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(54) Title: USE OF CYTOSOLIC PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE TO REDUCE TUMOR GROWTH

(57) Abstract: The present invention relates to the reduction of tumor growth. More specifically, the present invention relates to the use of cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) to reduce tumor growth. Moreover, the present invention relates to the detection of mutations in the gene, encoding cPHGPx to forecast the likelihood to develop cancer and/or to forecast the likelihood to develop metastasis and/or to determine the aggressiveness of the tumor.

METHODS AND MEANS TO REDUCE TUMOR GROWTH

The present invention relates to the reduction of tumor growth. More specifically, the present invention relates to the use of cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) to reduce tumor growth. Moreover, the present invention relates to the detection of mutations in the gene, encoding cPHGPx, to forecast the likelihood to develop cancer and/or to forecast the likelihood to develop metastasis and/or to determine the aggressiveness of the tumor

The oxidant – antioxidant balance of a cell regulates to an important extent the different cellular and metabolic functions. A disturbance of the redox state by external and/or internal stimuli may result in a disturbance of cell functions (Finkel, 1998). As a non-limiting example, reactive oxygen intermediates (ROI) are essential for cell proliferation, but at the same time, the cells need defense mechanisms to avoid oxidative stress and to inhibit ROI induced apoptosis (Gottlob *et al.*, 2001).

Cells do produce considerable amounts of ROI. Those ROI may cause damage to the DNA both in a direct or in an indirect way – the latter mainly by degradation of lipid hydroperoxydes (ROOH). The role of ROI-mediated DNA damage to the transformation of a normal cell to an immortalized cell, which is the first step in tumorgenesis, has been demonstrated (Finkel and Holbrook, 2000; Moos *et al.*, 2003). However, the role of ROI in tumor growth is far less documented.

In nearly all solid tumors, there is an imbalance between O_2 delivery and O_2 consumption, which causes hypoxia (Hockel and Vaupel, 2001). Indeed, tumor cells show an increased oxygen consumption (Dunn, 1997), while, by the tumor growth, the distance to the nearest blood vessels is increasing (Vaupel *et al.*,2001). Cells respond to hypoxia with a stabilization of hypoxia-inducible-factor-1α (HIF1α). In this hypoxia response, the mitochondria and the production of ROI play an essential role (Chandel *et al.*, 2000). Apart from the induction by HIF-1α of genes involved in angiogenesis, hypoxia also induces apoptosis and hence promotes the outgrowth of tumor cells with increased resistance to apoptosis (Piret *et al.*, 2002; Schmaltz *et al.*, 1998).

Another important stimulus for ROI production in tumor cells is inflammation. An indication for an active tumor-promoting role of inflammatory mediators such as eicosanoids is given by the increase in expression of cyclooxygenase (COX) and lipoxygenase (LOX) in an important number of tumors (Asano *et al.*, 2002;Gupta *et al.*, 2001; Nie and Honn, 2002; Shureiqi and Lipmann, 2001) and by the fact that the chemoprotective role of specific COX and LOX inhibitors is being demonstrated (Gunning *et al.*, 2002; Gupta and Dubois, 2001). The tumor inducing mechanism of the COX reaction products (mainly prostaglandin E_2 (PGE_2), formed from arachidonic acid) include the inhibition of apoptosis, the stimulation of angiogenesis, the promotion of metastasis and the stimulation of tumor cell proliferation. Recently, it has been

shown that also leukotrienes can stimulate the proliferation of tumor cells and repress apoptosis.

The transformation of arachidonic acid by COX and LOX is controlled, amongst others, by PHGPx. Indeed, during studies of the arachidonic acid metabolism, it was shown that overexpression of cytosolic PHGPx (cPHGPx) is inhibiting the activity of COX and LOX (Chen *et al.*, 2002; Imai *et al.*, 1998). However, the role of cPHGPx in tumor formation has never been described.

Surprisingly we found that overexpression of cPHGPx and tumor growth is both retarding the onset of tumor formation, as well as inhibiting the growth of the tumor. Moreover, overexpression of cPHGPx is far more efficient in this respect than known COX and LOX inhibitors, such as the COX-2 inhibitor meloxicam. Taking into account that cPHGPx is far less toxic than the known COX and LOX inhibitors, this finding opens several possibilities for new cancer treatments.

A first aspect of the invention is the use of the cPHGPx gene, or a functional fragment thereof, to modulate the onset of tumor formation and/or tumor growth. Preferably, said tumor is chosen from the group of fibrosarcoma, melanoma, lymphoma, breast, lung and colon cancer and brain metastases. Most preferably, said tumor is a brain metastasis. Preferably, said use is an overexpression of the gene, or a functional fragment thereof, and said modulation is a retardation of the onset and/or a reduction in tumor growth. A preferred functional fragment is the coding sequence of the gene. Indeed, said coding sequence may be placed after a strong inducible or constitutive promoter, to facilitate overexpression of cPHGPx. Alternatively, said coding sequence may be operably linked to a tissue specific promoter, or to a promoter that is induced in tumor cells, such as a hypoxia induced promoter.

These constructs can be delivered to the tumor cells using gene therapy. Vectors and methods for gene therapy are known to the person skilled in the art, and include, but are not limited to ex vivo and in vivo gene therapy, and adenoviral, retroviral and lentiviral vectors.

Alternatively, the overexpression of endogenous cPHGPx may be induced by the addition of a compound. Indeed, it is know that several compounds are inducing the cPHGPx gene. As a non-limiting example, the cPHGPx gene is induced by superoxide radicals and/or by singlet oxygen. Therefore, said compounds can be used to induce the cPHGPx gene, as well as compounds that result in superoxide radical formation and/or singlet oxygen formation. WO02078718 describes compositions, methods, apparatuses and systems for singlet oxygen delivery. Said singlet oxygen can be used for the treament of tumors. However, it is clearly mentioned that in this case, the singlet oxygen is believed to produce toxic effects on the cells of the tumor through oxidation and/or free radical reactions. Induction of cPHGPx, which may occur at lower concentration of singlet oxygen than the induction of the above-mentioned toxic reactions, is not considered.

Alternatively, cPHGPx may be induced by the addition of selenium, or by tertiary-butylhyperoxide. Said induction may be combined with other forms of overexpression of cPHGPx, as described above.

As overexpression of the cPHGPx gene will result in an increased amount of cPHGPx, another aspect of the invention is the use of cPHGPx protein to delay the onset of tumor formation, and/or to reduce tumor growth.

Another aspect of the invention is the use of the cPHGPx gene, or a functional fragment thereof, to increase the efficacy of an existing tumor therapy. In one preferred embodiment, said therapy is an anti-angiogenic therapy. Anti-angiogenic therapy is known to the person skilled in the art, and includes, but is not limited to treatment with Avastin, Vitaxin, Herceptin or TNF. Preferably, said anti-angiogenic treatment is a TNF treatment. In another preferred embodiment, said therapy is the photodynamic therapy. In these cases the use of the cPHGPx gene may occur during the cancer treatment, or alternatively it may be phased, by giving first the tumor therapy, followed by induction and/or overexpression of the cPHGPX gene, or delivering the cPHGPx protein to the tumor, or vice versa. In case of the photodynamic therapy, said therapy is preferably applied first, followed by the use of the cPHGPx gene according to the invention.

Still another aspect of the invention is the use of the PHGPx gene, or a functional fragment thereof, for diagnosis related to cancer and malignancy. Preferably, said diagnosis is the determination of the likelihood to develop cancer and/or the determination of the likelihood to develop metastasis and/or the determination of the aggressiveness of the tumor. Even more preferably, said cancer is chosen from the group of fibrosarcoma, melanoma lymphoma, breast, lung and colon cancer and brain metastases. Most preferably, said cancer is brain metastasis. The diagnosis may be related to the expression level of the cPHGPx gene, which can be tested both on RNA or on protein level, or it may be related to mutations which affect either the expression level of the gene or the activity of the protein. Indeed, as the expression of cPHGPx is retarding the onset of tumor formation and tumor growth, mutations in the gene may affect the tumor development. Said mutations may be affecting the expression of the gene, such as a mutation in the promoter region, or they may affect the activity of the gene, such as a mutation in the coding sequence. A functional fragment of the gene for use in diagnosis is every fragment that gives rise to specific primers, which can be used in, as a non-limiting example, polymerase chain reaction (PCR) or hybridization experiments. Preferably, said fragments are used in small nuclear polymorphism (SNP) analysis. Methods for SNP analysis are known to the person skilled in the art.

Alternatively, said diagnosis may be carried out by the use of antibodies against the cPHGPx protein, or against a specific domain of said protein. Indeed, the expression level can be tested by measuring the protein in a quantitative way, by immunological techniques such as ELISA,

or mutations in the protein can be detected by binding or loss of binding of an antibody directed against the domain carrying the mutation. Alternatively, cPHGPx mRNA levels may be measured. Methods to measure mRNA levels are known to the person skilled in the art and include, but are not limited to quantitative PCR and quantitative micro array analysis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Influence on tumor growth by constitutive cytosolic overexpression of cPHGPx in L929 fibrosarcoma cells inoculated in nu/nu mice. 2 10^5 cells were injected subcutaneously at day 0 (n = 5 mice per group). Neo indicates cells in which only the selection plasmid has been injected. Those cells are indicated as "neo-cells"

Figure 2: Effect of cPHGPx-transfectants on the tumor growth of neo-cells. Cell were injected subcuteaneously in nu/nu mice at day 0. The amount of neo-cells was kept constant at 2 10^5 cells. (n = 10 mice per group)

Figure 3: Effect of neo-cells on the tumor growth of cPHGPx-transfectants (n = 5 mice per group)

Figure 4: Expression of the key enzymes of the eicosanoid metabolism and of the receptors of PGE_2. Expression was measured using Q-PCR with SYBR green. The specificity of the primers was checked by a melting curve.

Figure 5: Role of COX-2 in cPHGPx related tumor growth inhibition. The effects of meloxicam on neo tumor growth (A) and of 16,16dmPGE_2 on cPHGPx tumorgrowth (B) were studied. The insert shows the effect of the combination of meloxicam and 16,16dmPGE_2 on neo (A) and on cPHGPx (B) tumor growth. 2 10^5 tumor cells/ mouse were injected subcutaneously. The treatment with meloxicam was done with daily intraperitoneal injection at a concentration of 3mg/kg body weight; treatment with 16,16dmPGE_2 was also by intra peritoneal injections, 3 times a week, using 10µg per mouse.

Figure 6: Expression of COX-2 in neo and cPHGPx cells after hypoxia or after hypoxia followed by reoxygenation. RNA was prepared after 6 hours or 16 hours of hypoxia (1% O_2), or after hypoxia followed by reoxygenation for 1 hour, at 20% O_2. COX-2 mRNA expression was quantified with Q-PCR using SYBR green.

Figure 7: Role of 5-LOX in L929 induced tumor growth in mice. Effect of the 5-LOX inhibitor zyflo at 60 mg/kg daily on L929 tumor growth on L929 neo and L929 cPHGPx. The treatment was started at day 8 after injection of the L929 cells in the mice.

Figure 8: Role of COX-1 in L929 induced tumor growth in mice. Effect of the COX-1 inhibitor piroxicam at 7.5 mg/kg daily on L929 tumor growth on L929 neo and L929 cPHGPx. The treatment was started at day 8 after the injection of the L929 cells in the mice.

Figure 9: The influence of hypoxia on the expression of the enzymes involved in the transformation of archidonic acid in prostanoids in L929 cells. Samples were analyzed after 8, 15 and 30 hours of hypoxia.

Figure 10: Effect of hypoxia on the level of COX-2 protein in L929 neo and L929 cPHGPx cells. Samples were analyzed after 8, 15 and 30 hours of hypoxia.

Figure 11: Effect of hypoxia on the expression level of (A) COX-2, (B) COX-1, (C) PLA2 and (D) 5-LOX in B16BL6 neo and B16BL6 cPHGPx cells.

Figure 12: Effect of mouse TNFα on the growth of (A) B16BL6, (B) B16BL6 neo and (C) B16BL6 cPHGPx induced tumor growth in mice. The treatment with TNFα was started 10 days after injection with the B16BL6 cells. The graphs represent the average of 3 mice.

DEFINITIONS

Gene as used here includes both the promoter region of the gene as well as the coding sequence. It refers both to the genomic sequence (including possible introns) as well as to the cDNA derived from the spliced messenger, operably linked to a promoter sequence.

Coding sequence is a nucleotide sequence, which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

Operably linked refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the promoter sequence.

Nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog.

Promoter region of a gene as used here refers to a functional DNA sequence unit that, when operably linked to a coding sequence and possibly placed in the appropriate inducing conditions, is sufficient to promote transcription of said coding sequence.

The terms *protein* and *polypeptide* as used in this application are interchangeable. *Polypeptide* refers to a polymer of amino acids and does not refer to a specific length of the molecule. This

term also includes post-translational modifications of the polypeptide, such as glycosylation, phosphorylation and acetylation

Compound means any chemical of biological compound, including simple or complex organic and inorganic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof.

Tumor stimulating factor or *tumor promoting factor* as used here may be any compound that is obtained by the action of COX and LOX enzymes. Preferably said compounds are cytosolic lipidhydroperoxides.

The aggressiveness of the tumor as used here relates to the growth rate of the tumor tissue, faster growing tumors being considered as more aggressive.

EXAMPLES

Methods to the examples

Genetic constructions and cell lines; transfection procedures

- **cPHGPx**: pig phospholipid hydroperoxide glutathione peroxidase that is expressed in the cytosol - unigene name Gpx4
- **mβactin**: mouse β actin gene
- **neo**: neomycine resistance gene
- **L929 cPHGPx**: L929 fibrosarcoma cells that overexpress cPHGPx
  * Transfection: DNA-calciumphosphate co-precipitation with DNA-mixture consisting of carrier DNA (pSV23s), selectionplasmid (pSV2_neo) and pCAGGS_cPHGPx
  * cPHGPx in pCAGGS-vector under control of the β-actin/β-globin hybrid promotor
- **L929 neo**: L929 fibrosarcoma cells that are transfected only with the selectionplasmid
  * Transfection: DNA-calciumphosphate co-precipitation withDNA-mixture consisting of carrier DNA (pSV23s) and selectionplasmid (pSV2_neo)

- **B16BL6 cPHGPx**: B16BL6 melanoma cells that overexpress cPHGPx
  * Transfection: co-transfection using lipofectamine with DNA-mixture consisting of selectionplasmid (pSV2_neo) and pCAGGS_cPHGPx
  * cPHGPx in pCAGGS-vector under control of the β-actin/β-globin hybrid promotor
- **B16BL6 neo**: B16BL6 melanoma cells that are only transfected with the selection plasmid
  * Transfection: transfection using lipofectamine with only using selectionplasmid (pSV2_neo)

Mice strains used

- ***nu/nu mice:*** immunodeficient mice (no T-cells) used for tumor experiments with L929 cells
- **C57BL/6 mice**: immunocompetent mice used for tumor experiments with B16BL6 cells

Tumor experiments with L929 or B16BL6 cells.

- tumor cells are grown to subconfluency
- cells are dislodged with EDTA-Trypsine and washed 3 times with LPS-free PBS
- cells are resuspended at 1.10^6 cells/ml (L929) or 25000 cells/ml (B16BL6) in LPS-free PBS
- 200µl cell suspension (~ 2.10^5 L929 cells or 5000 B16BL6 cells) is injected subcutaneously in mice
- tumor diameters are measured 3 times / week

Treatments of mice.

COX- and LOX-inhibitors

Specific inhibitors of the COX and LOX enzymes that are expressed during tumor growth were used to study the role of these enzymes in L929 or B16BL6 tumor growth and their role as cPHGPx-target.
Administration to the mice bearing tumors happens intraperitonially or subcutaneously. These inhibitors were used separately or in combination.

COX- and LOX-products

The involvement of COX- and LOX-products in L929 and B16BL6 tumor growth was examined through administration of these products to mice injected with the tumor cells.

TNFα treatment

C57B1/6 mice were injected subcutaneously with 6.10^5 tumor cells (B16L6, B16L6 neo or B16L6 cPHGPx). 10 days after injection, TNFα treatment was started by applying paralesionally a daily dose of 1µg/mice. The treatment was continued for 10 days. The dose applied is about 7 times lower than the dose needed to obtain a clear tumor regression.

Study of metastasis.

Performed with B16BL6 melanoma model as these tumor cells are very well suited for study of metastasis because of their pigmentation with melanin. 2 strategies are followed comparing B16BL6 neo with B16BL6 cPHGPx:

1. injection of tumor cells in the rear limb of mice and amputation of this limb when a primary tumor is formed.
2. experimental metastasis: intravenous injection of tumor cells

Measurements of prostaglandin and leukotriene levels.

The production of prostaglandins and leukotrienes was measured with commercial available kits (Neogen Corporation).

Genomic DNA-isolation.

- performed on tumors excised at mean diameters of 4mm and 10mm
- genomic DNA-isolation is performed using Generation capture column DNA isolation Kit – Gentra Systems and following manufacturer's instructions

Real-time PCR.

RNA-isolation

- performed using either TRIzol (Invitrogen) or RNeasy-kit (Qiagen)
- DNase-treatment: with DNase I (Qiagen)

cDNA-synthesis

- reverse transcriptase enzyme used is Superscript II RNase H$^-$ (Invitrogen)

real-time PCR

- Sybr Green based real-time PCR (Eurogentec)

Hypoxia-experiments in vitro

Cells are cultured in a hypoxia chamber in which O_2-concentration can be controlled. The condition that is defined as hypoxia is 1% O_2 – 5% CO_2 – 94% N_2. Medium of the cells is overnight pre-incubated in these conditions and at the start of the experiment the medium of the cells is replaced with the pre-incubated medium. Normoxia is defined as 20% O_2 – 5%CO_2 – 75% N_2

Photodynamic Therapy

In vitro: The photosensitizer, hypericine, is added to the medium and cells are incubated for 10 hours in the dark. Next, cells undergo light stimulation with visible light.

In vivo: Hypericine is intravenously injected in the mice and a number of hours later the tumor is exposed to visible light.

Single Nucleotide Polymorphism (SNP)

SNP-analysis of cytosolic PHGPx-gene in human is performed comparing healthy volunteers (n = 25 – 30) and patients diagnosed with colon cancer (n = 25- 30) and brain metastases from breast and lung cancer. Specific primers are designed to amplify specific regions of the genomic DNA. Thereafter, this PCR-product is sequenced.

Example 1: Cytosolic overexpression of PHGPx inhibits tumor growth

The effect of several antioxidant enzymes on tumor growth has been tested. Overexpression of cytosolic PHGPx (cPHGPx) resulted in a significant decrease of both tumor formation and tumor growth (Figure 1). Due to the fact that L929 fibrosarcoma cells are only tumorigenic in nu/nu mice which are deficient in T-cells, it can be concluded that the tumor inhibition is independent from the adaptive immune system. The inhibitory effect may then be explained either by the fact that the cPHGPx transfectants do change the microenvironment of the tumor, which results in a tumor inhibition, or by the fact that said transfectants are unable to synthesize certain tumor stimulating factors. To check the first hypothesis, neo-cells (transfected with the selection plasmid only) and cPHGPx cells were mixed in vitro, using different amounts of cPHGPx cells, and the tumor growth was evaluated after inoculation of the cells or the cell mixtures in nu/nu mice. The results are presented in figure 2. The presence of cPGHPx cells has no influence on the tumor growth of the neo-cells, which proves that the inhibition by the overexpression of cPHGPx is not due to a change in microenvironment of the tumor, but rather to the absence of one or more tumor stimulating factors in the cPHGPx-transfectants. Experiments using overexpression of cytosolic CuZn superoxide dismutase or cytosolic catalase strongly suggested that O_2^- nor H_2O_2 had a direct influence on tumor growth. As a consequence, the tumor stimulating factor cannot be produced by the lipid peroxidation chain reaction, but should be formed enzymatically during the tumor growth, by the enzymatic action of COX and LOX. The possible tumorigenic effect of the COX and LOX products (prostaglandins and leukotrienes) as tumor stimulating factors has been described in a number of tumor types. Moreover, it is known that several tumor types do show an increased expression of COX or LOX (Asano *et al.* 2002; Gupta *et al.*, 2003; Nie and Honn, 2002; Shureiqi and Lippman, 2001) and that the use of COX and/or LOX inhibitors results in a decreased risk of cancer (Gunning *et al.*, 2002; Gupta and Dubois, 2001).

To study a possible positive effect of neo-cells on tumor growth of cPHGPx-cells, both cell types were mixed at a total amount of $2\ 10^5$ cells. This is the maximal amount where there is still a dose response of cells injected on tumor growth. From figure 3, it can be deduced that the growth of the mixed population is close to that of the neo-cells alone, and far higher than that of the cPHGPx cells alone. These results indicate that the neo-cells indeed do have a

tumor growth promoting effect on the cPHGPx cells, by releasing a tumor growth promoting factor.

The results were confirmed by analyzing the composition of the tumors at genomic DNA level, in tumors with a diameter of 4 and 10 mm. The presence of cPHGPx was quantified using Q-PCR (SYBR Green) and expressed as a ration on mβ-actin (Table 1). As an internal control, cells were mixed as in vitro culture and measured. In neo-cells, no cPHGPx can be detected, proving the specificity of the primers used. The in vitro results show an intermediate value for the ratio, compared with the results on pure neo- and pure cPHGPx-cells, proving the validity of the approach. Both in tumors of 4mm and 10mm induced by the mixed population, cPHGPx can be detected, be it at a lower ration in the bigger tumors.

It is important to note that at day 14, when the 4mm tumors were measured for the mixed population, no tumor formation could yet be observed for the cPHGPx cells. These results indicate that the onset of tumor formation can be complemented by the presence of neo-cells, but, as the ratio of cPHGPx lowers when the tumor becomes bigger, that the presence of neo-cells is not sufficient to restore the tumor growth in cPHGPx-cells.

	Neo			Mixed population			cPHGPx		
	culture	Tumor		culture	Tumor		culture	Tumor	
		Φ 4mm	Φ 10mm		Φ 4mm	Φ 10mm		Φ 4mm	Φ 10mm
cPHGPx/mb-actin	0,00	0,00	0,00	1,68 1,92	0,25 0,25	0,03 0,03	2,90	1,32	2,37
time	NR	Day 13	Day 20	NR	Day 14 Day 14	Day 27 Day 32	NR	Day 32	Day 44

Table 1: composition of the tumors, induced by a constant number of cells, either by pure neo-cells, pure cPHGPx cells, or a mixture of both. The mixture contained neo-cells and cPHGPx cells in a ratio 1/1. The results are expressed as a ratio of cPHGPx on mβ-actin, as determined by Q-PCR on genomic DNA.

NR: not relevant

Example 2: Expression of the enzymes of the eicanosoid metabolism

Overexpression of CuZnsuperoxide dismutase or catalase did not reduce the tumor growth, indicating that the tumor promoting factors were not originating from the lipid peroxidation chain reaction, but were formed enzymatically by the activity of COX and LOX. In order to identify which factors were blocked by cPHGPx overexpression, the key enzymes were studied using Q-PCR. The results are summarized in Figure 4.

Cytosolic phospholipase A_2 ($cPLA_2$), that liberates arachidonic acid from the membrane, is the only enzyme of the eicosanoid metabolism that is clearly expressed in vitro. In the tumor tissue, however, there was also a significant induction of COX-1, COX-2 and 5-LOX. 12-LOX is also induced in tumor tissue, but its expression is low in comparison with 5-LOX. These results indicate that in tumor tissue, both prostanoids (formed by COX) and leukotrienes (formed by 5-LOX) play an important role in the L929 induced tumor formation.

Due to the fact that eicosonoids may exert their effect partly in a paracrinic way, the expression of the membrane bound PGE_2- receptors was also studied (Figure 4D). Only expression of EP1 and EP4 can be detected. It is known that those receptors play a role in the development of colon cancer (Mutoh *et al.*, 2002; Watanabe *et al.*, 1999)

Example 3: Role of COX-2 in cPHGPx induced inhibition of tumor growth.

Although cPHGPx inhibits all COX and LOX isoforms, it is known that COX-2 is about 10 times more sensitive to cPHGPx inhibition than COX-1 or 5-LOX (Huang *et al.*, 1999). Therefore, the possible role of COX-2 as target for the cPHGPx induced inhibition of tumor growth was studied in detail. The COX-2 specific inhibitor meloxicam was added to see if the tumor growth is inhibited in a similar way as by cPHGPx overexpression. On the other hand, the PGE_2 analog 16,16-dimethyl-PGE_2 was added to check if the effect of cPHGPx overexpression on tumor growth could be neutralized. Indeed, it is know that that COX-2 induction is accompanied by an induction of the membrane bound PGE_2 synthesis, resulting in a preferential transformation of arachidonic acid in PGE_2 when COX-2 is up-regulated.

From Figure 5A, it can be concluded that meloxicam is only inhibiting tumor growth when the treatment is started at the moment that the tumor is already present. When the treatment is started at day 0, together with the injection of the tumor cells, no effect on tumor growth is seen.

Treatment with 16,16-dimethyl-PGE_2, starting from day 0, results in a faster onset of the tumor formation by injection with cPHGPx cells, and makes the tumor detectable at about the same time as for the control neo-cells (figure 5B). When 16,16-dimethyl-PGE_2 is applied together with meloxicam, the effect is neutralized.

The results indicate that COX-2 is indeed a target of cPHGPx. However, inhibition with meloxicam proofs to be less efficient than inhibition using cPHGPx overexpression, indicating that cPHGPx is working on several targets simultaneaously.

Example 4: Hypoxia as stimulus for COX-2 induction during tumor growth

Hypoxia is an important stimulus for COX induction (Schmedtje *et al.*, 1997). This is also seen in the L929 neo-cells (Figure 6). Hypoxia (1% O_2) gave a clear COX-2 induction; in neo-cells, treated with hypoxia followed by reoxygenation till 20% O_2, the effect is even slightly more

pronounced. However, in the cPHGPx overexpressing cells, no COX-2 induction can be noticed, neither after hypoxia, nor after hypoxia followed by reoxygenation. Taking into account that in tumors, hypoxia may occur starting from a tumor volume as small as 1 mm^3, the COX-2 repression, and the resulting decrease in angiogenesis and increased apoptosis may be an important factor in the slower tumor formation by cPHGPx overexpression.

Example 5: Role of COX-1 and 5-LOX in L929 induced tumor growth

As shown in example 3, inhibition of COX-2 by meloxicam is not sufficient to revert a L929 neo phenotype in an L929 cPHGPx phenotype in respect to tumor induction in mice. Therefore, the role of 5-LOX was studied in a comparable set up, using zyflo as specific 5-LOX inhibitor. The treatment with zyflo clearly slows down the tumor development of L929 neo induced tumors, while there is no effect for L929 cPHGPx induced tumor growth (Figure 7). This indicates that besides the COX-2 inhibition, also the 5-LOX inhibition by cPHGPx contributes to the tumor inhibition.

As COX-1 is the third enzyme of the eicosanoid metabolism which is expressed in L929 cells, the role of COX-1 inhibition by cPHGPx in tumor inhibition was also studied, using piroxicam as inhibitor. The results are summarized in Figure 8. The inhibitory effect of peroxicam on L929 neo tumor growth inhibition is limited and far less important than that of meloxicam and xyflo, thus indicating that although COX-1 is a target of cPHGPx the contribution of COX-1 to promotion of tumor growth is far less important than that of COX-2 and 5-LOX.

Example 6: Effect of hypoxia on COX-1, COX-2, PLA2 and 5-LOX expression in L929 neo and B16BL6 neo cells, compared with L929 cPHGPx and B16BL6 cPHGPx cells

L929 cells were placed in hypoxia conditions, and the effect on RNA and protein levels was studied. Samples were analyzed after 8, 15 and 30 hours of hypoxia.

As shown in Figure 9, there is a clear induction of COX-2 by hypoxia in L929 neo cells, whereas no induction is seen in L929 cPHGPx while on the contrary, COX-1 is only induced in L929 cPHGPx cells. There is no significant difference between the induction of PLA2 under normoxia and hypoxia. It is remarkable, however, that the expression of PLA2 in L929 cPHGPx is significantly lower than for L929 neo.

The effect on COX-2 expression was confirmed at the protein level (Figure 10). COX-2 is hardly detectable in L929 cPHGPx cells, even in hypoxia. In contrast COX-2 protein was clearly detectable in L929 neo cells following culture under hypoxia conditions.

Similar experiments were carried out on B16BL6 cells. In this case, the induction in B16BL6 neo cells of COX-2 by hypoxia was apparent only after 15 hours, similar to L929 cPHGPx. B16BL6 cPHGPx cells failed to upregulate COX-2 upon hypoxia. COX-1 has a similar evolution as COX-2, but in contrast to the L929 cells, B16BL6 cPHGPx cells show a low

induction by hypoxia compared with the neo control. The repression of PLA2 in B16BL6 cPHGPx cells is even more pronounced than for their L929 counterpart. Interestingly 5-LOX shows a similar evolution as COX-1 and COX-2, with a maximal induction after 15 hours of hypoxia. Again this induction is suppressed in B16BL6 cPHGPx cells (Figure 11).

Example 7: Effect of cPHGPx on the efficacy of TNFα tumor therapy

TNFα has a known anti-tumor activity by its anti-angiogenic activity on the tumor vasculature. However, it exerts also a tumor promoting effect due to its COX-2 inducing effect. This induction results in the production of tumor-promoting prostanoids. Therefore, high doses of TNFα are needed to obtain the desired anti-tumoral effect. As shown in Figure 12, inhibition of the COX-2 induction by cPHGPx results in a clear tumor reduction at lower dosage of TNFα. While the dose applied has no effect on the tumor development induced by B16BL6, nor on that induced by B16BL6 neo, there is a clear inhibition of tumor growth in the B16BL6 cPHGPx induced tumors. In one mouse, even a nearly complete regression was noticed.
This clearly indicates that cPHGPx expression enhances the efficacy of an anti-angiogenic treatment such as a TNFα therapy.

Example 8: Improved efficacy of photodynamic therapy by combination with cPHGPx

It is studied how the efficacy of photodynamic therapy (PDT) for cancer treatment can be improved through cPHGPx-overexpression. In PDT, a photosensitizer such as hypericine, which is preferentially taken up and retained by tumor cells, is exited by visible light. When returning to its ground state, hypericine generates superoxide radicals and/or singlet oxygen that promote tumor cell destruction (Agostinis *et al.*, 2002). Although PDT is an accepted anticancer modality, its application is limited to small, superficial tumors. The induction of survival mechanisms under suboptimal light stimulation is responsible for this limitation and involves the upregulation of COX-2 expression and PGE_2 production. Since cPHGPx-overexpression inhibits COX-2 activity as well as the induction of COX-2 in response to hypoxia, cPHGPx-overexpression suppresses this defense mechanism.
cPHGPx clearly suppresses COX-2 induction by PDT performed under suboptimal light stimulation. Combining cPHGPx with PDT extends the applicability of PDT to larger tumors and to a much wider range of tumor types.

Example 9: cPHGPx single-nucleotide polymorphism (SNP) analysis

So far, no literature data are available that correlate in any way cPHGPx with tumorigenesis. Yet, our results show that cPHGPx-overexpression suppresses tumor growth, therefore suggesting that the gene may act as a tumor suppressor gene. Most tissues display a low

cPHGPx-activity (Brigelius-Flohe *et al.*,2000). A mere 3-fold increase in cPHGPx activity is sufficient to repress tumor growth. Considering this narrow activity window, genetic polymorphisms that negatively affect the expression or activity level of the enzyme are likely to have direct consequences for the capacity of the enzyme to neutralize pro-tumor factors, generated during (chronic) inflammation. Such polymorphisms in fact predict an increased risk for developing cancer. *cPHGPx* SNPs are identified in a cohort of healthy volunteers (n = 25 to 30) and there is looked for aberrant frequencies of the identified SNPs in cancer patients, in first instance brain metastase from breast and lung cancers. SNPs deviating from the normal distribution are checked for their impact on cPHGPx enzymatic activity by gene transfection in human cell lines possessing low basal cPHGPx activity. This approach provides a genetic marker for the early identification of individuals at risk of developing cancer.

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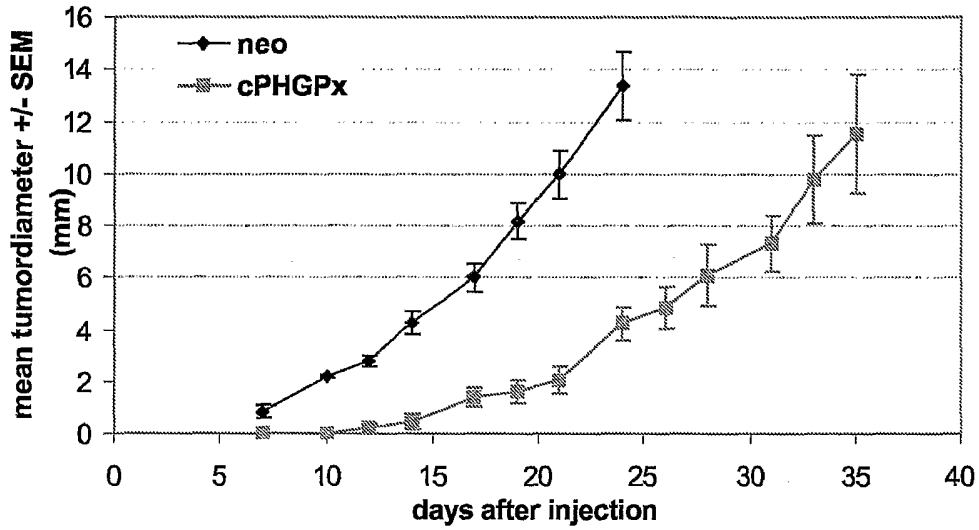
CLAIMS

1. The use of the cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene, or a functional fragment thereof, to modulate the onset of tumor formation and/or tumor growth.
2. The use of a compound, inducing the cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene, to delay the onset of tumor formation and/or to reduce tumor growth.
3. The use according to claim 2, whereby said compound is a superoxide radical and/or singlet oxygen
4. The use of cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) protein to delay tumor formation.
5. The use of cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) protein to reduce tumor growth.
6. The use of cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene to delay tumor formation.
7. The use of cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene to reduce tumor growth.
8. The use of the cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene, or a functional fragment thereof, to increase the efficacy of a tumor therapy.
9. The use of a compound, inducing the cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene to increase the efficacy of a tumor therapy.
10. The use of the cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) protein to increase the efficacy of a tumor therapy.
11. The use according to claim 8-10, whereby said tumor therapy is an anti-angiogenic therapy.
12. The use according to claim 11, whereby said anti-angiogenic therapy is a TNF treatment.
13. The use according to claim 8-10, whereby said tumor therapy is a photodynamic therapy.
14. The use of the cytosolic phospholipid hydroperoxide glutathione peroxidase (cPHGPx) gene, or a functional fragment thereof, for diagnosis related to cancer and malignancy.
15. The use according to claim 14, whereby said diagnosis is the determination of the likelihood to develop cancer
16. The use according to claim 14, whereby said diagnosis is the determination of the likelihood to develop metastasis
17. The use according to claim 14, whereby said diagnosis is the determination of the aggressiveness of a tumor

Fig. 1:


neo
cPHGPx
mean tumordiameter +/- SEM (mm)
days after injection
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8
10
12
14
16
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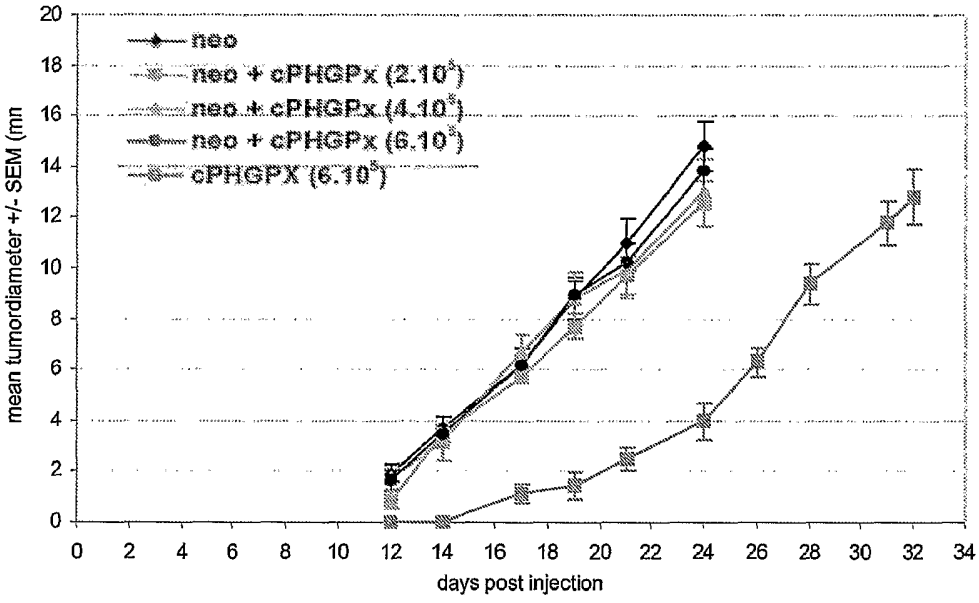
5
10
15
20
25
30
35
40

Fig. 2:


neo
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neo + cPHGPx (4.10⁵)
neo + cPHGPx (6.10⁵)
cPHGPX (6.10⁵)
mean tumordiameter +/- SEM (mm
days post injection
0
2
4
6
8
10
12
14
16
18
20
0
2
4
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8
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18
20
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26
28
30
32
34

Fig. 3:

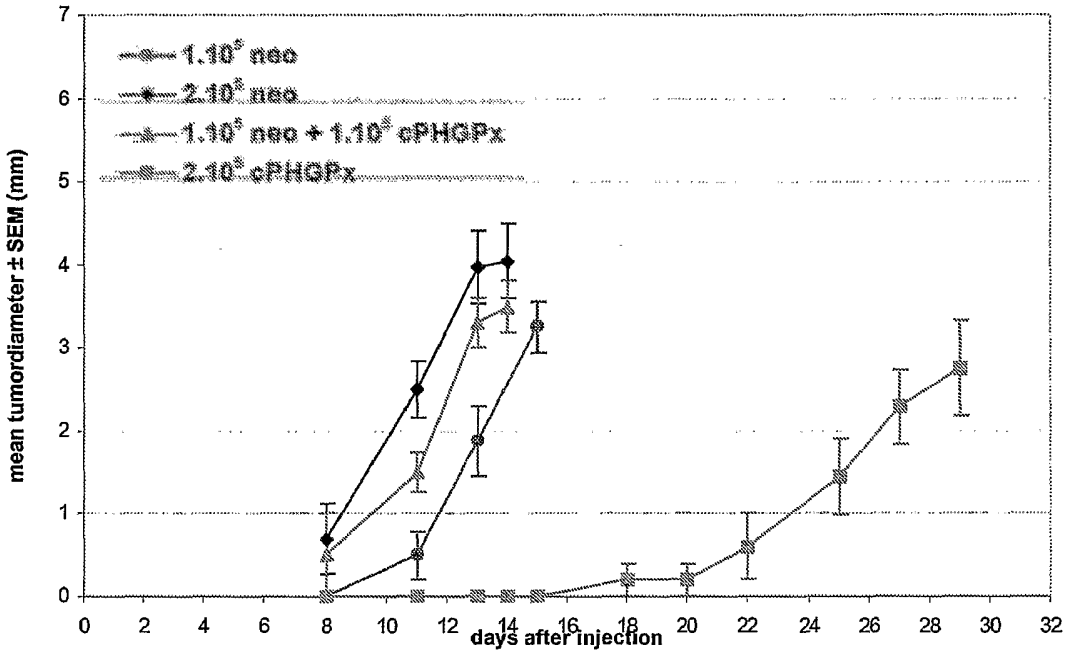
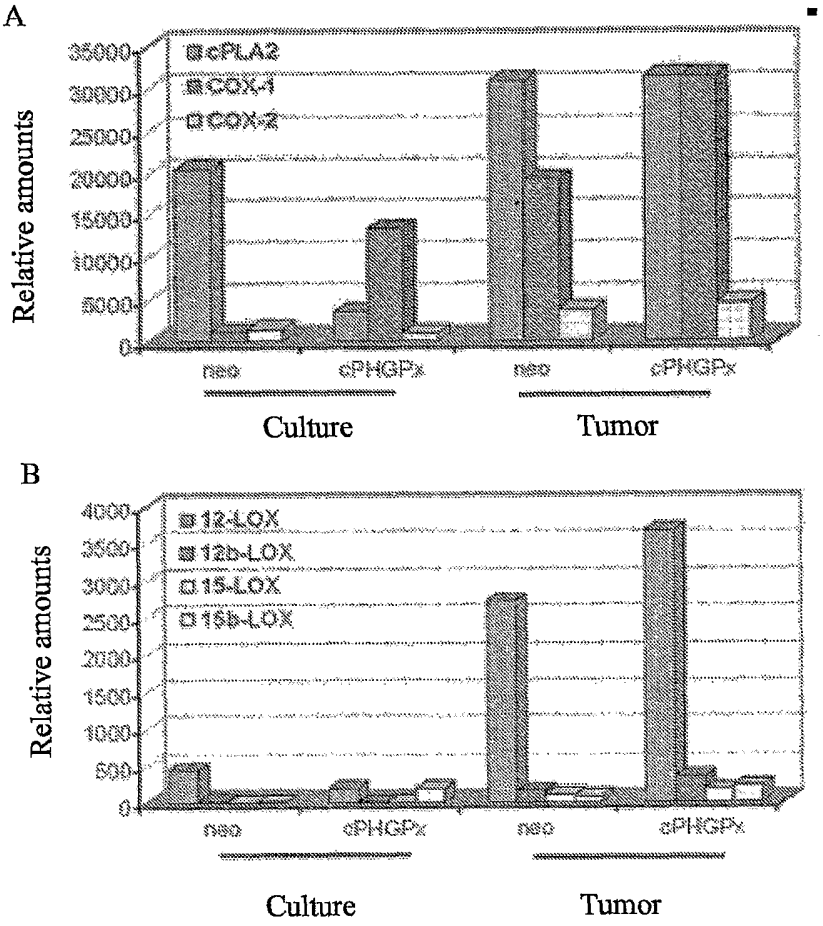

1.10^6 neo
2.10^6 neo
1.10^6 neo + 1.10^6 cPHGPx
2.10^6 cPHGPx
mean tumordiameter ± SEM (mm)
days after injection
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

Fig. 4:


A
cPLA2
COX-1
COX-2
Relative amounts
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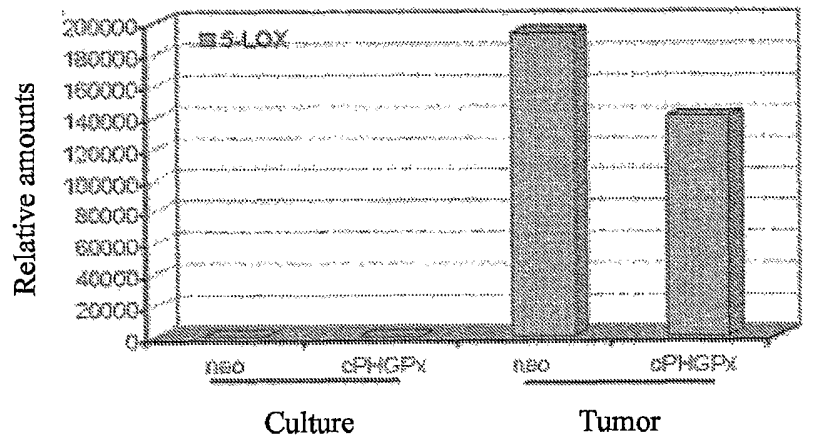
30000
25000
20000
15000
10000
5000
0
neo
cPHGPx
neo
cPHGPx
Culture
Tumor
B
12-LOX
12b-LOX
15-LOX
15b-LOX
Relative amounts
4000
3500
3000
2500
2000
1500
1000
500
0
neo
cPHGPx
neo
cPHGPx
Culture
Tumor

Fig. 4 C:


5-LOX
Relative amounts
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180000
160000
140000
120000
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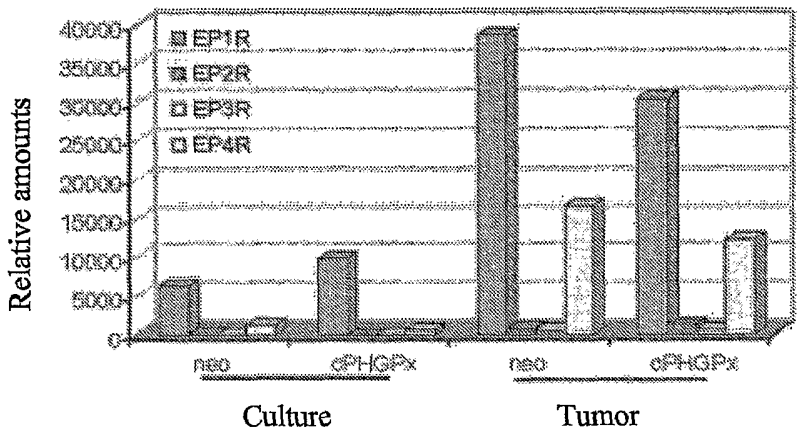
60000
40000
20000
0
neo
cPHGPx
neo
cPHGPx
Culture
Tumor

Fig. 4 D:


Relative amounts
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30000
25000
20000
15000
10000
5000
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EP2R
EP3R
EP4R
neo
cPHGPx
neo
cPHGPx
Culture
Tumor

Fig. 5:

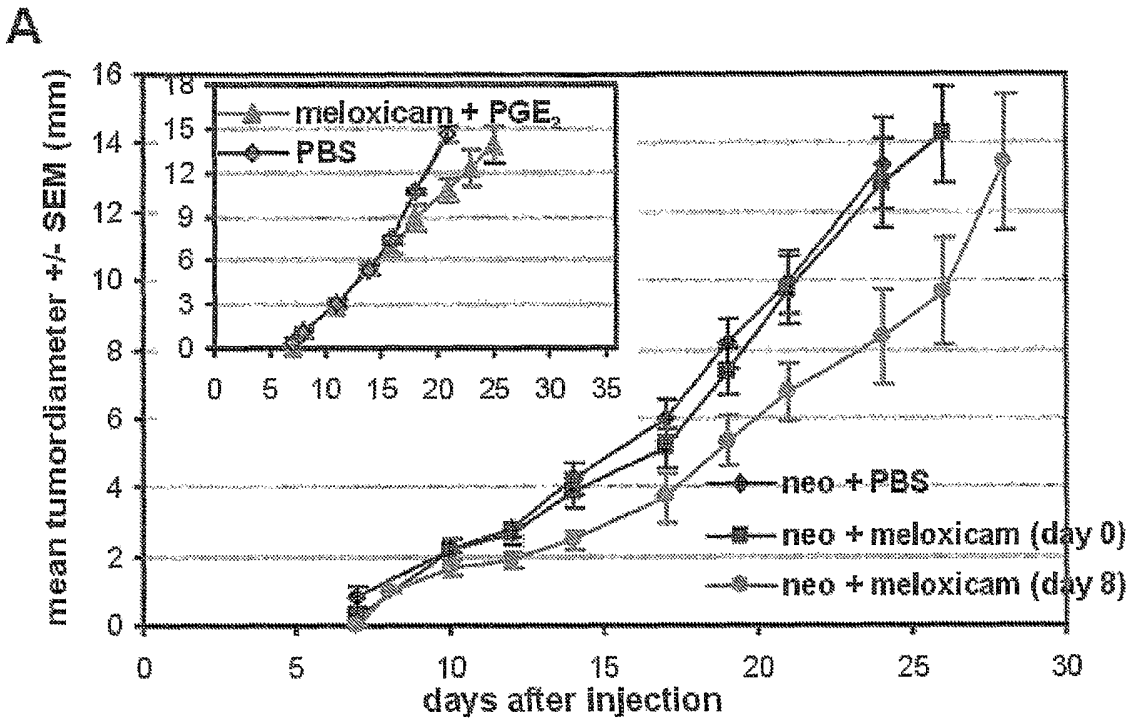

A
mean tumordiameter +/- SEM (mm)
days after injection
meloxicam + PGE2
PBS
neo + PBS
neo + meloxicam (day 0)
neo + meloxicam (day 8)

Fig. 5 (cont.):

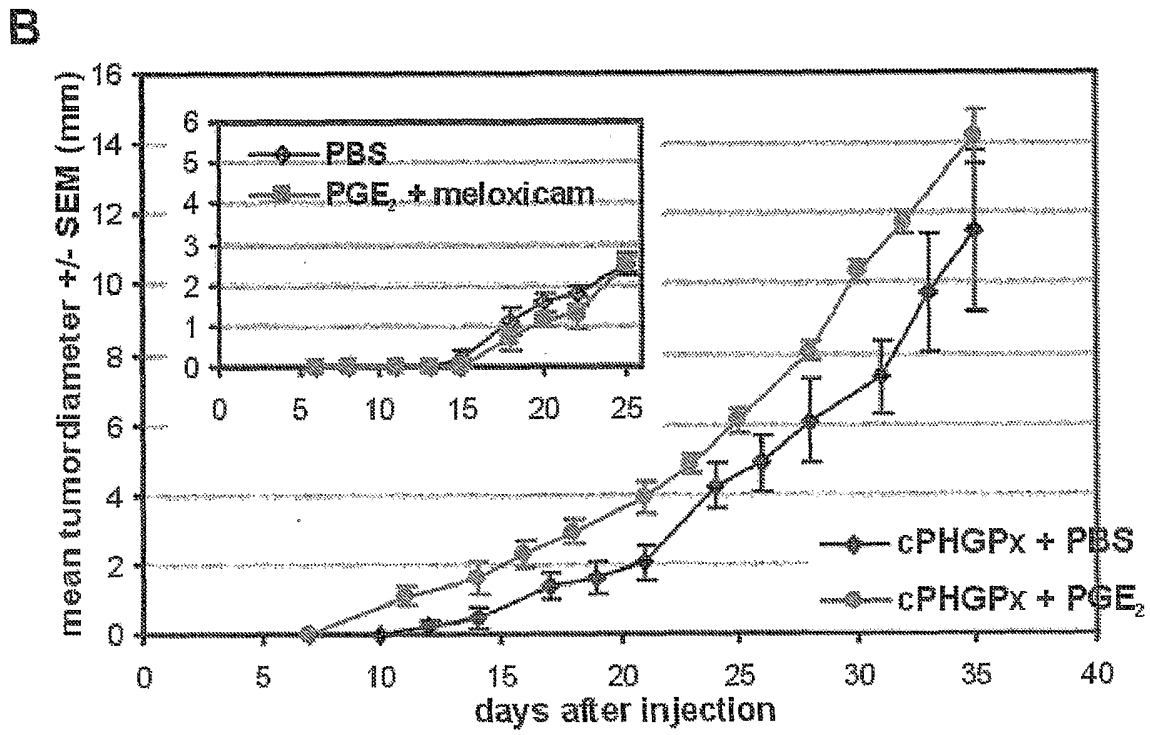


Fig. 6:

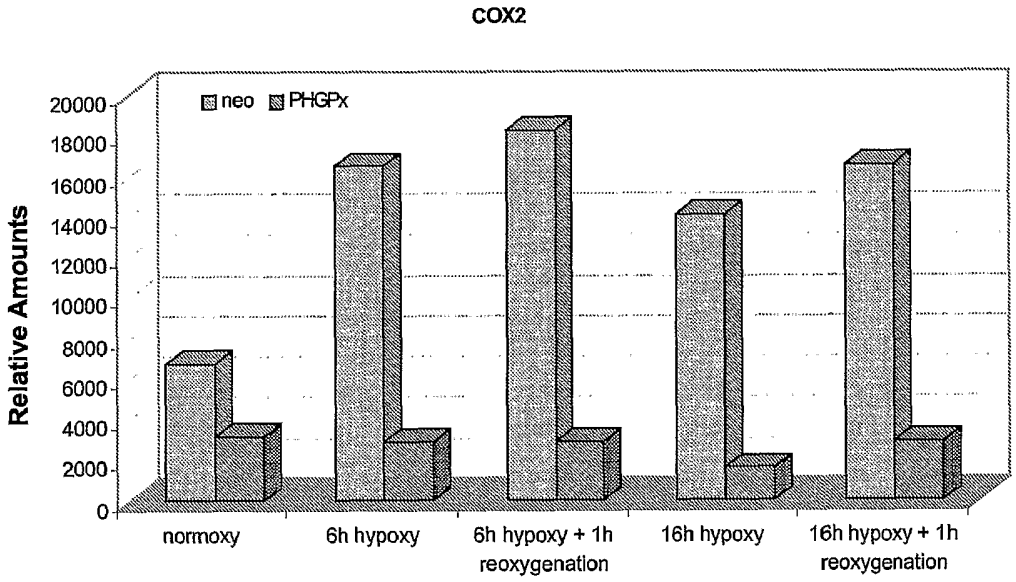


Fig. 7:

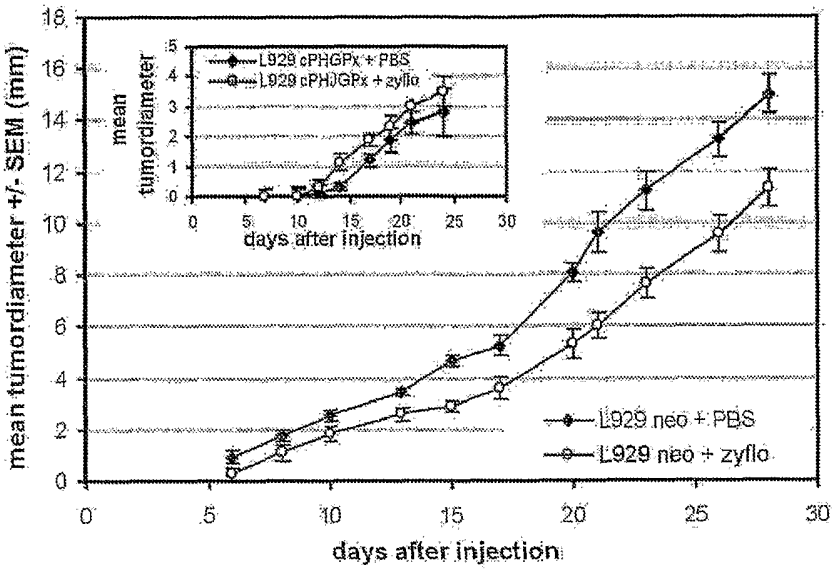

mean tumordiameter +/- SEM (mm)
days after injection
L929 neo + PBS
L929 neo + zyflo
mean tumordiameter
L929 cPHGPx + PBS
L929 cPHGPx + zyflo
days after injection

Fig. 8:

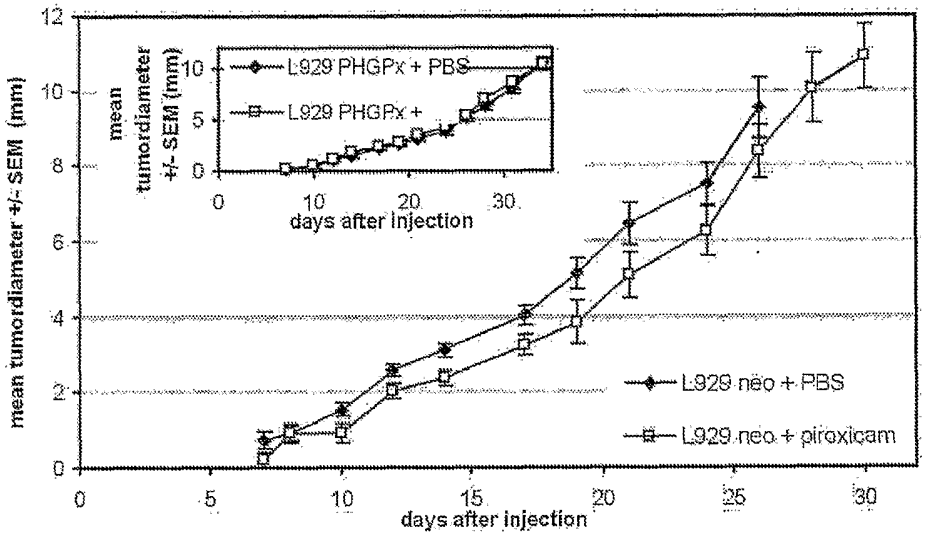
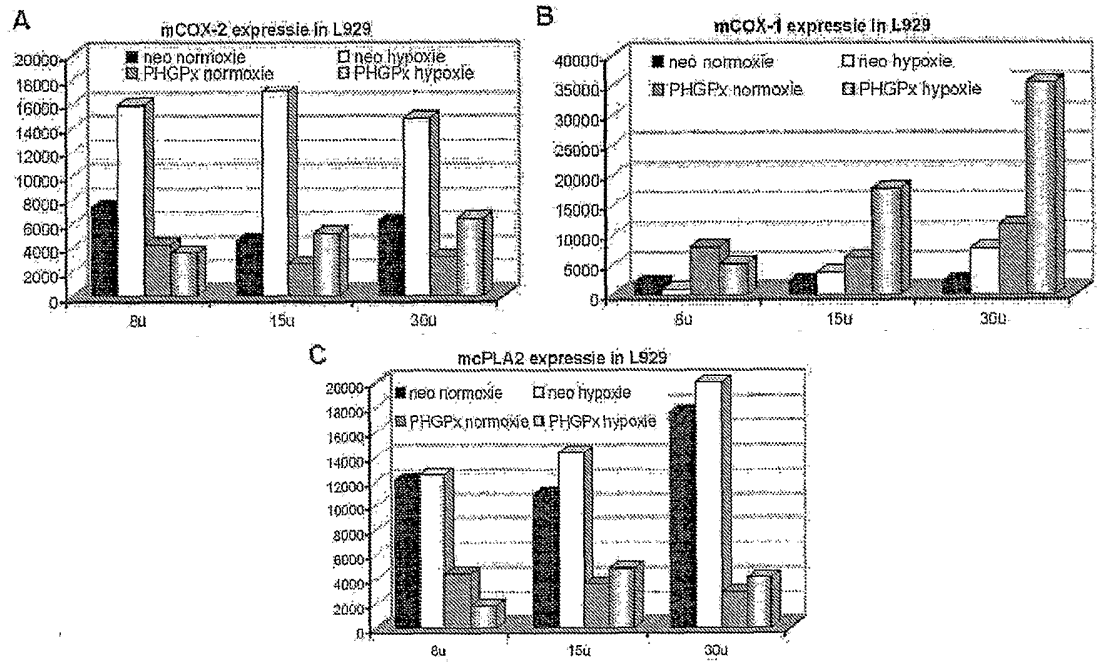

mean tumordiameter +/- SEM (mm)
days after injection
L929 neo + PBS
L929 neo + piroxicam
mean tumordiameter +/- SEM (mm)
L929 PHGPx + PBS
L929 PHGPx +
days after injection

Fig. 9:


A
mCOX-2 expressie in L929
neo normoxie
neo hypoxie
PHGPx normoxie
PHGPx hypoxie
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
8u
15u
30u
B
mCOX-1 expressie in L929
neo normoxie
neo hypoxie
PHGPx normoxie
PHGPx hypoxie
40000
35000
30000
25000
20000
15000
10000
5000
0
8u
15u
30u
C
mcPLA2 expressie in L929
neo normoxie
neo hypoxie
PHGPx normoxie
PHGPx hypoxie
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18000
16000
14000
12000
10000
8000
6000
4000
2000
0
8u
15u
30u

Fig. 10:

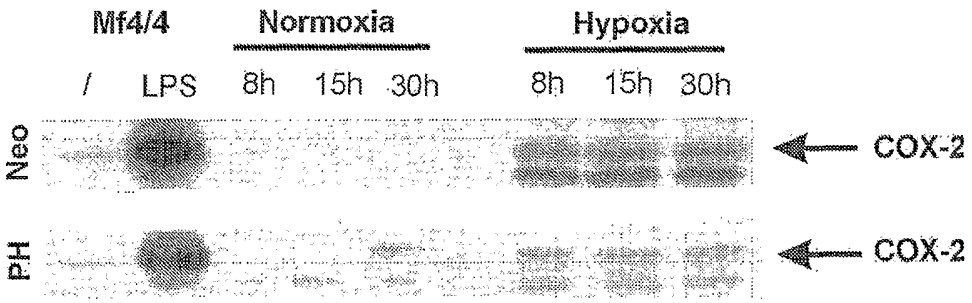

Mf4/4
Normoxia
Hypoxia
/
LPS
8h
15h
30h
8h
15h
30h
Neo
COX-2
PH
COX-2

Fig. 11:

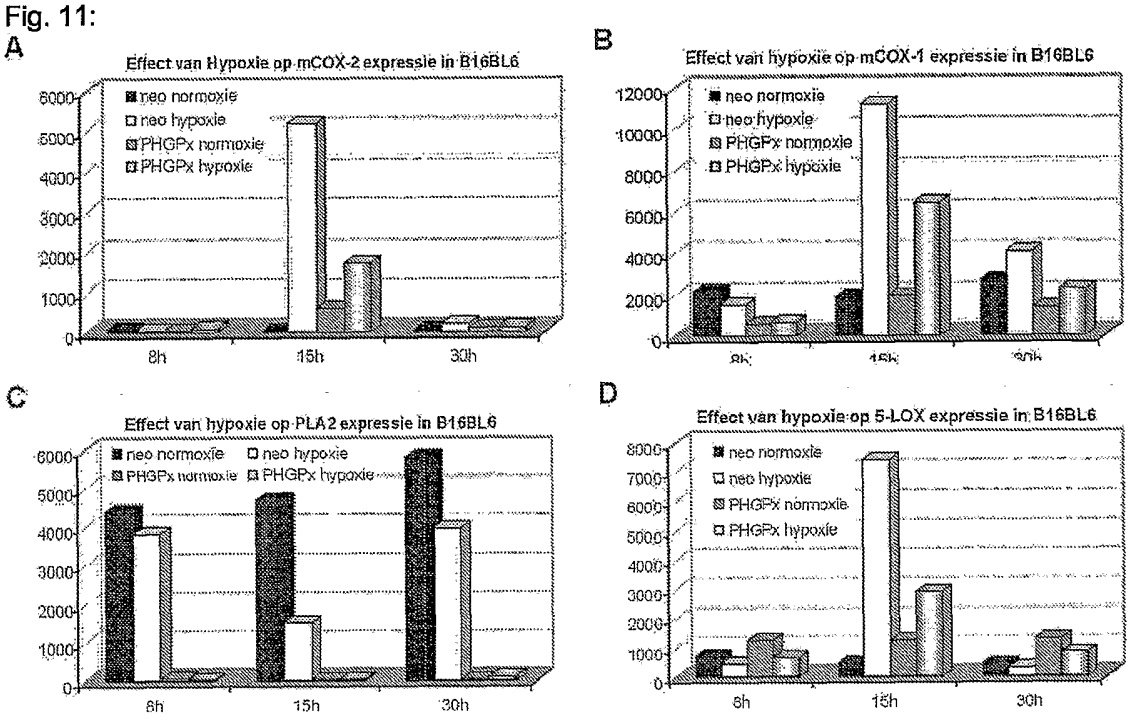
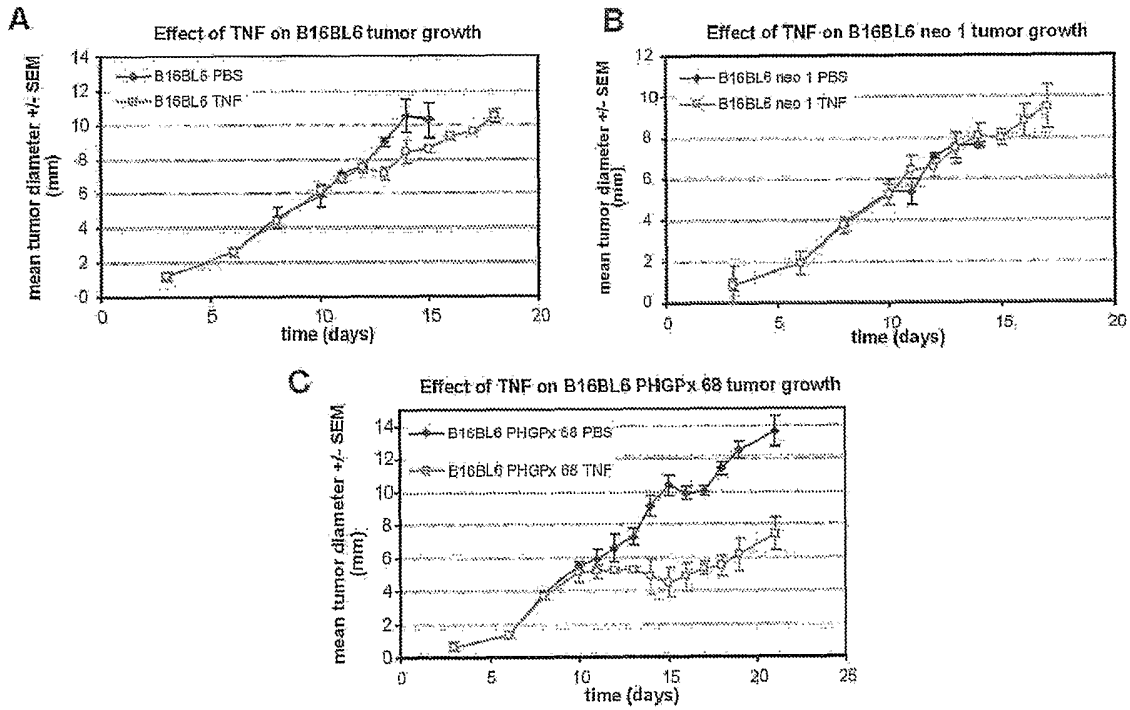

A
Effect van Hypoxie op mCOX-2 expressie in B16BL6
neo normoxie
neo hypoxie
PHGPx normoxie
PHGPx hypoxie
8h
15h
30h
B
Effect van hypoxie op mCOX-1 expressie in B16BL6
C
Effect van hypoxie op PLA2 expressie in B16BL6
D
Effect van hypoxie op 5-LOX expressie in B16BL6

Fig. 12:


A
Effect of TNF on B16BL6 tumor growth
B16BL6 PBS
B16BL6 TNF
mean tumor diameter +/- SEM (mm)
time (days)
B
Effect of TNF on B16BL6 neo 1 tumor growth
B16BL6 neo 1 PBS
B16BL6 neo 1 TNF
C
Effect of TNF on B16BL6 PHGPx 68 tumor growth
B16BL6 PHGPx 68 PBS
B16BL6 PHGPx 68 TNF

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PC T/EP2004/051216

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/44 A61K33/40 A61K49/00 A61P35/00 A61K38/19

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE, MEDLINE, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG HONG P ET AL: "Phospholipid hydroperoxide glutathione peroxidase induces a delay in G1 of the cell cycle." FREE RADICAL RESEARCH, vol. 37, no. 6, June 2003 (2003-06), pages 621-630, XP009022014 ISSN: 1071-5762 (ISSN print) page 621, paragraphs 1,2 page 628	1,4-7
X	WO 02/078718 A (HOWES RANDOLPH M) 10 October 2002 (2002-10-10) page 7, paragraph 30 - page 8, paragraph 33	2,3
A	page 3, paragraph 9	13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2004	05/10/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Deck, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOLBEN D H ET AL: "The diverse role of selenium within selenoproteins: a review." JOURNAL OF THE AMERICAN DIETETIC ASSOCIATION. UNITED STATES JUL 1999, vol. 99, no. 7, July 1999 (1999-07), pages 836-843, XP002263054 ISSN: 0002-8223 page 840, right-hand column, paragraph CANCER	2
A	page 838, right-hand column, last paragraph	1-17
X	EP 0 750 911 A (LIFE SCIENCE LABS INC) 2 January 1997 (1997-01-02) claims 1,2	2
A	WO 02/072626 A (BRIELMEIER MARKUS ;HAHN MEITNER INST BERLIN GMBH (DE); CONRAD MARC) 19 September 2002 (2002-09-19) claim 40 the whole document	1-17
Y	WANG HONG P ET AL: "Phospholipid hydroperoxide glutathione peroxidase protects against singlet oxygen-induced cell damage of photodynamic therapy" FREE RADICAL BIOLOGY AND MEDICINE, vol. 30, no. 8, 15 April 2001 (2001-04-15), pages 825-835, XP002263055 ISSN: 0891-5849 page 834, left-hand column, last paragraph	8-10,13
Y	LIN F ET AL: "SELENOPEROXIDASE-MEDIATED CYTOPROTECTION AGAINST MEROCYANINE 540-SENSITIZED PHOTOPEROXIDATION AND PHOTOKILLING OF LEUKEMIA CELLS" CANCER RESEARCH, vol. 52, no. 19, 1992, pages 5282-5290, XP001156229 ISSN: 0008-5472 page 5289, right-hand column, last paragraph	8-10,13
A	SUN Q ET AL: "EFFECT OF SELENIUM ON HUMAN PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE EXPRESSION AND HOST CELL SUSCEPTIBILITY TO LIPID HYDROPEROXIDE-MEDIATED INJURY" BIOCHEMISTRY AND MOLECULAR BIOLOGY INTERNATIONAL, ACADEMIC PRESS, LONDON, GB, vol. 42, no. 5, August 1997 (1997-08), pages 957-963, XP008014177 ISSN: 1039-9712 the whole document	

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MAIORINO M ET AL: "(47) PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE" METHODS IN ENZYMOLOGY, ACADEMIC PRESS INC, SAN DIEGO, CA, US, vol. 186, 1990, pages 448-457, XP000921458 ISSN: 0076-6879 the whole document	
Y	US 5 632 982 A (FOK KATHERINE S ET AL) 27 May 1997 (1997-05-27) claims 1,5,6	8-12
Y	US 2002/106348 A1 (FENG LI ET AL) 8 August 2002 (2002-08-08) paragraphs '0009!, '0088!; claims 1,2,10	8-12

INTERNATIONAL SEARCH REPORT

national application No.
PCT/EP2004/051216

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

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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

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

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-13 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.
Although claims 14-17 are directed to a diagnostic method practised on the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

formation on patent family members

Inte nal Application No
PC /EP2004/051216

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