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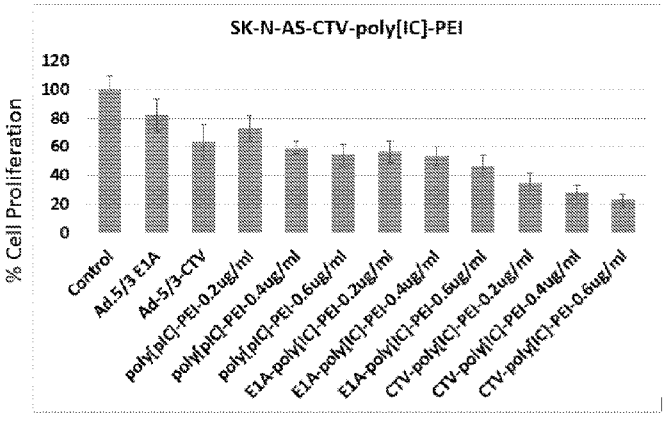
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(54) Title: COMBINATION THERAPY FOR NEUROBLASTOMA USING MDA-7/IL-24 WITH THERAPEUTIC AGENTS

FIGURE 17



(57) Abstract: This invention discloses methods of treating neuroblastoma using Ad.5/3-*CTV*. In one embodiment, the present invention provides a method of treating neuroblastoma in a subject using Ad.5/3-*CTV*. In another embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma tumor using Ad.5/3-*CTV*. In one embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma cell using Ad.5/3-*CTV*. In yet another embodiment, the present invention provides a method of inducing apoptosis or toxic autophagy in a neuroblastoma cell using Ad.5/3-*CTV*. In one embodiment, the present invention provides a method of maintaining remission of neuroblastoma in a subject which has been treated for neuroblastoma. In one embodiment, the methods described herein further comprise a step of administering to the subject or cell a therapeutic agent selected from the group consisting of poly[IC]-PEI, doxorubicin, and any other therapeutic agents that promote toxic autophagy and/or apoptosis.

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INTERNATIONAL PATENT APPLICATION UNDER THE PATENT COOPERATION TREATY

To all whom it may concern:

Be it known that Paul B. FISHER, Praveen BHOOPATHI, Luni EMDAD, Swadesh K. DAS and Devanand SARKAR

have invented certain new and useful improvements in

COMBINATION THERAPY FOR NEUROBLASTOMA USING MDA-7/IL-24 WITH THERAPEUTIC AGENTS

of which the following is a full, clear and exact description.

COMBINATION THERAPY FOR NEUROBLASTOMA USING MDA-7/IL-24 WITH THERAPEUTIC AGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Serial No. 62/315,126, filed March 30, 2016, the entire content of which is incorporated herein by reference into this application. This application also cites various publications, the entire contents of which are incorporated herein by reference into this application.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made at least in part with government support under R01 CA097318 and P30 CA016059 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] This invention relates to a method of treating neuroblastoma using Ad.5/3-*CTV*, in combination with other therapeutic agents that promote toxic autophagy and/or apoptosis. In one embodiment, the therapeutic agent is poly[IC]-PEI, doxorubicin, or any other agents that promote toxic autophagy and/or apoptosis. In one embodiment, the present invention provides a method which inhibits the growth of a neuroblastoma cell, inhibits the growth of a neuroblastoma tumor, and/or induces apoptosis and/or toxic autophagy in a neuroblastoma cell. In another embodiment, the present invention provides a method of maintaining remission of neuroblastoma in a subject after treatment.

BACKGROUND OF THE INVENTION

[0004] Neuroblastoma is the most frequent extracranial solid tumor in children under five years of age, affecting 1 in 7000 children. It is speculated that these tumors develop as a result of rapid proliferation of neuroblasts during fetal growth (1). Neuroblastoma arises along the sympathetic nervous system and adrenal medulla (2) from neural crest cells of sympathetic origin. They represent heterogeneous masses, both clinically and biologically. When occurring in infants, neuroblastoma may relapse unpredictably in a majority of cases, while in older patients these tumors frequently persist as benign ganglioneuromas (2). At the time of diagnosis, approximately half of the patients are assumed to be high-risk due to distant metastasis. Neuroblastoma can be classified into four stages. In stage I and II, disease is confined to the primary lesion. Stage III

and IV are characterized by disease outside the primary lesion. Once neuroblastoma attains advanced stage (III or IV), it expands persistently even when subjected to rigorous multimodal therapies (1, 3). Presentation of tumors at advanced stages combined with the absence of surgical options culminates in very poor patient prognosis. Although some improvements in the overall cure rate of neuroblastomas have been realized using intensive multimodality therapies these therapies promote significant short- and long-term toxicities (1, 3, 4). Only about 2% of neuroblastoma patients with stage III or IV remain disease free with relapse occurring shortly after completing chemotherapy, indicating a negligible effect of these agents long-term (5). Therapy failures are believed to be a consequence of development of resistance, underscoring the need for less toxic and more efficient therapeutic strategies (6). Accordingly, a comprehensive knowledge of mechanisms governing proliferation, differentiation, and cell death may expand the understanding of the molecular pathogenesis of neuroblastoma, which may result in novel biologically-based therapies diminishing toxicity and maximizing efficacy.

[0005] Melanoma- differentiation-associated Gene 7/interleukin-24 (*mda-7/IL-24*) is a unique member of the IL-10-related cytokine gene family (7) displaying wide spectrum anti-tumor activity in diverse cancers without harming normal cells or tissues (8, 9). *mda-7/IL-24* was initially cloned using subtraction hybridization combined with induction of cancer cell terminal differentiation (10). Forced expression of *mda-7/IL-24* in cancer cells promotes direct cancer toxicity through induction of apoptosis or toxic autophagy (11) and indirect antitumor effects through inhibition of angiogenesis (8, 12), promoting antitumor immune responses (8), sensitization of cancer cells to radiation- and chemotherapy-induced killing (13), and by promoting potent 'antitumor bystander activity' through autocrine/paracrine secretion (14). *mda-7/IL-24* displays nearly universal antitumor properties *in vitro* and *in vivo* in almost every cancer context (15, 16), which led to successful entry into clinical trials (17, 18). These properties of *mda*-7/IL-24 make it a potential candidate gene for the treatment of neuroblastoma, where adenovirus administration in a single human neuroblastoma cell line (SH- SY5Y) inhibited both *in vitro* and *in vivo* xenograft growth (19). To enhance the utility of *mda- 7/IL-24* for gene therapy of cancer, a conditionally replication-competent Ad carrying *mda-7/IL-24* (20) is employed. In this *Cancer Terminator Virus* (*CTV*) (21) adenoviral replication is controlled by the promoter of a cancer-selective rodent gene, progression elevated gene-3 (*PEG-3*) (22). To enhance even further the utility of the *CTV*, the adenovirus has been engineered to more effectively infect cancer cells, creating tropism modified chimeric *CTVs* (23).

[0006] Adenoviruses (Ads) use CAR (Coxsackie-Adenovirus Receptors) to infect normal and cancer cells, however cancer cells express varying levels of CAR on their cell surface. To improve the low efficiency of Ad infection of tumor cells, "tropism modification" approaches

have been developed (23). One such vector Ad.5/3 displayed equal efficacy when compared with wild type Ad.5, thereby providing an expanded range of utility for Ad.5/3, in both low and high CAR expressing cells (23, 24). For that reason, a modified Ad.5/3-*CTV* (Ad.5/3-*PEG*-E1A-*mda-7)* was used to evaluate therapeutic applications in human neuroblastoma cells.

[0007] A previously unrecognized pathway involved in *mda-7/IL-24*-mediated induction of caspase-independent apoptosis induction in neuroblastoma cells was recently described.

[0008] This pathway involves modulation of Apoptosis-inducing factor (AIF) expression and translocation into the nucleus of neuroblastoma cells that is mediated through induction of Ataxia telangiectasia mutated (ATM) followed by phosphorylation and nuclear translocation of histone γ-H2AX into the nucleus. These findings provide new insights into the mechanism of action of a near ubiquitous cancer-suppressing gene supporting its' applications for potential therapy of neuroblastoma.

[0009] This invention demonstrated a new cell death pathway in neuroblastoma which is triggered by *mda-7/IL-24* through ATM-mediated activation of H2AX and AIF resulting in caspase-independent apoptosis. This pathway is unique to neuroblastoma cells, since this effect was not evident in human breast carcinoma or melanoma cells.

[0010] To further enhance the therapeutic potential of Ad.5/3-*CTV* on neuroblastoma, this invention further developed a combination therapy for neuroblastoma using *mda-7/IL-24* and other therapeutic agents including poly[IC]-PEI, and doxorubicin. Polyinosine–polycytidylic acid, Poly[IC] is a synthetic dsRNA directly activating dendritic cells and triggering natural killer (NK) cells to kill tumor cells, which establishes its immuno-modulatory function (59, 60). It is commonly considered an anti-viral agent, which has shown anti-viral activity through mimicking the effect of authentic viral RNA. The anti-viral role is primarily attributed to the induction of type I IFNs and downstream stimulated genes (60). Poly[IC] has been used for more than four decades as a synthetic dsRNA mimic to boost the immune system in an IFN-dependent manner (51). Naked poly[IC] was shown to induce cell death in neuroblastoma when used at a very high concentration (52, 53). Unfortunately, clinical trials with naked poly[IC] showed poor poly[IC] stability and interferon (IFN) induction, and no detectable antitumor effect (51).

[0011] How poly(IC) is delivered is critical in defining its function. Cytoplasmic delivery of poly[IC] as a complex with Polyethylenimine (PEI), poly[(IC)-PEI, has profound effects on cancer cell growth, inducing apoptosis and toxic autophagy, and promoting potent immune modulating activities (61-63). It was found that, when naked Poly[IC] was combined with polyethylenimine (PEI), which allows cytoplasmic delivery of poly[IC], the combination poly[IC]-PEI induced more effective therapeutic responses than naked poly[IC] (51, 50). The molecular mechanisms for induction of apoptosis and toxic autophagy as a final end-point phenotype occur by both common

and different pathways in specific cancer indications. However, in different cancers depending on the delivery, poly[IC]-PEI promotes different sets of gene expression changes to induce the endpoint phenotype of apoptosis and toxic autophagy. For example, in melanoma, poly[IC]-PEI is involved in the recruitment of ATG-5, inducing MDA-5 which is linked to induction of toxic autophagy (61, 62). In breast cancer, poly[IC]-PEI promotes induction of apoptosis through the regulation of MDA-5 (62). In pancreatic cancer, poly[IC]-PEI represses XIAP and survivin expression and activates an immune response by inducing MDA-5, RIG-I, and NOXA (63). Considering the above studies as examples, unique molecular mechanisms of apoptosis and toxic autophagy induction have been established in different cancer types. Indeed, there are some overlapping mechanisms associated with poly[IC]-PEI-mediated anti-cancer effects, at least in some contexts the apoptotic/toxic autophagy effects partially depend on MDA-5 induction.

[0012] Cisplatin and doxorubicin are routinely used in neuroblastoma patients as part of a combination therapeutic regime (54-56). Doxorubicin induces different apoptotic pathways that can include AIF in specific cancer cells, but not MDA-5.

[0013] MDA-7/IL-24 is a unique gene that displays cancer-selective apoptosis inducing capabilities in a wide spectrum of tumors (64). Intracellular delivery of MDA-7/IL-24 is achievable through multiple approaches including viral-mediated gene delivery, administration of a recombinant protein or direct introduction of an expression plasmid. Viral-mediated *mda-7/IL-24* gene expression results in robust expression which elicits an autocrine/paracrine loop and regulates its own transcription and translation. A wide variety of signaling pathways are activated by MDA-7/IL-24 that facilitate the induction of apoptosis (as well as toxic autophagy) (64). These include stress protein expression, induction of reactive oxygen species, switching protective to toxic autophagy, etc. There are currently no reports suggesting the direct induction of MDA-5 by MDA-7/IL-24 to induce apoptosis. Also, there are no reports available that make any *a priori* predictions of induction of apoptosis by MDA-7/IL-24 through direct induction of NOXA, XIAP. In Neuroblastoma, MDA-7/IL-24-mediated apoptosis is unique, at least in the context of our present understanding of how this cytokine promotes apoptosis/toxic autophagy in diverse cancer cells, by inducing AIF, ATM and γ-H2AX (67).

[0014] Synergy occurs when a greater than anticipated additive effect is observed when agents are used in combination (this can be measured using the approach described by Chou and Talalay method which was used in our studies (57). This can occur when two molecules operate through overlapping molecular mechanisms, which at times can be distinct but show amplified effects when used in combination. A synergistic effect can be observed when one molecule sensitizes the signaling pathway, which is being used by another molecule. Thus, theoretically a synergistic effect between two molecules may occur when both molecules ***a)*** display similar modes of action

(at a molecular level), ***b)*** function through overlapping pathways, or ***c)*** promote two distinct pathways, but one pathway sensitizes the action of the other compound. Based on the literature, both MDA-7/IL-24 and poly[IC]-PEI display two distinct pathways in mediating apoptosis or toxic autophagy in different cancer cells. Accordingly, a synergistic effect of MDA-7/IL-24 and poly(IC)-PEI in neuroblastoma could not have been predicted *a priori*. Based on the proposed modes of action, we assumed a potential additive effect, but rather observed a synergistic effect in the context of Ad.5/3-*CTV* (encoding mda-7/IL-24) and poly[IC]-PEI.

[0015] Furthermore, a synergistic effect of combining MDA-7/IL-24 with a second compound is not a general phenomenon. This is evident from the present experiments in neuroblastoma, where three compounds were tested in combination with MDA-7IL-24. MDA-7/IL-24 only synergized with poly[IC]-PEI and doxorubicin, but not with cisplatin. The mathematical model (using the Chou and Talalay method) also indicated that the synergistic effect varied between the two compounds tested, i.e., poly[IC]-PEI and doxorubicin. Poly[IC]-PEI was the most potent synergistic molecule with MDA-7/IL-24 so far tested. Thus, overall the invention is not only novel but also important in terms of displaying enhanced anti-neuroblastoma activity, which can in principle be used to maximize clinical outcomes for treating patients with neuroblastoma.

[0016] In summary, this invention provides a selective novel approach to target neuroblastoma cells for growth suppression and apoptosis by means of a cancer-selective conditionally replication competent toxic adenovirus expressing *mda-7/IL-24*. This invention further provides a novel combination therapy for neuroblastoma using *mda-7/IL-24* and other therapeutic agents that promote toxic autophagy and/or apoptosis, including poly[IC]-PEI, and doxorubicin.

SUMMARY OF THE INVENTION

[0017] This invention discloses methods of treating neuroblastoma using Ad.5/3-*CTV* in combination with other therapeutic agents that promote toxic autophagy and/or apoptosis.

[0018] In one embodiment, the present invention provides a method of treating neuroblastoma in a subject using Ad.5/3-*CTV*. In another embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma tumor using Ad.5/3-*CTV*.

[0019] In one embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma cell using Ad.5/3-*CTV*. In yet another embodiment, the present invention provides a method of inducing apoptosis in a neuroblastoma cell using Ad.5/3-*CTV*. In yet another embodiment, the present invention provides a method of inducing toxic autophagy in a cancer cell using Ad.5/3-*CTV*.

[0020] In one embodiment, the present invention provides a method of maintaining remission of neuroblastoma in a subject that has been treated for neuroblastoma.

[0021] In one embodiment, the methods described herein further comprise a step of administering to the subject or cell a therapeutic agent selected from the group consisting of poly[IC]-PEI, and doxorubicin.

BRIEF DESCRIPTION OF THE FIGURES

[0022] Figures 1A and 1B show inhibition of neuroblastoma cell growth by Ad.5/3-*CTV*. In **Figure 1A**, Neuroblastoma cells were infected with either Ad.5/3-Null (25 pfu), Ad.5/3-*E1A* (25 pfu) or Ad.5/3-*CTV* (12.5 or 25 pfu) for 72 hours and cell lysates were evaluated by western blotting for E1A and MDA-7/IL-24 protein using specific antibodies. In **Figure 1B**, Neuroblastoma cells were plated in 96-well plates in quadruplicate and infected with virus as above for the indicated times. Cell growth was measured using MTT assay and shown as relative proliferation rate compared with control cells. *, $p<0.01$ versus control.

[0023] Figures 2A-2C show induction of apoptosis in neuroblastoma cells by Ad.5/3-*CTV*. In **Figure 2A**, neuroblastoma cells were cultured in 8-well chamber slide and treated with 25 pfu of Ad.5/3-Null or Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 72 hours. Cells were fixed and TUNEL assays were performed. Data presented as TUNEL positive cells in a defined microscopic field as compared with un-treated control cells. In **Figure 2B**, neuroblastoma cells were infected as above for 72 hours and were collected and subjected to FACS analysis with propidium iodide staining for DNA content and data presented in a graphical manner from three independent experiments. Columns: mean of triplicate experiments. Bars: S.D., *, $p<0.001$ versus control. In **Figure 2C**, neuroblastoma cells were treated as described above for 72 hours. Cells were collected and western blot analysis was performed for PARP using specific antibody and β-Actin served as loading control.

[0024] Figures 3A-3C show induction of caspase-independent cell death in neuroblastoma cells by Ad.5/3-*CTV*. In **Figure 3A**, neuroblastoma cells were infected with 25 pfu of Ad.5/3-Null or Ad.5/3-*E1A* or with the indicated dose of Ad.5/3-*CTV* for 72 hours. Cells were collected and western blot analysis was performed for caspase-3 and caspase-9 using specific antibodies and □-Actin served as loading control. Staurosporine served as a positive control for caspase 3 activation. In **Figure 3B**, neuroblastoma cells were treated as above for 72 hours, collected and caspase-3 activation assays were performed according to the manufacturer's protocol. Staurosporine served as a positive control. Results represent three independent experiments displayed in a graphical manner. Columns: mean of triplicate experiments; bars, S.D. (C) Neuroblastoma cells were pre-treated with 20 μM Z-VAD-FMK and were infected as above for 72 hours. Cells were collected and western blotting analysis was performed for PARP using

specific antibody and β-Actin served as loading control. Results are representative of three independent experiments.

[0025] Figures 4A-4D show promotion of AIF-mediated cell death in neuroblastoma cells by Ad.5/3-*CTV*. In **Figure 4A**, neuroblastoma cells were infected with 25 pfu of Ad.5/3-Null or Ad.5/3-*E1A* or with the indicated dose of Ad.5/3-*CTV* for 72 hours. Cells were collected and western blotting analysis was performed for AIF using specific antibodies and β-Actin served as loading control. Results are representative of three independent experiments. In **Figure 4B**, neuroblastoma cells were cultured in 8-well chamber slide and treated as described as above in **Figure 4A** for 72 hours. These cells were then subjected to immunofluorescence analysis of AIF using anti-AIF antibody and Alexa Flour-594 secondary antibody (red fluorescence). Nuclei were stained with DAPI (blue fluorescence). Fluorescent cells were visualized and photographed from 10 different fields and representative images are shown. In **Figure 4C,** subcellular distribution of AIF was determined using western blot analysis. Neuroblastoma cells were infected with 25 pfu of Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 72 hours. The cytoplasmic and nuclear fractions were isolated and examined by western blotting for AIF using specific antibodies. HDAC3 served as loading control for nuclear extract and β-Actin served as loading control for cytoplasmic extracts. In **Figure 4D,** neuroblastoma cells were pre-treated with AIF inhibitor and infected with 25 pfu of Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 48 hours. Cells were collected and western blotting analysis was performed for AIF and PARP using specific antibodies and β-Actin served as loading control. Results are representative of three independent experiments.

[0026] Figures 5A-5E show that Ad.5/3-*CTV*-induced AIF-mediated cell death requires ATM and γ-H2AX phosphorylation. Neuroblastoma cells were infected with 25 pfu of Ad.5/3-Null or Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 72 hours. In **Figure 5A,** cells were collected and western blotting was performed for γ-H2AX and H2AX using specific antibodies and β-Actin served as loading control. In **Figure 5B,** Western blotting was performed for determining pATM and ATM protein levels using specific antibodies and β-Actin served as loading control. In **Figure 5C,** neuroblastoma cells were untreated or treated overnight with KU-60019 (3 μM) and infected with 25 pfu Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 48 hours. Cells were collected and western blotting was performed for MDA-7/IL-24, pATM, γ-H2AX, AIF and PARP using specific antibodies and β-Actin served as loading control. Results are representative of three independent experiments. In **Figure 5D,** neuroblastoma cells were pre-treated with AIF inhibitor and infected with 25 pfu of Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 48 hours. Cells were collected and western blotting analysis was performed for pATM using specific antibodies and β-Actin served as loading control. Results are

representative of three independent experiments. In **Figure 5E,** neuroblastoma cells were untreated or treated over night with KU-60019 (3 μM) and infected with 25 pfu Ad.5/3-Null or Ad.5/3-*E1A* or the indicated dose of Ad.5/3-*CTV* for 48 hours. Cells were fixed and TUNEL assays were performed. TUNEL positive cells were counted and data presented as TUNEL positive cells per microscopic field in a graphical manner, columns, average of TUNEL positive cells per 5 different microscopic fields; bars, S.D. *, $p<0.001$ versus control; @, $p<0.01$ versus Ad.5/3-*CTV* alone at corresponding doses.

[0027] Figures 6A-6C shows that intratumoral injections of Ad.5/3-*CTV* induce AIF-mediated cell death and inhibit human neuroblastoma xenograft tumor growth. NB1691 human neuroblastoma cells were implanted subcutaneously in both flanks of nude mice and left-sided tumors were treated with 8 intratumoral injections including mock (solvent), Ad.5/3-*E1A* or Ad.5/3-*CTV* as described in Materials and Methods. A total of 6 animals were studied in each group. Once the control animals' tumors reached maximum allowable limit, tumors were collected fixed in formalin and embedded in paraffin. In **Figure 6A,** tumor volumes from the left and right flank were quantified and the results are presented in a graphical manner. Line represents average of all the tumor volumes of the group at the indicated time points: Bars, S.D. *, $p<0.05$ versus control; **, $p<0.001$ versus control. In **Figure 6B,** formalin fixed paraffin embedded tissue sections were stained for H&E and TUNEL as per standard protocol; representative images of the indicated treatment groups are shown. In **Figure 6C,** immunohistochemical analysis of MDA-7/IL-24, AIF, γ-H2AX and pATM from tumor sections as described in Materials and Methods. Representative sections shown.

[0028] Figure 7 shows the induction of AIF-mediated cell death in Ad.5/3-*CTV*-treated neuroblastoma cells by ATM and γ-H2AX phosphorylation. Schematic representation of Ad.5/3-*CTV*-induced cell death in neuroblastoma cells.

[0029] Figures 8A-8B show the expression of adenoviral receptors in neuroblastoma cells. In **Figure 8A,** neuroblastoma cells were cultured at 60-70% confluence and cells were collected and stained for CAR, CD46 and Desmoglein surface receptors using FACS analysis. The results are presented as percent positive cells from the total cell population. Columns represent 3 independent experiments and bars, S.D. In **Figure 8B,** cells were collected at 60-70% confluence and western blotting was performed for CAR, CD46 and Desmoglein using specific antibodies.

[0030] Figure 9 shows that Ad.5/3-*CTV* induces pro-apoptotic molecules and downregulates anti-apoptotic molecules. Neuroblastoma cells were infected with either Ad.5/3-Null (25 pfu). Ad.5/3-*E1A* (25 pfu) or Ad.5/3-*CTV* (12.5 or 25) for 72 hours, cells were collected and western blotting analysis was performed for pJNK, JNK, BCL-2, BAX, and BCL-xL using specific antibodies and β-Actin served as loading control. Results are representative of three independent experiments.

[0031] Figure 10 shows that Ad.5/3-*CTV* induces caspase-independent cell death in neuroblastoma cells. Neuroblastoma cells were infected with Ad.5/3-Null (50 pfu), Ad.5/3-*E1A* (50 pfu) or Ad.5/3-*CTV* (25 or 50 pfu) for 72 hours. Cell lysates were made and western blotting analysis was performed for caspase-8 using specific antibody. β-Actin served as the loading control.

[0032] Figure 11 shows that Ad.5/3-*CTV* enhances p53 levels in neuroblastoma cells. Neuroblastoma cells were infected with Ad.5/3-Null (25 pfu), Ad.5/3-*E1A* (25 pfu) or Ad.5/3-*CTV* (12.5, 25 or 50 pfu) for 72 hours. Cells were collected and western blotting was performed for p53 using specific antibodies and β-Actin served as loading control. Results are representative of three independent experiments.

[0033] Figure 12 shows the nuclear translocation of γ–H2AX in Ad.5/3-*CTV*-treated neuroblastoma cells. Neuroblastoma cells were cultured in 8-well chamber slides and treated with Ad.5/3-Null (25 pfu), Ad.5/3-*E1A* (25 pfu) or Ad.5/3-*CTV* (25 or 50 pfu) for 72 hours. These cells were then subjected to immunofluorescence analysis of γ–H2AX distribution using anti-γ–H2AX antibody and Alexa Fluor-594 secondary antibody (red fluorescence). Nuclei were stained with DAPI (blue fluorescence). Fluorescent cells were visualized and photographed from 10 different fields and a representative image is shown in this figure.

[0034] Figure 13 shows that Ad.5/3-*CTV* infection in neuroblastoma cells promotes nuclear translocation of pATM. Neuroblastoma cells were cultured in 8-well chamber slide and treated with Ad.5/3-Null (25 pfu), Ad.5/3-*E1A* (25 pfu) or Ad.5/3-*CTV* (25 or 50 pfu) for 72 hours. These cells were then subjected to immunofluorescence analysis of pATM distribution using anti-pATM antibody and Alexa Fluor-594 secondary antibody (red fluorescence). Nuclei were stained with DAPI (blue fluorescence). Fluorescent cells were visualized and photographed from 10 different fields and a representative image is shown in this figure.

[0035] Figure 14 shows that ATM inhibitor rescues neuroblastoma cells from Ad.5/3-*CTV*-induced cell death. Neuroblastoma cells were untreated or treated over night with either KU-55933 (5 μM) and infected with Ad.5/3-Null (25 pfu), Ad.5/3-*E1A* (25 pfu) or Ad.5/3-*CTV* (25 or 50 pfu) for 48 hours, cells were fixed and TUNEL assays were performed. TUNEL positive cells were counted per microscopic field and data presented as TUNEL positive cells in a graphical manner, columns, average of TUNEL positive cells per 5 different microscopic fields; bars, S.D. *, $p<0.001$ versus control; @, $p<0.01$ versus Ad.5/3-*CTV* alone at corresponding doses.

[0036] Figure 15 shows the effect of Ad.5/3-*CTV* on MDA-7/IL-24 and AIF expression in breast cancer and melanoma cells. Breast cancer (MDA-MB-231 and ZR-751) or melanoma (C8161 and SK-Mel) cells were either untreated (Control) or infected with Ad.5/3-*E1A* (25 pfu) or Ad.5/3-

CTV (12.5 or 25 pfu) for 72 hours and cell lysates were assessed for MDA-7/IL-24 or Akt expression by western blotting analysis. β-Actin served as loading control.

[0037] Figure 16 shows absorbance determined in MTT assay under different treatments. SK-N-AS cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of poly[IC]-PEI for further 24 hours. MTT assay was then performed according to the standard protocol.

[0038] Figure 17 shows % cell proliferation under different treatments. SK-N-AS cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of poly[IC]-PEI for further 24 hours. MTT assay was then performed according to the standard protocol. Control values were adjusted to 100% and calculated other values accordingly.

[0039] Figure 18 shows absorbance determined in MTT assay under different treatments. SK-N-SH cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of poly[IC]-PEI for further 24 hours. MTT assay was then performed according to the standard protocol.

[0040] Figure 19 shows % cell proliferation under different treatments. SK-N-SH cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of poly[IC]-PEI for further 24 hours. MTT assay was then performed according to the standard protocol. Control values were adjusted to 100% and calculated other values accordingly.

[0041] Figure 20 shows the combination index for Ad.5/3 CTV-poly[IC]-PEI.

[0042] Figure 21 shows Western Blot analysis on Ad. 5/3 CTV-poly[IC]-PEI. About $2X10^6$ Neuroblastoma cells (SK-N-AS or SK-N-SH) cells were cultured for 24 hours. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for

24 hours and then treated poly[IC]-PEI (0.5ug/ml) for further 24 hours. Cells were then collected, lysed for protein isolation and used for western blot analysis.

[0043] Figure 22 shows absorbance determined in MTT assay under different treatments. SK-N-AS cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of doxorubicin for further 24 hours. MTT assay was then performed according to the standard protocol.

[0044] Figure 23 shows % cell proliferation under different treatments. SK-N-AS cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Doxorubicin for further 24 hours. MTT assay was then performed according to the standard protocol. Control values were adjusted to 100% and calculated other values accordingly.

[0045] Figure 24 shows absorbance determined in MTT assay under different treatments. SK-N-SH cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Doxorubicin for further 24 hours. MTT assay was then performed according to the standard protocol.

[0046] Figure 25 shows % cell proliferation under different treatments. SK-N-SH cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Doxorubicin for further 24 hours. MTT assay was then performed according to the standard protocol. Control values were adjusted to 100% and calculated other values accordingly.

[0047] Figure 26 shows the combination index for Ad.5/3 CTV-Doxorubicin.

[0048] Figure 27 shows Western Blot analysis on Ad.5/3 CTV-Doxorubicin. About $2X10^6$ Neuroblastoma cells (SK-N-AS or SK-N-SH) were cultured for 24 hours. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for

24 hours and then treated with of doxorubicin (5uM) for further 24 hours. Cells were then collected, lysed for protein isolation and used for western blot analysis.

[0049] Figure 28 shows absorbance determined in MTT assay under different treatments. SK-N-AS cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Cisplatin for further 24 hours. MTT assay was then performed according to the standard protocol.

[0050] Figure 29 shows % cell proliferation under different treatments. SK-N-AS cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Cisplatin for further 24 hours. MTT assay was then performed according to the standard protocol. Control values were adjusted to 100% and calculated other values accordingly.

[0051] Figure 30 shows absorbance determined in MTT assay under different treatments. SK-N-SH cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Cisplatin for further 24 hours. MTT assay was then performed according to the standard protocol.

[0052] Figure 31 shows % cell proliferation under different treatments. SK-N-SH cells were cultured for 24 hours (with 60-70% confluency), cells were then trypsinized and about 5,000 cells were plated in each well of 96 well plate. 8 wells for each treatment were used for performing MTT assay. Once the cell are attached the cells were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with indicated doses of Cisplatin for further 24 hours. MTT assay was then performed according to the standard protocol. Control values were adjusted to 100% and calculated other values accordingly.

[0053] Figure 32 shows the combination index for Ad.5/3 CTV-Cisplatin.

[0054] Figure 33 shows Western Blot analysis on Ad.5/3 CTV-Cisplatin. About $2X10^6$ Neuroblastoma cells (SK-N-AS or SK-N-SH) were cultured for 24 hours. Once the cell are attached they were treated with Ad.5/3 E1A (12.5 IU) or Ad.5/3 CTV (12.5 IU) cultured for 24 hours and then treated with Cisplatin (7.5uM) for further 24 hours. Cells were then collected, lysed for protein isolation, and used for western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

[0055] This invention relates to methods of treating neuroblastoma using Ad.5/3-*CTV* in combination with other therapeutic agents that promote toxic autophagy and/or apoptosis.

[0056] Advanced stages of neuroblastoma, the most common extracranial malignant solid tumor of the central nervous system in infants and children, are refractive to therapy. Ectopic expression of melanoma differentiation associated gene-7/Interleukin-24 (*mda-7/IL-24*) promotes broad-spectrum antitumor activity *in vitro*, *in vivo* in pre-clinical animal models and in a Phase I clinical trial in patients with advanced cancers, including melanoma and various carcinomas, without harming normal cells. *mda-7/IL-24* exerts cancer-specific toxicity (apoptosis or toxic autophagy) by promoting ER stress and modulating multiple signal transduction pathways regulating cancer cell growth, invasion, metastasis, survival and angiogenesis. To enhance cancer-selective expression and targeted anti-cancer activity of *mda-7/IL-24*, a tropism-modified *Cancer Terminator Virus (*Ad.5/3-*CTV)* was created, which selectively replicates in cancer cells producing robust expression of *mda-7/IL-24.* It was found that Ad.5/3-*CTV* induces profound neuroblastoma anti-proliferative activity and apoptosis in a caspase 3/9-independent manner both *in vitro* and *in vivo* in a tumor xenograft model. Ad.5/3-*CTV* promotes these effects through a unique pathway involving apoptosis inducing factor (AIF) translocation into the nucleus. Inhibiting AIF rescued neuroblastoma cells from Ad.5/3-*CTV*-induced cell death, whereas pan-caspase inhibition failed to promote survival. Ad.5/3-*CTV* infection of neuroblastoma cells increased ATM phosphorylation instigating nuclear translocation and increased γ–H2AX, triggering nuclear translocation and intensified expression of AIF. These results were validated further using two ATM small molecule inhibitors that attenuated PARP cleavage by inhibiting γ–H2AX, which in turn inhibited AIF changes in Ad.5/3-*CTV*-infected neuroblastoma cells. Taken together, in this invention, a novel pathway for *mda-7/IL-24*-induced caspase-independent apoptosis in neuroblastoma cells mediated through modulation of AIF, ATM and γ-H2AX has been elucidated. Novel therapeutic methods for neuroblastoma using *mda-7/IL-24* are also developed.

[0057] Neuroblastoma is a heterogeneous clinical entity, ranging from subgroups that have a very favorable prognosis with a high probability of spontaneous regression to those that display a very poor prognosis despite aggressive therapies (1, 25). Considering this conundrum and the high incidence of recurrence in advanced stages of neuroblastoma (stage III and IV) (4, 6), defining appropriate strategies for treating this cancer particularly in advanced stages is a priority. Presently, a broad-spectrum anti-tumor protein MDA-7/IL-24 (11, 12, 16) delivered by a tropism-modified chimeric *cancer terminator virus* (Ad.5/3-*CTV*) (23, 43) was evaluated in neuroblastoma cells. Ad.5/3-*CTV* induced decreased neuroblastoma cell growth and increased

apoptosis *in vitro* and decreased tumor growth *in vivo*, supporting potential applications for the therapy of this cancer. Mechanistic studies uncovered a new pathway by which *mda-7/IL-24* can promote apoptosis in cancer cells, i.e., through induction and translocation of AIF into the nucleus.

[0058] *mda-7/IL-24* shows potent antitumor activity that is mediated through multiple pathways in diverse cancers (7, 16). Mechanisms of *mda-7/IL-24* toxicity include ER stress and tumoral cell apoptosis by suppression of anti-apoptotic Bcl-2 family members (26, 44), which was also evident in this study. Previously, MDA-7/IL-24 treatment has been shown to increase Reactive oxygen species (ROS) production in many cancer types (18, 27). It is well known that ROS generation is closely associated with early stages of apoptosis and mitochondrial dysfunction (28). Earlier reports suggest that ROS generation, together with Cyt C release from mitochondria, promote cell death (29, 30). This results in increased permeability of the outer mitochondrial membrane, decreasing transmembrane potential, and activation of AIF (45) eventually inducing caspase-independent apoptosis (31). Ad.5/3-*CTV*-delivered *mda-7/IL-24* to neuroblastoma cells increased the levels of AIF. The present results show, in agreement with previously published data, that AIF is translocated into the nucleus inducing caspase-independent cell death in *mda-7/IL-24* overexpressed neuroblastoma cells. This was further confirmed using AIF and pan-caspase inhibitors. Inhibition of AIF by small molecule inhibitor attenuated PARP cleavage, inhibiting cell death in neuroblastoma cells upon treatment with Ad.5/3-*CTV*. In addition, applying a pan-caspase inhibitor did not alter *mda-7/IL-24*-induced PARP cleavage; further validating caspase-independent cell death induced by this cytokine in neuroblastoma cells.

[0059] Cells respond to DNA damage by phosphorylating a variant of the H2A protein family, H2AX (32). H2AX assists chromatin to facilitate DNA repair by providing binding sites for downstream repair factors (33). AIF is a flavoprotein that is in the mitochondrial inter-membrane space and performs a major role in mediating caspase-independent cell death (34, 35). Upon receiving a cell death stimuli, AIF is cleaved within the mitochondria by calpains and cathepsins (36), released into the cytosol possibly through a mitochondrial permeability transition pore, and translocated into the nucleus where it induces chromatin condensation and DNA fragmentation through complex formation with H2AX and cyclophilin A (37). A variety of apoptotic stimuli have been documented to induce AIF mitochondria-to-nucleus translocation including DNA damaging agents, hypoxia/ischemia, oxidative stress and excitotoxins (such as glutamate) (38). However, the signaling pathways that cause AIF nuclear translocation have not been fully elucidated. In this regard, the effects of Ad.5/3-*CTV* on key apoptotic proteins, AIF and poly (ADP-ribose) polymerase-1 (PARP1), which constitute a relatively novel, yet crucial pathway of caspase-independent apoptosis in MDA-7/IL-24 treatment, was studied.

[0060] H2AX, a member of the histone H2A family is characterized by a phosphorylable SQE motif in its C-terminal tail (39, 45). It is also established that DNA fragmentation induces phosphorylation of H2AX histone at serine 139 (40). Even though H2AX is mainly associated with DNA-damage repair and DNA packaging, it is also a key regulator of programmed cell death (41, 45). To decipher the mechanism by which MDA-7/IL-24 leads to AIF nuclear translocation, the levels of γ-H2AX in Ad.5/3-*CTV* treated cells were assessed, and increased activation levels of H2AX were found. It is known that ATM is a primary kinase involved in the phosphorylation of H2AX and also that ATM is one of the earliest kinases to be activated in the cellular response to double-strand breaks. Activation of ATM, H2AX and AIF translocation following Ad.5/3-*CTV* treatment was found, and these cellular modifications were confirmed by pharmacological inhibition of ATM and AIF. Data obtained through these studies indicate that ATM activation is important in triggering H2AX phosphorylation and AIF activation leading to caspase-independent cell death in Ad.5/3-*CTV* treated neuroblastoma cells. In contrast to neuroblastoma cells, infection of human breast cancer (MDA-MB-231 and ZR-751) and melanoma (C8161 and SK-Mel) cells with Ad.5/3-*CTV* did not enhance AIF expression (Fig. 15). Precisely how ATM is activated in MDA-7/IL-24-mediated apoptosis in neuroblastoma cells is a key question requiring further experimentation. One recent study by Baritaud et al. revealed the significance of ATM and DNA-PK in regulating γ-H2AX in AIF-mediated caspase-independent necroptosis (45). In particular, they showed that ATM inhibition prevents the H2AX phosphorylation observed after MNNG addition and, subsequently, blocked AIF-mediated cell death. This is in agreement with the present observations that ATM small molecule inhibitors attenuated Ad.5/3-*CTV*-induced PARP cleavage and H2AX phosphorylation, and inhibited AIF changes in neuroblastoma cells (Fig. 5C). Conversely, an AIF small molecule inhibitor reduced Ad.5/3-*CTV*-induced ATM phosphorylation and cell death (Fig. 4D and 5D) in neuroblastoma cells. Taken together, the present results suggest that ATM and AIF are functionally related in a positive feedback loop in which they regulate each other in neuroblastoma cells following Ad.5/3-*CTV* infection (Fig. 7). Another recent study reported that treatment with pro-oxidant resulted in caspase-independent, AIF-dependent apoptosis in ATM-null primary CLL tumors (42). All the results suggest that ATM and AIF can work independently or together to induce caspase-independent cell death.

[0061] In conclusion, a new cell death pathway has been demonstrated for the first time, and it is triggered by *mda-7/IL-24* through ATM-mediated activation of H2AX and AIF resulting in caspase-independent apoptosis (Fig. 7) that appears unique to neuroblastoma cells, since this effect was not evident in human breast carcinoma or melanoma cells (Fig. 15). Support for this stems from three lines of experimental evidence: (1) inhibition of AIF using AIF-inhibitors

decreased MDA-7/IL-24-mediated apoptosis; (2) inhibition of caspases using a pan-caspase inhibitor did not block MDA-7/IL-24-induced cell death; and (3) inhibition of ATM altered the levels of AIF, resulting in inhibition of cell death in MDA-7/IL-24 overexpressing neuroblastoma cells. Accordingly, the use of Ad.5/3-*CTV*, which displays cancer-specific viral replication combined with robust production and secretion of MDA-7/IL-24, to selectively induce cytolysis in neuroblastoma cells may represent a potentially viable treatment option for this aggressive cancer.

[0062] This invention further provides a combination therapy using Ad.5/3-*CTV* and other therapeutic agents including poly[IC]-PEI, doxorubicin and any other therapeutic agents that promote toxic autophagy and/or apoptosis. This could include radiation, inducers of reactive oxygen species and specific DNA damaging chemotherapeutic agents. The results indicated that a combination treatment using Ad.5/3-*CTV* and poly[IC]-PEI induced synergistic growth inhibition in neuroblastoma cells (Fig. 16-20) and the growth inhibition is likely caused by apoptosis (Fig 21). There was no report on the combined use of the two agents at the time of the invention, and the synergy observed herein is significant and unexpected because Ad.5/3-*CTV* and poly[IC]-PEI involve distinct apoptotic pathways (Ad.5/3-*CTV* induces apoptosis through AIF and ATM in neuroblastoma cells, while poly[I:C] induces apoptosis through XIAP, MDA-5, NOXA and/or RIG-I in different cancer cells). For doxorubicin, the results indicated that a combination treatment using Ad.5/3-*CTV* and doxorubicin induced strong additive growth inhibition in neuroblastoma cells (Fig. 22-26) and the growth inhibition is likely caused by apoptosis (Fig 27). For cisplatin, it was observed that the combined use of Ad.5/3-*CTV* and cisplatin did not generate any observable synergistic or additive growth inhibition in neuroblastoma cells under the tested range of dosages (Fig. 28-33). Therefore, not all chemotherapeutic agents can provide synergistic effects, and to the best knowledge of inventors, this is of the first time that the combination and the treatment method therein are reported.

[0063] Poly(IC)-PEI allows cytoplasmic delivery of poly[IC]. The unique combination of Ad.5/3-CTV through expression of viral replication and expression of mda-7/IL-24 and the toxic autophagy and apoptosis inducing agent poly(IC)-PEI in the therapy of neuroblastoma. The mechanisms of action of these two agents are different and the combining agents that induce the respective pathways result in toxic autophagy and apoptosis in neuroblastoma. Although the combination of doxorubicin with *mda-7/IL-24* has been shown potentiate apoptosis induction in colon cancer and hepatocarcinomas, there is no report indicating that the combination of Ad.5/3-CTV-Doxorubicin promotes apoptosis in neuroblastoma.

[0064] The combination therapeutic approach introduced here may have significant clinical value as a primary treatment or as a secondary approach for treating children with neuroblastoma. Every

year about 700 children are diagnosed with neuroblastoma, which is considered one of the most common solid tumours of early childhood (usually occurring in babies or young children of 1-2 years old). The most effective therapeutic options include radiation, chemotherapy and surgery. However, despite these approaches, the five-year survival rate for high risk patients (or in Stage IV) is only 40-50%. Additionally, current approaches of treatment may have long-lasting negative effects including cardiovascular, slower growth, changes in intellectual functions with learning issues, and the potential of developing a second cancer such as leukaemia. Thus, the present combination therapy which requires a significantly lower level of drugs will be beneficial for long-term treatment. The combination therapy is also useful in achieving long term remission of neuroblastoma. For example, a subject having neuroblastoma can be initially treated with a combination of Ad.5/3-*CTV* and poly[IC]-PEI, followed by poly[IC]-PEI alone for maintenance of remission. Furthermore, since drug resistance is not anticipated, patients could receive the combination therapy more than once if necessary.

[0065] In one embodiment, the present invention provides a method of treating neuroblastoma in a subject, the method comprising a step of administering to the subject an effective amount of a nucleic acid Ad.5/3-*CTV* comprising an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24. In one embodiment, the therapeutic agent is selected from the group consisting of poly[IC]-PEI, and doxorubicin.

[0066] In one embodiment, Ad.5/3-*CTV* of the present invention is a nucleic acid molecule containing an adenovirus vector Ad.5/3 and the gene of *mda-7/IL-24* as described in Dash (20). In another embodiment, Ad.5/3-*CTV* of the present invention is a nucleic acid molecule containing an adenovirus vector Ad.5/3 and the gene of *mda-7/IL-24* as described in WO2014093270. In another embodiment, Ad.5/3-*CTV* of the present invention comprises one or more of the sequences of SEQ ID NO.1-3.

[0067] In one embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma tumor in a subject, the method comprising a step of administering to the subject an effective amount of Ad.5/3-*CTV* comprising an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24.

[0068] In one embodiment, the present invention provides a method of inducing apoptosis or toxic autophagy in a neuroblastoma cell, the method comprising a step of administering to the cell, or contacting the cell with, an effective amount of Ad.5/3-*CTV* comprising an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24. In one embodiment, the present invention provides a method of inducing apoptosis and toxic autophagy in a cancer cell, the method comprising a step of administering to the cell, or contacting the cell with, an effective amount of Ad.5/3-*CTV* comprising an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24.

[0069] In one embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma cell, the method comprising a step of administering to the cell, or contacting the cell with, an effective amount of Ad.5/3-*CTV* comprising an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24.

[0070] In one embodiment of the present invention, the expression of MDA-7/IL-24 induces apoptosis in a neuroblastoma cell through the modulation of AIF, ATM and γ-H2AX.

[0071] In one embodiment, methods described herein further comprise a step of administering an effective amount of a molecule selected from the group consisting of poly[IC]-PEI, and doxorubicin.

[0072] In one embodiment, the present invention provides a method of treating neuroblastoma in a subject, the method comprising a step of administering to the subject an effective amount of Ad.5/3-*CTV* and a molecule selected from the group consisting of poly[IC]-PEI and doxorubicin.

[0073] In one embodiment, the present invention provides a method of inhibiting the growth of a neuroblastoma cell, the method comprising a step of administering to the cell, or contacting the cell with, an effective amount of Ad.5/3-*CTV* and a molecule selected from the group consisting of poly[IC]-PEI, and doxorubicin. In another embodiment, the inhibition of the growth of the neuroblastoma cell is significantly higher than the inhibition resulting from treatment using either Ad.5/3-*CTV* or the molecule alone.

[0074] In one embodiment, the present invention provides a method of maintaining remission of neuroblastoma in a subject that has been treated with Ad.5/3-*CTV* and poly[IC]-PEI, or with Ad.5/3-*CTV* and poly[IC]-PEI, the method comprising a step of administering an effective amount of poly[IC]-PEI or doxorubicin to the subject to maintain remission of neuroblastoma.

[0075] In one embodiment, the method described herein is used as a primary treatment or a secondary treatment for treating a subject with neuroblastoma.

[0076] In one embodiment, the concentration of poly[IC]-PEI to be administered ranges from 0.05 μg/ml to 5 μg/ml. In another embodiment, the concentration of poly[IC]-PEI ranges from 0.2 to 2.5 μg/ml. In yet another embodiment, the concentration of poly[IC]-PEI ranges from 1 ng/ml to 100 μg/ml. In one embodiment, the amount of poly[IC]-PEI ranges from 1 ng to 100 μg per dose. In yet another embodiment, the dose of poly[IC]-PEI ranges from 1 ng to 100 μg per kilogram of body weight.

[0077] In one embodiment, the concentration of doxorubicin to be administered ranges from 1 to 20 μM. In another embodiment, the concentration of doxorubicin to be administered ranges from 5 to 10 μM. In yet another embodiment, the dose of doxorubicin ranges from 0.1 mg to 20 mg per kilogram of body weight. In yet another embodiment, the amount of doxorubicin ranges from 1 mg to 500 mg per dose.

[0078] In one embodiment, Ad.5/3-*CTV* is administered at the same time as poly[IC]-PEI, or doxorubicin. In another embodiment, poly[IC]-PEI, or doxorubicin is administered 12-96 hours after the administration of Ad.5/3-*CTV*. In another embodiment, poly[IC]-PEI, or doxorubicin is administered 24-96 hours after the administration of Ad.5/3-*CTV*.

[0079] In one embodiment of the method described herein, the dose of Ad.5/3-*CTV* ranges from 1 plaque-forming unit (pfu) to 500 pfu. In another embodiment, the dose of Ad.5/3-*CTV* ranges from 6.25 pfu to 50 pfu. In another embodiment, the dose of Ad.5/3-*CTV* is 5, 10, 25, 30, 40, 50 or 75 pfu. In yet another embodiment of the method described herein, the amount of Ad.5/3-*CTV* ranges from $1x10^2$ to $1x10^{15}$ pfu per ml or per dose. In yet another embodiment, the amount of Ad.5/3-*CTV* ranges from $1x10^4$ to $1x10^6$ pfu per ml or per dose. In one embodiment, the dose of Ad.5/3-*CTV* ranges from $1x10^6$ to $1x10^{10}$ pfu per kilogram of body weight. In yet another embodiment, the dose of Ad.5/3-*CTV* is lower than the LD_{50} (minimum dose of Ad.5/3-*CTV* that kills 50% of the subjects).

[0080] In one embodiment of the method described herein, the dose of Ad.5/3-*CTV* ranges from 0.1 international unit (IU) to 100 IU. In another embodiment, the dose Ad.5/3-*CTV* ranges from 2.5 IU to 50 IU. In another embodiment, the dose of Ad.5/3-*CTV* is 5, 7.5, 10, 12.5 or 25 IU. In yet another embodiment of the method described herein, the amount of Ad.5/3-*CTV* ranges from 1 IU to 100 IU per dose. In yet another embodiment, the dose of Ad.5/3-*CTV* ranges from 0.1 IU to 1.5 IU per kilogram of body weight. In yet another embodiment, the dose of Ad.5/3-*CTV* is lower than the LD_{50} (minimum dose of Ad.5/3-*CTV* that kills 50% of the subjects).

[0081] In one embodiment of the method described herein, the neuroblastoma cell is selected from the group consisting of SK-N-AS, SK-N-SH and NB1691.

[0082] In one embodiment, the present invention provides a combination of Ad.5/3-*CTV* and an agent for use in a method of treating cancer in a subject, wherein said Ad.5/3-*CTV* and agent produce a synergy in inducing cancer cell growth suppression, toxic autophagy and apoptosis. In one embodiment, said Ad.5/3-*CTV* comprises an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24. In yet another embodiment, said agent is selected from the group consisting of poly[IC]-PEI, doxorubicin, and other agents capable of promoting toxic autophagy and/or apoptosis.

[0083] In one embodiment of the combination, the expression of MDA-7/IL-24 induces apoptosis in the cancer cells through the modulation of AIF, ATM and γ-H2AX.

[0084] In one embodiment of the combination, Ad.5/3-*CTV* and the agent are to be administered at the same time. In yet another embodiment, the agent is to be administered 12-96 hours after the administration of Ad.5/3-*CTV*.

[0085] In one embodiment of the combination, the cancer is neuroblastoma. In yet another embodiment, the cancer is melanoma, glioblastoma, prostate cancer, breast cancer, colorectal cancer, lung cancer or pancreatic cancer.

[0086] In one embodiment of the combination, the agent is poly[IC]-PEI. In yet another embodiment, the agent is doxorubicin.

[0087] In one embodiment of the combination, the agent induces one or more of MDA-5, NOXA and RIG-I.

[0088] In one embodiment, the present invention provides a method of treating cancer in a subject, comprising administering to the subject an effective amount of Ad.5/3-*CTV* and an agent, wherein said Ad.5/3-*CTV* and agent produce a synergy in inducing cancer cell growth suppression and apoptosis. In one embodiment, the Ad.5/3-*CTV* comprises an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24. In yet another embodiment, the agent is selected from the group consisting of poly[IC]-PEI, doxorubicin, and other agents capable of promoting toxic autophagy and/or apoptosis. In one embodiment of the method, the expression of MDA-7/IL-24 induces apoptosis in the cancer cells through the modulation of AIF, ATM and γ-H2AX.

[0089] In one embodiment of the method, Ad.5/3-*CTV* and agent are administered at the same time. In yet another embodiment, the agent is administered 12-96 hours after the administration of Ad.5/3-*CTV*.

[0090] In one embodiment of the method, the cancer is neuroblastoma. In yet another embodiment, the cancer is melanoma, glioblastoma, prostate cancer, breast cancer, colorectal cancer, lung cancer or pancreatic cancer.

[0091] In one embodiment of the method, the agent is poly[IC]-PEI. In yet another embodiment, the agent is doxorubicin. In one embodiment of the method, the agent induces one or more of MDA-5, NOXA and RIG-I.

[0092] This invention will be better understood by reference to the examples which follow. However, one skilled in the art will readily appreciate that the examples provided are merely for illustrative purposes and are not meant to limit the scope of the invention which is defined by the claims following thereafter.

[0093] Throughout this application, it is to be noted that the transitional term "comprising", which is synonymous with "including", "containing" or "characterized by", is inclusive or open-ended, and does not exclude additional, un-recited elements or method steps.

RESULTS

CTV expressing *mda-7/IL-24* inhibits neuroblastoma cell proliferation *in vitro*

[0094] To examine *mda-7/IL-24* overexpression on neuroblastoma tumor growth *in vitro* and *in vivo* a genetic approach was used with a tropism modified *Cancer Terminator Virus*

(Ad.5/3-*CTV*) (23, 26), i.e., a conditionally replicating adenovirus that ectopically expresses *mda-7/IL-24*. Adenovirus entry is cell surface receptor dependent, so the receptor status of CAR (for Ad.5), desmoglein and CD46 (for Ad.3) of the three neuroblastoma cell lines, SK-N-AS, NB1691 and SK-N-SH, used in this study was determined. CAR expression was variable with similar lower levels in SK-N-AS and NB1691 cells and higher levels in SK-N-SH cells (Figs. 8A and 8B). CD46 was expressed at a comparable level in all three-cell lines and Desmoglein levels followed a similar pattern as CAR, with highest expression in SK-N-SH cells. When transgene expression was monitored (MDA-7/IL-24 and E1A) following Ad.5/3-*CTV* infection, all three neuroblastoma cell lines expressed the expected transgenes as compared to mock and Ad.5/3-Null infected cells (Fig. 1A). A dose-dependent increase in MDA-7/IL-24 protein levels was seen in Ad.5/3-*CTV* infected cells compared to controls. To confirm that the increased levels of MDA-7/IL-24 protein represented an up-regulation of *mda-7/IL-24* mRNA transcription, the mRNA transcript levels in the Ad.5/3-*CTV*-treated cells were assessed. Ad.5/3-*CTV* treated neuroblastoma cells showed a strong increase in *mda-7/IL-24* mRNA levels when compared to mock, Ad.5/3-null, or Ad.5/3-*E1A* treated cells (data not shown). Next, the effects of Ad.5/3-*CTV* on neuroblastoma cell growth using MTT assays were assessed. Although Ad.5/3-*E1A* inhibited cell proliferation to some extent through its oncolytic activity, forced expression of *mda-7/IL-24* using Ad.5/3-*CTV* resulted in the greatest dose-dependent decrease in cell proliferation compared to other treatments (Fig. 1B).

Ad.5/3-*CTV* induces cell death in neuroblastoma cells

[0095] Ectopic expression of *mda-7/IL-24* induces apoptosis in a wide-array of cancer types (16). Overexpression of *mda-7/IL-24* in the neuroblastoma cell lines increased TUNEL positive cells compared to that of cells treated with mock, Ad.5/3-Null or Ad.5/3-*E1A* treatment (Fig. 2A). Further confirmation of apoptosis-induction was shown by FACS analysis where the sub-G1 population (DNA content) was enhanced in Ad.5/3-*CTV*-infected cells. DNA frequency distribution histograms in which the sub-G1 region corresponded to apoptotic cells indicated that Ad.5/3-*CTV* increased the number of apoptotic cells to 40-50% in SK-N-AS, 35-50% in SK-N-SH and 40-50% in NB1691 cells, as compared with 5% in Ad.5/3-Null-infected controls or ~20% in Ad.5/3-*E1A* infected cells (Fig. 2B). PARP cleavage was also evident by Western blotting analysis predominantly in Ad.5/3-*CTV*-infected neuroblastoma cells (Fig. 2C). The expression of *mda-7/IL-24*-downstream molecules and signals involved in cell death following infection of neuroblastoma cells with Ad.5/3-*CTV* was also analyzed. In many cancers, *mda-7/IL- 24* is known to enhance pro-apoptotic genes and decrease anti-apoptotic genes in the Bcl-2 family of proteins (44). A similar expression profile was found in neuroblastoma cells following Ad.5/3-*CTV* infection, i.e., enhancement of BAX and P-JNK

(pro-apoptotic proteins) and decreased expression of BCL-2 and BCL-xL (anti-apoptotic proteins) (Fig. 9). Of note, these changes were only evident following Ad.5/3-*CTV* infection and not with Ad.5/3-null or Ad.5/3-*E1A* infection of neuroblastoma cells.

***mda-7/IL-24* induces caspase-independent apoptosis *in vitro* in neuroblastoma cells**

[0096] Although *mda-7*/IL-24 has well established tumor-suppressor and apoptosis-promoting properties in a broad spectrum of human cancer cells, the molecular mechanism by which *mda-7/IL-24* induces apoptosis is quite diverge and involves different pathways depending on the tumor type (44). Caspase-dependent apoptosis is the common modality of programmed cell death (46), so whether *mda-7/IL-24*-induced cell death in neuroblastoma was caspase dependent was determined. Treatment of neuroblastoma cells with Ad.5/3-*CTV* did not induce caspase-3 or caspase-9 activation when analyzed by Western blotting or using a luminescence-based assay (Figs. 3A and 3B). To determine a potential involvement of the extrinsic apoptosis pathway, caspase 8 expression following Ad.5/3-*CTV* infection was monitored, and no activation in these cell lines was found (Fig. 10). In an additional confirmatory study, neuroblastoma cells were treated with z-vad fmk, a pan-caspase inhibitor, prior to Ad.5/3-*CTV* infection and cultured for an additional 48 hours. The cells were then analyzed via western blotting for PARP cleavage, which demonstrated similar PARP cleavage with or without treatment with the pan-caspase inhibitor prior to Ad.5/3-*CTV* infection (Fig. 3C). This result further supports the caspase- independent cell death mechanism following ectopic expression of MDA-7/IL-24 in neuroblastoma cells.

***mda-7/IL-24*-mediated apoptosis in neuroblastoma involves AIF activation and nuclear translocation**

[0097] The tumor suppressor p53 plays an important role in suppressing tumorigenesis by inducing genomic stability, cell cycle arrest or apoptosis (47). Apoptosis inducing factor (AIF) is a mitochondrial protein, which, when translocated to the nucleus, results in apoptosis, mainly in a caspase-independent context through the induction of chromatin condensation and DNA fragmentation (48). p53 is an established regulator of AIF in caspase-independent cell death and AIF can contribute to p53-mediated cell death (49). p53 levels were modestly increased in a dose-dependent manner following Ad.5/3-*CTV* infection as compared with the mock or Ad.5/3- Null-infected cells (Fig 11). AIF levels were increased in a dose-dependent manner in Ad.5/3-*CTV*-infected neuroblastoma cells (Fig. 4A) suggesting the potential involvement of AIF in caspase-independent cell death.

[0098] Previous studies suggest that BCL-2 proteins facilitate the insertion of BAK or BAX into the mitochondrial membrane to form functional oligomers, which result in depolarization of the inner mitochondrial membrane and subsequent AIF nuclear translocation, which promotes

caspase-independent apoptosis (50). To determine if *mda-7/IL-24* exerts similar effects on AIF nuclear translocation, immunostaining and cell fractionation methods were used. AIF displayed a granular pattern in the mitochondria of untreated controls, whereas after treatment with Ad.5/3-*CTV* AIF was detected in the nucleus (Fig. 4B). This result was confirmed using cellular fractionation, which clearly showed AIF accumulation in nuclear lysates and concomitant decreased levels in the cytoplasmic fraction following infection with Ad.5/3-*CTV* (Fig. 4C).

[0099] Ad.5/3-*CTV*-induced, AIF-mediated cell death in neuroblastoma cells was confirmed using an AIF inhibitor, N-Phenylmaleimide. Treatment with the AIF inhibitor at a concentration of 50 μM/L for 1 hour prior to treatment with Ad.5/3-*CTV* for 48 hours reduced cell death as monitored by FACS analysis (data not shown). This phenomenon was further confirmed by western blotting analysis for PARP cleavage. Treatment with an AIF-inhibitor prior to Ad.5/3-*CTV* infection resulted in decreased PARP cleavage (Fig. 4D). Collectively, these results indicate that Ad.5/3-*CTV* induces caspase-independent AIF-mediated apoptosis in neuroblastoma cells.

ATM-γ–H2AX axis mediates AIF-induced cell death in neuroblastoma cells

[0100] The mechanism that regulates AIF induction and nuclear translocation leading to caspase-independent apoptotic functions is not well understood. Previous studies suggest that γ-H2AX plays a pivotal role in AIF-mediated necroptosis in MEFs (45). To test this hypothesis, the effects of Ad.5/3-*CTV* on expression and activation of H2AX in SK-N-AS and NB1691 neuroblastoma cells were checked. Ad.5/3-*CTV* infection increased the levels of H2AX as well as phosphorylation of H2AX (γ-H2AX) when compared to control, Ad.5/3-Null, or Ad.5/3-*E1A* infected cells (Fig. 5A and Fig. 12). To further decipher the molecular mechanism of H2AX phosphorylation, the levels and activation of ATM in Ad.5/3-*CTV* treated SK-N-AS, SK-N-SH and NB1691 neuroblastoma cells were evaluated. There were increased ATM levels as well as phosphorylation of ATM when compared to control, Ad.5/3-Null or Ad.5/3-*E1A* treated cells (Fig. 5B and Fig. 13). In total, these results indicate that AIF-mediated caspase-independent apoptosis requires ATM-induced histone H2AX phosphorylation in Ad.5/3-*CTV* treated neuroblastoma cells.

[0101] To confirm further the involvement of ATM as an upstream regulator of H2AX- and AIF-mediated cell death in Ad.5/3-*CTV* treated neuroblastoma cells, small molecule ATM inhibitors, KU-60019 and KU-55933, for blocking the ATM kinase were used. ATM phosphorylates numerous proteins at specific positions, including H2AX at S139 (γ-H2AX). Neuroblastoma cells were treated over night with either KU-60019 (3 μM) or KU-55933 (5 μM, Fig. 14) prior to Ad.5/3-*CTV* infection. Treatment with KU60019 prior to Ad.5/3-*CTV* infection inhibited the phosphorylation of ATM thereby inhibiting γ-H2AX in SK-N-AS and NB1691 cells (Fig. 5C). This also resulted in decreased PARP cleavage reflecting decreased

apoptosis, which was confirmed by TUNEL assay (Fig. 5D and Fig. 14). Taken together, these results indicate that ATM acts as an upstream regulator of H2AX phosphorylation, which results in AIF-mediated cell death in neuroblastoma cells following treatment with Ad.5/3-*CTV*.

mda-7/IL-24* inhibits neuroblastoma tumor growth *in vivo

[0102] To directly evaluate the effect of *mda-7/IL-24* delivered by Ad.5/3-*CTV* on tumor growth *in vivo*, NB1691 cells were implanted subcutaneously on both sides of nude mice. The tumors on the left flank were challenged with intra-tumoral injections of Ad.5/3-Null, Ad.5/3-*E1A*, or Ad.5/3-*CTV (mda-7/IL-24* transducing virus*)*. Tumor growth was monitored in mice by measuring tumors every alternate day with Vernier calipers. There was a significant decrease in tumor volume in mice treated with Ad.5/3-*CTV* compared with mice treated with Ad.5/3-Null or Ad.5/3-*E1A* (Fig. 6A). Although Ad.5/3-*E1A* reduced tumor growth to some extent on the injected left side, it was evident that "*bystander activity*" in the non-injected right tumor was only observed in animals in which the left tumors were treated with Ad.5/3-*CTV* (Fig. 6). These results confirm previously published data that MDA-7/IL-24 exhibits potent anti-tumor "*bystander activity*". To determine whether MDA-7/IL-24 caused AIF-mediated apoptosis *in vivo*, tumor sections were immunoassayed for MDA-7/IL-24, pATM, γ–H2AX and AIF. Apoptotic content was determined by TUNEL analysis. Consistent with the present *in vitro* observations, tumor sections from Ad.5/3-*CTV*-treated mice showed increased staining for MDA-7/IL-24, pATM, γ–H2AX and AIF (Fig. 6C). Furthermore, the apoptotic index of tumor cells quantified by the number of TUNEL staining positive cells increased with Ad.5/3-*CTV* treatment (Fig. 6B).

Ad.5/3-CTV in combination with poly[IC]-PEI induces synergistic growth inhibition in neuroblastoma (NB) cells

[0103] NB cell lines (SK-N-AS and SK-N-SH) were infected with Ad.5/3 E1A (12.5 IU) or Ad.5/3-*CTV* (12.5 IU) and cultured for 24 hours. Next, these cells were treated with various doses of poly[IC]-PEI for another 24 hours. Cell proliferation was determined with MTT assays and graphs were plotted (Figs. 16-19). Treatment with Ad.5/3-*CTV* alone resulted in 30-40% growth inhibition in both tested cell lines. Poly[IC]-PEI treatment alone resulted in 20-40% cell growth inhibition. Interestingly, the combination of poly[IC]-PEI at different concentrations with a fixed low concentration of Ad.5/3-*CTV* resulted in a strong significant synergistic inhibition in cell proliferation with a combination index (CI) of (0.15-0.40) (Fig. 20). The CI was determined using Chou and Talalay method (57). The inhibition in cell growth by combination treatment of Ad.5/3-*CTV and* Poly[IC]-PEI is likely caused by apoptosis induction as evidenced by increased PARP cleavage in the combination treatment group as compared to the single treatment group (Fig. 21).

Ad.5/3-CTV in combination with Doxorubicin induces strong additive growth inhibition in neuroblastoma (NB) cells

[0104] NB cell lines (SK-N-AS and SK-N-SH) were infected with Ad.5/3 E1A (12.5 IU) or Ad.5/3-*CTV* (12.5 IU) and cultured for 24 hours. Next, these cells were treated with various doses of doxorubicin for another 24 hours. Cell proliferation was determined with MTT assays and graphs were plotted (Figs. 22-25). Infection with Ad.5/3-*CTV* alone resulted in 30-40% growth inhibition in both tested cell lines. Doxorubicin treatment alone resulted in 40-50% cell growth inhibition. Interestingly, combination of Doxorubicin at different concentrations with a fixed low concentration of Ad.5/3-*CTV* resulted in a strong additive/synergistic inhibition in cell proliferation with a CI index of (0.52-0.59) (Fig. 26). The combination index was determined using the Chou and Talalay method (57). The inhibition in cell growth by combination treatment of Ad.5/3-*CTV* and Doxorubicin is likely caused by apoptosis induction as evidenced by increased PARP cleavage in combination treatment groups as compared to single treatment group (Fig. 27).

Ad.5/3-CTV in combination with Cisplatin did not enhance the therapeutic efficacy of either agent alone in neuroblastoma (NB) cells

[0105] NB cell lines (SK-N-AS and SK-N-SH) were infected with Ad.5/3 E1A (12.5 IU) or Ad.5/3-*CTV* (12.5 IU) and cultured for 24 hours. Next, these cells were treated with various doses of Cisplatin for another 24 hours. Cell proliferation was determined with MTT assays and graphs were plotted (Figs. 28-31). Infection with Ad.5/3-*CTV* alone resulted in 30-40% growth inhibition in both tested cell lines. Cisplatin treatment alone resulted in 10-30% cell growth inhibition. However, combination of Cisplatin at different concentrations with a fixed low concentration of Ad.5/3-*CTV* did not enhance the therapeutic efficacy of either agent alone. Additionally, the CI determined using the Chou and Talalay method (57) suggests that this CI is antagonistic in nature with a CI value ranged from 0.9-1.4 (Fig. 32). Moreover, the Western blot analysis for PARP cleavage (a marker of apoptosis induction) shown in Fig. 33 did not show any further increase in PARP cleavage when Ad.5/3-CTV was added to Cisplatin.

[0106] In sum, the above studies suggest a novel viral-chemical combination approach which could provide enhanced treatment options for early stage and advanced neuroblastoma. The immune stimulating properties of *mda-7/IL-24* suggest that the combination approach will have even further enhanced activity when used *in vivo* through augmentation of immune response to the tumor cells (12, 59).

Materials and Methods

Cells and Reagents

[0107] Human neuroblastoma cancer cell lines SK-N-AS and SK-N-SH were obtained from ATCC (Manassas, VA) and NB1691 cells were obtained from Dr. Alan Houghton of St. Jude

Children's Research Hospital (Memphis, TN). NB1691 cell line was authenticated using the "CellCheck" service provided by the Research Animal Diagnostic Laboratory and compared with initial STR profile generated by the collaborator (IDEXX BioResearch). The cumulative culture length of the cells (SK-N-SH and SK-N-AS) was less than 6 months after recovery. Early passage cells were used for all experiments. All the cell lines were frequently tested for mycoplasma contamination using a mycoplasma detection kit from Sigma. SK-N-AS cells were cultured in DMEM with non-essential amino acids, SK-N-SH cells were cultured with RPMI 1640 and NB1691 cells were cultured with DMEM (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS), 50-units/mL penicillin, and 50 µg/mL streptomycin (Life Technologies Inc., Frederick, MD). Cells were incubated in a humidified 5 % CO2 atmosphere at 37 °C. The antibodies specific for AIF (#4642), ATM (#2873), pATM (ser 1981, #13050), BCL-2 (#2876), BAX (2772), PARP (#9542), Caspase 3 (#9662), Caspase 8 (#9746), H2AX (#7631) and γ-H2AX (ser 139, #9718) (Cell signaling Technology, Boston, MA), MDA-7/IL-24 (#K101,GenHunter, Nashville, TN), HRP-conjugated secondary antibodies (Dako, Carpinteria, CA), β-Actin (#NB600-501, Novus Biologicals, Inc., Littleton, CO), were used in this study. The other materials used in this study were Transcriptor First Strand cDNA Synthesis Kit, In Situ Cell Death Detection Kit, Fluorescein (#11684795910, Roche Applied Science, Indianapolis, IN), MTT cell growth assay kit (#CT02, Millipore Corporation, Billerica, MA). KU60019 (#SML1416) and KU-55933 (#SML-1109) (Sigma, St Louis, MO).

Transfection with plasmids

[0108] All transfection experiments were performed with FuGene HD transfection reagent according to the manufacturer's protocol (Roche, Indianapolis, IN). Briefly, plasmid/siRNA was mixed with FuGene HD reagent (1:3 ratio) in 500 µL of serum free medium and left for 30 min to allow for complex formation. The complex was then added to the 100-mm plates, which had 2.5 mL of serum-free medium (2 µg plasmid per mL of medium). After 6 hours of transfection, complete medium was added, and cells were cultured for another 24 h (1).

Cell proliferation assay (MTT assay)

[0109] Cell growth rate was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as a measurement of mitochondrial metabolic activity as described earlier (8). Cells were treated with mock, Ad.5/3-Null, Ad.5/3-*E1A* or the indicated doses of Ad.5/3-*CTV* and incubated at 37 °C. After 0–96 h, MTT reagent was added, and cells were incubated for 4 h at 37 °C. After removing the medium, formazan crystals were dissolved in DMSO, absorbance at 550 nm was read using a microplate spectrophotometer and the results were expressed graphically.

Terminal deoxy nucleotidyl transferase-mediated nick labeling (TUNEL) assay

[0110] Induction of apoptosis in Neuroblastoma cancer cells as well as in xenograft tumor tissue sections of mice treated with mock, Ad.5/3-Null, Ad.5/3-*E1A* or Ad.5/3-*CTV* was evaluated using TUNEL enzyme reagent following the manufacturer's instructions and as described previously (50). Briefly, 5×10^3 Neuroblastoma cancer cells were cultured and treated with mock, Ad.5/3-Null, Ad.5/3-*E1A* or Ad.5/3-*CTV* for 72 hours, fixed in 4 % paraformaldehyde in PBS for 1 hour at room temperature (RT), and permeabilized with 0.1 % Triton-X 100 in 0.1 % sodium citrate in PBS for 2 min (for cells) or 10 min (for tissue sections) on ice. The samples were incubated in TUNEL reaction mixture in a humidified atmosphere at 37 °C for 1 hour in the dark. Images were captured with an Olympus research fluorescence microscope attached to a CCD camera and cells were counted. The positive-staining apoptotic cells were counted from 5 microscopic fields per tumor tissue from 3 animals per treatment.

Western blotting

[0111] Western blotting analysis was performed as described previously (50). Briefly, Mock, Ad.5/3-Null, Ad.5/3-*E1A* or Ad.5/3-*CTV*-treated neuroblastoma cancer cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin. Equal amounts of total protein fractions of lysates were resolved by SDS-PAGE and transferred to PVDF membranes. The blot was blocked with 5% non-fat dry milk/5% BSA and probed overnight with primary antibodies followed by HRP-conjugated secondary antibodies. An ECL system was used to detect chemiluminescent signals. All blots were re-probed with β-Actin antibody to confirm equal loading.

***In vivo* studies**

[0112] To directly evaluate the effect of Mock, Ad.5/3-Null, Ad.5/3-*E1A* or Ad.5/3-*CTV* on tumor growth *in vivo*, 5 X 10^6 NB1691 cells were subcutaneously implanted on both flanks of 4-to 6-week-old athymic nude mice. The tumors on the left flank were challenged with 8 intra-tumoral injections of Mock, Ad.5/3-Null, Ad.5/3-*E1A* or Ad.5/3-*CTV* for three weeks (3 injections for 2 weeks and 2 injections in the last week) after 7 days post tumor cell implantation when the tumors reached palpable sizes. Tumor growth was monitored in mice by measuring tumor size with calipers on each flank every alternate day until completion of the experiment. Each treatment group had two sets of animals. One set was sacrificed 2 days after the final dose of treatment (1 mouse from each group) and another set (N=5) was followed until the control tumor group reached a point where it needed to be sacrificed according to the IACUC protocol. After completion of the experiment the tumors were fixed and the sections were used for immunohistochemical analysis.

Statistical analysis

[0113] All data are presented as mean ± standard deviation (S.D.) of at least three independent experiments, each performed at least in triplicate. One-way analysis of variance (ANOVA) combined with the Tukey post hoc test of means was used for multiple comparisons. Statistical differences are presented at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Combination study of Ad.5/3-*CTV* and other therapeutic agents

[0114] Neuroblastoma cell lines (SK-N-AS and SK-N-SH) were infected with Ad.5/3 E1A (12.5 IU) or Ad.5/3-*CTV* (12.5 IU) and cultured for 24 hours. Next, these cells were treated with various doses of poly[IC]-PEI, Doxorubicin, or Cisplatin for another 24 hours. Cell proliferation was determined with MTT assays and graphs were plotted. Combination index (CI) of each combination treatment was determined using Chou and Talalay method (57). Western blotting analysis was performed to compare the PARP cleavage between the combination treatment group and the single treatment group.

[0115] In addition to poly[IC]-PEI, and Doxorubicin, which were studied in this invention, other therapeutic agents that are known to have effects on neuroblastoma cells can be used in combination with Ad.5/3-*CTV*. Potential therapeutic agents or therapeutic means to be used in combination with the present invention include but are not limited to the following: radiation; reactive oxygen inducing compounds; GD2 binding antibody (such as dinutuximab/Unituxin); granulocyte-macrophage colony-stimulating factor (GM-CSF); interleukin-2 (IL-2); 13-cis-retinoic acid (RA); immunotherapy; checkpoint inhibitors, cyclophosphamide or ifosfamide, carboplatin, vincristine, Adriamycin, etoposide, topotecan, busulfan and melphalan; mcl-1 inhibitors; heat shock protein-90 inhibitors; proteasome inhibitors (such as MG132), and other agents which are potentially effective on neuroblastoma or other cancers.

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What is claimed is:

1. A combination of Ad.5/3-*CTV* and an agent for use in a method of treating cancer in a subject, wherein said Ad.5/3-*CTV* and agent produce a synergy in inducing cancer cell growth suppression, toxic autophagy and apoptosis.

2. The combination of claim 1, wherein said Ad.5/3-*CTV* comprises an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24.

3. The combination of claim 1, wherein said agent is selected from the group consisting of poly[IC]-PEI, doxorubicin, and other agents capable of promoting toxic autophagy and/or apoptosis.

4. The combination of claim 2, wherein the expression of MDA-7/IL-24 induces apoptosis in the cancer cells through the modulation of AIF, ATM and γ-H2AX.

5. The combination of claim 1, wherein said Ad.5/3-*CTV* and agent are to be administered at the same time.

6. The combination of claim 1, wherein said agent is to be administered 12-96 hours after the administration of Ad.5/3-*CTV*.

7. The combination of any one of claims 1-6, wherein said cancer is neuroblastoma.

8. The combination of any one of claims 1-6, wherein said cancer is melanoma, glioblastoma, prostate cancer, breast cancer, colorectal cancer, lung cancer or pancreatic cancer.

9. The combination of claim 7, wherein said agent is poly[IC]-PEI.

10. The combination of claim 7, wherein said agent is doxorubicin.

11. The combination of claim 3, wherein said agent induces one or more of MDA-5, NOXA and RIG-I.

12. A method of treating cancer in a subject, comprising administering to the subject an effective amount of Ad.5/3-*CTV* and an agent, wherein said Ad.5/3-*CTV* and agent produce a synergy in inducing cancer cell growth suppression and apoptosis.

13. The method of claim 12, wherein said Ad.5/3-*CTV* comprises an adenoviral vector Ad.5/3 and a nucleic acid expressing MDA-7/IL-24.

14. The method of claim 12, wherein said agent is selected from the group consisting of poly[IC]-PEI, doxorubicin, and other agents capable of promoting toxic autophagy and/or apoptosis.

15. The method of claim 13, wherein the expression of MDA-7/IL-24 induces apoptosis in the cancer cells through the modulation of AIF, ATM and γ-H2AX.

16. The method of claim 12, wherein said Ad.5/3-*CTV* and agent are administered at the same time.

17. The method of claim 12, wherein the agent is administered 12-96 hours after the administration of Ad.5/3-*CTV*.

18. The method of any one of claims 12-17, wherein said cancer is neuroblastoma.

19. The method of any one of claims 12-17, wherein said cancer is melanoma, glioblastoma, prostate cancer, breast cancer, colorectal cancer, lung cancer or pancreatic cancer.

20. The method of claim 18, wherein said agent is poly[IC]-PEI.

21. The method of claim 18, wherein said agent is doxorubicin.

22. The method of claim 14, wherein said agent induces one or more of MDA-5, NOXA and RIG-I.

FIGURE 1A

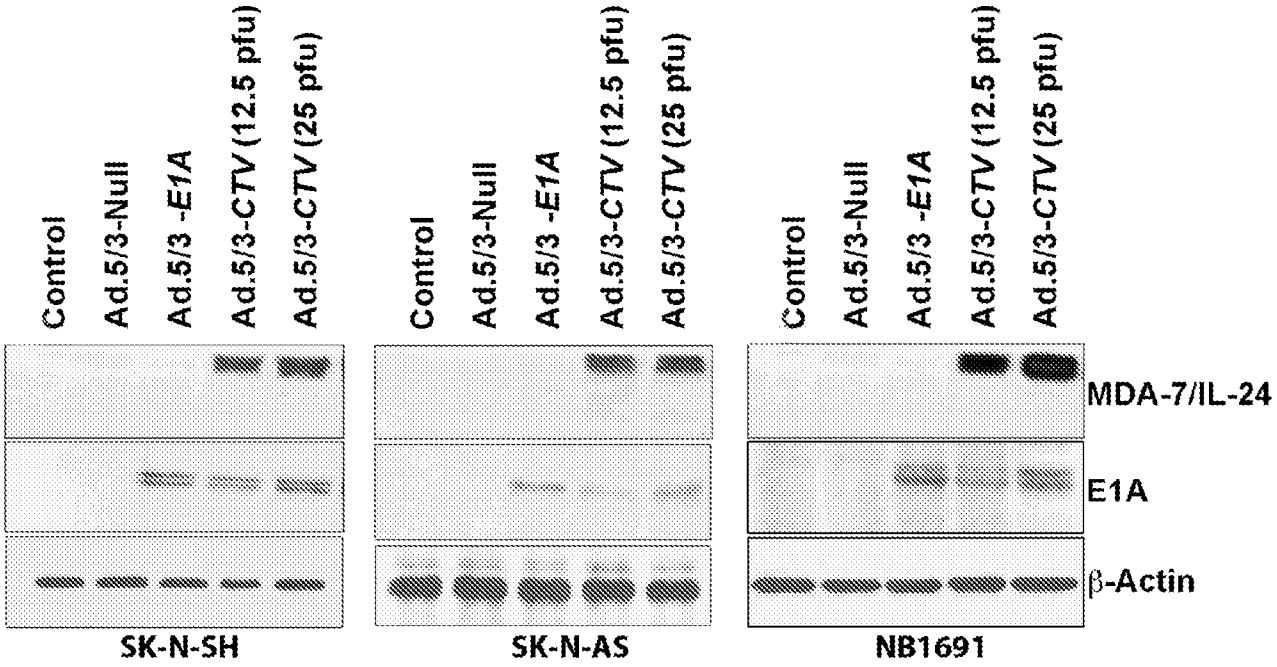
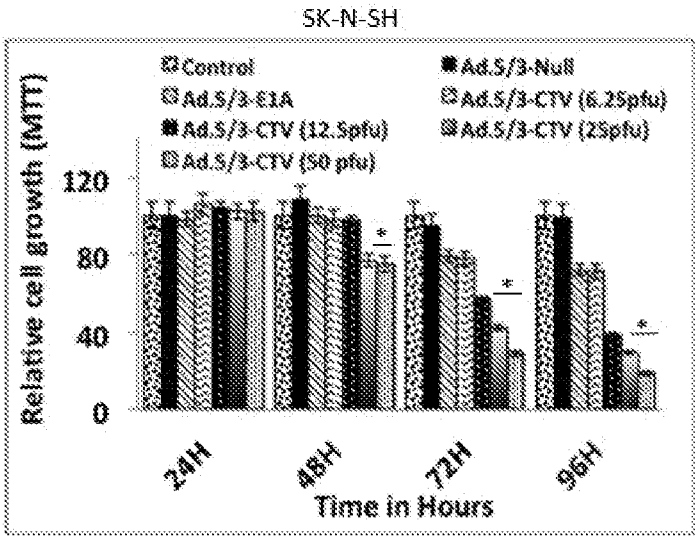
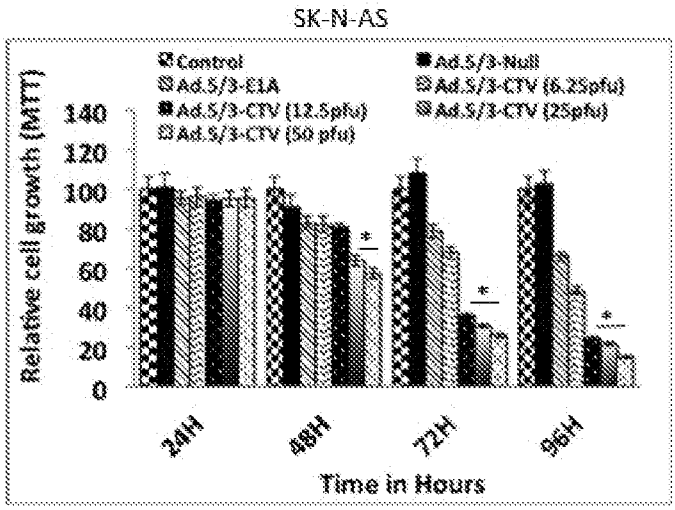

Control
Ad.5/3-Null
Ad.5/3 -E1A
Ad.5/3-CTV (12.5 pfu)
Ad.5/3-CTV (25 pfu)
MDA-7/IL-24
E1A
β-Actin
SK-N-SH
SK-N-AS
NB1691

FIGURE 1B


SK-N-SH
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (6.25pfu)
Ad.5/3-CTV (12.5pfu)
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50 pfu)
Relative cell growth (MTT)
120
80
40
0
*
*
*
24H
48H
72H
96H
Time in Hours


SK-N-AS
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (6.25pfu)
Ad.5/3-CTV (12.5pfu)
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50 pfu)
Relative cell growth (MTT)
140
120
100
80
60
40
20
0
*
*
*
24H
48H
72H
96H
Time in Hours

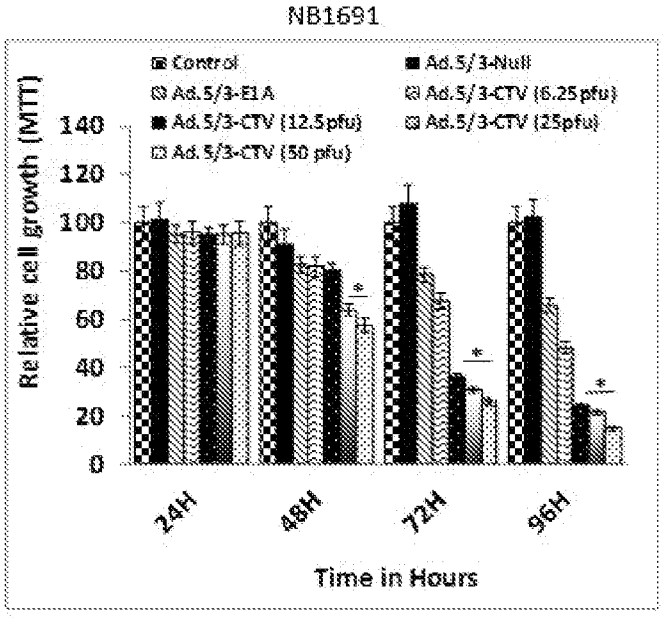

NB1691
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (6.25pfu)
Ad.5/3-CTV (12.5pfu)
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50 pfu)
Relative cell growth (MTT)
140
120
100
80
60
40
20
0
*
*
*
24H
48H
72H
96H
Time in Hours

FIGURE 2A

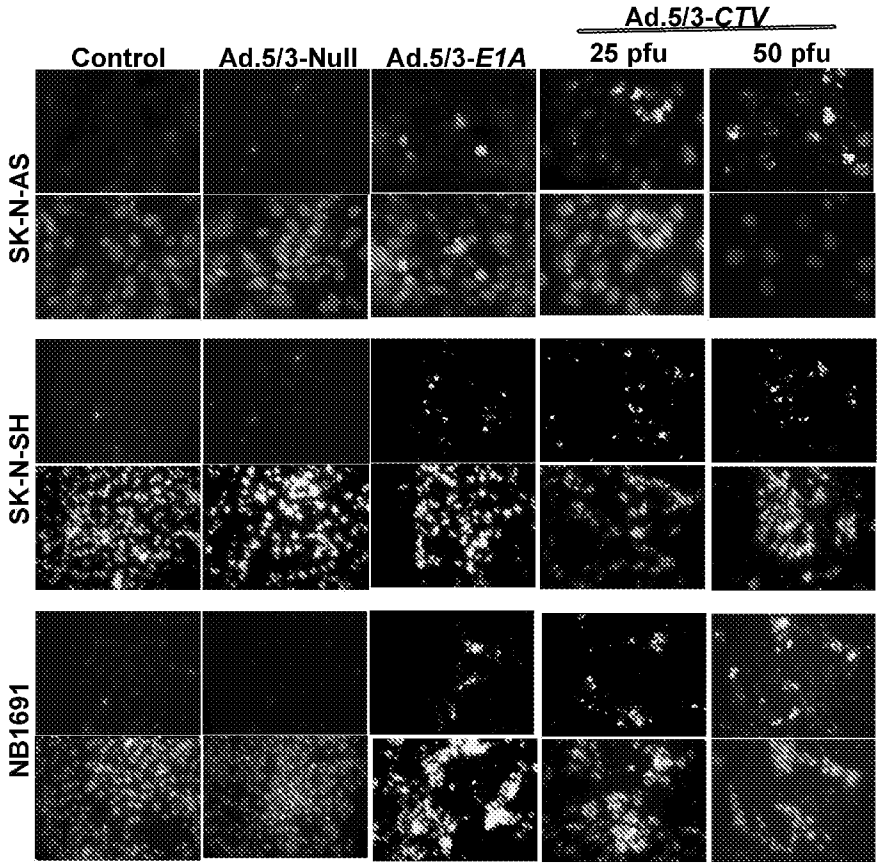


FIGURE 2B

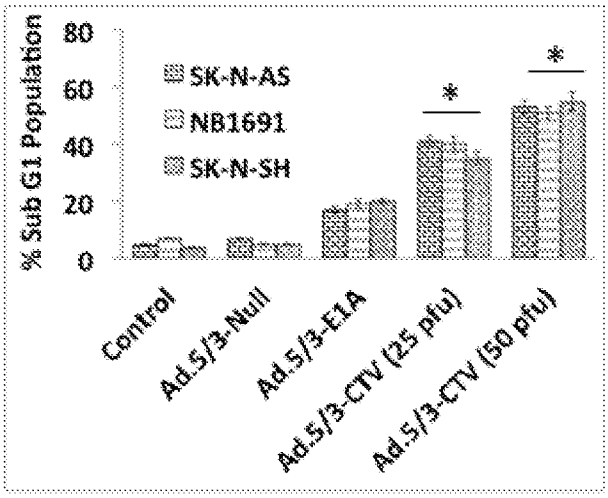


FIGURE 2C

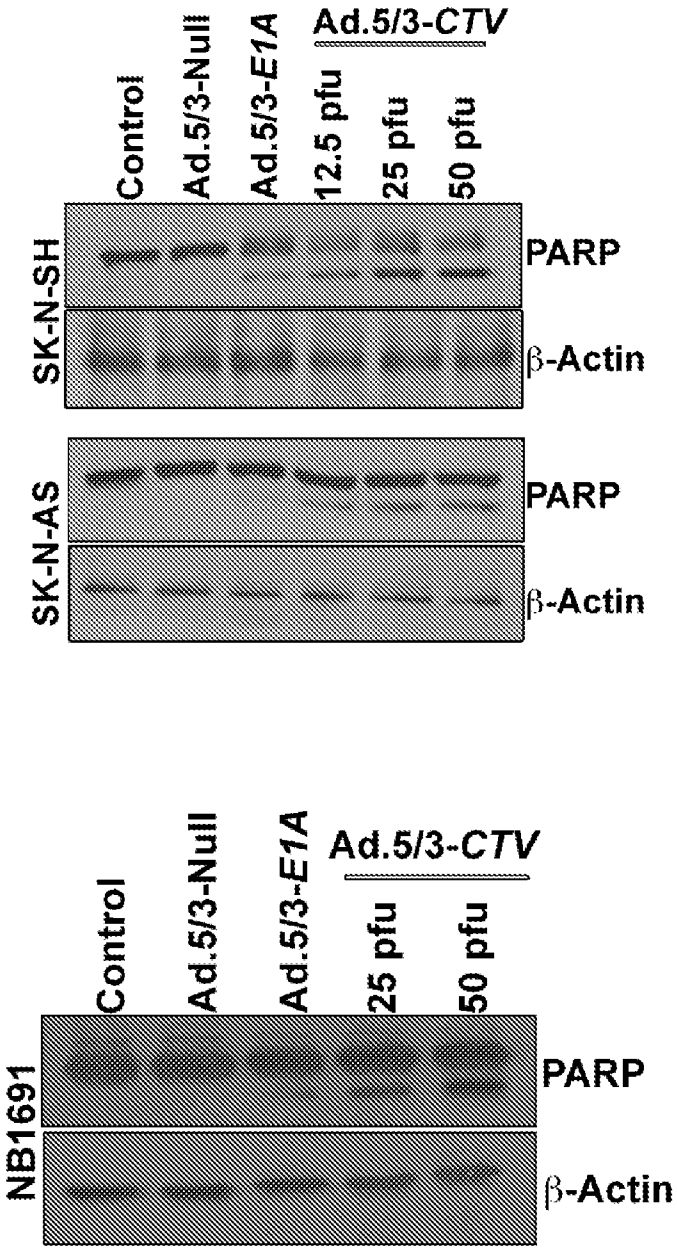

Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV
12.5 pfu
25 pfu
50 pfu
SK-N-SH
PARP
β-Actin
SK-N-AS
PARP
β-Actin
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV
25 pfu
50 pfu
NB1691
PARP
β-Actin

FIGURE 3A

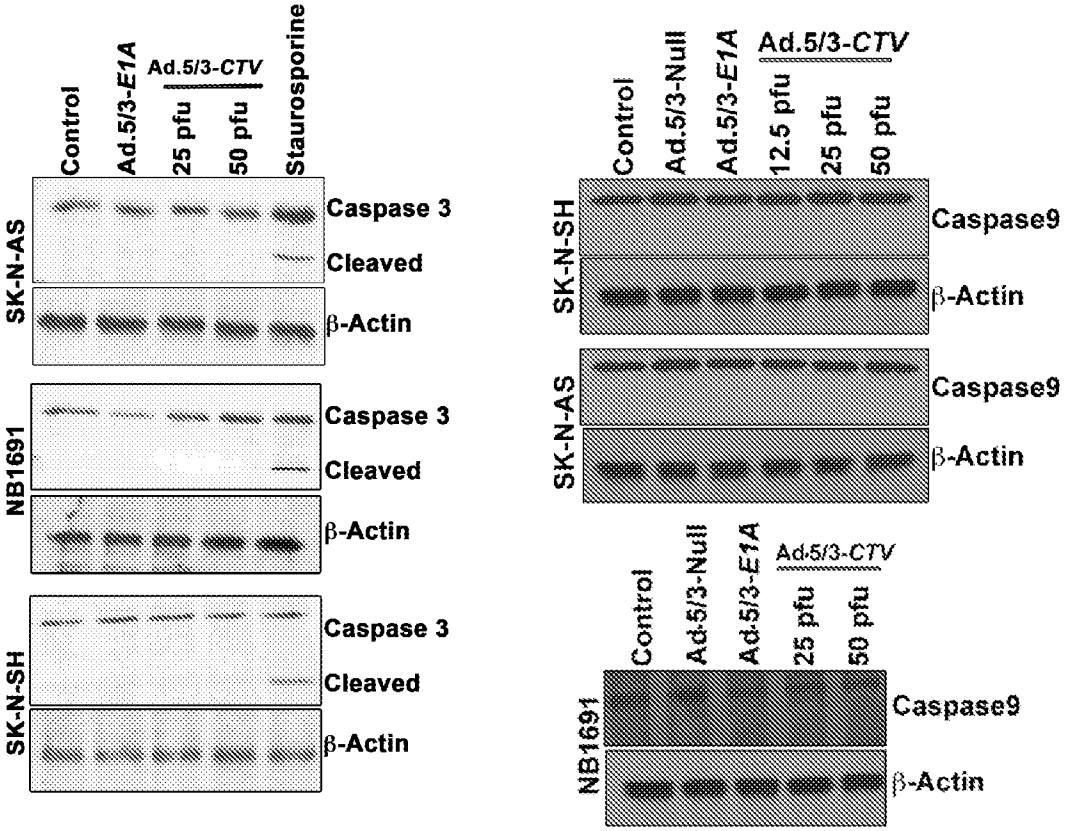

Control
Ad.5/3-E1A
Ad.5/3-CTV
25 pfu
50 pfu
Staurosporine
SK-N-AS
Caspase 3
Cleaved
β-Actin
NB1691
Caspase 3
Cleaved
β-Actin
SK-N-SH
Caspase 3
Cleaved
β-Actin
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV
12.5 pfu
25 pfu
50 pfu
SK-N-SH
Caspase9
β-Actin
SK-N-AS
Caspase9
β-Actin
Control
Ad-5/3-Null
Ad-5/3-E1A
Ad-5/3-CTV
25 pfu
50 pfu
NB1691
Caspase9
β-Actin

FIGURE 3B

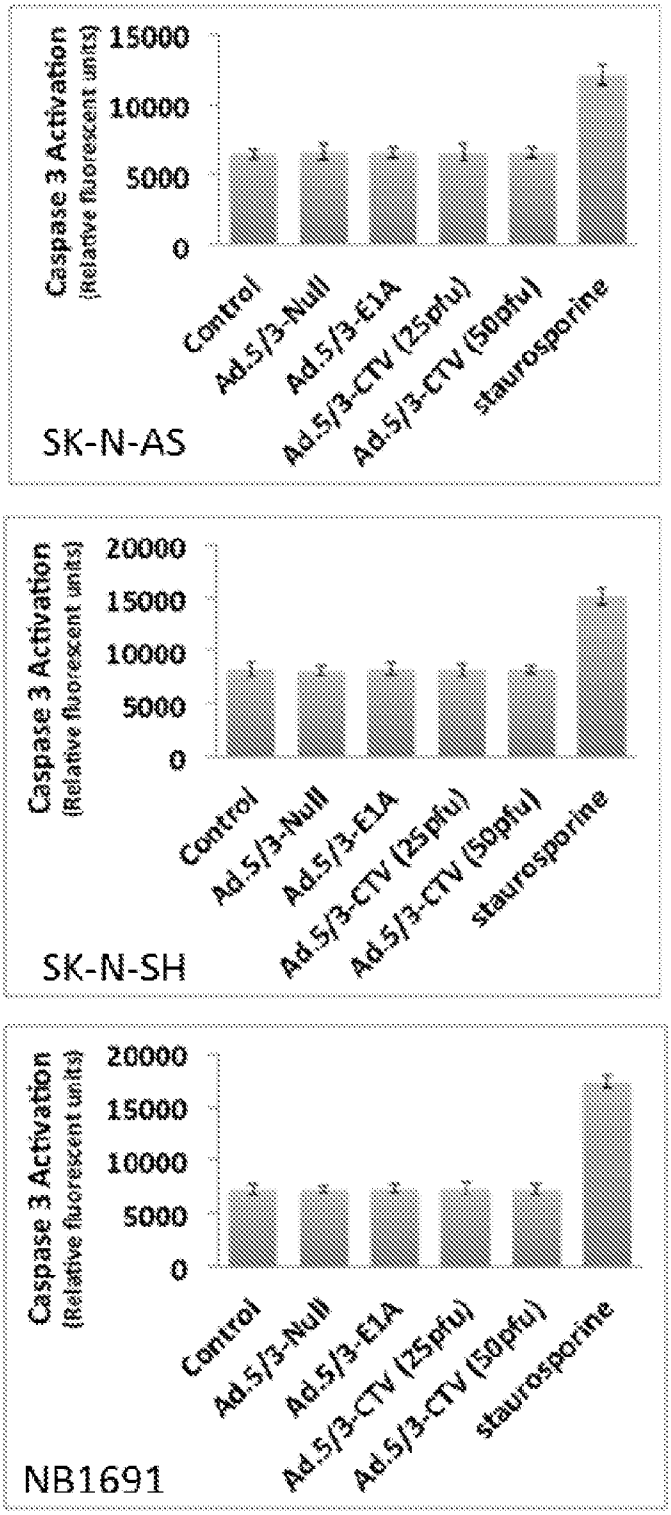
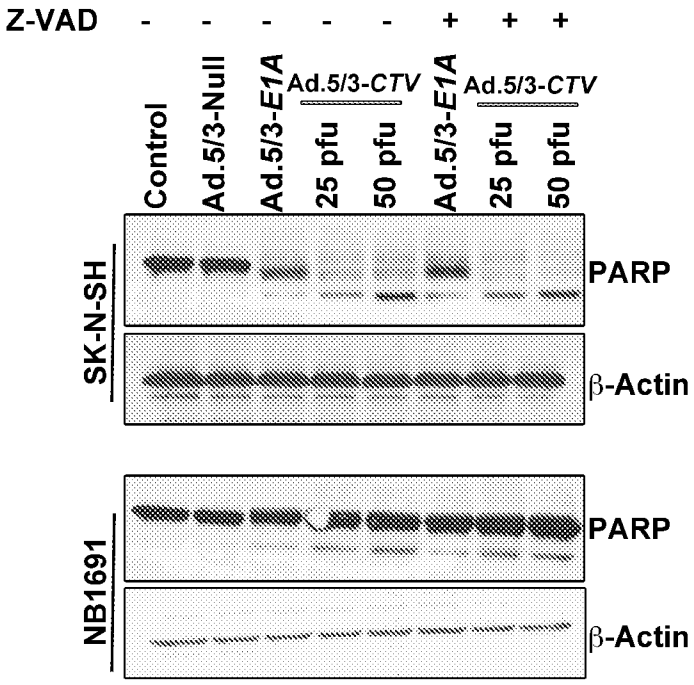

Caspase 3 Activation
(Relative fluorescent units)
15000
10000
5000
0
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50pfu)
staurosporine
SK-N-AS
Caspase 3 Activation
(Relative fluorescent units)
20000
15000
10000
5000
0
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50pfu)
staurosporine
SK-N-SH
Caspase 3 Activation
(Relative fluorescent units)
20000
15000
10000
5000
0
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50pfu)
staurosporine
NB1691

FIGURE 3C


Z-VAD - - - - - + + +
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV
25 pfu
50 pfu
Ad.5/3-E1A
Ad.5/3-CTV
25 pfu
50 pfu
SK-N-SH
PARP
β-Actin
NB1691
PARP
β-Actin

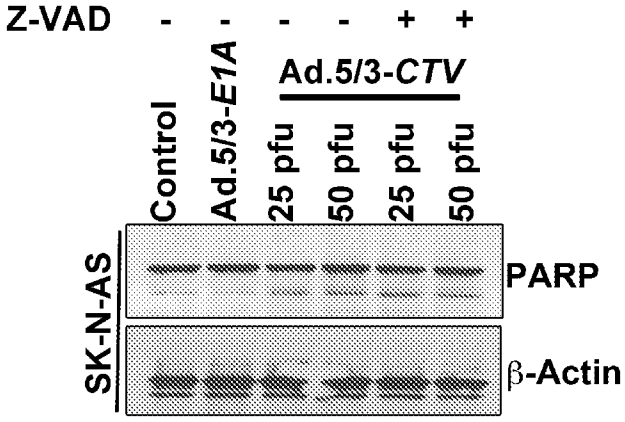

Z-VAD - - - - + +
Control
Ad.5/3-E1A
Ad.5/3-CTV
25 pfu
50 pfu
25 pfu
50 pfu
SK-N-AS
PARP
β-Actin

FIGURE 4A

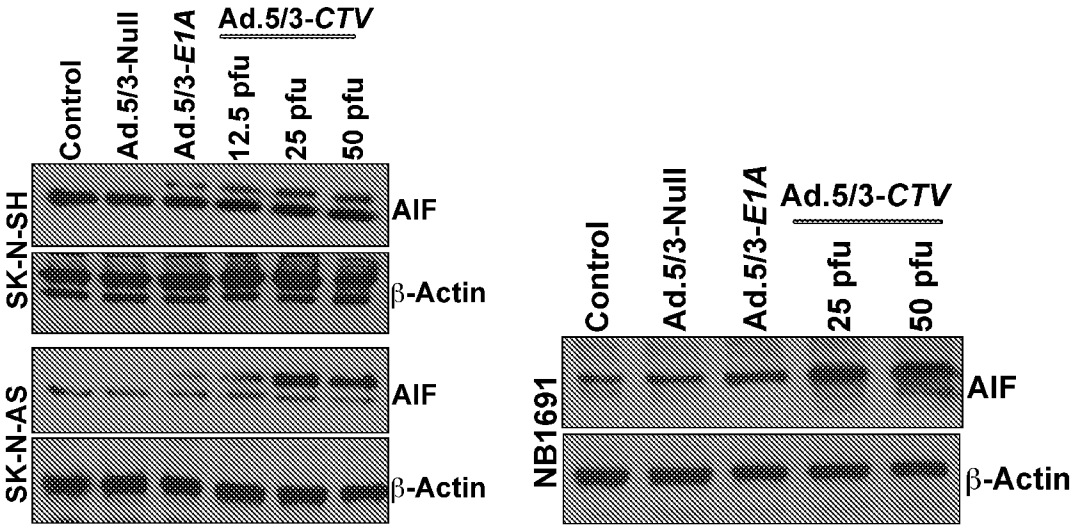


FIGURE 4B

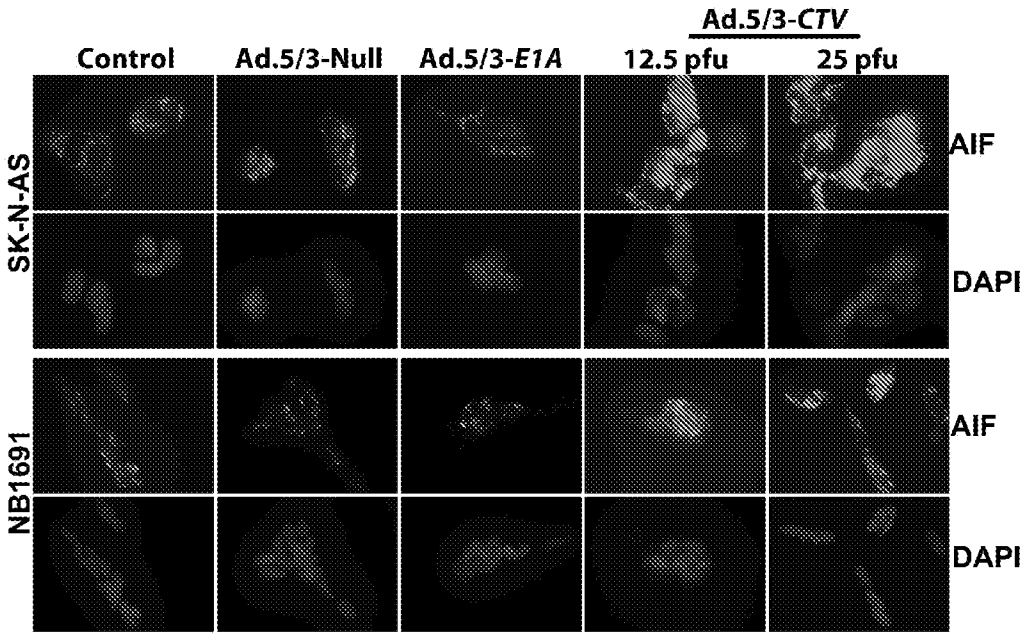


FIGURE 4C

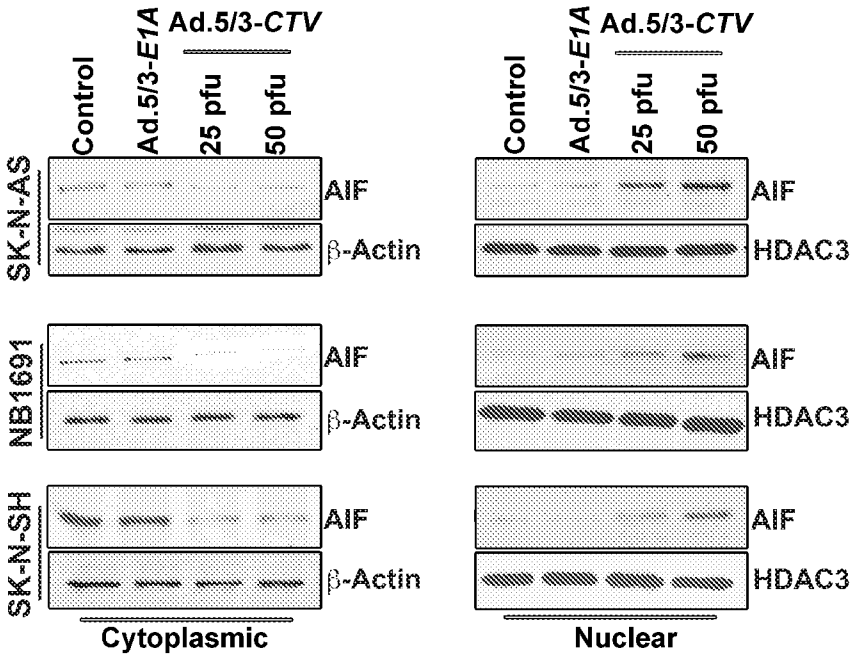


FIGURE 4D

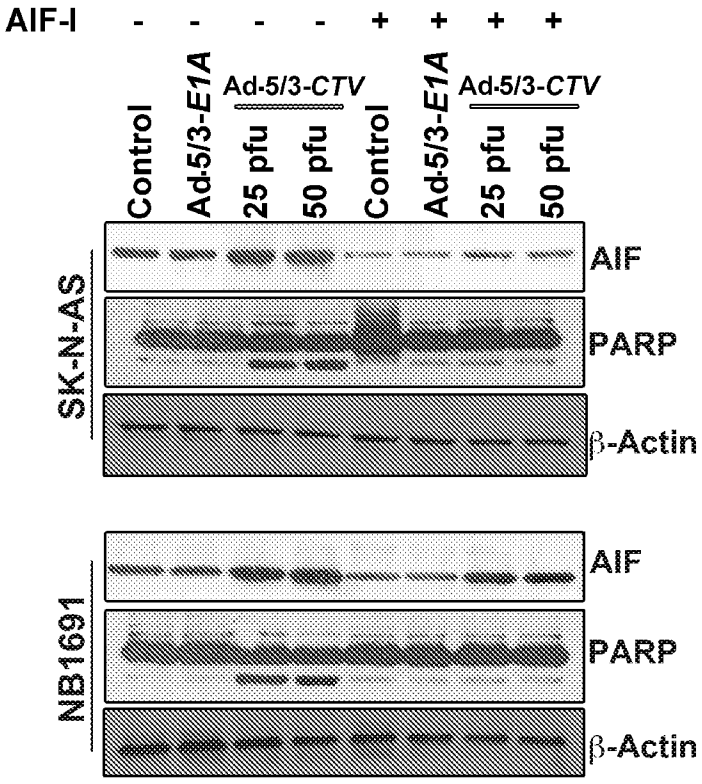


FIGURE 5A

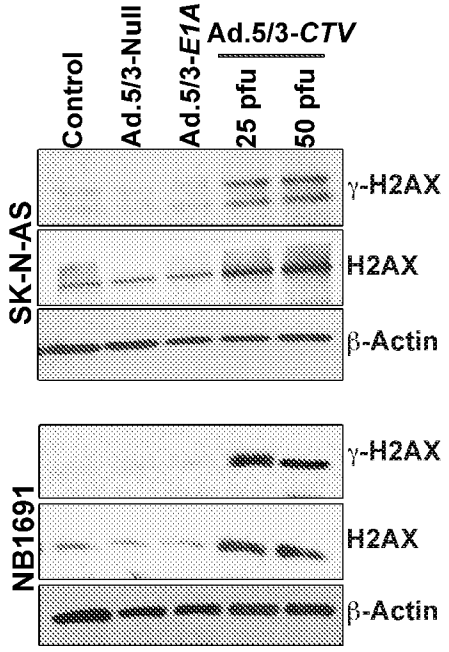


FIGURE 5B

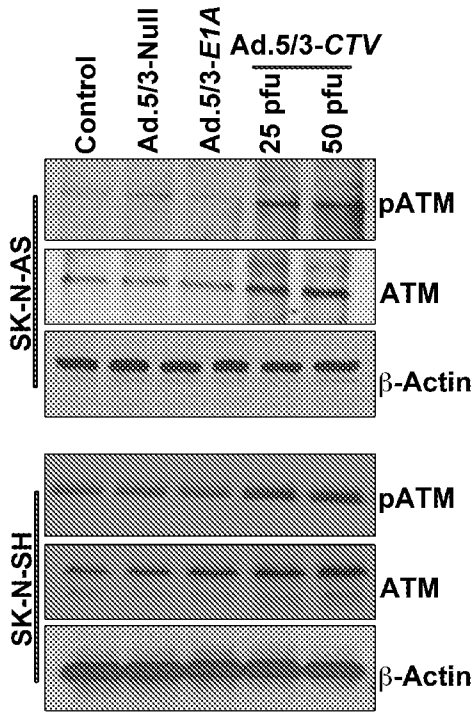


FIGURE 5C

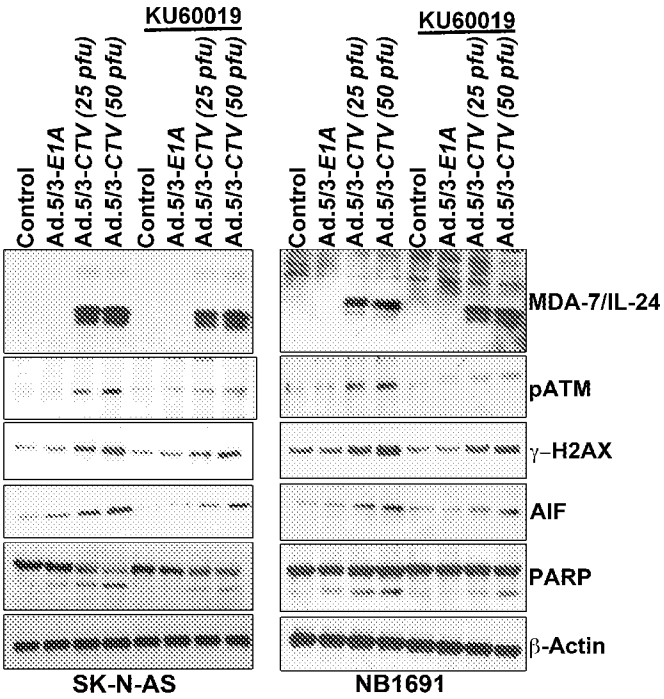


FIGURE 5D

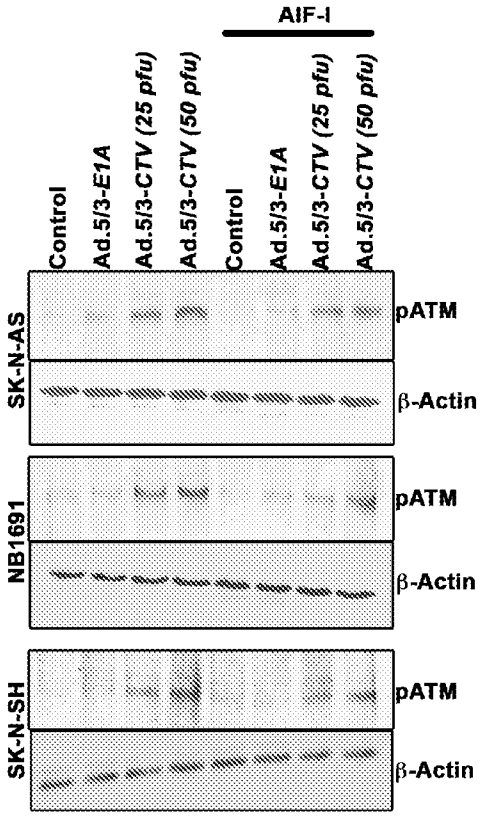


FIGURE 5E

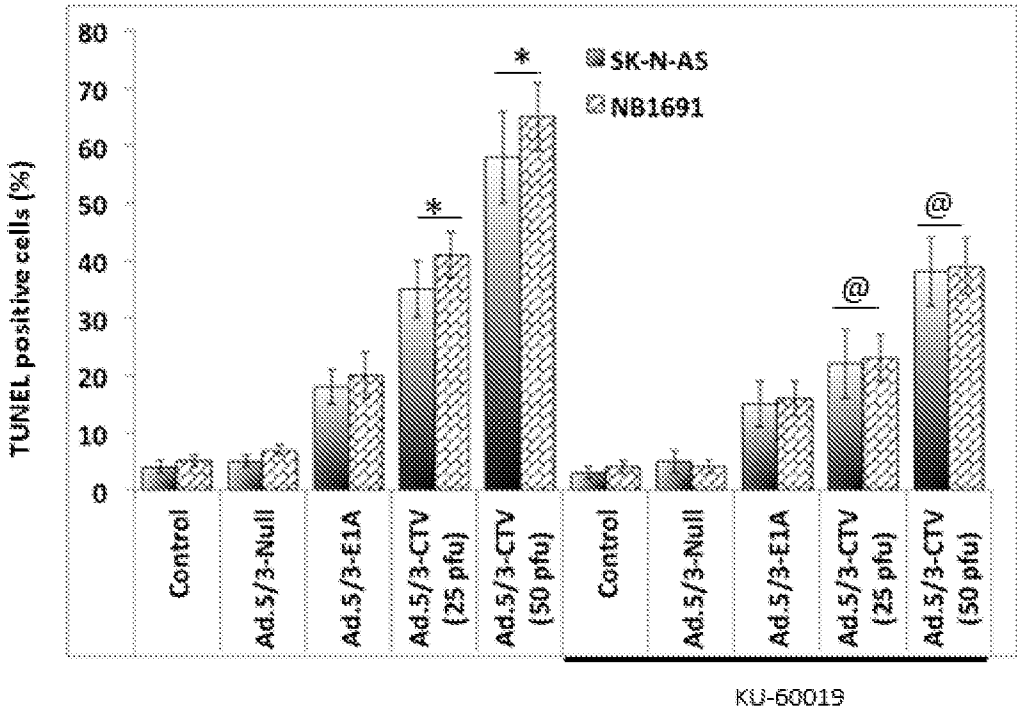


FIGURE 6A

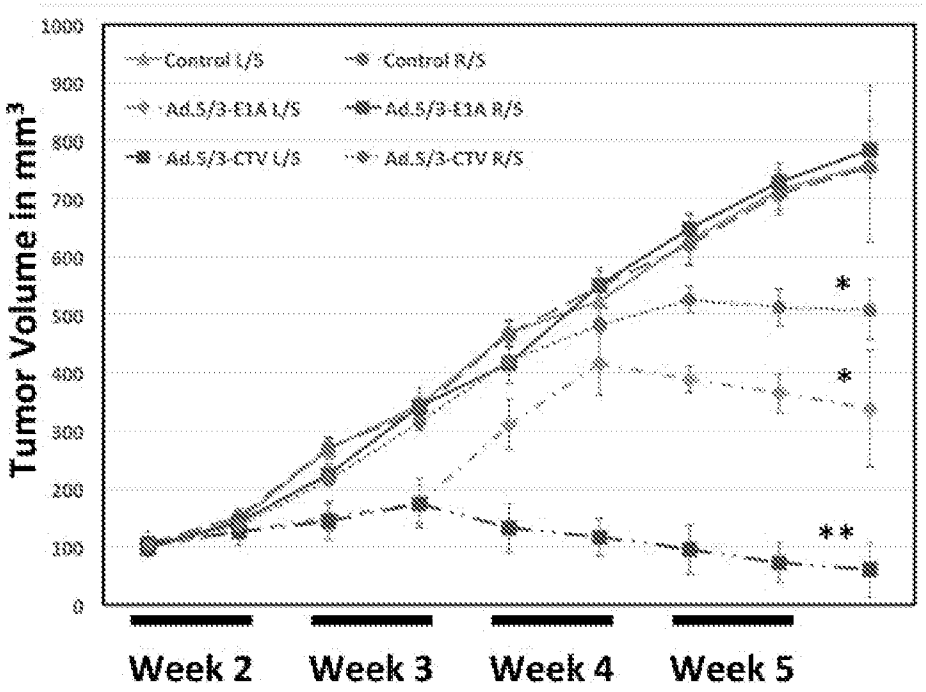


FIGURE 6B

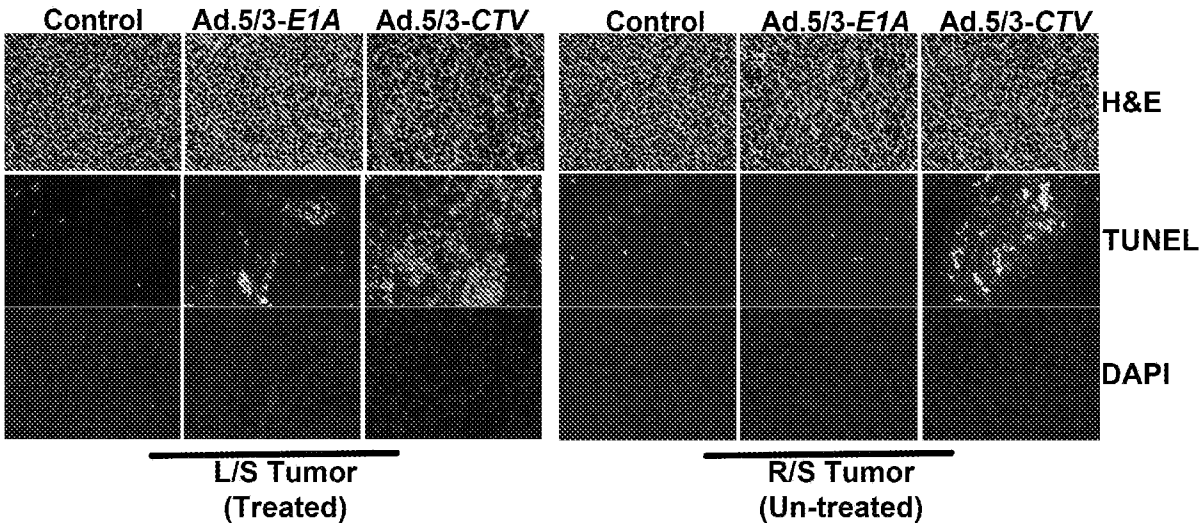


FIGURE 6C

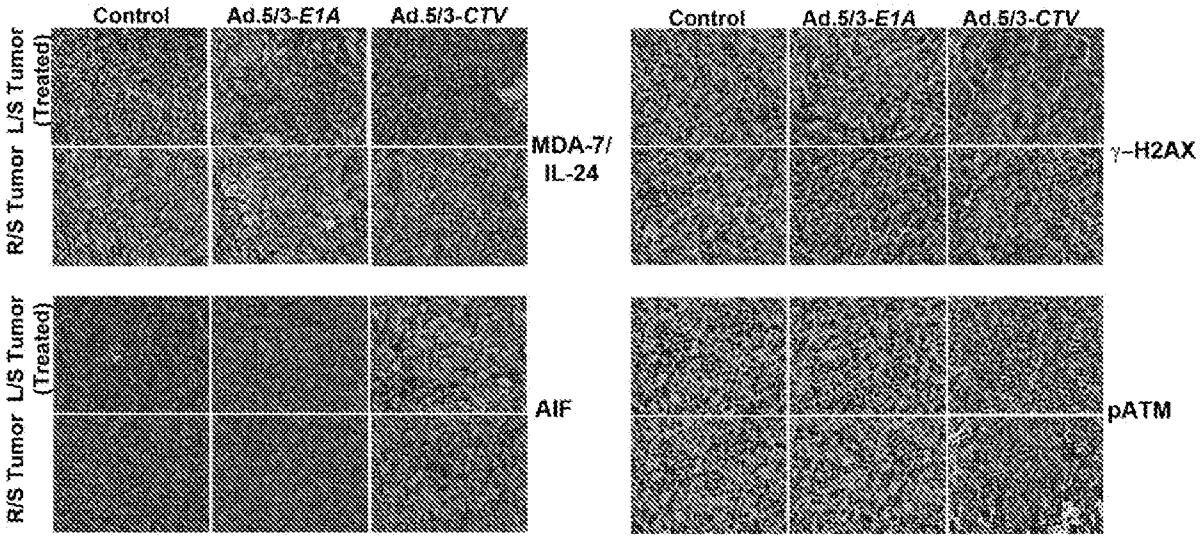


FIGURE 7

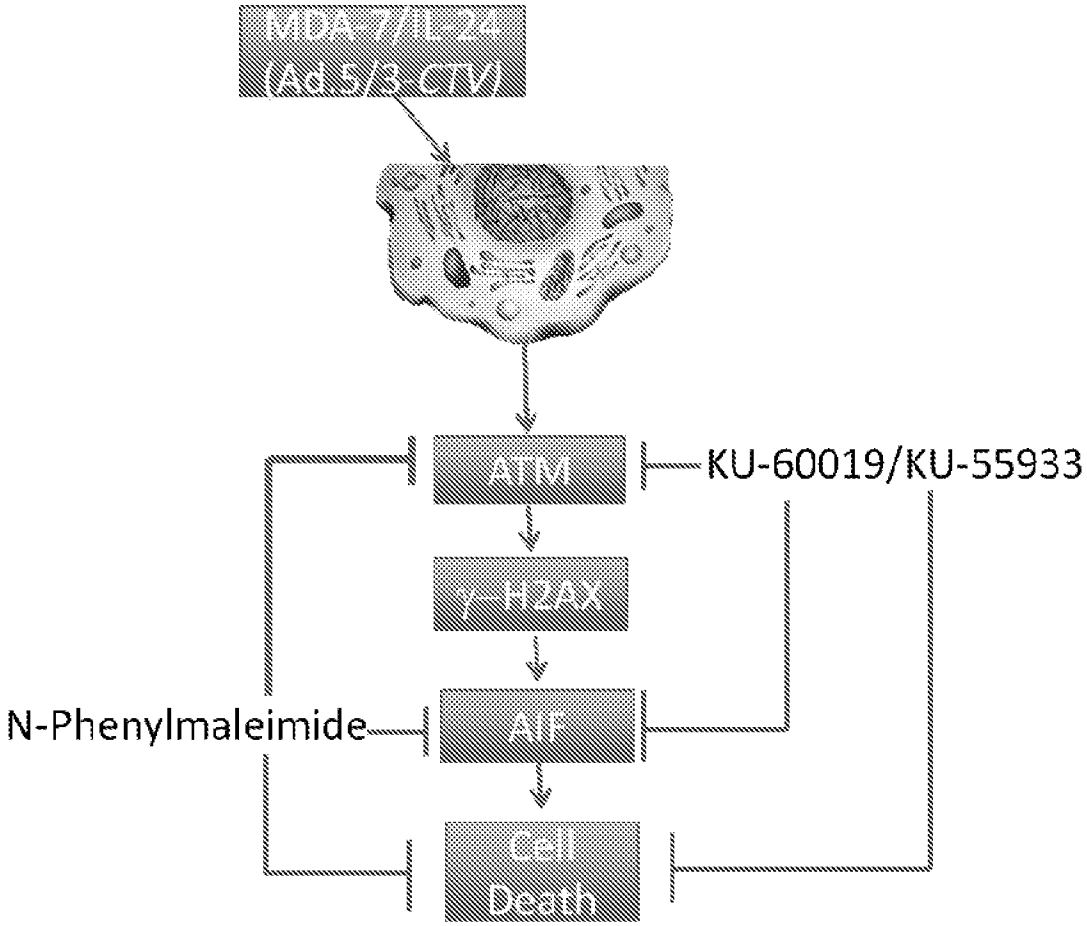

MDA-7/IL-24
(Ad.5/3-CTV)
ATM
KU-60019/KU-55933
γ-H2AX
N-Phenylmaleimide
AIF
Cell
Death

FIGURE 8A

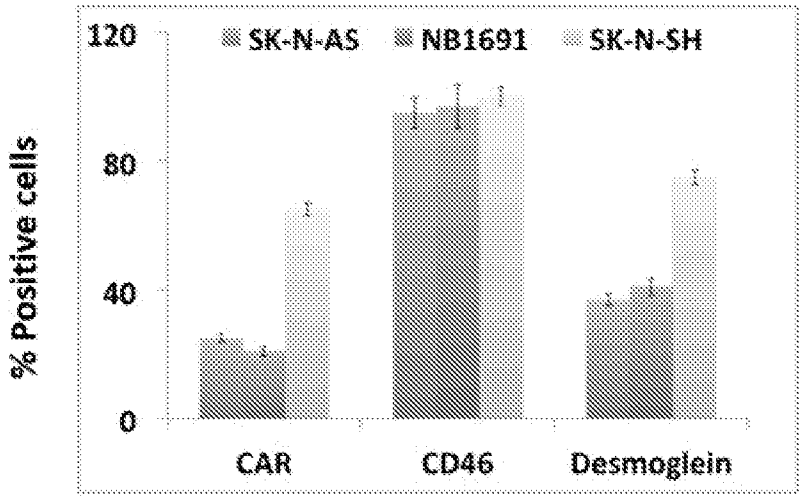


FIGURE 8B

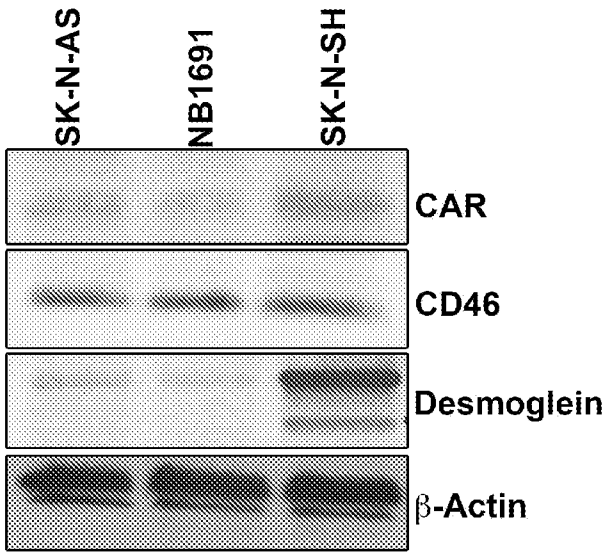


FIGURE 9

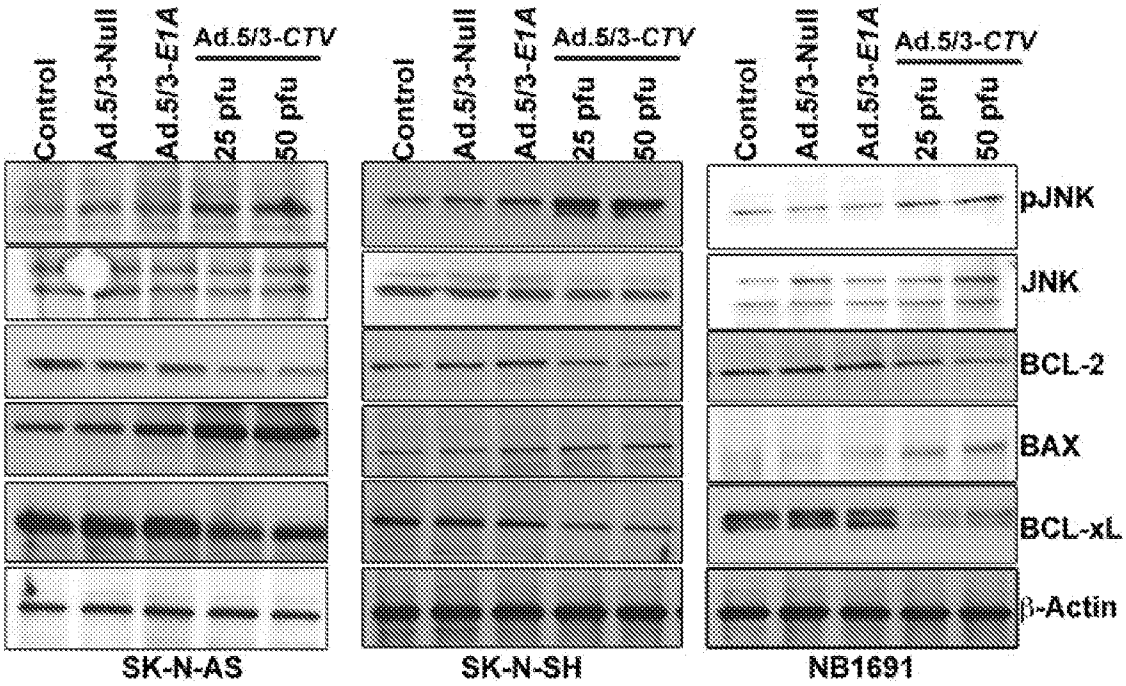


FIGURE 10

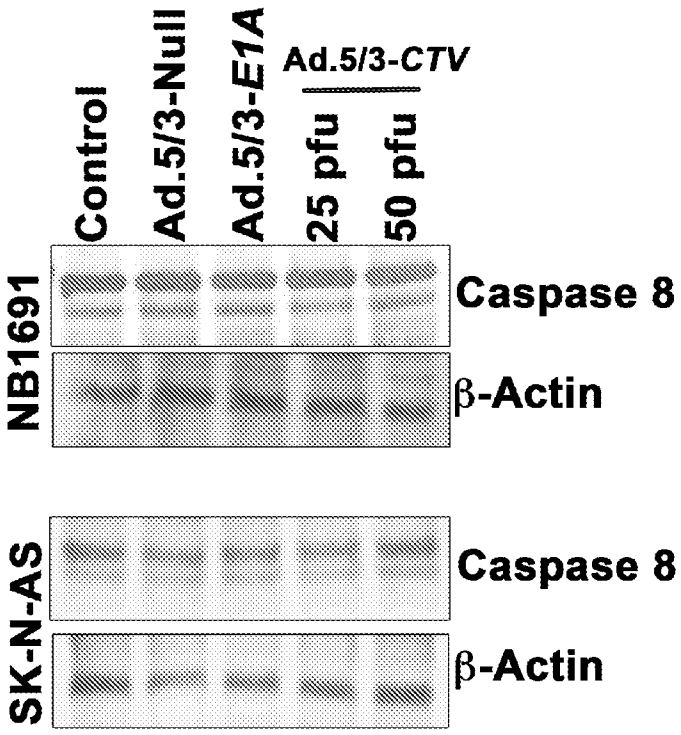
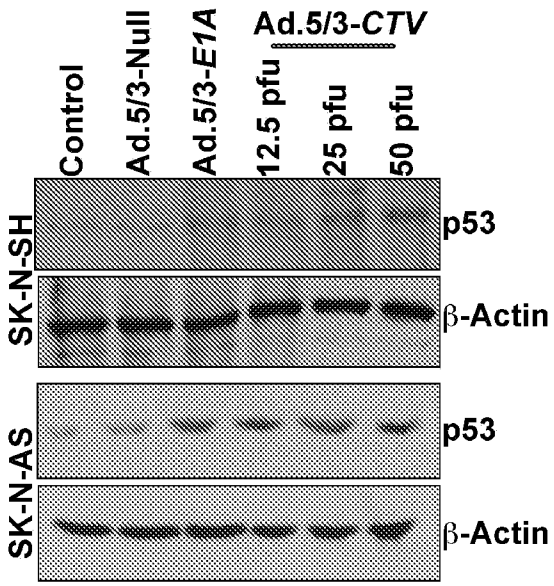


FIGURE 11


Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV
12.5 pfu
25 pfu
50 pfu
SK-N-SH
p53
β-Actin
SK-N-AS
p53
β-Actin

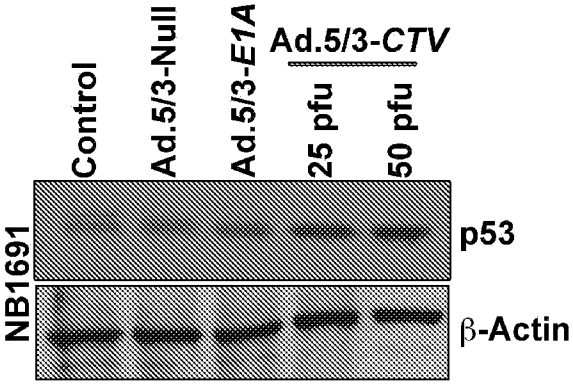

Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV
25 pfu
50 pfu
NB1691
p53
β-Actin

FIGURE 12

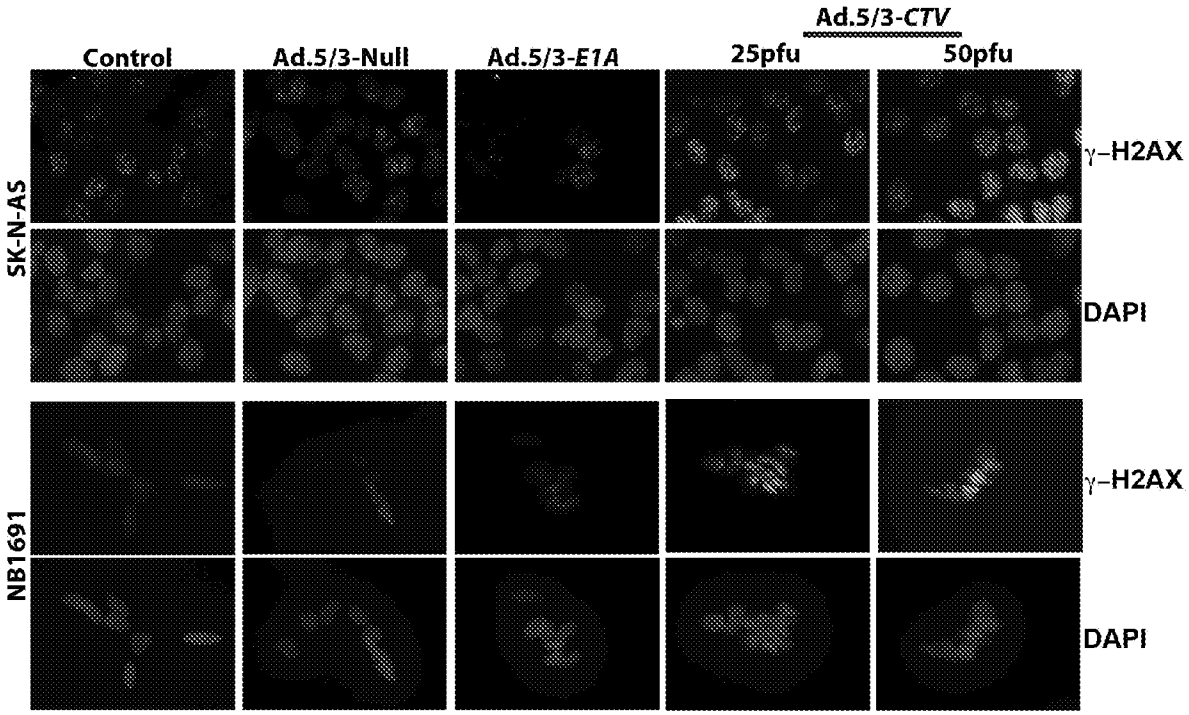


FIGURE 13

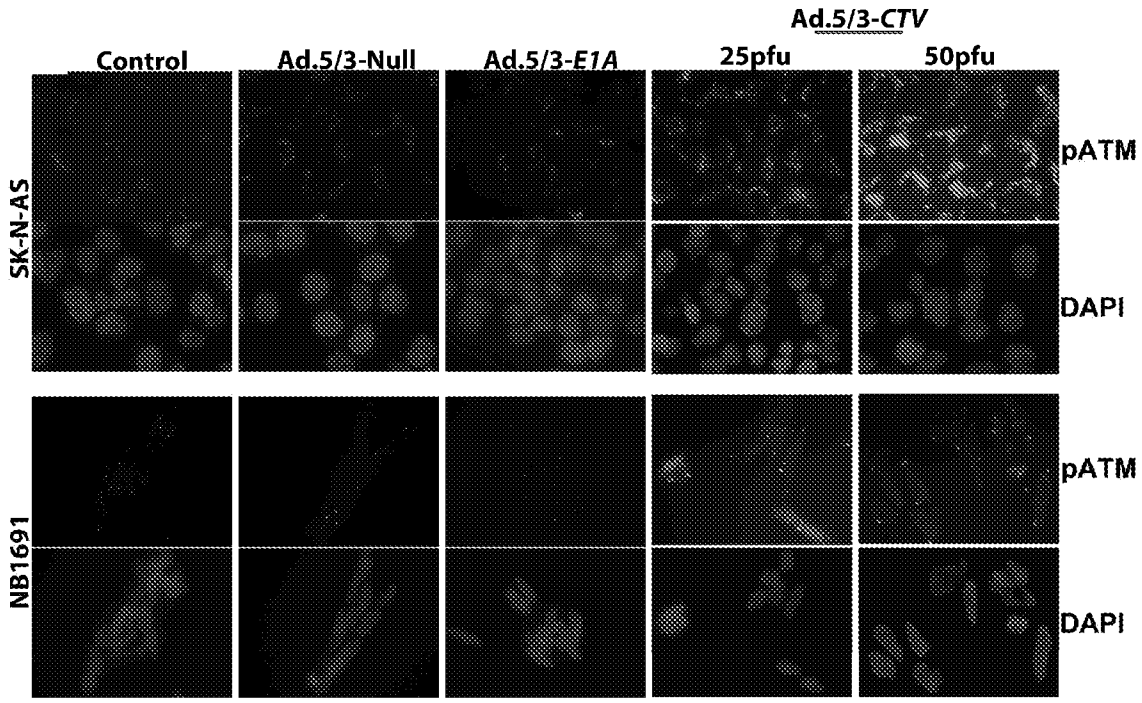


FIGURE 14

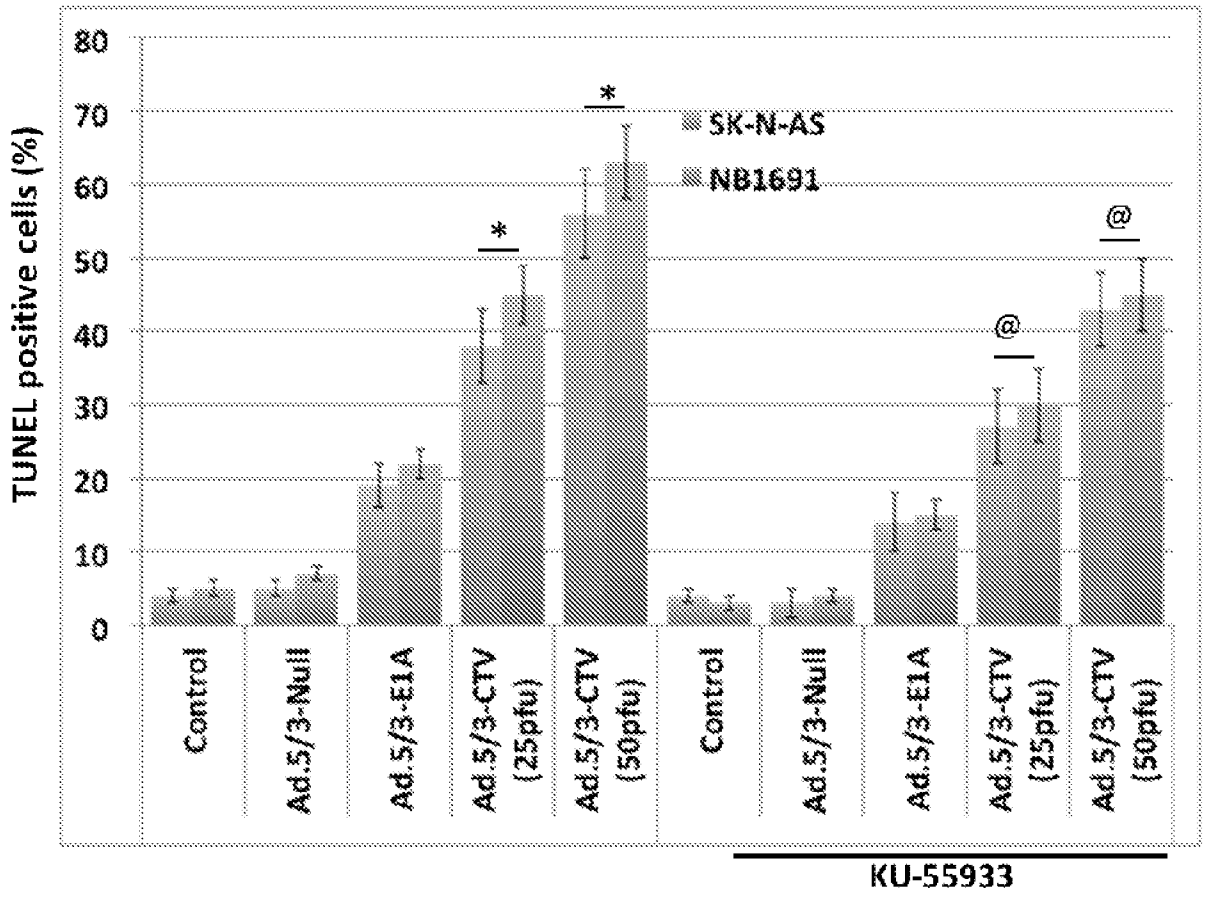

TUNEL positive cells (%)
80
70
60
50
40
30
20
10
0
SK-N-AS
NB1691
*
*
@
@
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50pfu)
Control
Ad.5/3-Null
Ad.5/3-E1A
Ad.5/3-CTV (25pfu)
Ad.5/3-CTV (50pfu)
KU-55933

FIGURE 15

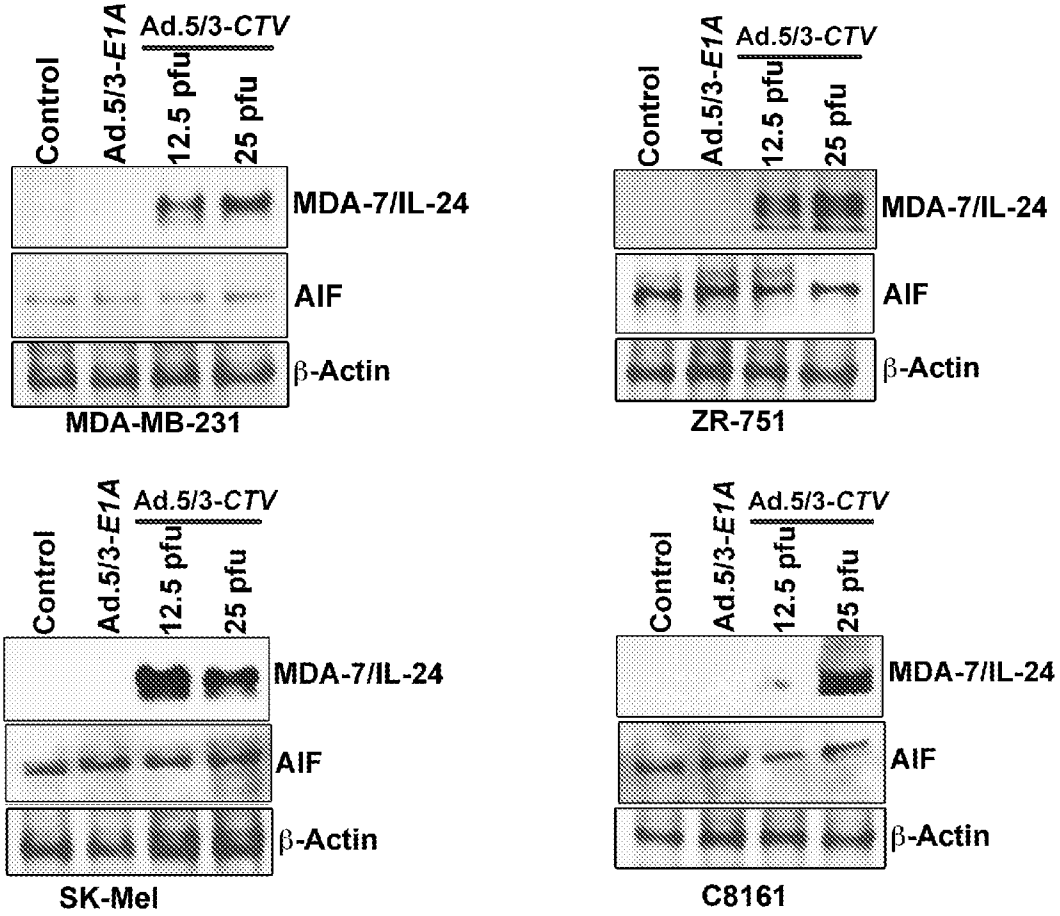

Control
Ad.5/3-E1A
Ad.5/3-CTV
12.5 pfu
25 pfu
MDA-7/IL-24
AIF
β-Actin
MDA-MB-231
ZR-751
SK-Mel
C8161

FIGURE 16

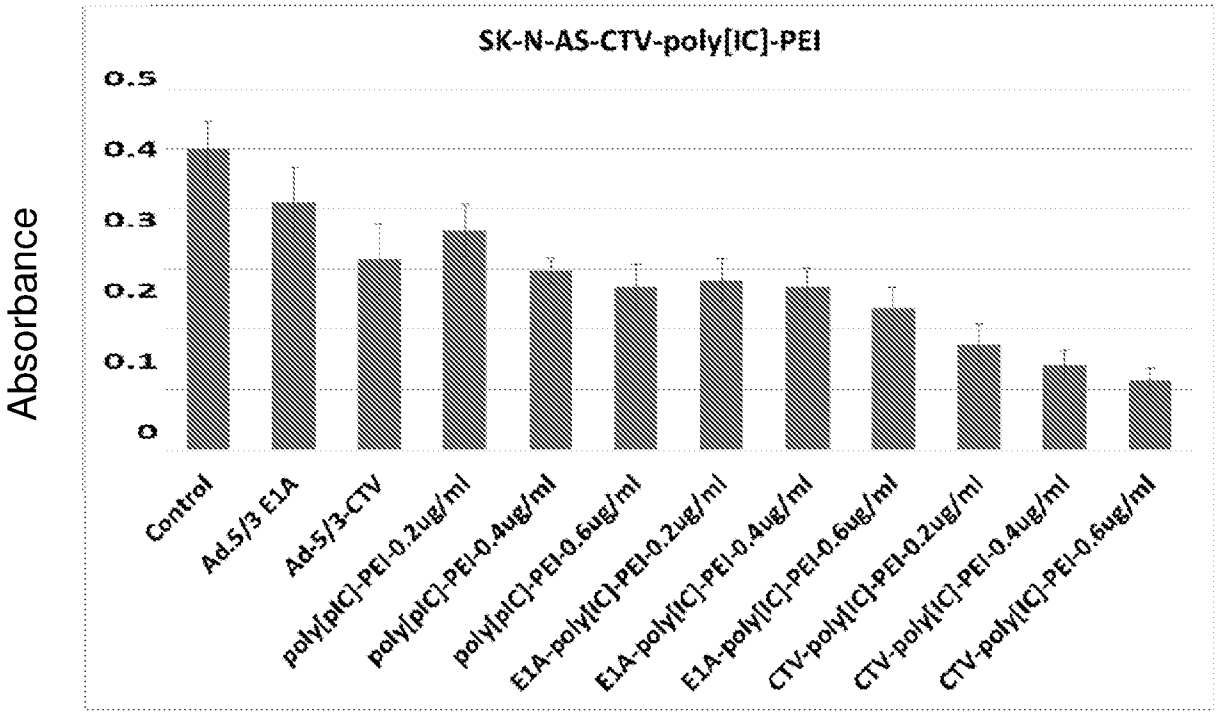


FIGURE 17

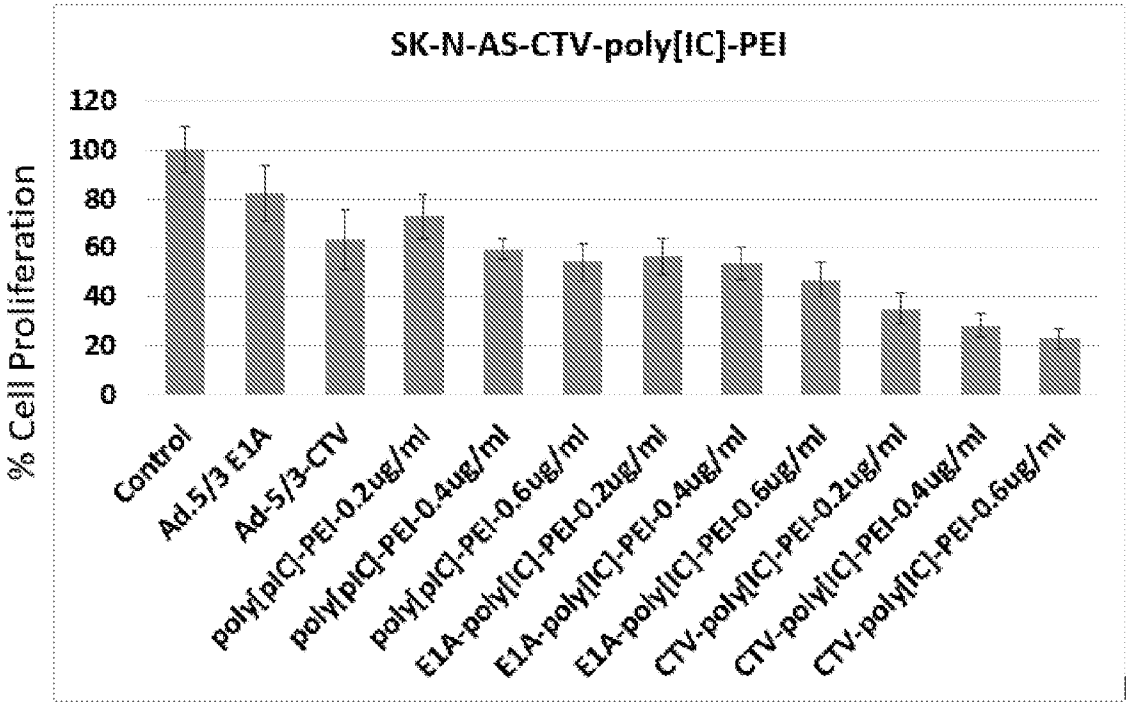


FIGURE 18

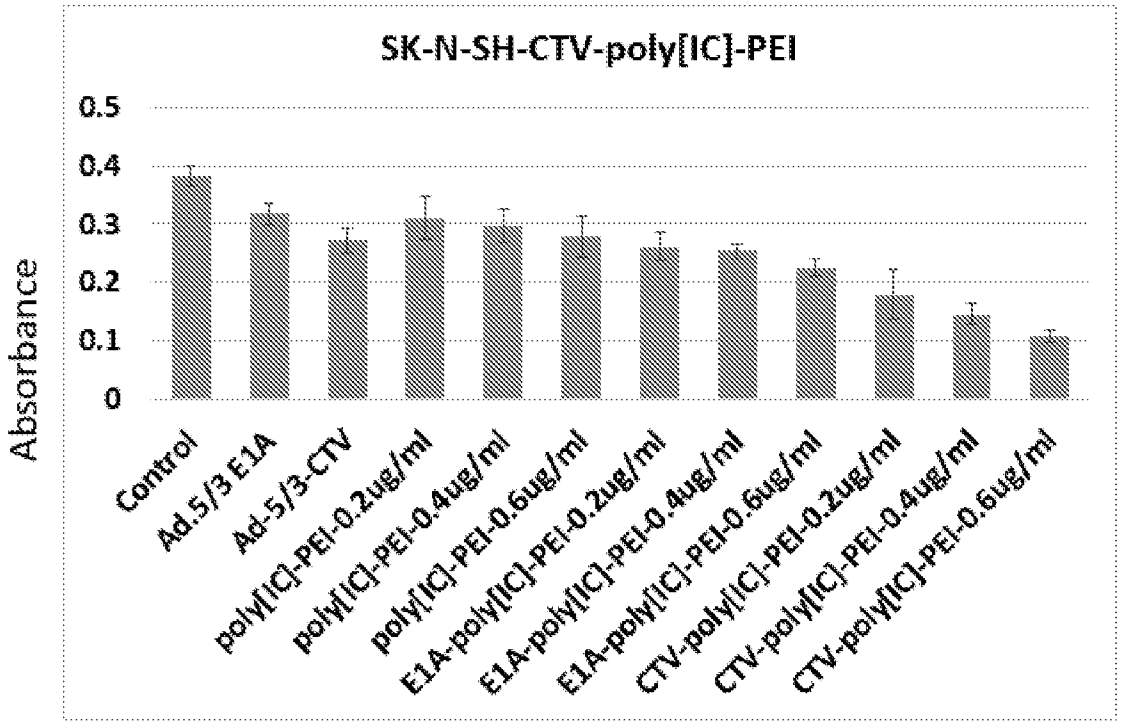


FIGURE 19

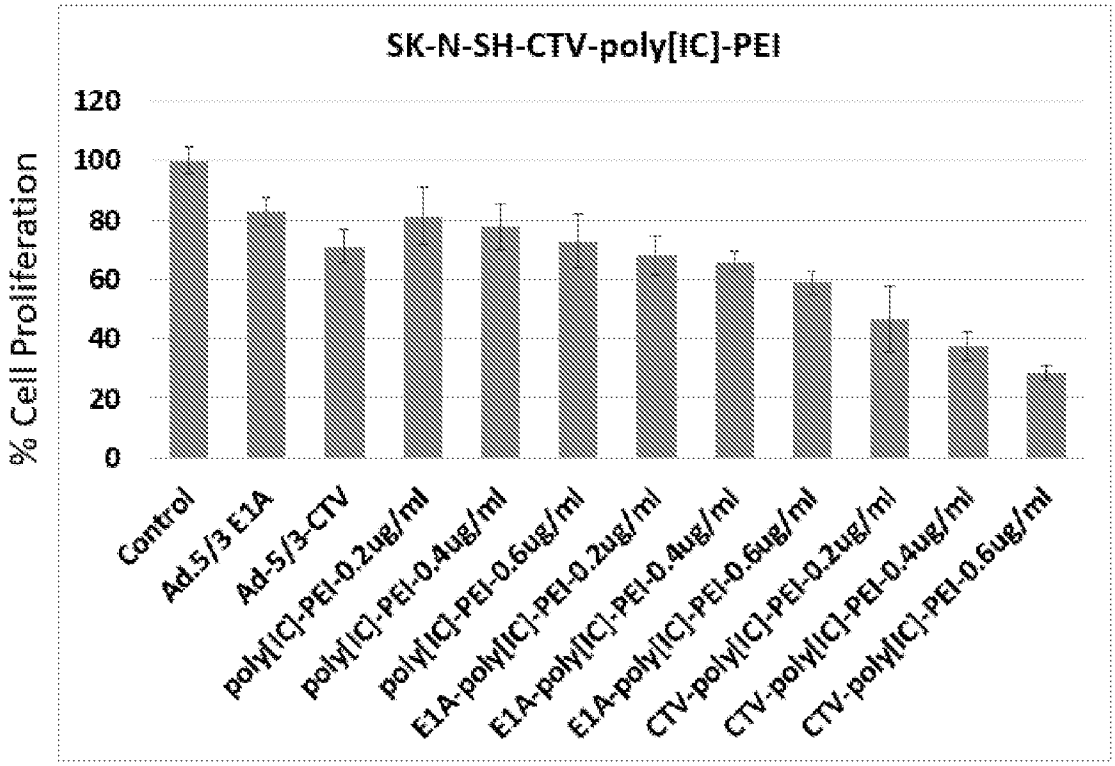
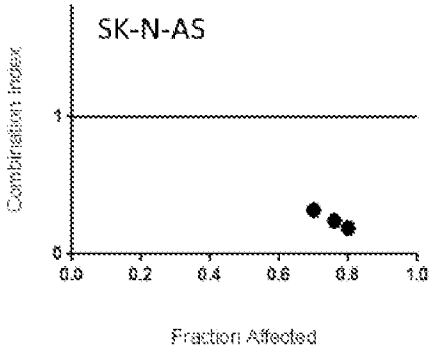
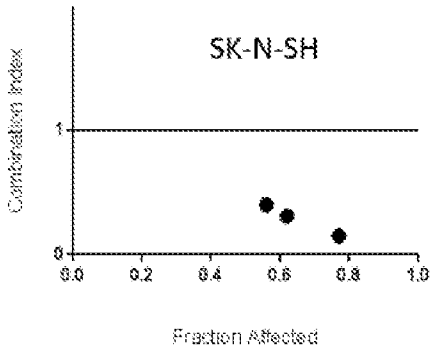


FIGURE 20

Combination Index for CTV-poly[IC]-PEI





Total Dose	Fa	CI Value
12.5IU+0.2ug/ml	0.7	0.32840
12.5 IU+0.4ug/ml	0.76	0.24377
12.5 IU+0.6ug/ml	0.8	0.19497

Total Dose	Fa	CI Value
12.5IU+0.2ug/ml	0.56	0.40
12.5 IU+0.4ug/ml	0.62	0.31
12.5 IU+0.6ug/ml	0.77	0.15

Fa- Fraction Affected and CI-Combination Index
Chou-Talalay combination index

FIGURE 21

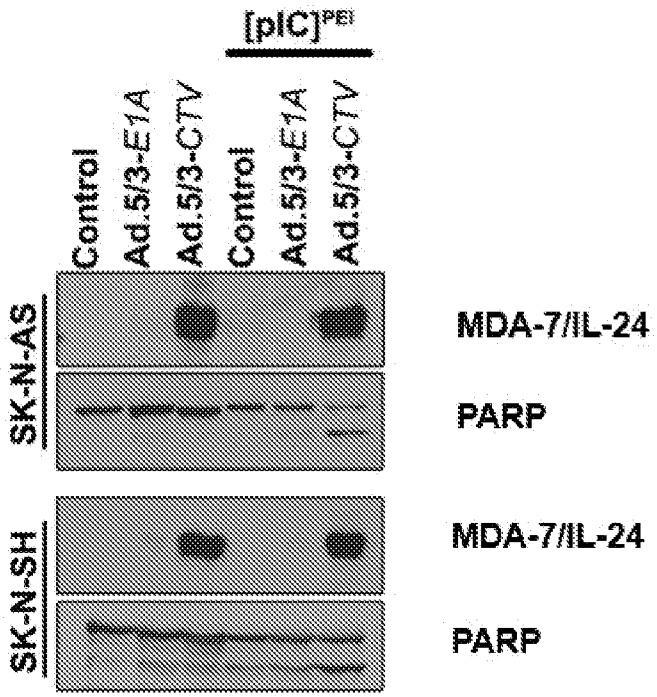


FIGURE 22

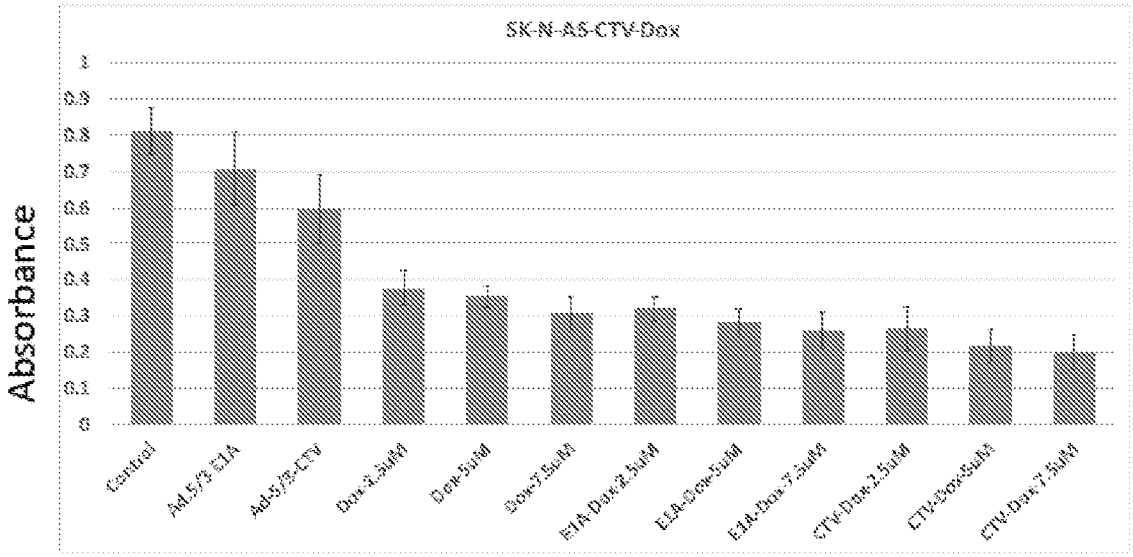


FIGURE 23

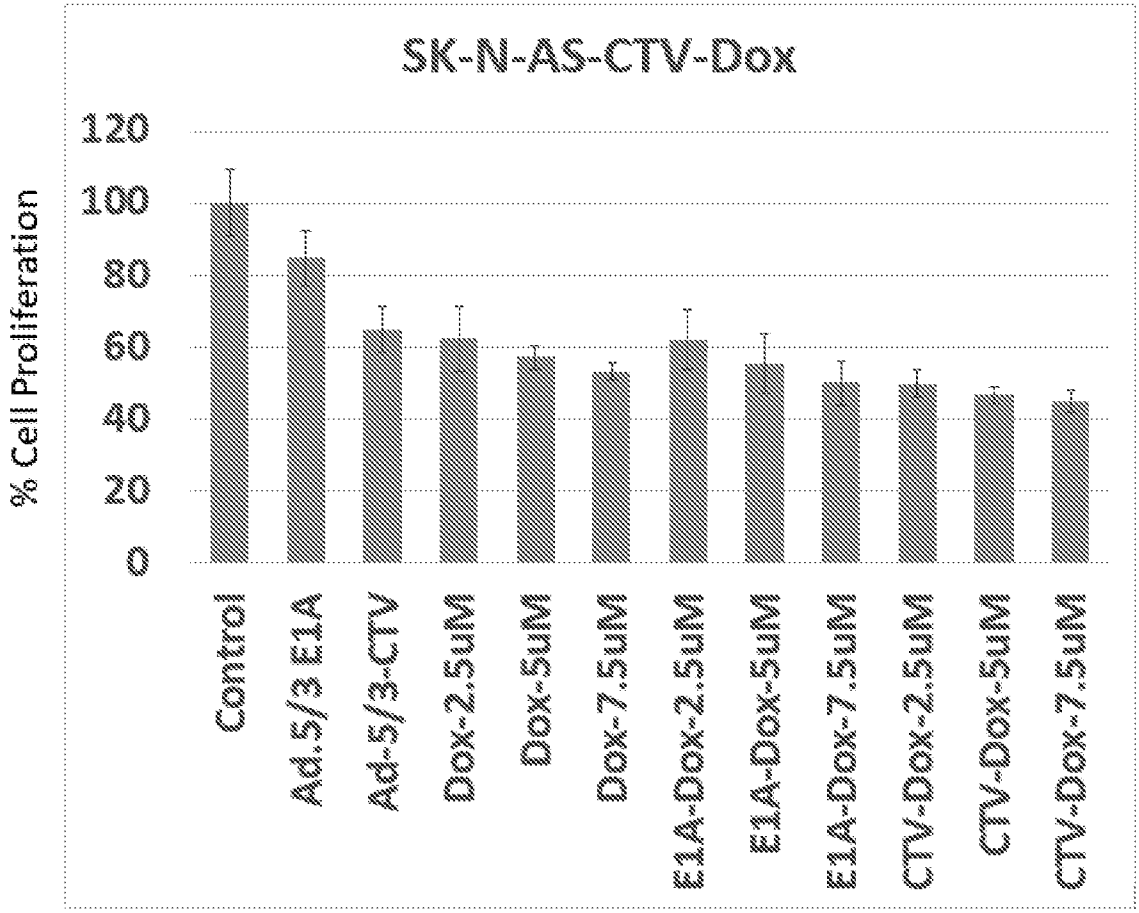


FIGURE 24

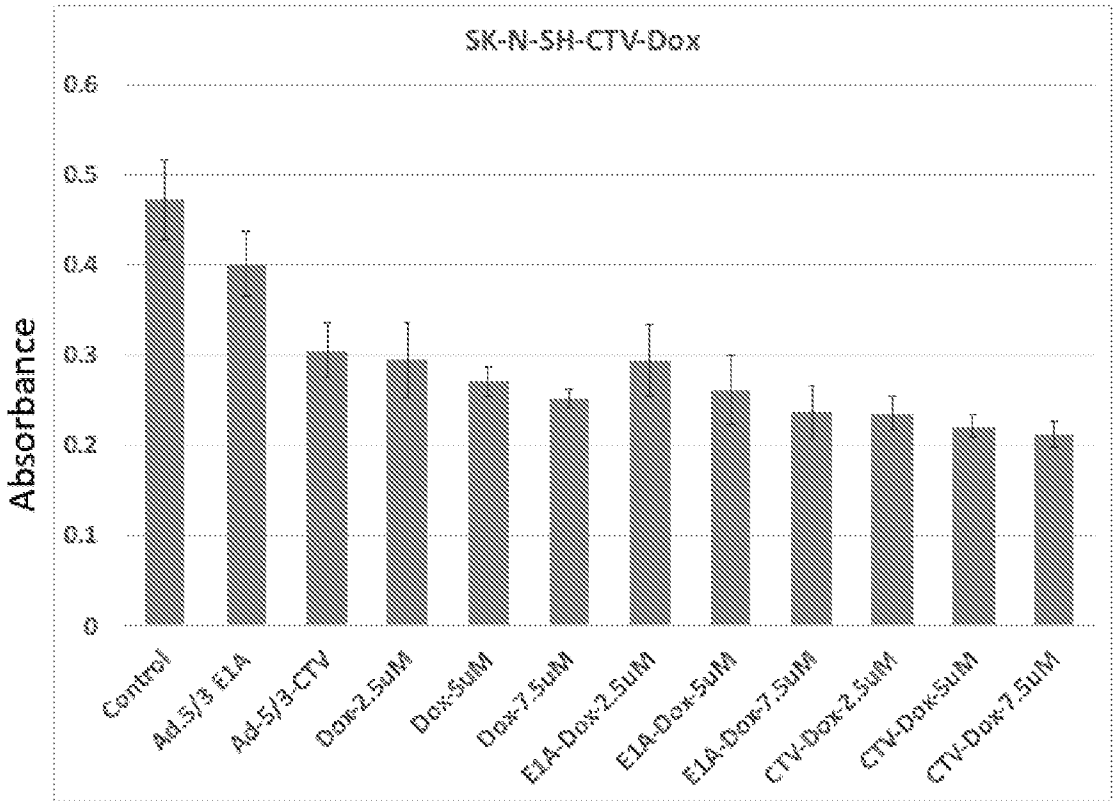


FIGURE 25

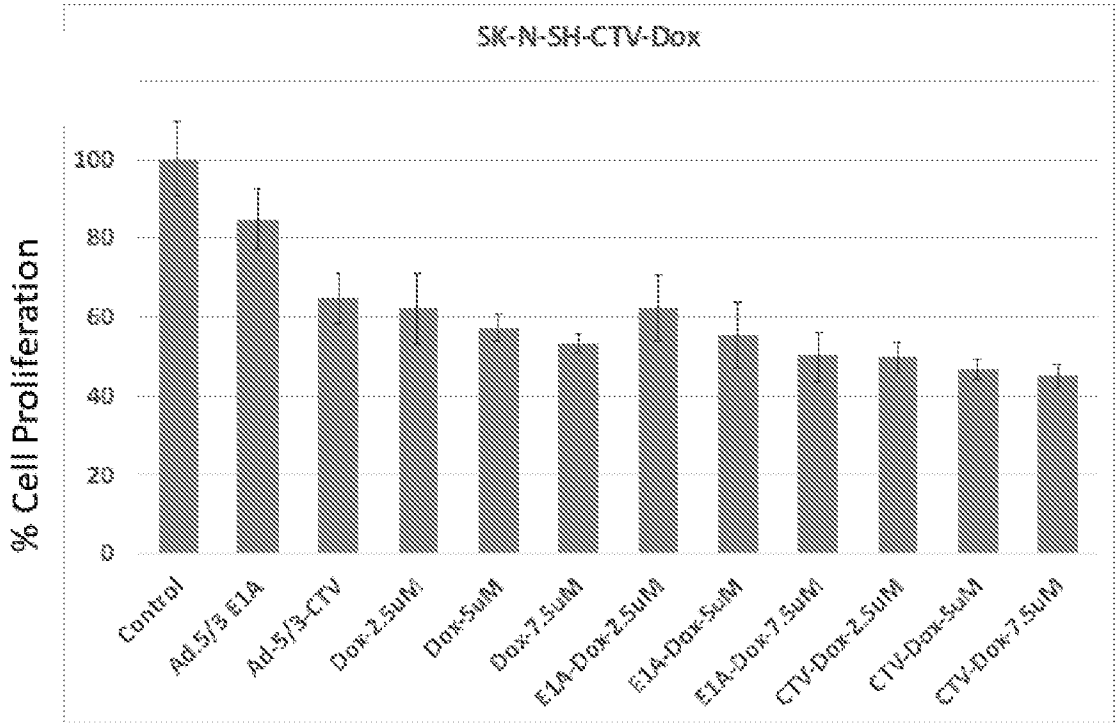
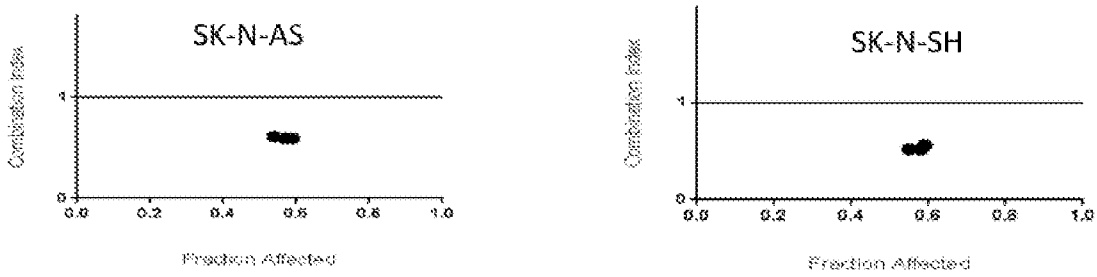


FIGURE 26

Combination Index for CTV-Doxorubicin



Total Dose	Fa	CI Value
12.5 IU+2.5uM	0.54	0.61804
12.5 IU+5uM	0.57	0.59024
12.5 IU+7.5uM	0.59	0.59396

Total Dose	Fa	CI Value
12.5 IU+2.5uM	0.55	0.52
12.5 IU+5uM	0.58	0.52
12.5 IU+7.5uM	0.59	0.56

Fa- Fraction Affected and CI-Combination Index
Chou- Talalay combination index

FIGURE 27

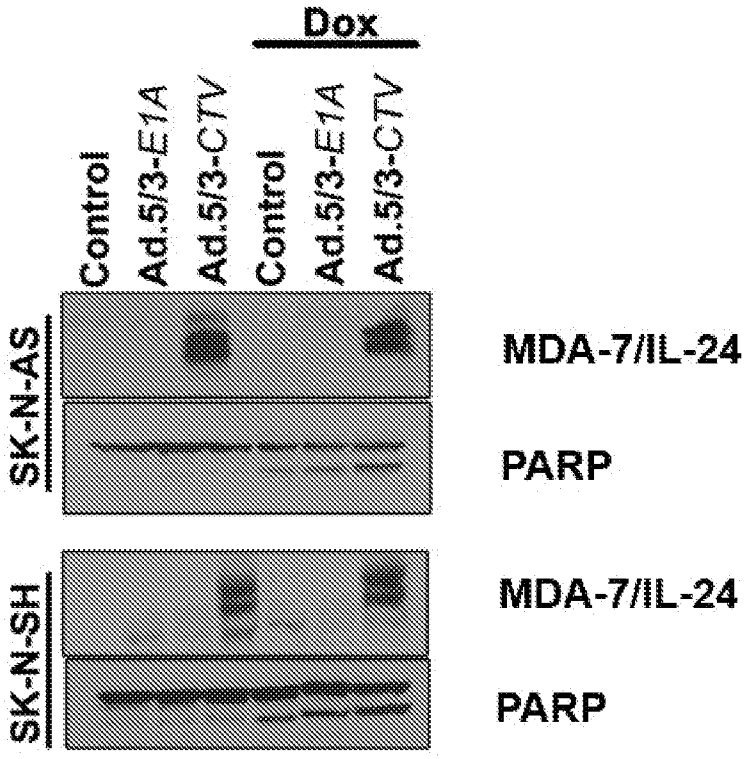


FIGURE 28

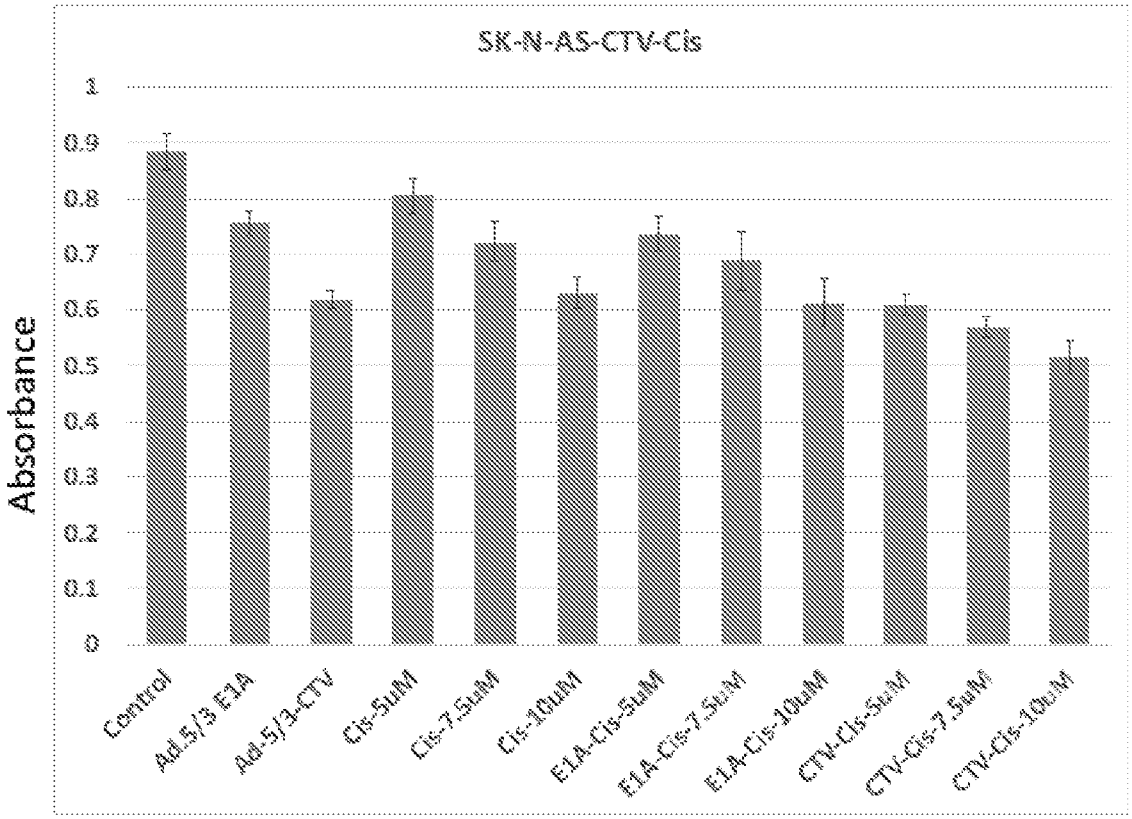


FIGURE 29

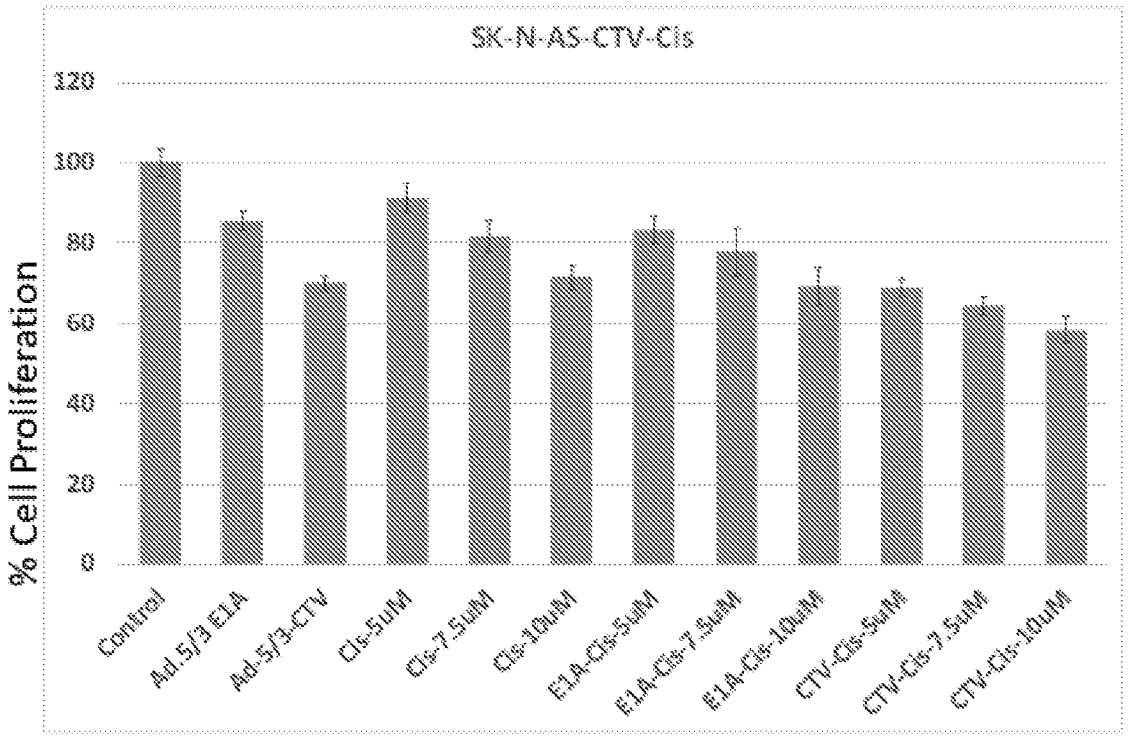


FIGURE 30

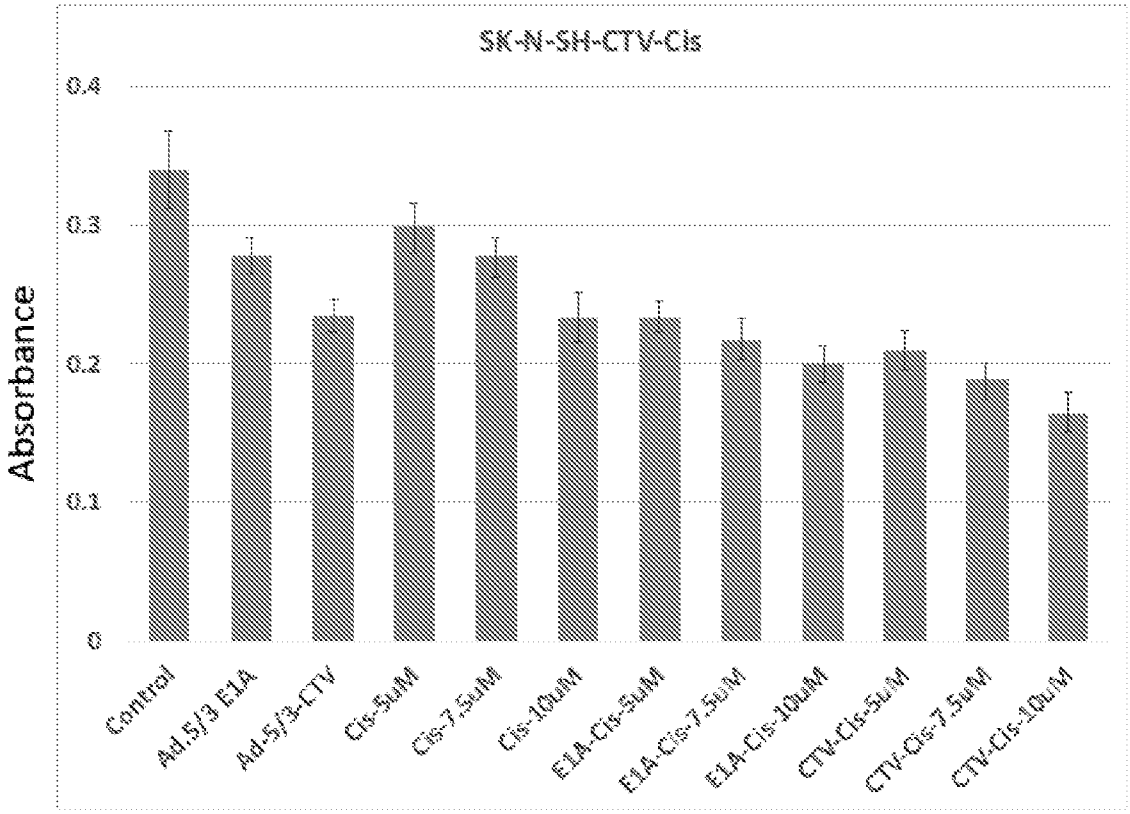


FIGURE 31

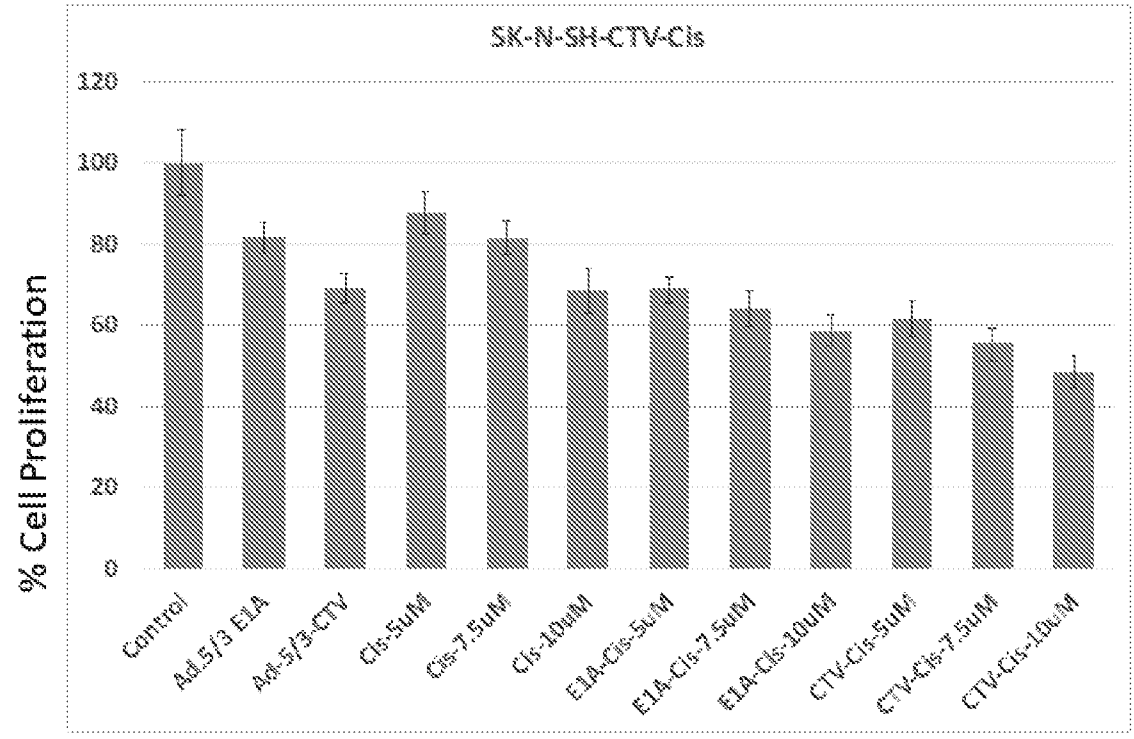
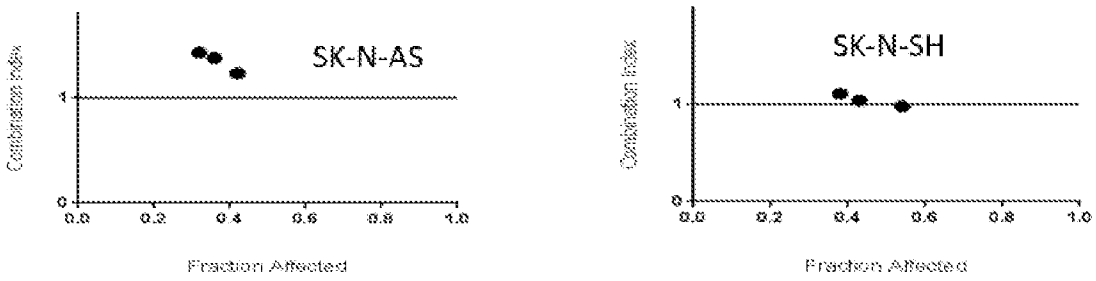


FIGURE 32

Combination Index for CTV-Cisplatin

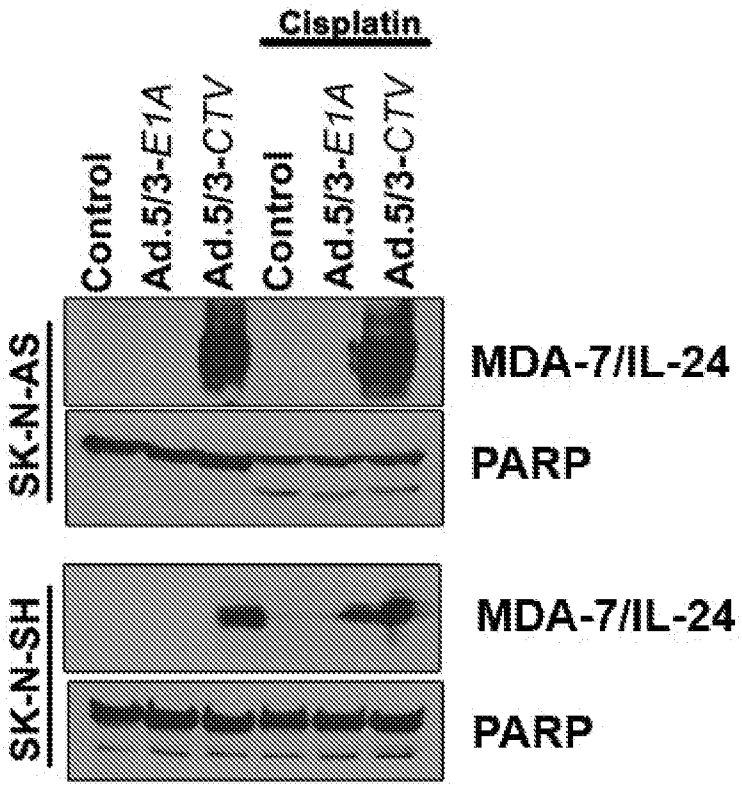


Total Dose	Fa	CI Value
12.5 IU+5uM	0.32	1.43260
12.5 IU+7.5uM	0.36	1.38685
12.5 IU+10uM	0.42	1.23349

Total Dose	Fa	CI Value
12.5 IU+5uM	0.38	1.11
12.5 IU+7.5uM	0.43	1.04
12.5 IU+10uM	0.54	0.98

Fa- Fraction Affected and CI-Combination Index
Chou-Talalay combination index

FIGURE 33



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/025056

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/20; A61K 48/00; A61P 35/00; C07K 14/54; C12N 15/86 (2017.01)

CPC - A61K 38/20; A61K 48/00; C07K 14/54; C12N 15/86; C12N 2799/022 (2017.02)

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)

See Search History document

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

USPC - 424/85.2; 424/93.2; 435/375; 435/455; 536/23.5 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/112470 A1 THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 18 September 2008 (18.09.2008) entire document	1-8, 10-19, 21, 22
Y	US 2016/0008413 A1 (VIRGINIA COMMONWEALTH UNIVERSITY) 14 January 2016 (14.01.2016) entire document	1-19, 21, 22
Y	EP 2708236 A1 (MEDIZINISCHE UNIVERSITÄT WIEN) 19 March 2014 (19.03.2014) entire document	1, 7, 9
Y	WU et al. "Intraperitoneal administration of poly(I:C) with polyethylenimine leads to significant antitumor immunity against murine ovarian tumors," Cancer Immunol Immunother, 28 April 2011 (28.04.2011), Vol. 60, Pgs. 1-20. entire document	1, 7, 9
A	WO 2014/197535 A1 (VIRGINIA COMMONWEALTH UNIVERSITY) 11 December 2014 (11.12.2014) entire document	1-22

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

* Special categories of cited documents:

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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
19 July 2017	14 AUG 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)