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(57) Abstract: This invention relates to the stabilization of p53 in cells, thereby inducing either cell cycle arrest or apoptosis or both and the consequence of such stabilization on the treatment of cancer. Specifically, the use of acridine and its derivatives in stabilizing p53 through blockage of its ubiquitination, thereby inducing cell cycle arrest or apoptosis in a cell; and the use of these compounds in the treatment of cancer.



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ACRIDINE ACTIVATION OF p53 AND USES THEREOF

FIELD OF INVENTION

5 [0001] This invention is directed to the stabilization of p53 in cells, thereby inducing either cell cycle arrest or apoptosis or both and the consequence of such stabilization on the treatment of cancer. Specifically, the use of acridine and its derivatives in stabilizing p53 through blockage of its ubiquitination, thereby inducing cell cycle arrest or apoptosis in a cell; and the use of these compounds in the treatment of cancer.

BACKGROUND OF THE INVENTION

10 [0002] The tumor suppressor protein p53 plays an important role in tumorigenesis and cancer therapy. Expression of p53 induces either a stable growth arrest or programmed cell death (apoptosis). Epidemiological data demonstrated that p53 is mutated in over half of all human tumors. Among the
15 remaining tumors, although wild-type p53 is expressed, the pathways of p53-mediated cell cycle arrest or apoptosis are defective due to virus infection, MDM2 overexpression, ARF or ATM deficiency. In the clinic, the functional status of p53 has been related to prognosis, progression and therapeutic response of tumors. Tumor cells containing wild-type p53 are usually more sensitive than those bearing mutant p53. All these characteristics make p53 an important molecular target for tumor suppression and drug development

20 [0003] The regulation of p53 activity is mainly post-translational. Stabilization is an essential step for p53 to function efficiently in response to cellular stresses or checkpoints. Under physiological conditions, p53 is expressed at low or undetectable levels with a half-life of approximately 10-20 minutes in most cells. This rapid degradation is at least in part mediated by the ubiquitination pathway following the interaction
25 between MDM2 and the amino-terminus of p53. DNA damage, caused by UV light or ionizing radiation, results in stabilization of endogenous p53 through a series of physiological responses, including ATM/ATR activation, phosphorylation of p53 and blockage of the binding of MDM2 to the p53 N-terminus. Activation of some oncogenes like c-myc, ras, or E2F1 also stabilizes p53 by the ARF-MDM2 pathway, in which case ARF binds to MDM2 and releases p53 from MDM2 association.

30 [0004] CP-31398, which was previously identified as a mutant p53 conformation modifying drug, was shown to be able to stabilize p53 through a unique pathway, which is different from what occurs after DNA-damage. CP-31398 blocks p53 ubiquitination, but does not induce phosphorylation of ser15 or ser20 on p53.

[0005] There is therefore a need to identify more potent p53 stimulators capable of either destabilizing mutated endogenous p53 tumor suppressor, or inactivating oncogenes associated with the stabilization of p53.

SUMMARY OF THE INVENTION

[0006] In one embodiment, the invention provides a method of stabilizing p53 in a cell, comprising contacting the cell with an acridine, or acridine derivative, wherein said acridine or acridine derivative block the ubiquitination, without phosphorylation of ser15 ser20 or both on p53, thereby stabilizing p53 in a cell.

[0007] In another embodiment, the invention provides a method of inducing cell cycle arrest or apoptosis in a cell comprising contacting said cell with an acridine or acridine derivative, wherein said acridine or acridine derivative induces transcriptional activity for p53, thereby inducing p53-induced cell cycle arrest or apoptosis.

[0008] In one embodiment, the invention provides a method of inducing p53 transcriptional activity in a tumor xenograft, comprising contacting said tumor xenograft with a composition comprising an acridine, an acridine derivative, or a combination thereof, thereby inducing transcriptional activity of p53.

[0009] In another embodiment, the invention provides a method of treating cancer in a subject, comprising administering to said subject an effective amount of a pharmaceutical preparation comprising an acridine derivative, wherein said acridine derivative stabilizes p53 in a cancer cell, inducing cell cycle arrest or apoptosis, thereby treating said cancer.

[00010] In one embodiment, the invention provides a method of blocking acridine, or acridine derivative – induced cell cycle arrest or apoptosis in a cell, comprising knocking out the expression of Bax gene, thereby knocking out a p53 target and a key cell death inducer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows Structures of acridine derivatives used, (1) N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine, (2) N'- Acridin- 9-yl-N,N –diethyl -propane-1,3-diamine, (3) Acridin-9-yl(3-morpholin-4-yl-

propyl)-amine, (4) Acridin-9 -yl [3-(4-methyl-piperazin-1-yl)-propyl]amine, (5) 9-Aminoacridine, (6) Acridine Orange, (7) Quinacrine, (8), Amsacrine

Figure 2 shows acridine derivatives induce high levels of p53 protein and activate its transcriptional activity. **A:** HCT116 cells were treated with CP-31398-like acridine derivatives using various concentrations as indicated. Cells were harvested after 12 hours of treatment and subjected to SDS-PAGE and Western blot with antibodies against p53 and its targets, p21 and MDM2. Ran was used as a loading control. **B:** HCT116 cells were treated with acridine derivatives and immunoblotted for p53, MDM2 and p21. c: control, 1: 9-aminoacridine (100 μ M), 2: quinacrine (20 μ M), 3: acridine orange (30 μ M), 4: amsacrine (50 μ M). **C:** H460 cells were treated with acridine derivatives for 12 hours prior to analysis by p53 Western blot. c: control, 1: CP-1 (5 μ M), 2: CP-2 (5 μ M), 3: 9-aminoacridine (100 μ M), 4: quinacrine (20 μ M), 5: acridine orange (30 μ M), 6: amsacrine (25 μ M). **D:** HCT116 cells, containing a p53 reporter, PG-13-luc, were treated with CP-1 over a broad range of concentrations for 12 hours and subjected to western blot for p53 or its targets, MDM2, DR5, p21. The bottom panel shows bioluminescent imaging of p53 transcriptional activity

Figure 3 shows bioluminescent imaging of p53 transcriptional activity induced by acridine derivatives. **A:** HCT116/PG-13 cells were treated with acridine derivatives for 12 hours. D-luciferin was added at a concentration of 100 mg/ml and images are taken by Xenogen imaging system. From the top panel, CP-31398, CP-1, CP-2, CP-3, CP-4. **B:** HCT116/PG13 were treated with acridine derivatives, respectively, amsacrine (25 μ M), CP-1 (5 μ M), CP-2 (5 μ M), acridine orange (30 μ M), quinacrine (20 μ M), and 9-aminoacridine (100 μ M). After 12 hours of treatment, bioluminescence was imaged and the fold-induction was calculated. Controls included no treatment and ADR (adriamycin, 1 μ M), CP-31398 (15 μ g/ml).

Figure 4 shows acridine derivatives block p53 ubiquitination. HCT116 cells were treated with the proteasomal inhibitor, MG132 (10 μ M) alone, or together with acridine derivatives, for 4 hours. Cell lysates were subjected to SDS-PAGE. P53 and its ubiquitinated forms were immunoblotted using the p53 monoclonal antibody DO-1.

Figure 5 shows Acridine derivatives activate p53 differently from DNA damage. **A:** Acridine derivatives do not induce phosphorylation of p53 at ser15 or ser20. HCT116 cells were treated with acridine derivatives for 8 hours, and adriamycin was used as a control. The phosphorylation status of p53 at ser15 or ser20 was detected by specific antibodies. **B:** Dissociation of MDM2 is not involved in acridine derivative-induced p53 stabilization. HCT116 cells were treated with quinacrine (20 μ M) or CP-1 (5 μ M) respectively for 6

hours, and adriamycin was used as a control. Cells were collected and lysed and immunoprecipitated with anti-p53 antibodies (FL-393). The IP products were subjected to SDS-PAGE and probed with anti-p53 and anti-MDM2 antibodies

5 **Figure 6** shows Acridine derivatives induce p53-dependent cell death. A: HCT116, HCT116 /p53^(-/-) and HCT/bax^(-/-) cells were treated with CP-1 at various concentrations for 20 hours. Cells were harvested and stained with PI for a flow cytometry assay. B: HCT116, HCT116/p53^(-/-) and HCT/bax^(-/-) cells were treated with amsacrine (25 μ M), CP-2 (5 μ M), acridine orange (20 μ M), quinacrine (20 μ M) and 9-aminoacridine (100 μ M). 20 hours after treatment, cells were harvested and subjected to a flow cytometry assay.

10 **Figure 7** shows CP-1 and quinacrine induce p53 transcriptional activity *in vivo* in a mouse tumor xenograft model. HCT116/PG-13 cells, carrying a firefly luciferase gene under the control of 13 p53 response elements, were inoculated subcutaneously at the right flank at a dose of 2×10^6 cells in 0.2 ml of PBS. As a control, HCT116/pGL-2 cells, expressing a firefly luciferase gene under the control of SV40 promoter, were
15 injected into the left flank. Bioluminescent imaging was performed before the injection of drugs and 12 hours after. A: CP-1, 50 mg/kg, i.p., B: quinacrine, 100 mg/kg, i.p.

DETAILED DESCRIPTION OF THE INVENTION

20 [00011] In one embodiment, acridine derivatives elicit a strong p53 activation through a mechanism different from that induced by DNA-damage and induce apoptosis of tumor cells. Acridine derivatives induce in another embodiment, p53 transcriptional activity. In one embodiment, p53 stabilization induced by these compounds was found to be mediated by the basic R1 structure, acridine.

25 [00012] The accumulation of p53 is an early and critical step for p53 activation. Under physiological conditions, p53 is under strict control with a very short half-life, in part due to the MDM2-p53 auto-regulatory feedback loop. MDM2, a transcriptional targets of p53, binds to the N-terminal transactivation domain of p53 with its p53-binding domain while the RING domain of MDM2 catalyses the ubiquitination of p53. The ubiquitinated p53 is then degraded by the 26S proteasome either in the cytoplasm or in the
30 nucleus. In the case of DNA damage, p53 is phosphorylated by a number of protein kinases including ATM/ATR, or Chk2. Phosphorylation of the amino-terminus of p53 is crucial in one embodiment for the DNA-damage induced stabilization of p53, by blocking binding of MDM2 to p53. As indicated in an embodiment of the invention, acridine derivatives induce similar levels of p53 stabilization by inhibition of p53 ubiquitination, but through a mechanism different from DNA-damage induced p53 stabilization. In

another embodiment, acridine derivatives do not induce phosphorylation at the amino-terminus of p53 and do not block the binding of MDM2 to p53. In one embodiment, there is an alternative pathway of p53 stabilization that involves blockade of ubiquitination. The term "p53" refers in one embodiment to the gene that encodes the nuclear phosphoprotein p53, which is involved in the regulation of fundamental biological processes in cell proliferation and cell death. This protein is also responsible in another embodiment for mediating cytotoxicity of anticancer therapy, and has been shown to act as a tumor-suppressor protein.

[00013] According to this aspect of the invention and in one embodiment, the invention provides a method of stabilizing p53 in a cell, comprising contacting the cell with an acridine, or acridine derivative, wherein said acridine or acridine derivative block the ubiquitination, without phosphorylation of ser15 ser20 or both on p53, thereby stabilizing p53 in a cell.

[00014] In one embodiment, elevated levels of p53 transcriptional targets, p21 and MDM2, following exposure of the cells to acridine or acridine derivatives according to the methods of the invention, indicate that the stabilized p53 protein is transcriptionally active.

[00015] In one embodiment, intercalation of DNA by acridine derivatives activates a unique p53 response, which is different from DNA damage induced by ionizing radiation, UV light exposure, or DNA-damaging agents.

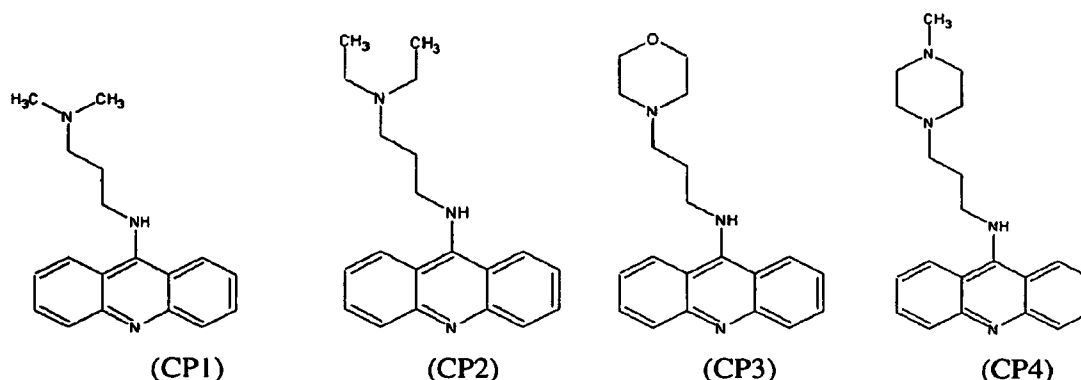
[00016] In one embodiment, "contacting" a cell with a substance refers to (a) providing the substance to the environment of the cell (e.g., solution, in vitro culture medium, anatomic fluid or tissue) or (b) applying or providing the substance directly to the surface of the cell, in either case so that the substance comes in contact with the surface of the cell in a manner allowing for biological interactions between the cell and the substance.

[00017] In one embodiment, ubiquitin-mediated proteolysis is an important pathway of non-lysosomal protein degradation which controls in another embodiment, the timed destruction of cellular regulatory proteins such as, p27, p53, p300, cyclins, E2F, STAT-1, c-Myc, c-Jun, EGF receptor, I κ B α , NF- κ B and β -catenin. Ubiquitin is an evolutionary highly conserved 76-amino acid polypeptide which is abundantly present in all eukaryotic cells. The ubiquitin pathway leads to the covalent attachment of a poly-ubiquitin chain to target substrates which are then degraded by the multi-catalytic proteasome complex. Initially the ubiquitin activating enzyme (E1), forms a high energy thioester with ubiquitin which is, in turn, transferred to a reactive cysteine residue of one of many ubiquitin conjugating enzymes (Ubcs or E2s). The final

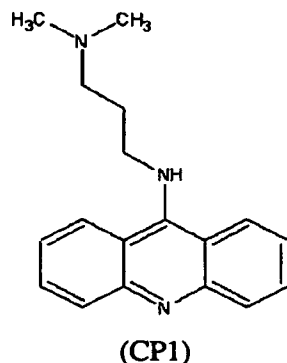
transfer of ubiquitin to an ϵ -amino group of a reactive lysine residue in the target protein occurs in a reaction that may or may not require an ubiquitin ligase (E3) protein. The large number of ubiquitin ligases ensures the high level of substrate specificity.

[00018] In one embodiment, immunoprecipitation of p53 show that MDM2 association in cells treated with quinacrine or CP-1 according to the methods and compositions of the invention, is considerably less than associated p53-MDM2 co-immunoprecipitate in cells treated with adriamycin (figure 5B). In one embodiment, acridine derivatives block p53 ubiquitination through a mechanism different from what occurs following DNA-damage.

[00019] The acridine or acridine derivatives used in the methods or compositions of the invention is N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1) in one embodiment, or N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4), 9-Amino-acridine, Acridine Orange, Quinacrine, Amsacrine or a combination thereof in other embodiments. In another embodiment, the N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4) are represented by the following formula:



[00020] In one embodiment, the acridine derivative used in the methods of the invention is (CP1), represented by the formula:



or amsacrine in another embodiment.

5 [00021] The most well-documented biochemical property of p53 is its ability to transcriptionally activate genes. Of 7,202 transcripts induced by p53 expression prior to the onset of apoptosis, only 14 (0.19%) are found at markedly higher levels in p53-expressing cells than in control cells. The genes encoding these transcripts are termed PIGS (p53-induced genes).

10 [00022] Activation of p53 leads in one embodiment to cell death in acridine drug-exposed cells. According to this aspect of the invention and in one embodiment, the invention provides a method of inducing cell cycle arrest or apoptosis in a cell comprising contacting said cell with an acridine or acridine derivative, wherein said acridine or acridine derivative induces transcriptional activity for p53, thereby inducing p53-induced cell cycle arrest or apoptosis.

15 [00023] In one embodiment, the cell contacted by acridine or acridine derivative according to the methods of the invention is a tumor cell, or in another embodiment, a cell wherein apoptosis, or cell cycle arrest are inhibited due to instability of p53. In one embodiment, apoptosis, or cell cycle arrest are not desirable attributes of the cell sought to be affected with the methods and compositions of the invention, such as cell required for angiogenesis following skin grafting in one embodiment. In another embodiment, prolonged exposure to acridine or acridine derivatives-containing pigments and dyes induces apoptosis in exposed cells. In one embodiment, it is desirable to ameliorate the effects of prolonged exposure to acridine by cells.

20 [00024] In one embodiment, knockout of p53 reduces greatly the sensitivity to treatment by acridine derivatives. Bax, a p53 target, plays in one embodiment, an essential role in acridine derivative-induced apoptosis, since Bax-null cells undergo primarily a growth arrest response with depletion of their S-phase fraction rather than apoptosis. This is due to the presence of other p53 targets, such as p21 in one embodiment, or p300 in another embodiment, that could induce cell cycle arrest.

[00025] In one embodiment, the tumor cell treated with the methods and compositions of the invention, is a solid tumor cell. In another embodiment, the cell cycle arrest or apoptosis is impaired due to p53 instability.

5 [00026] There are two major classes of cell cycle regulation events: DNA damage events and dependency events. DNA damage events delay cell cycle transitions from G₁ to S and from G₂ to M, thereby providing more time for DNA repair. Essential components of the G₁ checkpoint include ATM, p53, RB, Chk2, and p21^{Waf1} (a downstream target of p53). DNA damage activates ATM kinase, which phosphorylates p53 and Chk2, leading to the induction and activation of p53. In turn, p53 transactivates p21^{Waf1}, which inhibits the
10 G₁ cyclin-dependent kinases that normally inactivate RB, and thereby represses the E2F transcription factors that initiate S phase. In one embodiment, damage to cellular DNA initiates increased expression of p53 which leads to arrest of the cell cycle. The interruption permits DNA repair to occur before the cell resumes the cell cycle and normal cell proliferation. If repair of the DNA is not successful, the cell then undergoes apoptotic cell death. In another embodiment, when p53 mutates, DNA damaged cells are not arrested in G₁
15 and DNA repair does not take place. The failure to arrest DNA-damaged cells is repeated in subsequent cell cycles permitting and contributes to tumor formation and cancer. The gene encoding p53 is mutated in more than half of all human tumors, suggesting that inactivation of the function of the p53 protein is critical for tumor development.

20 [00027] The N-terminus of p53 (residues 1-90 of the wild-type p53 sequence) encodes its transcription activation domain, also known as transactivation domain. The sequence-specific DNA binding domain has been mapped to amino acid residues 90-289 of wild-type p53. C-terminal to the DNA binding domain, p53 contains a tetramerization domain. This domain maps to residues 322-355 of p53. Through the action of this domain p53 forms homotetramers and maintains its tetrameric stoichiometry even when bound to DNA.

25 [00028] The p53-inducible p21^{WAF1/CIP1} gene encodes a protein which binds to and inhibits a broad range of cyclin–cyclin-dependent kinase complexes, which promote cell cycle progression. Thus, the consequence of p21^{WAF1/CIP1} activity in one embodiment is growth arrest, which is evident in another embodiment, following exposure of cells to DNA-damaging agents such as γ radiation or adriamycin. In one embodiment,
30 DNA damage brings about p21^{WAF1/CIP1}-induced growth arrest via transcriptional upregulation of p21^{WAF1/CIP1} by the p53 tumor suppressor gene. In one embodiment, p53 deficient cells exposed to γ radiation fail to exhibit either induction of p21^{WAF1/CIP1} expression or G₁ arrest. In another embodiment, the p21^{WAF1/CIP1} promoter region has been shown to contain two conserved p53-binding sites through which p53 can regulate p21^{WAF1/CIP1} transcription.

[00029] In one embodiment, the methods of the invention used with p53 tumor suppressor expressing cell lines, including non-small cell lung carcinoma cell line, ovarian cancer cell line and human foreskin fibroblast HFF cells show that acridine derivatives activated wild-type p53 in all the cell lines. The term "tumor suppressor" refers in one embodiment to a gene involved in normal control of cellular growth and division which when inhibited contributes to tumor development. Representative examples of tumor suppressor genes include the RB gene isolated from a region deleted in retinoblastoma cells, the WT1 gene isolated from 11p3, which is occasionally deleted in Wilms' tumor types, the NF1 gene involved in neurofibromatosis, and the p53 gene, which has been found to be associated with a wide variety of tumors.

[00030] In one embodiment, the invention provides a method of inducing p53 transcriptional activity in a tumor xenograft, comprising contacting said tumor xenograft with a composition comprising an acridine, an acridine derivative, or a combination thereof, thereby inducing transcriptional activity of p53.

[00031] The elevated levels of two p53 transcriptional targets, p21 and MDM2, indicates that the stabilized p53 protein is transcriptionally active.

[00032] In another embodiment, the invention provides a method of treating cancer in a subject, comprising administering to said subject an effective amount of a pharmaceutical preparation comprising an acridine derivative, wherein said acridine derivative stabilizes p53 in a cancer cell, inducing cell cycle arrest or apoptosis, thereby treating said cancer.

[00033] In another embodiment, the tumor cell treated using the methods and compositions of the invention, is a solid cancer tumor, which is an osteosarcoma in one embodiment, or soft tissue sarcoma, breast tissue sarcoma, ovarian carcinoma, cervical carcinoma, oral squamous cell carcinoma, brain tumor, esophageal cancer, colorectal carcinoma, bladder cancer, urothelial carcinoma, leukemia, large B cell lymphoma, non-small cell lung carcinoma, prostate carcinoma, renal carcinoma, pancreatic carcinoma, melanoma, or a combination thereof in other embodiments. In one embodiment, the invention provides a method of treating breast tissue carcinoma in a subject, comprising administering to the subject an effective amount of acridine, or an acridine derivative, in an amount therapeutically effective to induce p53 stabilization in the tumor cells, thereby inducing cell cycle arrest at high dosage, or apoptosis at low dosage. A "tumor cell" refers in one embodiment to a neoplastic cell. A tumor cell may be benign, i.e. one that does not form metastases and does not invade and destroy adjacent normal tissue in one embodiment, or malignant, i.e. one that invades

surrounding tissues in another embodiment; and is capable of producing metastases, may recur after attempted removal, and is likely to cause death of the host.

[00034] In one embodiment, the term "treatment" refers to any process, action, application, therapy, or the like, wherein a subject, including a human being, is subjected to medical aid with the object of improving the subject's condition, directly or indirectly. In another embodiment, the term "treating" refers to reducing incidence, or alleviating symptoms, eliminating recurrence, preventing recurrence, preventing incidence, improving symptoms, improving prognosis or combination thereof in other embodiments.

[00035] "Treating" embraces in another embodiment, the amelioration of an existing condition. The skilled artisan would understand that treatment does not necessarily result in the complete absence or removal of symptoms. Treatment also embraces palliative effects: that is, those that reduce the likelihood of a subsequent medical condition. The alleviation of a condition that results in a more serious condition is encompassed by this term.

[00036] In another embodiment one may irradiate the localized tumor site with DNA damaging radiation such as X-rays, UV-light, gamma -rays or even microwaves. Alternatively, the tumor cells may be contacted with a DNA damaging agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound, such as adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like.

[00037] The terms "therapeutically effective amount" refers in one embodiment to known treatments at dosages and for periods of time effective to reduce tumor cell growth. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal or intrarectal. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μ M to about 100 μ M. For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. Preferably, a total dosage of acridine or acridine derivative will range from about 0.001 mg per patient per day to about 200 mg per kg body weight per day.

[00038] In one embodiment, disruption of p53 function strongly correlates with tumorigenesis. p53 inactivation of various types is found in over 50% of all human cancers, with p53 gene mutations being the prevalent mechanism. Viral oncoproteins inactivate p53 protein by complex formation as seen with simian virus 40 large T antigen, adenovirus type 5 E1B protein, human papilloma virus type 16/18 E6 protein, and hepatitis B virus X-protein. In 30% of soft-tissue sarcomas, p53 protein is inactivated by binding to overexpressed mdm-2 protein, which abrogates p53-mediated transactivation.

[00039] In another embodiment, altered cellular localization of p53 protein inhibits p53 function. In one embodiment of primary human tumors, cytoplasmic sequestration of w/t p53 protein with concomitant nuclear exclusion is found, such as in inflammatory breast cancer and in neuroblastoma (NB), in colon carcinoma and in another embodiment, in malignant melanoma. In colon carcinoma, cytoplasmic accumulation of p53 correlates in one embodiment with unfavorable prognosis.

[00040] In one embodiment, in addition to the acridine or acridine derivatives used in the methods of the invention for the treatment of tumors associated with cancer, the invention provides for co-administering an effective cancer-treating amount of an anti-cancer chemotherapeutic agent, such as 10-hydroxycamptothecin in one embodiment, or adriamycin, or 5-fluorouracil, cisplatin, carboplatin, camptothecins, doxorubicin, cyclophosphamide, cloposide, or a combination thereof in other embodiments. The term "co-administering" refers in one embodiment to the administration of two or more compounds simultaneously, or non-simultaneously as a cocktail in one embodiment, or discretely in another embodiment. The term "chemotherapeutic" refers in another embodiment, to a pharmaceutical, drug, medication, or compound that is used to treat cancer, a cancerous cell, a tumorigenic cell, or a tumor.

[00041] The terms "DNA-damage inducing agent" and "cancer chemotherapeutic agent" refers in one embodiment antineoplastic compounds that are capable of interfering with DNA synthesis at any stage of the cell cycle. As a practical matter, such activity can be inferred by the observation of cell apoptosis. Examples of such agents include but are not limited to alkylating agents (e.g., mechlorethamine, chlorambucil, cyclophosphamide, mephalan, or ifosfamide), S-phase specific antimetabolites (e.g., folate antagonists, purine antagonists, or cytarabine), plant alkaloids (e.g., vinblastine, vincristine, or podophyllotoxins), antibiotics (e.g., doxorubicin, bleomycin, or mitomycin), nitrosureas (e.g., carmustine, or lomustine), inorganic ions (such as cisplatin). Etoposide and cisplatin are other chemotherapy drugs that are known to activate p53 by causing DNA damage and are contemplated for use in the invention.

[00042] In one embodiment, acridine or acridine derivatives used in the methods of the invention is administered in conjunction with one or more chemotherapeutic agents effective against the particular cancer such as gemcitabine or 5-FU, if pancreatic cancer is being treated, tamoxifen or paclitaxel, if breast cancer is to be treated, leuprolide or other anti-androgens, if prostate cancer is involved, and the like in other
5 embodiments.

[00043] Any suitable route of administration may be employed for providing the patient with an effective dosage of acridine or acridine derivative used in the methods and compositions of the invention. While it is possible that, for use in therapy, acridine or acridine derivative used in the methods and compositions of the
10 invention may be administered as the pure chemical, as by inhalation of a fine powder via an insufflator, in one embodiment the acridine, acridine derivative or their combination is present as a pharmaceutical pharmaceutical preparation or formulation. The invention thus further provides a pharmaceutical formulation comprising acridine or acridine derivative used in the methods and compositions of the invention or an analog thereof, together with one or more pharmaceutically acceptable carriers therefor and,
15 optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be 'acceptable' in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof, such as a human patient or domestic animal.

[00044] Pharmaceutical formulations include those suitable for oral or parenteral (including in other
20 embodiments intravenously, intraarterially, intratumorically or intramuscularly) administration. Forms suitable for parenteral administration also include forms suitable for administration by inhalation or insufflation or for nasal, or topical (including buccal, rectal, vaginal and sublingual) administration. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of
25 bringing into association the active compound with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, shaping the product into the desired delivery system.

[00045] Pharmaceutical formulations suitable for oral administration may be presented as discrete unit
30 dosage forms such as hard or soft gelatin capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or as granules; as a solution, a suspension or as an emulsion; or in a chewable base such as a synthetic resin or chicle for ingestion of the agent from a chewing gum. The active ingredient may also be presented as a bolus, electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrants,

or wetting agents. The tablets may be coated according to methods well known in the art, i.e., with enteric coatings.

[00046] The methods of the present invention involves in one embodiment, administering to a subject a pharmaceutical preparation comprising the acridine, acridine derivative or their combination. The pharmaceutical preparation can comprise the acridine, acridine derivative or their combination alone or can further include a pharmaceutically acceptable carrier and can be in solid or liquid form such as tablets, powders, capsules, pellets, solutions, suspensions, elixirs, emulsions, gels, creams, or suppositories, including rectal and urethral suppositories. Pharmaceutically acceptable carriers include gums, starches, sugars, cellulosic materials, and mixtures thereof. The pharmaceutical preparation containing the acridine, acridine derivative or their combination can be administered to a subject by, for example, subcutaneous implantation of a pellet; in a further embodiment, the pellet provides for controlled release of acridine, acridine derivative or their combination over a period of time. The preparation can also be administered by intravenous, intra-arterial, or intramuscular injection of a liquid preparation, oral administration of a liquid or solid preparation, or by topical application. Administration can also be accomplished by use of a rectal suppository or a urethral suppository. The pharmaceutical preparation can also be a parenteral formulation; in one embodiment, the formulation comprises a liposome that includes a complex of acridine, acridine derivative or their combination such as, for example, N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl] amine (CP4), 9-Amino-acridine, Acridine Orange, Quinacrine, Amsacrine or a combination thereof.

[00047] Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives.

[00048] The compounds according to the invention may also be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form, obtained by aseptic isolation of sterile

solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

[00049] For topical administration to the epidermis, the compounds may be formulated as ointments, creams or lotions, or as the active ingredient of a transdermal patch. Suitable transdermal delivery systems are disclosed, for example, in A. Fisher et al. (U.S. Pat. No. 4,788,603), or R. Bawa et al. (U.S. Pat. Nos. 4,931,279; 4,668,506 and 4,713,224). Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents.

[00050] For topical administration to body surfaces using, for example, creams, gels, drops, and the like, the acridine, acridine derivative or their combinations or their physiologically tolerated derivatives such as salts, esters, N-oxides, and the like are prepared and applied as solutions, suspensions, or emulsions in a physiologically acceptable diluent with or without a pharmaceutical carrier. In another embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*).

[00051] Formulations suitable for topical administration in the mouth include unit dosage forms such as lozenges comprising active ingredient in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base such as gelatin and glycerin or sucrose and acacia; mucoadherent gels, and mouthwashes comprising the active ingredient in a suitable liquid carrier.

[00052] When desired, the above-described formulations can be adapted to give sustained or controlled release of the active ingredient employed, e.g., by combination with certain hydrophilic polymer matrices, e.g., comprising natural gels, synthetic polymer gels or mixtures thereof. The polymer matrix can be coated onto, or used to form, a medical prosthesis, such as a stent, valve, shunt, graft, or the like.

[00053] Controlled or sustained release compositions include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms, protective coatings, protease inhibitors or

permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood
5 following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less
10 frequently or in lower doses than with the unmodified compound.

[00054] In yet another embodiment, the pharmaceutical composition can be delivered in a controlled release system. For example, the agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may
15 be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989). In another embodiment, polymeric materials can be used. In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984). Preferably, a controlled
20 release device is introduced into a subject in proximity of the site of inappropriate immune activation or a tumor. Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990).

[00055] Pharmaceutical formulations suitable for rectal administration wherein the carrier is a solid are in
25 one embodiment, presented as unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art, and the suppositories may be conveniently formed by admixture of the active compound with the softened or melted carrier(s) followed by chilling and shaping in molds.

[00056] Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams,
30 gels, pastes, foams or sprays containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

[00057] For administration by inhalation, the compounds according to the invention are conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an

aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

5 [00058] Alternatively, for administration by inhalation or insufflation, the compounds according to the invention may take the form of a dry powder composition, for example, a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges or, e.g., gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflator.

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[00059] Further, as used herein "pharmaceutically acceptable carriers" are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, 15 vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may 20 also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

[00060] The pharmaceutical preparations of the invention can be prepared by known dissolving, mixing, granulating, or tablet-forming processes. For oral administration, the acridine, acridine derivative or their combinations or their physiologically tolerated derivatives such as salts, esters, N-oxides, and the like are 25 mixed with additives customary for this purpose, such as vehicles, stabilizers, or inert diluents, and converted by customary methods into a suitable form for administration, such as tablets, coated tablets, hard or soft gelatin capsules, aqueous, alcoholic or oily solutions. Examples of suitable inert vehicles are conventional tablet bases such as lactose, sucrose, or cornstarch in combination with binders like acacia, cornstarch, gelatin, or with disintegrating agents such as cornstarch, potato starch, alginic acid, or with a 30 lubricant such as stearic acid or magnesium stearate. Examples of suitable oily vehicles or solvents are vegetable or animal oils such as sunflower oil or fish-liver oil. Preparations can be effected both as dry and as wet granules. For parenteral administration (subcutaneous, intravenous, intraarterial, or intramuscular injection), the acridine, acridine derivative or their combinations or their physiologically tolerated derivatives such as salts, esters, N-oxides, and the like are converted into a solution, suspension, or

emulsion, if desired with the substances customary and suitable for this purpose, for example, solubilizers or other auxiliaries. Examples are: sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycols or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

[00061] The preparation of pharmaceutical compositions which contain an active component is well understood in the art. Typically, such compositions are prepared as an aerosol of the polypeptide delivered to the nasopharynx or as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, or pH buffering agents which enhance the effectiveness of the active ingredient.

[00062] An active component can be formulated into the composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[00063] The acridine, acridine derivative or their combination may precede or follow a DNA damaging agent treatment by intervals ranging from minutes to weeks. Protocols and methods are known to those skilled in the art. DNA damaging agents or factors are known to those skilled in the art and refer to any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage, such as gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use include, e.g., adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C,

cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide.

5 [00064] In one embodiment, colon adenocarcinoma cell are treated with CP-1 (5 μ M), CP-2 (5 μ M), asmacridine (25 μ M), acridine orange (20 μ M), quinacrine (20 μ M) and 9-aminoacridine (100 μ M) respectively. After 20 hours of drug treatment, wild-type colon adenocarcinoma cell show much higher levels of apoptosis as measured by analysis of sub-G1 cells. In in another embodiment, colon adenocarcinoma cell wherein Bax expression was knocked out, were quite resistant to the treatments, indicating that the mitochondrial pathway plays in another embodiment, an important role in the mechanism of induced apoptosis by acridine, acridine derivative or their combination.

[00065] According to this aspect of the invention and in one embodiment, the invention provides a method of blocking acridine, or acridine derivative – induced cell cycle arrest or apoptosis in a cell, comprising
15 knocking out the expression of Bax gene, thereby knocking out a p53 target and a key cell death inducer.

[00066] In another embodiment, knocking out the expression of Bax gene according to the methods of the invention is done by expression of an RNA antisense to the DNA or mRNA of said Bax gene; or by expression of a ribozyme specific for the mRNA of said Bax gene; or by contacting the cell with siRNA
20 specific for Bax mRNA.

[00067] In one embodiment, Bax gene expression is through the use of anti-sense RNA. The term "anti-sense RNA" refers in one embodiment to an RNA molecule that is capable of forming a duplex with a second RNA molecule. Thus a given RNA molecule is said to be an anti-sense RNA molecule with respect to a
25 second, complementary or partially complementary RNA molecule, i.e., the target molecule. An anti-sense RNA molecule may be complementary to a translated or an untranslated region of a target RNA molecule. The anti-sense RNA need not be perfectly complementary to the target RNA. Anti-sense RNA may or may not be the same length of the target molecule; the anti-sense RNA molecule may be either longer or shorter than the target molecule. For artificial antisense RNA expression, part of a target gene is transcribed in one
30 embodiment in the opposite orientation so that the RNA from the target gene can anneal and form a double-stranded RNA with the antisense RNA, thereby inhibiting the translation of the mRNA. Antisense DNA and RNA has been used to inhibit gene expression in many instances. Many modifications, such as phosphorothioates, have been made to antisense oligonucleotides to increase resistance to nuclease

degradation, binding affinity and uptake (Cazenave et al. 1989; Sun et al. 1989; McKay et al. 1996; Wei et al. 1996).

[00068] In another embodiment, the inhibition of Bax gene expression is through the use of Bax-specific ribozyme for the mRNA of the Bax gene. The term "Bax-specific ribozyme" refers in one embodiment to a nucleic acid sequence that comprises a ribozyme catalytic domain sequence in combination with a nucleic acid sequence that is complementary to and binds to an RNA transcript of a Bax gene. A number of ribozymes are known, virtually any one of which may be used in conjunction with the present invention. In one embodiment, ribozyme catalytic domains from hammerhead ribozymes and from hairpin ribozyme structures are used. In other embodiments, ribozyme sequences from RNaseP, hepatitis delta virus, avocado sunblotch viroid virus, lucerne transient streak, and tobacco ringspot virus are used. In one embodiment, the Bax-specific ribozyme will comprise a ribozyme catalytic domain linked to a nucleic acid sequence that is complementary to and binds to an RNA transcript of a Bax gene; in other embodiments, the ribozyme will comprise a nucleic acid sequence that directs binding to a Bax gene. The Bax-gene nucleic acid sequence may be linked either to the 5' or to the 3' end of the ribozyme sequence, in one embodiment the ribozyme sequence is linked at each end to a Bax-gene nucleic acid sequence. In this manner, this Bax-specific sequences will flank the ribozyme catalytic sequence.

[00069] In one embodiment, the inhibition of Bax gene expression is through the use of siRNA specific for the Bax-gene. In one embodiment, the term "siRNA" refers to RNA interference, which in one embodiment refers to the process of sequence-specific post-transcriptional gene silencing in animals, mediated by short interfering RNAs (siRNAs). In another embodiment, the process of post-transcriptional gene silencing is an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes. Such protection from foreign gene expression evolved one embodiment, in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or in another embodiment, from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA of viral genomic RNA. In one embodiment, the presence of dsRNA in cells triggers the RNAi response.

[00070] By the term "conserved", amino acid sequences comprising the peptides of this invention remain in one embodiment, essentially unchanged throughout evolution, and exhibit homology among various species producing the protein.

[00071] The presence of long dsRNAs in cells stimulates in another embodiment, the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in one embodiment, in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are in another embodiment about 21 to about 23 nucleotides in length and
5 comprise about 19 base pair duplexes. Small RNAs function in one embodiment, by base-pairing to complementary RNA or DNA target sequences. When bound to RNA, small RNAs trigger RNA cleavage in another embodiment, or translational inhibition of the target sequence in another embodiment. When bound to DNA target sequences, small interfering RNAs mediate in one embodiment, DNA methylation of the target sequence. The consequence of these events, in one embodiment, is the inhibition of gene expression.

10 [00072] In one embodiment, the siRNA of a Bax gene exhibit substantial complementarity to its target sequence. In another embodiment, "complementarity" indicates that the oligonucleotide has a base sequence containing an at least 15 contiguous base region that is at least 70% complementary, or in another embodiment at least 80% complementary, or in another embodiment at least 90% complementary, or in
15 another embodiment 100% complementary to an-at least 15 contiguous base region present of a target gene sequence (excluding RNA and DNA equivalents). (Those skilled in the art will readily appreciate modifications that could be made to the hybridization assay conditions at various percentages of complementarity to permit hybridization of the oligonucleotide to the target sequence while preventing unacceptable levels of non-specific hybridization.). The degree of complementarity is determined by
20 comparing the order of nucleobases making up the two sequences and does not take into consideration other structural differences which may exist between the two sequences, provided the structural differences do not prevent hydrogen bonding with complementary bases. The degree of complementarity between two sequences can also be expressed in terms of the number of base mismatches present in each set of at least 15 contiguous bases being compared, which may range from 0-3 base mismatches, so long as their
25 functionality for the purpose used is not compromised.

[00073] In one embodiment, the siRNA of a Bax gene, is sufficiently complimentary to its target sequence. "Sufficiently complementary" refers in one embodiment to a contiguous nucleic acid base sequence that is capable of hybridizing to another base sequence by hydrogen bonding between a series of complementary
30 bases. In another embodiment, complementary base sequences may be complementary at each position in the base sequence of an oligonucleotide using standard base pairing (e.g., G:C, A:T or A:U pairing) or may contain one or more residues that are not complementary using standard hydrogen bonding (including abasic "nucleotides"), but in which the entire complementary base sequence is capable of specifically hybridizing with another base sequence under appropriate hybridization conditions. Contiguous bases are at least about

80% in one embodiment, or at least about 90% in another embodiment, or about 100% complementary to a sequence to which an oligonucleotide is intended to specifically hybridize in another embodiment. Appropriate hybridization conditions are well known to those skilled in the art, can be predicted readily based on base sequence composition, or can be determined empirically by using routine testing (e.g., See
5 Sambrook et al., Molecular Cloning. A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

[00074] The term "nucleic acid" refers in one embodiment to a polymer or oligomer composed of nucleotide units (ribonucleotides, deoxyribonucleotides or related structural variants or synthetic analogs thereof)
10 linked via phosphodiester bonds (or related structural variants or synthetic analogs thereof). Thus, the term refers to a nucleotide polymer in which the nucleotides and the linkages between them are naturally occurring (DNA or RNA), as well as various analogs, for example and without limitation, peptide-nucleic acids (PNAs), phosphoramidates, phosphorothioates, methyl phosphonates, 2-O-methyl ribonucleic acids, and the like. In one embodiment, the siRNAs used in the compositions and methods of the invention, are
15 nucleic acid sequences.

[00075] As can be readily appreciated by one of ordinary skill in the art, the methods and pharmaceutical compositions of the present invention are particularly suited to administration to a mammal, preferable a human subject.

[0001] The term "about" as used herein means in quantitative terms plus or minus 5%, or in another embodiment plus or minus 10%, or in another embodiment plus or minus 15%, or in another embodiment plus or minus 20%.

[0002] The term "subject" refers in one embodiment to a mammal including a human in need of therapy for, or susceptible to, a condition or its sequelae. The subject may include dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice and humans. The term "subject" does not exclude an individual that is normal in all respects.

[0003] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Materials and Methods:

Reagents

[0004] CP-31398 was kindly provided by Farzan Rastinajad at Pfizer Inc., Groton, CT. Amsacrine (A9809), acridine orange (A6014), quinacrine (Q3251), and 9-aminoacridine (A3773) are from Sigma (St. Louis, MO). Doxorubicin is from GensiaSicor Pharmaceuticals (Irvine, CA). D-luciferin is purchased from Biotium, Inc. (Hayward, CA).

Synthesis of 9-chloroacridine

[0005] Acridone(Acros) was mixed with POCl₃ at 1:10 (W/V) and heated with reflux for 1hr. After cooling, 30% aqueous ammonia was added drop-wise until the solution turned basic, at which point the mixture was extracted 3 times with chloroform. The organic layers were combined and dried with MgSO₄, filtered, and then rotary evaporated. The resulting solid was then recrystallized overnight at -20°C in chloroform/hexane, producing long, pale yellow crystals. The mother liquor was filtered away, the crystals were weighed and product was confirmed via Electrospray Mass Spectrometry (ES-MS) and Nuclear Magnetic Resonance spectroscopy (NMR).

Synthesis of, N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine, N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine, Acridin-9-yl(3-morpholin-4-yl-propyl)-amine, and Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine

[0006] 9-chloroacridine, as synthesized above, was mixed with phenol in an approximate 1:3 molar ratio along with 4 ml dry benzene. Then a 1 mmole excess of either 3-dimethylaminopropylamine(Aldrich), 3-diethylaminopropylamine(Aldrich), 3-morpholin-4-yl-propylamine(Aldrich) or 3-(4-methyl-piperazin-1-yl)-propylamine(Pfaltz and Bauer) was added respectively. The mixture was then heated at reflux for 1hr. After cooling the mixture was diluted with 15mL of dichloromethane and 15 mL of 2M NaOH was added. The aqueous layer was extracted 3 times with 15 ml of dichloromethane. The organic layers were combined and dried with MgSO₄. The solution was rotary evaporated and the resulting solid was dissolved in diethyl ether and then flushed with HCl gas or HCl ether, at which point a yellow precipitate was observed. The ether was filtered away and the remaining solid was then recrystallized using methanol/ethyl acetate at -20°C, producing short, yellow crystals. All products were confirmed by ES-MS and NMR .

Cell lines

[0007] Tumor cell lines with wild-type p53 were used, including the H460 non-small cell lung carcinoma cell line, the PA-1 ovarian cancer cell line, the HCT116 colon adenocarcinoma cell lines and the human foreskin fibroblast HFF cells. H460, PA-1 and HFF were obtained from American Type Culture Collection

(ATCC, Rockville, Maryland). HCT116/p53(-/-), HCT116/p53(-/-) and HCT116/bax(-/-) cells are from Bert Vogelstein (Johns Hopkins University, Baltimore, MD).

Antibodies

[0008] Anti-p53 antibodies, DO-1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for detection of p53 and its ubiquitinated forms, FL293 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for p53 immunoprecipitation. Anti-MDM-2 (AB-1) was from Calbiochem/Oncogene Science (Boston, MA) for MDM2 immunoblot. Anti-phospho-p53 antibodies against phosphorylation of ser15, ser20 were obtained from Cell Signaling Technology (Beverly, MA).

Western and Northern blotting

[0009] For Western blotting, cells were collected and protein concentration was quantified by the Bio-Rad protein assay prior to SDS-PAGE. Proteins were transferred to a PVDF membrane (Immunobilon-P, Millipore Corporation, Bedford, Massachusetts) by a semi-dry transfer apparatus from Bio-Rad Laboratories (Hercules, California). The membranes with transferred proteins were blotted with 10% W/V non-fat dry milk and then subjected to the primary antibody and subsequently secondary antibodies, which are labeled by horseradish peroxidase, or infrared (IR). Signals were visualized by either ECL (Amersham Pharmacia Biotech, England, UK) and exposed to an X-ray film, or scanned by the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

In vivo ubiquitination assay

[00010] Cells were grown in a 6-well plate to 80% confluency. CP-31398 was added at a final concentration of 15 µg/ml and incubated at 37 °C for 1 hr. Then MG-132, a proteosomal inhibitor (Sigma, St. Louis, MO), was added at the final concentration of 10 µM and incubated for 4 hr. The plate with cells was placed on ice and boiled SDS-sample buffer was added after washing twice with ice-cold PBS. Lysates were collected for SDS-PAGE and ubiquitinated p53 proteins were detected by immunoblotting with the DO-1 antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, California).

Flow cytometry assay

[00011] Cells in a 6-well plate were trypsinized and collected in 15 ml centrifuge tubes. The collected cells were ethanol fixed and stained with propidium iodide (Sigma, St. Louis, MO). The DNA content of the stained cells was then measured using an Epics Elite flow cytometer (Beckman-Coulter, Fullerton, CA).

Example 1: Acridine derivatives activate p53

[00012] It has been shown that CP-31398 can activate p53 in tumor cells in a unique pathway that is different from the known p53 activation pathways that occur following DNA damage. In order to identify other potent p53 stimulators with the potential for greater bioavailability, four novel compounds were synthesized with structural similarity to CP-31398, based on the active forms of compounds previously described. All four compounds have the same R1 group, which is acridine, and a three carbon linker. However a different R2 group—dimethyl, diethyl, morpholine, and methyl piperazine was linked to each compound, respectively. It was reasoned that both of the respective R1 groups, a quinazoline ring for CP-31398 and the acridine ring for our synthesized compounds would have similar properties given that both heterocycles are flat, rigid, efficient intercalators, in addition to being hydrophobic. As such, it was anticipated, that their similar structures would impart similar activities. The chemical names of these compounds are N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine, N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine, Acridin-9-yl(3-morpholin-4-yl-propyl)-amine, and Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine. The 2D structures of these compounds are shown in figure 1. as the compounds were termed CP-1, CP-2, CP-3, and CP-4 respectively

[00013] HCT116 cells were treated with various concentrations of the compounds to test their ability to stabilize p53 protein. As shown in figure 2, after 12 hours, p53 levels increased in cells treated with each of these four compounds. The elevated levels of two well-known p53 transcriptional targets, p21 and MDM2, indicated that the stabilized p53 protein was transcriptionally active.

[00014] Similar activation of p53 by these four compounds prompted the investigation of whether the R1 group may play a fundamental role in p53 stabilization rather than the linker or R2 group. The basic structure of R1 is acridine, whose derivatives have not only been widely used against malaria and some bacterial infections, but also against cancer. To confirm the hypothesis, several other acridine derivatives were tested for their p53 activation potential. These include 9-Aminoacridine, a basic structure of acridine, acridine orange, which has been used for DNA/RNA staining and malaria and bacteria diagnosis, quinacrine, an anti-malaria drug, and amsacrine, an anti-leukemia drug. Figure 2B shows that all these acridine derivatives stabilized p53 protein. The p53 targets, p21 and MDM2 were subsequently elevated in acridine compound-treated cells. These drugs were further tested using other human wild-type p53 expressing cell lines, including the H460 non-small cell lung carcinoma cell line, the PA-1 ovarian cancer cell line and human foreskin fibroblast HFF cells. Acridine derivatives activated wild-type p53 in all the cell lines we tested (figure 2C).

[00015] To further confirm the activation of p53 transcriptional activity, a bioluminescent imaging assay was performed using HCT116 cells stably expressing a p53 reporter, PG13-luc, which contains 13 p53 response elements. All four compounds synthesized and other acridine derivatives were found to elicit a p53 transcriptional response. Of interest, the p53 stabilized by the compounds at higher concentrations possessed lower transcriptional activity (figure 2A and 2D). p53 was stabilized to a high level at a dose of 20 μ M to 40 μ M of CP-1, while p53 targets, MDM2, DR5, and p21 were not increased at these doses. A p53 reporter assay also showed the same pattern (figure 2D, bottom panel). Surprisingly, as compared to CP-31398, the acridine derivatives activated the p53 reporter at much lower doses (figure 3A). This may in part address the poor bioavailability of the parental CP-31398 compound.

Example 2: Acridine derivatives block p53 ubiquitination

[00016] In order to explore the mechanism of p53 stabilization by acridine derivatives, p53 ubiquitination status in treated cells was first checked. As shown in figure 4, treatment with the proteasome inhibitor MG-132, which inhibits the degradation of ubiquitinated p53, resulted in accumulation of ubiquitinated p53 proteins in a characteristic pattern of ladders with increasing molecular weight. Co-treatment with acridine derivatives prevented the formation of ubiquitinated p53 ladders.

Example 3: Acridine derivatives block p53 ubiquitination through a mechanism different from what occurs following DNA-damage

[00017] DNA-damage induced blockage of p53 ubiquitination provides a major mechanism for p53 stabilization, which proceeds through phosphorylation of the amino-terminus of p53 and dissociation from MDM2, a major p53 E3 ubiquitin ligase. However, CP-31398 stabilizes p53 in a different way that does not involve phosphorylation of p53 or dissociation of MDM2. In order to clarify whether acridine derivatives act in a similar manner as CP-31398, or as DNA-damaging agents, the phosphorylation of the amino-terminus of p53 was first determined. Figure 5A shows that acridine derivatives did not induce p53 phosphorylation at ser15 and ser20 at the dose that induced high levels of p53 protein, while the DNA-damaging agent, adriamycin, induced a high level of p53 phosphorylation at both the sites. Immunoprecipitation of p53 showed MDM2 association in the cells treated with quinacrine or CP-1, while considerably less MDM2 was co-immunoprecipitated with p53 in cells treated with adriamycin (figure 5B). It was therefore concluded that acridine derivatives block p53 ubiquitination through a mechanism different from what occurs following DNA-damage, while being similar to CP-31398.

Example 4: Acridine derivatives induce apoptosis in a p53- and bax-dependent manner

[00018] It was further determined, whether acridine derivatives can induce cell cycle arrest or apoptosis. HCT116 cells were treated with CP-1 (5 μ M), CP-2 (5 μ M), asmacridine (25 μ M), acridine orange (20 μ M), quinacrine (20 μ M) and 9-aminoacridine (100 μ M) respectively. After 20 hours of drug treatment, HCT116 wild-type cells showed much higher levels of apoptosis as measured by analysis of sub-G1 cells. In contrast, p53 knockout HCT116 cells, HCT/p53(-/-), showed significantly reduced levels of apoptosis after treatment with acridine derivatives. HCT116/Bax(-/-) cells were quite resistant to the treatments, indicating that the mitochondrial pathway plays an important role in the mechanism of acridine derivative-induced apoptosis. Of particular interest, in the case of CP-1 shown in figure 6A, higher concentrations induced less apoptosis. This may partly be due to the fact that p53 stabilized by higher concentration of CP-1 is less effective in terms of transcription as shown in figure 2D. It is also possible that higher concentrations of the acridine derivatives activate cell survival pathway(s) to keep the cell from dying.

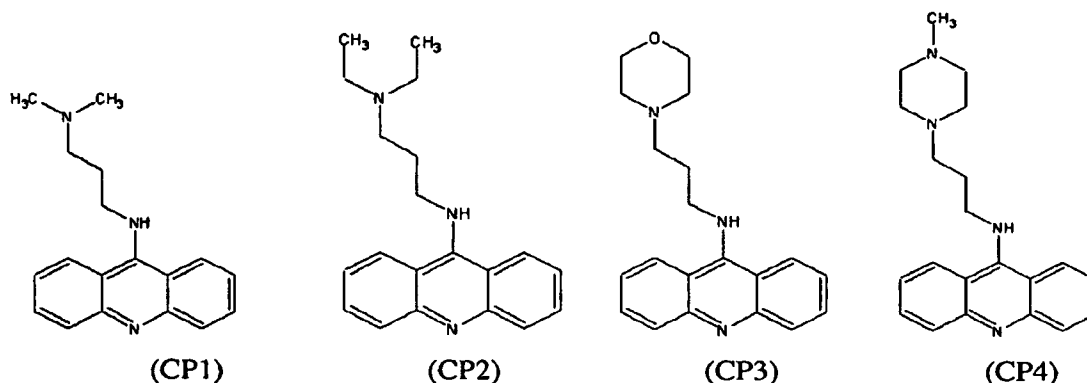
Example 4: CP-1 and quinacrine induce p53 transcriptional activity in mouse tumor xenografts

[00019] The anti-tumor activity of acridine derivatives has been intensively evaluated. Whether these compounds can induce p53 transcriptional activity in tumor cells was investigated *in vivo*. HCT116/PG-13 cells²⁷ that stably express a firefly luciferase gene under the control of 13 p53 response elements were injected subcutaneously in the right flank of nude mice at a dose of 2×10^6 cell in 0.2 ml of PBS. As a control, HCT116/pGL-2 cells, expressing a firefly luciferase gene under the control of the SV40 promoter that is not responsive to the p53 activation, were injected in the left flank. At 24 hours after injection of the cells, the mice were imaged and the bioluminescence intensities of both sides were measured. Then CP-1 and quinacrine were injected intraperitoneally at a dose of 50 mg/kg and 100 mg/kg respectively. At 12 hours after drug injection, the bioluminescence intensity of the tumor sites was imaged. As shown in figure 7, the bioluminescence intensity of the tumor on the right dramatically increased as compared to that on the left following drug exposure. The ratio change of the bioluminescence intensity between the right and the left flanks indicates that p53 was induced to activate transcription. The doses of the drugs were well tolerated by the mice for 5 days of observation.

[00020] The foregoing has been a description of certain non-limiting preferred embodiments of the invention. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

What is claimed is:

1. A p53 stabilizing composition, comprising an acridine derivative.
2. The composition of claim 1, wherein said acridine derivative is N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4), 9-Amino-acridine, Acridine Orange, Quinacrine, Amsacrine or a combination thereof.
3. The composition of claim 2, wherein said N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4) are represented by the following formula:



4. The composition of claim 1, comprising a carrier, excipient, flow agent, processing aid, a diluent or a combination thereof.
5. The composition of claim 4, wherein said carrier, excipient, lubricant, flow aid, processing aid or diluent is a gum, a starch, a sugar, a cellulosic material, an acrylate, calcium carbonate, magnesium oxide, talc, lactose monohydrate, magnesium stearate, colloidal silicone dioxide or mixtures thereof.
6. The composition of claim 4, comprising a binder, a disintegrant, a buffer, a protease inhibitor, a surfactant, a solubilizing agent, a plasticizer, an emulsifier, a stabilizing agent, a viscosity increasing agent, a sweetener, a film forming agent, or any combination thereof.

7. The composition of claim 1, wherein said composition is in the form of a pellet, a tablet, a capsule, a solution, a suspension, a dispersion, an emulsion, an elixir, a gel, an ointment, a cream, or a suppository.

8. The composition of claim 1, wherein said composition is in a form suitable for oral, intravenous, intraaortical, intratumoral, intramuscular, subcutaneous, parenteral, transmucosal, transdermal, or topical administration.

9. The composition of claim 1, wherein said composition is a controlled release composition.

10. The composition of claim 1, wherein said composition is an immediate release composition.

11. The composition of claim 1, wherein said composition is a liquid dosage form.

12. The composition of claim 1, wherein said composition is a solid dosage form.

13. The composition of claim 1, wherein said acridine derivative is present at a dosage of 0.01-100 μ M.

14. The composition of claim 13, wherein the dosage is between about 25 to about 100 μ M

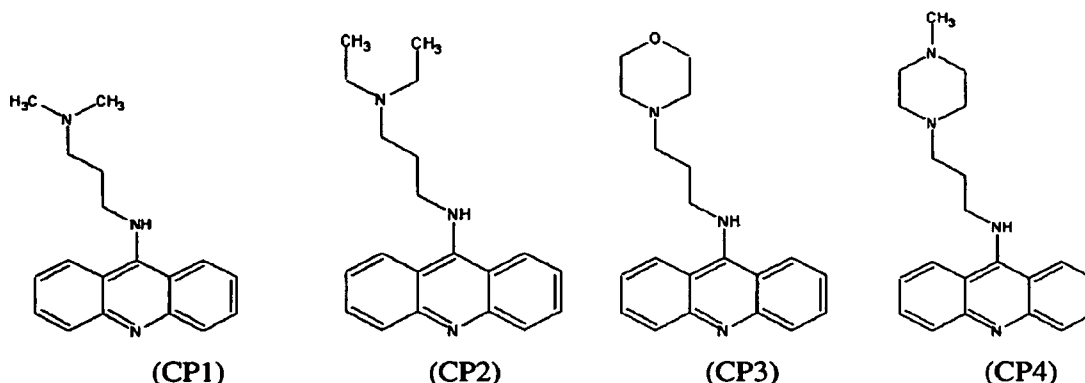
15. The composition of claim 13, wherein the dosage is between about 50 and about 100 μ M

16. A method of inducing cell cycle arrest or apoptosis in a cell comprising contacting said cell with an acridine derivative.

17. The method of claim 16, wherein said acridine derivative is N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'- Acridin- 9-yl-N,N -diethyl -propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4), 9-Amino-acridine, Acridine Orange, Quinacrine, Amsacrine or a combination thereof.

18. The method of claim 16, wherein said N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'- Acridin- 9-yl-N,N -diethyl -propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-

amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4) are represented by the following formula:



5

19. The method of claim 16, wherein said cell is a tumor cell.

20. The method of claim 16, wherein said cell cycle arrest or apoptosis is impaired due to p53 instability.

10

21. A method of stabilizing p53 expression in a cell, comprising contacting said cell with the composition of claim 1.

22. A method of treating cancer in a subject, comprising administering to said subject an effective amount of the composition of claim 1.

15

23. The method of claim 22, wherein the cancer is a solid tumor.

24. The method of claim 23, wherein said tumor is osteosarcoma, soft tissue sarcoma, breast tissue sarcoma, ovarian carcinoma, cervical carcinoma, oral squamous cell carcinoma, brain tumor, esophageal cancer, colorectal carcinoma, bladder cancer, urothelial carcinoma, leukemia, large B cell lymphoma, non-small cell lung carcinoma, prostate carcinoma, renal carcinoma, pancreatic carcinoma, melanoma, or a combination thereof.

20

25. The method of claim 22, comprising co-administering an effective cancer-treating amount of an anti-cancer chemotherapeutic agent.

25

26. The method of claim 24, wherein the cancer chemotherapeutic agent is 10-hydroxycamptothecin, adriamycin, or 5-fluorouracil, isplatin, carboplatin, camptothecins, doxorubicin, cyclophosphamide, cloposide, or a combination thereof.

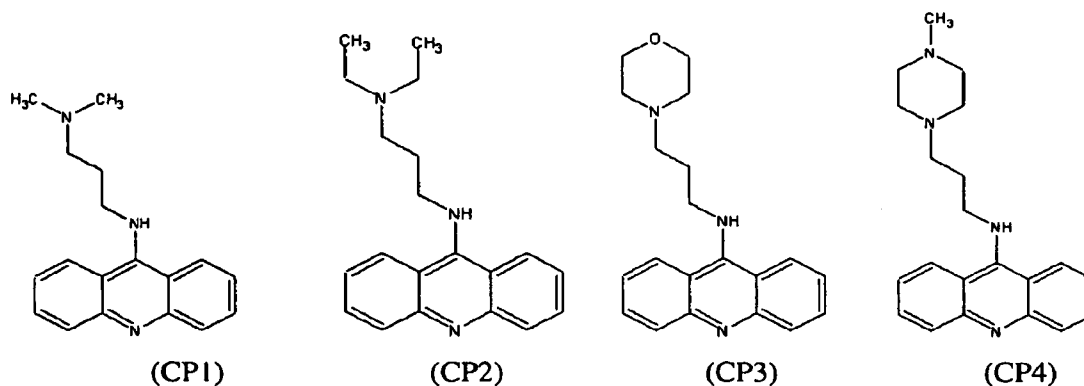
27. The method of claim 25 wherein the composition of claim 1 and anticancer chemotherapeutic agent are administered as a cocktail.

28. The method of claim 25 wherein the composition of claim 1 and said anticancer chemotherapeutic agent are administered in separate unit dosage forms.

29. A method of treating cancer in a subject, comprising administering to said subject an effective amount of a pharmaceutical preparation comprising an acridine derivative, wherein said acridine derivative stabilizes p53 in a cancer cell, inducing cell cycle arrest or apoptosis, thereby treating said cancer.

30. The method of claim 29, wherein said acridine derivative is N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4), 9-Amino-acridine, Acridine Orange, Quinacrine, Amsacrine or a combination thereof.

31. The method of claim 16, wherein said N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine (CP1), N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine (CP2), Acridin-9-yl(3-morpholin-4-yl-propyl)-amine (CP3), Acridin-9-yl[3-(4-methyl-piperazin-1-yl)-propyl]amine (CP4) are represented by the following formula:



32. The method of claim 29, wherein said administering comprises intravenously, intraarterially, intratumorically or intramuscularly injecting in said subject said pharmaceutical preparation in liquid form.

5 33. The method according to claim 29, wherein said administering comprises orally administering to said subject a liquid or solid preparation containing said pharmaceutical preparation.

34. The method according to claim 29, wherein said administering comprises topically applying to skin surface of said subject said pharmaceutical preparation.

10 35. The method according to claim 29, wherein said pharmaceutical preparation is selected from the group consisting of a pellet, a tablet, a capsule, a solution, a suspension, an emulsion, an elixir, a gel, a cream, and a suppository.

15 36. The method according to claim 35, wherein said suppository is a rectal suppository or a urethral suppository.

37. The method according to claim 29, wherein said pharmaceutical preparation is a parenteral formulation.

20 38. The method of claim 29, wherein the cancer is a solid tumor.

25 39. The method of claim 38, wherein said tumor is osteosarcoma, soft tissue sarcoma, breast tissue sarcoma, ovarian carcinoma, cervical carcinoma, oral squamous cell carcinoma, brain tumor, esophageal cancer, colorectal carcinoma, bladder cancer, urothelial carcinoma, leukemia, large B cell lymphoma, non-small cell lung carcinoma, prostate carcinoma, renal carcinoma, pancreatic carcinoma, melanoma, or a combination thereof.

30 40. The method of claim 29, comprising co-administering an effective cancer-treating amount of an anti-cancer chemotherapeutic agent.

41. The method of claim 40, wherein the cancer chemotherapeutic agent is 10-hydroxycamptothecin, adriamycin, or 5-fluorouracil, isplatin, carboplatin, camptothecins, doxorubicin, cyclophosphamide, cloposide, or a combination thereof.

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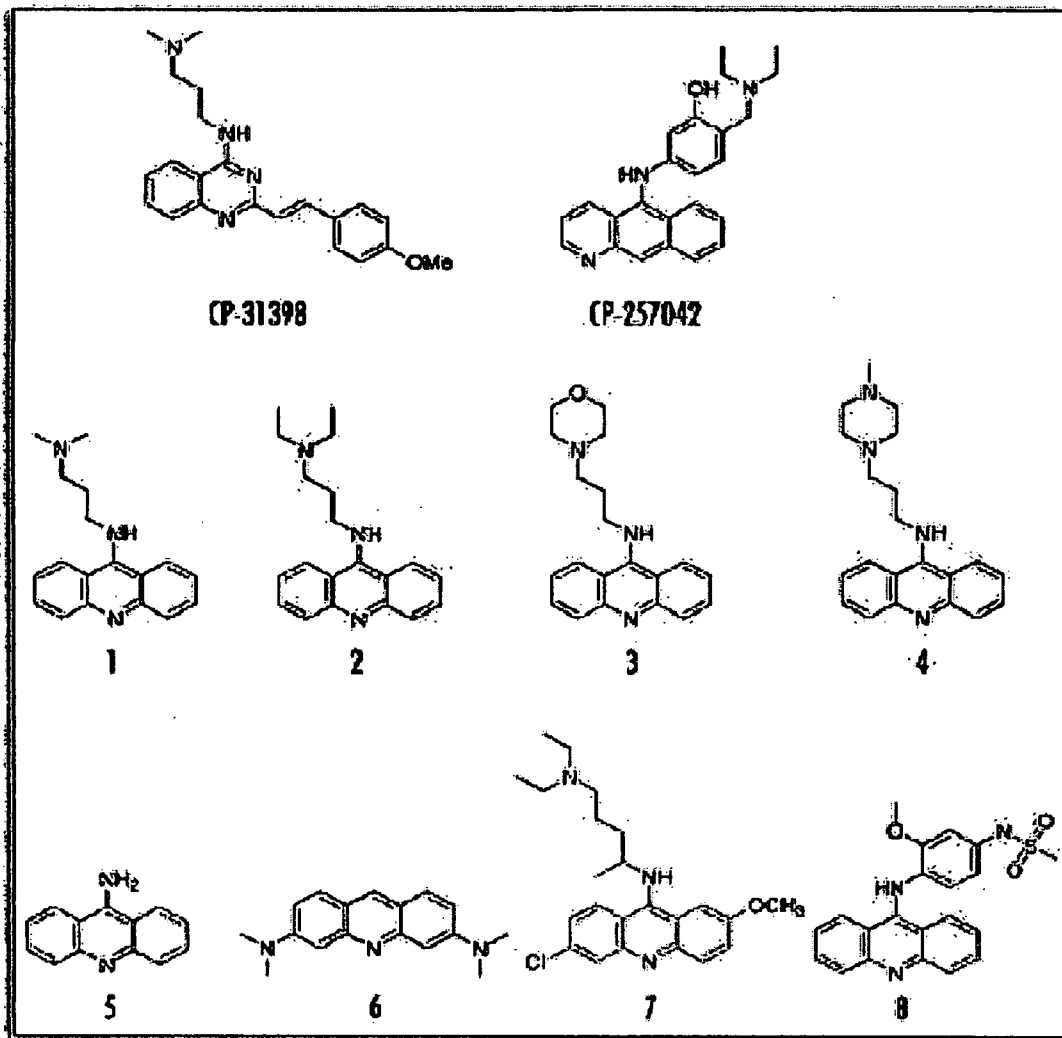


Figure 1

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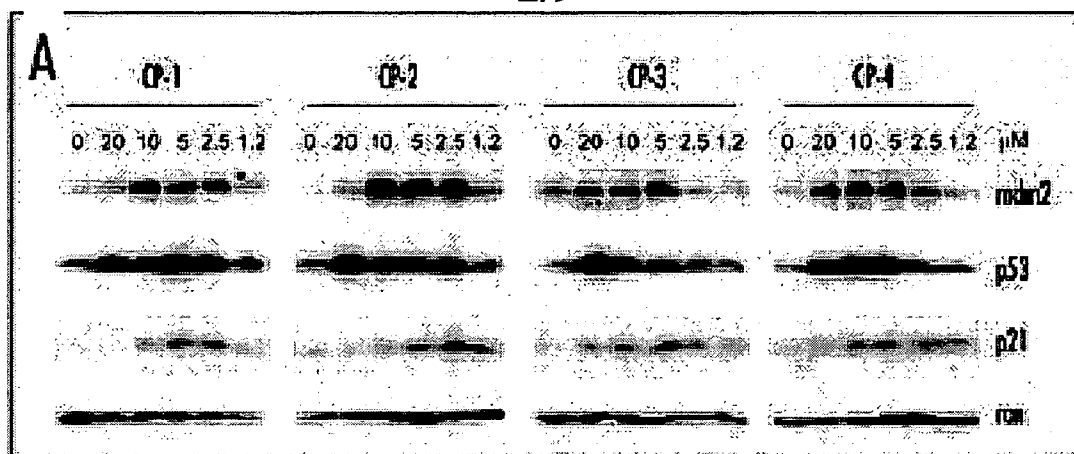
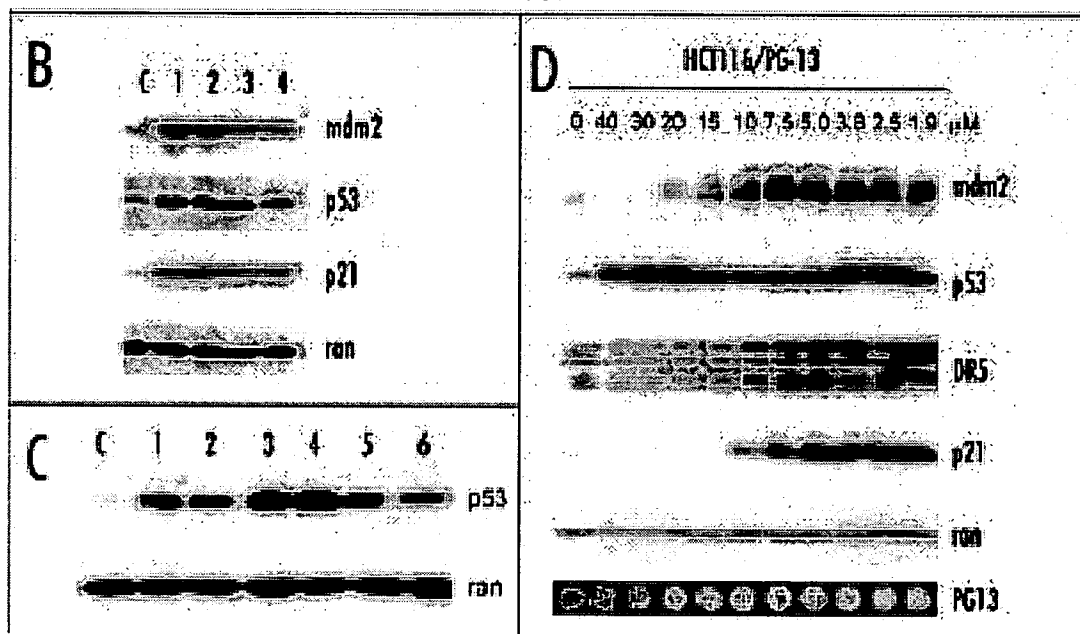


Figure 2a

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Figures 2b, 2c, and 2d

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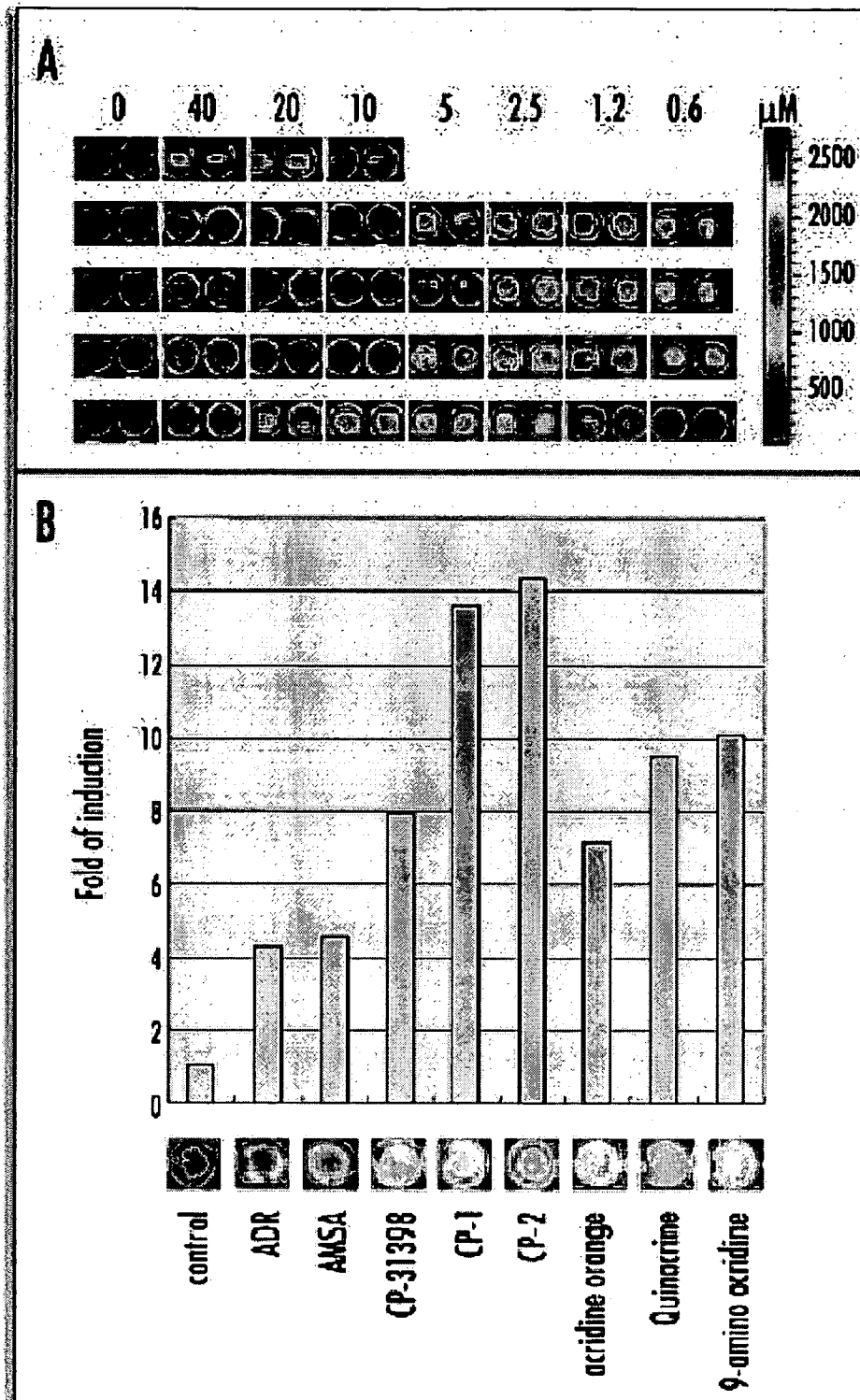
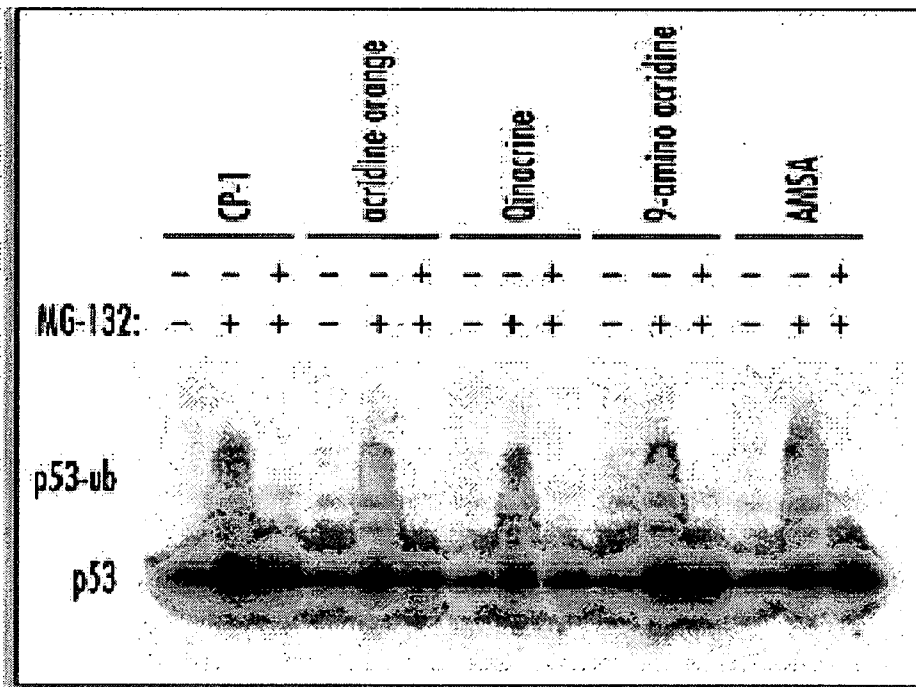


Figure 3

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Figure 4



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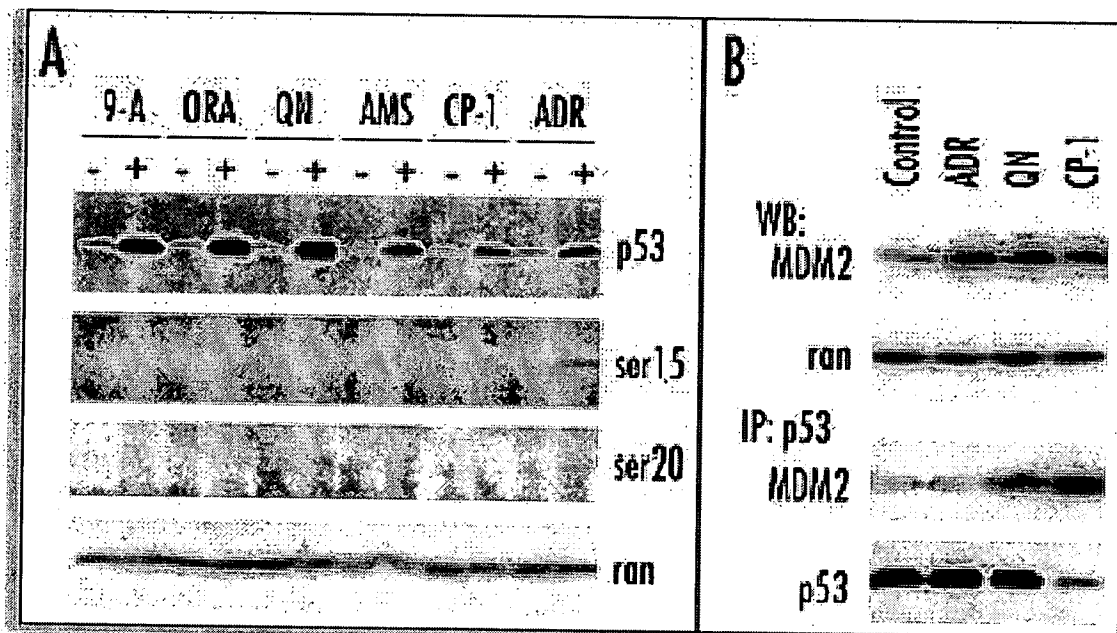


Figure 5

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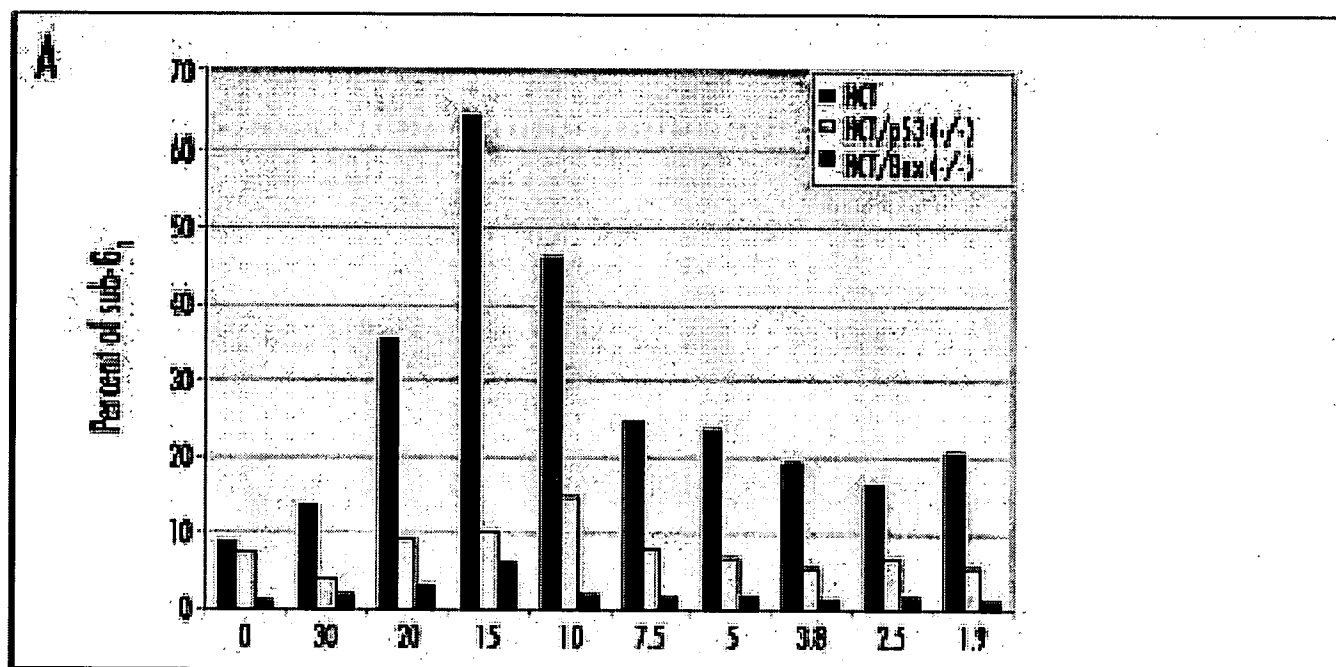


Figure 6a

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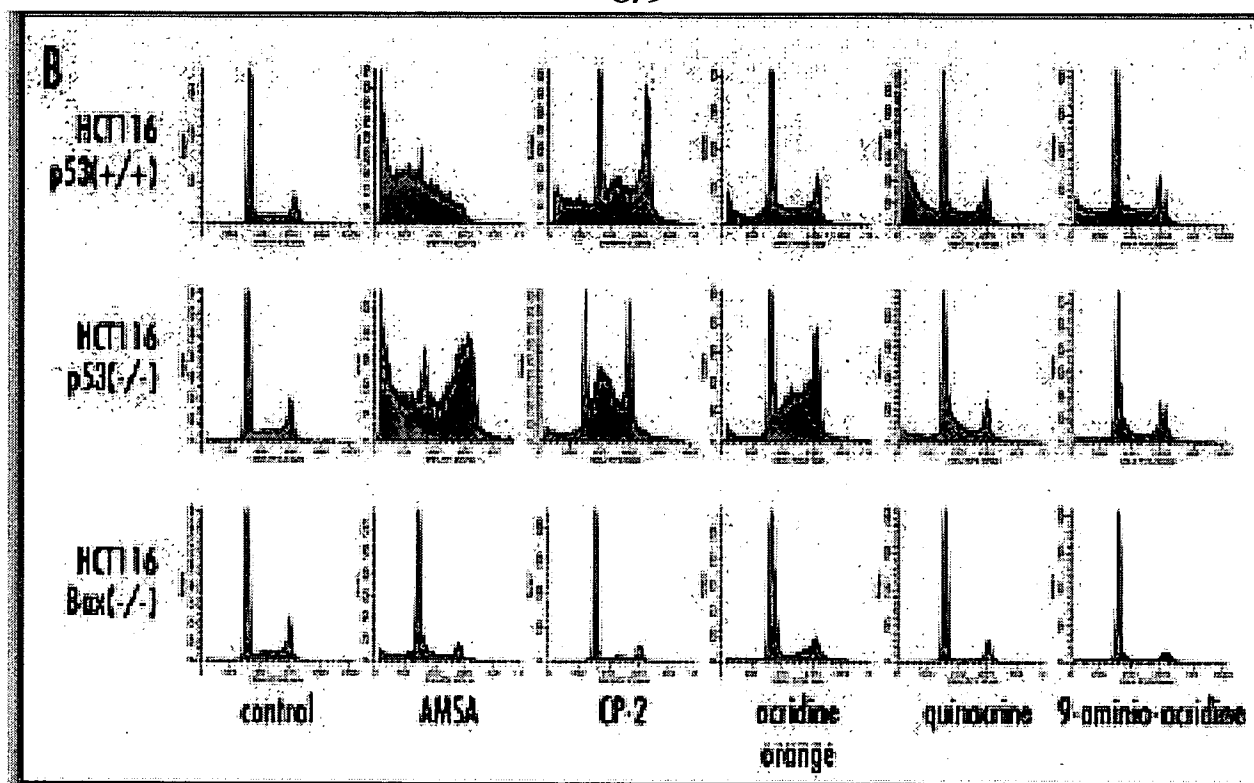


Figure 6b

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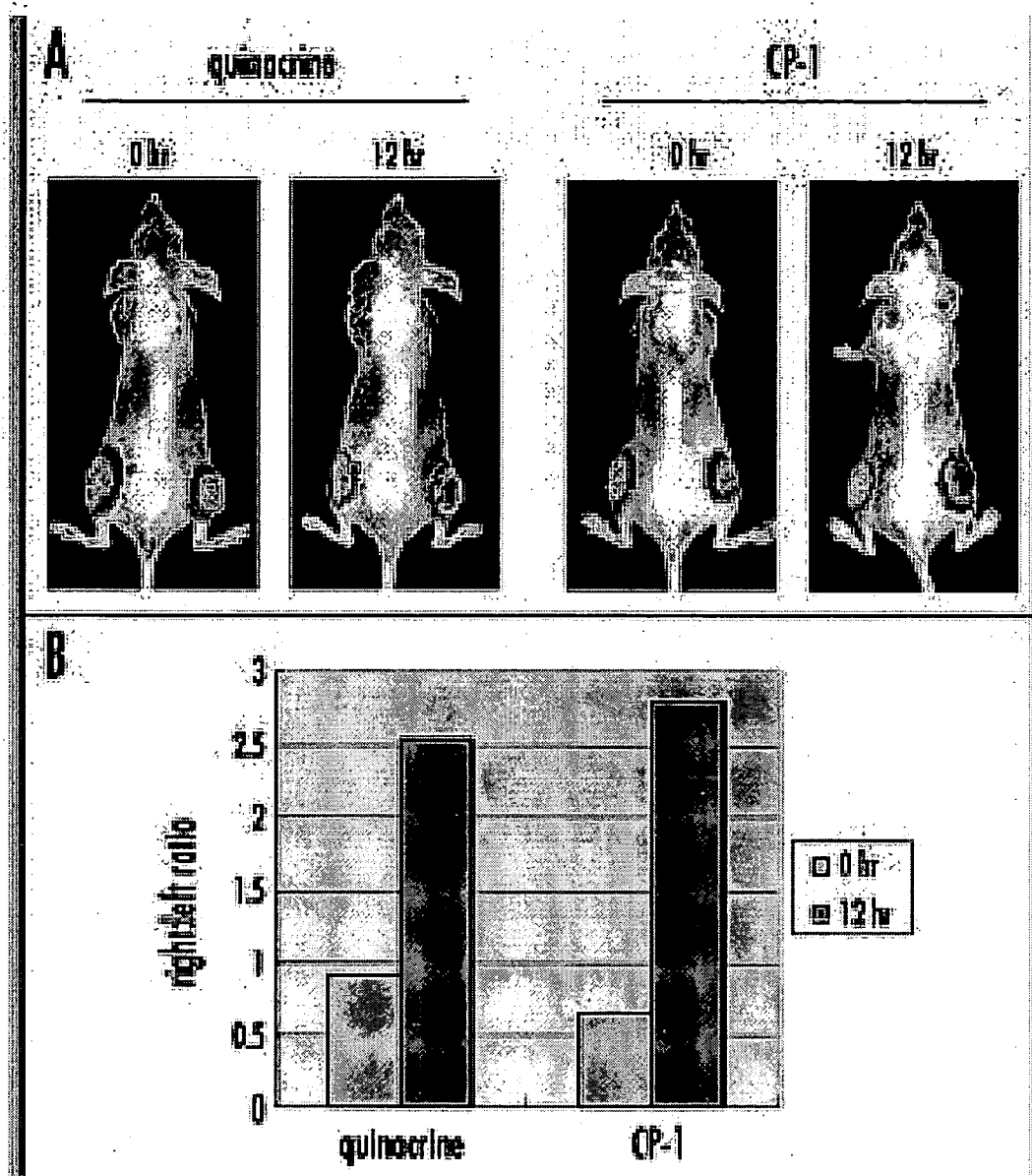


Figure 7