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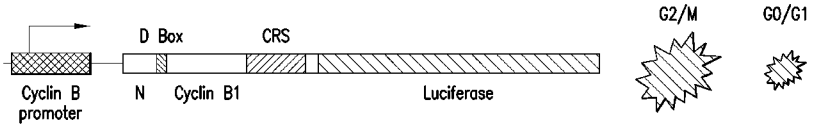


FIG. 1

(57) Abstract: Disclosed herein is a fusion protein comprising of 173 amino acid residues of the N terminus of cyclin B1 (including the Destruction Box and Cytoplasmic Retention Signal) fused in-frame with the luciferase reporter gene. Expression of the fusion protein is driven by the cyclin B1 promoter and mimics expression of the endogenous cyclin B1 gene. Following addition of the substrate luciferin, increased levels of the luciferase reporter gene can be used to monitor cell cycle arrest at the G2/M phase. Importantly, this invention may be used in vivo to monitor luciferase expression and G2/M cell cycle arrest in living animals. A broad aspect of the invention provides a method of detecting cell death in a target cell comprising transfecting the target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of the fusion protein; introducing the target cell with a substrate specific for the fusion protein for a period of time sufficient to detect a signal, wherein the generation of a signal indicates that the cell has arrested at the G2/M transition; and detecting a signal.

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TITLE OF THE INVENTION

# GENERATION OF MITOTIC PHASE REPORTER FOR IN VIVO BIOLUMINESCENCE IMAGING IN LIVING ANIMALS

## TECHNICAL FIELD

The present invention relates to a novel, non-destructive and dynamic process for determining mitotic phase arrest in living cells and animals.

## BACKGROUND OF THE INVENTION

Eukaryotic cell division proceeds through a highly regulated cell cycle comprising consecutive phases termed G1, S, G2 and M. Disruption of the cell cycle or cell cycle control can result in cellular abnormalities or disease states such as cancer which arise from multiple genetic changes that transform growth-limited cells into highly invasive cells that are unresponsive to normal control of growth. Transition of normal cells into cancer cells can arise though loss of correct function in DNA replication and DNA repair mechanisms. All dividing cells are subject to a number of control mechanisms, known as cell-cycle checkpoints, which maintain genomic integrity by arresting or inducing destruction of aberrant cells. Investigation of cell cycle progression and control is consequently of significant interest in designing anticancer drugs. (Flatt, P. M. and Pietenpol, J. A. Drug Metab. Rev., (2000), 32(3-4), 283-305; Buolamwini, J. K. Current Pharmaceutical Design, (2000), 6, 379-392).

As a consequence, accurate determination of cell cycle status is a key requirement for investigating cellular processes that affect the cell cycle or are dependent on cell cycle position. Such measurements are particularly vital in drug screening applications where:

i) substances which directly or indirectly modify cell cycle progression are desired, for example, for investigation as potential anti-cancer treatments;

ii) drug candidates are to be checked for unwanted effects on cell cycle progression; and/or

iii) it is suspected that an agent is active or inactive towards cells in a particular phase of the cell cycle.

Traditionally, cell cycle status for cell populations has been determined by flow cytometry using fluorescent dyes which stain the DNA content of cell nuclei (Barlogie, B. et al, Cancer Res., (1983), 43(9), 3982-97). Flow cytometry yields quantitative information on the DNA content of cells and hence allows determination of the relative numbers of cells in the G1,

S and G2+M phases of the cell cycle. However, this analysis is a destructive non-dynamic process and requires serial sampling of a population to determine cell cycle status with time. Furthermore, standard flow cytometry techniques examine the total cell population in the sample and yield limited data on individual cells, thereby precluding the study of cell cycle status of different cell types that may be present within the sample under analysis.

A further disadvantage of flow cytometry techniques relates to the indirect, and inferred assignment of cell cycle position of cells based on DNA content. Since the DNA content of cell nuclei varies through the cell cycle in a reasonably predictable fashion, i.e. cells in G2 or M have twice the DNA content of cells in G1, and cells undergoing DNA synthesis in S phase have an intermediate amount of DNA, it is possible to monitor the relative distribution of cells between different phases of the cell cycle. However, the technique does not allow precision in determining the cell cycle position of any individual cell due to ambiguity in assigning cells to G2 or M phases. Further imprecision arises from inherent variation in DNA content from cell to cell within a population which can preclude precise discrimination between cells which are close to the boundary between adjacent phases of the cell cycle. Additionally, variations in DNA content and DNA staining between different cell types from different tissues or organisms require that the technique is optimized for each cell type, and can complicate direct comparisons of data between cell types or between experiments (Herman, Cancer (1992), 69(6), 1553-1556). Thus, while flow cytometry maybe suitable for examining the overall cell cycle distribution of cells within a population, it is unavailable for monitoring the precise cell cycle status of an individual cell over time.

EP 798386 describes a method for the analysis of the cell cycle of cell sub-populations present in heterogeneous cell samples. This method uses sequential incubation of the sample with fluorescently labeled monoclonal antibodies to identify specific cell types and a fluorochrome that specifically binds to nucleic acids. This permits determination of the cell cycle distribution of sub-populations of cells present in the sample. However, as this method utilizes flow cytometry, it still yields only non-dynamic data and requires serial measurements to be performed on separate samples of cells to determine variations in the cell cycle status of a cell population with time following exposure to an agent under investigation for effects on cell cycle progression.

Cell cycle regulators are known to play an important role in cell cycle progression (See Pines, J., Nature Cell Biology, (1999), 1, E73-E79). For example, at specific cell cycle stages some proteins translocate from the nucleus to the cytoplasm, or vice versa, and some are rapidly degraded. For details of known cell cycle control components and interactions, see Kohn, Molecular Biology of the Cell (1999), 10, 2703-2734.

One of the most extensively characterized cell cycle regulators in human cells is cyclin B1, temporal and spatial expression and destruction of which controls cell transition from G2 to M and its exit from M. Cyclin B1 expression is driven by a cell cycle phase specific promoter which initiates expression at the end of S phase and peaks during G2. Once expressed, this protein constantly shuttles between the nucleus and the cytoplasm during the G2 phase, but it is primarily cytoplasmic because the rate of its export is much greater than its import. At the start of mitosis, cyclin B1 rapidly translocates into the nucleus, when its rate of import substantially increases, and its export decreases, in a phosphorylation dependent manner. Thus, the localization of cyclin B1 in the cell can be used to mark the transition from G2 phase to mitosis. Once a cell reaches metaphase, or, more accurately, when the spindle assembly checkpoint is satisfied, cyclin B1 is very rapidly degraded. Cyclin B1 destruction continues throughout the following G1 phase but stops once cells begin DNA replication. These events have been visualized in real time by micro-injection of fluorescently labeled cyclin B1 into living cells (See Clute and Pines, Nature Cell Biology, (1999), 1, 82-87).

The controlling elements which regulate temporal expression and destruction have been elucidated in a number of studies. Biosynthesis of cyclin B1 has been shown to be controlled at the level of transcription by a promoter sequence that confines expression to the late S and G2 phases of the cell cycle (Piaggio et al, Exp. Cell. Research, (1995), 216, 396-402; Cogswell et al, Mol. Cell. Biology, (1995), 15, 2782-2790). Destruction of cyclin B1 at the appropriate time in M phase has been shown to be controlled by a 9 amino acid sequence, termed the destruction box (D-box) which targets the protein for proteolysis via ubiquitinylation. Expression of a *Drosophila* cyclin B-GFP fusion protein driven by a constitutive polyubiquitin promoter (Huang and Raff, EMBO Journal, (1999), 18(8), 2184-2195) has shown that fluorescently-tagged cyclin B mimics the behaviour of endogenous cyclin B in being degraded at the end of metaphase. Studies (Hagting et al, Current Biology, (1999), 9, 680-689) using human cyclin B1-GFP have shown that temporal changes in cytoplasmic and nuclear localization of cyclin B1 with cell cycle progression is dependent on a nuclear export signal (NES), phosphorylation of which leads to nuclear import.

A number of methods have been described which make use of certain components of the cell cycle control mechanisms to provide procedures which analyze or exploit cell proliferation status.

WO 00/29602 describes use of a cyclin A promoter to drive expression of GFP as a selectable marker for dividing transgenic stem cells to allow dividing cells to be isolated from a background of non-dividing cells by fluorescence activated cell sorting. While this method may allow for the identification and selection of cells which have progressed past a certain stage in the cell cycle, it does not yield information on the cell cycle status of any given cell, other than

historical information that the cell has or has not passed through the G2 phase of the cell cycle at some time in the past.

U.S. Pat. No. 6,048,693 aims to describe a method of screening for compounds affecting cell cycle regulatory proteins, wherein expression of a reporter gene is linked to control elements which are acted on by cyclins or other cell cycle control proteins. In this method, temporal expression of a reporter gene product is driven in a cell cycle specific fashion and compounds acting on one or more cell cycle control components may increase or decrease expression levels. Since the assay system contains no elements which provide for the destruction of the reporter gene product nor for destruction of any signal arising from the reporter gene, the method cannot yield information on the cell cycle position of any cells in the assay and reports only on general perturbations of cell cycle control mechanisms.

U.S. Pat. No. 5,849,508 and U.S. Pat. No. 6,103,887 describe methods for determining the proliferative status of cells by use of antibodies which bind to cyclin A. These methods provide means for determining the percentage of proliferating cells in a test population relative to a control population.

U.S. Pat. No. 6,159,691 relates to a method for assaying for putative regulators of cell cycle progression. In this method, nuclear localization signals (NLS) derived from the cell cycle phase specific transcription factors DP-3 and E2F-1 are used to assay the activity of compounds which act as agonists or antagonists to increase or decrease nuclear localisation of an NLS fused to a detectable marker.

Traditionally, researchers have studied the cell cycle using traditional reporter molecules that require the cells to be fixed or lysed. For example Hauser and Bauer (Plant and Soil, 2000, 226, p 1-10) used β-glucuronidase (GUS) to study cell division in a plant meristem and Brandeis and Hunt (EMBO J., 1996, vol 15, pp 5280-5289) used chloramphenical acetyl transferase (CAT) fusion proteins to study variations in cyclin levels. Although these methods provide a means of studying the cell cycle position of a particular cell (using GUS) or the average cell cycle status of a population of cells (using CAT) both methods are destructive. Neither method allows the repeated analysis of a specific cell over time and they are therefore not suitable to follow the progression of a cell through the cell cycle.

None of the preceding methods provide means for determining the cell cycle status of an individual cell or a population of cells. Consequently, there remains a need for a method that enables one to precisely determine cell cycle position of a single living cell non-destructively, allowing the same cell to be repeatedly interrogated over time, and which enables the study of the effects of agents having potentially desired or undesired effects on the cell cycle. It is desirable for cell cycle position to be determined from a probe controlled directly by cell cycle

control components, rather than indirectly through DNA content or other indirect markers of cell cycle position as described above (U.S. Pat. No. 7,235,401 describes a nucleic acid sequence encoding a fluorescent reporter protein operably linked to and under control of a cyclin B1 promoter, a cyclin B1 destruction box (D-box) and a cyclin B1 cytoplasmic retention sequence (CRS) to provide means of determining cell cycle status for individual living mammalian cells in a non-destructive process).

The present invention aims to overwrite aforementioned drawbacks relative to cell cycle determination by providing a bioluminescent reporter to non-invasively image cyclin B1 transcriptional regulation as well as post-translational shuttling and degradation, and further to monitor the mitotic phase of the cell cycle.

## SUMMARY OF THE INVENTION

Embodiments of this invention are made available by the development of a fusion protein comprising 173 amino acid residues of the N terminus of cyclin B1 fused in-frame with a luciferase reporter gene - cyclinB1-Luciferase fusion protein. Expression of the fusion protein is driven by the cyclin B1 promoter and mimics expression of the endogenous cyclin B1 gene. Following addition of the substrate luciferin, increased levels of the luciferase reporter gene can be used to monitor cell cycle arrest at the G2/M phase. Importantly, this invention may be used in vivo to monitor luciferase expression and G2/M cell cycle arrest in living animals.

A broad aspect of the invention provides for a method of detecting cell cycle arrest at the G2/M phase in a target cell. The method proposes transfecting the target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of the fusion protein, introducing the transfected target cell with a substrate specific for the fusion protein for a period of time sufficient to detect a signal, wherein the generation of a signal indicates that said cell has arrested at the G2/M transition. This is followed by detecting the signal as a determination of cell cycle arrest.

The above assay can also be used to detect cell death.

In another aspect the assays described herein may be repeated over time wherein the presence of a signal at any one or more time points is indicative of an apoptotic event while the absence impels the conclusion that the cell arrest did not occur. Consequently, iterative assays over two or more time pots area is also encompassed.

A method of detecting apoptotic events in a target cell is also contemplated comprising transfecting the target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of said fusion protein; introducing into the target cell a substrate specific for the fusion protein for a period of time sufficient to detect a signal, wherein the generation of a signal indicates that said cell has arrested at the G2/M transition; wherein the absence of signal indicates a cellular apoptotic event.

Another aspect of the invention provides for a method of detecting cyclinB1 sub-cellular localization in a target cell comprising:

(i) transfecting the target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of the fusion protein;

(ii) introducing said target cell with a substrate specific for the fusion protein for a period of time sufficient to detect a signal; and

(iii) detecting a signal, wherein localization of signal inside the nucleus indicates cyclinB1 sub-cellular localization as evidenced by transition from G2 to mitosis.

In another aspect, the invention provides a method for determining the efficacy of a test compound in promoting G2/M cell cycle arrest or apoptosis in a target cell consisting of a cyclinB1-Luciferase fusion protein comprising transfecting the target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of the fusion protein; introducing into the target cell a substrate specific for the fusion protein for a period of time sufficient to detect a signal; and detecting a signal as indicative of the therapeutic efficacy of the test compound in inducing one of a cell death, cell cycle arrest or an apoptotic event.

In yet another aspect, the invention provides a method for determining the efficacy of a test compound in promoting G2/M cell cycle arrest or apoptosis in a target cell using any one or more of the above mentioned assays.

Another aspect of the invention provides a method for determining the therapeutic efficacy of a chemical compound or biological agent in treating hyper-proliferative disorders characterized by aberrant cell division comprising contacting the test compound with a target cell previously transfected with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of the fusion protein; and having introduced into it a substrate specific for the fusion protein for a period of time sufficient to detect a signal; and detecting a signal and correlating the signal strength as indicative of the therapeutic efficacy of the test compound in inducing one of a cell death, cell cycle arrest or an apoptotic event.

Transgenic animals are also contemplated.

## BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 shows a schematic drawing of the mitotic reporter. A fusion protein of the N-terminus of cyclin B fused to luciferase is driven by a cyclin B promoter. The N-terminal domain of cyclin B1 contains a conserved 9 amino acid motif (RTALGDIGN) called the destruction box (D-box) that is necessary for cyclin B1 ubiquitination and subsequent degradation. The cytoplasmic retention sequence (CRS) region is responsible for nuclear/cytoplasmic shuttling of cyclin B. In the G2/M phase of cell cycle, the reporter is stabilized, and is degraded during G0/G1 phase. The N-terminus of cyclin B1 was amplified by PCR and ligated into the pGL3 vector. The promoter of cyclin B was inserted to the upstream of N cyclin B-luciferase fusion sequence.

Figure 2 displays induction of cyclinB-Luc reporter following treatment of cells with Nocodazole, a mitotic phase blocking reagent. Cell cycle block of polyclonal HeLa cells stably transfected with pGL3-cyclinB-Luc (HeLa-cyclinB-Luc) was achieved by growth in media containing 0.2 mM mimosine or 500 nM Nocodazole for 18 hours. Mimosine arrested 80% of cells in late G1 phase, while nocodazole arrested 65% of cells in G2/M phase. Luciferase assay showed that mimosine down-regulated the cyclinB-Luc reporter activity by 60%, while nocodazole induced cyclinB-Luc activity in a dose-dependent manner. Normalized relative luciferase units (RLU) are shown. Western blots show that nocodazole induced dose-dependent up regulation of cyclinB-Luc protein, consistent with the induction of endogenous cyclin B1.

Figure 3 describes induction of cyclinB-Luc by anti-mitotic anti-cancer drugs *in vitro*. HeLa-cyclinB-Luc cells were incubated with two anti-cancer drugs, taxotere and taxol (anti-mitotic drugs), in the indicated concentrations for 24 hr, and *in vitro* imaging was acquired. Quantitative analysis showed that both taxotere and taxol induced the cyclinB-Luc reporter in a dose-dependent manner.

Figure 4 displays induction of cyclinB-Luc by taxotere and taxi in hollow fibers *in vitro*. HCT116 human colon carcinoma cells stably transfected with a pGL3-cyclinB-Luc vector (HCT116-cyclinB-Luc) were filled in hollow fibers and treated with either taxotere or taxol at 100 nM for 24 hr. Uncontaminated pure tumor cells retrieved from hollow fibers were applied to cell cycle assay with flow cytometry. Both taxotere and taxol arrested HCT116-cyclinB-Luc

cells in G2/M phase. Bioluminescence images of hollow fibers filled with HCT116-cyclinB-Luc cells showed cyclinB-Luc induction with taxotere treatment.

Figure 5 presents data showing that mitotic arrest of HCT116-cyclinB-Luc cells by taxotere induces cyclinB-Luc reporter in hollow fibers *in vivo*. Nude mice bearing hollow fibers filled with HCT116-cyclinB-Luc cells were either vehicle-treated or treated with taxotere (i.p., 20 mpk). Bioluminescence images were acquired at the time points indicated. Taxotere treatment induced cyclinB-Luc 2~2.3 fold at 24 hr and 48 hr. Cell cycle distribution was also determined by flow cytometry with cells retrieved from hollow fibers at 48 hr after taxotere treatment.

Figure 6 describes induction of cyclinB-Luc by taxotere in HCT116-cyclinB-Luc subcutaneous tumors *in vivo*. Nude mice bearing HCT116-cyclinB-Luc subcutaneous tumors were either vehicle-treated or treated with taxotere (i.p., 20 mpk). Bioluminescence images were acquired at the time points indicated.

Figure 7 shows upregulation of cyclin B1 in tumors treated with taxotere. 48 hr after taxotere treatment (i.p., 20 mpk), subcutaneous tumors were removed and paraffin-embedded sections were stained with cyclin B1 antibody. Quantitation showed significantly higher cyclin B1 positive cells in taxotere-treated tumors than vehicle treated tumors.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention consists of 173 amino acid residues of the N terminus of cyclin B1 (including the Destruction Box and Cytoplasmic Retention Signal) fused in-frame with the luciferase reporter gene. Expression of this fusion protein is driven by the cyclin B1 promoter and mimics expression of the endogenous cyclin B1 gene. Following addition of the substrate luciferin, increased levels of the luciferase reporter gene can be used to monitor cell cycle arrest at the G2/M phase. Importantly, this invention may be used in vivo to monitor luciferase expression and G2/M cell cycle arrest in living animals.

Methods of detecting biological activities and substances using bioluminescent proteins have recently been developed. For example, protein phosphorylation events can be detected using fusion proteins containing GFP (see, e.g., U.S. Pat. No. 5,958,713) or luciferase, aequorin and obelin (see, e.g., U.S. Pat. No. 5,683,888). Light-generating moieties have been introduced into mammals to specifically localize events such as parasite infection (see, e.g., U.S. Pat. No. 5, 650,135).

A key advance in the biomedical arts has been the discovery of bioluminescent protein moieties, e.g. luciferase which can be expressed in diverse mammalian cell types and

thus act as detectable signals for biological signal transduction pathways and events. An increased understanding of how signal transduction events and changes in gene expression regulate cell cycle progression in mammalian cells has also been emerging. Indeed, alterations in the activity of these pathways leading to altered expression of cell cycle regulators may underlie the initiation and/or progression of diseases such as cancer.

Luciferase is a useful bioluminescent moiety of the invention. Members of the luciferase family have been identified in a variety of prokaryotic and eukaryotic organisms. Luciferase and other enzymes involved in the prokaryotic luminescent (lux) systems, as well as the corresponding lux genes, have been isolated from marine bacteria in the *Vibrio* and *Photobacterium* genera and from terrestrial bacteria in the *Xenorhabdus* genus, also called photorhalodus. An exemplary eukaryotic organism containing a luciferase system (luc) is the North American firefly *Photinus pyralis*. Firefly luciferase has been extensively studied, and is widely used in ATP assays. cDNAs encoding luciferases from *Pyrophorus plagiophthalamus*, another species, click beetle, have been cloned and expressed (See, Wood et al., 1989, *Science* 244:700-702). This beetle is unusual in that different members of the species emit bioluminescence of different colors. Four classes of clones, having 95-99% similarity with each other, were isolated. They emit light at 546 nm (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm (orange).

Luciferases requires a source of energy, such as ATP, NAD(P) H, and the like, and a substrate, such as luciferin, decanal (bacterial enzymes) or coelentrizine and oxygen. The substrate luciferin must be supplied to the luciferase enzyme in order for it to luminesce. Thus, a convenient method for providing luciferin is to express not only the luciferase but also the biosynthetic enzymes for the synthesis of the substrate decanal. Oxygen is then the only extrinsic requirement for bioluminescence, in bacteria expressing these proteins from the Lux operon. In the present embodiment, enzyme activity of the cyclin B1-luciferase fusion protein can be monitored following intraperitoneal (IP) injection of the substrate luciferin into living animals.

The selection of a light-generating polypeptide moiety of the light-generating fusion protein should be done so as to produce light capable of penetrating animal tissue such that it can be detected externally in a non-invasive manner. The ability of light to pass through a medium such as animal tissue (composed mostly of water) is determined primarily by the light's intensity and wavelength.

A first factor governing detectability of light through a layer of tissue is the intensity of emitted light. The more intense the light produced in a unit volume, the easier the light will be to detect. The intensity of light produced in a unit volume depends on the spectral

characteristics of individual bioluminescent polypeptide moieties, and on the concentration of those moieties in the unit volume. Accordingly, schemes that place a high concentration of bioluminescent polypeptide moieties in or on an entity (such as high-efficiency loading of a liposome or high-level expression of a light-generating fusion protein in a cell) typically produce brighter light-generating fusion proteins (LGPs), which are easier to detect through deeper layers of tissue, than schemes which conjugate, for example, only a single LGM onto each entity.

A second factor governing detectability through a layer of tissue is the wavelength of the emitted light. Water may be used to approximate the absorption characteristics of animal tissue, since most tissues are composed primarily of water. It is well known that water transmits longer-wavelength light (in the red range) more readily than it does shorter wavelength light. Accordingly, bioluminescent polypeptide moieties which emit light in the range of yellow to red (550-1100 nm) are typically preferable to those which emit at shorter wavelengths. However, excellent results can be achieved in practicing the present invention with LGMs that emit in the range of 486 nm, despite the fact that this is not an optimal emission wavelength.

Transgenic Animals.

In another aspect, the present invention includes transgenic animals containing a heterologous (or exogenous) gene construct or transgene encoding a light-generating fusion protein or complex of proteins.

The preparation of a transgenic mammal requires introducing a nucleic acid construct that will be used to express a nucleic acid encoding a light-generating fusion protein into an undifferentiated cell type, e.g., an embryonic stem (ES) cell. The ES cell is then injected into a mammalian embryo, where it will integrate into the developing embryo. The embryo is then implanted into a foster mother for the duration of gestation.

Embryonic stem cells are typically selected for their ability to integrate into and become part of the germ line of a developing embryo so as to create germ line transmission of the heterologous gene construct. Thus, any ES cell line that has this capability is suitable for use herein. One mouse strain that is typically used for production of ES cells is the 129J strain. A preferred ES cell line is murine cell line D3 (American Type Culture Collection catalog no. CRL 1934). The cells are cultured and prepared for DNA insertion using methods well known in the art, such as those set forth by Robertson (Robertson, In: *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach,* E. J. Robertson, ed., IRL Press, Washington, D.C., 1987.). Insertion of the nucleic acid construct into the ES cells can be accomplished using a variety of methods well known in the art including for example, electroporation, microinjection, and calcium phosphate treatment.

The term "transgene" is used herein to describe genetic material that has been or is about to be artificially inserted into the genome of a mammalian cell, particularly a mammalian cell of a living animal. The transgene is used to transform a cell, meaning that a permanent or transient genetic change, preferably a permanent genetic change, is induced in a cell following incorporation of an heterologous nucleic acid, such as DNA. A permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. Of interest are transgenic mammals, e.g. cows, pigs, goats, horses, etc., and particularly rodents, e.g., rats, mice, etc. Preferably, the transgenic animals are mice.

Transgenic animals comprise a heterologous nucleic acid sequence present as an extrachromosomal element or stably integrated in all or a portion of its cells, especially in germ cells. Unless otherwise indicated, it will be assumed that a transgenic animal comprises stable changes to the germline sequence. During the initial construction of the animal, "chimeras" or "chimeric animals" are generated, in which only a subset of cells have the altered genome. Chimeras are primarily used for breeding purposes in order to generate the desired transgenic animal. Animals having a heterozygous alteration are generated by breeding of chimeras. Male and female heterozygotes are typically bred to generate homozygous animals.

The heterologous gene is usually either from a different species than the animal host, or is otherwise altered in its coding or non-coding sequence. The introduced gene may be a wild-type gene, naturally occurring polymorphism, or a genetically manipulated sequence, for example having deletions, substitutions or insertions in the coding or non-coding regions. Where the introduced gene is a coding sequence, it is usually operably linked to a promoter, which may be constitutive or inducible, and other regulatory sequences required for expression in the host animal. By "operably linked" is meant that a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules, e.g., transcriptional activator proteins, are bound to the regulatory sequence(s). The transgenic animals of the invention can comprise other genetic alterations in addition to the presence of the heterologous gene. For example, the host's genome may be altered to affect the function of endogenous genes (e.g., endogenous cyclin B1), contain marker genes, or other genetic alterations such as are described in the Examples.

Knockouts and Knockins

Although not necessary to the operability of the invention, the transgenic animals described herein may comprise alterations to endogenous genes in addition to the genetic alterations described above. For example, the host animals may be either "knockouts" and/or "knockins" for a target gene(s) as is consistent with the goals of the invention (e.g., the host

animal's endogenous cyclin B1 may be "knocked out" and/or the endogenous bioluminescent fusion protein "knocked in". Knockouts have a partial or complete loss of function in one or both alleles of an endogenous gene of interest (e.g., cyclin B1). Knockins have an introduced transgene with altered genetic sequence and/or function from the endogenous gene. The two may be combined, for example, such that the naturally occurring gene is disabled, and an altered form introduced. For example, it may be desirable to knockout the host animal's endogenous cyclin B1 gene, while introducing an exogenous bioluminescent fusion protein gene (e.g., a human transgene encoding the N-terminal region of cyclin B1 operably linked to luciferase).

In a knockout, preferably the target gene expression is undetectable or insignificant. For example, a knock-out of the cyclin B1 gene means that function of cyclin B1 has been substantially decreased so that expression is not detectable or only present at insignificant levels. This may be achieved by a variety of mechanisms, including introduction of a disruption of the coding sequence, e.g., insertion of one or more stop codons, insertion of a DNA fragment, etc., deletion of coding sequence, substitution of stop codons for coding sequence, etc. In some cases the exogenous transgene sequences are ultimately deleted from the genome, leaving a net change to the native sequence. Different approaches may be used to achieve the "knock-out". A chromosomal deletion of all or part of the native gene may be induced, including deletions of the non-coding regions, particularly the promoter region, 3' regulatory sequences, enhancers, or deletions of gene that activate expression of cyclin B1 genes. A functional knock-out may also be achieved by the introduction of an anti-sense construct that blocks expression of the native genes (See, e.g., Li and Cohen (1996) *Cell* 85:319-329). "Knock-outs" also include conditional knockouts, for example where alteration of the target gene occurs upon exposure of the animal to a substance that promotes target gene alteration, introduction of an enzyme that promotes recombination at the target gene site (e.g. Cre in the Cre-lox system), or other method for directing the target gene alteration postnatally.

A "knockin" of a target gene means an alteration in a host cell genome that results in altered expression or function of a native target gene. Increased (including ectopic) or decreased expression may be achieved by introduction of an additional copy of the target gene, or by operatively inserting a regulatory sequence that provides for enhanced expression of an endogenous copy of the target gene. These changes may be constitutive or conditional, i.e. dependent on the presence of an activator or represser. The use of knockin technology may be combined with production of exogenous sequences to produce the transgenic animals of the invention.

The heterologous gene construct includes a nucleic acid encoding a light-generating fusion protein or complex of proteins. The heterologous gene construct can also encode for various accessory proteins required for the functional expression of the light-generating protein,

as well as selection markers and enhancer elements. Examples of accessory proteins include the biosynthetic enzymes for the synthesis of the luciferase substrate decanal. A selection marker can be any nucleic acid sequence that is detectable and/or assayable. Examples of selection markers include positive selection markers and negative selection markers. Positive selection markers include drug resistance genes; e.g., neomycin resistance genes or hygromycin resistance genes, or beta-galactosidase genes. Negative selection markers, e.g., thymidine kinase gene, diphtheria toxin gene and ganciclovir are useful in the heterologous gene construct in order to eliminate embryonic stem (ES) cells that do not undergo homologous recombination. The selection marker gene is usually operably linked to its own promoter or to another strong promoter from any source that will be active or can easily be activated in the cell into which it is inserted; however, the marker gene need not have its own promoter attached as it may be transcribed using the promoter of the light-generating fusion protein gene to be suppressed. In addition, the marker gene will normally have a polyA sequence attached to the 3' end of the gene; this sequence serves to terminate transcription of the gene.

"Enhancer elements" include nucleic acid sequences that are bound by polypeptides associated with transcription, and are usually in cis with the nucleic acid encoding a light-generating fusion protein. Examples of enhancer elements include cyclic AMP response elements (CRE), serum response elements (SRE), nuclear factor B (NF-κB), activator protein 1 (AP-1), serum response factor (SRF), and p53 binding sites. These enhancer elements may further include a TATA box.

The heterologous gene construct may be constitutively expressed in the transgenic mammal. The gene construct may be expressed in specific tissues, e.g., the construct is under the control of a tissue-specific promoter.

The invention includes a transgenic mouse containing a heterologous gene construct encoding a light-generating fusion protein, where the fusion protein contains 173 amino acid residues of the N-terminus of cyclin B1 and luciferase, which allows M phase arrested cells to be imaged and distinguished from normoxic tissue. For example, in a mammal such as a mouse, inducible expression and translation of a nucleic acid encoding a light-generating fusion polypeptide that includes a degradable fragment of cyclin B1 and luciferase allows the detection of M phase arrested cells, by detecting and localizing the light generated by the luciferase.

The gene construct is under the control of the endogenous cyclin B1 promoter, and thus the transcriptional regulation is cell cycle dependent. Activation of the promoter during M phase results in increased expression of the gene construct encoding the light-generating fusion proteins and the accessory proteins, if present. If the activation occurs only in a part of the animal, only cells in that part will express the light-generating protein.

Imaging of Light-generating Fusion Proteins

Light-generating fusion proteins produced within a stably transfected cell or within cells of a transgenic animal are capable of being imaged or detected by a variety of means well known in the art. Since the imaging, or measuring photon emission from the subject, may last up to tens of minutes, the subject is desirably immobilized during the imaging process. Imaging of the light-generating polypeptide moiety involves the use of, e.g., a photodetector capable of detecting extremely low levels of light--typically single photon events--and integrating photon emission until an image can be constructed. Examples of such sensitive photodetectors include devices that intensify the single photon events before the events are detected by a camera, and cameras (cooled, for example, with liquid nitrogen) that are capable of detecting single photons over the background noise inherent in a detection system.

Once a photon emission image is generated, it is typically superimposed on a "normal" reflected light image of the subject to provide a frame of reference for the source of the emitted photons (i.e., localize the light-generating fusion proteins with respect to the subject) . A "composite" image formed by the superimposition of the photon emission image on the reflected light image is then analyzed to determine the location and/or amount of a target in the subject.

Light-generating fusion proteins that have localized to their intended sites in a subject may be imaged in a number of ways. Guidelines for such imaging, as well as specific examples, are described below.

Localization of Light-generating fusion proteins. In the case of "targeted" conjugates, that is, conjugates which contain a targeting moiety--a molecule or feature designed to localize the conjugate within a subject or animal at a particular site or sites, localization refers to a state when an equilibrium between bound, "localized", and unbound, "free" entities within a subject has been essentially achieved. The rate at which such an equilibrium is achieved depends upon the route of administration. For example, a conjugate administered by intravenous injection to localize thrombi may achieve localization, or accumulation at the thrombi, within minutes of injection. On the other hand, a conjugate administered orally to localize an infection in the intestine may take hours to achieve localization.

Alternatively, localization may simply refer to the location of the entity within the subject or animal at selected time periods after the entity is administered. In a related aspect, localization of, for example, injected tumors cells expressing a light-generating moiety, may consist of the cells colonizing a site within the animal and forming a tumor mass.

By way of another example, localization is achieved when an entity becomes distributed following administration. For example, in the case of a conjugate administered to

measure the oxygen concentration in various organs throughout the subject or animal, the conjugate becomes "localized", or informative, when it has achieved an essentially steady-state of distribution in the subject or animal.

In all of the above cases, a reasonable estimate of the time to achieve localization may be made by one skilled in the art. Furthermore, the state of localization as a function of time may be followed by imaging the light-emitting conjugate according to the methods of the invention.

The "photodetector device" used should have a high enough sensitivity to enable the imaging of faint light from within a mammal in a reasonable amount of time, and to use the signal from such a device to construct an image.

In cases where it is possible to use light-generating moieties which are extremely bright, and/or to detect light-generating fusion proteins localized near the surface of the subject or animal being imaged, a pair of "night-vision" goggles or a standard high-sensitivity video camera, such as a Silicon Intensified Tube (SIT) camera (e.g., from Hammamatsu Photonic Systems, Bridgewater, N.J.), may be used. More typically, however, a more sensitive method of light detection is required.

In extremely low light levels the photon flux per unit area becomes so low that the scene being imaged no longer appears continuous. Instead, it is represented by individual photons which are both temporally and spatially distinct form one another. Viewed on a monitor, such an image appears as scintillating points of light, each representing a single detected photon. By accumulating these detected photons in a digital image processor over time, an image can be acquired and constructed. In contrast to conventional cameras where the signal at each image point is assigned an intensity value, in photon counting imaging the amplitude of the signal carries no significance. The objective is to simply detect the presence of a signal (photon) and to count the occurrence of the signal with respect to its position over time.

At least two types of photodetector devices, described below, can detect individual photons and generate a signal which can be analyzed by an image processor. Reduced-Noise Photodetection Devices achieve sensitivity by reducing the background noise in the photon detector, as opposed to amplifying the photon signal. Noise is reduced primarily by cooling the detector array. The devices include charge coupled device (CCD) cameras referred to as "backthinned", cooled CCD cameras. In the more sensitive instruments, the cooling is achieved using, for example, liquid nitrogen, which brings the temperature of the CCD array to approximately-120°C. "Backthinned" refers to an ultra-thin backplate that reduces the path length that a photon follows to be detected, thereby increasing the quantum efficiency. A

particularly sensitive backthinned cryogenic CCD camera is the "TECH 512", a series 200 camera available from Photometrics, Ltd. (Tucson, Ariz.).

"Photon amplification devices" amplify photons before they hit the detection screen. This class includes CCD cameras with intensifiers, such as microchannel intensifiers. A microchannel intensifier typically contains a metal array of channels perpendicular to and co-extensive with the detection screen of the camera. The microchannel array is placed between the sample, subject, or animal to be imaged, and the camera. Most of the photons entering the channels of the array contact a side of a channel before exiting. A voltage applied across the array results in the release of many electrons from each photon collision. The electrons from such a collision exit their channel of origin in a "shotgun" pattern, and are detected by the camera.

Even greater sensitivity can be achieved by placing intensifying microchannel arrays in series, so that electrons generated in the first stage in turn result in an amplified signal of electrons at the second stage. Increases in sensitivity, however, are achieved at the expense of spatial resolution, which decreases with each additional stage of amplification. An exemplary microchannel intensifier-based single-photon detection device is the C2400 series, available from Hamamatsu.

Image Processors process signals generated by photodetector devices which count photons in order to construct an image which can be, for example, displayed on a monitor or printed on a video printer. Such image processors are typically sold as part of systems which include the sensitive photon-counting cameras described above, and accordingly, are available from the same sources. The image processors are usually connected to a personal computer, such as an IBM-compatible PC or an Apple Macintosh (Apple Computer, Cupertino, Calif.), which may or may not be included as part of a purchased imaging system. Once the images are in the form of digital files, they can be manipulated by a variety of image processing programs (such as "ADOBE PHOTOSHOP", Adobe Systems, Adobe Systems, Mt. View, Calif.) and printed.

The Detection Field Of The Device is defined as the area from which consistent measurements of photon emission can be obtained. In the case of a camera using an optical lens, the detection field is simply the field of view accorded to the camera by the lens. Similarly, if the photodetector device is a pair of "night vision" goggles, the detection field is the field of view of the goggles.

Alternatively, the detection field may be a surface defined by the ends of fiber-optic cables arranged in a tightly-packed array. The array is constructed to maximize the area covered by the ends of the cables, as opposed to void space between cables, and placed in close proximity

to the subject. For instance, a clear material such as plexiglass can be placed adjacent the subject, and the array fastened adjacent the clear material, opposite from the subject.

With higher-intensity LGPs, photodiode arrays may be used to measure photon emission. A photodiode array can be incorporated into a relatively flexible sheet, enabling the practitioner to partially "wrap" the array around the subject. This approach also minimizes photon loss, and in addition, provides a means of obtaining three-dimensional images of the bioluminescence. Other approaches may be used to generate three-dimensional images, including multiple detectors placed around the subject or a scanning detector or detectors.

It will be understood that the entire animal or subject need not necessarily be in the detection field of the photodetection device. For example, if one is measuring a light-emitting conjugate known to be localized in a particular region of the subject, only light from that region, and a sufficient surrounding "dark" zone, need be measured to obtain the desired information.

Immobilizing the subject. In those cases where it is desired to generate a two-dimensional or three-dimensional image of the subject, the subject may be immobilized in the detection field of the photodetection devices during the period that photon emission is being measured. If the signal is sufficiently bright that an image can be constructed from photon emission measured in less than about 20 milliseconds, and the subject is not particularly agitated, no special immobilization precautions may be required, except to insure that the subject is in the field of the detection device at the start of the measuring period.

If, on the other hand, the photon emission measurement takes longer than about 20 msec, and the subject is agitated, precautions to insure immobilization of the subject during photon emission measurement, commensurate with the degree of agitation of the subject, need to be considered to preserve the spatial information in the constructed image. For example, in a case where the subject is a person and photon emission measurement time is on the order of a few seconds, the subject may simply be asked to remain as still as possible during photon emission measurement (imaging). On the other hand, if the subject is an animal, such as a mouse, the subject can be immobilized using, for example, an anesthetic or a mechanical restraining device.

In cases where it is desired to measure only the total amount of light emanating from a subject or animal, the subject does not necessarily need to be immobilized, even for long periods of photon emission measurements. All that is required is that the subject be confined to the detection field of the photodetector during imaging. It will be appreciated, however, that immobilizing the subject during such measuring may improve the consistency of results obtained, because the thickness of tissue through which detected photons pass will be more uniform from animal to animal.

Applications: Localization of Tumor Cells

The growth and metastatic spread of transformed cells in a subject may be monitored using methods and compositions of the present invention. In particular, in experiments where an animal is implanted with cyclin B1-Luciferase stably transfected cells, expression of the light generating fusion protein could be used to both define the boundaries of the tumor, and to determine whether cells from the primary tumor mass have migrated and colonized distal sites.

In a related aspect, images utilizing tumor-localizing LGPs, such as those described above, may be generated at selected time intervals to monitor tumor growth, progression and metastasis in a subject over time. Such monitoring may be useful to record results of anti-tumor therapy, or as part of a screen of putative therapeutic compounds useful in inhibiting tumor growth or metastasis.

In the practice of the invention, the tissue and the light-generating fusion protein can be contacted in vitro, such as where one or more biological samples (e.g., blood, serum, cells, tissue) are arrayed on a substrate under tissue culture conditions known by those in the art to preserve the viability of the tissue and then the fusion protein is added to the tissue culture. In a preferred embodiment of the methods of the invention the tissue is mammalian tissue, in particular human tissue.

Another aspect of the invention provides methods for determining cell cycle arrest, cancer or apoptosis in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of agents (e.g., drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (e.g., the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.) Yet another aspect of the invention pertains to monitoring the influence of agents (e.g., drugs, compounds) on cell cycle arrest, cancer or infection in clinical trials.

The invention also encompasses kits for detecting the presence of cell cycle arrest, cancer or apoptosis in a biological sample. For example, the kit can comprise the fusion protein of the invention packaged in a suitable container. The kit can further comprise instructions for using the kit to detect cell cycle arrest, cancer or apoptosis.

Thus, the diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with cell cycle arrest, cancer or apoptosis. Furthermore, the prognostic assays described herein can be used to determine whether a subject should be administered an agent (e.g., an agonist, antagonist,

peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with abnormal cell cycle arrest, cancer or apoptosis.

The invention further provides a method for testing a compound for activity in promoting G2/M cell cycle arrest, cancer treatment, and apoptosis. The method (also referred to herein as a "screening assay") can be used for identifying modulators, i. e., candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other drugs) that promote G2/M cell cycle arrest or apoptosis. The invention also includes compounds identified in the screening assays described herein.

The test compounds of the invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds. See, e.g., Lam, 1997. *Anticancer Drug Design* 12: 145.

Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art and can be screened with any of the assays of the invention. Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt, et al., 1993. *Proc. Natl. Acad. Sci. U.S.A.* 90: 6909; Erb, et al., 1994. *Proc. Natl. Acad. Sci. U.S.A.* 91: 11422; Zuckermann, et al., 1994. *J. Med. Chem.* 37: 2678; Cho, et al., 1993. *Science* 261: 1303; Carrell, et al., 1994. *Angew. Chem. Int. Ed. Engl.* 33: 2059; Carell, et al., 1994. *Angew. Chem. Int. Ed. Engl.* 33: 2061; and Gallop, et al., 1994. *J. Med. Chem.* 37: 1233.

Libraries of compounds may be presented in solution (e.g., Houghten, 1992. *Biotechniques* 13: 412-421), or on beads (Lam, 1991. *Nature* 354: 82-84), on chips (Fodor, 1993. *Nature* 364: 555-556), bacteria (Ladner, U.S. Pat. No. 5,223,409), spores (Ladner, U.S. Pat. No. 5,233,409), plasmids (Cull, et al., 1992. *Proc. Natl. Acad. Sci. USA* 89: 1865-1869) or on phage (Scott and Smith, 1990. *Science* 249: 386-390; Devlin, 1990. *Science* 249: 404-406; Cwirla, et al., 1990. *Proc. Natl. Acad. Sci. U.S.A.* 87: 6378-6382; Felici, 1991. *J. Mol. Biol.* 222: 301-310; Ladner, U.S. Pat. No. 5, 233,409.).

The invention further pertains to novel agents identified by the aforementioned screening assays and uses thereof in pharmaceutical compositions for treatments as described herein. The pharmaceutical compositions of the invention comprise the novel agents combined with a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" is

intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (i.e., topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL® (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many

cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4, 522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

EXAMPLES

**Antibodies and chemicals**. Rabbit polyclonal anti-luciferase was purchased from Sigma, rabbit polyclonal cdk1 (C-19) and mouse monoclonal anti-cyclinB1 were purchased from Santa-Cruz Laboratory. Nocodazole and mimosine was purchased from Sigma.

**Generation of cyclin B1 reporter, pGL3-cyclinB-Luc**. The following primers (forward primer: 5'-GCGCAAGCTTGCCACCATGGCGCTCCGAGTCACCAGGAA, (SEQ ID. NO. 1) Reverse primer: 5'-GCGCCCATGGTCACATATTCACTACAAAGGTTTGG) (SEQ ID. NO. 2) were used to amplify the N-terminal of cyclin B1 (173 amino acids) by PCR. The PCR-amplified product was digested with Hind III/NcoI and ligated into pGL3-control vector (Promega) already cut with the same restriction enzymes. The resulted plasmid was named pGL3-NB-Luc. Then, cyclin B promoter excised from previously generated vector pGL3-cycBpro-Luc was used to replace the SV40 promoter in the pGL3-NB-Luc vector. The newly

generated vector is an N-terminus of cyclin B1-luciferase fusion protein construct controlled by cyclin B promoter.

**Tissue culture and transfection.** Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, for HeLa cells) or McCoy's 5A (for HCT116 cells) supplemented with 10% fetal bovine serum (FBS). To make stable cell lines, HeLa or HCT116 cells were cotransfected with 5 μg of pGL3-cyclinB-Luc and 0.5 μg of empty pcDNA3 (Invitrogen). 24 hours later, transfected cells were selected and maintained by growth in media containing G418 (1 mg/ml). Monoclonal cell lines were established with single cell deposition method.

**Cell cycle analysis.** Subconfluecnt HeLa-cyclinB-Luc cells were blocked in late G1 by growth in media containing mimosine or nocodazole for 18 hours. Then, the cells were lysed for luciferase assay or fixed with ice-cold 70% ethanol for FACS analysis. Fixed cells were incubated in PBS containing 69 μM propidium iodide and 20 μg/ml RNAse A for 30 minutes at 37°C. DNA content per nucleus was analyzed using a FACScan flow cytometer.

**Luciferase assay**. Luciferase assay system (Promega) was used according to the manufacturer's instructions. Cells were lysed by rocking in Passive Lysis Buffer (Promega) for 15 minutes at room temperature. 10 μl of cell extract was assayed using a Lumat LB9507 luminometer (Berthold Technologies). Luciferase values for stable cell lines were normalized to total protein concentration.

**Hollow fiber assay and tumor xenograft.** Growth of cells in hollow fibers was done essentially as described previously. Briefly, a hollow fiber was filled with cells (5 X $10^6$ cells/ml) and cut into 1.5 cm pieces that were sealed at both ends. For *in vitro* studies hollow fibers were placed in 6 well culture dishes containing DMEM with 10% FBS before adding anti-cancer drugs. For *in vivo* studies, hollow fibers were implanted subcutaneously in Nu/Nu mice using an 11 gauge trochar inserted through a neck incision under anesthesia (ketamine 140 mg/kg and xylazene 12 mg/kg given by IP injection). For tumor xenograft study, approximately 5 x $10^6$ cells in 0.2 ml PBS were injected subcutaneously per site into the flanks of Nu/Nu nude mice under anesthesia. All the animal experiments described in this paper were approved by the Animal Care and Use Committee of Merck.

**Bioluminescence imaging**. For in vitro studies D-luciferin was added to tissue culture media (final concentration 50 μg/ml). 5 minutes later photons were counted using the IVIS™ imaging system (Xenogen) according to the manufacturer's instructions. Data were analyzed using Living Image software (version 2.52; Xenogen). For *in vivo* studies, mice were administered D-luciferin (90 mg/kg) by intraperitoneal injection. Ten minutes later photons were counted and analyzed as above.

WHAT IS CLAIMED IS:

1. A method of detecting cell cycle arrest at the G2/M phase in a target cell comprising:

(i) transfecting said target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of said fusion protein;

(ii) introducing into said target cell a substrate specific for the fusion protein for a period of time sufficient to detect a signal, wherein the generation of a signal indicates that said cell has arrested at the G2/M transition; and

(iii) detecting a signal.

2. A method of detecting apoptotic events in a target cell comprising:

(i) transfecting said target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of said fusion protein;

(ii) introducing into said target cell a substrate specific for the fusion protein at various time points and under conditions sufficient to generate a signal

(iii) correlating said signal with an apoptotic event, wherein the absence of the signal indicates a cellular apoptotic event.

3. A method of detecting cyclinB1 sub-cellular localization in a target cell comprising:

(i) transfecting said target cell with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of said fusion protein;

(ii) introducing into said target cell a substrate specific for the fusion protein for a period of time sufficient to detect a signal; and

(iii) detecting said signal, wherein localization of said signal inside the nucleus indicates transition from G2 to mitosis.

4. A method for determining the efficacy of a test compound in promoting G2/M cell cycle arrest or apoptosis in a target cell comprising

(i) contacting said test compound with a target cell previously transfected with a nucleic acid construct that encodes a cyclinB1-Luciferase fusion protein under conditions favoring expression of said fusion protein and further comprising a substrate specific for the fusion protein;

(ii) detecting a signal; and

(iii) correlating signal strength with the efficacy of said test compound in promoting one of cell death, cell cycle arrest or apoptosis in said target cell.

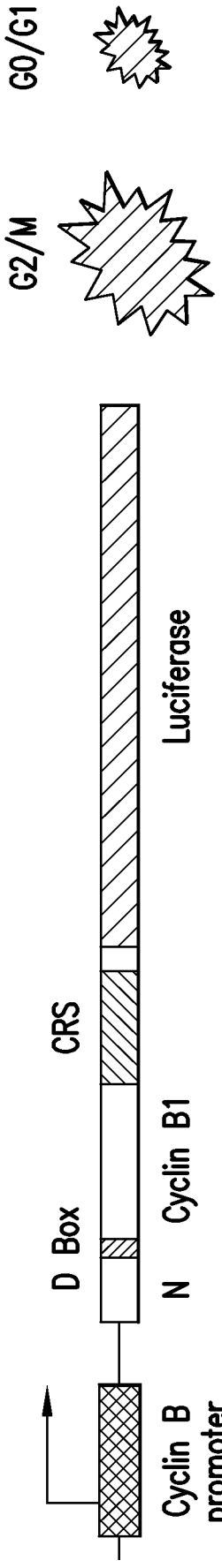
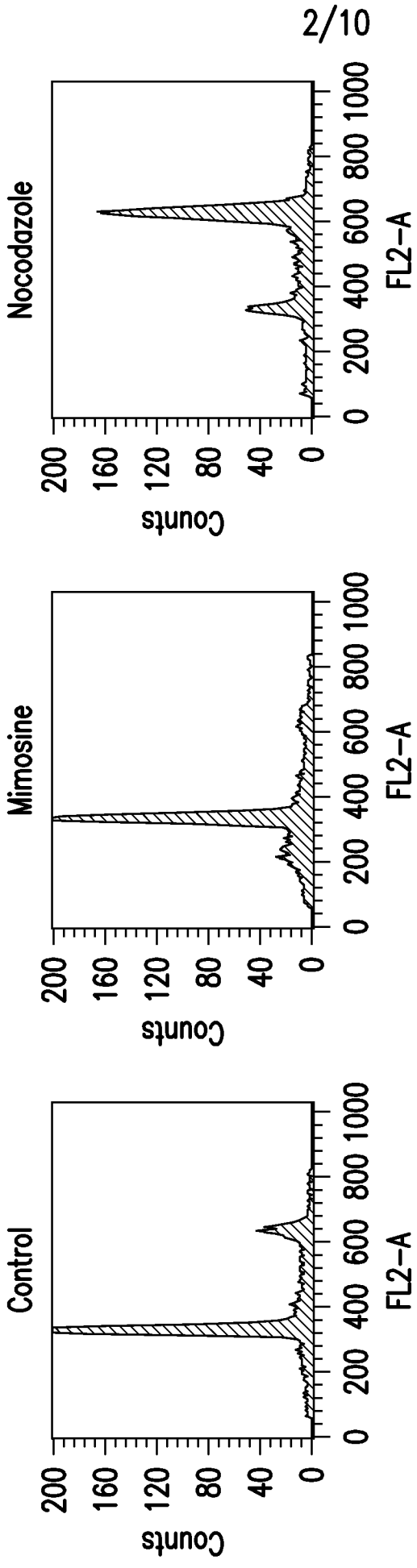



FIG. 1




| | Sub G1 | G1 | S | G2/M |
|---|---|---|---|---|
| Control | 4 | 75 | 8 | 13 |
| Mimosine | 5 | 90 | 3 | 2 |
| Nocodazole | 3 | 15 | 12 | 70 |

FIG.2A

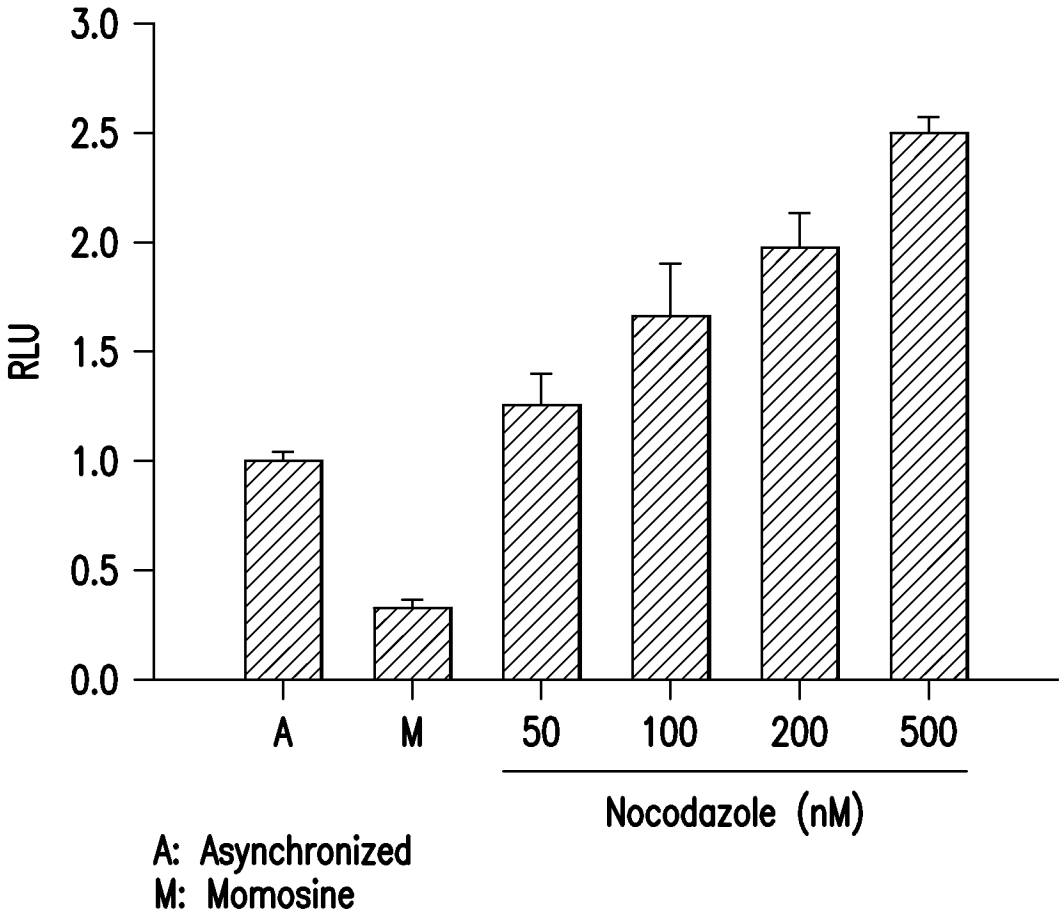

3.0
2.5
2.0
1.5
1.0
0.5
0.0
RLU
A
M
50
100
200
500
Nocodazole (nM)
A: Asynchronized
M: Mornosine

FIG.2B

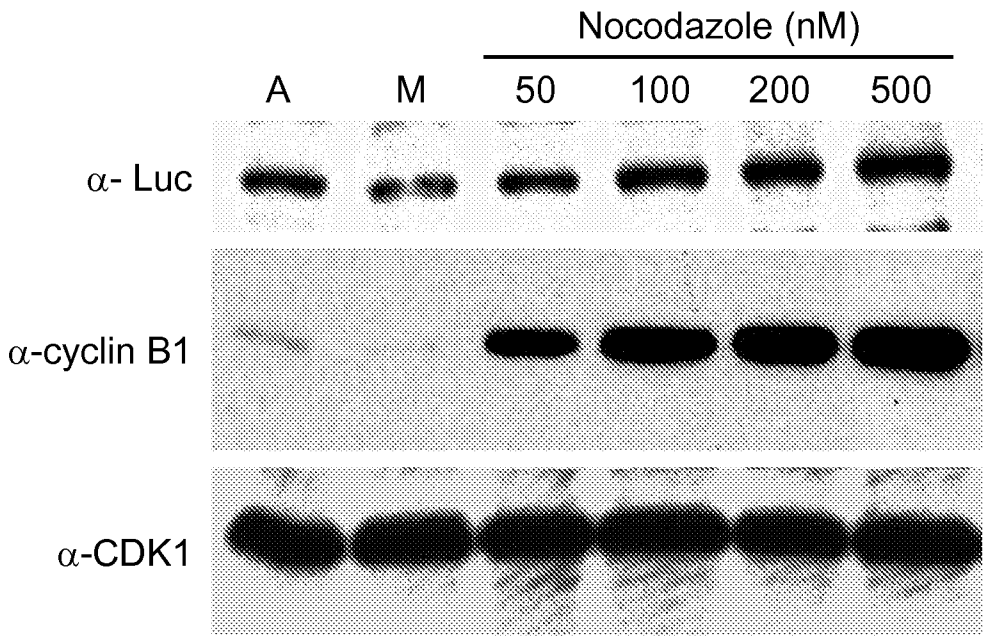



FIG.2C

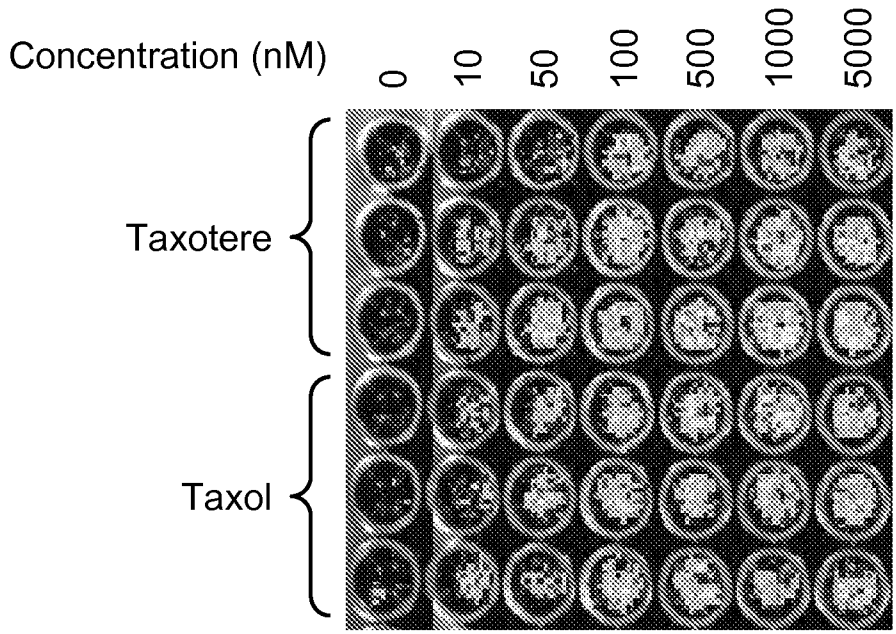

Concentration (nM)
0
10
50
100
500
1000
5000
Taxotere
Taxol

FIG.3A

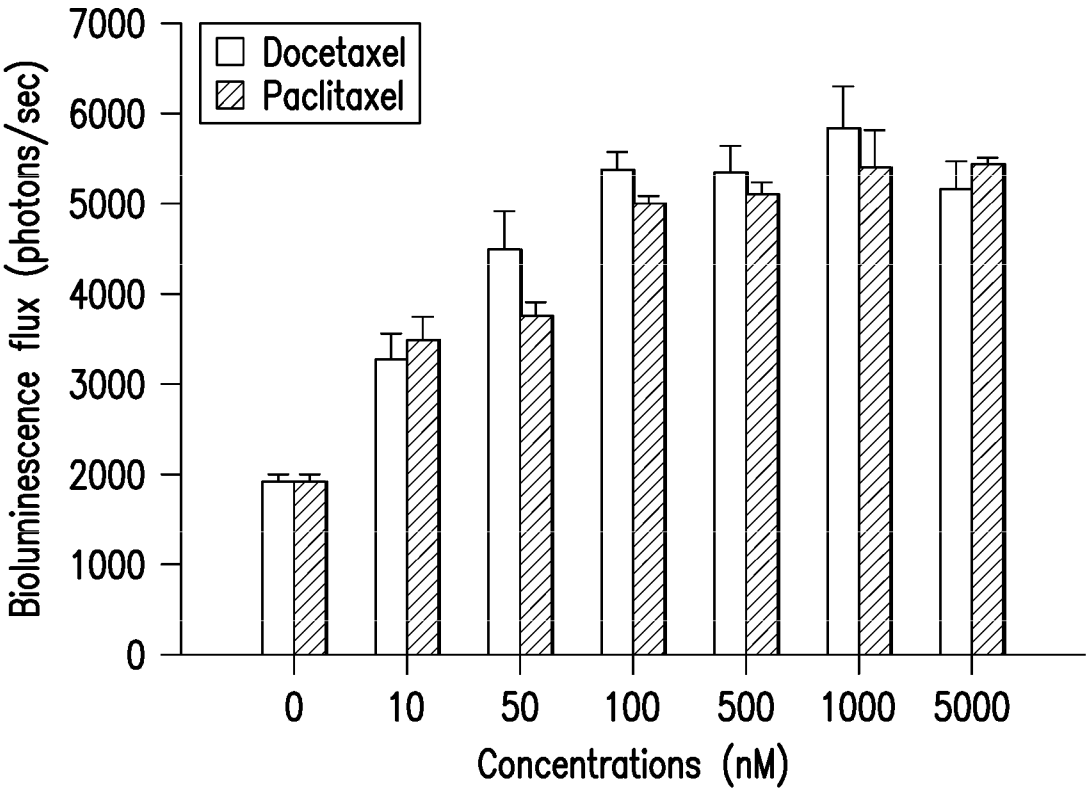
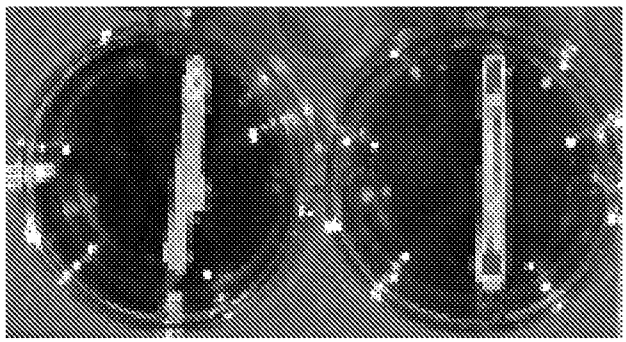

Docetaxel
Paclitaxel
Bioluminescence flux (photons/sec)
7000
6000
5000
4000
3000
2000
1000
0
0
10
50
100
500
1000
5000
Concentrations (nM)

FIG.3B



Vehicle Taxotere

FIG.4

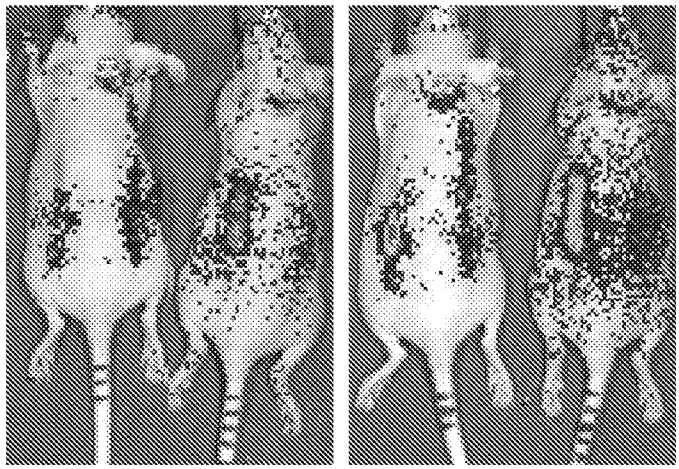

Before
48 hr

FIG.5A

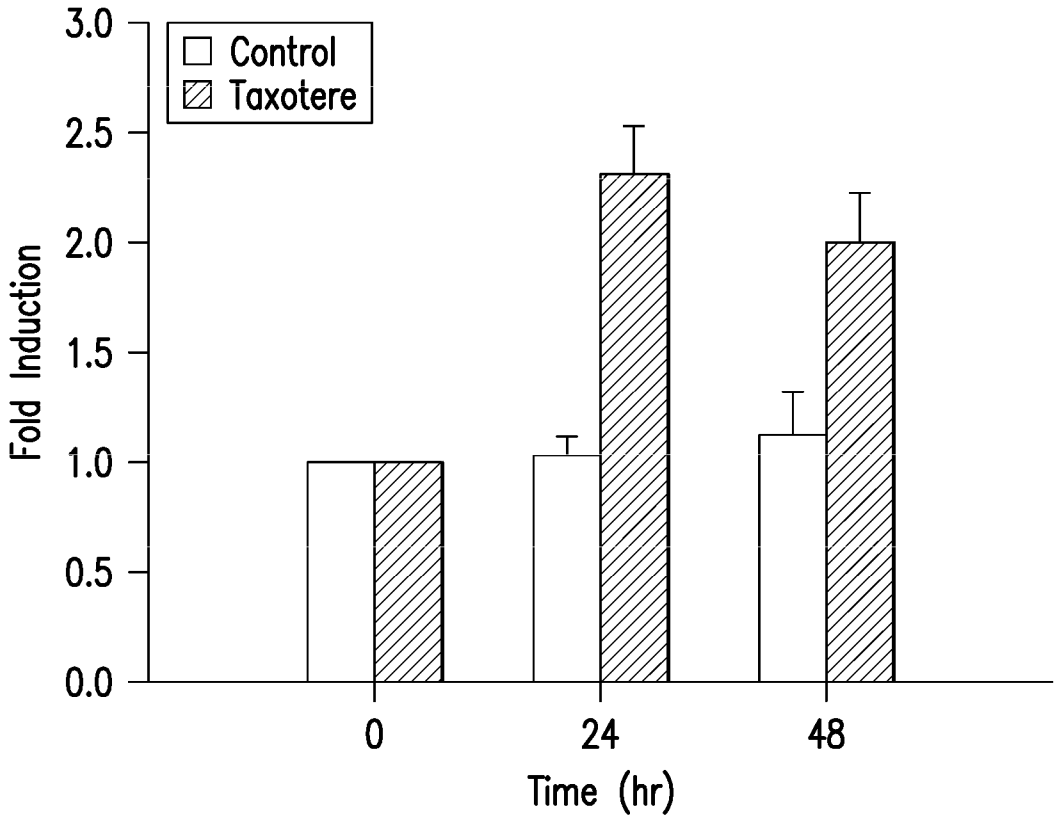

Control
Taxotere
Fold Induction
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
24
48
Time (hr)

FIG.5B

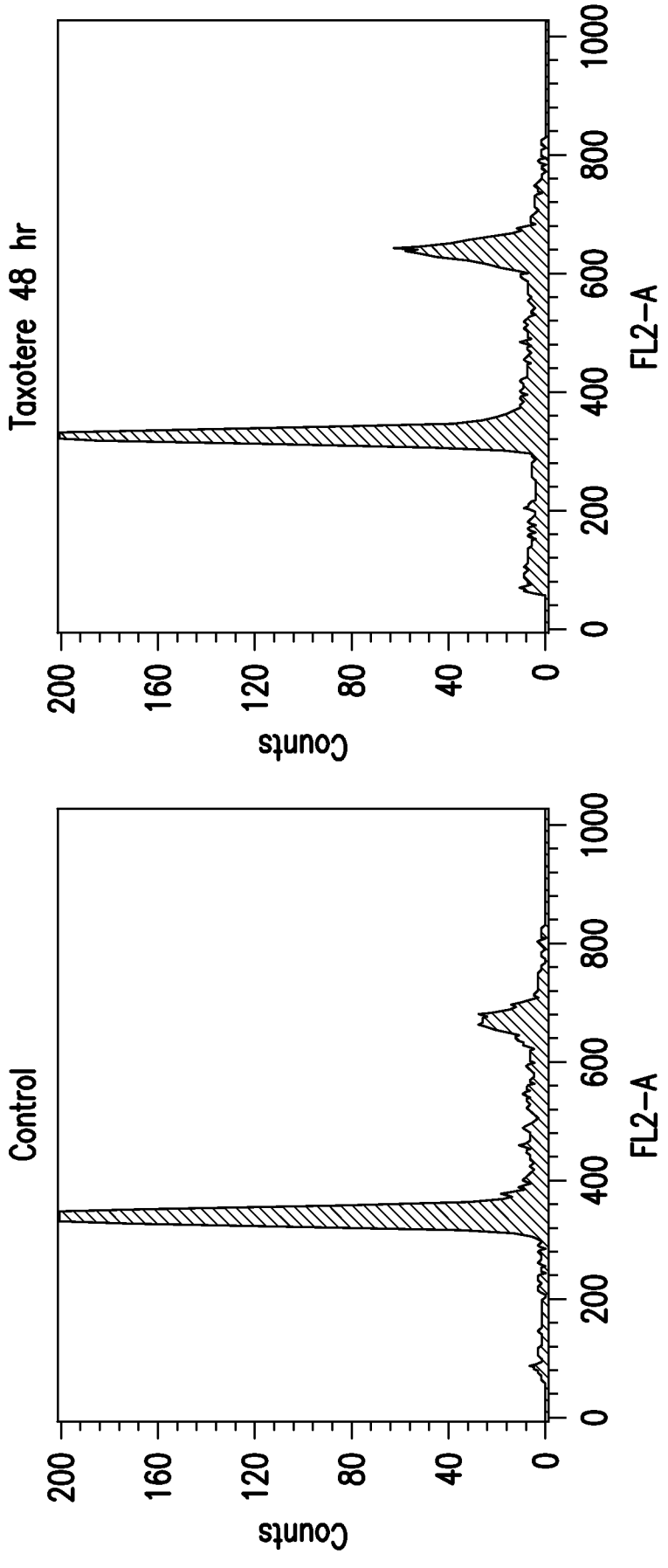



FIG.5C

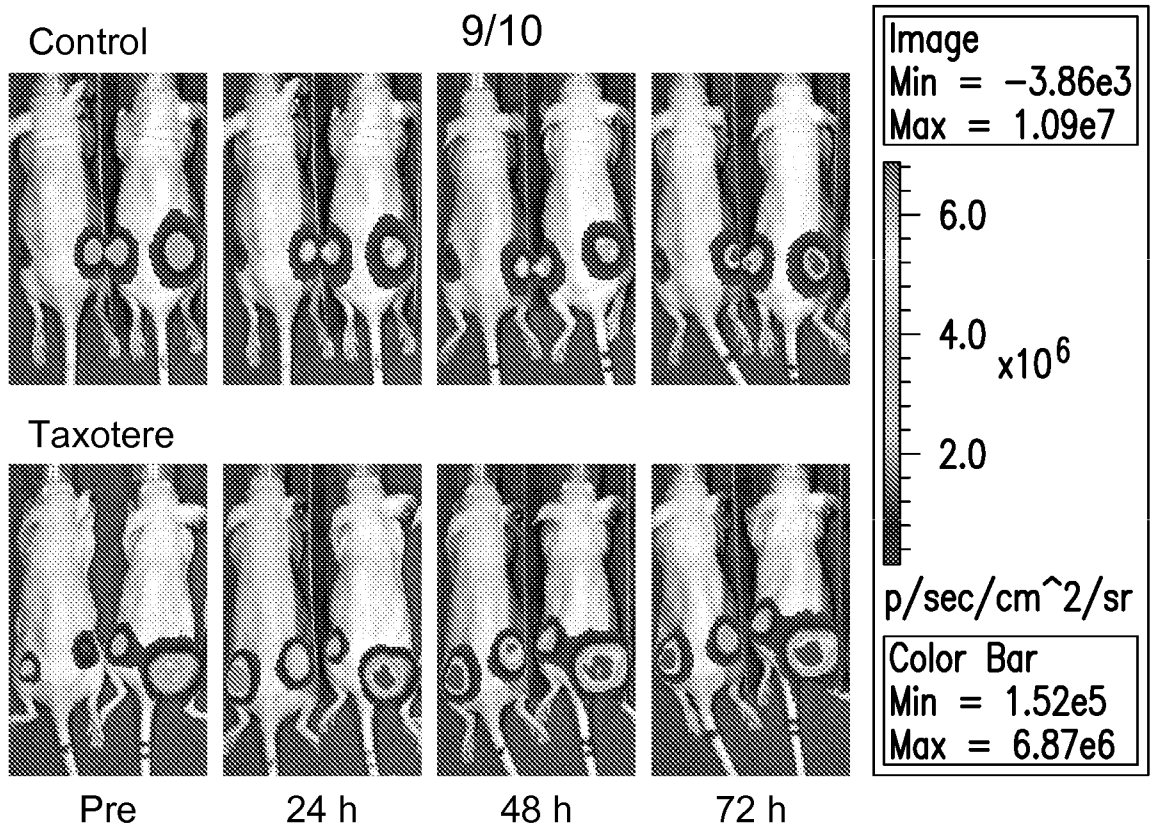

Control
Taxotere
Pre
24 h
48 h
72 h
Image
Min = -3.86e3
Max = 1.09e7
6.0
4.0
2.0
x10^6
p/sec/cm^2/sr
Color Bar
Min = 1.52e5
Max = 6.87e6

FIG.6A

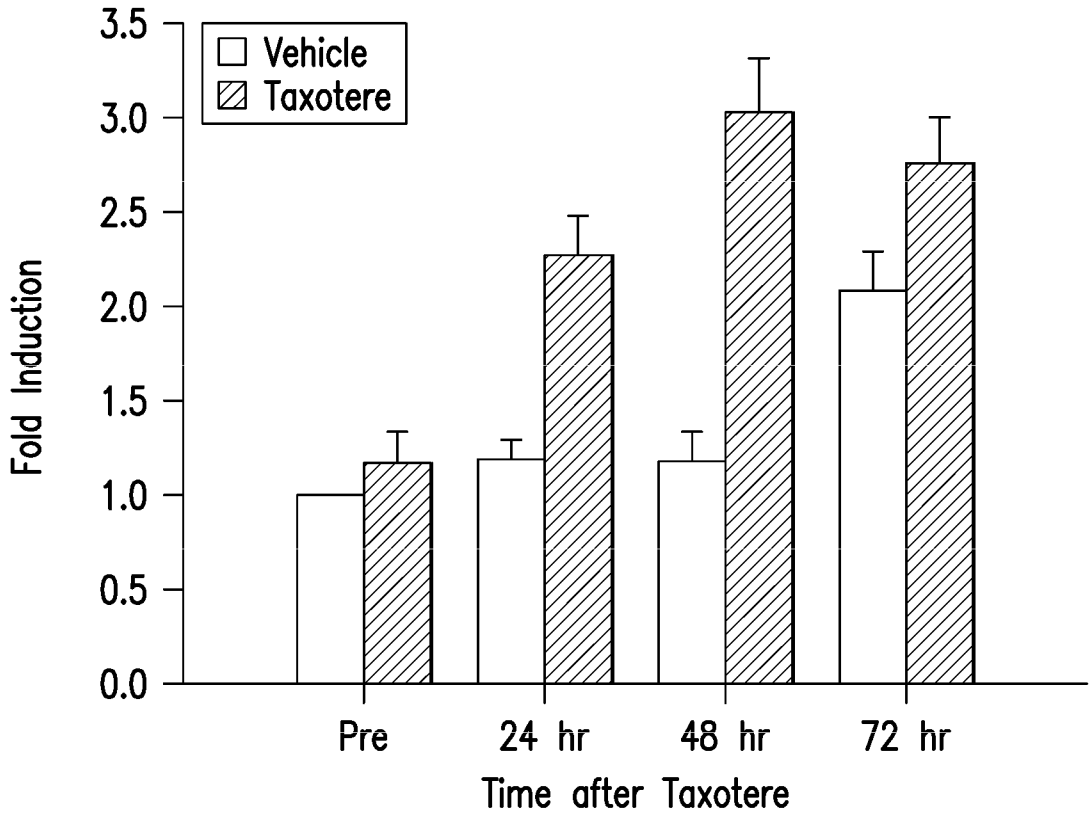

Vehicle
Taxotere
Fold Induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
24 hr
48 hr
72 hr
Time after Taxotere

FIG.6B

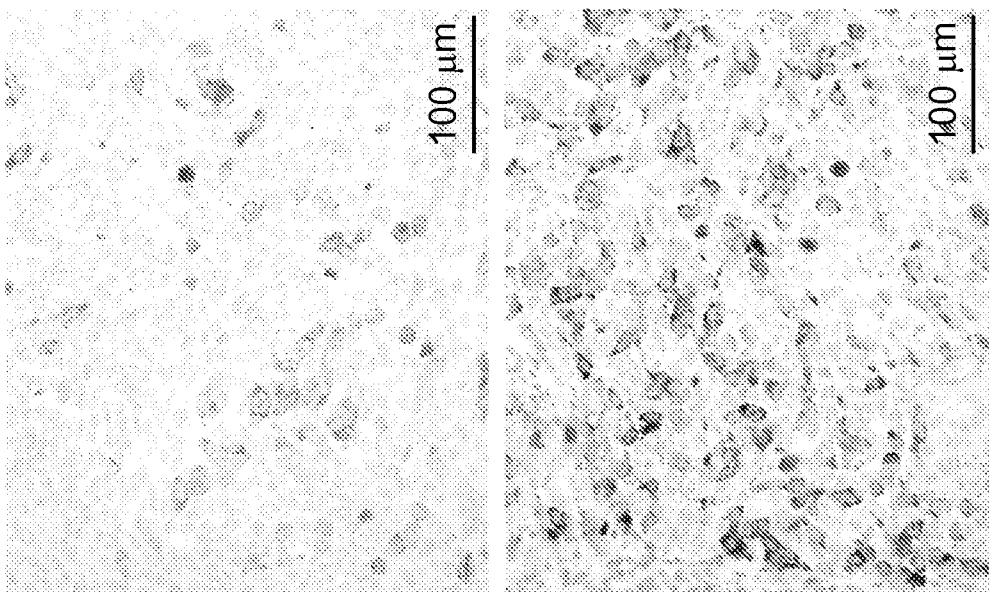

100 µm
100 µm

FIG.7A

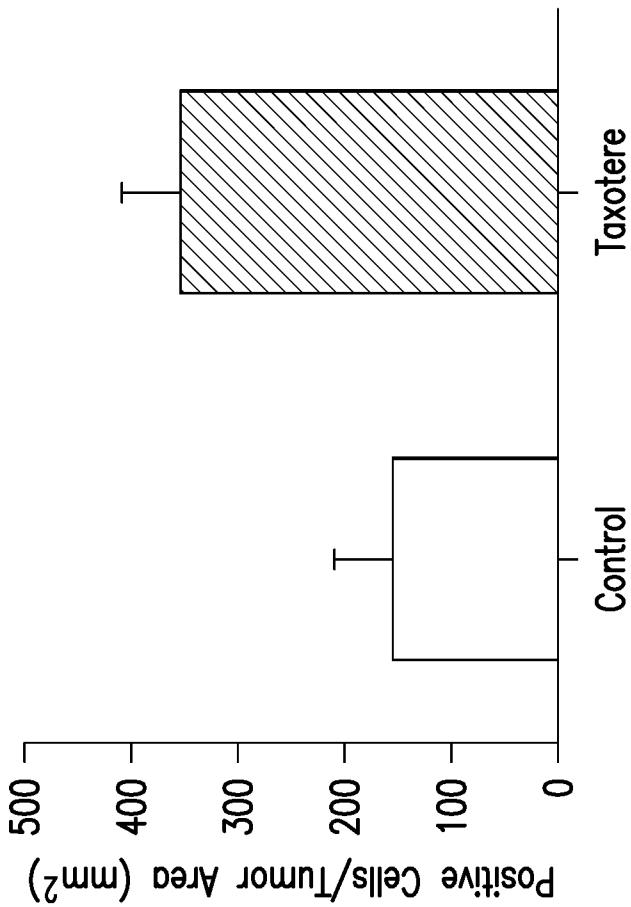

Positive Cells/Tumor Area (mm2)
500
400
300
200
100
0
Control
Taxotere

FIG.7B

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/58515

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/00;C12P 21/06;C12P 21/04; A61K 39/00(2009.01)
USPC - 435/320.1; 424/192.1; 435/69.1; 435/69.7
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 15/00;C12P 21/06;C12P21/04;A61K39/00(2009.01)
USPC - 435/320.1; 424/192.1; 435/69.1; 435/69.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
435/325 514/44R 536/23.4 536/23.5 424/9.6 424/178.1 800/8 800/3 530/350

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(GPB,USPT,USOC,EPAB,JPAB); Google scholar: detect, detecting, detected, sense, sensed, isolated, detection, measure, measurement, measured, measuring, level, reporter, luciferase, fluoresc$, cyclinB1, CCNB, CCNB1, cyclin B1, cyclin B, mitotis, G2/M, arrest, arrest$, apoptosis, cell cycle

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 2003/0059764 A1 (RAVKIN et al.) 27 March 2003 (27.03.2003) para [0118]-[0119], [0133], [0135]-[0138], [0142], [0186], [0209], [0298]-[0306], [0598], [0605] | 1-4 |
| Y | US 2007/0238144 A1 (HANCOCK et al.) 11 October 2007 (11.10.2007) para [0016]-[0019], [0022], [0026]-[0028] | 1-4 |

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
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"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 05 November 2009 (05.11.2009) | 16 NOV 2009 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-3201 | Authorized officer:<br>Lee W. Young<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |