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(54) Title: TUMOR DETECTION BY IMAGING AND THERAPY OF TUMORS

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(57) Abstract: A method for detecting or monitoring tumor cells in an individual is provided which is based on routinely used imaging techniques such as magnetic resonance imaging (MRI) and ultrasound techniques. The detection and monitoring of the cells is carried out non-invasively by externally monitoring the level of oxygenation of the cells (measured as BOLD images) and blood flow to the cells (measured by Doppler ultrasound) in a tested tissue of the individual. The method may be used to identify regions in an individual that does not have a pre-known tumorigenic lesion as well as to detect regions having a high probability of comprising cells in a pre-suspected lesion of an individual (which may, for example be a region in where there is a high probability of existence of primary tumor cells based on analysis by routinely used methods). The method comprises use of an agent which administration to the individual or contact with the cells results in enhancement of the oxygen consumption of the cells detectable by the various imaging techniques. The activating agent may, for example, activate the cells by binding to a receptor specifically expressed by the cells. In addition, methods for evaluating the efficacy of anti-tumorigenic treatments based on measuring blood oxygenation levels and blood flow levels to the cells by imaging techniques are also provided. Furthermore, methods for enhancing the efficacy of anti-tumorigenic therapeutic treatments including chemotherapeutical drugs, antibodies, and irradiation treatments are provided which involve administration to the treated individual of an amount of an agent which enhances the oxygenation of the target cells and the blood flow to the target cells in the treated individual. The efficacy of chemical contrast agents used in conjunction with MRI or ultrasound is also enhanced by the provided method.

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TUMOR DETECTION BY IMAGING AND THERAPY OF TUMORS

FIELD OF THE INVENTION

The present invention concerns methods for the detection of tumor foci by imaging and methods for enhancing the efficacy of anti-tumorigenic treatments as well as treatments of other diseases. The invention also concerns compositions for use in such methods and kits for carrying them out.

PRIOR ART

The following is a list of references which are intended for better understanding of the background of the present invention:

LIST OF PRIOR ART

Ghoussoub, R.A.D. *et al.*, *Cancer,* **85**:1513, (1998).

Kaplan O. *et al.*, *J. Biol. Chem.,* **265**:13641 (1990).

Kaplan O. *et al.,* FEBS Letters., **406**:175 (1997).

Muruganandham M. *et al.*, *Int. J. Radiat. Onco. Biol. Phys.* **43** :413, (1999).

Nagy, J.J.S *et al.*, *J. Surg. Oncol.*, **60**:95, (1995).

Netti P.A., *et al., Proc. Natl. Acad. Sci. U.S.A.,* **96**:3137, (1999)

Ogawa, S., and Lee T..M.,. *Magn Reson Med.,* **16:**9, (1990).

Rong. S, *Proc. Natl. Acad. Sci. USA,* **91**;4731, (1994).

Rosen E.M. *et al., Adv. Cancer Research,* **67**:257 (1994)

Takeda A, *et al., J. Chemother.,* **11**:137, (1999).

Tolnay E., *et al., Cancer Res. Clin. Oncol.*, **124**:291 (1998).

Yang R *et al., J. Cardiovasc. Pharmacol.*, **30**:294 (1997).

BACKGROUND OF THE INVENTION

Imaging of tumors is based on structural analysis of mammography, ultrasound and magnetic resonance (MR) images. In order to increase the resolution of such images, contrast agents are often added which make the blood vessels more clear and the tumor site more visible. An endogenous contrast agent, the blood, is also used in magnetic resonance imaging (MRI) techniques and is known as blood oxygen level dependent (BOLD) contrast. Changes in oxygenation of the blood can be observed as signal changes in BOLD MRI measurements. The presence of deoxyhaemoglobin, which is a paramagnetic molecule, in a tested blood vessel, causes a darkening of the obtained image while oxyhaemoglobin, which is diamagnetic, does not produce the same dephasing effect (Ogawa, S., and Lee T.M., 1990).

The dependency between magnetic properties and MR signals give rise to a method for measuring activation using MRI, commonly known as functional magnetic resonance imaging (fMRI). Using this technique, flow of oxygenated blood into an activated region (e.g. a specific region of the brain) increases the BOLD signal from this region.

During the development of the tumor, through a mechanism termed angiogenesis, the tumor becomes substantively vasculated. As the tumor grows in size, a large part of the blood flow reaches the outer part of the tumor, while its inner part receives much less blood flow. Various agents have been used for increasing tumor blood flow and vascular permeability of a tumor in the attempt to enhance the effectivity of various anti-tumorigenic treatments. Angiotensine (APII) and tumor necrosis factor (TNF) were, for example, used to try and enhance the activity of monoclonal anti-tumor antibody immunoconjugates (Takeda, A. *et al.*, 1999). All of the known agents used for this purpose caused non-specific vasodilatation of blood vessels.

Angiotensine was also shown to enhance general tumor microvascular pressure leading to an increase in uptake of specific antibody in the tumor (Netti P.A. *et al.*, 1999).

Non-specific haemodynamic changes were also shown to be induced by other agents such as, for example, by the calcium-channel blocker, diltiazem (Muruganandham M. *et al.*, 1999). BOLD contrast MR imaging measurements show the intensity enhancement after treatment with diltiazem, which was used as a radiosensititator for tumor radiotherapy. The increase in tumor blood flow caused by diltiazem was non-specific.

Various growth factors and cytokines such as epidermal growth factor (EGF) and TNF-α, were shown to induce substantive increase in glucose utilization of various cancer cells (Kaplan, O., *et al.*, 1990 and Kaplan, O. *et al.*, 1997). Such increase in glucose utilization requires an increase in supply of oxygen.

A specific growth factor, Hepatocyte Growth Factor/Scattering Factor (HGF/SF) and its receptor Met (tyrosine kinase) have been shown to be involved in normal mammary development as well as in tumor development and progression (Rong. S. *et al.*, 1994). Specifically, Met and HGF/SF have been shown to be involved in breast cancer (Nagy J. *et al.*, 1995 and Ghoussoub, R.A.D. *et al.*, 1998). More than 20% of primary breast tumors show strong expression of the Met receptor and several studies have shown that Met is highly expressed on metastatic tumors. Met-HGF/SF signaling and Met amplification, over expression or activation have been associated with a variety of additional human cancers including sarcomas, brain tumors, renal carcinomas, ovary carcinoma, prostate cancers and thyroid cancers.

The effect of the Met receptor and its ligand on tumor growth was shown to be by increasing division rate of the tumorgenic cells and/or by increasing angiogenesis of the tumor (Rosen, E.M., *et al.,* 1995 and Tolnay E., *et al.,* 1998). HGF/SF was also shown to decrease mean arterial pressure and to increase the heart rate in conscious instrumented rats (Yang *et al.,* 1997).

SUMMARY OF THE INVENTION

In accordance with the present invention, it was surprisingly shown that injection of an agent which activates specific receptors expressed by tumor cells in a tumor to an individual having such a tumor, enables to detect the presence of such tumor cells in the individual using imaging methods. The activation of the tumor cells results in a substantive *in* vivo increase of oxygen consumption by active tumor cells. The increase in oxygen consumption leads to an increase in blood flow to the tumor which, in itself is detectable by imaging methods such as Doppler ultrasound. In addition, the increase in blood flow results in increased oxygenation in the area of the tumor cells which is detectable by imaging methods such as functional MRI(fMRI). The blood flow and blood oxygenation will be, at times, referred to as *"blood hemodynamics"*. The detection of tumor cells in an individual according to the invention is thus based on the specific physiological reaction of the tumor to the administered agent and, for the first time, detection of the changes in the tumor by imaging techniques. Specifically, it was shown that injection of Hepatocyte Growth Factor/Scattering Factor (HGF/SF) to mice bearing the DA3 mammary carcinoma tumor, resulted in *in* vivo enhancement of BOLD images and changes in Doppler ultrasound flow measurements in regions comprising cells which express high levels of the HGF/SF receptor. The changes in blood hemodynamics is specific to those areas in which there are cells expressing high levels of the receptor which the injected agent activates. Other areas in which the specific receptor was expressed at a low level or not expressed at all did not show such changes in blood hemodynamics. Thus, as opposed to agents and methods which non-specifically enhance blood flow to tumors by vasodilatation mechanisms, the method of the present invention enables to change the blood hemodynamics specifically in regions in which there are cells which physiologically react to the activating agent in a manner which is detectable by imaging techniques.

It is to be noted that the differences in blood hemodynamics following administration of the agent may vary in different regions of the tumor. Typically, for example, there is an enhancement in the blood flow to the tumor cells in the tumor center and an increase in blood oxygenation (BOLD measurements) in this area of the tumor while there is a decrease in the level of blood flow in the tissue surrounding and adjacent to the tumor and at times in the cells present in the tumor periphery. A decrease in oxygenation levels in these peripheral regions is also typically found.

Thus, in accordance with the present invention, a relatively simple and short term method for detecting or monitoring tumor cells is provided. By using routinely used imaging techniques such as MRI and ultrasound techniques, the detection of tumor cells is carried out non-invasively by externally monitoring the level of oxygenation (measured as BOLD images) and blood flow (seen by Doppler ultrasound measurements) to the cells in a tested tissue of the individual. This is highly advantageous, since there is no need in invasively obtaining a specimen from the individual and the tests may be repeatedly carried out over various periods of time without subjecting the individual to undesired side effects and with minimal inconvenience to the patient.

The above findings showing *in vivo* detectable enhanced oxygenation and blood flow levels of activated tumor cells open the way for identification of regions having a high probability of containing tumor cells in an individual.

By a first embodiment of the invention the method is used to identify such regions in an individual that does not have a pre-known tumorigenic lesion. Such identification is based on comparing measured oxygenation and blood flow in a tested tissue before and after administration of an agent to the tested individual.

The term *"the level of oxygenation"* relates to the amount or concentration of oxygen in the blood reaching the cells in the tested tissue. Changes in the level of oxygenation result from changes in the level of oxygen consumption of the cells. In accordance with the invention, the oxygenation level is detected by any of the known imaging methods such as functional magnetic resonance imaging (fMRI)

using any of the devices routinely used in these methods and is measured as alterations in BOLD imaging signals.

Where the level of oxygen consumption of the tumor cells is measured by functional MRI experiments typically, in each experiment, a series of a number of images of the tested tissue are obtained before administration of the activating agent to the individual, which are used as a base line. Following injection of the activating agent, an additional series of BOLD images are obtained and the results are typically analyzed by one of the following ways:

(i) by ROI analysis. A region of interest (ROI) is defined and a time lapse analysis is carried out by using appropriate computer programs such as an "*Omnipro*" computer or "*Matlab*" programming. These programs provide a graph which describes the average signal intensity in the ROI at different time points.

(ii) by Pixel analysis – in this method, the percentage of changes in signal intensity (including increase and decrease) is calculated for each pixel in the image using two time intervals in the experiment. The analysis is carried out using an appropriate computer program such as, for example, "*Matlab*" programming. A base line is calculated using an average value measured over a period of several minutes before injection of the activating agent (and possibily following injection of a control agent such as e.g. saline), an average value of the peak region is calculated on the basis of measurements over several minutes following a period of time after administration of the activating agent. The following formula is used for this analysis:

Average value based on measurements following administration of activating agent - average value based on measurements following administration of control agent / average value based on measurements following administration of control agent x 100.

Such an analysis is carried out for each individual pixel and a map of the percentage of changes of the signal following administration of the activating agent is then obtained. The map may be converted to a colored look-up table.

The term *"blood flow"* relates to the amount of blood flowing into the close vicinity of the tumor cells and to the tumor cells themselves in the blood vessels surrounding them. In certain areas around the tumor the change following administration of the agent in accordance with the invention may be elevation of the blood flow to the tumor, while in other areas of the tumor or its surrounding the effect of the agent administered may be a reduction in the blood flow. The blood flow may be measured by Doppler ultrasound imaging which measures volume and velocity of the blood flowing to the cells. This technique is especially suitable for screening of a large number of regions of an individual, and in wide-scale screening of a population.

Where the blood flow is measured in order to detect a region of a tested individual having a high probability of comprising tumor cells, an average value is calculated for detected regions based on measurements over several minutes following a period of time after administration of the activating agent as compared to measurements over the same period of time following administration of a control agent.

Thus, in accordance with a first diagnostic aspect of the present invention, a method is provided for identifying regions having a high probability of containing tumor cells in a tested tissue of an individual, said method comprising the steps of:

(i) measuring the level of oxygenation and/or blood flow in a tested tissue of a tested individual;

(ii) administering to said individual an agent which enhances oxygen consumption and/or blood flow of said tumor cells;

(iii) measuring the level of oxygenation and/or blood flow of said tested tissue; and

(iv) comparing the level of oxygenation and/or blood flow measured in (i) to the level of oxygen consumption and/or blood flow, respectively measured in (iii);

wherein a level of oxygenation and/or in blood flow in said tissue measured in (iii) being substantively higher than the level of oxygenation and/or blood

flow measured in (i) in the tested tissue, indicating a high probability that said tested tissue contains tumor cells.

In accordance with one embodiment of this aspect of the invention, the level of oxygenation and/or blood flow which is measured before administration of the agent, may be measured following administration of a control agent.

The *"control agent"* may be any physiologically tolerant agent which has no influence on blood oxygenation or blood flow in the treated individual such as, for example, saline.

The *"agent"* used in accordance with the invention may be any substance which, when coming in contact with tumor cells, results eventually in enhancement of the tumor cells oxygen consumption that will result in higher oxygenation and in a change in the blood flow to the tumor cells. To test if an agent falls within the scope of this definition, the increase in oxygen consumption of cells following their contact with the tested agent *in vitro* may be measured by any of the known *in vitro* methods. Typically, oxygen consumption can be measured *in vitro* by perfusion experiments, in which the oxygen content in the perfusion medium is compared to the oxygen content in the effluent medium of the cells (see Examples). Such an agent may, for example, be a ligand of a receptor expressed by the cells, an antibody directed against such receptors or other components of the cells, as well as derivatives and analogues of such substances which maintain their ability to cause enhanced oxygen consumption in tumor cells. In addition, it is possible to test the effect of such an agent *in vivo* by measuring changes in BOLD images and Doppler ultrasound images in a tissue after administration of the agent *in vivo*.

It is to be noted that the amount of the activating agent necessary for obtaining the desired enhancement in oxygenation and/or blood flow level to the tumor cells which may be required to enable detection by any of the imaging techniques mentioned above, is a very low amount which is below the amount of activating agent which may cause a long-term activation effect in the tumor. The amount of the agent is typically close to the physiological level of said agent,

which, for example, in the case of HGF/SF is an amount similar to that which is freed into the bloodstream in an individual having a typical cut or injury.

The administered agent may be administered to the individual by any of the known administration modes, such as intravenously (i.v.), per os, subcutaneously (s.c.), interperitoneally (i.p.) locally, etc. Typically, the agent is adminstered i.v. and the oxygenation level of the tested tissue is then measured at various time intervals following its injection.

The term "*substantively higher*" should be understood to mean a level of oxygenation of the tested tissue following administration of the agent which is statistically significantly higher than the level of the oxygenation of the same tested tissue before administration of the agent to the individual.

Typically, in order to determine if the level of oxygenation and/or blood flow to the tumor cells measured following activation with the activation agent is substantively different than the level of the same parameters before activation, a cut-off value is predetermined on the basis of a large number of measurements of these parameters prior to and following administration of the activating agent in non-malignant tissue. These measurements may be accumulated at a database which may be updated from time to time on the basis of accumulation of additional measurements. The cut-off value is expressed as a percentage of enhancement or reduction of the signal following administration of the activating agent. The measured value of the tested tissue is calculated as described above, and its value is then analyzed in comparison to the predetermined cut-off value. The cut-off value is determined for a specific set of parameters (e.g. the type of activating agent and/or kind of imaging technique used) and can be used as a basis of comparison to the calculated value of the tested tissue under the same parameters. Wherein the value of the calculated index of the tested tissue differs from the predetermined cut-off value, there is a high probability that the tested tissue contains tumor cells. In certain cases, the predetermined cut-off value may be a range of values having a low cut-off value and a high cut-off value, to which the calculated value of the tested tissue is compared.

In accordance with one embodiment of this aspect of the invention, the enhanced oxygen consumption of tumor cells and/or enhanced blood flow to the tumor cells following administration of said agent to the tested individual is a result of activation of said tumor cells. Such activation may be a result of binding of a specific ligand to its receptor which initiates a signal pathway in the cells resulting in their activation. Alternatively, activation of the cells may be as a result of their contact with any other substance which activates the cells by any cellular pathway. The term *"activation"* of cells renders any change that occurs within the cells as compared to their inactivated condition including metabolic changes, over-expression of a DNA sequence, or a protein activation of signal transduction pathways, effecting ion channels, etc. Such activation is manifested in enhanced oxygen consumption resulting in enhanced oxygenation, alteration of the BOLD signal and/or a change in the blood flow level of the tumor cells which is measured by any of the methods mentioned above. In accordance with an additional embodiment of this aspect of the invention, the activation of the tumor cells is obtained by activation of a specific receptor expressed by the cells. A typical example of such receptors is various growth factor receptors expressed on the membrane of tumor cells. The activation of the receptor may be direct or indirect activation. Direct activation is a result of contact of a specific ligand or its derivatives or any other substance which is capable of specific binding to the receptor (e.g. a specific ligand or antibody) resulting in activation of the receptor. Indirect activation of a receptor may be caused by any substance which does not specifically bind to the receptor, but its contact with the tumor cells results, eventually, in activation of said receptor. Activating agents may also be substances which activate such receptors indirectly by elevating the level of receptor ligands in the blood which, in turn, activate one or more receptors.

In some cases, the tumor cells may express more than one receptor in which case several receptors may be activated one at a time or several at a time. The activation of more than one receptors may be obtained by administration of several different agents, each activating (directly or indirectly) one or more receptors or,

alternatively, by administering an agent which activates more than one of the receptors. Combined or sequential activation of more than one receptor on the tumor cells may enhance the signal detected by the various imaging techniques.

Various tyrosine kinase receptors such as the Met, erbII and others are expressed on breast cancer tumors as well as other kinds of tumors at a higher level than their expression on normal cells. In accordance with an additional embodiment of the invention, the tumor cells present in a tested tissue may be activated by activation of such tyrosine kinase receptors by an agent which directly or indirectly activates the receptors in a manner which is manifested in enhanced oxyge consumption of the activated cells, which may be detected by an imaging technique.

As mentioned above, in accordance with the invention, more than one kind of tyrosine kinase receptor may be activated on the tumor cells either by a single administered agent or by a combination of several agents and the combined activation of several tyrosine kinase receptors may further enhance the oxygen consumption of the tumor cells and as a result increase the signal detected by the imaging technique.

The Met tyrosine kinase growth factor receptor and its ligand HGF/SF have been shown to be over-expressed in breast tumors. In addition, Met expression has been shown to be upregulated in metastatic breast tumors, as compared to the primary breast tumor. Moreover, Met-HGF/signalling and Met amplification, over expression or activation by point mutation have been shown to be associated with a variety of human cancers including sarcomas, brain tumors, renal carcinoma, ovary carcinoma, prostate cancer and thyroid cancer. Being over expressed in primary metastatic tumors, the Met receptor is a good candidate for being activated by an agent in a manner which can be selectively detected by imaging techniques in accordance with the invention.

In accordance with a second diagnostic aspect of the invention, the method of the invention is used for detecting regions having a high probability of comprising tumor cells in a pre-suspected lesion of an individual. Such a suspected

lesion may be a region in which there is a high probability of existence of primary tumor cells based on analysis by routinely used methods such as histological methods, radioimmunological methods, etc. Such a suspected region may also be in an individual having a high risk of developing a primary tumor due, for example, to hereditary risk factors, exposure to carcinogenic agents, etc. In addition, such a lesion may be a region in which there is high probability of existence of a secondary metastatic tumor such as in an individual who had a preliminary tumor which was treated or irradicated by routine methods such as irradiation, chemotherapy or surgery. In accordance with this aspect, areas which are considered "*non-malignant*" areas (in which there is a very low probability of the existence of malignant cells) are determined and the suspected lesion is compared to these areas. The level of blood oxygenation and blood flow is compared between the suspected lesion and the non-malignant lesion before administration of the agent in accordance with the invention, and the two areas are compared once more following administration of the agent to the tested individual. Thus, the enhanced blood oxygenation and difference in blood flow following administration of the agent in the suspected lesion is compared to the increase in blood oxygenation and/or blood flow following administration of the agent in the non-malignant areas of the same individual. When the method measures the increase in blood oxygenation levels, this will typically be carried out by fMRI. When the blood flow to the tumor cells is measured, this will typically be carried out by Doppler ultrasound imaging.

Thus, in accordance with one embodiment of this aspect of the invention, a method is provided for detection of a region with a high probability of comprising tumor cells in a suspected tissue of an individual comprising:

(i) measuring the level of blood oxygenation in said suspected tested tissue;

(ii) measuring the level of blood oxygenation in a non-malignant tested tissue of said individual;

(iii) administering to said individual an agent which enhances the level of blood oxygen consumption of said tumor cells, thus enhancing blood oxygenation;

(iv) measuring the level of blood oxygenation in said suspected tested tissue;

(v) measuring the level of blood oxygenation in said non-malignant tissue;

(vi) comparing the level of blood oxygenation measured in (iv) to the level of blood oxygenation measured in (i) and calculating a ratio (a).;

(vii) comparing the level of blood oxygenation measured in (v) to the level of blood oxygenation measured in (ii) and calculating a ratio (b); and

(viii) comparing the calculated ratio (a) to the calculated ratio (b); a ratio (a) higher than a ratio (b) indicating a high probability of the existence of tumor cells in said suspected tissue.

In accordance with this embodiment of the invention, a method is further provided for detection of a region with a high probability of comprising tumor cells in a suspected tissue of an individual comprising:

(i) measuring the level of blood flow in said suspected tested tissue;

(ii) measuring the level of blood flow in a non-malignant tested tissue of said individual;

(iii) administering to said individual an agent which enhances the level of blood flow of said tumor cells;

(iv) measuring the level of blood flow in said suspected tested tissue;

(v) measuring the level of blood flow in said non-malignant tissue;

(vi) comparing the level of blood flow measured in (iv) to the level of blood flow measured in (i) and calculating a ratio (c).;

(vii) comparing the level of blood flow measured in (v) to the level of blood flow measured in (ii) and calculating a ratio (d); and

(viii) comparing the calculated ratio (c) to the calculated ratio (d); a ratio (d) different than a ratio (d) indicating a high probability of the existence of tumor cells in said suspected tissue.

In accordance with a preferred embodiment of the invention, the values measured in the malignant and non-malignant tissues are analyzed with respect to a cut-off value or range of cut-off values based on measurements of the non-malignant tissue as described above. A large number of measurements of the

enhancement of blood oxygenation or the changes in blood flow in the non-malignant tissues may serve as a basis for calculating such a cut-off value. A ratio is calculated on the basis of the measured values of each of these parameters before and after administration of the agent in accordance with the invention in the suspected area are than compared to the predetermined cut-off value or values. A calculated ratio of oxygenation which is higher than the cut-off value calculated for this parameter or a calculated ration of blood flow which differs from the cut-off value calculated for this parameter indicates a high probability that tumor cells exist in the suspected lesion.

As mentioned in the previous aspect of the invention, in this aspect as well, the enhancement in oxygen consumption of the tumor cells and thus the enhancement in blood oxygenation of the tumor in the suspected tissue may be as a result of activation of the tumor cells by the administered agent. Such activation may be as a result of activation of a receptor expressed by the tumor cells activated by an agent which activates a receptor directly or indirectly. By a preferred embodiment, such a receptor is a tyrosine kinase receptor and the agent in the composition of the invention will be an agent which is capable of activating tyrosine kinase receptors directly or indirectly. Such an agent may, for example, be HGF/SF, Her2/ neu ligand, EGF etc. In accordance with the invention, the tumor cells may at times be actived by more than one agent. Thus for example, two different ligands may be used, each activating a different receptor on the cells.

By a preferred embodiment, the tyrosine kinase receptor is a Met receptor. In this case, the agent in the composition will be any agent which directly or indirectly activates the Met receptor. Direct activators may be the ligand HGF/SF and derivatives and analogues of HGF/SF which specifically bind to the receptor and activate it. Indirect activators may, for example, be substances such as Heparin which administration results in an increase in the level of HGF/SF and eventually in the activation of the Met receptor and the cells expressing it. Other examples of agents which activate the Met receptor

and may be used in accordance with the invention are specific ligands and antibodies which bind and activate the Met receptor.

By a preferred embodiment of this aspect of the invention, the tumor cells are metastatic breast cancer tumor cells. In accordance with the present typical practice, following detection and removal of a primary breast tumor, the patient undergoes an additional operation during which lymph nodes are removed and checked for the existence of metastatic tumor cells. This procedure, which is very important for determination of disease staging, is very difficult for the patient to recover from and also only provides sample information from the several lymph nodes which were removed. In accordance with this aspect of the invention, the existence of areas having a high probability of comprising metastatic breast tumor cells in all the regional lymph nodes of such a patient may easily be detected without the need for further operating or hospitalization. Additional distal metastasis may also be discovered using this method. Since in the lymph node the non-malignant cells express very low levels of the Met protein, it is especially advantageous to use the method in accordance with the invention, wherein an agent which activates the Met receptor in the tumor cells is used.

In addition, in accordance with this aspect of the invention, the method is used in an individual following removal of a primary tumor and during chemotherapy or radiation treatment. In such patients, secondary metastic tumors are usually not removed, but are expected to shrink and disappear following such treatment. In accordance with this aspect of the invention, the method of the invention may be used at various times during the administration of such treatment to monitor the existence of secondary metastatic cells in such an individual and/or the response of such cells to the treatment.

Furthermore, where the tested individual is an individual suffering from breast cancer, the method of the invention may be used as a confirmation procedure of currently used diagnostic methods such as mammography.

As mentioned above, the detection of areas in a lesion having high probability of comprising mammary tumor cells may also be based on activation of

additional tyrosine kinase receptors other than the Met receptors such as the erb II receptor which is found to be over-expressed in about 20%-30% of breast cancer patients.

In accordance with an additional aspect of the invention, compositions are provided for use in any of the above methods of the invention. Such compositions comprise as an active ingredient an effective amount of at least one agent which enhances the oxygen consumption of tumor cells present in the tested tissue. Wherein the composition is used for a method in which the agent administered to the individual, is such which can activate said tumor cells, the agent/s in the composition will be agents capable of activating tumor cells. Wherein the composition is used for a method in which the activated tumor cells express a certain receptor, the agents in the composition will be such which can activate said receptor directly or indirectly on the tumor cell.

Such compositions will also comprise a pharmaceutically acceptable carrier which may be any of the carriers known in the art. Such carriers may be, for example, soluble physiologically acceptable carriers such as saline, PBS, etc. or solid state carriers such as, for example, latex beads. In some cases, the composition may also comprise additional non-active substances such as diluents.

By yet an additional aspect of the invention, kits are provided for carrying out any of the above methods. Such a kit will typically comprise at least one agent which enhances the oxygen consumption of the tumor cells to be detected, at least one predetermined cut-off value, optionally an algorithm for calculating a diagnostic index and instructions for use. By one embodiment, said kit may contain means for being connected to a computerized database comprising a collection of measured values and cut-off values which are periodically updated.

In addition to the above diagnostic applications, the present invention provides also the following therapeutic aspects.

In accordance with the invention, it has been shown that *in vitro* activation of perfused mammary adenocarcinoma cells by the HGF/SF ligand induced modifications in the glucose metabolism and oxygen consumption of the cells. The

metabolic changes and mainly the increased oxygen consumption of the cells was detectable using NMR measurements and various biochemical assays. These findings led to the realization that the change of oxygen consumption of tumor cells *in* vitro following various treatments may be used as a basis for studying susceptibility of such tumors to various treatments and drugs. Indeed, in accordance with the invention, it has also been found that anti-cancer drugs enhance the oxygen consumption of tumor cells *in* vitro.

In accordance with this embodiment, the present invention thus provides a method for testing the sensitivity of tumor cells to a specific therapeutic drug or treatment comprising:

(i) obtaining tumor cells from said individual and growing said cells *in vitro;*

(ii) measuring the level of oxygen consumption of said tumor cells *in vitro*;

(iii) treating said tumor cells with the tested anti-tumorigenic treatment or drug;

(iv) measuring the level of oxygen consumption in said tumor cells following treatment with said anti-tumorigenic treatment or drug;

wherein a higher level of oxygen consumption in (iv) compared to the level of oxygen consumption in (i) indicating sensitivity of the tumor cells to the tested anti-tumorigenic treatment or drug.

Furthermore, in accordance with the invention it was realized that the change in blood oxygenation or blood flow of tumor cells which is detectable by imaging techniques may enable testing susceptibility of the tumor in an individual to an intended chemotherapeutic drug or to a potential anti-tumorigenic treatment. For this purpose, the individual may be treated with a low dose of the intended drug, and the change in blood flow to the tumor cells and/or the enhancement in blood oxygenation levels of the tumor in an individual may be analyzed by appropriate imaging techniques. This pre-treatment testing will enable to prevent non-efficient chemotherapeutic treatments and the side effects associated with such treatments and will enable to treat the individual with a chemotherapeutic drug to which the specific tumor in the individual is most sensitive.

Thus the invention further provides a method for testing the sensitivity of a tumor in an individual to a tested anti-tumorigenic treatment or drug comprising:

(i) measuring the level of blood oxygenation of said tumor and/or of blood flow to said tumor in said individual;

(ii) administering a low dose of said tested anti-tumorigenic treatment or drug to said individual;

(iii) measuring the level of blood oxygenation of the tumor and/or of blood flow to the tumor in said individual;

wherein a higher level of measured blood oxygenation and/or blood flow in (iii) as compared to the level of measured oxygen in (i) indicating susceptibility of said tumor to the tested tumorigenic treatment or drug.

The term "*low dose*" is to be understood as a dose of anti-tumorigenic treatment or drug which is sufficient to enhance the hemodynamics of the tumor and which is not higher than the dose intended for treatment of the tested individual. The dose to be used may be determined by a person versed in the art relatively easily, for example, by carrying out several preliminary experiments *in vitro*, in which the target tumor cells are contacted with various doses of the intended antitumorgenic treatment or drug.

As mentioned with regards to the previous aspect of the invention, in accordance with this embodiment as well, a cut-off value or range of cut-off values may be determined on the basis of preliminary experiments in which the intended anti-tumorigenic treatment or drug is contacted with tumor cells which are not susceptible to said drug or treatment. The measured levels of oxygen consumption of the tumor cells *in vitro*, or of the hemodynamics of the tumor cells *in vivo* before and after administration of the tested anti-tumorigenic treatment or drug may then be compared to the cut-off values, wherein measured levels substantially differing from the cut-off values indicate sensitivity of the tumor cells to the tested anti-tumorigenic treatment or drug.

In accordance with an additional embodiment of the invention, it has been realized that the method of the invention is especially suitable for screening for

susceptibility to inhibitors of receptors which are intended to be used as therapeutic drugs. Such receptor inhibitors may, for example, be small molecules, peptides or antibodies which inhibit the expression or activity of such receptors, antibodies directed against such receptors, etc. The common method today to screen patients for susceptibility to such inhibitors is by measuring expression levels of the receptors themselves in biopsies obtained from the individual to be treated containing target cells expressing the receptors against which the inhibitors are intended. However, this method is not applicable in many cases such as, for example, wherein the target cells are metastatic tumor cells, and the results of this method are very often not sufficiently indicative. In such cases, patients are treated for a long period of time with a drug which has no effect on the target cells before it is realized that the treatment is non-effective and that alternative treatment should be searched for.

In accordance with the present invention, in order to evaluate whether an intended drug which is an inhibitor of a receptor may be effective in a patient, first a ligand of the receptor is administered to the individual and the effect of the ligand on the oxygen consumption of the cells expressing the receptor is tested by fMRI and Doppler ultrasound imaging as described above. In case the administered ligand does not change the oxygen consumption of the cells, it is then most likely that it will not be effective to use an inhibitor of the receptor to which the ligand binds as a therapeutic treatment in the tested individual. However, if administration of the ligand of the receptor results in changes in the oxygen consumption of the target cells, the intended inhibitor drug is then administered to the individual in conjunction with the activating ligand and the effect of the inhibitor on the level of oxygen consumption of the target cells by the ligand is measured again using the above-mentioned methods. Wherein the ligand itself resulted in reduction of the oxygen consumption of the cells, an inhibitor which will elevate the oxygen consumption of the cells to the normal level will be regarded as a potentially effective drug. Wherein the ligand itself enhanced the oxygen consumption of the target cells, an inhibitor which will reduce the oxygen consumption of the target

cells to the normal level will be regarded as a potentially effective drug. In other words, a receptor inhibitor which has a good chance of being effective in the treatment of the tested individual is one which has a contrasting effect on the oxygen consumption of the target cells as compared to the effect of the ligand on the same target cells. Any receptor inhibitors may be evaluated by this method such as, for example, tyrosine kinase receptor inhibitors such as inhibitors of EGF receptor, Her2 receptor (the target of Herceptine), inhibitors or hormone receptors such as estrogen receptors, etc. By a preferred embodiment, the tested inhibitor will be a Met receptor inhibitor.

The above method is suitable for testing receptor inhibitors of receptors which are involved in a wide scope of disorders and diseases. By a preferred embodiment, the receptor inhibitors are such which are intended to be used as anti-cancer drugs.

Thus, in accordance with this aspect of the invention, a method is provided for screening for inhibitors of receptors having potential in the treatment of disorders and diseases involving target cells expressing receptors inhibited by said tested inhibitor comprising:

(i) measuring the level of blood oxygenation of the cells expressing said receptor and/or blood flow to said cells in said individual;

(ii) administering a ligand capable of binding to said receptor to said individual;

(iii) measuring the level of blood oxygenation of said cells and/or of blood flow to said cells in said individual;

(iv) wherein there is no substantial difference in the measurements of (i) and (iii) above, said tested receptor inhibitor is determined to be non-suitable for treatment of said individual and wherein the measurements in (iii) is substantially different than those in (i), then:

(v) repeating steps (i) – (iii) above and administrating said tested inhibitor together with the ligand and measuring the level of blood oxygenation and/or blood flow to said cells; and

(vi) wherein the measurement in (v) is substantially different than the measurement in (iii), said tested inhibitor is a potentially effective drug for use in said individual.

The term *"substantially different"* should be understood to mean a level which is statistically different than the control level as determined by known statistical methods.

By an additional embodiment, a method is provided for direct screening of a receptor inhibitor as a potentially effective drug. In such a case, the effect of the receptor inhibitor on the oxygen consumption of the target cells is measured and compared to the level of the blood consumption of the cells before their contact with the inhibitor. Wherein the level of blood consumption of these cells contacted with the receptor inhibitor is substantially different than the level of the oxygen consumption of the cells before their contact with the inhibitor, the tested inhibitor is most likely to be potentially effective as a drug.

The invention thus further provides a method for screening for inhibitors of receptors having a potential in the treatment of disorders and diseases involving target cells expressing receptors inhibited by said tested inhibitor comprising:

(i) measuring level of blood oxygenation of the cells expressing said receptor and/or of blood flow to said cells in said individual;

(ii) administering at the receptor inhibitor to said individual;

(iii) measuring the level of blood oxygenation of said cells and/or of blood flow to said cells in said individual;

(iv) wherein there is a substantial difference in the measurement of (iii) and (i) above, said tested receptor inhibitor may have a potential as a drug for the treatment of said disorders and diseases.

The present invention further provides a kit for evaluating the efficacy of inhibitors of receptors in the treatment of disorders or diseases involving target cells expressing receptors inhibited by said tested inhibitor comprising at least one ligand capable of binding to said receptor; at least one inhibitor of said receptor to

be tested, optionally predetermined cut-off values of the level of blood oxygenation and blood flow of said target cells and instructions for use.

In accordance with an additional embodiment of the therapeutic aspect of the invention, it has been realized that the enhanced oxygen consumption of tumor cells and blood flow to tumor cells following their activation in accordance with the methods of the invention is useful for increasing tumor permeability and thus susceptibility to various anti-tumorigenic treatments and drugs, such as chemotherapeutical drugs, antibodies and radiation. As mentioned above, in accordance with the invention, it has been shown that following administration of an activating agent to an individual having a tumor, there was a dramatic increase in the blood flow to the tumor cells in the tumor center, while there is a decrease in hemodynamic factors in the tissue surrounding the tumor and in its periphery. Thus, in accordance with this embodiment of the invention, the activating agent is administered to an individual as an auxiliary treatment in conjunction with the anti-tumorigenic treatment or drug. An activating agent may be administered to the individual prior to, simultaneously with, or following the administration of the anti-tumorigenic treatment to achieve the maximal enhancement of the therapeutic effect of the administered drug or therapy.

In accordance with this embodiment, the present invention provides an auxiliary composition for administration in conjunction with an anti-tumorigenic treatment administered to an individual for the treatment of a tumor, said composition comprising as an active ingredient an agent which enhances the oxygenation of said tumor cells and/or changes the blood flow to said tumor cells and a pharmaceutically acceptable carrier.

The invention further provides a method for enhancing the efficacy of an anti-tumorigenic treatment administered to an individual having a tumor comprising administering to said individual an effective amount of an agent which enhances the oxygenation of said tumor and/or changes the blood flow to said tumor cells in conjunction with said anti-tumorigenic treatment.

In accordance with this aspect of the invention, it was furthermore realized that the efficacy of irradiation treatment in an individual suffering from a tumor may be enhanced by administrating to the individual an activating agent which enhances the oxygenation of said tumor cells in conjunction with the irradiation treatment. Thus, the invention further provides a method for enhancing the efficacy of an anti-tumorigenic irradiation treatment administered to an individual having a tumor comprising administering to said individual an effective amount of an agent which enhances the oxygenation of said tumor in conjunction with said anti-tumorigenic irradiation treatment.

In accordance with the above embodiments, the term *"effective amount"* should be understood to mean an amount which when administered to the individual enhances the anti-tumorigenic effect of the drug, antibody or irradiation. The effective amount may readily be determined by a person versed in the art by preliminary measurements on tumor cells *in vitro* in conjunction with the intended anti-tumorigenic treatment as well as such measurements *in vivo*.

Moreover, in accordance with the invention it has also been realized that increasing tumor permeability by enhancing the oxygen consumption of the tumors and thus the blood flow to the tumor cells in accordance with the invention, enables to enhance the efficacy of chemical contrast agents used in conjunction with magnetic resonance imaging (MRI) or ultrasound.

A chemical contrast medium is a substance, which is introduced into the body to change the contrast between the tissues. MRI contrast agents (also called paramagnetic agents) are used to help provide a clear picture during MRI. Such agents are administered by injection into the vein before or during MRI to help diagnose problems or diseases of the brain or the spine, and to help diagnose problems in other parts of the body, such as the bones and joints, breast, liver, soft tissues, and uterus. A typical chemical contrast media is a complex of a paramagnetic metal ion such as gadolinium (Gd). However, many paramagnetic metal ions are toxic. To decrease their toxicity, these metal ions are typically

complexed with other molecules or ions to prevent them from complexing with molecules in the body. Other molecules are used as contrast agents in intrasound.

Following injection of a contrast agent, the concentration of the contrast agent increases in the tissues, and then starts to decrease as it is eliminated from the tissues. In general, a contrast enhancement is obtained by one tissue having a higher affinity or vascularity than another. Most tumors for example have a greater Gd uptake than the surrounding tissues, causing a larger T_1 signal.

Since the efficacy of these agents greatly depends on their penetration to the tumor, an efficient blood supply to the tumor will improve their action. In accordance with the invention it has been shown that the i.v. injection of HGF/SF or Heparin increases the blood flow specifically to the tumor. This led to the realization that when injected in conjunction with HGF/SF or Heparin the contrast agent will be delivered to the tumor more efficiently. This will increase the MRI or ultrasound signal difference between tumor and surrounding tissue, enabling a better sensitivity and specificity in tumor imaging.

The present invention thus provides a method for enhancing the efficacy of chemical contrast substances used during magnetic resonance imaging of cells (MRI) or ultrasound comprising administering said chemical contrasts medium in conjunction with an activating agent capable of enhancing the level of blood oxygenation of said cells and/or blood flow to the cells.

In accordance with a preferred embodiment, the above method is used for enhancing the efficacy of chemical contrast substances used during MRI or ultrasound of an individual having a tumor and said activating agent is an agent capable of enhancing the level of blood oxygenation of the tumor cells or blood flow to the tumor cells.

In addition, preferably, the activating agent is an agent which activates the tumor cells by activation of at least one cellular receptor expressed by the tumor cells such as tyrosine kinase receptors, an example being the Met tyrosine kinase receptor.

The present invention also provides an auxiliary composition for administration in conjunction with chemical contrast substances used during MRI or ultrasound of cells, said composition comprising as an active ingredient an agent which enhances the oxygenation of said cells and/or changes the blood flow to said cells and a pharmaceutically acceptable carrier.

In all the above-mentioned embodiments, the term *"in conjunction"* should be understood to mean administration of the agent either prior to, simultaneously to or following the said treatment being either the anti-tumorigenic chemotherapeutical drugs, the irradiation treatment or contrast agents.

EXAMPLES

The various aspects of the invention will now be illustrated by the following non-limiting examples with occasional reference to the attached figures.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a photograph showing a series of BOLD measurements obtained from BALB/c mice which were injected with DA3 into their mammary glands and later treated first with saline and then with HGS/SF. The measurements were taken in one minute intervals for 55 mins. The percentage of BOLD alteration was calculated comparing base line (average of 7 measurements after saline injection) to peak reaction (average of 7 measurements 30 mins after HGF/SF injection).

Fig. 1A shows a transverse BOLD image through the mouse.

Fig. 1B shows negative alteration of signal intensity level superimposed on the first BOLD image.

Fig. 1C shows positive alteration of signal intensity level superimposed on the first BOLD image. A substantive increase in blood oxygenation level is seen in the liver(1) where Met is highly expressed. The kidneys(2) and tumor(3) show similar levels of reaction to HGF/SF while lungs(4) show no reaction to HGF/SF.

Fig. 2 is a photograph showing differential alteration of blood oxygenation in the tumor as measured by BOLD images of mice treated as explained in Fig. 1

above. The percentage of BOLD alteration was calculated as described in Fig. 1 above.

Fig. 2A shows an axial BOLD image of the tumor.

Fig. 2B shows positive (+4% - +250% - green) and negative (-80% - -4% - red) alteration of signal intensity levels superimposed on the BOLD image.

Fig. 2C shows time lapse analysis of the elevation of the BOLD signal. X axis is time in seconds and Y axis is average values of signal intensity in ROI.

Fig. 2D shows a reduction of the BOLD signal intensity level superimposed on the BOLD image. The X and Y axes are like in Fig. C.

Fig. 3 is a photograph showing a series of ultrasound/Doppler measurements in 2-5 min. intervals for 20-30 mins. which were obtained from mice treated as explained in Fig. 1 above.

The measurements were taken before HGF/SF injection (upper row), after 3 mins. (2^{nd} row) and after 8, 15 and 20 mins. after HGF/SF injection (rows 3-5, respectively).

MATERIALS AND METHODS

In vitro experiments

Materials

All chemicals were purchased from Sigma Chemical (S. Louis, USA) unless otherwise indicated, and were of highest available purity. HGF/SF was produced as previously described (Rong, S. *et al.*, *Cell Growth Differ* **4**:563-9, 1993).

Tumor cells

D1-DMBA-3 is a cell line derived from a poorly differentiated mammary adenocarcinoma induced in BALB/C mice by dimethylbenzanthracene. Limiting dilution cloning produced the cell line designated DA3 (Fu, Y., *et al., Cancer Res.*, **50**:227-234, 1990). DA3 cells were grown in DMEM (GibcoBRL Gaithersburg, MD) supplemented with 10% heat - inactivated fetal calf serum

(FCS) (GibcoBRL, Gaithersburg, MD), penicillin-streptomycin-nystatine and L-glutamine, under 5% CO_2 environment. The growth medium contained 11 mM glucose. For the NMR experiments the cells were grown to approximately 90% confluency, harvested with 0.25% trypsin-0.05% EDTA, centrifuged at 4°C at 1000 x g for 5 min., and washed twice with medium.

Biological effects of HGF/SF: ruffling and scatter assay

To analyze the effect of HGF/SF on membrane ruffling a rapid CLSM time lapse photography of untreated and treated DA3 cells was performed. CLSM DIC images of untreated and HGF/SF-treated cells after 10 min and 10 min + 0.05 sec were acquired. Using the CLSM program we subtracted the 10 min images from the 10 min + 0.05 sec images. The differences between images were calculated and then areas where changes occurred were superimposed on the corresponding 10-min time image.

Scatter assay was carried out as previously described (Rosen, E., *et al., In vitro Cell Dev. Biol.*, **25**:163-73, 1993). Assay medium (DMEM with 5% calf serum, GIBCO) with or without HGF/SF was added in 150 µl to 96 well plates (Costar). Cells were added in 150 µl assay medium and incubated overnight. Cells were fixed, air dried, stained with Giemsa and examined for scattering (spreading and dispersion of epithelial colonies).

Perfusion method

Cell perfusion mandates attaching cells to a matrix to prevent washing with the effluent or clogging the filters. In these studies we used the method of cellular embedding in sodium alginate microcapsules (Narayan, K.S., *et al., NMR Biomed*, **3**23-6, 1990). This method is very suitable for NMR studies of normal and tumor human cells. $1.0\pm0.2 \times 10^8$ cells were used in each experiment. The cells were harvested as described above and the pellet was mixed with equal volume of 2.5% (w/v in PBS) sodium alginate. The mixture was manually extruded, under minimal pressure, through a 25-gauge needle, onto the surface of

0.1 M $CaCl_2$ solution. The small drops gelled into capsules which were immediately washed three times in growth medium. The capsules were isolated by decantation, transferred to a 10 mm screw cap NMR tube, and perfusion was promptly initiated. The capsules were not tightly packed in order to ensure optimal perfusion. Average procedure length was 15-20 min., and the time in $CaCl_2$ was kept below 5 min. The perfusion was performed through an insert with inlet and outlet tubing, and the volume of the perfusion chamber was 2 ml. The perfusion solution flowed from the opening of the inlet near the bottom of the tube through the packed alginate capsules, and the outflow was directed through opening in the insert to the outlet tubing. A constant flow of 0.9 ml/min. in a single pass mode was maintained by a peristaltic pump throughout all experiments, and the temperature was maintained at 37°C. The perfusion solution contained 5.5 mM of glucose, similar to glucose physiological concentration, unless otherwise indicated. In each experiment control perfusion with ^{31}P NMR recording was carried out for about 90 min., to ensure metabolic stability of the cells, before adding the HGF/SF to the perfusion solution.

Magnetic Resonance Imaging (MRI)

MRI experiments were performed on a 1.9T whole body MRI system (2T Prestige, Elscint) using small shoulder surface coil (14 cm in diameter) Gradient echo images were acquired with TR/TE 0/11 msec. Slice thickness of 2 mm, field of view 4 cm, 128 x 128 matrix size, i.e. in plane resolution of 300 mm.

Magnetic resonance spectroscopy and data analysis

NMR spectra were recorded on a Bruker ARX-500 spectrometer equipped with a quadro-nuclei software controlled probe. ^{31}P spectra were recorded at 202.46 MHz, with broad band proton decoupling. The temperature of the sample inside the magnet was maintained at 37°C using a thermocouple. Each spectrum was an accumulation of 400 scans, with 1.85 s repetition time and 45° flip angle. ^{31}P chemical shifts were determined by standardizing GPC to 0.49 ppm (Navon,

G., *et al., Proc. Natl. Acad. Sci.*, USA, 74:87-91, 1977). The spectra were analyzed on a SGI Data Station, and acquisition and processing parameters were identical throughout all experiments. Quantification was performed by measuring the heights of the peaks that had 20 Hz line broadening. Under these conditions there was no significant nuclear Overhauser effect. The intracellular pH was determined from the intracellular Pi peak. Although using phosphate-free medium would facilitate measuring this peak, we use phosphate containing medium in these experiments in order to keep the conditions as physiological as possible. Moreover, previously we have found continuous depletion of NTP when cells were perfused with phosphate-free medium (unpublished results). A reference, 50 mM preweighed tetrametaphosphate (TMP), within a sealed capillary in the perfusion tube, was used for quantitative analyses. ^{13}C NMR spectra were recorded in cells perfused with [$^{13}C_1$]-glucose-enriched medium, at 125.7 MHz, with proton decoupling. Each spectrum was an accumulation of 200 transients, with 1s repetition time and 45° flip angle. Quantification was performed by measuring relative integrals of the peaks. Peaks were assigned by standardizing glucose to 94.7 ppm. A reference, preweighed dioxane solution, within a capillary in the perfusion tube was used for quantitative analyses of ^{13}C-containing metabolites, taking into account the 1.1% natural abundance of ^{13}C nucleus.

Protein determination in the embedded cells

Cell number in each experiment is standardized for quantification of the results. Since substantial loss of cells might occur during alginate capsule preparation and initiation of perfusion, cell counts are not always precise. Therefore, protein level determination, rather than cell counts, was performed. The proteins were extracted by treating the cells (in the alginate capsules) with a high temperature base. Protein content was measured by the bicinchoninic acid assay, which is based on the reaction of protein with Cu^{+2}, and

spectrophotometric quantitation at 562nm. Absorbance measurements were performed with an ELISA reader (Elx808 BioTEC Instruments, Inc.)

Glucose consumption assays

$25x10^4$ cells were plated in 25-cm^2 flasks in 10 ml growth medium. When the cells reached 50, 5% confluency the medium was replaced by 11 mM glucose DMEM with 80u/ml of HGF/SF. 24 hours later samples of medium were taken for glucose measurements, and a cell count was performed in one flask. After 48 hours of HGF/SF stimulation glucose levels were measured again, and cell counts were performed in the remaining flasks. Glucose concentrations were determined by the hexokinase enzymatic assay, utilizing the coupled enzyme reaction catalysed by hexokinase and glucose-6-phosphate dehydrogenase, and measuring the product, NADH, at 340 nm. Cell counts were performed by trypsinization and suspension of the cells in trypan blue.

Oxygen consumption measurements

The cells were embedded in alginate capsules and were perfused as described above. Two three-way valves were inserted in the inflow and the effluent tubes, in close proximity to the perfusion chamber. After 90 min of perfusion for stabilization, baseline control samples were collected at 10 min intervals for an hour. Then, 400 u/ml HGF/SF were added to 50 ml of medium, which were perfused for 1 hour, followed by perfusion with medium for additional 120 min. At each time point samples were withdrawn from the inflow medium and the effluent. The samples were kept in closed syringes without air, and the measurements were promptly done with a Stat 1 Profile instrument (NOVA Biomedical) Oxygen consumption, in addition to pCO_2 and pH changes, were calculated from the difference between the measurements of the inflow medium and the effluent.

Functional imaging of mitochondria

Confocal laser-scanning microscopy was used to quantify the mitochondrial autofluorescence changes of NAD(P)H and flavoproteins in unfixed saponin-permeabilized DA3 cells. The changes were detected by ratio imaging of autofluorescence intensities of fluorescent flavoproteins and NAD(P)H as previously described (Kuznetsov, A.V., *et al.*, *J. Cell Biol.*, **140**:1091-9, 19__. The NAD(P)H signal was co-localized with MitoTracker™ Red mitochondrial marker (molecular probes, Or, USA). In brief, coverslip - 8 well Lab-Tek chamber slides (Nunc, Denmark) were seeded with 10^4 cells per well in medium containing 10% FCS for 24h. Cells were washed twice and incubated in measurement medium (consisting of 110 mM mannitol, 60 mM KCl, 10 mM KH2 PO4, 5 mM MgCl2, 0.5 mM Na2 EDTA, and 60 mM Tris-HCl, pH 7.4). Saponin treatment was performed by a 30-min incubation of the cells in relaxing solution-ethanesulfonic acid, 3 mM KH2 PO4 , 9.5 mM MgCl2 , 5 mM ATP, 15 mM phosphocreatine, pH 7.1) containing 50 mg/ml saponin as previously described. The cells were treated with 80u of HGF/SF for 0 to 20 min. The NAD(P)H fluorescence was excited at 325 nm using the CLSM UV laser. The flavoprotein fluorescence-was excited at 488 nm using a 75-mW argon ion laser (model OMI-532 AP; Omnichrome). The emission wavelengths 450 (NAD(12)H fluorescence) or 520 nm (flavoprotein fluorescence) were collected by the CLSM PMTs using a 410 Zeiss (Oberkochen, Germany) confocal laser scanning microscope (CLSM) with the following configuration: 25 mW HeNe lasers, Krypton Argon UV laser lines. For control of NAD(P)H elevation the cells were treated with 1mM octanoylcarnitine and 5 mM malate. For control of NAD(P)H down-regulation the cells were treated with 1 mM ADP.

To calculate the ratio between the green and red fluorescence we used the percentage positive area (PPA) image analysis procedure previously described. In short, PPA was calculated as a ratio between the positive stained area and the total cellular area. The positive stained area was determined by measuring the fluorescence intensity of the image, which is above the positive cutoff intensity.

Positive cutoff intensities were determined based on the fluorescence intensity histogram for each staining. Total cellular area was determined by measuring the fluorescence intensity above the surrounding background of the image, and depicts the cellular auto-fluorescence. The PPA calculation allows for the quantitative comparison of protein expression by cell populations or in tissue sections. The PPA data shown represents the calculated average of at least three different CLSM fields. Statistical significance was calculated using the Student T test in Microsoft Excel (Microsoft, Redmond, WA).

Images were printed using Codonics dye sublimation color printer. When comparing fluorescence intensity, we used identical parameters for each image (*e.g.*, scanning line, laser light, contrast brightness).

Statistics

Each series of NMR and oxygen consumption measurements was repeated at least three times, and the results are expressed as means SD of these experiments. Statistical analyses were performed with the paired, double-tailed, Student's t test (p 0.05).

***In vivo* experiments**

Tumor induction

Cells were trypsinized, washed twice and resuspended in PBS. $5x10^4$ cells in 0.2 ml PBS were injected into the mammary gland of Balb/c mice. Tumor formation was monitored twice weekly.

Anesthesia

Mice were injected with solution that contained 1mg/ml Xylazine and 10mg/ml Imalgene 1000. 100 μl per 10gr body weigh were injected, if needed another 50μl were injected after 1 hour. Catheter was inserted intra-peritoneal to the mice, so that additional injection was done without moving the mouse out of the MR devise.

Heparin injection

Heparin was injected i.v. to mice in doses of 6, 3, 0.3 or 0.003 units per mice (Intramed Heparin Sodium Injection – Pharmacare Limited). The doses of Heparin used in accordance with the invention are non-hemolytic doses.

MRI Experiments

Anesthetize mice were held on a surface with tape to prevent any movement during MRI measurements. Catheter was inserted IV in the mice tail, so that injection is done without moving the mouse out of the MR machine. 0.1 ml Saline was injected and 15 minutes later HGF/SF was injected. A series of BOLD measurements in intervals of about 1 minute started after the saline injection and proceed for about 55 min.

For general image of the mouse T1 weighted and T2 weighted measurements were done before injections.

Analysis of MRI experiments

A series of BOLD images was the result of each experiment. This series included about 10 images before HGF/SF injection that were used as base line, and about 40 images after HGF/SF injection. We analyzed these results in one of two ways:

1) ROI analysis – This analysis was done on "Omnipro" computer or "Matlab" programming. We defined an region of interest (ROI), and a time lapse analysis was done. The resulting graph described the average signal intensity in the ROI (Y axis)at the different time points (X axis).

2) Pixels analysis – This analysis was done using "Matlab" programming. We calculated percentage of changes in signal intensity (both increase and decrease), using two time intervals in the experiment. For base line we used the average value of 7 minutes after saline injection (before HGF/SF injection), and

for peak reaction we used 7 minutes about 30 minutes after HGF/SF injection. The following formula was used.

1. **Avg. HGF injection - Avg. saline injection X 100**
   Avg. saline injection

This analysis applied to each individual pixel and resulted in a map of the percentage of changes of the signal following HGF/SF injection. We converted this map to colored look – up table.

Ultra-sound experiments:

The mice injected with the DA3 cells were taken to ultra-sound experiments, there was no need for anesthesia. A series of Doppler measurements were taken before (for 10 minutes) and after (for 30 minutes) HGF/SF injection.

The measurements were performed in **ACUSON 128XP Computer Sonography System,** with **Art** 7.5 mHz probe using the Doppler modes.

RESULTS:

1. ***In vitro* tissue culture experiments**:

1.1 **Induction of Met phosphorylation and ruffling in DA3 cells**

Met expression in DA3 cells was determined by Western blot (WB) analysis with SP260 rabbit anti-peptide antibody. High levels of $p140^{met}$ were detected (Fig. 1A lane 1). This band was not evident in the presence of SP260 immunizing peptide (Fig. 1A lane 2), confirming the specificity of the antibody. The influence of HGF/SF on Met phosphorylation was determined by immunoprecipitation (IP) using SP260 followed by WB analysis using anti-phosphotyrosine antibody (anti-pTyr). Low levels of phosphorylated Met were detected in the untreated DA3 cells (Fig. 1A lane 3). A 5-minute exposure to HGF/SF increased Met phosphorylation (Fig 1A lane 4). Thus, Met is present and is rapidly phosphorylated in response to HGF/SF treatment.

Motility and invasiveness are characteristics of malignant cells. To analyze the effect of HGF/SF on membrane ruffling we compared rapid CLSM time lapse photography of untreated and treated DA3 cells. CLSM images of HGF/SF-treated and untreated cells after 10 min (Fig. 1A D - F) and 10 min + 0.05 sec were acquired (Fig. 1A A and C). In the HGF/SF treated cells extensive formation of motile cell surface protrusions was observed. No significant ruffling was observed in untreated cells (see arrow in fig. 1). We performed 10 experiments to determine number of cells exhibiting membrane ruffling for at least 50 cells per experiment. 95% of the HGF/SF-treated cells exhibit membrane ruffling while only 4% of the untreated cells exhibited ruffling.

After 24 h DA3 cells which were treated with HGF/SF displayed fibroblast-like "scattered" morphology (Fig 1B ?) as compared to the epithelial appearance of untreated cells (Fig 1B ?). This change in morphology is similar to that of HGF/SF-treated MDCK cells (the classical model for HGF/SF-induced cell motility exhibiting disruption and scattering of epithelial cell colonies. These results show that HGF/SF treatment induces rapid Met phosphorylation followed by rapid membrane ruffling and motility of the cell.

1.2 The effect of HGF/SF on glucose consumption of DA3 cells

The effect of HGF/SF on glucose consumption in tissue culture conditions was also examined. DA3 cells were plated in tissue culture flasks. When confluency was approximately 50% the growth medium was replaced with medium containing 2g/l glucose, with or without HGF/SF. 24 and 48 hours later samples from the medium were tested for glucose concentration using hexokinase enzymatic assay kit (Sigma) according to manufacturer's instructions, and cell number in one flask was counted using trypan blue. Glucose consumption levels after 48 hours are displayed as mg/dL/million cells. Consistent with the 13C NMR results, we found that HGF/SF significantly increased glucose consumption in the cultured DA3 cells, from 79.85 ± 12.2 to 126.0 ± 5.2 mg/dL/million cells ($P<0.0001$).

1.3 Effect of HGF/SF on oxygen consumption of DA3 cells

Cellular metabolism is usually associated with oxygen consumption and lactate, carbon dioxide and acids production. We measured HGF/SF induced alteration in those cellular metabolisms in a perfusion system.

Oxygen consumption was measured by comparing oxygen content between perfusion medium and effluent. Measurements were done in the present and absent of HGF/SF. We found that HGF/SF treatment increased oxygen consumption by 20.8% compared to control untreated cells. Based on the solubility of oxygen in aqueous solutions at 37°C, perfusion rate and protein content, oxygen consumption was determined to be 0.58 0.02 mole/hour/mg protein (control cells) and 0.71 0.03 mole/hour/mg protein (HGF/SF-stimulated cells ($p<0.05$).

The same measurements were performed for carbon dioxide production. Surprisingly, we found that HGF/SF treatment decreased carbon dioxide production by 26% (Table 1). In the control cells the pH decreased, the decrease was smaller in the presence of HGF/SF by 21.8%.

In order to evaluate the time scale of the above noted changes, two more experiments were carried out in which samples were collected at 2 min. intervals for the first 10 min after HGF/SF had entered the perfusion tube (Fig. 7). As can be seen these effects are very fast; they were apparent after 6- 8 min and after 10 min they nearly reached a steady state that was maintained over 2 hours after termination of HGF/SF perfusion.

1.4 Modifications in glucose metabolism using NMR measurements of perfuse DA3 cells

^{31}P NMR - ^{31}P NMR spectra of perfused DA3 murine mammary cancer cells are shown in Fig. 2. The peaks were assigned according to previously published data, including perchloric acid extraction studies (22, 24, 31). It should be noted that the effects of HGF/SF were assessed in the same sample from which the control spectrum was acquired. Perfusions with 5 μg HGF/SF induced

remarkable elevations of the PC and GPC signals compared to the control ($p<0.05$). Similar effects were also noted following treatment with a low HGF/SF concentration, but the magnitude of the signals was smaller (data not shown). HGF/SF stimulation was also associated with a small increase in the NTP peaks (ATP is a principal component of these signals), and had no effects on the intracellular pH.

2-Deoxyglucose is a metabolic inhibitor that competes with glucose on transport into the cells, and once entering cells is phosphorylated by hexokinase to 2-DG-6P which undergoes no further metabolism (10, 16). We used 2-DG as a "probe" to measure this initial step of glycolysis. The cells were perfused with DMEM (containing 5.5 mM glucose) into which 5 mM 2-DG were added, and the accumulation of 2-DG-6P was followed serially (Fig. 3A). Because 2-DG treatment causes energy deprivation, and the cells are no longer in homeostasis, it was necessary to perform the control and the HGF/SF stimulation experiments with different samples. 2-DG and HGF/SF had some effects on all the ^{31}P signals and a reference was mandatory. The signal of 2-DG-6P overlaps the PME peaks and measuring its integrals may be inaccurate. Therefore we used peak heights with line broadening of 20 Hz. The results were normalized according to the reference and protein contents. The accumulation of 2-DG-6P is shown in Fig. 3B. The rates of the phosphorylation reaction were calculated using the equation: Initial rate = $\ln 2 \times I_\infty / t_{1/2}$, where I_∞ is the magnitude of the final 2-DG-6P accumulation, and $t_{1/2}$ is the time that was taken to reach one-half of this magnitude. The I_∞ in three different control experiments was $92.2 \pm 10.5 \times 10^{-3}$ normalized integral/mg protein, and following HGF/SF administration it was $125.3 \pm 9.8 \times 10^{-3}$ normalized integral/mg protein in three identical experiments ($p< 0.05$). HGF/SF significantly increased 2-DG phosphorylation rate, from $2.2 \pm 0.4 \times 10^{-3}$ normalized integral/mg protein/min in the control perfusions to $3.5 \pm 0.3 \times 10^{-3}$ normalized integral/mg protein/min after HGF/SF stimulation ($p<0.05$).

^{13}C NMR - The effects of HGF/SF on cellular glucose uptake and lactate production were studied by continuous ^{13}C NMR measurements. The following protocol was used: cells were perfused with [$^{13}C_1$]-glucose enriched medium and control ^{13}C spectra were continuously recorded (Fig. 4a). After a complete washout for 60 min with medium, HGF/SF was perfused for 60 min. A second series of [$^{13}C_1$]-glucose enriched medium was then recorded (Fig. 4b). Thus, in each experiment the measurements of control and HGF/SF effects were performed in the same sample. It is noteworthy that in 20-25 min both glucose and lactate reached plateau levels that represent a steady state of glucose utilization and glycolysis.

In order to quantify cellular glucose utilization, its concentration in the perfusion chamber should be determined. Glucose is continuously transported into the cells, and once inside them it immediately undergoes phosphorylation. Therefore calculation based only on its concentration in the perfusion solution, and on the volume of the cells and capsules may lead to erroneous results. Indeed, glucose NMR-detected levels at steady state are lower than the results obtained based on these estimations. In order to solve this problem we used the glucose transport inhibitor phloretin dissolved in DMSO (Fig 4C). The final concentration of the DMSO in the perfusion solution was 0.2%. We have previously shown that DMSO at this concentration had no effects on cellular viability or on NMR spectra. Addition of phloretin to the perfusion solution increased glucose levels by 47.7±6.1% compared to controls of the same samples (Fig. 5A). In cells perfused with [$^{13}C_1$]-glucose-enriched medium and phloretin there was no lactate signal (Fig. 5B), indicating that phloretin completely inhibited glycolysis. The amount of lactate formed in the control perfusions was 70.2±8.6% of the increase of the glucose in the phloretin perfusions. Thus, 70% of glucose metabolism is in the glycolytic pathway in which lactate is the end product- this is the glycolytic pathway portion of the entire glucose metabolism of DA3 cells. The kinetic

studies demonstrated significant effects of HGF/SF on glucose uptake and lactate accumulation; it reduced the steady state levels of glucose and elevated those of lactate, compared to the control measurements (Fig. 5). Thus, lactate signals increased from 0.66±0.05 normalized integrals/mg protein in the control perfusions to 0.79±0.06 normalized integrals/mg protein following HGF/SF ($p<0.05$). ^{13}C NMR of the effluent, which was collected after perfusion, showed similar pattern, i.e. higher levels of lactate and lower levels of glucose following HGF/SF treatment compared to the control perfusions (data not shown). The differences in the lactate signals are therefore not a consequence of extracellular lactate transport inhibition, but reflect HGF/SF effects on its formation as the final product of glycolysis.

SUMMARY OF THE *IN VITRO* EXAMPLES

Activating Met signal transduction by HGF/SF induced rapid phosphorylation of the receptor followed by ruffling and scattering of the DA3 cells. Using NMR measurements of perfused DA3 cells at physiological conditions, and traditional biochemical assays, modifications in glucose metabolism and oxygen consumption during the same time frame were shown.

The principal changes, which were induced by HGF/SF, were increased glucose utilization and glycolysis, concomitant with increased oxygen consumption and decreased carbon dioxide production. The mechanism and pathways of these effects were delineated by CLSM which showed that HGF/SF activation also induced increased mitochondrial activity using NAD(P)H fluorescence that measures oxygenation of the mitochondrial NAD system.

2. ***In vivo* experiments in mice**:

2.1 Functional MRI scans before and after HGF/SF injection to DA3 bearing mice

DA3 cells were injected into the mammary glands of Balb/c mice as described above. The tumor bearing mice were injected IV with saline and 15 minutes later received an injection of HGF/SF. A series of BOLD measurements in 1 minute intervals for 55 minutes were obtained. Functional MRI scans, before and after HGF/SF injection, of coronal sections of a mouse that included longitudinal section of the tumor as well as liver, lungs and kidneys were obtained and can be seen in Fig. 1a. Analysis of the pixel activation was calculated as the percentage of BOLD alteration comparing base line (average of 7 measurements after saline injection), to peak reaction (average of 7 measurements 30 min. after HGF/SF injection). Analysis of BOLD signal revealed certain areas exhibiting decreased signal such as seen in Fig 1b. These areas were particularly evident in the areas that surrounded the tumor. The analysis of BOLD signal also revealed areas exhibiting increased signal such as those shown in Fig. 1c. The most significant elevation in the BOLD signal was shown in organs expressing high levels of Met such as the liver. Organs that express low levels of Met, such as lungs and muscles, did not exhibit significant elevation of BOLD signal following HGF/SF injection. In the kidneys, which express moderate levels of Met, BOLD activation levels resembled those of the tumor.

2.2 BOLD image, pixel activation and ROI analysis of the tumor

DA3 tumors obtained in Balb/c mice as described above, were analyzed at a higher resolution by focusing on the tumor. Analysis of the pixel activatin was carried out as described in 2.1 above. Fig 2 shows the BOLD image (Fig. 2a), pixel activation (Fig.2b) and ROI analysis (Fig. 2c-d) of the tumor. In areas primarily bordering the tumor, the signal dramatically decreases (red). On the other hand, areas within the tumor predominantly exhibit increased BOLD signal (green) (Fig.2b).

The ROI graphs display time course of BOLD signal in different regions of the tumor before and after HGF/SF injection. The X-axis denotes time in seconds, and the Y-axis shows normalized fMRI signal strength. Fig 2c describes time course derived from ROI that included most of the internal region of the tumor (green area). This ROI clearly demonstrates the increase in the BOLD signal typical of Met-expressing tumors, following injection of HGF/SF. Fig 2d shows ROI in the tumor margin (red region) in which the BOLD signal decreases.

2.3 Ultra-sound Doppler signal following HGF/SF injection in DA3 tumor bearing mice

Doppler analysis detects blood vessels and alteration in blood flow. In these experiments (Fig 3) we measured the changes in blood vessel appearance and hemodinamics in the tumor and adjacent areas. A large vessel surrounding the tumor near the skin was clearly visible before HGF/SF injection, whereas inside the tumor, blood vessels were sparsely detected (Fig. 3 - upper row). Following HGF/SF injection (Fig.3 middle and lower rows), the external vessel nearly disappeared while inside the tumor the number and size of vessels detected increased significantly. Furthermore, the regions connecting the tumor to the leg or to the abdomen appeared very clearly following HGF/SF injection. This alteration in blood hemodynamics continued for about 10 minutes, after which the surrounding vessels exhibited recovery to their previous size.

2.4 Functional MRI Scan before and after heparin injection to DA3 tumor bearing mice

fMRI analysis of tumors of the DA3 tumor bearing mice following injection of heparin were carried out as described above. The analysis revealed an increase in oxygenation levels of the tumor. The increase in oxygenation level was in the tumor core, while a decrease in oxygenation level was seen in the tumor margin. The percentage of increase in oxygenation level of the tumor were dose responsive being about 4% to 5% when 6 units (U) of heparin was administered to the mice, a

smaller increase when 3U were injected and an almost undetectable increase when 0.3 units were administered to the mice. In addition, as compared to the increase in oxygenation of the tumors in mice injected with HGF/SF, a delay is seen in the increase in oxygenation of the tumors following injection of heparin. Thus, an increase in oxygenation levels is fMRI BOLD measurements in tumors of mice receiving heparin administration. The kinetics of the increase in oxygenation levels of the tumor indicate that the effect caused by heparin is most likely mediated by HGF/SF.

SUMMARY OF *IN VIVO* EXAMPLES

The above *in vivo* results show that the internal region of the tumor reacts differently to HGF/SF injection than its marginal regions. In addition, these results clearly show that the enhanced requirements for oxygen in the tumor, following direct (e.g. HGF/SF) or indirect (E.G. Heparin) activation of the tumor cells via activation of receptors expressed by the cells can easily and effectively be monitored using routine imaging techniques such as fMRI and Ultrasound Doppler.

CLAIMS:

1. A method for identifying regions having a high probability of containing tumor cells in a tested tissue of an individual, said method comprising the steps of:

(i) measuring the level of oxygenation and/or blood flow in a tested tissue of a tested individual;

(ii) administering to said individual an agent which enhances oxygenation and/or blood flow of said tumor cells;

(iii) measuring the level of oxygenation and/or blood flow of said tested tissue; and

(iv) comparing the level of oxygenation and/or blood flow measured in (i) to the level of oxygen consumption and/or blood flow, respectively measured in (iii);

wherein a level of oxygenation and/or in blood flow in said tissue measured in (iii) being substantively higher than the level of oxygenation and/or blood flow measured in (i) in the tested tissue, indicating a high probability that said tested tissue contains tumor cells.

2. A method for identifying regions having a high probability of containing tumor cells in a tested tissue of an individual, said method comprising the steps of:

(i) measuring the level of oxygenation and/or blood flow in a tested tissue of a tested individual following administration of a control agent;

(ii) administering to said individual an agent which enhances oxygenation and/or blood flow of said tumor cells;

(iii) measuring the level of oxygenation and/or blood flow of said tested tissue; and

(iv) comparing the level of oxygenation and/or blood flow measured in (i) to the level of oxygen consumption and/or blood flow, respectively measured in (iii);

wherein a level of oxygenation in said tissue and/or in blood flow measured in (iii) being substantively higher than the level of oxygenation

and/or blood flow measured in (i) in the tested tissue, indicating a high probability that said tested tissue contains tumor cells.

3. A method according to Claims 1 or 2, wherein said level of oxygenation is measured by fMRI and BOLD measurements.

4. A method according to any of Claims 1-3, wherein said level of blood flow is measured by Doppler ultrasound measurement.

5. A method according to any of Claims 1-4, wherein the measurements in step (i) are a large number of measurements and a cut-off value is calculated on the basis of said measurements and wherein in step (iv) the level of oxygenation and blood flow measured in (iii) is compared to said cut-off value, a level substantially higher than the cut-off value indicating a high probability that said tested tissue contains tumor cells.

6. A method according to any one of Claims 1-5, wherein said agent is an agent which activates said tumor cells.

7. A method according to Claim 6, wherein said activation is obtained by activation of at least one cellular receptor expressed by said tumor cells.

8. A method according to Claim 7, wherein said at least one receptor is a tyrosine kinase receptor.

9. A method according to Claim 8, wherein said tyrosine kinase receptor is the Met tyrosine kinase receptor.

10. A method according to Claim 9, wherein said agent is HGF/SF or heparin.

11. A method for detecting of a region with a high probability of comprising tumor cells in a suspected tissue of an individual comprising:

(i) measuring the level of blood oxygenation in said suspected tested tissue;

(ii) measuring the level of blood oxygenation in a non-malignant tested tissue of said individual;

(iii) administering to said individual an agent which enhances the level of blood oxygenation of said tumor cells;

(iv) measuring the level of blood oxygenation in said suspected tested tissue;

(v) measuring the level of blood oxygenation in said non-malignant tissue;

(vi) comparing the level of blood oxygenation measured in (iv) to the level of blood oxygenation measured in (i) and calculating a ratio (a).;

(vii) comparing the level of blood oxygenation measured in (v) to the level of blood oxygenation measured in (ii) and calculating a ratio (b); and

(viii) comparing the calculated ratio (a) to the calculated ratio (b); a ratio (a) higher than a ratio (b) indicating a high probability of the existence of tumor cells in said suspected tissue.

12. A method for detecting of a region with a high probability of comprising tumor cells in a suspected tissue of an individual comprising:

(i) measuring the level of blood flow in said suspected tested tissue;

(ii) measuring the level of blood flow in a non-malignant tested tissue of said individual;

(iii) administering to said individual an agent which enhances the level of blood flow of said tumor cells;

(iv) measuring the level of blood flow in said suspected tested tissue;

(v) measuring the level of blood flow in said non-malignant tissue;

(vi) comparing the level of blood flow measured in (iv) to the level of blood flow measured in (i) and calculating a ratio (c).;

(vii) comparing the level of blood flow measured in (v) to the level of blood flow measured in (ii) and calculating a ratio (d); and

(viii) comparing the calculated ratio (c) to the calculated ratio (d); a ratio (d) different than a ratio (d) indicating a high probability of the existence of tumor cells in said suspected tissue.

13. A method according to Claim 11, wherein said level of blood oxygenation is measured by fMRI BOLD measurements.

14. A method according to Claim 12, wherein said blood flow is measured by Doppler ultrasound measurement.

15. A method according to any of Claims 11-14, wherein the measurements in step (ii) are in non-malignant tested tissues of a large number of individuals and are calculated into a cut-off value and wherein the calculated ratio (a) (b) (c) and (d)

are compared to said cut-off value, a measured calculated ratio higher than the cut-off value indicating a high probability of the existence of tumor cells in said suspected tissue.

16. A method according to any of Claims 11-15, wherein said agent is an activating agent of the tumor cells.

17. A method according to Claim 16, wherein said activation is obtained by activation of at least one receptor expressed by the tumor cells.

18. A method according to Claim 17, wherein said receptor is a tyrosine kinase receptor.

19. A method according to Claim 18, wherein said tyrosine kinase receptor is a Met tyrosine kinase receptor.

20. A method according to Claim 19, wherein said activating agent is HGF/SF.

21. A method according to Claim 19, wherein said activating agent is heparin.

22. A method according to any of the previous claims, wherein said tumor cells are breast cancer tumor cells.

23. A method according to Claim 22, wherein said activating agent is a ligand of the Met tyrosine kinase receptor.

24. A method according to Claim 23, wherein said activating agent is HGF/SF.

25. A method according to Claim 23, wherein said agent is heparin.

26. A method according to Claim 22, wherein said individual is an individual in which a primary breast tumor has been removed.

27. A pharmaceutical composition comprising as an active ingredient an effective amount of an agent which enhances the oxygen consumption of tumor cells, said enhancement being detectable by imaging techniques; and a pharmaceutically acceptable carrier.

28. A pharmaceutical composition according to Claim 27, wherein said imaging techniques are fMRI BOLD and Doppler ultrasound measurements.

29. A pharmaceutical composition according to Claims 27 or 28, for identifying regions having a high probability of containing tumor cells in a tested tissue of an individual.

30. A pharmaceutical composition according to any of Claims 27-29, wherein said agent is an agent which activates tumor cells.

31. A pharmaceutical composition according to Claim 30, wherein said activation is obtained by activation of at least one cellular receptor expressed by said tumor cells.

32. A method according to Claim 31, wherein said at least one receptor is a tyrosine kinase receptor.

33. A pharmaceutical composition according to Claim 32, wherein said tyrosine kinase receptor is the Met tyrosine kinase receptor.

34. A pharmaceutical composition according to Claim 33, wherein said agent is HGF/SF.

35. A pharmaceutical composition according to Claim 33, wherein said agent is heparin.

36. A pharmaceutical composition according to any of Claims 27-35, wherein said tumor cells are breast cancer tumor cells.

37. A kit for carrying out the method of any of Claims 1-26, comprising an agent which enhances the oxygen consumption of tumor cells, at least one predetermined cut-off value, optionally an algorithm for calculating a diagnostic index and instructions for use.

38. A kit in accordance with Claim 37, further comprising means for being connected to a computerized database comprising a collection of measured values and cut-off values which are periodically updated.

39. A kit according to Claim 37 or 38, wherein said agent is an agent which activates said tumor cells.

40. A kit according to Claim 39, wherein said activating agent is an agent which activates at least one cellular receptor expressed by said tumor cells.

41. A kit according to Claim 40, wherein said at least one receptor is a tyrosine kinase receptor.

42. A kit according to Claim 41, wherein said tyrosine kinase receptor is a Met tyrosine kinase receptor.

43. A method according to Claim 42, wherein said agent is HGF/SF.

44. A kit according to Claim 42, wherein said agent is heparin.

45. A kit according to any of Claims 37-44, wherein said tumor cells are breast cancer tumor cells.

46. A method for testing the sensitivity of tumor cells to a specific therapeutic drug or treatment comprising:

(i) obtaining tumor cells from said individual and growing said cells *in vitro;*

(ii) measuring the level of oxygen consumption of said tumor cells *in vitro*;

(iii) treating said tumor cells with the tested anti-tumorigenic treatment or drug;

(iv) measuring the level of oxygen consumption in said tumor cells following treatment with said anti-tumorigenic treatment or drug;

wherein a higher level of oxygen consumption in (iv) compared to the level of oxygen consumption in (i) indicating sensitivity of the tumor cells to the tested anti-tumorigenic treatment or drug.

47. A method according to Claim 46, wherein the measurements obtained in (ii) are from a large number of samples and a cut-off value is calculated and wherein the level of oxygen consumption measured in (iv) is compared to said cut-off-value a higher level of measured oxygen consumption indicating sensitivity of the tumor cells to the tested anti-tumorigenic treatment or drug.

48. A method for testing the sensitivity of a tumor in an individual to a tested anti-tumorigenic treatment or drug comprising:

(i) measuring the level of blood oxygenation of said tumor and/or of blood flow to said tumor in said individual;

(ii) administering a low dose of said tested anti-tumorigenic treatment or drug to said individual;

(iii) measuring the level of blood oxygenation of the tumor and/or of blood flow to the tumor in said individual;

wherein a higher level of measured blood oxygenation and/or blood flow in (iii) as compared to the level of measured oxygen in (i) indicating susceptibility of said tumor to the tested tumorigenic treatment or drug.

49. A method according to Claim 48, wherein the measurements in (i) are from a large number of tested individuals and are calculated into a cut-off value and wherein the measured level of blood oxygenation of the tumor and/or of the blood flow in (iii) is compared to said cut-off value, a higher level of measured blood oxygenation and/or blood flow indicating susceptibility of said tumor to the tested tumorigenic treatment or drug.

50. A method according to Claim 48 or 49, wherein the level of blood oxygenation is measured by fMRI or BOLD measurements and the level of blood flow to the tumor is measured by Doppler ultrasound methods.

51. A method for screening of inhibitors of receptors having potential in the treatment of disorders and diseases involving target cells expressing receptors inhibited by said tested inhibitor comprising:

(i) measuring the level of blood oxygenation of the cells expressing said receptor and/or blood flow to said cells in said individual;

(ii) administering a ligand capable of binding to said receptor to said individual;

(iii) measuring the level of blood oxygenation of said cells and/or of blood flow to said cells in said individual;

(iv) wherein there is no substantial difference in the measurements of (i) and (iii) above, said tested receptor inhibitor is determined to be non-suitable for treatment of said individual and wherein the measurements in (iii) are substantially different than those in (i), then:

(v) repeating steps (i) – (iii) above and administrating said tested inhibitor together with the ligand and measuring the level of blood oxygenation and/or blood flow to said cells; and

(vi) wherein the measurement in (v) is substantially different than the measurement in (iii), said tested inhibitor is a potentially effective drug for use in said individual.

52. A method according to Claim 50, wherein the level of blood oxygenation is measured by fMRI or BOLD measurements and the level of blood flow is measured by Doppler ultrasound measurements.

53. A method according to Claims 51 and 52, wherein said inhibitor is an inhibitor of a tyrosine kinase receptor.

54. A method according to Claim 53, wherein said tyrosine kinase receptor is a Met receptor.

55. A method according to any of Claims 51-54, wherein said disease is cancer.

56. A method for screening for inhibitors of receptors having a potential in the treatment of disorders and diseases involving target cells expressing receptors inhibited by said tested inhibitor comprising:

(i) measuring level of blood oxygenation of the cells expressing said receptor and/or of blood flow to said cells in said individual;

(ii) administering at the receptor inhibitor to said individual;

(iii) measuring the level of blood oxygenation of said cells and/or of blood flow to said cells in said individual;

(iv) wherein there is a substantial difference in the measurement of (i) and (iii) above, said tested receptor inhibitor may have a potential as a drug for the treatment of said disorders and diseases.

57. A kit for evaluating the efficacy of inhibitors of receptors in the treatment of disorders or diseases involving target cells expressing receptors inhibited by said tested inhibitor comprising at least one ligand capable of binding to said receptor; at least one inhibitor of said receptor to be tested, optionally predetermined cut-off values of the level of blood oxygenation and blood flow of said target cells and instructions for use.

58. An auxiliary composition for administration in conjunction with an anti-tumorigenic treatment administered to an individual for the treatment of a tumor, said composition comprising as an active ingredient an agent which enhances the oxygenation of said tumor cells and/or changes the blood flow to said tumor cells and a pharmaceutically acceptable carrier.

59. An auxiliary composition according to Claim 58, wherein said anti-tumorigenic treatment is a chemotherapeutic drug or an antibody binding to said tumor cells.

60. An auxiliary composition according to Claim 58, wherein said anti-tumorigenic treatment is an anti-tumorigenic irradiation treatment.

61. A method for enhancing the efficacy of an anti-tumorigenic treatment administered to an individual having a tumor comprising administering to said individual an effective amount of an agent which enhances the oxygenation of said tumor cells and/or changes the blood flow to said tumor cells in combination with said anti-tumorigenic treatment.

62. A method according to Claim 61, wherein aid anti-tumorigenic treatment is an anti-tumorigenic chemotherapeutic drug or an antibody binding to said tumor cells.

63. A method according to Claim 61, wherein said anti-tumorigenic treatment is an anti-tumorigenic irradiation treatment.

64. A method for enhancing the efficacy of chemical contrast substances used during magnetic resonance imaging of cells (MRI) or ultrasound comprising administering said chemical contrasts medium in conjunction with an activating agent capable of enhancing the level of blood oxygenation of said cells and/or blood flow of the cells.

65. A method according to Claim 64, wherein said cells are tumor cells.

66. A method according to Claim 64 or 65, wherein said activation of the cells is obtained by activation of at least one cellular receptor expressed by the cells.

67. A method according to Claim 66, wherein said receptor is a tyrosine kinase receptor.

68. A method according to Claim 67, wherein said tyrosine kinase receptor is a Met receptor.

69. An auxiliary composition for administration in conjunction with chemical contrast substances used during MRI or ultrasound of cells, said composition comprising as an active ingredient an agent which enhances the oxygenation of said cells and/or changes the blood flow to said cells and a pharmaceutically acceptable carrier.

Fig. 1A

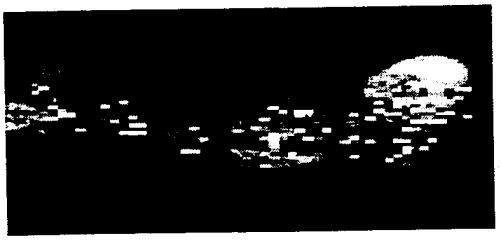

Fig. 1B

Fig. 1C

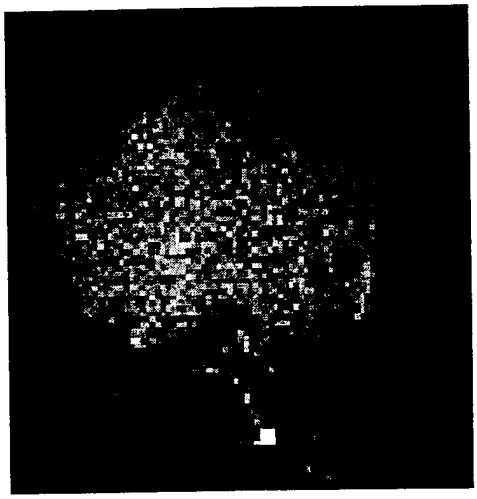

Fig. 2A

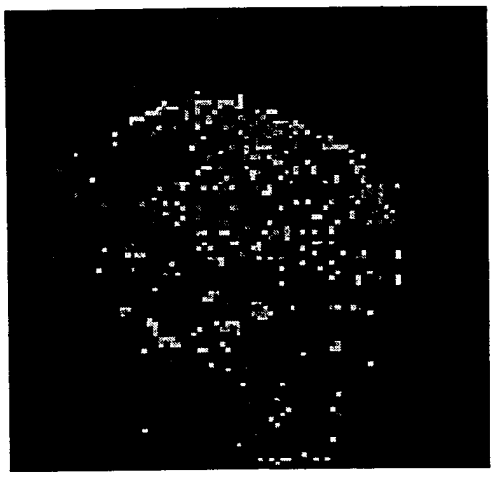

Fig. 2B

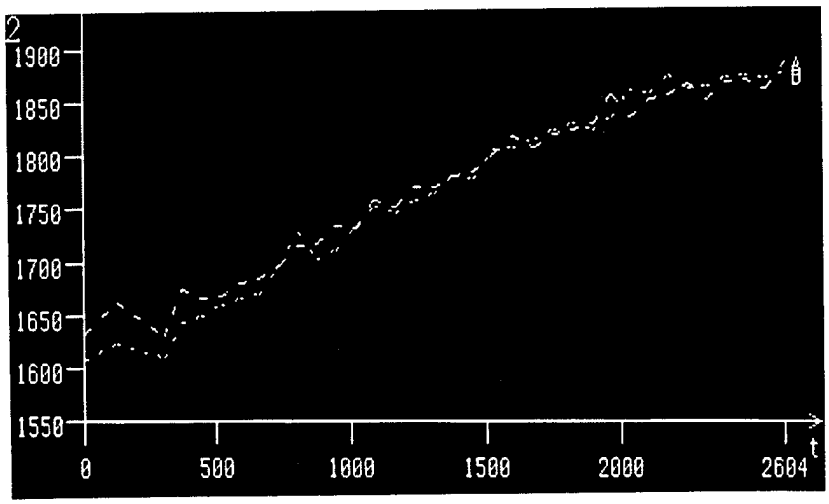

Fig. 2C

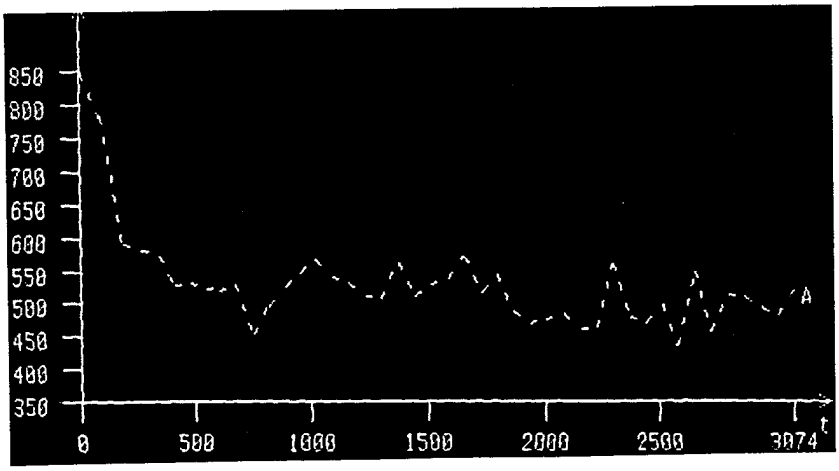

Fig. 2D

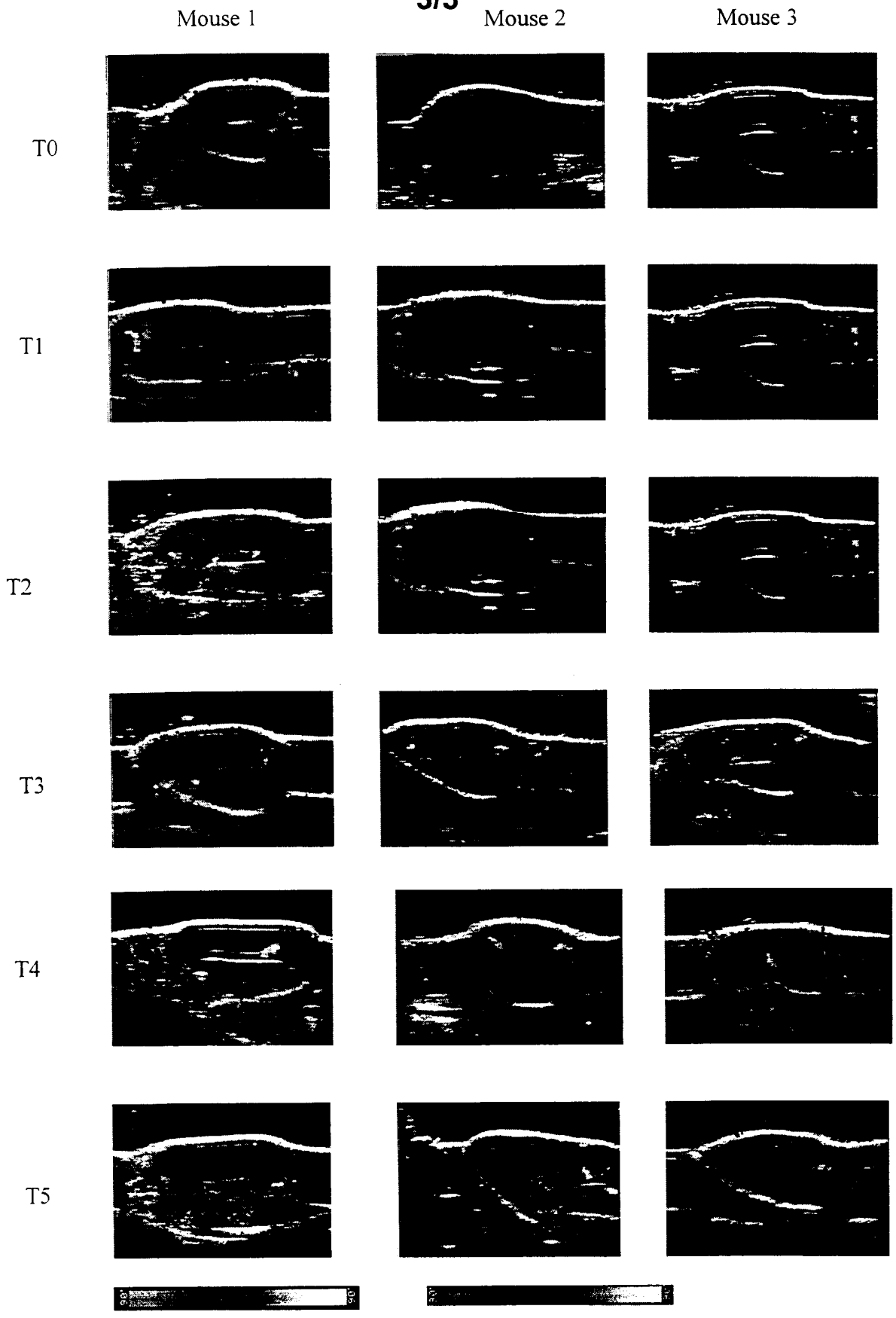

Fig. 3

专利名称(译)	通过肿瘤的成像和治疗进行肿瘤检测		
公开(公告)号	EP1221977A2	公开(公告)日	2002-07-17
申请号	EP2000969788	申请日	2000-10-20
[标]申请(专利权)人(译)	FBIT		
申请(专利权)人(译)	FBIT LTD.		
当前申请(专利权)人(译)	FBIT LTD.		
[标]发明人	TSARFATY ILAN SHAHARABANY MIRIAM ABRAMOVITCH RINAT KUSHNIR TAMMAR TSARFATY GALIA ITZCHAK YACOV		
发明人	TSARFATY, ILAN SHAHARABANY, MIRIAM ABRAMOVITCH, RINAT KUSHNIR, TAMMAR TSARFATY, GALIA ITZCHAK, YACOV		
IPC分类号	G01N33/50 A61B5/055 A61B5/145 A61B8/06 A61B10/00 A61K31/00 A61K45/06 A61K49/00 A61K49/14 A61P35/00 G01N33/15		
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优先权	132531 1999-10-22 IL		
外部链接	Espacenet		

摘要(译)

提供了一种用于检测或监测个体中的肿瘤细胞的方法，其基于常规使用的成像技术，例如磁共振成像（MRI）和超声技术。通过外部监测个体的测试组织中的细胞氧合水平（测量为BOLD图像）和血液流向细胞（通过多普勒超声测量），非侵入地进行细胞的检测和监测。该方法可以用于识别个体中不具有预先已知的致瘤性病变的区域，以及检测具有在个体的预先怀疑的病变中包含细胞的高概率的区域（例如，可以是基于常规使用的方法分析的原发肿瘤细胞存在概率很高的区域。该方法包括使用对个体给药或与之接触的药剂细胞导致通过各种成像技术可检测的细胞的氧消耗增加。例如，活化剂可以通过与细胞特异性表达的受体结合来活化细胞。此外，还提供了用于评估基于通过成像技术测量血液氧合水平和血流水平的抗肿瘤发生治疗功效的方法。此外，提供了用于增强抗肿瘤发生治疗处理（包括化学治疗药物，抗体和辐射治疗）的功效的方法，其涉及向治疗个体施用一定量的增强靶细胞氧合作用和血液流动的药剂。受治疗个体中的靶细胞。结合MRI或超声波使用的化学造影剂的功效也通过提供的方式得到增强方法。