing by RNA interference (RNAi) technique made possible to define the roles of the identified genes in resistance to the anticancer drugs 5-FU and oxaliplatin. One of the genes silenced in the US patent number 8,232,085 B2 was the BTK gene. The results of experiments on this gene made it possible to reveal that functional silencing of the BTK, carried out by both RNA interference (RNAi) or a BTK (LFMA13) protein inhibitor, induces sensitization of resistant tumor cells to various chemotherapeutic agents alone or in combination (including 5FU and oxaliplatin).

In particular, a new isoform has been identified for the protein encoded by the BTK gene, with molecular weight comprised between 65-68kDa, that is shorter than the

previously known BTK isoform with molecular weight of 77 kDa.

The BTK gene has always been known to be expressed only in bone marrow derived cells, such as B cells, mast cells, platelets and erythroid progenitors. Instead the aforementioned patent led to the discovery that the new shorter BTK  
5 protein isoform is expressed in several carcinoma cell lines.

High tumor heterogeneity explains the different responses to chemotherapy, thus making difficult and complex the oncologists' choice of chemotherapy. Usually patients are offered a standard therapy which may be followed by personalized therapy proposed by the oncologist, based on its own experience over the years  
10 and on data reported in the literature.

In this kind of scenario, it is essential to develop tests that can identify/measure markers with theranostic value, that is tests enabling the oncologist to provide patients with an appropriate and personalized therapy in order to achieve maximum benefit for both the patient and the National Health System (SSN).

15 WO 2011/123103 describes a method to evaluate the ability of a chemotherapeutic drug to induce apoptosis in a cancer cell line. Apoptosis is a well known programmed cell death process normally used by organisms to eliminate cells that are too old or damaged.

Cells undergo several morphological changes in the course of apoptosis, until  
20 fragmentation into vesicles, termed apoptotic bodies, which are phagocytosed by macrophages. The method described in patent WO 2011/123103 makes use of the MICK (microculture kinetic) assay in order to assess whether cancer cells derived from known tumor cell lines undergo apoptosis in response to a particular drug which is known to be active against one or more types of cancer. Such assay  
25 evaluates the morphological changes of the cell membrane that take place in cancer cells after administration of a drug, by means of temporal kinetics. Therefore this method is substantially, if not almost exclusively, addressed to screen for candidate anticancer drugs in known tumor cell lines.

Therefore the technical task of the present invention is to eliminate the drawbacks  
30 of the prior art, in particular to allow identification of anticancer drugs or their combination to which patient's cells are sensitive, and identification of patients in whom chemotherapy is ineffective due to the development of drug resistance.

## SUMMARY OF THE INVENTION

Therefore the invention relates to a method to assess sensitivity or resistance of at least one cancer cell to at least one anticancer drug, at least one therapeutically active molecule capable of inhibiting growth (proliferation and/or metabolism) or of inducing death of said tumor cell, and/or capable of restoring in resistant tumor cells the sensitivity to said at least one anticancer drug, and at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule, comprising the steps of:

- 5 a) incubate the tumor sample (solid or liquid tumor biopsy), isolated from a patient, in saline solution and/or culture medium supplemented with antibiotics and antifungals;
- b) in the case of solid tumor biopsy, mechanically disrupt cells of the tumor sample obtained from step a) followed by enzymatic digestion to obtain cells to be seeded  
15 in the subsequent step;
- c) seed the cells obtained in step b) in cell culture dishes and treat a first portion of said cells with said at least one anticancer drug, a second portion of said cells with said at least one therapeutically active molecule, a third portion of said cells with said at least one combination of said anticancer drugs, a fourth portion of said  
20 cells with said at least one combination of said therapeutically active molecules, a fifth portion of said cells with said at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule, a sixth portion of said cells is cultured without addition of drugs (untreated cells, NT);
- d) quantify cell growth (proliferation and/or metabolism) and/or viability and/or  
25 death of the first, second, third, fourth, fifth and sixth portions of said cells of step c) by exposure to a reagent selective for live cells or a reagent selective for metabolically active cells or a dedicated reagent for detection of energy consumption;
- e) analyze data obtained from step d) to classify cells according to their response  
30 to the treatment with an anticancer drug and/or a therapeutically active molecule.

Therefore another aspect of the invention relates to a method to determine sensitivity or resistance of at least one tumor cell to a combination of at least one

anticancer drug and at least one therapeutically active molecule able to inhibit growth (proliferation and/or metabolism) or induce death of said tumor cell and/or able to restore in resistant tumor cells the sensitivity to said at least one anticancer drug, comprising the steps of:

- 5 a) incubate the tumor sample isolated from a patient (solid or liquid tumor biopsy) in saline solution and/or culture medium supplemented with antibiotics and antifungals;
- b) in the case of solid tumor biopsy, mechanically disrupt cells of the tumor sample obtained from step a) followed by enzymatic digestion to obtain cells to be seeded  
10 in the subsequent step;
- c) seed cells obtained from step b) in cell culture dishes and treat said cells with said at least one combination of said anticancer drugs, with said at least one combination of said therapeutically active molecules, with said at least one combination of said at least one anticancer drug and said at least one  
15 therapeutically active molecule;
- d) quantify growth (proliferation and/or metabolism) and/or viability and/or death of said cells of step c) by exposure to a selective reagent for live cells, or to a selective reagent for metabolically active cells, or to a dedicated reagent for detection of energy consumption;
- 20 e) analyze data obtained from step d) to classify cells according to their response to the treatment with an anticancer drug and/or a therapeutically active molecule.

The methods surprisingly allow the development of a personalized therapy for the patient. Cancer patients very often require a personalized and targeted therapy due to tumor type, genetics and heterogeneity. Therefore the possibility to obtain,  
25 through the methods according to the present invention, a rapid and reliable response about treatment success, makes possible considerable cost and time saving, in addition to being an advantage for the patient.

Therefore the methods according to the present invention make possible, by a surprisingly efficient and reliable single test lasting few hours, to determine  
30 whether cancer cells of the cancer patient are sensitive or resistant to an anticancer drug, to a therapeutically active drug or to combinations thereof, with obvious saving of time and costs.

In the present invention, when using the definition:

- "tumor cell" it is meant to include neoplastic cells of any tumor stage or classification, derived from epithelial tumors, mesenchymal tumors, hematological tumors or tumors of the nervous tissue, in particular cells from colon carcinoma, breast cancer, ovarian cancer, stomach cancer, lung carcinoma, renal carcinoma, thyroid carcinoma or pancreatic carcinoma, but also circulating cancer cells, sarcomas and melanomas;

- "anti-cancer drug" it is meant to comprise classical chemotherapeutic drugs such as 5-fluorouracil (5-FU), irinotecan or capecitabine, oxaliplatin and other platinum coordination compounds; and

- "therapeutically active molecule" refers to a molecule capable of inhibiting growth (proliferation and/or metabolism) or inducing death of said tumor cell and/or capable of restoring in resistant cancer cells the sensitivity to said at least one anticancer drug. Examples of such molecules are biological drugs such as monoclonal antibodies, preferably Cetuximab, Panitumumab, Bevacizumab and kinase inhibitors such as gefitinib, erlotinib, vemurafenib, sorafenib, ibrutinib and dasatinib;

- "first portion", "second portion", "third portion", "fourth portion", "fifth portion" and "sixth portion" refers to six equal amounts of a pool of cells to be defined depending on cell type, anticancer drug and therapeutically active molecule involved.

#### **DESCRIPTION OF THE FIGURES**

The invention will now be described in detail and further characteristics and advantages of the invention will become more clear in the following description that is meant to be provided as an indicative but non-limitative example in reference to attached Figures 1 to 14.

Figure 1 shows the results obtained with the method according to the present invention from colon carcinoma-derived cell lines HCT116 and HCT116p53KO known to be chemo-sensitive and chemo-resistant, respectively. Cell lines were treated with 5-fluorouracil (5-FU) and the effect of the drug was detected by exposure to a reagent which is excluded selectively from live cells (Trypan blue).

Evaluation of the sensibility/resistance to 5FU by using the Trypan Blue test.

Sensitive cells (HCT116) e resistant cells (HCT116p53KO) have been treated with 200  $\mu$ M 5FU for 72hs and are then counted after being colored with Trypan Blue (selective for dead cells) \*Bunz et al JCI 1999.

5 Figure 2 shows the results obtained with the method according to the present invention from colon carcinoma-derived cell lines, chemo-sensitive HCT116 and chemo-resistant HCT116p53KO. Cell lines were treated with 5-FU and the effect of the drug was detected upon exposure to a dedicated reagent for detection of energy consumption (ATP).

10 Evaluation of the sensibility/resistance to 5FU by using the test which evaluates the energy consumption (Cell Titer Glo Luminescent Cell Viability Assay).

Sensitive cells (HCT116) e resistant cells (HCT116p53KO) have been treated with 200  $\mu$ M 5FU for 72hs before undergoing the test.

15 Figure 3 shows the results obtained with the method according to the present invention from colon carcinoma-derived cell lines, chemo-sensitive HCT116 and chemo-resistant HCT116p53KO. Cell lines were treated with 5-FU and the effect of the drug was detected by a selective reagent for metabolically active cells (MTT).

20 Evaluation of the sensibility/resistance to 5FU by using the test which evaluates the metabolic activity (MTT assay ). Sensitive cells (HCT116) e resistant cells (HCT116p53KO) have been treated with 200  $\mu$ M 5FU for 72hs before undergoing the test. HCT116 NT vs HCT116 5FU  $p < 0.05$ ; HCT116p53KO NT vs HCT116p53KO 5FU  $p > 0.05$

25 Figure 4 shows the results obtained with the method according to the present invention from the chemoresistant colon carcinoma-derived cell lines HCT116p53KO, DLD-1 and SW480. Cell lines were treated with 5-FU and the effect of the drug was detected by exposure to a reagent which is excluded selectively from live cells (Trypan blue).

Evaluation of the sensibility/resistance to 5FU by using the Trypan Blue test.

30 HCT116p53KO, DLD-1, SW480 resistant cells are treated with 200  $\mu$ M 5FU for 72hs and then cells are counted after Trypan Blue colouring.



Figure 5 shows the results obtained with the method according to the present invention from the chemoresistant colon carcinoma-derived cell lines HCT116p53KO, DLD-1 and SW480. Cell lines were treated with Oxaliplatin (OxPt) and the effect of the drug was detected by exposure to a reagent which is excluded selectively from live cells (Trypan blue).

Evaluation of the sensibility/resistance to 5FU by using the Trypan Blue test.

HCT116p53KO, DLD-1, SW480 resistant cells are treated with 50 uM OxPt for 72hs and then cells are counted after Trypan Blue colouring.

Figure 6 shows the results obtained with the method according to the present invention from the chemoresistant colon carcinoma-derived cell lines HCT116p53KO, DLD-1 and SW480. Cell lines were treated with a combination of 5FU and OxPt and the effect of the drug was detected by exposure to a reagent which is excluded selectively from live cells (Trypan blue).

Evaluation of the sensibility/resistance to 5FU by using the Trypan Blue test.

HCT116p53KO, DLD-1, SW480 resistant cells are treated with the combination of 200 uM 5FU + 50 uM OxPt for 72hs and then cells are counted after Trypan Blue colouring.

Figure 7 shows the results obtained with the method according to the present invention from the chemo-resistant colon carcinoma-derived cell line HCT116p53KO after treatment with a combination of 5FU and Dasatinib. The effect of the drug was detected by exposure to a reagent which is excluded selectively from live cells (Trypan blue).

Evaluation of the sensibility/resistance to 5FU by using the Trypan Blue test.

HCT116p53KO resistant cells with 200 uM 5FU, 50 nM Dasatinib or with the combination of both for 72hs and cell count after Trypan Blue colouring.

Figure 8 shows the results obtained with the method according to the present invention from the chemo-resistant colon carcinoma-derived cell line HCT116p53KO after treatment with "biological" drugs antagonist of Epidermal Growth Factor (Cetuximab and Panitumumab) and Vascular Endothelial Growth Factor (bevacizumab). The effect of the drug was detected upon exposure to a selective reagent for metabolically active cells (MTT).

Evaluation of the sensibility/resistance to biological drugs by using the test which evaluates the energy consumption (Cell Titer Glo Luminescent Cell Viability Assay). HCT116p53KO chemioresistent cells have been treated with Cetuximab 10 µg/ml , Panitumumab 75 µg/ml, Bevacizumab 25 µg/m for 72hs before  
5 undergoing the test. Cetuximab vs NT  $p>0.05$ ; Panitumumab vs NT  $p>0.05$ ; .  
Bevacizumab vs NT  $p>0.05$

Figure 9 shows the results obtained with the method according to the present invention from the chemo-resistant colon carcinoma-derived cell line HCT116p53KO after treatment with "biological" drugs antagonist of Epidermal  
10 Growth Factor (Cetuximab and Panitumumab) and Vascular Endothelial Growth Factor (bevacizumab). The effect of the drug was detected by exposure to a selective reagent for live cells (Calcein assay).

Evaluation of the sensibility/resistance to biological drugs by using the test which evaluates the metabolic activity (MTT assay ). HCT116p53KO chemioresistent  
15 cells have been treated with Cetuximab 10 µg/ml , Panitumumab 75 µg/ml,  
Bevacizumab 25 µg/m for 72hs before undergoing the test. Cetuximab vs NT  $p>0.05$ ; Panitumumab vs NT  $p>0.05$ ; . Bevacizumab vs NT  $p>0.05$

Figure 10 shows the results obtained with the method according to the present invention from the chemo-resistant ovarian carcinoma-derived cell line SKOV3  
20 after treatment with classical chemotherapeutic agents (carboplatin, paclitaxel) and "biological" drugs antagonist of Vascular Endothelial Growth Factor (bevacizumab). The effect of the drug was detected upon exposure to a selective reagent for metabolically active cells (MTT).

Dose-response curve of the ovary carcinoma cell line SKOV3 for Paclitaxel,  
25 Carboplatino and Bevacizumab

Figure 11 shows the distribution of cell samples from colon tumors from patients tested with the method according to the present invention, who were treated only with 5-FU. The effect of the drug was detected by exposure to a reagent selective for metabolically active cells (MTT).

30 Distribution of delta (D) values obtained as the difference between the average of triplicate samples for the untreated and the average of triplicate samples for treatment in 135 colon samples. Each symbol represents one patient.

On the left are the delta (D) of samples found to be resistant with Student's T test, on the right are the delta of samples found to be sensitive according to the same test .

Figure 12 shows the distribution of delta values obtained as difference between the average of triplicate samples for the untreated condition and the average of triplicate samples for the treated condition in 135 colon samples treated with 5FU, Ibrutinib or with a combination of the two drugs. Drug effect was detected by exposure to a reagent selective for metabolically active cells (MTT).

Distribution of delta (D) values obtained as the difference between the average of triplicate samples for the untreated and the average of triplicate samples for treatment in colon samples.

On the left are the delta (D) of samples found to be resistant with Student's T test, on the right are the delta of samples found to be sensitive according to the same test .

Figure 13 shows the distribution of delta values obtained as difference between the average of triplicate samples for the untreated condition and the average of triplicate samples for the treated condition in 135 colon samples treated with 5FU, Dasatinib or with a combination of the two drugs. Drug effect was detected by exposure to a reagent selective for metabolically active cells (MTT).

Distribution of delta (D) values obtained as the difference between the average of triplicate samples for the untreated and the average of triplicate samples for treatment in 25 colon samples.

On the left are the delta (D) of samples found to be resistant with Student's T test, on the right are the delta of samples found to be sensitive according to the same test .

Figure 14 shows the distribution of delta values obtained as difference between the average of triplicate samples for the untreated condition and the average of triplicate samples for the treated condition in 29 samples from the ovary. Drug effect was detected by exposure to a reagent selective for metabolically active cells (MTT).

Distribution of delta (D) values obtained as the difference between the average of triplicate samples for the untreated and the average of triplicate samples for treatment in 29 ovary samples

On the left are the delta (D) of samples found to be resistant with Student's T test, on the right are the delta of samples found to be sensitive according to the same test .

#### **DETAILED DESCRIPTION OF THE INVENTION**

The invention therefore relates to a method for determination of the sensitivity or resistance of at least one cancer cell to at least one anti-tumor drug, at least one therapeutically active molecule capable of inhibiting growth (proliferation and/or metabolism) or of inducing death of said tumor cell and/or capable of restoring in resistant tumor cells the sensitivity to said at least one anticancer drug, and at least to one combination of said at least one anticancer drug and said at least one therapeutically active molecule, comprising the steps of:

a) incubate the tumor sample (solid or liquid tumor biopsy), isolated from a patient, in saline solution and/or culture medium supplemented with antibiotics and antifungals;

b) in the case of solid tumor biopsy, mechanically disrupt cells of the tumor sample obtained from step a) followed by enzymatic digestion to obtain cells to be seeded in the subsequent step;

c) seed the cells obtained in step b) in cell culture dishes and treat a first portion of said cells with said at least one anticancer drug, a second portion of said cells with said at least one therapeutically active molecule, a third portion of said cells with said at least one combination of said anticancer drugs, a fourth portion of said cells with said at least one combination of said therapeutically active molecules, a fifth portion of said cells with said at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule, a sixth portion of said cells is cultured without addition of drugs (untreated cells, NT);

d) quantify cell viability and/or death of the first, second, third, fourth, fifth, sixth portion of said cells from step c) by exposure to a reagent selective for live cells or to a reagent selective for metabolically active cells or to a reagent dedicated to detection of energy consumption;

e) analyze data obtained from step d) to classify cells according to their response to the treatment with an anticancer drug and/or a therapeutically active molecule.

Therefore another aspect of the invention relates to **a method to** determine sensitivity or resistance of at least one tumor cell to a combination of at least one anticancer drug and at least one therapeutically active molecule able to inhibit growth (proliferation and/or metabolism) or induce death of said tumor cell and/or able to restore in resistant tumor cells the sensitivity to said at least one anticancer drug, comprising the steps of:

a) incubate the tumor sample from solid or liquid tumor biopsy, isolated from a patient, in saline solution and/or culture medium supplemented with antibiotics and antifungals;

b) in the case of solid tumor biopsy, mechanically disrupt cells of the tumor sample obtained from step a) followed by enzymatic digestion to obtain cells to be seeded in the subsequent step;

c) seed cells obtained from step b) in cell culture dishes and treat said cells with said at least one combination of said anticancer drugs, with said at least one combination of said therapeutically active molecules, with said at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule;

d) quantify growth (proliferation and/or metabolism) and/or viability and/or death of said cells of step c) by exposure to a selective reagent for live cells, or to a selective reagent for metabolically active cells, or to a dedicated reagent for detection of energy consumption;

e) analyze data obtained from step d) to classify cells according to their response to the treatment with an anticancer drug and/or a therapeutically active molecule.

A further aim of the invention was to develop a method to determine tumor sensitivity/resistance, thus allowing not only development of personalized therapy but also an evaluation of the evolution of tumor resistance to drugs.

The invention advantageously provides a simple and effective method applicable to any type of tumor and cell line, allowing for determination of the most suitable therapy for certain tumor processes starting from the biological material taken from the patient, and selection of the most appropriate treatment for the case under

analysis.

In a preferred form of realization, the step d) for quantification of cell growth (proliferation and/or metabolism) and/or viability and/or death of the method according to the present invention is advantageously performed by exposure to a reagent selective for live cells or to a reagent selective for metabolically active cells or a dedicated reagent for detection of energy consumption.

In particular, in the method according to the invention, the step of quantitative analysis of metabolism to verify metabolically active cells and quantify total metabolism is realized by MTT assay (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) comprising a colorimetric reagent and a step of measurement of spectrophotometric absorbance at a wavelength of 570 nm.

The phase of quantitative analysis of cell energy consumption (or energy metabolism), a quantitative analysis correlated with cell viability, is realized by the Cell Titer Glo Luminescent Cell Viability Assay.

Preferably, the step of quantitative analysis of cell viability is performed by the Calcein-AM assay (acetoxymethyl Calcein) comprising a pro-fluorescent compound, and the phase of fluorimetric measurement of the emission is carried out at a wavelength of 535nm.

In a further form of realization of the present method, to quantify cell viability and/or death, it is possible to expose the cells to a reagent that is selectively excluded from living cells, such as trypan blue (stains dead cells).

In a further form of realization, step e) for data analysis of the method according to the present invention is carried out by statistical analysis of the data obtained from step d) for categorization of cells into classes of response to treatment selected from the group consisting of :

- a) cells sensitive to said at least one anticancer drug;
- b) cells resistant to said at least one anticancer drug;
- c) cells sensitive to said at least one therapeutically active molecule;
- d) cells resistant to said at least one therapeutically active molecule;
- e) cells resistant to said at least one anticancer drug whose resistance is reverted by the action of said at least one therapeutically active molecule;
- f) cells resistant to said at least one anticancer drug whose resistance is not

reverted by the action of said at least one therapeutically active molecule;  
g) cells sensitive to said at least one anticancer drug whose sensitivity is not increased in the presence of said at least one therapeutically active molecule;  
h) cells sensitive to said at least one anticancer drug whose sensitivity increases in the presence of said at least one therapeutically active molecule.

The methods according to the present invention allow for determination of sensitivity or resistance of a tumor cell also in function of spectrophotometric measurement evaluating the absorbance (OD) (average value of a triplicate): i) of samples of tumor cells treated (T) with an anticancer drug and/or a therapeutically active molecule and/or a combination thereof, ii) of samples of tumor cells not treated (NT) with a drug or a combination of drugs, followed by exposure to one of said dedicated detection reagents (in particular MTT or calcein). A Student's T test value  $\leq 0.05$  from three identical replicas (triplicate) of the NT sample and of the T sample of tumor cells from the same patient identifies cancer cells sensitive to a specific treatment. A Student's T test value  $\geq 0,05$  from three identical replicas (triplicate) of the NT sample and of the T sample of tumor cells from the same patient identifies cancer cells resistant to a specific treatment.

In fact, in a further form of realization for sensitive samples, the delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) for not treated (NT) cancer cells and treated (T) cancer cells from sensitive samples ( $p \leq 0.05$ ) are preferably  $\geq 0.04$ , more preferably from  $+0.04$  to  $+0.4$ .

In yet a further form of realization, for the resistant samples, the delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells for resistant samples ( $p \geq 0.05$ ) are preferably  $\leq +0.02$ , more preferably from  $+0.02$  to  $-0.2$ .

In yet a further form of realization, the method also identifies tumors containing a mixed population of sensitive and resistant cells wherein the delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated cancer cells (NT) and treated cancer cells (T) are preferably comprised between  $+0.04$  and  $+0.02$ .

In the methods according to the present invention, measurement of absorbance is

carried out by spectrophotometric or fluorimetric measurement, preferably at a wavelength from 400 to 700 nm, preferably at 570 nm (MTT assay) or 535nm (calcein assay).

In the methods according to the present invention, said at least one tumor cell is a cell derived from epithelial, mesenchymal tumors, hematological tumors or tumors of the nervous tissue. In particular said cell is a carcinoma, in particular from colon cancer, breast cancer, ovarian cancer, stomach cancer, lung cancer, kidney cancer, thyroid cancer or carcinoma of the pancreas, but also circulating cancer cells, sarcomas and melanoma of any stage or tumor class.

In a preferred form of realization said at least one tumor cell is a colon or ovarian carcinoma cell.

In a preferred form of realization of the invention, said at least one anticancer drug advantageously includes classical chemotherapeutic drugs (alkylating drugs, base analogues, intercalators, microtubule inhibitors, nitrogen mustards, etc.) or biological drugs or kinase inhibitors.

Preferably said classical chemotherapy drugs comprise but are not limited to 5-fluorouracil (5-FU), irinotecan or capecitabine, oxaliplatin and other platinum coordination compounds, said biological drugs comprise but are not limited to monoclonal antibodies, preferably Cetuximab, Panitumumab, Bevacizumab and said kinase inhibitors comprise gefitinib, erlotinib, vemurafenib, sorafenib, ibrutinib and dasatinib.

In a preferred form of realization, said therapeutically active molecule is Dasatinib or ibrutinib.

Said cancer cell is advantageously a colon carcinoma cell and said at least one anticancer drug is 5FU, said at least one therapeutically active molecule is dasatinib or ibrutinib and said combination of said at least one anticancer drug and said at least one therapeutically active molecule is selected from 5-fluorouracil and dasatinib or 5-fluorouracil and ibrutinib.

Said cancer cell is advantageously an ovarian cancer cell and said at least one anticancer drug is Paclitaxel or Carboplatin, and said at least one therapeutically active molecule is bevacizumab and said combination of anticancer drugs is Paclitaxel + Carboplatin and said combination of said at least one anticancer drug



and said at least one therapeutically active molecule is selected from Paclitaxel, and Carboplatin, and bevacizumab and combinations of two of Paclitaxel, Carboplatin or bevacizumab or Paclitaxel, Carboplatin and bevacizumab.

The methods for determination of sensitivity or resistance of human tumor cells  
5 may include one or a combination of chemotherapeutic drugs and may be used to test new drugs and/or combinations of drugs. The study of new cancer drugs is in fact updated and expanded daily with new activities of new molecules to be tested on each individual patient. The methods according to the present invention have the advantage that they make possible the rapid testing of each new molecule  
10 alone or in combination, thereby allowing an effective targeted therapy for the patient.

In a further form of realization, in the methods according to the present invention, said tumor sample of step a) is pre-stored at a temperature comprised between 0-8 °C for a storage time above 5 hours and a maximum duration of 7 days, and at a  
15 temperature comprised between 0-24 °C for a storage time up to a maximum of 5 hours.

In a further form of realization, in the methods according to the present invention, said physiological saline solution and/or said culture medium of step a) are supplemented with antibiotics and antifungals in a range comprised between 1-5%  
20 of the final volume of penicillin, streptomycin, gentamicin, metronidazole and amphotericin.

In a further form of realization, in the methods according to the present invention, said physiological saline solution and/or said culture medium of step a) are supplemented with penicillin from 100 to 500 U/ml, streptomycin and gentamycin  
25 from 100 to 500 micrograms/ml, metronidazole from 1 to 5 micrograms/ml and amphotericin from 5 to 25 micrograms/ml.

In a further form of realization, in the methods according to the present invention, in case of solid tumor biopsies, cells are grown on a support for cell trofism comprising collagen or type I collagen or type IV collagen or laminin or vitronectin  
30 or fibronectin.

In a further aspect, the invention relates to a method for determining sensitivity or resistance of at least one cancer cell to at least one anti-cancer drug, comprising

the steps of: - *ex vivo* collection of a tumor sample to be tested from a patient;

- Storage of the tumor sample in saline solution and/or culture medium supplemented with antibiotics and antifungals to prevent contamination until the next phase of disruption;
- in the case of solid tumors, mechanical disruption followed by enzymatic digestion of the tumor sample in order to obtain cells for the subsequent seeding step and preparation of a support substrate for cell trofism in multi-well plates for culturing the so obtained cells;
- seeding cells in multi-well plates so coated in culture medium supplemented with antibiotics/antifungals, support factors and growth factors;
- treatment of plated cells with said at least one anticancer drug;
- execution on treated and untreated cells of a test to quantify cell growth (proliferation and/or metabolism), viability and/or death by exposure to a reagent selective for live cells or to a reagent selective for metabolically active cells or to a dedicated reagent for detection of energy consumption;
- quantitative analysis of cell viability or metabolism of treated and untreated cells reacting with the reagent or measurement of energy consumption of treated and untreated cells reacting with the reagent, by spectrophotometric or fluorimetric measurement of absorbance or emission;
- statistical analysis of data obtained by absorbance measurement by comparison of the absorbance value of not treated cells with cells treated in presence of said at least one anticancer drug, for the following phase of categorization into classes of response to treatment;
- categorization into classes of response to treatment selected from: a) cells sensitive to said at least one anticancer drug; b) cells resistant to said at least one anticancer drug; c) mixed population of sensitive and resistant cells.

Therefore in a still further aspect of the invention relates to a method to determine sensitivity or resistance of at least one cancer cell to at least one therapeutically active molecule capable of inhibiting growth (proliferation and/or metabolism) or inducing death of said tumor cell and/or restoring anticancer drug sensitivity in resistant tumor cells, comprising the steps of: - *ex vivo* collection from a patient of a tumor sample to be tested;- storage of the tumor sample in saline solution and/or culture medium supplemented with antibiotics and antifungals to prevent contamination until the subsequent phase of disruption;
- in the case of solid tumors, mechanical disruption followed by enzymatic digestion of the tumor

sample in order to obtain cells for the subsequent seeding step and preparation of a support substrate for cell trofism in multi-well plates for culturing the so obtained cells; - seeding cells in multi-well plates so coated in culture medium supplemented with antibiotics/antifungals, support factors and growth factors; -  
5 treatment of plated cells with said at least one anticancer drug; - execution on treated and untreated cells of a test to quantify cell growth (proliferation and/or metabolism), viability and/or death by exposure to a reagent selective for live cells or to a reagent selective for metabolically active cells or to a dedicated reagent for detection of energy consumption; - quantitative analysis of cell viability or  
10 metabolism of treated and untreated cells reacting with the reagent or measurement of energy consumption of treated and untreated cells reacting with the reagent, by spectrophotometric or fluorimetric measurement of absorbance or emission; - statistical analysis of data obtained by absorbance measurement by comparison of the absorbance value of not treated cells with cells treated in  
15 presence of said at least one anticancer drug, for the following phase of categorization into classes of response to treatment, - categorization into classes of response to treatment selected from: a) cells sensitive to said at least one anticancer drug; b) cells resistant to said at least one anticancer drug; c) mixed population of sensitive and resistant cells.

20 The preferred aspects indicated in the present detailed description for the first two methods of the invention according to claims 1 and 2, are also preferred in the two above described methods.

As it will be evident from the following experimental part, the methods, particularly those in claims 1 and 2, surprisingly allow development of personalized therapy for  
25 the patient. Cancer patients very often require a personalized targeted therapy due to tumor type, genetics and heterogeneity that change from patient to patient. Therefore the possibility to obtain a rapid and reliable response, by the methods according to the present invention, about the best treatment and its potential effectiveness, allows considerable cost and time saving, in addition to being an  
30 advantage for the patient.

Therefore the methods according to the present invention make possible, in a surprisingly efficient and reliable single test, to determine if the cancer patient has

cancer cells sensitive or resistant to an anticancer drug, a therapeutically active drug or a combination thereof, with obvious time and cost saving.

Therefore below are provided examples of realization in order to illustrate the present invention.

5 **EXAMPLES**

**Example 1**

**PROTOCOL**

The method that is object of the present invention has been devised on colon cancer and ovarian cancer as an example, without being limited to this.

10 This method may be applicable to other cancers, not only tumors of epithelial origin and circulating cancer cells.

In the case of a solid tumor, the tissue sample containing tumor cells is taken over by the pathologist, after surgery, removed and transferred to a test tube containing DMEM medium supplemented with antibiotics and antifungals in a range  
15 comprised between 1 and 5% of the final volume of penicillin, streptomycin, gentamicin, metronidazole and amphotericin. Preferably penicillin can be added in a range from 100 to 500 U/ml, streptomycin and gentamicin in a range from 100 to 500 micrograms/ml, metronidazole in a range from 1 to 5 micrograms/ml and amphotericin in a range from 5 to 25 micrograms/ml. The tissue is subsequently  
20 rinsed in saline solution (HBSS: Hank's balanced salts solution) and transferred to fresh DMEM medium with antibiotics comprised in a range between 1 and 5%. Storage conditions of the tumor sample vary depending on the time required for the sample to travel from where it is taken to the laboratory. In case of storage above 5 hours, the sample is maintained at a temperature between 0-8 °C for a  
25 maximum of 7 days. Instead in case of storage for a maximum of 5 hours, the sample is maintained at a temperature comprised between 0-24 °C.

Antibiotics and antifungals are required in the medium to eradicate the bacterial charge possibly present in the sample. This crucial step made possible to reduce the number of contaminated samples. Tumor cells derived from tissue samples  
30 from colon and ovary are at high risk for contamination due to the presence of bacteria, and keeping samples in 5% solution of broad-spectrum antibiotics has made possible to minimize the probability of contamination.

The percentage of antibiotics and antifungals vary depending on the site of the tumor under analysis, ranging from the 1% standard dose used in any cell culture up to 7%. Preferably the range is comprised between 1% and 5%. After a storage time at a temperature comprised between 0 and 8 °C, the sample is rinsed with  
5 HBSS and cut with sterile scissors until it is reduced to fragments of less than one millimeter. Fragments are subjected to enzymatic digestion in 10 ml of trypsin/EDTA for a time ranging from a minimum of 1 hour to a maximum of 1 hour and 30 minutes, depending on the consistency of the tissue.

After the time of enzymatic digestion, the suspension is filtered through 40 micron  
10 nylon filters by crushing the cell suspension with a syringe plunger to facilitate disaggregation of pieces and passage through the filter. The filtrate containing the cells is collected in a centrifuge tube with the addition of HBSS and centrifuged for 10 min at 1000 rpm. After centrifugation the supernatant is removed and the pellet containing the cells is resuspended in 2 ml of lysis solution (0.84% ammonium  
15 chloride) and left at room temperature (T) for 10 minutes. After the lysis time, 5 ml of DMEM medium are added (to stop the reaction by dilution) and the suspension is centrifuged at 1000 rpm for 10 minutes. The supernatant is again removed and the pellet is resuspended in 1 ml of complete medium: DMEM with 2% antibiotics (penicillin, streptomycin, metronidazole, gentamicin and amphotericin)  
20 supplemented with 20% fetal bovine serum and growth factors (EGF 20 ng/ml in case of tumors of epithelioid origin). 10 µl of cell suspension is withdrawn and cells are counted in a Burker chamber with 10 µl of Trypan-blue dye to discriminate live cells. Multi-well plates are coated with collagen (extracted in the laboratory from rat tail) at least 3 hours prior to plating the cells prepared as above from the tumor  
25 biopsy. This collagen does not interfere with the functional MTT assay.

Other type of substrates tested for cell adhesion were type I collagen, laminin, vitronectin and fibronectin. All tests performed with the above listed substrates showed compatibility with the experiment; in particular, in the response tests to treatment with chemotherapeutic or therapeutic molecules, measuring viability or  
30 cell metabolism, the results were mutually comparable and compatible with those observed when using collagen prepared in the laboratory by rat tail extraction and used in previous experiments.

Cells are seeded in a 96-well plate at a minimum concentration of 20,000 cells per well in 100 microliters of complete medium, in triplicate for each treatment. Cell concentration may vary up to a maximum of 40.000/60.000 cells per well. After approximately 1 hour from seeding, cells are treated with the drugs of interest.

5 Treatment of colon carcinoma is with 5FU (e.g., treatment with 5FU 200  $\mu$ M), ibrutinib (e.g., treatment with 20  $\mu$ M ibrutinib) and Dasatinib (e.g., treatment with Dasatinib at different concentrations ranging from 2 to 100 nM). Treatment of cells from ovarian cancer is with Paclitaxel, Carboplatin and Bevacizumab at final concentrations of 500 nM, 10-100 nM, 50 micrograms/ml, respectively.

10 After 48-72 hours treatment, depending on the drug of interest, each well receives 100  $\mu$ l of a solution obtained by dissolving a solution of Thiazolyl Blue Tetrazolium Bromide (MTT) in DMEM medium at a concentration of 1 mg/ml. The plate is transferred back to the incubator for 3 hours, which is the time required for metabolically active cells to convert the tetrazolium ring of the MTT molecule into a  
15 purple color insoluble salt (formazan salt) that precipitates inside the cells. At the end of the incubation period the plate is centrifuged for 10 min at 2000 rpm. After centrifugation, 100  $\mu$ l of supernatant is removed from each well and 150  $\mu$ l of absolute ethanol is added by vigorous resuspension followed by plate stirring until complete solubilization of the formazan granules (about 15 minutes). Finally the  
20 plate is centrifuged for 10 min at 2000 rpm, 100  $\mu$ l are removed from each well and transferred to a new plate for spectrophotometric reading at 570 nm. The absorbance value is proportional to the number of metabolically active cells present in the well.

#### Example 2: DRUG TREATMENT

25 The tumor cells *ex vivo collected* from patients' tumor biopsies are treated with a single drug or a combination of anticancer drugs in order to assess sensitivity/resistance. Colon carcinoma cells were tested for sensitivity/resistance to classical chemotherapeutic agents (e.g., 5-FU, oxaliplatin, irinotecan). Tumor cells derived from ovarian cancer were in turn tested for sensitivity/ resistance to the  
30 chemotherapeutic agents Paclitaxel and Carboplatin as well as to Bevacizumab.

In case of colon and ovary carcinoma it is possible to test and will be tested other anticancer drugs such as those belonging to the families i) of the so-called

biological drugs (monoclonal antibodies, e.g. Cetuximab, Panitumumab, Bevacizumab), ii) of kinase inhibitors (e.g. gefitinib, erlotinib, vemurafenib, sorafenib, ibrutinib, dasatinib), iii) of classical chemotherapeutic agents (e.g. 5-FU, oxaliplatin, irinotecan). In addition to chemotherapeutic agents, other molecules can be tested with therapeutic activity such as molecules capable of inhibiting cell cycle progression or inducing cancer cell death or restoring sensitivity to chemotherapeutic agents in resistant tumor cells. As an example, Dasatinib and Ibrutinib are among these molecules without being limited to these. Dasatinib is a multikinase inhibitor clinically used for the treatment of adult chronic myeloid leukemia (CML), positive for the Philadelphia chromosome (Ph+), in chronic phase, accelerated phase or blast phase with resistance or intolerance to prior therapy including Imatinib mesylate, for the treatment in adults affected by acute lymphoblastic leukemia (ALL) and for the treatment of CML with lymphoid blast phase with resistance or intolerance to prior therapy. Ibrutinib is a specific BTK inhibitor still undergoing clinical trials for treatment of chronic lymphocytic leukemia. As it is well known from the literature, Dasatinib inhibits the Abl/Src fusion protein, SRC and also BTK (Hantschel et al., Proc Natl Acad Sci USA, 2007, vol 104:13283; Rix et al., Blood 2007, 110: 4055). In consideration of the literature data and of recent discoveries highlighted in patent US 8,232,085 B2, Dasatinib and Ibrutinib were tested for treatment of carcinoma-derived cells, in particular colon carcinoma expressing the shorter BTK protein isoform (65-67 kDa). Such therapeutically active molecules have surprisingly shown in colon carcinoma cells both their own anti-tumor activity and an activity that restores sensitivity to an anti-cancer drug in colon cancer cells resistant to 5FU.

### 25 Example 3: FUNCTIONAL ASSAY

Experiments involved the use of MTT assay, which is based on a metabolic reaction that correlates with cell viability. In these experiments, the MTT assay was used to assess viability of cancer cells in presence of a chemotherapeutic drug or of other drugs and/or therapeutic molecules, or the combination thereof. Assays other than MTT can be used to measure cell metabolism. The MTT assay is widely used to assess cell viability. It is a simple, accurate and reproducible assay. It consists of a colorimetric assay that exploits the ability of mitochondrial

dehydrogenases to cleave the tetrazolium ring of the MTT molecule (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) in a formazan salt. To perform the assay, cells are grown on a multi-well plate and triplicate samples are treated with different drugs for the time of interest. At the end of treatment, the MTT solution (initially yellow) is added to the cells and, after three hours (time required for formation of the formazan salt, with a violet color), the absorbance is measured using a plate reader at 570 nm (violet color detection). The quantity of formazan that is measured is proportional to the number of live cells. Therefore the assay is quantitative.

In a second alternative form of realization of the present invention, the detection assay can also consist of a colorimetric assay measuring energy consumption of live cells (ATP consumption). In particular the CellTiter-Glo® assay has been used that allows determination of the number of viable cells in culture, based on quantification of the ATP content. The assay makes use of the luciferin/luciferase principle and, in presence of a specific luminescence filter, can be read at 3600 gain and 0.5 seconds.

Alternatively, the assay can be read at a wavelength ranging from 500 to 650 nm.

In a third alternative form of realization of the present invention, the assay can also consist of a colorimetric assay measuring activity of cellular esterases. In particular, the assay employed is the Fluorescent cell counting kit (Sigma) based on the ability of cellular esterases to hydrolyze calcein-AM (3',6'-Di(O-acetyl)-2',7'-bis[N,N-bis(32(carboxymethyl)aminomethyl)]-fluorescein, tetraacetoxymethyl ester) in calcein. The amount of (fluorescent) calcein produced is proportional to the number of live cells. Fluorescent emission is measured by use of a plate reader (fluorimeter) at 535 nm (excitation at 485 nm).

In a fourth alternative form of realization of the present invention, the assay can even be any one of the systems for read-out of cellular death, based on the use of vital dyes, detection of release of intracellular enzymes, cellular metabolism, loss of cell adhesion, morphological parameters or on specific systems for detection of apoptosis or necrosis (Kepp, 2011).

#### Example 4: SET UP OF THE METHOD

Set up of the assay, in order to optimize experimental conditions and transpose



them to primary cultures of tumor cells, was initially performed on colon carcinoma derived cell lines. A chemosensitive cell line, HCT116, and 3 chemoresistant lines, HCT116p53KO, DLD-1 and SW480, have been used. In various experiments, different methods were used to evaluate viability/mortality of untreated cells, cells  
5 treated with 5-FU or other drugs commonly used for colon cancer treatment (5FU, OxPt, Cetuximab, Panitumumab, Bevacizumab) or drugs currently used for other cancer pathologies (Dasatinib). Drug concentration was selected based on dose-response curves carried out on available colon carcinoma lines. The same procedure can be carried out for all drugs and/or therapeutically active molecules  
10 of interest, and on all commercial lines from the different carcinomas, in order to identify the most effective concentrations. The best results in terms of treatment efficacy with a chemotherapeutic agent were obtained after 72 hours of combined treatment with 200 $\mu$ m 5-FU and 50 $\mu$ M OxPt, 50nM Dasatinib, 20 $\mu$ M Ibrutinib, 10 $\mu$ g/ml Cetuximab, 75 $\mu$ g/ml Panitumumab, 25 $\mu$ g/ml Bevacizumab.

15 The ovarian cancer-derived cell line SKOV-3 is used to set up the assay and the experimental conditions for ovarian cancer cells. SKOV-3 cells are used to test the concentrations of Paclitaxel, Bevacizumab and Carboplatin. The best results in terms of treatment efficacy with chemotherapy are obtained with 500nM Paclitaxel, 50 $\mu$ g/ml Bevacizumab and 100nM Carboplatin.

20 Data obtained and shown in Figure 1 and 2 were analyzed statistically by ANOVA with Tukey's post-hoc test (significance set at 0.05). Figures 1-3 show results of experiments comparing the response to 5FU of the chemosensitive HCT116 line and the chemoresistant HCT116p53KO line. Cell mortality/viability was quantitatively assessed by exposure to a reagent selective for live cells (trypan  
25 blue, Figure 1), quantitative analysis of energy consumption (Cell Titer Glo Luminescent Cell Viability Assay, Figure 2), an assay evaluating metabolic activity (MTT assay, Figure 3). In each case, assays confirmed that 5-FU treatment induces significant reduction of viability in the chemosensitive cell line HCT116, whereas it fails to induce significant reduction of cell viability in the HCT116p53KO  
30 line which was thereby confirmed to be chemoresistant. Effectiveness of these assays to assess the response to anticancer drugs was further confirmed by treating with 5FU (Figure 4), OxPt (Figure 5) and a combination of 5FU+OxPt

(Figure 6) the DLD-1 and SW480 cell lines (derived from colon carcinoma and known from the literature to be drug-resistant). Figure 7 shows reversal of 5FU resistance in the HCT116p53KO line by the combined use of 5FU and Dasatinib. In the chemoresistant HCT116p53KO line, lack of response to so-called  
5 "biological" drugs (Cetuximab, Panitumumab and Bevacizumab) was ultimately assessed, obtaining similar results from the Calcein-AM assay (Figure 8) and MTT assay (Figure 9).

Subsequently, the MTT assay was validated on tumor cells freshly prepared from *ex vivo* samples of patients from the operating rooms. A total number of 135  
10 samples were used. Setup of the best possible cell culture conditions required several initial trials with the use of 6 tumor samples.

The following conditions were identified:

- 1) from time of collection to time of use, the biopsy should be kept in saline solution or in culture medium containing high doses of different antibiotics and  
15 antifungals;
- 2) several washes of the tumor in large volumes of saline solution containing high doses of different antibiotics and antifungals are required to prevent contamination during the test;
- 3) isolation of cells from the biopsy is performed by mechanical disruption followed  
20 by enzymatic treatment with trypsin;
- 4) optimal concentration of cells for seeding was determined to be  $2 \times 10^4$ /well;
- 5) well coating with collagen or other suitable substrate should be performed to support cell trofism. This procedure should be carried out from 20 minutes to 3 hours before cell seeding, depending on substrate type.
- 25 6) DMEM supplemented with fetal calf serum and growth factors was found to be the optimal culture medium for cell growth in the case of colon carcinoma.

#### Example 5: RESULTS

Colon carcinoma:

The following samples were obtained: 151 biopsies from tumor tissue.

30 16 samples were used to set up and validate the MTT assay in order to assess, with respect to viability, the cellular response without treatment and upon 5FU treatment.

135 samples were used to evaluate the response to 5FU treatment. Of those, 68 samples were used to evaluate the response to 5FU treatment in absence and presence of Ibrutinib, and 25 samples were used to evaluate the response to 5FU treatment in absence and presence of Dasatinib.

#### 5 Ovarian cancer

The same protocol was used for ovarian cancer, with 28 samples obtained for primary tumor and two samples for metastatic tumor. All samples were treated with paclitaxel, carboplatin, and bevacizumab.

Ovarian cancer cells proved to be easily cultured *in vitro*, with viability beyond 72  
10 hours of culture, as shown by mean absorbance values (0.356 vs 0.214 in untreated samples from colon).

#### Example 6A: ASSESSMENT OF 5FU SENSITIVITY

The graph in Figure 11 shows the distribution of responses of 135 tumor samples treated with 5FU alone (200 $\mu$ M). For each sample of tumor cells, 5FU sensitivity  
15 was assessed by the MTT assay comparing the absorbance values of triplicates by the Student's T test relative to untreated cells (control) ( $p < 0.05$ ).

The absorbance (OD) (average value of a triplicate) was assessed for: i) the sample of tumor cells treated (T) with 5FU; ii) the sample of not treated (NT) tumor cells. A Student's T test value  $\leq 0.05$  from three identical replicas (triplicate) of the  
20 NT sample and of the T sample of tumor cells from the same patient identifies cancer cells sensitive to a specific treatment. A Student's T test value  $\geq 0.05$  from three identical replicas (triplicate) of the NT sample and of the T sample of tumor cells from the same patient identifies cancer cells resistant to a specific treatment.

Delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells in  
25 sensitive samples ( $p \leq 0.05$ ) are preferably  $\geq 0.04$ .

Delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated cancer cells (T) in resistant samples ( $p \geq 0.05$ ) are preferably  $\leq 0.02$ .

30 The method also identifies tumors containing a mixed population of sensitive and resistant cells wherein delta values ( $\Delta$ ) obtained as the difference between mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated

(T) cancer cells are preferably comprised between +0.04 and +0.02.

Example 6B: EVALUATION OF RESPONSES TO TREATMENT WITH 5FU ALONE OR IN COMBINATION WITH IBRUTINIB AND DASATINIB.

5 Figure 12 shows the distribution of responses to 5FU alone, Ibrutinib alone, and to the combination Ibrutinib + 5FU in 68 samples analyzed. Figure 13 shows the distribution of responses to 5FU alone, Dasatinib alone, and to the combination Dasatinib + 5FU in 25 samples analyzed.

As for example 5, the absorbance (OD) (average value of a triplicate) is assessed  
10 for: i) the sample of 5FU treated (T) cancer cells; ii) the sample of not treated (NT) cancer cells. A Student's T test value  $\leq 0.05$  from three identical replicas (triplicate) of the NT sample and of the T sample of tumor cells from the same patient identifies cancer cells sensitive to a specific treatment. A Student's T test value  $\geq 0,05$  from three identical replicas (triplicate) of the NT sample and of the T sample  
15 of tumor cells from the same patient identifies cancer cells resistant to a specific treatment.

Delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells in sensitive samples ( $p \leq 0.05$ ) are preferably  $\geq 0:04$ .

20 Delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated cancer cells (T) in resistant samples ( $p \geq 0.05$ ) are preferably  $\leq +0:02$ .

The method also identifies tumors containing a mixed population of sensitive and resistant cells wherein delta values ( $\Delta$ ) obtained as the difference between mean  
25 absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells are preferably comprised between +0.04 and +0.02.

Example 7: EVALUATION OF RESPONSES OF THE OVARIAN SAMPLES TO DIFFERENT TREATMENTS

30 Figure 10 shows dose-response curves relative to the different drugs tested on the SKOV-3 cell line, in order to select the most appropriate drug concentration. In

light of this, concentrations of 500nM Paclitaxel, 50 µg/ml Bevacizumab and 100nM Carboplatin were selected.

Figure 14 shows results obtained for the different treatments on 29 ovarian samples. In light of the above observations, the tumors analyzed can be divided  
5 into subtypes based on their sensitivity/resistance.

As with examples 5 and 6, the absorbance (OD) (average value of a triplicate) was measured for: i) the sample of 5FU treated (T) cancer cells; ii) the sample of not treated (NT) cancer cells. A Student's T test value  $\leq 0.05$  from three identical replicas (triplicate) of the NT sample and of the T sample of tumor cells from the  
10 same patient identifies cancer cells sensitive to a specific treatment. A Student's T test value  $\geq 0,05$  from three identical replicas (triplicate) of the NT sample and of the T sample of tumor cells from the same patient identifies cancer cells resistant to a specific treatment.

Delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells in  
15 sensitive samples ( $p \leq 0.05$ ) are preferably  $\geq 0:04$ .

Delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated cancer cells (T) in resistant samples ( $p \geq 0.05$ ) are preferably  $\leq +0:02$ .

The method also identifies tumors containing a mixed population of sensitive and  
20 resistant cells wherein delta values ( $\Delta$ ) obtained as the difference between mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells are preferably comprised between +0.04 and +0.02.

The advantages achieved by the method of the present invention are evident from  
25 the detailed description and from the above Examples. In particular, the results obtained show that the method for determination of sensitivity and resistance of an ex vivo cancerous cell toward a particular drug or combination of drugs is extremely effective and useful as it makes possible for the oncologist to set up from the beginning a potentially successful therapy, thereby proving to be  
30 extremely beneficial for both the patient, who is spared from various extremely invasive attempts of treatment without disease eradication, and for the NHS (SSN) that will sustain lower costs. At the same time, such method, being fast and

extremely easy to perform, can be conveniently carried out in any type of diagnostic or research laboratory.

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**CLAIMS**

1. A method to determine sensitivity or resistance of at least one cancer cell to at least one anticancer drug, at least one therapeutically active molecule capable of inhibiting the growth or inducing the death of said tumor cell and/or capable of restoring sensitivity to said at least one anticancer drug in resistant tumor cells, and to at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule, comprising the steps of:
- a) incubate the tumor sample (solid or liquid tumor biopsy), isolated from a patient, with saline and/or culture medium supplemented with antibiotics and antifungals;
  - b) in the case of solid tumor biopsy, mechanically disrupt cells of the tumor sample obtained from step a) followed by enzymatic digestion to obtain cells to be seeded in the subsequent step;
  - c) seed cells obtained from step b) in cell culture dishes and treat a first portion of said cells with said at least one anticancer drug, a second portion of said cells with said at least one therapeutically active molecule, a third portion of said cells with said at least one combination of said anticancer drugs, a fourth portion of said cells with said at least one combination of said therapeutically active molecules, a fifth portion of said cells with said at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule, a sixth portion of said cells is cultured without addition of drugs (untreated cells, NT);
  - d) quantify cell growth (proliferation and/or metabolism) and/or viability and/or death of the first, second, third, fourth, fifth and sixth portions of said cells of step c) by exposure to a reagent selective for live cells or a reagent selective for metabolically active cells or a dedicated reagent for detection of energy consumption;
  - e) analyze data obtained from step d) to classify cells according to the response to treatment with an anticancer drug and/or a therapeutically active molecule.
2. A method to determine sensitivity or resistance of at least one cancer cell to a combination of at least one anticancer drug and at least one therapeutically active molecule capable of inhibiting growth (proliferation and/or metabolism) or inducing

death of said cancer cell and/or capable of restoring sensitivity to said at least one anticancer drug in resistant cancer cells, comprising the steps of:

a) incubate the tumor sample (solid or liquid tumor biopsy), isolated from a patient, with saline and/or culture medium supplemented with antibiotics and antifungals;

5 b) in the case of solid tumor biopsy, mechanically disrupt cells of the tumor sample obtained from step a) followed by enzymatic digestion to obtain cells to be seeded in the subsequent step;

c) seed cells obtained from step b) in cell culture plates and treat said cells with said at least one combination of said anticancer drugs, with said at least one  
10 combination of said therapeutically active molecules, with said at least one combination of said at least one anticancer drug and said at least one therapeutically active molecule;

d) quantify growth (proliferation and/or metabolism) and/or viability and/or death of said cells of step c) by exposure to a selective reagent for live cells, or to a  
15 selective reagent for metabolically active cells, or to a dedicated reagent for detection of energy consumption;

e) analyze data obtained from step d) to classify cells according to their response to the treatment with an anticancer drug and/or a therapeutically active molecule.

20 3. Method according to any of claims 1 or 2 wherein, the step d) to quantify cell growth (proliferation and/or metabolism) and/or viability and/or death is carried out by exposure to a reagent selective for live cells or to a reagent selective for metabolically active cells or to a dedicated reagent for detection of energy consumption.

25

4. Method according to any of claims 1 to 3 wherein the step e) of data analysis is carried out by statistical analysis of data obtained from step d) for the categorization of cells into classes of treatment response selected from the group consisting of:

30 a) cells sensitive to said at least one anticancer drug;

b) cells resistant to said at least one anticancer drug;

c) cells sensitive to said at least one therapeutically active molecule;



- d) cells resistant to said at least one therapeutically active molecule;
- e) cells resistant to said at least one anticancer drug whose resistance is reverted by the action of said at least one therapeutically active molecule;
- f) cells resistant to said at least one anticancer drug whose resistance is not  
5 reverted by the action of said at least one therapeutically active molecule;
- g) cells sensitive to said at least one anticancer drug whose sensitivity is not increased in the presence of said at least one therapeutically active molecule;
- h) cells sensitive to said at least one anticancer drug whose sensitivity increases in the presence of said at least one therapeutically active molecule.

10

5. Method according to any of the claims 1 to 4 wherein delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells in sensitive samples ( $p \leq 0.05$ ) are preferably  $\geq 0.04$ , more preferably from +0.04 to +0.4.

15

6. Method according to any of the claims 1 to 5 wherein delta values ( $\Delta$ ) obtained as the difference between the mean absorbance (OD, average of triplicate) of not treated (NT) cancer cells and treated (T) cancer cells in resistant samples ( $p \geq 0.05$ ) are preferably  $\leq +0.02$ , more preferably from +0.02 to -0.2.

20

7. Method according to any of the claims 5 or 6, wherein said mean absorbance (OD) is measured with a spectrophotometer, preferably at a wavelength comprised between 400 and 800 nm.

25

8. Method according to any of the claims 1 to 7 wherein the step of quantitative analysis of metabolism is made by the MTT assay comprising a colorimetric reagent.

30

9. Method according to any of the claims 1 to 8 wherein said spectrophotometric measurement of absorbance is carried out at a wavelength of 570 nm.

10. Method according to any of the claims 1 to 9, wherein the step of quantitative

analysis of cell viability is performed by the Calcein-AM assay, comprising a pro-fluorescent reagent.

11. Method according to any of the claims 1 to 10, wherein said at least one  
5 cancer cell is a cell of colon carcinoma, breast carcinoma, ovarian cancer, stomach cancer, lung carcinoma, renal carcinoma, thyroid carcinoma or pancreatic cancer or a circulating cancer cell.

12. Method according to any of the claims 1 to 11, wherein said at least one  
10 anticancer drug comprises classical chemotherapeutic drugs or biological drugs or kinase inhibitors.

13. Method according to claim 12, wherein said classical chemotherapeutic drugs  
15 comprise 5-fluorouracil (5FU), irinotecan or capecitabine, oxaliplatin, and other platinum coordination compounds, said biological drugs include monoclonal antibodies, preferably Cetuximab, Panitumumab, Bevacizumab and said kinase inhibitors include gefitinib, erlotinib, vemurafenib, sorafenib, ibrutinib and dasatinib.

14. Method according to any of the claims 1 to 13, wherein said tumor sample of  
20 step a) is prior stored at a temperature between 0-8 °C, in case of storage for more than 5 hours and up to a maximum of 7 days, and at a temperature comprised between 0-24 °C in case of storage up to a maximum of 5 hours.

15. Method according to any of the claims 1 to 14, wherein said physiological  
25 saline solution and/or said culture medium of step a) are supplemented with antibiotics and antifungals in a range comprised between 1-5% of the final volume of penicillin, streptomycin, gentamicin, metronidazole, and amphotericin.

16. Method according to any of the claims 1 to 15, wherein said physiological  
30 saline solution and/or said culture medium of step a) are supplemented with penicillin from 100 to 500 U/ml, streptomycin and gentamycin from 100 to 500 micrograms/ ml, metronidazole from 1 to 5 micrograms/ml and amphotericin from

5 to 25 micrograms/ml.

Figure 1

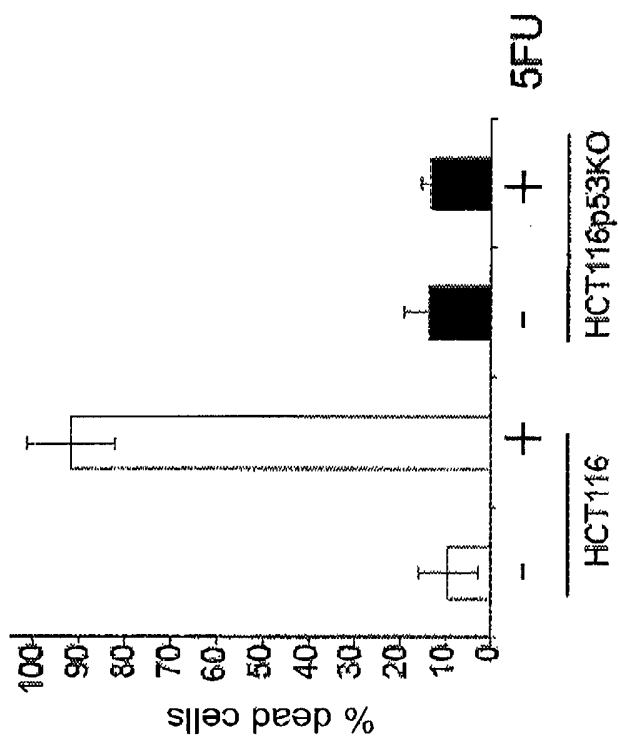


figure 2

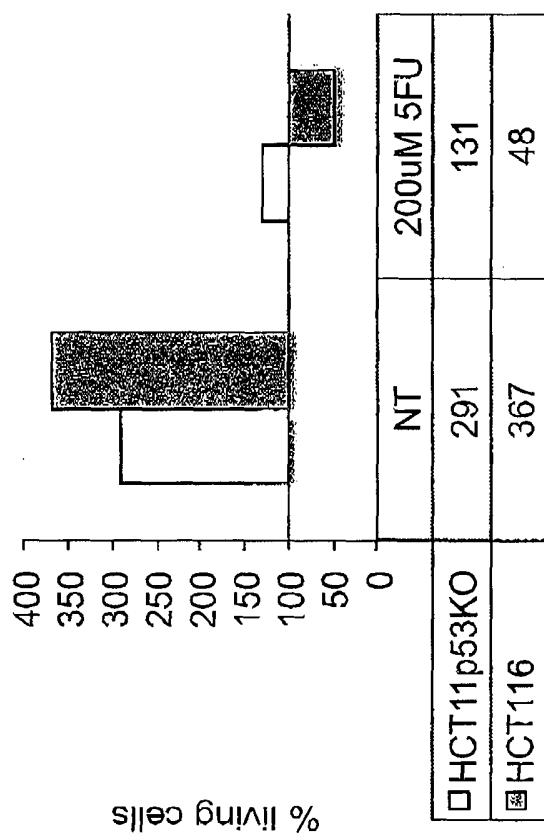


figure 3

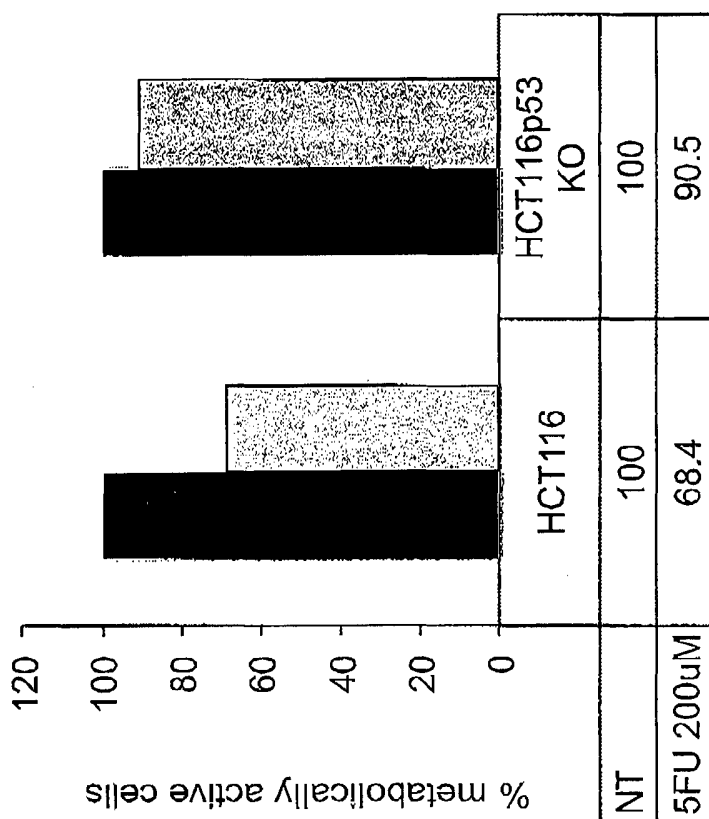


figure 4

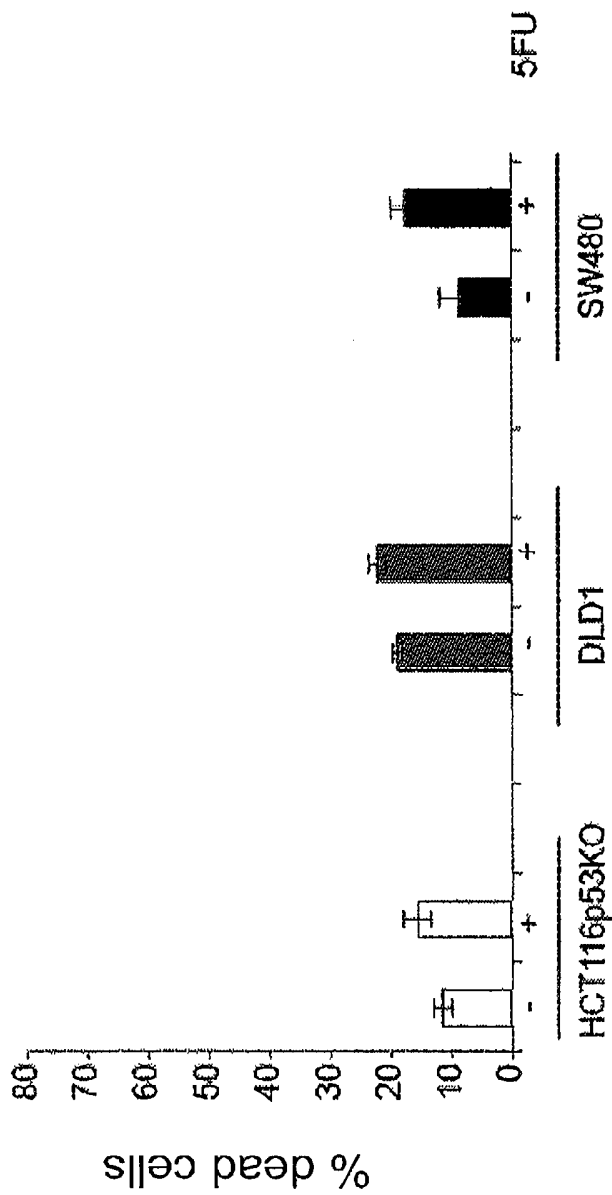


figure 5

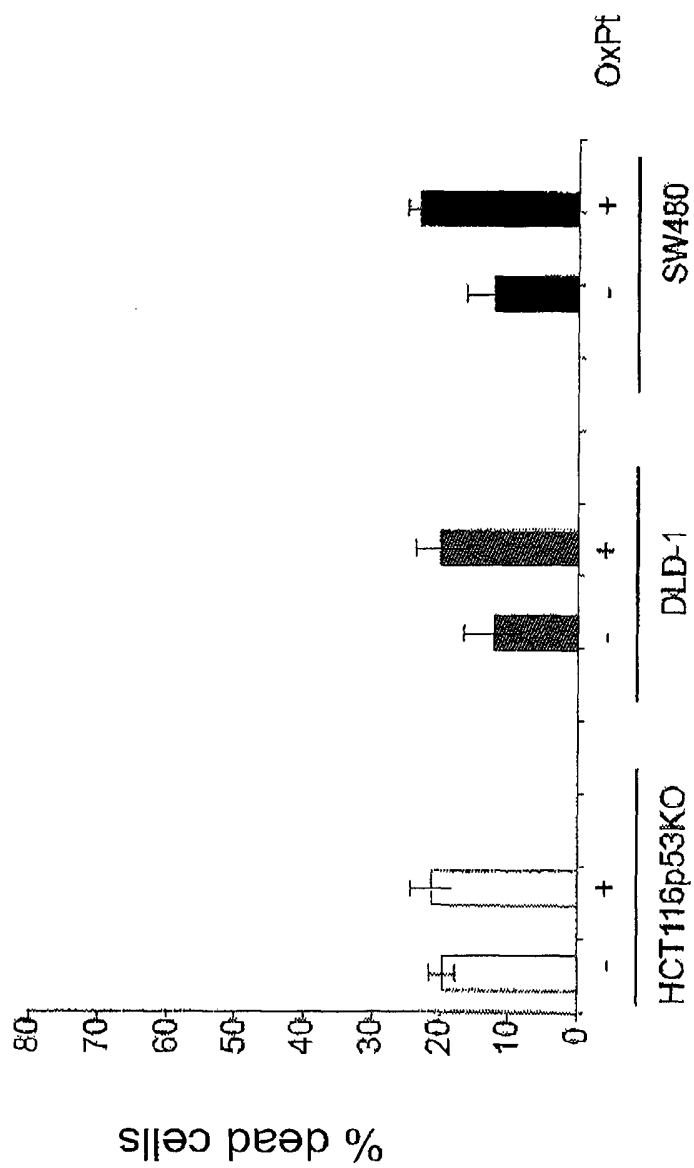
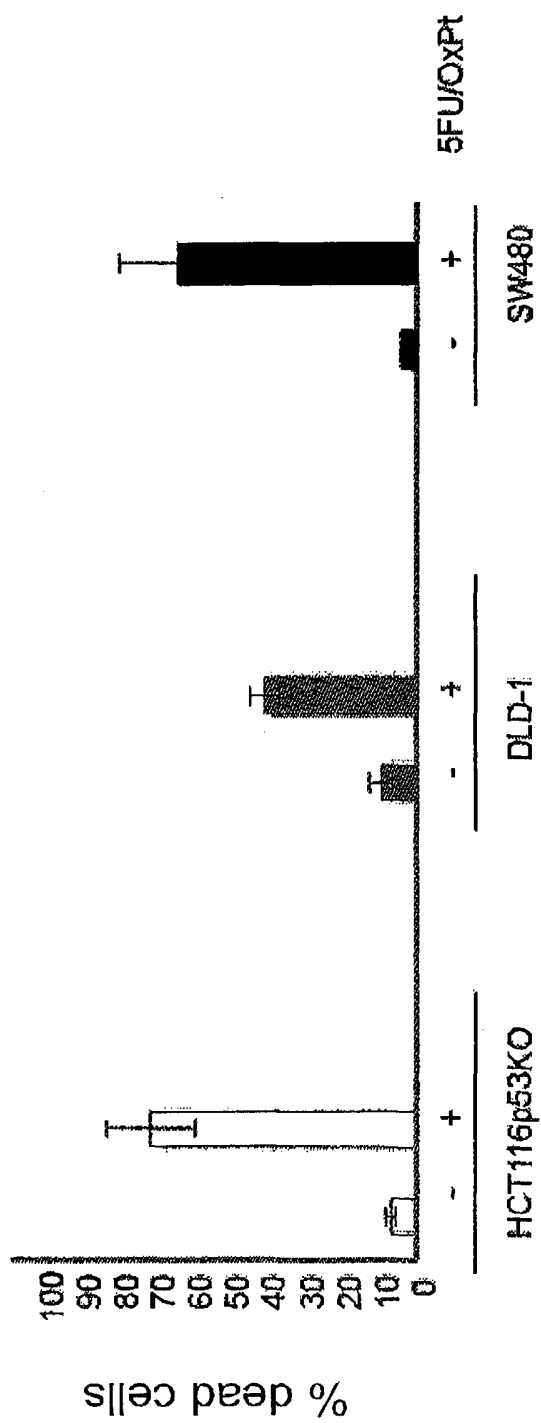




figure 6



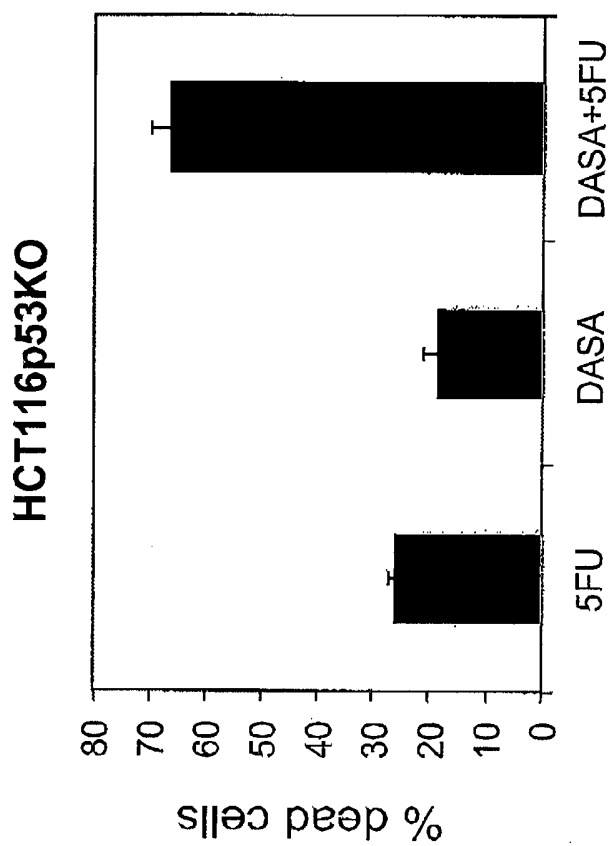


figure 7

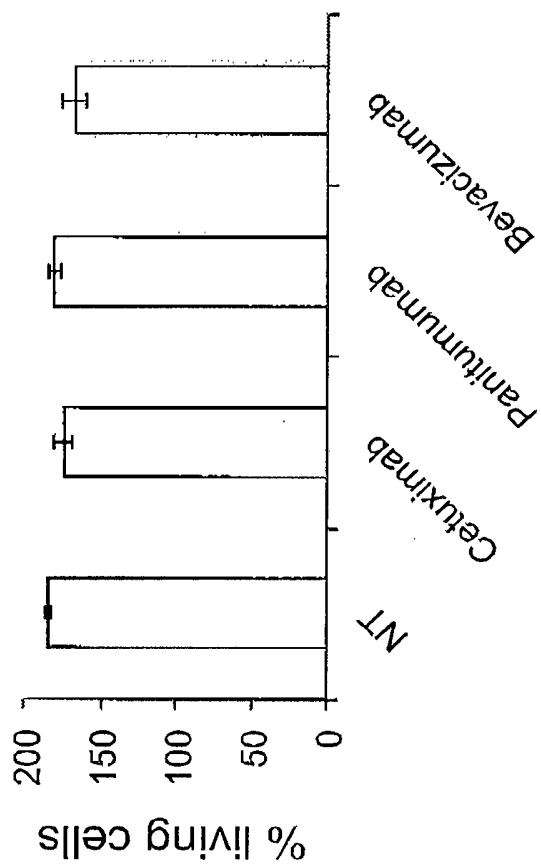
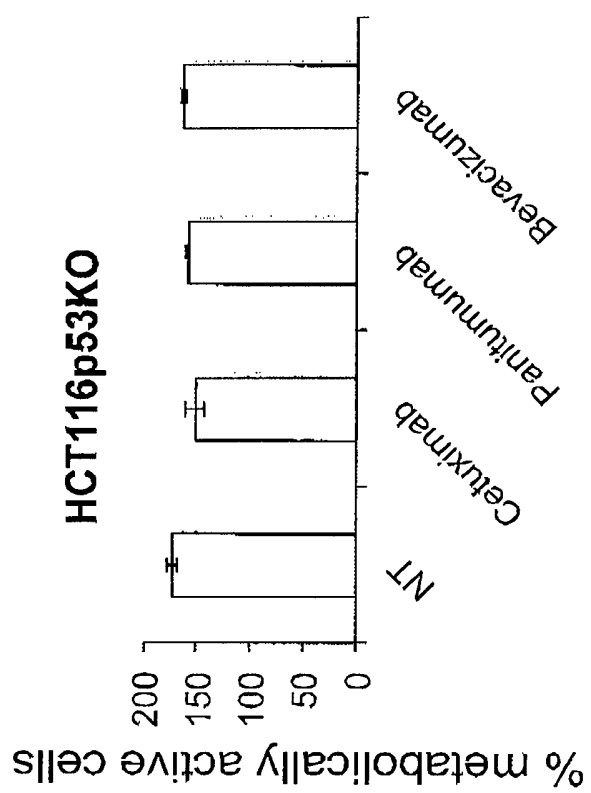


figure 8

figure 9



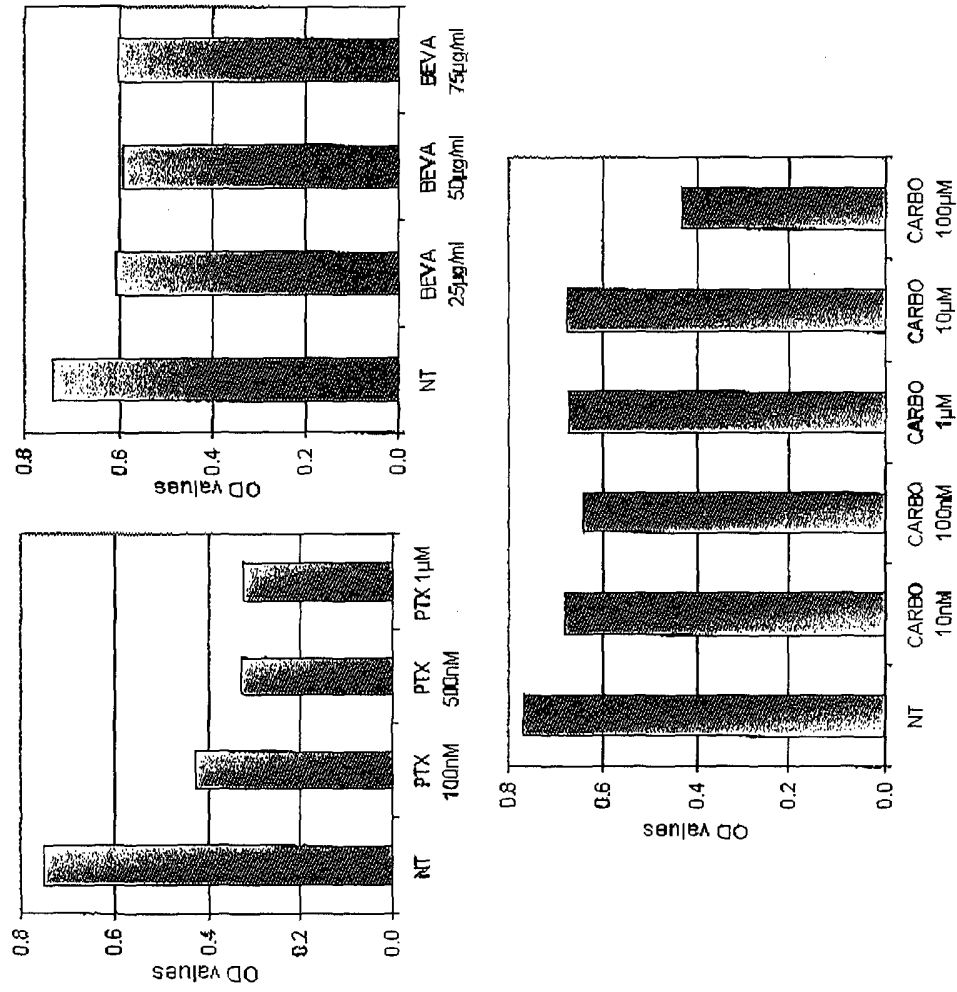


figure 10

5FU TREATED COLONIC SAMPLES

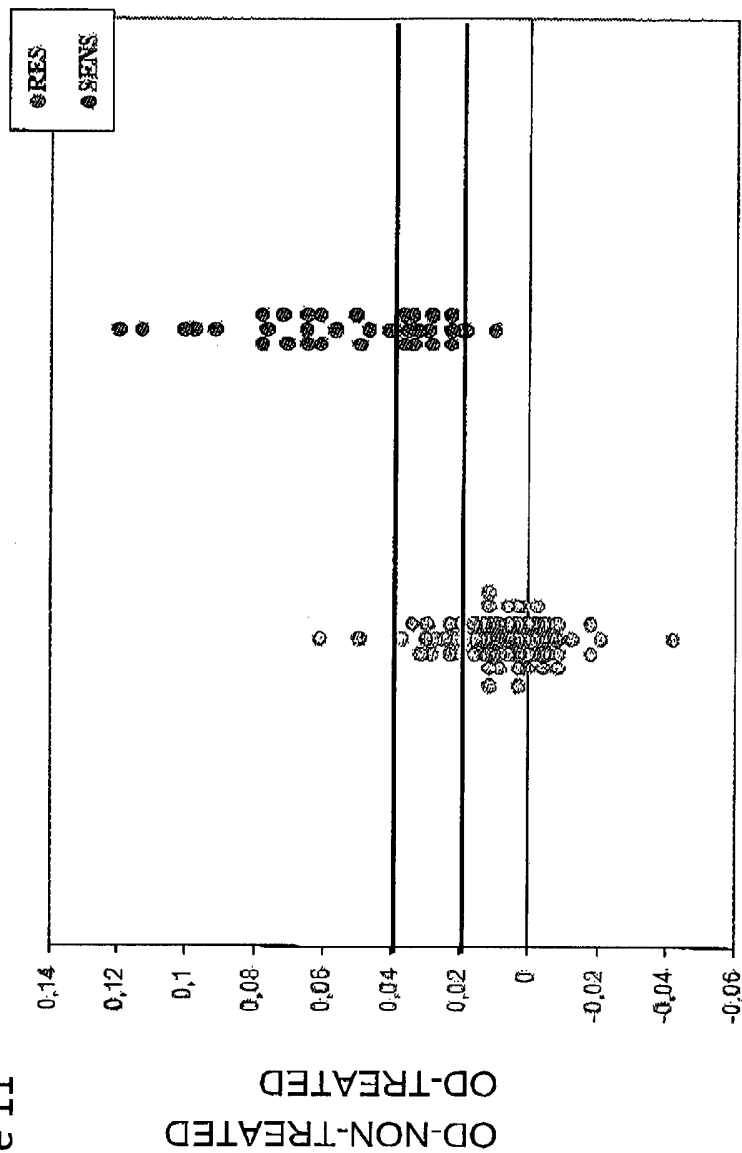


figure 11

COLONIC SAMPLES

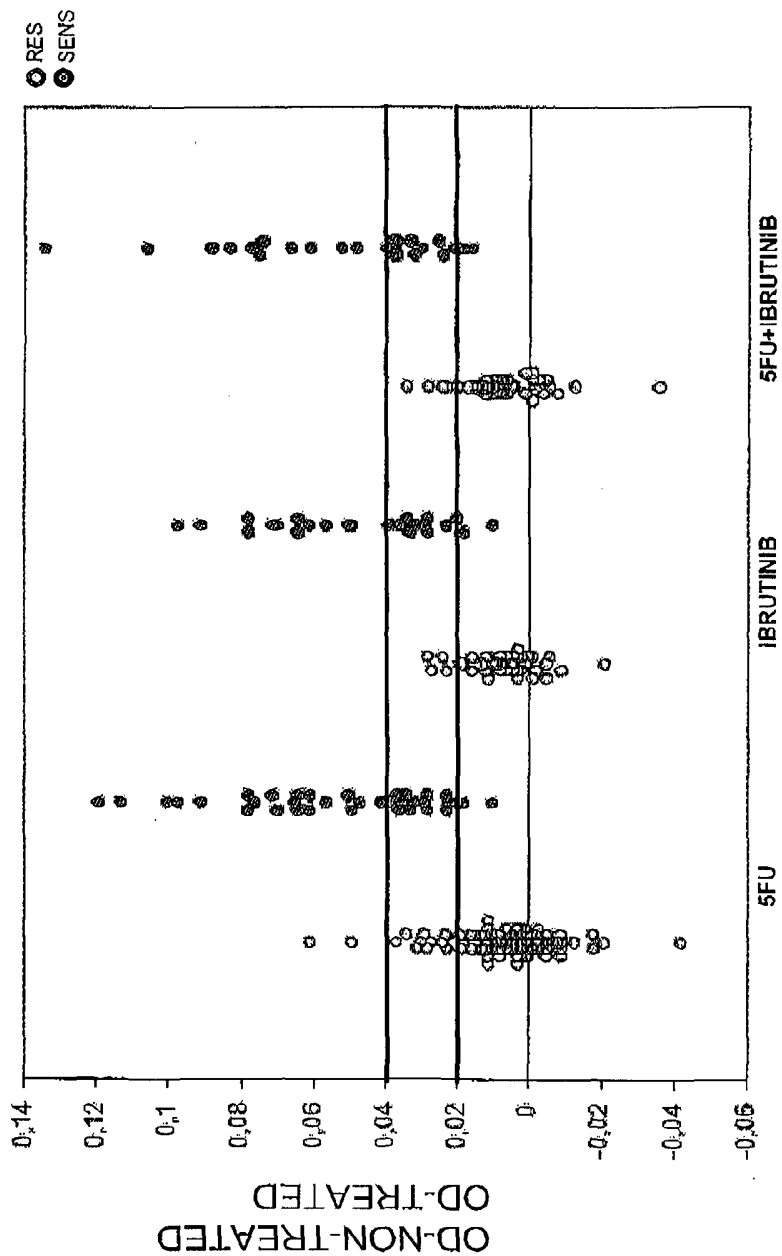


figure 12

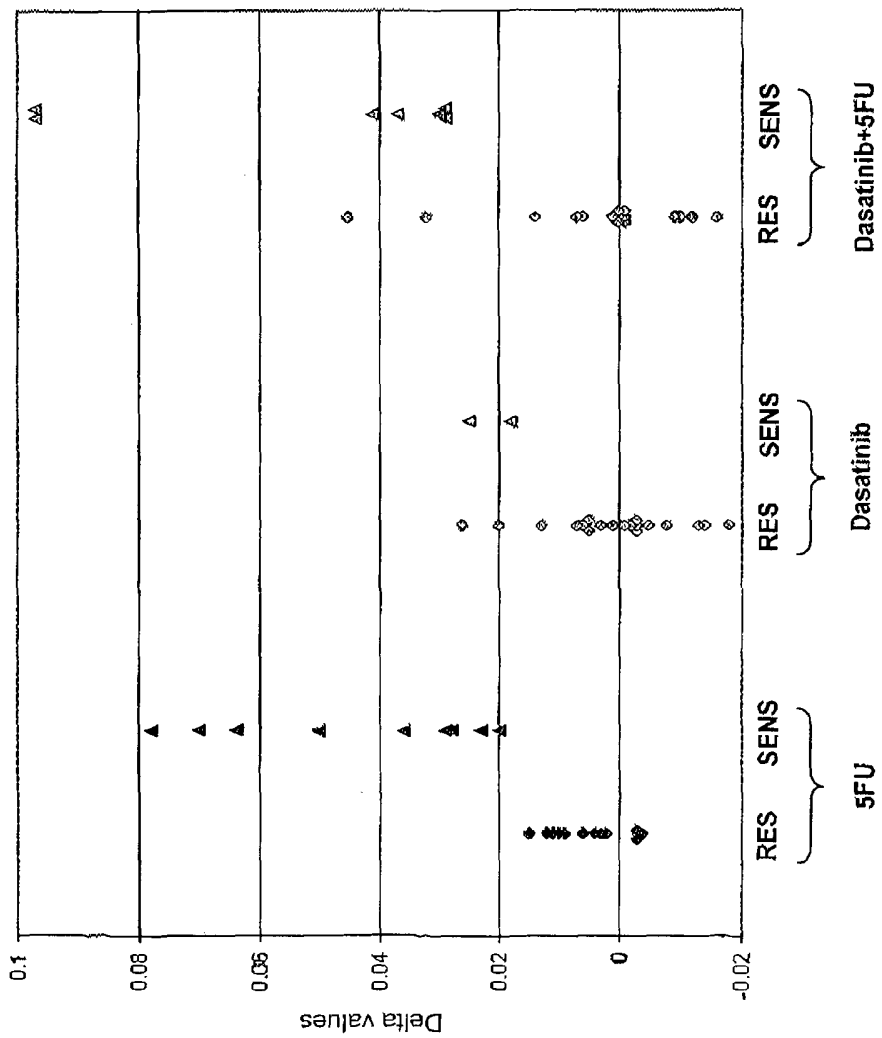


figure 13





**INTERNATIONAL SEARCH REPORT**

International application No  
PCT/IB2014/062303

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> INV. G01N33/50 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/207119 A1 (SUCHY SARAH L [US] ET AL) 25 August 2011 (2011-08-25) claims paragraph [0018] - paragraph [0023] paragraph [0033]	1-16
X	US 2009/104647 A1 (MI ZHIBAO [US] ET AL) 23 April 2009 (2009-04-23) claims paragraph [0022] - paragraph [0027]	1-16
Y	US 2005/100508 A1 (NICHOLS M J [US] ET AL) 12 May 2005 (2005-05-12) claims	1-16
Y	WO 03/040724 A1 (CELLCONTROL BIOMEDICAL LAB AG [DE]; WALDENMAIER DIRK [DE]; METZGER RAI) 15 May 2003 (2003-05-15) claims	1-16
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> 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 application or patent 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 <p align="center">15 August 2014</p>		Date of mailing of the international search report <p align="center">08/09/2014</p>
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer <p align="center">Routledge, Brian</p>

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No

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