The invention relates to the use of a gene or a gene product as a target for identifying a pharmacologically active compound, preferably for sensitizing a cancer cell for ionizing radiation. The invention further relates to methods for determining whether a compound is capable of sensitizing a cancer cell for ionizing radiation and to methods for increasing the susceptibility of a cell to radiation. Additionally, the invention relates to a compound for use in the treatment of an individual suffering from cancer, preferably wherein said treatment comprises irradiating said individual or part thereof with ionizing radiation. Furthermore, the invention relates to methods for determining the radiation sensitivity of a cancer in an individual. The preferred target of radiosensitization is GPR27.
Title: Means and method for increasing the sensitivity of cancers for radiotherapy.

The invention relates to the field of cancer, more in particular to the field of radiotherapy of cancer.

Radiotherapy (or radiation therapy; RT) is the most used treatment against cancer, in particular but not exclusively for prostate cancer, lung cancer, head and neck cancer, breast cancer, rectum cancer, oesophageal cancer, pancreatic cancer, cervical cancer, bladder cancer, lymphoma and brain tumors. Approximately 50% of all cancer patients are treated with RT. For many indications, radiotherapy is combined with other treatment modalities, such as surgery and chemotherapy. The biological basis for the therapeutic effects of RT is that the applied ionizing radiation (IR) causes damage to the cellular DNA. Cancer cells are generally more vulnerable to this damage than healthy cells.

For localized prostate cancer (PCa), the standard treatment is radiotherapy alone or in combination with prostatectomy. Moreover, for the treatment of advanced disease radiotherapy is also used as a palliative and adjuvant treatment. Advances in radiotherapy technology also allow precise irradiation of oligometastases with a curative intend. Radiotherapy of localized PCa can cure patients from the disease. However, in 30-40% of patients cancer recurs and progresses. In this case, a next cycle of treatment with irradiation is not possible anymore. Advanced prostate cancer has a very poor prognosis, emphasizing the need for more effective early local treatment, preventing advanced disease.

IR-induced DNA damage triggers a multitude of DNA damage response (DDR) signalling pathways in cells. These can result in cell cycle arrest, DNA damage repair and cell death. Differences in the functioning of these processes in different cells or under different conditions determine the final effect of a certain dose of IR. The irradiated cell could repair its damaged DNA and survive, continue to divide despite the damage, or die. The cause of cancer cells surviving irradiation could be intrinsic or acquired resistance of said cancer cells to irradiation. This
phenomenon is further referred to as "radioresistance". Mechanisms underlying radioresistance in various cancers are poorly understood. At present only few genes have been described to play a role in radiation response. These include e.g. ATM and DNA-PKcs, (Tannock et al. The Basic Science of Oncology (4th ed.), McGraw-Hill Ltd, New York (2005), pp. 77-99). ATM mutation is associated with high radiosensitivity. The DNA damage, more precisely DNA double-strand breaks (DSBs) induced by IR, trigger assembly of the MRN complex. The MRN complex binds to DSBs and activates ATM kinase activity, while ATM on its turn phosphorylates the DNA histone variant H2AX, γ-H2AX. The specific binding of γ-H2AX to the damaged DNA serves as a platform for binding of various proteins, including BRCA1, 53BP1 and MDC1 involved in the CDK independent DDR. This process initiates a signalling cascade which results in S-phase arrest and DNA synthesis blockage (Ribbalo et al. Mol Cell. 2004 Dec 3;16(5):715-24; Krempler et al Cell Cycle. 2007 Jul 15;6(14):1682-6). DNA-PKcs is involved in DNA damage repair, specifically in non-homologous end joining, NHEJ. After DSBs have been induced by IR they are recognized by the Ku70/Ku80 complex. This complex triggers binding of the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs. Ku70/Ku80 and DNA-PKcs form the DNA-PK complex that promotes binding of the Lig4/XRCC4/XLF complex to the damaged DNA ends resulting in the ligation of DNA ends and their repair. Cells deficient in any of the DNA-PK components are radiosensitive (Collis et al., Cancer Res. 63, 1550-1554, 2003; Cowell et al., Biochem. Pharmacol. 71, 13-20, 2005).

In addition, chemical compounds have been identified that cooperate with IR, resulting in increased killing of cancer cells. These compounds are referred to herein as "radiosensitizer drug", or "radiosensitizer" for short. Many chemotherapeutic drugs show stronger cancer cell killing effect when combined with IR. In this sense these chemotherapeutic drugs are considered radiosensitizers. Non-limiting examples of chemotherapeutic drugs with radiosensitizing property are 5-FU, platinum analogs such as cisplatin and oxaliplatin, gemcitabine and temozolamide. The mechanisms underlying the radiosensitization brought about by these compounds is usually poorly understood (reviewed in Katz et al., Int. J. Rad. Oncol. Biol. Phys. 73, 988-996, 2009). Many clinical trials have been done combining RT with chemotherapy. Meta-analyses
have shown that combination treatment is associated with modest clinical benefit and significantly increased toxicity to healthy tissue (Pignon et al., Lancet 355, 949-955, 2000; Bourhis et al, Semin. Oncol. 31, 822-826, 2004). Hence, there is a clear need for more effective and specific radiosensitizers.

The colony formation assay (CFA) is the golden standard assay commonly used to investigate radiosensitivity of cancer cells in vitro. The CFA is a cell survival assay that tests the ability of a single cell to grow into a colony after treatment. The radiosensitizing effect of a radiosensitizer can be described by its "radiosensitization factor" (RF), i.e., the ratio between the fraction surviving cancer cells (i.e., the surviving fraction, SF) after IR alone or IR plus an irrelevant compound or IR plus vehicle and the fraction surviving cancer cells after IR plus radiosensitizer. A RF of more than 1 indicates that a greater fraction of cancer cells is killed at the same IR dose. The RF can be different at different radiosensitizer concentration and IR dose. Another way to quantify the radiosensitizing effect is by calculating the "dose modifying factor" (DMF). The DMF is the ratio between the IR dose required to kill a certain fraction of cancer cells in the absence of the radiosensitizer and the IR dose required to kill the same fraction of cancer cells in the presence of the radiosensitizer. A DMF of more than 1 indicates that the same fraction of cancer cells is killed with a lower IR dose. The DMF can be different at different radiosensitizer concentration and fraction cancer cell survival. Patients with cancer are usually treated with low dose IR (i.e. up to 4 Gy) to minimize toxicity to healthy tissue. Multiple cycles of low dose IR may be given to increase the total dose IR. Hence, a radiosensitizer that exerts a radiosensitizing effect at low dose IR is particularly relevant.

Generally speaking, two types of radiosensitizers can be discriminated. The first type of radiosensitizer changes the properties of the irradiated cell to enhance DNA damage. Examples of this type of drug include molecules that increase or stabilize free radicals in the nucleus. The second type of radiosensitizer influences pathways that are involved in the response of cancer cells to IR. Non-limiting examples of this type of drug includes molecules that inhibit cell cycle arrest, DNA repair or cell death. This second type of radiosensitizer is referred to
as "biological radiosensitizer". The present invention relates in particular to biological radiosensitizers and to their targets (i.e., the cellular molecules whose function or activity is affected by the biological radiosensitizer) and to the genes encoding said targets.

Examples of previously identified biological radiosensitizers include e.g. inhibitors of the EGFR pathway, farnesyl transferase inhibitors, VEGF inhibitors, an ATM inhibitor and a DNA-PKcs inhibitor (reviewed in Katz et al., Int. J. Rad. Oncol. Biol. Phys. 73, 988-996, 2009). The experience with several of these compounds is discussed here. The PI3K inhibitor wortmannin induced sensitivity to IR in mouse tumors (Kim et al., J. Radiat. Res. 48, 187-95, 2007). Even though it proved an effective radiosensitizer in preclinical studies, its clinical use was limited by poor solubility, low stability, and high toxicity (Karve et al., Proc. Natl Acad Sci. USA 109, 8230-8235, 2012; Garcia-Echeverria et al., Oncogene 27, 5511-5526, 2008). Caffeine sensitizes tumor cells for RT through inhibition of ATM (Blasina et al., Curr. Biol. 9, 1135-1138, 1999; Sarkaria et al., Semin. Radiat. Oncol. 11, 316-327, 2001). Caffeine in combination with RT has been tested in 3 clinical trials with contradictory results (Takeuchi et al., Anticancer Res. 27, 3489-3495, 2007; Heilbrun et al., Am. J. Clin. Oncol. 26, 543-549, 2003; Tsuchiya et al., Anticancer Res. 20, 2137-4213, 2000). In preclinical experiments, tirapazamine (TPZ) sensitized head and neck, and cervix cancer cells to low dose irradiation (Richin et al. J. Clin. Oncol. 23, 79-87, 2005). However, in a phase III clinical trial, overall survival of head and neck cancer patients was not improved, compared to RT alone (Richin et al., J. Clin. Oncol. 28, 2989-2995, 2010). Farnesyl transferase regulates Ras activity at a posttranslational level. The treatment combination of RT and farnesyl transferase inhibitor FTI-277, L-744,832 and L-778,123 compounds resulted in significant reduction of xenograft tumor cell survival (Cohen-Jonathan et al. Radiat Res. 154, 125-32, 2000). The farnesyl transferase inhibitor L-778,123 entered a phase I clinical trial in combination with RT in HNSCC and NSCLC patients. In this study, 5 out of 9 patients had a complete response to RT and L-778,123 and no disease progression was observed 7-12 months after treatment. However, the results were difficult to interpret since none of the patients had a Ras mutation (Hahn et al., 2002. Clin. Cancer Res. 8, 1065-1072, 2002).
combination of RT with the inhibition of EGFR with Cetuximab induced complete response in a preclinical HNSCC xenograft model in mice (Nasu et al., Int. J. Radiat. Oncol. Biol. Phys. 51, 474-477, 2001). Moreover, this drug showed its radiosensitizing effect in a phase III trial with HNSCC patients (Bonner et al., N. Eng. J Med. 354, 567-578, 2006). However, the effect of Cetuximab on radiosensitivity is not better than the modest effect of cisplatin on radiosensitivity (Levy et al., Curr. Med. Res. Opin. 27, 2253-9, 2011). Manipulation of hypoxia is already for years under investigation as approach to sensitize tumor cells to RT. Preclinically, inhibition of VEGF with bevacizumab showed a decrease in tumor hypoxia and a better response to IR (Ceng et al., Cancer Res. 61, 2413-2419, 2001). Currently, bavacizumab is being used in cancer patients in combination with various chemotherapies and as an adjuvant drug in chemo-radiotherapy. However, observed effects are only slightly positive (Vredenburgh et al., Clin. Cancer Res. 17, 4119-24, 2011). Hence, there is a clear need to identify new targets and drugs for more effective sensitization of cancer cells to irradiation.

The emergence of high-throughput screening (HTS) and RNA interference (RNAi) technologies now allow comprehensive enterprises to identify new targets for radiosensitization. Several radiosensitization targets have been identified this way. For example, using 53BP1 focus formation shortly after IR as readout, Kolas et al. (Science 318, 1637-1640, 2007) identified RNF8 as a regulator of the DDR promoting radioresistance. Sudo et al. (Biochem. Biophys. Res. Comm. 364, 695-701, 2007) found that combining 4 Gy irradiation with silencing ATM, ATR, CCNG1, CDKNIA, CENPE, H3F3A, IL13RA1, TRIP11, UCC1, ZDHHC8, ZNF146, or ZNF354A reduced survival of HEK293 cells compared to irradiation alone. The effect of silencing these genes per se was not investigated. Higgins et al. (Cancer Res. 70, 2984-2993, 2010) identified POLQ as cancer-specific radioresistance gene by siRNA HTS on SQ20B laryngeal cancer cells and MRC5 normal fibroblasts. More recently, Ni et al. (J. Clin. Invest. 121, 2383-2390, 2011) performed an siRNA screen targeting 249 genes, primarily encoding DNA repair proteins, on DU145 prostate cancer cells. They identified 10 candidate targets, 6 of which were confirmed using the CFA and in a second prostate cancer cell line, LNCaP. Radiosensitizing effects, expressed by the DMF(0.1), i.e. the DMF at 10%
cell survival, were 1.3 for silencing NBN (also known as NBS1), RAD23B and RAD54L; and 1.6 for silencing DNA-PKcs, MAD2L2 and BRCA2.

Cancer cells can be sensitised to IR by silencing expression of genes involved in radioresistance by means of RNAi technology. Other means to accomplish this include the use of antisense RNA, RNA decoys, ribozymes or zinc finger proteins. Alternatively, new chemical drugs can be designed to inhibit the function or activity of the proteins encoded by said genes. Very recently, a useful tool for objectively prioritizing candidate targets for chemical drug development was presented (Patel et al., Nat. Rev. Drug Discov. 12, 35-50, 2013). Databases can be searched for already available drugs known to inhibit a candidate target protein. In particular drugs that are already approved for use in humans for a different purpose could relatively rapidly be developed as a new radiosensitizer. In addition, structural information on candidate target proteins can be retrieved to enable structure-based new drug design.

Summary of the invention

The invention provides the use of a gene of table 3a, or an RNA or protein encoded by the gene as a target for identifying a pharmacologically active compound. The use is preferably for identifying a pharmacological compound active in sensitizing a cancer cell for ionizing radiation.

The invention also provides a method for determining whether a compound is capable of sensitizing a cancer cell for ionizing radiation comprising (a) determining whether a compound is capable of inhibiting the activity of a gene of table 3a or an RNA or protein encoded by the gene, said method further comprising (b) determining whether the compound identified in step (a) is capable of sensitizing a cancer cell for ionizing radiation.

Further provided is a method for increasing the susceptibility of a cell to radiation, comprising introducing into the cell and/or contacting the cell with, a compound selected from:
- an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, an RNAi molecule (siRNA or miRNA) or a precursor thereof or a ribozyme capable of binding under stringent hybridization conditions to a gene or an mRNA gene product of the genes selected from the group consisting of genes of table 3a;

- a small molecule interfering with the biological activity of a gene product of a gene selected from the group consisting of genes of table 3a;

- a (glyco)protein, a hormone or other biologically active compound capable of interacting with a gene selected from the group consisting of genes of table 3a, or with a gene product thereof, and

- an antibody that inhibits the activity of a gene product of a gene selected from the group consisting of genes of table 3a.

The method preferably further comprises irradiating said cell with ionizing radiation. The cell is preferably irradiated with 1-6 Gy. The cell is preferably a cancer cell. In a preferred embodiment the cell is a prostate cell.

The invention further provides a compound selected from:

- an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, an RNAi molecule (siRNA or miRNA) or a precursor thereof or a ribozyme capable of binding under stringent hybridization conditions to a gene or an mRNA gene product of the genes selected from the group consisting of genes of table 3a;

- a small molecule interfering with the biological activity of a gene product of a gene selected from the group consisting of genes of table 3a;

- a (glyco)protein, a hormone or other biologically active compound capable of interacting with a gene selected from the group consisting of genes of table 3a or with a gene product thereof, and

- an antibody that inhibits the activity of a gene product of a gene selected from the group consisting of genes of table 3a,

for use in the treatment of an individual suffering from cancer.

The treatment preferably further comprises irradiating said individual or part thereof with ionizing radiation.
The invention further provides a compound selected from:
- an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, an RNAi molecule (siRNA or miRNA) or a precursor thereof or a ribozyme capable of binding under stringent hybridization conditions to a gene or an mRNA gene product of the genes selected from the group consisting of genes of table 3a,
- a small molecule interfering with the biological activity of a gene product of a gene selected from the group consisting of genes of table 3a,
- a (glyco)protein, a hormone or other biologically active compound capable of interacting with a gene selected from the group consisting of genes of table 3a or with a gene product thereof, and
- an antibody that inhibits the activity of a gene product of a gene selected from the group consisting of genes of table 3a,

for use in the manufacture of a medicament for treatment of an individual with ionizing radiation, whereby the compound sensitizes a cancer cell of the individual for the ionizing radiation.

The invention further provides a method for determining the radiation sensitivity of a cancer in an individual or predicting the response of a cancer in an individual to radiation therapy, comprising detecting a gene or gene product of a gene of table 3a in the blood and/or a cancer cell of said individual.
Brief description of the drawings

**Figure 1.** Irradiation dose response curves of prostate cancer cell lines PC-3 and DU-145, as measured using with two different assays, i.e. the colony formation assay (CFA; open squares) and the Acumen assay (closed triangles). The two assays yield similar curves that follow a linear-quadratic equation typical for irradiation dose-effects.

**Figure 2.** The effect of DNA-PKcs silencing on irradiation dose response of PC-3 cells, as measured using two different assays, i.e. the CFA (left panel) and the Acumen assay (right panel). Cells were transfected with siRNA against DNA-PKcs (siDNA-PKcs; closed squares) or with irrelevant control siRNA (siCon; open triangles) before irradiation. Both assays detect the radiosensitizing effect of DNA-PKcs silencing.

**Figure 3.** Confirmation screen for PC-3 cell survival with and without 4Gy IR and silencing of 45 primary hit siRNAs. (A) Cell numbers after gene silencing only (white bars) or after gene silencing followed by IR (black bars). (B) Relative cell survival after gene silencing and IR compared to gene silencing only.

**Figure 4.** Target validation by colony formation assay. Each panel shows the irradiation dose response curve for cells transfected with siRNA directed against a different candidate target gene as indicated (closed triangles) and the dose response curve for cells transfected with siRNA directed against an irrelevant control siRNA (open circles). Data are the mean results of two independent experiments.

**Figure 5.** IR dose response effects on colony formation of PC-3 cells stable transduced with lentiviral vectors expressing shRNAs silencing candidate radiation susceptibility genes. Cells were seeded, irradiated 0-6Gy and allowed to form colonies. Colonies were counted and survival curves were fitted using the linear quadratic equation on the average data of 2 independent experiments. Most panels show the results obtained with cells transduced with a negative control.
lentiviral vector (scr) and two independent cell cultures transduced with two different lentiviral vectors targeting a distinct mRNA sequence on the same target gene. The lower right panel shows the results obtained with cells transduced with a negative control lentiviral vector (scr) and three cell cultures transduced with different lentiviral vectors targeting a different target gene.

**Figure 6.** Silencing GPR27 sensitizes prostate cancer cells to fractionated irradiation. PC-3 and DU145 human prostate cancer cells stable transduced with a lentiviral vector expressing a short hairpin silencing GPR27 (black bars) or with a negative control lentiviral vector (white bars) were subjected to 1, 3 or 5 daily fractions of 2Gy IR. Cell survival was assessed using the CFA. Data are means ±SD of three (PC-3) or two (DU145) experiments performed in duplicate.

**Figure 7.** Rescue experiment validating GPR27-dependent radiation protection. PC-3 parental cells (WT; left panel) and PC-3 cells stable expressing a GPR27mut coding ORF carrying silent nucleotide mutations in the binding site of siGPR27 D-005562-02 (ORF; right panel) were transfected with siGPR27 D-005562-02 (open symbols) or non-targeting control siRNA (closed symbols). Cells were irradiated 0-6Gy and allowed to form colonies. Colonies were counted and survival curves were fitted using the linear quadratic equation on the average data of 2 independent experiments.

**Figure 8.** Cell cycle distribution of PC-3/LV-shSCR cells and PC-3/LV-shGPR27-ElI cells at 4, 16, 24 and 32 hours after 4Gy irradiation, as determined by FACS analysis. Results are the average of two independent experiments.
Detailed description of the invention

A gene or a product of a gene can be a target for identifying compounds that inhibit the expression of the gene, or that inhibit the activity of a gene product of the gene.

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in non-protein coding genes such as e.g. ribosomal RNA (rRNA), transfer RNA (tRNA) or small nuclear RNA (snRNA) genes, the product is a functional RNA. Several steps in the gene expression process may be modulated, including the transcription, RNA splicing and translation. Transcription can be inhibited by providing the cell with a compound that interferes with a protein that binds at or near the promoter/enhancer of the gene. Typical compounds are hormones. Nowadays it is very common to target the RNA that is produced by transcription of the gene. Non-limiting examples of such compounds are RNAi molecules, small nuclear RNA molecules (such as but not limited to miRNAs) that inhibit translation or destabilize the mRNA and exon-skip AON that interfere with the incorporation of a targeted exon in the mature mRNA. Exon-skipping can be used to delete specific domains from a protein but it can also be used to inactivate the target gene by skipping an exon that leaves an out of frame coding region and thereby reduce the amount of functional protein being produced.

The biological target for the drug or compound is most commonly a protein such as an enzyme, a receptor, transporter or channel.

The drug or compound typically interacts with the protein. The interaction can be engaging in a covalent bond. But more often the interaction is through non-covalent interactions. The interaction can also be reversible covalent, - in this case, a chemical reaction occurs between the stimulus and target in which the stimulus becomes chemically bonded to the target, but the reverse reaction also readily occurs in which the bond can be broken. The stimulus is permanently bound to the target when the interaction is through irreversible chemical bond formation. The interaction can lead to a steric hindrance or inactivation of the catalytic site. However, more often the interaction interferes with conformational changes in the biological target thereby acting as an inhibitor (prevention of a
conformational change into an active form) or activator (induction of an active conformational form. The term biological target or drug target is frequently used in pharmaceutical research to describe the native protein in the body whose activity is modified by a drug resulting in a desirable therapeutic effect. In this context, the biological target is often referred to as a drug target. The most common drug targets of currently marketed drugs include:

- proteins;
  - G-protein-coupled receptors (target of 50% of drugs); enzymes (especially protein kinases, proteases, esterases, and phosphatases);
  - ion channels; ligand-gated ion channels; voltage-gated ion channels;
  - nuclear hormone receptors; structural proteins such as tubulin; membrane transport proteins;

- nucleic acids
  - Typical nucleic acid targets are (pre-)mRNA, miRNA, promoters and enhancers.

Where herein reference is made to a gene or gene product of table 3a it is preferred that the gene or gene product is a gene or gene product of table 3a, more preferably of table 3b, more preferably of table 3c. In a particularly preferred embodiment the gene or gene product is a gene or gene product of table 3d. Preferably the gene is GPR27 or CPNE7. Preferably the gene product is a gene product of the gene GPR27 or the gene CPNE7. In a preferred embodiment the gene is the GPR27 gene. In a preferred embodiment the gene product is a gene product of the GPR27 gene.

The gene as target is preferably a gene of table 3b, or a gene as listed here GPR27; CPNE7; RNF8; RGNEF; HTR1E; CBL; CCNB1; GJB3; KCNQ3; OR2F1; RPL3L; FAM174B; ITPKA; DGAT2; SLC22A10; PDILT; HOXD3; IFNA7; MGC2705; ENTPD5; NLRP9; FBX04; PDCD4; CCK; MTX1; CLIC5; SIAH1; FZD1; FFAR1; KRTAP5-11; ANKRD34B; ZBTB43; MEIS1; MMP7; PEX16; ANXA13; USP33; CD163; MME; GPER; HIG2; PPP1R12B; MAEA; or EML5; The gene product as target is preferably a gene product of a gene of table 3b, or a gene product of a gene as listed here GPR27; CPNE7; RNF8; RGNEF; HTR1E; CBL; CCNB1; GJB3;
Preferably the gene as target is GPR27 or CPNE7. In a preferred embodiment the gene as target is the GPR27 gene.

The cancer can be any type of cancer. In a preferred embodiment the cancer is brain cancer, head/neck cancer, lung cancer, liver cancer, pancreatic cancer, skin cancer, gastrointestinal cancer, kidney cancer, ovarian cancer, breast cancer, prostate cancer or a cancer originating from the hemopoietic system. In a preferred embodiment the cancer is brain cancer, head/neck cancer, lung cancer, gastrointestinal cancer, or prostate cancer. In a particularly preferred embodiment the cancer is a prostate cancer.

The radiation is preferably ionizing radiation. Ionizing radiation typically encompasses alpha particles, beta particles, Gamma rays, X-rays, and in general any charged particle moving at relativistic speeds. Neutrons are considered ionizing radiation at any speed. Ionizing radiation includes some portion of the ultraviolet spectrum, depending on context. Radio waves, microwaves, infrared light, and visible light are normally considered non-ionizing radiation, although very high intensity beams of these radiations can produce sufficient heat to exhibit some similar properties to ionizing radiation, by altering chemical bonds and removing electrons from atoms. Ionizing radiation is typically quantified by the absorbed dose indicated in gray. The gray (symbol: Gy) is the SI derived unit of absorbed dose, specific energy (imparted) and of kerma. Such energies are typically associated with ionising radiation such as X-rays or gamma particles or with other nuclear particles. It is defined as the absorption of one joule of such energy by one kilogram of matter. The gray is always defined independently of any target material.

A population of cells, preferably cancer cells, is said to be sensitized to radiation, i.e. rendered more susceptible to radiation, when the death of (a...
proportion of) the cells can be induced at a lower radiation dose than prior to the radiosensitizing treatment. The factor by which the dose can be lowered by the radiosensitization treatment to achieve the same cell killing effect as is achieved without radiosensitization treatment is called the "dose modifying factor" or DMF. A population of cells is also said to be sensitized to radiation if the same dose of radiation induces a larger proportion of cells to die than prior to the radiosensitizing treatment. The fold increase in cell killing effect achieved by irradiation following radiosensitization treatment compared to irradiation alone is referred to here as the "radiosensitization factor " or RF. In a preferred embodiment the sensitization or increase in susceptibility is such that the DMF is at least 1.3 and/or the RF is at least 1.1. In a more preferred embodiment the sensitization or increase in susceptibility is such that the DMF is at least 1.6 and/or the RF is at least 1.3, even more preferably the DMF is at least 2.0 and/or the RF is at least 1.5, most preferably the DMF is at least 3.0 and/or the RF is at least 2.0. The sensitization or susceptibility is preferably measured by means of the CFA assay described in the examples.

The amount of radiation can be between 1 and 10 gray. The higher doses are typically so high that a lot of damage is done to non-carcinogenic surrounding tissue. As at high dose many irradiated cells die anyway there is less need for a radiosensitizing effect at high dose than at a lower dose. The invention provides compounds that induce profound radiosensitization also - and in particular - at low dose IR. The amount of radiation is preferably between 1 and 6 gray. In a particularly preferred embodiment the dose is between 2 and 4 gray. Cells may be irradiated in one single session or in several sessions. In the case of several sessions, it is preferred that the cells are irradiated with the preferred dose at every session. The exception is when the cells are irradiated in several sessions in one day. In that case the individual doses must be added up to arrive at the preferred total dose for the day.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring
amino acid polymers. Polypeptides can be modified, e.g., by the addition of carbohydrate residues to form glycoproteins. The terms "polypeptide", "peptide" and "protein" include glycoproteins and proteins comprising any other modification, as well as non-glycoproteins and proteins that are otherwise unmodified. A peptide is typically a polymer of up to 30 amino acids. Longer amino acid polymers are typically referred to as a polypeptide. A protein is a polypeptide or a collection of one or more polypeptides with zero, one or more peptides.

The terms "affecting the expression" and "modulating the expression" of a protein or gene, as used herein, should be understood as regulating, controlling, blocking, inhibiting, stimulating, enhancing, activating, mimicking, bypassing, correcting, removing, and/or substituting said expression, in more general terms, intervening in said expression, for instance by affecting the expression of a gene encoding that protein and/or of the gene product itself.

The terms "individual", "subject" or "patient" are used interchangeably herein and include, but are not limited to, an organism; a mammal, including, e.g., a human, non-human primate, mouse, pig, cow, goat, cat, rabbit, rat, guinea pig, hamster, horse, monkey, sheep, or other non-human mammal; and a non-mammal, including, e.g., a non-mammalian vertebrate, such as a bird (e.g., a chicken or duck), an amphibian and a fish, and a non-mammalian invertebrate. In the context of therapy it is preferred that the "individual", "subject" or "patient" is a human.

The term "homologous" as used herein refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared x 100. Homologous sequences (referred to herein as "homologues"), preferably have more than 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99,5 or more percent identity with one another. The genes provided herein are the human forms. The skilled person will appreciate that any mammalian and preferably human homologue is expressly intended to be included herein.

The term "pharmaceutically acceptable carrier" refers to a carrier for
administration of a therapeutic agent, such as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Pharmacologically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmacologically acceptable carrier.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent a disease or condition, in particular cancer, or to exhibit a detectable therapeutic or preventative effect. The effect can be detected by, for example, chemical markers or antigen levels, imaging such as by using MM, PET, SPECT or CT, or molecular, biochemical or histological examination of a tumor (biopsy) sample taken from the body of a patient. Therapeutic effects also include reduction in physical symptoms. The precise effective amount for a subject may depend e.g. upon the subject's size and health, the nature and extent of the condition, other treatments being given previously or simultaneously, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation and is within the judgment of the clinician.

For purposes of the present invention, an effective dose will be from about 0.01 mg/kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the polynucleotide, polypeptide, small molecule, or antibody compositions in the individual to which it is administered.
The term "functional fragment" refers to a shortened version of a protein, which is a functional variant or functional derivative. A "functional variant" or a "functional derivative" of a protein is a protein the amino acid sequence of which can be derived from the amino acid sequence of the original protein by the substitution, deletion and/or addition of one or more amino acid residues in a way that, in spite of the change in the amino acid sequence, the functional variant or derivative retains at least a part of at least one of the biological activities of the original protein that is detectable for a person skilled in the art. A functional variant is generally at least 50% homologous (preferably the amino acid sequence is at least 50% identical), advantageously at least 70% homologous and even more advantageously at least 90% homologous to the protein from which it can be derived. A functional variant may also be any functional part of a protein; the function in the present case being the capacity to inhibit the activity of a gene of table 3a or an RNA or protein encoded by said gene. Preferably the amino acid sequence differs from the native protein sequence mainly or only by conservative substitutions. More preferably the protein comprises an amino acid sequence having 90% or more, still more preferably 95%, sequence identity with the native protein sequence and optimally 100% identity with those sequences. "Functional" as used herein means functional in mammals, preferably human patients.

The term "antibody" includes reference to antigen-binding peptides and refers to antibodies, monoclonal antibodies, to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule. Examples of such peptides include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, complementarity determining regions (CDRs), VL (light chain variable region), VH (heavy chain variable region), and any combination of those or any other functional portion of an antibody peptide. The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). However, while various antibody fragments can be defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i.e., comprising constant
and variable regions from different species), humanized antibodies (i.e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e.g., bispecific antibodies); as well as single-domain antibodies, including nanobodies, engineered from e.g. heavy-chain only antibodies found in camelids or cartilaginous fishes.

The invention provides a (glyco)protein, a hormone or other biologically active compound capable of interacting with a gene selected from the group consisting of genes of table 3a, or with a gene product thereof. The (glyco)protein, a hormone or other biologically active compound preferably inhibits the expression of a gene selected from the group consisting of genes of table 3a, or inhibits the activity of a gene product thereof.

The invention provides as a therapeutic compound an antibody or derivative thereof (such as an scFv fragment, Fab fragment, chimeric antibody, bifunctional antibody, intrabody, and other antibody-derived molecule) directed against a polypeptide gene product of a gene of table 3a. The antibodies of the present invention have the effect of interfering with the function of the protein such that, for instance, the ligand-receptor interaction, transport or an enzyme function of the protein is blocked. Also generally envisioned herein is an antibody or derivative thereof that is a receptor antagonist.

The invention further provides as a compound an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, a morpholino, an RNAi molecule or a ribozyme binding under stringent conditions with a gene, or a mRNA of a gene, of table 3a described herein. The antisense molecule inhibits the expression of the respective gene or mRNA of the gene.

The term "stringent conditions" refers to conditions under which said antisense molecule will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to essentially no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. The term is used to indicate that the nucleotide sequence of the antisense molecule is essentially identical to the complement of a region of a gene, or a mRNA of a gene,
of table 3a. The term "essentially identical" is used to indicate that the antisense molecule is more than 90% identical, more preferred more than 95% identical, more preferred more than 99% identical to the complement of a region of a gene, or a mRNA of a gene, of table 3a.

Preferably, the above compounds of the invention are used as a medicament. Medicaments of the invention can suitably be used for the treatment of an individual suffering from cancer. Pharmaceutical compositions of the invention are particularly suited for sensitizing cancer cells to ionizing radiation. The pharmaceutical compounds of the invention are suited for the treatment of an individual suffering from cancer and in particular in combination with radiotherapy treatment of the cancer that the individual is suffering from.

In another aspect the invention further provides a pharmaceutical composition for sensitizing a cancer cell to radiation therapy comprising a compound according to the invention and a suitable excipient, carrier or diluent as explained above.

In one embodiment the compound that increases the susceptibility of a cell for radiation is a compound that inhibits the activity of a product of a gene selected from the group of genes of table 3a. A skilled person can use techniques known in the art to make such inhibitors based on the sequence information provided by the accession numbers in table 1. In one embodiment of this aspect, the inhibitor is an antibody and/or an antibody derivative directed against an expression product of a gene of table 3a. Therapeutic antibodies are for instance useful against gene expression products located on the cellular membrane and can be comprised in a pharmaceutical composition. Also, antibodies may be targeted to intracellular, e.g. cytoplasmic, gene products such as RNA's, polypeptides or enzymes, in order to modulate the activity of these products.

In a preferred embodiment of the present invention, the inhibitor is a small molecule capable of modulating the activity or interfering with the function of a protein expression product of a gene of table 3a.

Methods for identifying compounds such as small molecules that modulate the activity or interfere with the function of a protein expression product
of a gene of table 3a are known in the art. For example, kinase assays, phosphatase assays, protease assays, and the use of the reporter genes, are well known in the art and not further elaborated upon herein.

Libraries of compounds such as peptide libraries (e.g. LOPAP™, Sigma Aldrich), lipid libraries (BioMol), synthetic compound libraries (e.g. LOPAC™, SigmaAldrich) and natural compound libraries (Natural Product Library; TimTec) can be screened, preferably in high throughput screens.

In addition, or as an alternative, a fragment-based strategy is used to identify low molecular weight chemical fragments (also known as scaffolds or templates) from small compound libraries (Hajduk and Greer, 2007. Nature Reviews Drug Discovery 6: 211-219). Initial fragments are identified by direct binding techniques. Fragments that interact efficiently with a target protein are selected and combined into larger, more complex molecules to provide starting points for further lead optimisation. Various biophysical screening methods may be used including nuclear magnetic resonance (NMR) and X-ray crystallography to provide structural understanding of the binding of the ligand to the protein expression product of a gene of table 3a.

The binding affinity of a compound with an expression product of a gene of table 3a can be measured by methods known in the art, such as by surface plasmon resonance biosensors (Biacore), by saturation binding analysis with a labeled compound (e.g. Scatchard and Lindmo analysis), and by using a Fluorometric Imaging Plate Reader (FLIPR®) system. The binding affinity of a compound can be expressed as a dissociation constant (Kd). The dissociation constant, Kd, is a measure of how well a compound binds to the expression product and is equivalent to the compound concentration that is required to saturate exactly half of the binding-sites on the expression product. A preferred compound that inhibits a protein expression product of a gene of table 3a exhibits a binding affinity to the protein of at most 1 micromolar, more preferred at most 1 nanomolar, more preferred at most 0.1 nanomolar.
A preferred method may comprise a series of assays, each of which is designed to determine whether a drug candidate compound is indeed acting on the protein expression product of a gene of table 3a and to increase the radiation sensitivity of a cancer cell after adding the compound to the cell. For example, an assay designed to determine the binding affinity of a test compound to the protein expression product of a gene of table 3a, or a fragment thereof, may be combined with a method to determine whether the test compound interferes with the biological activity of the protein expression product, and/or a cellular assay. Methods for determining the biological activity of an expression product, e.g. a G-protein-coupled receptor, a kinase, a phosphatase or a protease are known in the art. In a cellular assay, the biological activity of a protein expression product of a gene of table 3a may be measured by determining the sensitivity of the cell upon IR in the presence and absence of a candidate compound. In such cellular assay, a host cell expressing a protein expression product is preferably a cell with endogenous expression of a protein expression product of a gene of table 3a. Compounds that were found active against the selected protein expression product of a gene of table 3a in a cellular assay are preferably tested at different concentrations to determine a dose-response curve.

The present invention further relates to a method for identifying a compound that sensitizes a cancer cell for ionizing radiation, comprising:

(a) contacting a compound with a protein expression product of a gene of table 3a;

(b) determining the binding affinity of the compound to the protein expression product;

(c) contacting a population of cells expressing said protein expression product with a compound that exhibits a binding affinity of at most 1 micromolar;

(d) irradiating the population of cells with ionizing radiation; and

(e) identifying a compound that sensitizes a cancer cell for ionizing radiation.
Once a compound has been identified that sensitizes a cancer cell for ionizing radiation, the performance of the compound may be improved by synthesizing analogues with improved potency, reduced off-target activities, and further physiochemical/metabolic properties suggestive of reasonable in vivo pharmacokinetics. This optimization is usually accomplished through chemical modification of the structure of the identified compound with modifications chosen by employing knowledge of the structure-activity relationship as well as structure-based design if structural information about the protein expression product of a gene of table 3a is available.

On a different level of inhibition, nucleic acids can be used to block the production of proteins by destroying the mRNA transcribed from a gene of table 3a. This can be achieved by antisense drugs, ribozymes or by RNA interference (RNAi). The present invention relates to antisense drugs, such as antisense RNA and antisense oligodeoxynucleotides, ribozymes and RNAi molecules, directed against a gene of table 3a.

Ribozymes

Trans-cleaving catalytic RNAs (ribozymes) are RNA molecules possessing endoribonuclease activity. Ribozymes are specifically designed for a particular target, and the target message must contain a specific nucleotide sequence. They are engineered to cleave any RNA species site-specifically in the background of cellular RNA. The cleavage event renders the mRNA unstable and prevents protein expression.

One commonly used ribozyme motif is the hammerhead, for which the substrate sequence requirements are minimal. Design of the hammerhead ribozyme is well known in the art, as is the therapeutic uses of ribozymes. Ribozymes can for instance be prepared and used as described in U.S. Pat. No. 5,254,678. Ribozyme cleavage of HIV-I RNA is described in U.S. Pat. No. 5,144,019; methods of cleaving RNA using ribozymes is described in U.S. Pat. No. 5,116,742; and methods for increasing the specificity of ribozymes are described in U.S. Pat. No. 5,225,337. Preparation and use of ribozyme fragments in a hammerhead or hairpin structure is also known in the art. Ribozymes can also be made by rolling
transcription.

The hybridizing region of the ribozyme may be modified or may be prepared as a branched structure. The basic structure of the ribozymes may also be chemically altered in ways familiar to those skilled in the art, and chemically synthesized ribozymes can be administered as synthetic oligonucleotide derivatives modified by monomeric units. In a therapeutic context, liposome mediated delivery of ribozymes improves cellular uptake.

Therapeutic and functional genomic applications of ribozymes requires knowledge of a portion of the coding sequence of the gene to be inhibited. Thus, for many genes, a nucleic acid sequence provides adequate sequence for constructing an effective ribozyme. A target cleavage site is selected in the target sequence, and a ribozyme is constructed based on the 5’ and 3’ nucleotide sequences that flank the cleavage site. Retroviral vectors are engineered to express monomeric and multimeric hammerhead ribozymes targeting the mRNA of the target coding sequence. These monomeric and multimeric ribozymes are tested in vitro for an ability to cleave the target mRNA. A cell line is stably transduced with the retroviral vectors expressing the ribozymes. The cells are analysed for inactivation of the target mRNA and/or reduction of the gene product of the target mRNA.

Antisense

Antisense polynucleotides are designed to specifically bind to RNA, resulting in the formation of RNA-DNA or RNA-RNA hybrids, with an arrest of DNA replication, reverse transcription or messenger RNA translation. Antisense polynucleotides based on a selected sequence can interfere with expression of the corresponding gene.

Antisense polynucleotides are typically generated within the cell by expression from antisense constructs that contain the antisense strand as the transcribed strand. Antisense polynucleotides will bind and/or interfere with the translation of the corresponding mRNA. As such, antisense may be used therapeutically to inhibit the expression of genes such as one or more of the genes of table 3a.

Antisense RNA or antisense oligodeoxynucleotides (antisense ODNs) can both be used and may also be prepared in vitro synthetically or by means of
recombinant DNA techniques. Both methods are well within the reach of the person skilled in the art. ODNs are smaller than complete antisense RNAs and have therefore the advantage that they can more easily enter the target cell. In order to avoid their digestion by DNase, ODNs and antisense RNAs may be chemically modified. For targeting to the desired target cells, the molecules may be linked to ligands of receptors found on the target cells or to antibodies directed against molecules on the surface of the target cells.

Antisense RNA includes reference to locked nucleic acid (LNA).

RNAi molecules

RNAi is based on the generation of short, double-stranded RNA (dsRNA) which activates a cellular process leading to a highly specific RNA degradation (Zamore et al., 2000. Cell 101: 25-33) and/or suppression of translation. For the purpose of the invention, the dsRNA molecules that activate RNAi and their precursors that are processed in a cell to generate dsRNA molecules that activate RNAi are referred to as "RNAi molecules". RNA interference is mediated by the generation of 18- to 23-nucleotide dsRNA molecules with 2 nucleotide-long 3' overhangs termed small interfering RNA (siRNA) duplexes. One strand of the siRNA duplex, the antisense or guide strand, is incorporated into a nuclease complex, called the RNA-induced silencing complex (RISC), which acts to destroy mRNAs that are recognized by the guide strand through base-pairing interactions. RNAi thus allows selective silencing of a gene on the basis of its sequence. RNAi molecules are thus double stranded RNAs (dsRNAs) that upon processing in a cell and incorporation in RISC are very potent in silencing the expression of their target gene. The invention provides RNAi molecules that are potent in silencing the expression of the radioresistance genes of the present invention.

Preferably, an RNAi molecule of the invention is an siRNA duplex that can activate an RNAi process in a cell directly through incorporation of its guide strand into RISC. Said siRNA duplex can also be a miRNA mimic, i.e., an siRNA comprising the guide strand of a miRNA. Alternatively, an RNAi molecule of the invention activates an RNAi process indirectly, because it is a precursor of a
molecule that can activate an RNAi process in a cell. Said precursor molecule is preferably a short hairpin RNA (shRNA) or a pre- or pri-miRNA or variant or analogue thereof. A short hairpin RNA (shRNA) typically comprises a 50-100 nucleotide long RNA molecule comprising two stretches of nucleotides that are complementary and can base-pair, whereby the two stretches are interconnected through a hairpin turn. The shRNA hairpin structure is cleaved by the cellular machinery into one or more 18-23 (typically 19) nucleotide-long double stranded siRNA molecules with 2 nucleotide-long 3’ overhangs with one of the strands exhibiting extensive complementary homology to a part of a mRNA transcript from a target gene. Said siRNA molecules activate the RNAi pathway and interfere with the expression of said target gene by specific mRNA degradation.

Expression of the shRNA can be driven by a polymerase II or polymerase III enhancer/promoter. Natural miRNA molecules are typically transcribed by polymerase II as pri-miRNA with a cap and poly-A tail and processed to short, 70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. These pre-miRNAs are then processed to mature double stranded miRNAs of about 18-25 nucleotides in the cytoplasm which silence gene expression via RNA interference, partly by specific RNA degradation and partly by suppressing translation. Pri-miRNAs and pre-miRNA molecules are also useful to silence the expression of a gene of table 3a according to the invention. Artificial miRNAs can be transcribed from any promoter, for example a polIII promoter, in a format analogous to that of a shRNA. They then differ from an shRNA in that the double-stranded region is not completely complementary. A preferred RNAi molecule according to the invention comprises a double stranded region of between 18 nucleotides and 25 nucleotides per strand. A most preferred RNAi molecule according to the invention comprises a double stranded region that has a length of 19 nucleotides after processing into a mature siRNA.

RNAi molecules are prepared by methods well known to the person skilled in the art. In general an isolated nucleic acid sequence comprising a nucleotide sequence which is substantially homologous to the sequence of at least one of the genes of table 3a and which is capable of forming a partially or fully double stranded (ds) RNA with (part of) the transcription product of said gene will
function as an RNAi molecule. The double stranded region may be in the order of between 10-250, preferably 10-100, more preferably 20-50 nucleotides in length. Although RNAi strategies rely on RNA-RNA hybrids, it is possible to provide cells with a single stranded antisense oligonucleotide specific for the target mRNA, and have it being broken down via the RNAi pathway. The RNAi molecule of the invention can thus be double or single stranded RNA.

Criteria that can be used to select one or more sequences of a target gene (the targeted region) for incorporation into the double-stranded part of the RNAi molecule are known in the art. For example, the targeted region is preferably located 50-100 nucleotides downstream of the start codon (ATG); is selected from an exon sequence since RNAi only works in the cytoplasm; sequences with > 50% G+C content or sequences with stretches of 4 or more nucleotide repeats are avoided as are sequences that share a certain degree of homology with another related or unrelated gene. Based on these or similar criteria, the skilled person is able to select one or more targeted regions of a target gene for generating the corresponding RNAi molecule. Testing one or more, preferably at least four, of the selected potential RNAi molecules for reducing expression of the corresponding target gene in a host cell, will provide the skilled person with one or more RNAi molecules that reduce expression of a target gene in a host cell.

Examples of RNAi molecules include siRNAs, shRNAs, miRNAs, pre-miRNAs, pri-miRNAs, and the like.

Antibodies and derivatives

The antibodies used in the present invention may be from any animal origin including birds, fish, and mammals (e.g., human, murine, donkey, sheep, rabbit, goat, guinea pig, camel, llama, dromedary, alpaca, shark, horse, or chicken). Preferably, the antibodies of the invention are human or humanized monoclonal antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries (including, but not limited to, synthetic libraries of immunoglobulin sequences homologous to human immunoglobulin sequences) or from mice that express antibodies from human genes.
For some uses, including in vivo therapeutic or diagnostic use of antibodies in humans and in vitro detection assays, it may be preferred to use human or chimeric antibodies. Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Human antibodies can be made by a variety of methods known in the art including phage display methods using antibody libraries derived from human immunoglobulin sequences or synthetic sequences homologous to human immunoglobulin sequences. See also U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893 and W098/16654, each of which is incorporated herein by reference in its entirety.

The antibodies to be used with the methods of the invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody. Additionally, the derivative may contain one or more non-classical amino acids.

In certain embodiments of the invention, the antibodies to be used with the invention have extended half-lives in a mammal, preferably a human, when compared to unmodified antibodies. Antibodies or antigen-binding fragments thereof having increased in vivo half-lives can be generated by techniques known to those of skill in the art (see, e.g., PCT Publication No. WO 97/34631).

In certain embodiments, antibodies to be used with the methods of the invention are single-chain antibodies, including scFv and single domain antibodies such as nanobodies. The design and construction of a single-chain antibody is well known in the art.

In certain embodiments, the antibodies to be used with the invention bind to an intracellular epitope, i.e., are intrabodies. An intrabody comprises at least a portion of an antibody that is capable of immuno-specific binding an antigen and preferably does not contain sequences coding for its secretion. Such antibodies will bind its antigen intracellular. In one embodiment, the intrabody comprises a single-chain Fv ("sFv"). In a further embodiment, the intrabody preferably does not encode an operable secretory sequence and thus remains within the cell.

Generation of intrabodies is well-known to the skilled artisan and is described for example in U.S. Patent Nos. 6,004,940; 6,072,036; 5,965,371, which are incorporated by reference in their entireties herein.
In one embodiment, intrabodies are expressed in the cytoplasm. In other embodiments, the intrabodies are localized to various intracellular locations. In such embodiments, specific localization sequences can be attached to the intranucleotide polypeptide to direct the intrabody to a specific location.

The antibodies to be used with the methods of the invention or fragments thereof can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in WO97/13844; and U.S. Patent Nos. 5,580,717, 5,821,047, 5,571,698, 5,780,225, and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described below. Techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication No. WO 92/22324.

It is also possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For a detailed discussion of the technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publication No. WO 98/24893. All references cited herein are are incorporated by reference herein in their entirety. In addition, companies such as Medarex, Inc. (Princeton, NJ), Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Recombinant expression used to produce the antibodies, derivatives or
analogs thereof (e.g., a heavy or light chain of an antibody of the invention or a portion thereof or a single chain antibody of the invention), requires construction of an expression vector containing a polynucleotide that encodes the antibody and the expression of said vector in a suitable host cell or even in vivo. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably, but not necessarily, containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, a heavy or light chain of an antibody, a heavy or light chain variable domain of an antibody or a portion thereof, or a heavy or light chain CDR, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy, the entire light chain, or both the entire heavy and light chains.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention or fragments thereof, or a heavy or light chain thereof, or portion thereof, or a single chain antibody of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.
A variety of host-expression vector systems may be utilized to express the antibody molecules as defined herein.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert.

These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc.

Once an antibody molecule to be used with the methods of the invention has been produced by recombinant expression, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. Further, the antibodies of the present invention or fragments thereof may be fused to heterologous polypeptide sequences described herein or otherwise known in the art to facilitate purification.

As stated above, according to a further aspect, the invention provides an antibody as defined above for use in therapy.

For therapeutic treatment, antibodies may be produced in vitro and applied to the subject in need thereof. The antibodies may be administered to a subject by any suitable route, preferably in the form of a pharmaceutical composition adapted to such a route and in a dosage which is effective for the
intended treatment. Therapeutically effective dosages of the antibodies required for decreasing the rate of progress of the disease or for eliminating the disease condition can easily be determined by the skilled person.

Alternatively, antibodies may be produced by the subject itself by using in vivo antibody production methodologies as described above. Suitably, the vector used for such in vivo production is a viral vector, preferably a viral vector with a target cell selectivity for a specific target cell referred to herein, preferably a brain cancer cell, a head/neck cancer cell, a lung cancer cell, a liver cancer cell, a pancreatic cancer cell, a skin cancer cell, a gastrointestinal cancer cell, a kidney cancer cell, an ovarian cancer cell, a breast cancer cell, a prostate cancer cell or a cancer cell originating from the hemopoietic system.

Therefore, according to a still further aspect, the invention provides the use of an antibody as defined above in the manufacture of a medicament for use in the treatment of a subject to achieve the said therapeutic effect. The treatment comprises the administration of the medicament in a dose sufficient to achieve the desired therapeutic effect. The treatment may comprise the repeated administration of the antibody.

According to a still further aspect, the invention provides a method of treatment of a human comprising the administration of an antibody as defined above in a dose sufficient to achieve the desired therapeutic effect. The therapeutic effect being the sensitation of a cancer cell in the human to radiation therapy.

The diagnostic and therapeutic antibodies are preferably used in their respective application for the targeting of kinases or phosphatases, which are often coupled to receptor molecules on the cell's surface. As such, antibodies capable of binding to these receptor molecules can exert their activity-modulating effect on the kinases or phosphatases by binding to the respective receptors. Also transporter proteins may be targeted with advantage for the same reason that the antibodies will be able to exert their activity-modulating effect when present extracellularly. The above targets, together with signalling molecules, represent preferred targets for the antibody uses of the invention as more effective therapy and easier diagnosis is possible thereby.

The diagnostic antibodies can suitably be used for the qualitative and quantitative detection of gene products, preferably proteins in assays for the
determination of altered levels of proteins or structural changes therein. Protein levels may for instance be determined in cells, in cell extracts, in tumor biopsies, in supernatants, or body fluids by for instance protein assays such as ELISA or RIA, Western blotting, flow cytometry, immunocytochemistry, immunohistochemistry and imaging technology (e.g., using confocal laser scanning microscopy).

A therapeutic compound such as an anti-sense molecule, a small molecule or a (glyco)protein, a hormone or biologically active compound, or a therapeutic effect as described herein refers to the radiation sensitizing effect of the compound or radiation sensitizing effect of the therapy. The invention does not exclude the possibility that apart from said radiation sensitizing effect, the compound may in the absence of radiation have a beneficial or therapeutic effect on it's own.
Delivery Methods

Once formulated, the pharmaceutical compositions of the invention can be administered directly to a subject; or delivered to cells in vitro.

Direct delivery of the compositions to a subject will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion or site of neoplastic growth. Other modes of administration include topical, oral, catheterized and pulmonary administration, suppositories, and transdermal applications, needles, and particle guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Generally, delivery of nucleic acids for both ex vivo and in vitro applications can be accomplished by, for example, gene transfer using a vector including a virus, dextran-mediated transfection, calcium phosphate precipitation, polybrene® mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well known in the art.

Various methods are used to administer the therapeutic composition directly to a specific site in the body, for example to a primary and/or secondary tumor. For example, a target location is located and the therapeutic composition injected in the target directly. Alternatively, arteries which serve target location are identified, and the therapeutic composition injected into such an artery, in order to deliver the composition directly into the target location. Image guidance by e.g. ultrasound, X-ray, or MRI imaging can be used to assist in certain of the above delivery methods.

Receptor-mediated targeted delivery of therapeutic compositions containing an antisense polynucleotide, subgenomic polynucleotides, or antibodies to specific tissues is also used. Receptor-mediated DNA or RNA delivery techniques are well known in the art. Particularly interesting molecules with high binding affinity and specificity are the so called "aptamers". Aptamers are nuclease-stabilized oligonucleotides with a secondary structure providing binding specificity (Ellington and Szostak, Nature 346, 818-822, 1990). They are considered as non-protein based alternatives for antibodies. They are manufactured chemically and
are therefore attractive reagents for therapeutic use. Many different aptamers with different binding specificities have already been produced. E.g., an aptamer with binding specificity for prostate specific membrane antigen (PSMA) was produced and shown to bind tightly to the extracellular part of PSMA on human prostate cancer cells (Lupoid et al., Cancer Res. 62, 4029-4033, 2002). Such aptamers can be covalently coupled to RNA, e.g. siRNA or shRNA, retaining aptamer binding specificity and siRNA gene silencing capacity. A chimeric RNA comprising an aptamer binding to PSMA and an siRNA directed against PLK1 was internalized selectively in PSMA-expressing cells and triggered silencing of PLK1. In addition, when delivered to mice carrying human prostate cancer xenograft tumors, the aptamer-siRNA inhibited tumor growth (McNamara et al., Nat. Biotechnol. 24, 1005-1015, 2006).

Pharmaceutical compositions containing antisense, ribozyme or RNAi polynucleotides are administered in a range of about 100 ng to about 200 mg of polynucleotides for local administration to a human subject. Concentration ranges of about 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of polynucleotides can also be used. Factors such as method of action and efficacy of uptake into cells and expression are considerations which will affect the dosage required for ultimate efficacy of the polynucleotides. Where greater expression is desired over a larger area of tissue, larger amounts of polynucleotides or the same amounts readministered in a successive protocol of administrations, or several administrations to different adjacent or close tissue portions of, for example, a prostate tumor in a prostate, may be required to effect a positive therapeutic outcome. In all cases, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect.

Diagnostic methods

The genes and gene products identified herein are associated with the sensitization of cancer cells to radiation and in particular ionizing radiation. The gene products of the genes of the table 3a and in particular the level of expression of the gene product in the cancer cell is therefore suitable for a diagnostic purpose. The level of expression of the gene product in the cancer cell, and in particular the
level of expression of the gene products of two or more of the genes is indicative for
the sensitivity of a cancer cell to radiation therapy. The invention thus further
provides a method for determining the radiation sensitivity of a cancer cell,
comprising determining the level of a gene product of one or more genes of table 3a
in said cancer cell and estimating the sensitivity of said cancer cell to said
radiation. This test is preferably performed prior to or together with a method for
sensitizing a cancer cell to radiation.

A method for increasing the sensitivity or susceptibility of a cancer cell
to radiation by providing a compound to said cancer cell is preferably combined
with a method for determining the expression of a gene of table 3a in said cancer
cell. In a preferred embodiment the compound inhibits the expression of a gene
and/or the activity of a gene product of said gene, of which it was established that
the cancer cell expresses it. It is preferred that the compound for sensitizing the
cancer cell to radiation, is directed towards inhibiting the expression of a gene, or
the activity of gene product of a gene, of which it was determined that it is
expressed in the cancer cell. Thus the invention further provides a method of
diagnosis of a cancer and a method for predicting the success of radiation therapy
for a specific cancer comprising determining the expression of a gene of table 3a in
said cancer cell, or the expression level of a gene product of a gene of table 3a in
said cancer cell.

The invention further provides a method for the determining the
radiation sensitivity of a cancer in an individual or predicting the utility of
radiation therapy of a cancer in an individual, comprising detecting a gene or gene
product of a gene of table 3a in the blood and/or a cancer cell of said individual.
Such a method may suitably be performed by using quantitative RT-PCR, RNA
massive parallel sequencing (RNAseq), a microarray (in particular comprising
specific binding partners that bind specifically to at least two biomarkers as
defined above bound to a solid support), by using tandem mass spectrometry (MS-
MS), by MALDI-FT mass spectrometry, MALDI-FT-ICR mass spectrometry,
MALDI Triple-quad mass spectrometry or immunoassay.

Kits of parts for performing a diagnostic method according to the
invention are also envisioned herein. Such kits comprise at least one gene product
as defined above or a specific binding partner that binds specifically to said gene
product, said kit of parts optionally further comprising one or more of the following:
- at least one reference or control sample;
- information on the reference value for the gene product;
- at least one test compound capable of binding to said specific binding partner;
- at least one detectable marker for detecting binding between said gene product and said specific binding partner.

Diagnostic reagents that bind specifically to a gene product as defined herein include an antibody or a nucleic acid molecule specifically hybridizing under stringent conditions to said gene product.

All reference referred to herein are incorporated by reference herein in their entirety.

The present invention will now be further illustrated in the Experimental part described below.
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<th>IR response compared to irrelevant control at least 2-fold</th>
<th>Single siRNA confirmed</th>
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Table 3a

GPR27; CPNE7; MAD2L2; RNF8; RGNEF; HTR1E; CBL; CCNB1; DNA-PKcs; GJB3; KCNQ3; OR2F1; RPL3L; FAM174B; ITPKA; DGAT2; SLC22A10; PDILT; HOXD3; IFNA7; MGC2705; ENTPD5; NLRP9; FBX04; PDCD4; CCK; MTXI; CLIC5; SIAH1; FZDI; FFAR1; KRTAP5-11; ANKR3D4B; ZBTB43; MEIS1; MMP7; PEX16; ANXA13; USP33; CD163; MME; GPER; HIG2; PPP1R12B; MAEA; EML5;

Table 3b

GPR27; CPNE7; RGNEF; HTR1E; CBL; CCNB1; GJB3; KCNQ3; OR2F1; RPL3L; FAM174B; ITPKA; DGAT2; SLC22A10; PDILT; HOXD3; IFNA7; MGC2705; ENTPD5; NLRP9; FBX04; PDCD4; CCK; MTXI; CLIC5; SIAH1; FZDI; FFAR1; KRTAP5-11; ANKR3D4B; ZBTB43; MEIS1; MMP7; PEX16; ANXA13; USP33; CD163; MME; GPER; HIG2; PPP1R12B; MAEA; EML5;

Table 3c

GPR27; CPNE7; RGNEF; HTR1E; CBL; CCNB1; GJB3; KCNQ3; OR2F1; RPL3L; FAM174B; ITPKA; DGAT2; SLC22A10;

Table 3d

GPR27; CPNE7;
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Table 4. Stable gene knockdown in PC-3 cells infected with lentiviral vectors expressing short hairpins. The table lists the shRNA insert sequence as well as the mRNA transcripts targeted by each lentiviral vector used from the TRC or GIPZ library. The percentage knockdown (% KD) obtained in individual stable transduced cell lines is also given.
Examples

Example 1. Development of a high-throughput assay to identify genes involved in cancer cell resistance to irradiation.

For a comprehensive identification of genes involved in resistance of cancer cells to irradiation, we designed a whole human genome siRNA library HTS method. For this, technical obstacles with respect to the radiation response readout assay had to be resolved. The colony formation assay (CFA) is not suitable for HTS. Therefore, we developed a new method that could be used to identify radiation susceptibility genes in a large scale high-throughput siRNA screen.

In this method, we count individual cells that survived irradiation and were allowed to proliferate for 5 days after irradiation. This is done by fixing the cells with 7% formaldehyde for 30 minutes, staining their DNA with Hoechst 33342 or DAPI for 30 minutes, and counting nuclei using the TTP LabTech Acumen eX3 laser-scanning microplate cytometer. Sometimes, PC-3 cells are situated too close to each other to be identified as a single object. By careful examination of these groups of cells we found that they consist of approximately 5 cells. Therefore, we defined 2 populations of objects detected; i.e. the single-cell population and the multiple-cell population. The single-cell population includes all objects that are 6-50um wide and 6-120um deep, while the multiple-cell population includes all objects that are 50.5-150um wide and 6-120um deep. The total cell number was defined as the sum of the single-cell population count plus the multiple-cell population count multiplied by 5. This assay is further referred to herein as the "Acumen assay". We measured the effect of different doses of irradiation ranging from 0 to 8 Gy on two prostate cancer cell lines, i.e. PC-3 cells and DU-145 cells, with the new method and compared the results to the traditional CFA. The irradiation was brought about by a Varian linear accelerator at a dose rate of 600 ME/minute. For both cell lines, the dose response curves produced by the Acumen assay were quite comparable to the CFA curves (Figure 1). The curves follow a
linear-quadratic equation typical for irradiation dose-effects. Hence, the Acumen assay recapitulates irradiation effects on cancer cell lines very well.

In order to investigate if the Acumen assay was suitable to detect radiosensitization by target gene silencing and to determine a most useful irradiation dose for HTS, we seeded 1,500 PC-3 cells per well in 96-well culture plates in 80ul RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). The next day, we transfected them with 20nM siRNA against the known irradiation susceptibility gene PRKDC, i.e. DNA-PKcs (Dharmacon Cat. No M-005030-01-0005) or with 20nM irrelevant control siRNA (Dharmacon Cat. No. D-001206-14-05) using 1/5,000 diluted Dharmafect 1 in a final volume of 100 microliter. This was done as follows. Per transfection, 1Oul O.luM siRNA in siRNA buffer (5x siRNA buffer, Dharmacon Cat. No. B-002000-UB-100, 5-times diluted in H20) was incubated for 5 minutes at room temperature; DharmaFECT1 transfection reagent was diluted 500-times in RPMI medium (without serum and antibiotics) and incubated for 5 minutes at room temperature. Next, lOul diluted siRNA was added to 1Oul diluted DharmaFECT1 and incubated for at least 20 minutes at room temperature.

Finally, 20ul of mixed siRNA/DharmaFECT1 was added to the cells, reaching a final volume of 1OOul. The next day, transfected PC-3 cells were trypsinized, counted and plated 250 and 500 cells per well in 6-well culture plates for the CFA assay or left in the 96-well plates for the Acumen assay. Two days after transfection, cells were irradiated at various doses as above. The radiosensitizing effect of PRKDC silencing on irradiation treatment was examined with both the CFA assay (read out 10 days after irradiation) and the Acumen assay (Figure 2). Radiosensitization by PRKDC silencing could be clearly detected using both assays. Upon silencing of PRKDC, irradiated PC-3 cells exhibited a lower survival fraction (SF) compared to cells treated with the irrelevant siRNA, even at low doses. The dose modifying factors (DMF), i.e., the relative difference in irradiation dose required to cause a particular surviving fraction, were comparable with the two assays at a range of surviving fractions. For HTS, we chose an irradiation dose of 4 Gy. At this dose, silencing PRKDC and the irrelevant control siRNA caused SF of 0.51 and 0.71, respectively. This corresponds to a RF of 1.4.
Example 2. Identification of target genes for radiation sensitization in PCS prostate cancer cells.

We used high-throughput RNAi screens to identify modulators of sensitivity to irradiation in cancer cells. We used PC-3 prostate cancer cells and the Dharmacom siARRAY whole human genome siRNA library. PC-3 cells were grown at 37°C and 5% CO2 in a humidified incubator in RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). The siRNA library consists of approximately 21,000 arrayed pools of 4 siRNAs each directed against a different human gene.

Two independent replicate genome-wide screens were performed with two sets of plates each. 1,500 cells were seeded per well in 96-well plates. The next day, cells were transfected with 20 nM siRNA using 0.02ul/well DharmaFECT 1 (Thermo Fisher Scientific). Two days later, one set of plates was irradiated with 4Gy on a Varian linear accelerator. The other set of plates was left untreated. Four days after irradiation, cell numbers were determined by nuclear counting using the Acumen assay with nuclear staining using Hoechst 33342 at 0.25ng/well. Data was normalized by correcting log-reads for both plate-specific and screen-specific effects. Subsequently, the treatment effect relative to that of negative controls was estimated by fitting a linear model to observations of each siRNA and all negative controls. p-values were subsequently corrected for multiple testing by using the Benjamini-Hochberg step-up false discovery rate (FDR).

Hits were selected according to the following criteria: siRNA pools that resulted in significantly less (FDR<0.1) PC-3 cells counted following treatment in combination with irradiation than in the absence of irradiation, and also significantly less (FDR<0.1) than following treatment with a combination of irrelevant control siRNA and irradiation were selected. The genes that are silenced by these siRNAs are probably supporting survival of PC-3 cells upon irradiation. These genes are thus potential targets to sensitize cancer cells, in particular prostate cancer cells, more particularly PC-3 prostate cancer cells to irradiation. These genes are listed in Table 1.
We selected 45 candidate target genes for further analysis. This included all 29 genes identified in the whole-genome screen with a FDR <5% plus 16 genes identified in the whole-genome screen with a FDR 5-10% that are considered "druggable" and thus represent good targets for chemical drug design.

These genes were re-examined in a secondary screen using the same procedure. For comparison, irrelevant control siRNA and siRNA against DNA-PKcs, a known radioresistance gene, were taken along. Due to the stringent hit selection criteria, DNA-PKcs had not been identified in the primary whole-genome screen. Figure 3a shows the cell numbers counted following siRNA transfection only or siRNA transfection with irradiation for all tested genes. Figure 3b shows the relative cell survival after gene silencing plus irradiation compared to gene silencing alone. As can be seen, silencing of 33 of the 45 tested genes resulted in substantially more cell growth inhibition upon irradiation than control siRNA transfection plus irradiation (87% cell survival), i.e. they exhibited a survival less than 79% corresponding to a RF >1.1. The radiosensitizing effect of silencing these genes was thus confirmed (see Table 1). Moreover, silencing of 17 candidate genes enhanced the inhibitory effect of 4Gy irradiation on cell survival and proliferation at least approximately 2-fold (see Table 1). These 17 candidate genes were subjected to further confirmation studies using 4 individual siRNAs directed to different sequences on the mRNA of each gene. The radiosensitizing effect of gene silencing could be confirmed with at least two different siRNAs for all 17 candidate target genes (2 out of 4 for one gene; 3 out of 4 for seven genes; and all 4 for nine genes; see Table 1). This shows that these genes are most likely genuine targets.

Example 3. Independent validation of target genes on PCS prostate cancer cells using the Colony Formation Assay.

We independently validated the radiosensitizing effect of silencing any of the 17 candidate target genes using a different assay. For this we used the Colony Formation Assay (CFA). This clonogenic assay is considered the golden standard assay in radioresistance research. Upon irradiation, cells are allowed to proliferate for 8-12 days and form colonies of at least 50 cells. Thus, they should survive irradiation and divide at least 6 times ($2^6 = 64$) to be counted in this assay.
The CFA thus recapitulates not only direct growth inhibitory effects of irradiation, but also disturbances in the cell division process due to unrepaired DNA damage becoming evident after multiple cell cycles.

The assay was performed as follows: 1,500 cells were plated in 96-well format plates. The next day, cells were transfected with 20nM siRNA using 0.02ul/well DharmaFECT 1 (Thermo Fisher Scientific) and incubated at 37°C. 24 hours after transfection cells were trypsinized and plated in 6-well plates at 250 or 500 cells/well depending on the irradiation dose (250 cells per well for unirradiated controls and irradiation at 1, 2, or 3 Gy and 500 cells/well for irradiation at 4, 5, 6 and 8Gy) and allowed to attach overnight. Each condition was set up in triplicate. On the next day, cells were irradiated using a Varian linear accelerator as described in Example 1. Eight days after irradiation, culture media were removed and cells were rinsed with PBS and fixed with 3.7% formaldehyde (Sigma Aldrich, UK) for 30-60 minutes. After fixation cells were washed with PBS and stained with 1:20 dilution Giemsa (Sigma Aldrich, UK) for 30-60 minutes. After staining with Giemsa, plates were washed with water and air dried. Colonies containing more than 50 cells were counted using a stereomicroscope. Figure 4 shows the irradiation dose response curves obtained. They represent the mean results of 2 independent experiments performed. Each panel shows the dose response curve for cells transfected with siRNA directed against a different candidate target gene and the dose response curve for cells transfected with siRNA directed against an irrelevant control siRNA. The latter serves as control for possible siRNA transfection related cytotoxicity. For comparison, cells transfected with siRNA against DNA-PKcs were included. As can be seen, silencing of 15 candidate target genes resulted in a decreased capacity of the cells to form colonies after irradiation, the exceptions being PDILT and SLC22A10. This thus validated these 15 candidate genes as genuine target genes for radiosensitization.

The CFA allows determining the so-called Dose Modifying Factor (DMF), i.e., the factor by which the irradiation dose can be lowered in combination with target gene silencing to achieve the same cancer cell killing effect as irradiation alone or irradiation combined with irrelevant control gene silencing. In
addition, it allows to calculate the Surviving Fraction (SF) i.e., the relative decrease in cell survival induced by a particular irradiation dose. The DMF and SF are most relevant at the clinically relevant low dose irradiation and consequent high proportion cell survival. Therefore, we determined the DMF(0.8), i.e., the DMF at 80% cell survival, and the SF2, i.e., the SF after 2Gy irradiation. Furthermore, by comparing the SF2 after gene silencing to the SF2 after irrelevant siRNA transfection, the RF at 2Gy was calculated. Table 2 gives the calculated SF2, RF2 and DMF(0.8) values for the 17 tested candidate target genes and DNA-PKcs and irrelevant siRNA controls. As can be seen, the radiosensitizing effect of silencing the 17 candidate target genes differed considerably. Importantly, the effect of silencing nine genes, i.e. CPNE7, GPR27, MAD2L2, RNF8, RGNEF, HTR1E, CBL, CCNB1 and KCNQ3 appeared stronger than silencing positive control gene DNA-PKcs, as evidenced by a higher RF2 and/or higher DMF(0.8). The effect of silencing GJB3 was similar to that of silencing DNA-PKcs. It was particularly interesting to note that silencing either GPR27 or CPNE7 had a profound effect on radiosensitivity of PC-3 cells. Their DMF(0.8), RF2 and SF2 values stood out compared to the other candidate target genes. To inhibit colony formation by 20%, i.e. DMF(0.8), the irradiation dose could be lowered by more than 4-fold; and irradiation at a clinically relevant dose of 2Gy decreased colony formation capacity from 70% to less than 30%. Hence, novel targets for radiosensitization were identified. For several of these, silencing had a more profound radiosensitizing effect than silencing a previously known target for radiosensitization.

Example 4. Independent validation of target genes on PC-3 prostate cancer cells with stable expression of short hairpin RNA.

We independently validated the radiosensitizing effect of silencing 7 of the candidate target genes that showed the strongest effect upon silencing using siRNA using independent RNAi molecules. To this end, PC-3 cells were stable transduced with short hairpin RNA (shRNA)-expressing lentiviral vectors (LV). For each target gene, multiple independent PC-3 derivative knockdown cell cultures were generated using distinct LV-shRNA constructs from the Open Biosystems TRC and
GIPZ libraries. As negative control, cells were transduced with LV-shSCR expressing a non-targeting shRNA sequence. As positive control, a cell culture expressing a shRNA against DNA-PKcs was made. Specific gene knockdown efficiencies were determined by quantitative RT-PCR. Only cultures with considerable gene knockdown were used for further experiments. Table 4 lists lentiviral shRNA vectors used and the knockdown efficiencies obtained. For MAD2L2 and CBL, a single culture with effective gene knockdown could be obtained; for 5 target genes, i.e., CCNBl, CPNE7, GPR27, RNF8, and RGNEF, two independent cultures with effective gene knockdown could be obtained. All cultures were analyzed for their radiation susceptibility using the CFA as described in example 3. LV-shRNA cell cultures were subjected to IR and analyzed for survival by CFA in two independent experiments. Figure 5 shows the mean CFA irradiation dose response curves obtained. As can be seen, radiosensitization by silencing every tested candidate target gene was validated with every distinct shRNA construct used. This thus further validated these 7 candidate genes as genuine target genes for radiosensitization.

Example 5. Expression of candidate radiation susceptibility genes in prostate tissue.

To study expression of CPNE7, GPR27, RNF8, RGNEF, MAD2L2, CBL and CCNBl in human prostate tissue at the for function most relevant protein level, we explored publicly available data provided in the Human Protein Atlas (www.proteinatlas.org). Although CPNE7, MAD2L2 and CBL were expressed in PC-3 cells, they were not or only at low levels expressed in human prostate cancer tissue. Hence, the relevance of targeting CPNE7, MAD2L2 or CBL to improve radiotherapy of prostate cancer is questionable. However, CBL is expressed at higher levels in other cancer types that are being treated with IR, such as glioma, lung cancer, and in particular lymphoma. CPNE7 is expressed in some colorectal cancers and in lymphoma. Thus, CBL and CPNE7 could be useful targets for radiosensitization in these cancers. In contrast, RGNEF and GPR27 were expressed in many different tissues and tumors, including prostate tumors, at moderate and strong levels, respectively. Interestingly, healthy human prostate
tissue was negative for RNF8, whereas this protein was expressed at moderate levels in prostate cancer tissue. Healthy human prostate tissue was also negative for CCNB1, while some prostate cancers expressed this protein at a moderate level. Thus, GPR27, RNF8, RGNEF and CCNB1 could all potentially be useful targets to improve radiotherapy of prostate cancer, with the latter two possibly adding tumor specificity. In terms of expression in tumor tissues, GPR27 clearly stood out. It is expressed at high levels in most tested breast, cervix, colorectal, endometrial, head&neck, liver, melanoma, ovarian, pancreatic, prostate, testis and thyroid cancers; and in at least moderate levels in carcinoid, glioma, lymphoma, skin, stomach and urothelial cancers. As shown in Examples 3 and 4, GPR27 also stood out in terms of radiosensitization upon gene silencing. Based on these findings, GPR27, the orphan G-protein Coupled Receptor 27, also known as Super Conserved Receptor Expressed in Brain 1 (SREB1), appears a particularly useful candidate target to improve radiotherapy of prostate cancer, and possibly other cancers as well.

Example 6. Silencing GPR27 sensitizes prostate cancer cells to fractionated irradiation.

In clinical practice, cancer patients are usually treated with IR given at multiple low-dose fractions. Often used treatment schedules consist of daily doses of 2Gy, given 5 days per week (on weekdays) for several weeks, reaching a total cumulative dose of more than 70Gy. Therefore, to further investigate the utility of silencing GPR27 to sensitize prostate cancer cells to IR, we subjected two different human prostate cancer cell lines, PC-3 and DU145, stable transduced with a lentiviral vector silencing GPR27, to fractionated low-dose IR. The lentiviral vector used for this purpose was TRCN0000008871 (see Table 4). Following 1, 3 or 5 daily fractions of 2Gy IR, cell survival was assessed using the CFA in comparison to survival of cells transduced with negative control vector LV-shSCR. Figure 6 shows that, as expected, multiple doses IR caused increased cell death in both prostate cancer cell lines. The cumulative effect of fractionated irradiation was more pronounced in PC-3 cells than in DU145 cells, suggesting that the latter cells were more effective in repairing the damage brought about by 2Gy IR. Importantly,
silencing GPR27 increased the toxicity of IR on both cell lines. In PC-3 cells, this effect was already evident after one cycle (40% versus 63% survival; p=0.04) and was also seen after 3 doses (12% versus 20% survival; p=0.04). After 5 cycles, hardly any surviving GPR27 knockdown cells (~3%) were observed. In DU145 cells, GPR27 silencing had little effect on single dose IR (51% versus 59% survival; n.s.), but increased toxicity became clearly apparent after three doses (10% versus 38% survival; p=0.02). Thus, silencing GPR27 sensitized two different human prostate cancer cell lines to a clinically relevant fractionated IR dosing schedule.

Example 7. Validation of GPR27-dependent radiation protection.

To corroborate that the observed effects of silencing GPR27 on radiation susceptibility are truly GPR27-dependent, we stably expressed a GPR27mut-coding ORF in PC-3 cells. GPR27mutORF was synthesized under contract by GeneArt Gene Synthesis (Life Technologies). This ORF carries the human GPR27 ORF sequence with silent nucleotide mutations in the binding site of siGPR27 with catalogue number D-005562-02 (i.e., the most effective individual GPR27-silencing siRNA that we identified). siGPR27 D-005562-02 targets the sequence 5'-CGGAGAAGAGGCTGTGCAA-3' in wild type GPR27. In GPR27mutORF, this sequence' was substituted for mutant sequence 5'-CGGAAAGAAGACTCTGTAA-3' (substitutions underlined). This abrogates silencing of GPR27mutORF by siGPR27 D-005562-02. Upon stable transfection of the GPR27mutORF expression plasmid, total GPR27/GPR27mut expression was increased approximately 2-fold, as shown by quantitative RT-PCR. PC-3 parental cells and PC-3-GPR27mutORF cells were transfected with siGPR27 D-005562-02 or non-targeting control siRNA (siNT).

Subsequently, cells were subjected to IR at doses up to 6Gy and cell survival was assessed using the CFA (Figure 7). Figure 7 left panel shows that, as before, GPR27 silencing sensitized PC-3 cells to IR (p=0.03; two-tailed t-test). In contrast, silencing GPR27 did not sensitize PC-3-GPR27mutORF cells to IR (Figure 7, right panel; difference not significant). Hence, sensitization to IR by GPR27 silencing was rescued by GPR27mutORF expression, validating GPR27 as a radiation susceptibility gene in PC-3 prostate cancer cells.
Example 8. Effects of GPR27 silencing on prostate cancer cell cycle after irradiation.

The effects of irradiation and GPR27 silencing on PC-3 cell cycle were assessed by FACS analysis. To this end, PC-3 cells transduced with a negative control lentiviral vector expressing shSCR; and PC-3 cells transduced with shGPR27-expressing lentiviral vector TRCN0000008871 (cell line Ell) were seeded 100,000 cells per well in 6-well-plates and 4Gy irradiated the next day. At 4, 16, 24 and 32 hours after irradiation, cells were harvested and incubated in propidium iodine (PI) solution (50ug/ml PI, 500ug/ml sodium citrate, 100ug/ml RNAse, 0.1% Triton X-100) for 30 minutes on ice. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Aggregates were gated out on the basis of fluorescence area and width. DNA histograms of single cells were prepared and analyzed for cell cycle distribution using ModFit LT. Two independent experiments were done and the results were averaged. Figure 8 shows that PC-3/LV-shSCR cells and PC-3/LV-shGPR27 cells had similar cell cycle distribution before irradiation. IR induced a transient loss of cells in Gl-phase and accumulation of cells in the G2-phase of cell cycle. This suggests activation of the G2/M cell cycle checkpoint to allow DNA damage repair. In PC-3/LV-shSCR cells, normal cell cycle distribution was fully recovered at 32 hours after irradiation. In PC-3/LV-shGPR27 cells, loss of cells in Gl-phase and accumulation of cells in the G2-phase was more profound than in PC-3/LV-shSCR cells (24% versus 37% Gl cells and 58% versus 47% G2/M cells at 16 hours) and an increased proportion of cells in G2-phase was still present at 32 hours post irradiation. This suggest that these cells have more difficulty recovering from IR-induced DNA damage, which is consistent with radiosensitization by GPR27 silencing.
Claims

1. Use of a GPR27 gene, or an RNA or protein encoded by the gene as a target for identifying a pharmacologically active compound active in sensitizing a cancer cell for ionizing radiation.

2. A method for determining whether a compound is capable of sensitizing a cancer cell for ionizing radiation comprising
   a. determining whether a compound is capable of inhibiting the activity of a GPR27 gene or an RNA or protein encoded by the gene, said method further comprising
   b. determining whether the compound identified in step a is capable of sensitizing a cancer cell for ionizing radiation.

3. A method for increasing the susceptibility of a cell to radiation, comprising introducing into the cell and/or contacting the cell with, a compound selected from:
   - an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, an RNAi molecule (siRNA or miRNA) or a precursor thereof or a ribozyme capable of binding under stringent hybridization conditions to a GPR27 gene or an mRNA gene product of the GPR27 genes
   - a small molecule interfering with the biological activity of a gene product of a GPR27 gene,
   - a (glyco)protein, a hormone or other biologically active compound capable of interacting with a GPR27 gene or with a gene product thereof, and
   - an antibody that inhibits the activity of a gene product of the GPR27 gene.

4. A method according to claim 3, further comprising irradiating said cell with ionizing radiation.

5. A method according to claim 4, wherein said cell is irradiated with 1-6 Gy.

6. A method according to claim 5, wherein said cell is a prostate cell.

7. A method according to any one of claims 3-6, wherein said cell is a cancer cell.

8. A compound selected from:
   - an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, an RNAi molecule (siRNA or miRNA) or a precursor thereof or a ribozyme capable of binding under stringent hybridization conditions to a GPR27 gene or an mRNA gene product of the gene
- a small molecule interfering with the biological activity of a gene product of the GPR27 gene,
- a (glyco)protein, a hormone or other biologically active compound capable of interacting with the GPR27 gene or with a gene product thereof, and
- an antibody that inhibits the activity of a gene product of the GPR27 gene, for use in the treatment of an individual suffering from cancer.

9. A compound selected from:
- an antisense molecule, in particular an antisense RNA or antisense oligodeoxynucleotide, an RNAi molecule (siRNA or miRNA) or a precursor thereof or a ribozyme capable of binding under stringent hybridization conditions to a GPR27 gene or an mRNA gene product thereof
- a small molecule interfering with the biological activity of a gene product of the GPR27 gene,
- a (glyco)protein, a hormone or other biologically active compound capable of interacting with the GPR27 gene or with a gene product thereof, and
- an antibody that inhibits the activity of a gene product of the GPR27 gene, for use in the treatment of an individual with ionizing radiation.

10. A method for determining the radiation sensitivity of a cancer in an individual or predicting the efficacy of radiation therapy of a cancer in an individual, comprising detecting a GPR27 gene or gene product thereof in the blood and/or a cancer cell of said individual.
FIG. 6

FIG. 7
FIG. 8
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search 15 May 2014

Date of mailing of the international search report 26/06/2014

Authorized officer

Nurmi, Jussi

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

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

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