A Priori Activation of Apoptosis Pathways of Tumor (AAAPT) Technology 1: Sensitization of Tumor Cells Using Targeted and Cleavable Apoptosis Initiators in Gastric Cancer.

Wu Han[1], Li Yan[1,2,] Raghu Pandurangi[3*]

[1]Department of Oncology, Zhongnan Hospital of Wuhan University, Hubei Key Laboratory of Tumor Biological Behaviors and Hubei Cancer Clinical Study Center, Wuhan, China

[2]Department of Peritoneal Cancer Surgery, Cancer Center of Beijing Shijitan Hospital, The Capital Medical University, Beijing, China.

3. Sci-Engi-Medco Solutions Inc. and Amplexi-LLC, 573, Lexington Landing Pl, St Charles, MO 63303-1750, raghuaa66@yahoo.com

Abstract

Cancer cells develop tactics to circumvent the interventions by desensitizing themselves to interventions. The principle route of desensitization includes activation of survival pathways (e.g. NF-kB, PARP) and downregulation of cell death pathways (e.g. CD95, ASK1). As a result, it requires high dose of therapy to induce cell death which, in turn damages normal cells through the collateral effects. Methods are needed to sensitize the low and non-responsive resistant tumor cells in order to evoke a better response from the current treatments. Current treatments including chemotherapy can induce cell death only in bulk cancer cells sparing low-responsive and resistant tumor cells. Here we report a novel tumor sensitizer derived from the natural Vitamin E analogue (AMP-001). The drug design is based on a novel "A priori activation of apoptosis pathways of tumor technology (AAAPT) which is designed to activate cell death pathways and inhibit survival pathways simultaneously. It involves an inbuilt targeting vector which targets tumor specific Cathepsin B, overexpressed by many cancers including gastric cancer. Our results indicate that AMP-001 sensitizes gastric cancer cells which resulted in expanding the therapeutic index of front-line chemotherapy doxorubicin both *in vitro* and *in vivo* nude mouse model. The synergy between AMP-001 and doxorubicin could pave a new pathway to use AMP-001 as a neoadjuvant to chemotherapy to achieve a better efficacy and reduced off-target toxicity.

Introduction

Tumor cells have a remarkable ability to circumvent endogenous and exogenous toxicities by deactivating cell death pathways and thereby desensitizing themselves to interventions[1]. Defects in apoptosis pathways (e.g. CD95, APO-1/Fas) would make tumor cells insensitive to chemotherapy[2]. For example, loss of CD95 signaling pathway resulted in the development of cancer[3] and resistance to chemotherapy[4]. Restoration of the apoptotic machinery with apoptosis inducing ligands (apoptogens) is an area of active investigation in the drug development. Several agents have been developed to activate TRAIL[5], to downregulate Bcl-2[6] and to restore function of mutated p53 in order to sensitize tumor cells. Similarly, mitogen activated protein kinase MAPK signaling pathways are also involved in desensitization of tumor cells. The advantage of sensitizing tumor technology is to evoke a better response from the existing treatments in terms of reducing the therapeutic dose without compromising efficacy and a potential reduction of dose related off-target toxicity. Consequently, tumor sensitizing agents, potentially can be used as neoadjuvant to chemotherapy to expand the therapeutic index of chemotherapy[7].

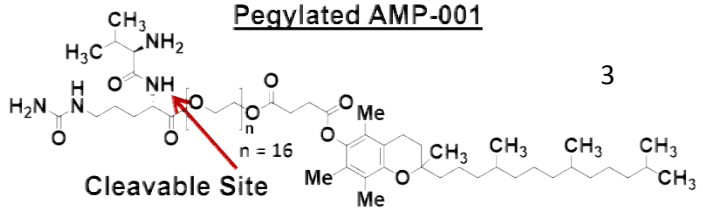
Tumor sensitivity to chemotherapy *in vivo* is shown to be dependent on spontaneous baseline tumor apoptosis index[8]. Aggressive or incurable cancers show low tumor apoptosis index (AI) and low sensitivity to chemotherapy compared to benign cancer[9]. In fact, the spontaneous levels of apoptosis are strong predictor of treatment response[10]. For example, low baseline apoptosis index (AI) of patient tumors (> 67%, see Fig 4 in ref 3g), is directly correlated to non-respondent patients to chemotherapy. Lower the baseline apoptosis index of tumor, least the response from chemotherapy and vice versa (Fig 3 in ref 3h). The overall 5-year survival rates for the patient group with high AI (> 0.97) and low AI (< 0.97, p =0.001) were 89.6 % and 69.2 % respectively[11] indicating that AI could be a potential biomarker of risk stratification of tumors/patients to see who responds better to which treatments.

We have developed a novel technology, “A priori Activation of Apoptosis Pathways of Tumor” (AAAPT) as a strategy to sensitize low responsive tumor cells in order to evoke a better response from chemotherapy[12]. The goal is to understand the molecular biology of desensitization tactics of tumor cells to

bypass the intervention by reactivating specific dysregulated apoptosis pathways selectively in tumor sparing normal cells. Since ubiquitous or systemic activation of apoptosis can induce undesirable neurodegeneration and myelosupression[13], targeting is essential[14].

Gastric cancer remains the second leading cause of cancer-related deaths in the world[15a]. Almost two-thirds of new diagnoses occur in developing countries with 42% in China alone, remaining high in developed countries[15b]. Although, surgery is the main treatment for regressing gastric cancer, the majority patients first diagnosed with gastric cancer have already presented local or distant metastasis. Thus, adjuvant or perioperative chemotherapy and molecule-targeted chemotherapy have been prescribed for gastric cancer, due to their proved benefits in controlling metastasis, reducing cancer recurrence and increasing overall survival[16]. Gastric cancer cells are also known to downregulate CD95 pathway which makes them insensitive to chemotherapy[17]. Hence, we investigate the tumor sensitizing potential of a leading AAAPT molecule AMP-001 in gastric cancer to evoke a better response or synergistic effect with a standard chemotherapy for example, doxorubicin. doxorubicin (dox) is a first-line anticancer agent which is widely used in clinical therapeutic regimens for a variety of cancers including gastric cancer. Nevertheless, the clinical application is limited due to the drug resistance and adverse effects, including cardiomyopathy, typhilitis, and acute myelotoxicity[18]. To decrease dox-induced dose-dependent toxicity, enhancing the effective therapeutic dox dose via combination with nontoxic or selective apoptotic inducing ligands has been investigated[19]. Here, we propose novel targeted tumor sensitizers which addresses how the cancer cells circumvent interventions by reactivating cell death pathways. Since desensitization tactics by cancer cells are successful in thwarting the efficacy of treatments, irrespective of nature of interventions (targets), a fundamental reversal of cancer cells tactics may have high impact on several modes of interventions (e.g. chemotherapy, radiation and immunotherapy). Here, we report to demonstrate the synergistic effect of AMP-001with doxorubicin *in vitro* and *in vivo* in a gastric cancer animal model.

Scheme 1:
Water Soluble Cathepsin B Cleavable Valine-Citrulline-Pegylated AMP-001



Rational Drug Design: The drug design involves pegylation of α-tocopheryl

succinate (apoptogen) with a dipeptide linker valine-citrulline which is cleavable by tumor specific Cathepsin B enzyme giving rise to a final molecule AMP-001(Scheme 1). This design provided impetus for a) targeting apoptogen to cancer cells using tumor specific biomarker Cathepsin B sparing normal cells, b) releasing the drug near tumor sites through the release of drug by cleaving at valine-citrulline link , c) pegylation to make it water soluble, d) enhancing the bioavailability of AMP-001and e) to keep it intact as a pro-drug in blood circulation for a long time to reach tumor sites. The rationale behind using cathepsin B cleavable linkers is based on the better safety profile of Cathepsin B cleavable prodrugs doxorubicin[20a], paclitaxel[20b] and antibodies[20c] (Seattle Genetics) compared to unmodified drugs. Cathepsin B is known to a) be a tumor specific biomarker and b) cleave valine-citrulline substrate[21]. For example, high delineation of tumor from the surrounding tissues by cathepsin B sensitive optical probes[22] shows that this enzyme is restricted to invading cancers. Cathepsin B cleaves AMP-001 at the citrulline-OH bond to release pegylated apoptogen at tumor sites.

Previous studies suggested that α-tocopheryl succinate could destroy mitochondrial function and cause the oxidative damage, resulting in over production of reactive oxygen species (ROS) and apoptosis. Although, parent molecule α-tocopheryl succinate (α-TOS) showed promising anti-tumor properties *in vitro*, studies in an immunocompetent mouse *in vivo* model showed that α-TOS was not only ineffective at the published doses, but resulted in severe side effects due to lack of targeting[23]. Elevated ROS levels can render cancer cells more sensitive to agents that cause oxidative stress[24-25]. There are many anti-cancer drugs such as daunorubicin[26a], cisplatin[26b], paclitaxel[26c] and trisenox[26d] that can augment ROS production in cancer cells, despite their inability to target cancer cells leading to non-specific side effects.

Here, we examined the effect of combining AMP-001 and doxorubicin for a potential synergistic effect in human gastric cancer both *in vitro* and *in vivo*. These results indicate that low dose pretreatment of tumor *in vivo* prepares tumor to make it sensitive to doxorubicin by showing a cumulative and significant tumor regression compared to individual drugs. A potential mechanism of action is being suggested based on the limited data.

Materials and Methods

Cell Culture and Treatment

The human gastric cancer cell lines MGC-803 and SGC-7901 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37℃.

Reagents

The novel compound AMP-001 was designed and synthesized by Sci-Engi-Medco Solutions Inc (SEMCO). AMP-001 was dissolved in distilled water and stored at -20℃ until use. The drug doxorubicin was purchased from Sigma-Aldrich Biotechnology. For all AMP-001/DOX combinational treatment, cancer cells were pretreated with AMP-001 before doxorubicin was added into the culture. All above micromolecular reagents were dissolved and saved according to manufacturer specifications.

Cell Survival and Growth Assay

The detection of cell viability and cell growth were performed with cholecystokinin-8 (CCK-8) assay (Dojindo, Japan). Briefly, 6000 viable gastric cancer cells were seeded in 96-well plates. After specific treatment, each well was mixed with 10 μl CCK-8 and incubated for additional 1h. The OD values were detected at an absorbance of 450nm.

Flow Cytometry Analysis

MGC-803 and SGC-7901 were placed in 12 well plates overnight, and then treated with AMP-001. Cells were then harvested, washed twice with pre-cold PBS, and evaluated for apoptosis by double staining with FITC conjugated Annexin V and Propidium Iodide (PI) kit (Multi Sciences) for 30 min in dark. For cell cycle, harvested cells were labeled with PI (5 mg/ml) in the presence of binding buffer (Multi Sciences) in darkness for 30 min. For JC-1 assay, the treated cells were incubated with JC-1 (Beyotime Institute of

Biotechnology, Nanjing, China) for 30min at 37℃, and then washed twice with PBS. Beckman flow cytometer was used for final detection. Flow Jo vX.0.7 software was used to analyze the data.

Measurement of intracellular ROS level

The intracellular ROS level was measured by flow cytometry as described previously[24]. Briefly, 2×10^5 cells were placed in 12 well plates, allowed to attach overnight, and exposed to the treatment needed. Cells were stained with 10μM DCFH-DA (Sigma) at 37℃ for 30 min with serum free culture. Cells were washed twice with pre-cold PBS and photographed or collected for fluorescence analysis using Beckman flow cytometer. In some experiments, cells were pretreated with 5mM NAC for 2h prior exposure to compounds and analyzed for ROS generation.

Cell Mitochondria Isolation

Fractionation of intracellular mitochondria was performed with Cell Mitochondria Isolation Kit (Beyotime Institute of Biotechnology, Nanjing, China), according to the manufacturer's protocols. First, 1×10^7 cells were collected and homogenized. Density gradient centrifugation at 600g for 10min was applied to remove cellular debris, and supernatants were centrifuged at 11000g for 10min at 4℃ to separate mitochondrial fractions. Then, supernatants containing cytoplasmic proteins were saved and the obtained mitochondria pellet was lysated for further immunoblotting.

Western Blotting

Cells were washed with cold PBS twice and prepared in RIPA lysis buffer, and western blot analysis was performed as described previously[25]. Specific primary antibodies used were as follows: anti-Bcl-2 (50E3), Caspase-9 (C9), Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, Phospho-p44/42 (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2), Phospho-SAPK/JNK (Thr183/Tyr185), JNK2 antibodies were purchased from Cell Signaling Technology (USA). Anti-Bax antibody was purchased from Abcam. Anti-Cytochrome C and COX IV were purchased from Beyotime Biotechnology. Antibody against PARP1 was obtained from Proteintech and Caspase-3 from ABclonal Technology. Anti-GAPDH and β-ACTIN were

147 purchased from Aspen (China). After incubating with the fluorescein-conjugated secondary antibody, the
148 membranes were detected using an Odyssey fluorescence scanner (Li-Cor, Lincoln, NE).

149 **Transmission Electron Microscopy (TEM)**

150 The treated cells were fixed in ice-cold 2% glutaraldehyde, scraped from the plates and post-fixed in
151 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol
152 (30%-100%), and embedded in Epon-812 monomer and polymerized. Ultrathin sections were cut with a
153 diamond knife mounted in a Reichart ultramicrotome, contrasted with uranyl acetate and lead citrate, and
154 examined in a Hitachi HT7700 transmission electron microscope operated at 120 kV[26].

155 **In Vivo Xenograft Assay**

156 Six-week-old athymic BALB/cA nu/nu female mice were purchased from Weitonglihua Laboratory
157 (Beijing, China) and maintained in an Animal Biosafety Level 3 Laboratory at the Animal Experimental
158 Center of Wuhan University. All animal experiments were performed according to the Wuhan University
159 Animal Care Facility and National Institutes of Health guidelines. Approximately 5×10^6 MGC-803 cells
160 were harvested and suspended in 200μl of PBS and Matrigel (BD Bio-science) (1:1) and injected
161 subcutaneously into the right flank of each mouse. After two weeks xenotransplantation, mice were
162 respectively randomized into four groups and treated as follow: AMP-001 (10mg/kg i.p. every other day
163 for 3weeks), DOX (1mg.kg i.p. every other day for 3 weeks), their combination, or saline as untreated
164 vehicle. The size of subcutaneous tumors and mice weight were recorded every two days. The tumor volume
165 (V) was calculated according to the formula $V=0.5\times l\times w^2$, where l is the greatest diameter and w is the
166 diameter at the point perpendicular to l. At the end of treatment, mice were sacrificed, and the tumors were
167 removed and used for immunohistochemical staining.

168 **Immunohistochemical Staining**

169 The xenograft tumor slides were incubated with the following primary antibodies: anti-CD31 was
170 purchased from ABclonal and anti-Ki67 from Cell Signaling Technology (USA). Anti-rabbit or anti-mouse

peroxidase-conjugated secondary antibody (ABclonal) and diaminobenzidine colorimetric reagent solution purchased from Dako (Carpinteria, CA) were used. The staining processes were according to standard methods.

TUNEL Assay

The level of tumor tissue apoptosis in vivo was determined using an TdT-mediated dUTP nick end labeling in situ apoptosis detection kit (Roche, USA) according to the manufacturer's protocol. The pictures were photographed by fluorescence microscopy at a 200x magnification.

Cardiotoxicity Assay

The data were collected from Ionic Transport Assays Inc. In brief, the adult human heart cell line was created by reprogramming an adult human fibroblast cell line by retroviral expression of the reprogramming factors *sox7, oct4, nanog,* and *lin28* using MMLV viral constructs. Cardiomyocytes were derived from this engineered stem cell clone line as follows. Stem cell aggregates were formed from single cells and cultured in suspension in medium containing zebrafish bFGF (basic fibroblast growth factor) and fetal bovine serum. Upon observation of beating cardiac aggregates, cultures were subjected to blasticidin selection at 25 ug/ml to enrich the cardiomyocyte population. Cardiomyocyte aggregate cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum during cardiomyocyte selection through the duration of the culture prior to cryopreservation. At 30 to 32 days of culture the enriched, stem cell-derived cardiomyocytes were subjected to enzymatic dissociation using 0.5% trypsin to obtain single cell suspensions of purified cardiomyocytes, which were >98% cardiac troponin-T (cTNT) positive. These cells (iCell® Cardiomyocytes) were cryopreserved and stored in liquid nitrogen before delivery to Ionic Transport Assays from Cellular Dynamics International, Madison, WI.

Cells were plated into 6 well plates that percolated with 0.1% gelatin. This was defined as culture day 1 for the purpose of this study. Cell plating media was changed at day 3 to cell maintenance media and cell

maintenance media subsequently was changed three times a week. Day 5-7 cells were re-suspended with trypsin and re-plated as desired density (>10,000) at 96 well plate which percolated with 0.1% gelatin.

Sample Preparation: 3.75 mg AMP-001 was dissolved into 0.25 ml water to create a 10 mM stock solution. This stock solution was added to maintenance medium for a final concentration 500 µM which was then diluted to 200 µM, 100 µM, 10 µM and 1 µM AMP-001 solution. 3 mg Dox (Tocris, Cat No. 2252, Cas No.25316-40-9, MW=579.99) was dissolved in 0.5 ml water to create a 10 mM stock solution. This stock was stored in desiccators at room temperature. This stock solution was added to maintenance medium with 200 µM AMP-001 for a final concentration 20 µM which was then diluted to, 10 µM, 1 µM, 0.1 µM and 0.01 µM Dox solution. The CCK-8 kit used in these experiments to determine IC-50 values is a nontoxic, highly sensitive colorimetric assay for the determination of cell viability in cell proliferation and cytotoxicity assays. Raw data were measured on a Spectra Max micro-96 well plate reader and plotted using Prism 5. Transformation, normalization and nonlinear regression were used to analyze data. According best-fit values were used to obtain IC-50. DMSO concentrations less than 1% had no effect on cell viability

Statistical Analysis

All experiments were performed at least three times. Data are presented as means ± SD. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad, SanDiego, CA). One-way ANONVA and Student's t-test were applied to determine statistical significance. A value of $p<0.05$ was considered statistically significant.

Results

1.0 Synthesis of AMP-001

The general synthesis of cathepsin B cleavable peptide conjugation with pegylated apoptogen and/or other apoptogens is accomplished through Fmoc chemistry to protect N end of peptide with Boc and then

couple it with tocopherol derivatives using DCC in DMF. This was further cleaved by TFA and purified using HPLC method. Cathepsin cleavable compounds have been synthesized using the proprietary peptide synthesis technology. In brief, dipeptide valine-citrulline was synthesized using a microwave peptide synthesizer. The resin (containing 0.25 mmol of peptide anchors) was deprotected using piperidine resulting in the formation of the primary amine. The carboxylic acids of the Fmoc protected amino acids (1 mmol) were activated using COMU and conjugated to the primary amines of the growing peptide on the resin. The process of deprotection, activation and conjugation was repeated until the desired peptide was synthesized. Purification of the peptide linked pegylated apoptogen (AMP-001) was performed using a semi-preparative Kromasil C18, 5u column with a flow rate of 5.0 mL/min. HPLC solvents were 0.1 % TFA acetonitrile (solvent A) and 0.1% TFA in water (solvent B) to get off- white powder. The initial gradient A: B, t =0, 10: 90 and t = 30 100 % B. Analytical data: HPLC, R_f = 14.3, single peak with a purity 96.62 %. The compound is characterized using MS with a molecular ion peak at 1967 corresponding to M+H peak. The compound was soluble in water (100mg/mL).

2.0 Cytotoxicity of AMP-001 in Gastric Cancer Cell Lines

To investigate the cytotoxicity of AMP-001 on human gastric cancer cells, cell lines SGC-7901 and MGC-803 were treated with different concentration of AMP-001 *in vitro* for 48 hours and cell viability was detected using CCK-8 assay. As shown in Fig. 1A-B, AMP-001 inhibited the growth of SGC-7901 and MGC-803 cells respectively in a concentration dependent manner. The IC_{50} values of AMP-001 against SGC-7901 and MGC-803 were 14.25 and 16.91 μM, respectively (Fig 1C-D). Cell proliferation rates were also decreased in a dose and time dependent manner as shown in Fig. 1E-F respectively.

Fig 1. A& C: Viability assay for AMP-001 in Gastric cancer cells SGC-7901 and MGC-803 respectively, B&D: IC50 curves, E&F: Concentration and time dependent cell proliferation assay for AMP-001.

3.0 AMP-001 Induces Apoptosis and Potentiates Doxorubicin-Induced Apoptosis in Gastric Cancer Cells.

The pro-apoptotic effect of AMP-001 was assessed using Annexin V/ staining assay. As shown in Figure 2, two gastric cancer cell lines SGC-7901 and MGC-803 showed significant apoptosis after 48h treatment with IC-50 dose of AMP-001. Although AMP-001 itself was able to induce cell apoptosis, the aim is to use it as a tumor sensitizer in conjunction with chemotherapy (e.g. doxorubicin) to see the potential synergy with it. This will enable us to extend the sensitizing potential of AMP-001 to other FDA approved chemotherapeutics used to treat other kinds of cancers. We, thus investigated whether the low dose of AMP-001 could be used in combination with doxorubicin, a widely used chemotherapeutic drug in the treatment of gastric cancer. MGC-803 and SGC-7901 cells were pre-incubated with or without AMP-001 (10 μM) for 8 hrs. and then treated with DOX (1 μM) for 48 hrs. before analyzed by FACS. Pre-incubation of gastric cancer cells with AMP-001 for 8 hours rendered cancer cells much more susceptible to doxorubicin induced cytotoxicity at a concentration at which AMP-001 itself did not cause substantial cell death. Fig 3A shows pictures of gastric cancer cells by transmission electron microscopy before and after AMP-001 treatment where the combination of doxorubicin and AMP-001 showed lower number of cancer cells compared to individual AMP-001 (10 μM) or doxorubicin (1 μM). This was confirmed by the flow-cytometric analysis of quantification of apoptosis, showing that AMP-001 enhanced early cellular damage induced by doxorubicin in MGC-803 and SGC-7901 cells by several folds (Fig 3B). In addition, combination treatment of cancer cells with 10μM AMP-001 significantly reduced the IC_{50} of doxorubicin in SGC-7901 and MGC-803 cells (~ 6.68 and 9.68 fold reduction, respectively) when compared with doxorubicin only treatment (Fig 4 A-B), thus, establishing the synergy between doxorubicin and AMP-001. These results will have a broader connotation that potentially, one can reduce the chemotherapeutic dose clinically without compromising efficacy and in the process, could reduce potentially dose related off-target toxicity. In other words, it is possible to expand the therapeutic index of chemotherapeutics when AMP-001 is used as a neoadjuvant to chemotherapeutics which are currently used for treating cancer.

Fig 2: A-B: Pro-apoptotic Effect of IC-50 dose of AMP-001 in SGC-7901(14 μM) and MGC-803 (17μM) Gastric Cancer Cells Using Flow Cytometry with Annexin and PI Staining. AMP-001 increased cell death significantly in gastric cancer cells.

Fig 3A: Synergy of AMP-001 (10 μM) with Doxorubicin (1 μM) in SGC-7901 and MGC-803 respectively, assessed through transmission microscopy. Combination of AMP-001 and Doxorubicin yielded cumulative cell death by an order of magnitude compared to individual drug treatments. 3B: Synergy of AMP-001 (10 μM) with Doxorubicin (1 μM) corroborated with FACS data. The combination of AMP-001 and doxorubicin showed higher cell death compared to individual drugs.

Fig 4: Quantitative Drift of IC_{50} value for the combination of AMP-001 and Doxorubicin in A) SGC-7901 and B) in MGC-803 cancer cells respectively.

4.0 AMP-001 Enhances DOX-induced Apoptosis through Mitochondrial Dysfunction

As AMP-001 could induce human gastric cancer cells apoptosis at a decent dose, the potential mechanism of cell death through reactive oxygen species (ROS) accumulation was investigated. As shown in Fig. 5 A-B, pretreatment with AMP-001 for 24h before doxorubicin treatment in SGC-7901 and MGC-803 cells caused a significant increase in DCF-reactive ROS than doxorubicin alone. It is well known that the excessive ROS generation causes oxidative stress and impairs membrane potential, resulting in mitochondrial dysfunction[28-29]. Hence, analysis of mitochondrial potential was carried out using polychromatic dye JC-1 (5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidoazolylcarbocyanino iodide) which forms red-orange clusters with high membrane potential mitochondria. while low membrane potential mitochondria show green staining. The results revealed a significant decrease in mitochondrial membrane potential through the enhanced monomer green fluorescence post combinational treatment (12.1%) compared to individuals AMP-001 (2.1%) and doxorubicin (5.7%) (Fig 5C).

Fig 5: A-B: Enhancement of potentiation of reactive oxygen species (ROS) for the combination of AMP-001 and doxorubicin compared to individual drugs, C: Decrease in mitochondrial potential (enhancement of green fluorescence) using JC-1 dye in SGC-7 and MGC-803 cancer cells respectively.

5.0 AMP-001 and Doxorubicin Synergistically Induce Sustained Mitogen Activated Protein Kinase (MAPK) and CD 95 Pathways Activation

The mitogen activated protein kinase (MAPK) signaling plays a critical role in the outcome and the sensitivity to anticancer therapies. MAPK signaling is also associated with various cellular stress and stimuli and has been shown to contribute to induction of apoptosis[30]. P38 MAPK, c-jun N-terminal kinase (JNK) and extracellular-regulated kinase 1/2 (ERK1/2) are the three major kinases in MAPK family. We thus examined whether AMP-001 could enhance the apoptosis inducing effects of doxorubicin through MAPK signaling activation. The activated status of MAPKs, ERK and p-38MAPK was characterized using MAPK antibodies that recognize the dually phosphorylated peptide sequence representing the catalytic core of active MAPK enzymes. In all experiments, cells were preincubated with 10 μM of AMP-001for 8 hrs. before adding doxorubicin and analyzed post 24 hrs. The results revealed clear increases in p-p38 MAPK, p-JNK and p-ERK activity following the combination treatment compared with the control and with AMP-001 alone (Fig 6A). It is to be noted that AMP-001 and doxorubicin have incremental effect on these activities. However, in combination with doxorubicin, there is a significant enhancement in the intensity of the downstream phosphorylated bands. These results suggested that MAPK pathways could play important role in doxorubicin/AMP-001-induced apoptosis. For CD 95 pathway activation assessment, Fas resistant MDA-MB-231 TNBC cells were treated with different concentrations of AMP-001 (5, 10 and 50 μM) and assess the expression of CD95 (43 KDa) by Western blotting. Isolation of membrane proteins after cell lysis showed concentration dependent CD95 expression compared to untreated control (Fig. 6B). Translocation of CD95 from cytosol to the membrane is a common observation for CD95 Trail synergy[31] which influences the cellular sensitivity to Fas death receptor pathway.

Fig 6A: Potential Mechanism of Action for AMP-0012. Activation of MAPKs signaling pathway during AMP-001 and Doxorubicin combination treatment induced apoptosis, 6B: Expression of CD95 band in post treated triple negative breast cancer cells MDA-MB-231 by AMP-001. CD95 pathways is associated with the sensitization of tumor cells.

6.0 AMP-001 Amplifies the Therapeutic Effect of Dox in a Xenograft Gastric Cancer Model in Nude Mice

The sensitizing potential of AMP-001 has paved the way for evoking a better response from chemotherapy, particularly doxorubicin *in vitro*. Hence, it is may be essential if the same thing is true in an *in vivo* situation. Doxorubicin either alone or in combination with AMP-001 were injected i.p in a xenograft gastric cancer model in nude mice. MGC-803 cells were implanted subcutaneously in the right flank of nude mice. After a week, the mice were randomized into 4 groups and treated as described in the experimental protocol. Animals were sacrificed after 3 weeks treatment. We found that low dose (10 mg/Kg) AMP-001 alone did not inhibit the growth of the tumor at that dose compared to control, while doxorubicin alone (1mg/Kg) inhibited tumor growing to a certain degree. However, the combination of AMP-001 and doxorubicin was more effective in reducing tumor burden (Fig 7A). The tumor volume and tumor weight in the combinational group was significantly lower than either doxorubicin or AMP-001. (Fig 7B, $p < 0.001$). Additionally, all mice did not undergo obvious body weight loss or abnormal symptoms in drug treatments (Fig 7D). In alternate studies on AMP-001 in triple negative breast cancer model, we have shown that high dose of AMP-001 (~200 mg/Kg) alone can have significant tumor regression with no observable toxicity, despite higher dose[12]. Similarly, in the current studies also, there was no evident histopathological abnormalities were observed in the vital organs such as heart, liver and kidney estimated by HE staining (Fig 8). Ki67 (marker for cell proliferation) and CD31 (marker for micro vessel density) were also examined by immunohistochemistry in the excised tumor sections. Although, doxorubicin also downregulated the Ki67 expression, the combination group was most effective similar to the tumor regression studies. The trend of CD31 expression was consistent with Ki67 (Fig 8). In order to determine whether AMP-001/Dox combination treatment effectively promotes apoptosis in tumors, sections of

342 excised tumors were also subjected to TUNEL assay. The results showed AMP-001 together with
343 doxorubicin treatment had more TUNEL positive cells compared to either doxorubicin or AMP-001 (Fig
344 8). In summary, the combination of doxorubicin and AMP-001 yielded a superior response compared to
345 either AMP-001 or doxorubicin in xenograft gastric cancer animal model.

346 Fig 7: Synergy betwwen AMP-001 and Doxorubicin in xenograft gastric cancer nude mouse model:
347 A: Dose dependent tumor size for untreated control, AMP-001, Doxorubicine and Combination of AMP-
348 001 and Doxorubicin, B: Classic tumor V curve for tumor regression, C: Statistical tumor weight reduction
349 comparison, D: Mouse body weight measurements post treatment.

350 Fig 8: Representative immunohistochemical analysis of CD31, Ki67 and apoptosis in tumor sections
351 Ex Vivo by TUNEL staining by combination of AMP-001 and Doxorubicin

352 **7.0 Cardiotoxicity of AMP-001 Compared to Dox**: The photomicrograph of cells in control experiment
353 when the iPSCs are treated with 0.8% DMSO (Fig 9A) showed normal, beating cardiomyocytes. The
354 treatment of the same cells with 50 0r 100 mM of AMP-001 did not change the morphology of
355 cardiomyocytes, while 10 mM of dox induced significant cell death, shown by the disintegration of
356 cardiomyocytes (Fig 9C). This pattern of normal beating for cardiomyocytes was seen at all
357 concentrations including 250 μM AMP-001 (Fig 9D). AMP-001 showed IC-50 greater than 250 μM,
358 compared to dox at 9.6 μM (25 times less, Fig 9F) which indicates a better safety profile for AMP-
359 001. Even FDA approved drug (Sorafinib ~ 40 μM) has lower IC-50 compared to AMP-001 (Fig 9E).
360 It is to be noted that IC-50 for cardiomyocytes has to be high (measure of safety) in contrast with IC-
361 50 for cancer cells which should be low (measure of potency). AMP-001 also showed a high potency
362 for cancer cells since IC-50 for MDA-MB-231 triple negative breast cancer cells to be 32 μM (Fig
363 9E). In essence, AMP-001 could be a potent antitumor agent with a better safety profile compared to
364 dox.

Fig 9. Photomicrographs of treated with A) control DMSO, B) 50 □M AMP-001, C) 10 μM Dox, D) 100 μM AMP-001 and E) IC-50 measurements for Vitamin E, AMP-001, Sorafinib, Dox in induced pluripotent stem cell cardiomyocytes (iPSc) compared with AMP-001 in MDA-MB-231 triple negative breast cancer cells and F) IC-50 of Dox in iPSc cells.

8.0 Discussion

Due to the lack of early detection, human gastric cancer remains as the second most common cause of death from cancer worldwide[32]. Chemotherapies have become inevitable despite high side effects. However, the therapeutic efficacy decreases when cancer cells develop resistance to chemotherapeutics. Combinational chemotherapies have been a mainstay in the treatment of disseminated malignancies for a long time[33-35] and have been widely used for minimizing acquired resistance, enhancing cell-intrinsic drug synergy with chemotherapy or for maximizing cumulative drug dose[36a]. One of the main reasons for tumor cells to develop resistance is downregulation of cell death pathways by cancer cells to apoptosis. New methods are required to activate these dysregulated cell death pathways and to sensitize the low-responsive resistant tumor cells which are otherwise non-responsive to any therapy.

The present study proposes targeting the chemotherapeutics using a tumor specific biomarker Cathespin B. Cathepsin B is overexpressed in many cancers including gastric cancer making it a unique biomarker of cancer to which drugs can be targeted[21]. Valine-citrulline dipeptide is one such linker which is cleavable by Cathespsin B[20]. The drug design AMP-001 has to take into consideration the release of apoptogen (apoptosis inducing ligand) at tumor sites in order to be selective to cancer cells. The pegylation of the basic apoptogen core is expected to increase water solubility and make it stable in the circulatory blood for a long time to reach the tumor[37]. The parent molecule α-tocopheryl succinate (α-TOS), although showed high promise in both *in vitro* and *in vivo*, low solubility in water and a serious toxicity in immunocompetent animal model limited its utility[23]. This observation could explain the lack of clinical

388 product so far for α-TOS. This prompted us to redesign apoptogen keeping in mind with a targeting moiety,
389 selectivity and high solubility in water.

390 Most clinically used chemotherapeutics are not selective to cancer. Moreover, these drugs do not
391 address the fundamental aberrations of cancer cells ability to circumvent interventions by dysregulating cell
392 death pathways and desensitize themselves to intervention, irrespective of nature and/or mode of
393 intervention. Hence, our first approach was to sensitize the low or non-responsive tumor cells by activating
394 CD95 pathway, one of the major dysregulated cell death pathways. The second approach was to take
395 advantage of activation technology to ascertain if it entitles synergy with the standard chemotherapeutics.
396 The significant decrease of IC_{50} for the combination of doxorubicin and AMP-001 for gastric cancer cells
397 (Fig 3-4) reveals that lower dose of doxorubicin could be clinically useful if AMP-001 is used as
398 neoadjuvant to chemotherapy. The tumor cells were pretreated for 6-12 hrs. depending upon cancer cell
399 type and allow the activation of CD95 before we administer chemotherapy. Several vitamin E (VE)
400 analogues, such as α-TOS, α-TOH and VES have been documented as combinations with
401 chemotherapeutics, such as doxorubicin, statins[38], gefitinib[39], celecoxib[40], SU11274[41], GW966[42], oridonin[43]
402 and baicalein[44] for potential synergetic effects. However, our leading compound AMP-001 is different from
403 the others in terms of ability to target cancer cells sparing normal cells. Activation of cell death pathways
404 in normal cells, for example in brain leads to neuro degenerative diseases[13] and hence, targeting is essential.

405 Our preclinical studies *in vitro* and *in vivo* revealed that AMP-001 has dual effects; at low dose it acts
406 as a tumor sensitizer inducting a reasonable cell death while, at higher doses it shows anti-tumorigenicity
407 on its own with little or no observable toxicity in non-target organs[12]. However, for our current studies we
408 used AMP-001 as a tumor sensitizer. The main objective is to enhance the efficacy of the currently used
409 chemotherapeutics using AMP-001 as a neoadjuvant to chemotherapy. The evidence showed that AMP-
410 001 synergized with doxorubicin and thereby, circumvents the cells which are resistant to doxorubicin in
411 human gastric cancer cells. Since doxorubicin is a first-line chemotherapeutic agent in clinical cancer
412 treatments but limited in its use due its cardiotoxicity, the combination of doxorubicin and AMP-001 may

413 be more effective in the clinical setting compared to doxorubicin alone in order to reduce the off-target
414 toxicity and to overcome cancer drug resistance.

415 In order for a potential clinical translation of AAAPT technology, we have studied the tumor regression
416 *in vivo* using triple negative breast cancer xenograft model so that the synergistic potential of AMP-001
417 could be extendable to *in vivo* situations. Doxorubicin has been combined with many other drugs for a
418 potential synergistic effects[19]. However, AMP-001 has a unique ability to sensitize tumor cells at low dose,
419 while inducing tumor regression at higher doses. In our alternate studies, we have shown that at 200mg/Kg
420 dose in a triple negative breast cancer xenograft model tumor regression was more than 80 percent
421 compared to control[12](supplementary data Fig. 1) Here, in this study we have shown that the combination
422 of AMP-001 and doxorubicin showed a synergistic tumor regression ($p < 0.005$) compared to individual
423 drugs (Fig 7). We have fixed AMP-001 at a lower dose and used it as a sensitizing agent rather than as an
424 anti-tumor agent. The combination treatment also showed non-significant changes in weight loss suggesting
425 a favorable toxicity (safety) profile for the combination. Conventional doxorubicin treatment, despite being
426 anti-tumorigenic riddles with cardiotoxicity issues. Hence, we conducted studies on the relative toxicity
427 profile of AMP-001 and doxorubicin in human induced pluripotent stem cell cardiomyocytes (iPSCs) using
428 a proprietary assay developed by Ionic Transport Assays Inc. Adult human induced pluripotent stem
429 cell-derived (hIPSC) cardiomyocyte technology, such as iCell ^{R} Cardiomyocytes, offers the
430 opportunity to accelerate the development of new therapeutic agents by providing a relevant human
431 target for efficacy and safety without going through the costly animal studies. Our results show that
432 the conventional chemotherapy such as doxorubicin and sorafinib showed 9.9 μM and 40 μM
433 respectively while, AMP-001 was greater than 250 μM (Fig 9F and E) indicating a better safety profile
434 for AMP-001. It is to be noted that AMP-001 showed lower IC-50 (32 μM) for TNBC cells indicating
435 that the efficacy is as good as many FDA approved chemotherapeutics. The IC-50 values corroborated
436 well with the photomicrograph of cardiomyocytes treated with doxorubicin showing significant cell
437 death compared to control DMSO and 100 μMAMP-001 (Fig 10 C Vs A and D).

438 The possible mechanism of AMP-001 synergetic effect was also explored. Combination of AMP-001
439 and doxorubicin induced increased intracellular ROS level, determined by the shift of DCFCA fluorescence
440 peak to right (Fig 5A-B). The selectivity of AMP-001 to cancer is due to the targeting vector valine-
441 citrulline dipeptide which is cleaved by tumor specific Cathepsin B. However, recent studies on ROS
442 mediated damage to cells also indicates, the kinetics of repair is almost twice faster in normal cells
443 compared to cancer cells[46]. Thus, enhancing ROS selectively in cancers could be a better strategy since
444 normal cells gets repaired faster than cancer cells and targeting helps minimizing affecting normal cells.
445 Previous studies have demonstrated how vitamin E analogues generate ROS species which led to cell
446 apoptosis. One possibility is that vitamin E analogues are known to interact with the UbQ-binding sites
447 resulting in destroying mitochondrial electron doxorubicin chain[45-48]. The shift of red fluorescence to green
448 fluorescence for combined doxorubicin and AMP-001 (Fig 6B) indicates the significant reduction in the
449 mitochondrial potential making mitochondria in cancer cells as a potential target.

450 In our studies, we found combinational drugs seem to affect mitochondria membrane potential and
451 triggered Bcl_2/Bax dependent mitochondria apoptotic cascade more effectively than the mono-drug
452 treatment. With the elevated cell stress induced by drugs, Mitogen Activated Protein Kinase (MAPK)
453 signaling pathway was also activated (Fig 7).

454 MAPKs represent a family of kinases that transduce diverse extracellular stimuli including
455 proapoptotic agents) to the nucleus via kinase cascades to regulate proliferation, DNA synthesis arrest,
456 differentiation and apoptosis phenomena. MAPKs are activated through phosphorylation of specific
457 threonines and tyrosines by dual specificity kinases via a four-step kinase cascade. There are three well-
458 defined MAPK pathways in mammalian cells: the ERK1/ERK2 cascade and the stress-activated JNK
459 MAPK cascades. Our studies document that either AMP-001 or doxorubicin triggered apoptosis involves
460 ERK1, MEK1, and JNK1. Both ERK1/2 and JNK1 were activated after drug treatment and were
461 documented by detection of the active (phosphorylated) forms of these kinases using antibodies specific
462 for the active enzyme. However, the combination of AMP-001 and doxorubicin yielded a stronger band in

463 Western Blotting which is corroborated well with the other *in vitro* data such as reduction in IC-50,
464 enhancement of ROS and disintegration of mitochondrial permeability. In other words, these data prove
465 that AMP-001 is synergistic with doxorubicin to enhance its efficacy.

466 In essence, multiple pathways are involved for the synergistic effect of AMP-001 with doxorubicin. It
467 is important to note that the dysregulation of cell death pathways is manifested in many cancers including
468 gastric cancer through the downregulation of CD95[3]. The appearance of band in Western Blotting at 43
469 KDa seems to explain the activation of CD95 pathway which has been shown to sensitize tumor cells[31].
470 Hence, activation of CD95 pathway selectively is presumed to sensitize tumor cells. Studies on cis-platin
471 in testicular cancer, for example concludes that loss of activation of CD95 pathway resulted in developing
472 resistance to cis-platin[4]. Further studies are necessary to confirm this hypothesis.

473 We can only speculate the potential mechanism of action based on our limited studies reported in our
474 patent disclosure which includes a) release of AIF1 from mitochondria Fig (2 Supplementary), b) reduction
475 of downstream Bcl2/Bax ratio (Fig 3A-B, Supplementary data), NF-kB inhibition (Fig 4-5, supplementary
476 data), PARP cleavage (Fig 6, supplementary data) and generation of reactive oxygen species (Fig 7, ROS,
477 data). Based on the limited data, it could be hypothesized that both nucleus and mitochondria may be
478 involved. ROS, generated by AMP-001/002 is presumably translocated Bax into mitochondria (data not
479 shown) triggering NF-kB inhibition which, in turn induces cell death presumably through α-TNF related
480 apoptosis inducing ligand (TRAIL) or interferons (IFNs). Similarly, release of apoptosis inducing factor
481 (AIF-1) from mitochondria to cytosol and subsequent reduction of downstream Bcl2/Bax proteins triggers
482 irreversible cell death through caspase independent pathway. On the other hand, cell death CD95 activation
483 is expected to mediate cell death through FADD/procaspase and caspase 3/7 pathway. Cleavage of DNA
484 repair enzyme PARP augments cell death and established to sensitize both CSCs and resistant tumor cells.
485 This contrasts with doxorubicin which is shown to hyperactivate NF-kB and PARP and impairs
486 mitochondrial respiratory chain complex I leading to cell death in cardiomyocytes. Thus, inhibition of NF-
487 kB pathway and PARP cleavage protects cardiomyocytes reducing the potential cardiotoxicity. A potential
488 mechanism of action is described in Fig 10.

Fig 10: Potential mechanism of AMP-001 mediating synergy with Doxorubicin.

Conclusions

Since cancer is a group of diseases, several targets and pathways are involved in the progression of the disease. Although, conventional drugs apart from nonspecific chemotherapeutics target specific target (s), each drug works with specific targets/pathways. The combination of AAAPT leading molecule with a conventional chemotherapy brings multiple pathways involved in cancer cells desensitization process.

In summary, the effects of novel vitamin E analogue AMP-001 is as a preclinical candidate is described. AMP-001 enhanced doxorubicin induced apoptosis in human gastric cancer cells via ROS-dependent mitochondrial apoptotic pathway and MAPK pathway. The efficacy of doxorubicin antitumor activity can be amplified by combining it with AMP-001, which permits lower dose use in patients without affecting the positive clinical outcome. These effects are confirmed both *in vitro* and *in vivo*. Our current studies indicated that AMP-001 might be a potential candidate in synergetic with the existing FDA approved chemotherapeutic drugs for treatment of gastric cancer. Ultimately, the combination treatments success depends on how low dose can be made effective in a clinical situation, reduced overall toxicity and improved patient compliance. Further studies are planned to optimize the dose combination as to which one has higher efficacy and low toxicity compared to the existing treatments.

Acknowledgements

Part of this research was supported by the grant SBIR NIH R43CA183353.

Conflict of interest

The authors declare no competing financial interest.

References

1. (a) Friesen C, Fulda S and Debatin KM; Deficient activation of the CD95 (APO- 1/Fas) system in drug-resistant cells., Leukemia, 1997, **11**: 1833–1841.
2. Meyn RE, Stephens LC, Hunter NR, et al: Apoptosis in murine tumors treated with chemotherapy agents. Anti-Cancer Drugs 1995, 6:443-450.
3. Krammer PH, The tumor strikes back. New data on expression of the CD95 (APO-1/Fas) receptor/ligand system may cause paradigm changes in our view on drug treatment and tumor immunology. Cell Death Differentiation, 1997, **4**: 362–364.
4. U Ramp, M Dejosez, C Mahotka, B Czarnotta, T Kalinski, M Wenzel, I Lorenz, M Müller, P Krammer, HE Gabbert and CD Gerharz. Deficient activation of CD95 (APO-1/Fas)-mediated apoptosis: a potential factor of multidrug resistance in human renal cell carcinoma, British Journal of Cancer, 2000, **82** (11), 1851–1859.
5. Tomasetti M, Rippo MR, Alleva R, Moretti S, Andera L, Neuzil J, Procopio A (2004) □-Tocopheryl succinate and TRAIL selectively synergise in apoptosis induction in human malignant mesothelioma cells. Br J Cancer, 90, 1644–1653.
6. Wuchter C, Karawajew L, Ruppert V, Büchner T, Schoch C, Haferlach T, Ratei R, Dörken B, Ludwig WD. Clinical significance of CD95, Bcl-2 and Bax expression and CD95 function in adult de novo acute myeloid leukemia in context of P-glycoprotein function, maturation stage, and cytogenetics. Leukemia. 1999 (12):1943-53.
7. Lijian Shao, Lixian Wu, Daohong Zhou, Sensitization of tumor cells to cancer therapy by molecularly targeted inhibition of the inhibitor of nuclear factor κB kinase, *Transl Cancer Res* 2012;1(2):100-108.
8. Smets L.A. Programmed cell death (apoptosis) and response to anti-cancer drugs. Anti-Cancer Drugs, 1994, 5, 3-9.

9. Suparna Bonthala Wedam et.al;. Antiangiogenic and Antitumor Effects of Bevacizumab in Patients With Inflammatory and Locally Advanced Breast Cancer, J Clin Oncol 2006, 24:769-777 (Low apoptosis index of patient tumors correlated to patient response to Bevacizumab, **See Fig 4**).

10. Eun Ji Chung, Jinsil Seong, Woo Ick Yang, Tchan Kyu Park, Jae Wook Kim, Chang Ok Suh and Gwi Eon Kim. Spontaneous Apoptosis as a Predictor of Radiotherapy in Patients with Stage IIB Squamous Cell Carcinoma of the Uterine Cervix, Acta Oncologica 1999, 38, 449–454.

11. Hiroko Kuriyama, Kathleen R. Lamborn, Judith R. O'Fallon, N. Iturria, Thomas Sebo, Paul L. Schaefer, Bernd W. Scheithauer, Jan C. Buckner, Nagato Kuriyama, Robert B. Jenkins, and Mark A. Israel. Prognostic significance of an apoptotic index and apoptosis/proliferation ratio for patients with high-grade astrocytomas**,** Neuro-Oncology*,* 2002, *4, 179–186.*

12. Raghu Pandurangi, Amol Karwa, Tomasetti, Hari Nakshatri, Jean-Luc Vanderheydon, Robert Gillies and Cynthia Ma et.al; A priori activation of apoptosis pathways by cathepsin cleavable apoptogens: Implications for Fas resistant and triple negative breast cancer treatment. Cancer Research, 2018 (Submitted). (b) A priori Activation of Apoptosis Pathways of Tumor Technology (AAAPT) for Enhancing Tumor Cell Response to Anticancer Agents, PCT Filed, Jan 2016, PCT/US16/68554.

13. Mark P Mattson, Apoptosis in neurodegenerative disorders, Nature, 2000, 120-129. (b) Hainaut, Pierre, Plymoth, Amelie. Targeting the hallmarks of cancer: towards a rational approach to next-generation cancer therapy, Current Opinion in Oncology, January 2013 - Volume 25 - Issue 1 - p 50–51.

14. Madduri Srinivasarao, Chris V. Galliford & Philip S. Low, Principles in the design of ligand-targeted cancer therapeutics and imaging agents, Nature Reviews Drug Discovery*,* 14, 203–219*,* (2015).

15. a) J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, Int J Cancer, 127 (2010) 2893-2917, b) D.G. Power, D.P. Kelsen, M.A. Shah, Advanced gastric cancer--slow but steady progress, Cancer Treat Rev, 2010. 36 384-392.

16. a) T. Waddell, M. Verheij, W. Allum, D. Cunningham, A. Cervantes, D. Arnold, O. European Society for Medical, O. European Society of Surgical, R. European Society of, Oncology, Gastric cancer:

ESMO-ESSO-ESTRO clinical practice guidelines for diagnosis, treatment and follow-up, Eur J Surg Oncol, 40 (2014) 584-591, b) J.T. Mullen, D.P. Ryan, Neoadjuvant chemotherapy for gastric cancer: what are we trying to accomplish?, Ann Surg Oncol, 2014, 21,13-15.

17. a) Fas-Related Apoptosis in Gastric Adenocarcinoma, SC Lim. Oncol Rep 2003, 10 (1), 57-63, , b) Fas Receptor Counterattack Against Tumor-Infiltrating Lymphocytes in vivo as a Mechanism of Immune Escape in Gastric Carcinoma, Koyama et al. J Cancer Res Clin Oncol 127 (1), 20-26. 1 2001.
18. a) S.E. Mirski, J.H. Gerlach, S.P. Cole, Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin, Cancer research, 1987, 47, 2594-2598. b)R.A. Pathan, B.K. Singh, K.K. Pillai, K. Dubey, Naproxen aggravates Doxorubicin-induced cardiomyopathy in rats, Indian J Pharmacol, 2010, 42, 44-49.
19. a) X. Zhang, X. Peng, W. Yu, S. Hou, Y. Zhao, Z. Zhang, X. Huang, K. Wu, Alpha-tocopheryl succinate enhances Doxorubicin-induced apoptosis in human gastric cancer cells via promotion of Doxorubicin influx and suppression of Doxorubicin efflux, Cancer Lett, 2011, 174-181, b) O.A. Bamodu, W.C. Huang, D.T. Tzeng, A. Wu, L.S. Wang, C.T. Yeh, T.Y. Chao, Ovatodiolide sensitizes aggressive breast cancer cells to doxorubicin, eliminates their cancer stem cell-like phenotype, and reduces Doxorubicin-associated toxicity, Cancer Lett, 2015, 125-134, c) P. Zou, Auranofin induces apoptosis by ROS-mediated ER stress and mitochondrial dysfunction and displayed synergistic lethality with poperlongumine in gastric cancer., Oncotarget, (2015, 6(34):36505-21.
20. a) G.M. Dubowchik, R.A. Firestone, L. Padilla, D. Willner, S.J. Hofstead, K. Mosure, J.O. Knipe, S.J. Lasch, P.A. Trail, Cathepsin B-labile dipeptide linkers for lysosomal release of Doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity, Bioconjug. Chem. 2002, 13 855–869, b) Liang Liang, Song-Wen Lin, Wenbing Dai, Jing-Kai Lu, Ting-Yuan Yang, Yu Xiang, Yang Zhang, Run-Tao Li, Qiang Zhang; Novel cathepsin B-sensitive paclitaxel conjugate: Higher water solubility, better efficacy and lower toxicity, Journal of Controlled Release 2012, 160, 618–629, C) May S. Kung Sutherland, Russell J. Sanderson et.al; Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by

peptide-linked anti-CD30-Auristatin Conjugates, The Biological Chemistry, 2007, 281, 15, 10540–10547.

21. a) Roshy S, Sloane BF, Moin K. Pericellular cathepsin B and malignant progression. Cancer Metastasis Rev. 2003 (2-3), 271-86. (b) Lopez-Otin C, Matrisian LM. Emerging roles of proteases in tumor suppression. Nat Rev Cancer. 2007; 7: 800-8. (c) Ebert MP, Kruger S, Fogeron ML, Lamer S, Chen J, Pross M, et al. Overexpression of cathepsin B in gastric cancer identified by proteome analysis. Proteomics. 2005; 5: 1693-704. (d) Kruszewski WJ, Rzepko R, Wojtacki J, Skokowski J, Kopacz A, Jaskiewicz K, et al. Overexpression of cathepsin B correlates with angiogenesis in colon adenocarcinoma. Neoplasma. 2004; 51: 38-43. (e) Kawada A, Hara K, Kominami E, Kobayashi T, Hiruma M, Ishibashi A. Cathepsin B and D expression in squamous cell carcinoma. Br J Dermatol. 1996; 135: 905-10.
22. Arig Ibrahim-Hashim, Jonathan W. Wojtkowiak, Maria de Lourdes Coelho Ribeiro, Veronica Estrella, Kate M. Bailey, Heather H. Cornnell, Robert A. Gatenby, and Robert J Gillies. Free Base Lysine Increases Survival and Reduces Metastasis in Prostate Cancer Model. J Cancer Sci Ther. 2011, 19; Suppl 1(4), 1-12.
23. Ireland DJ, Kissick HT, Beilharz MW. Alpha-Tocopheryl succinate: toxicity and lack of anti-tumor activity in immuno-competent mice, Food Chem Toxicol. 2008, 46 (2):508-12.
24. a) D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, Nat Rev Drug Discov, 2009, 8, 579-591, b) T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer research, 1991, 51 794-798.
25. a) V. Nogueira, N. Hay, Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy, Clin Cancer Res, 2013, 19, 4309-4314, b) D.A. Gewirtz, A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin, Biochem Pharmacol, 1999, 57, 727-741.

611 26. a) daunorubicin-ROS P. Bragado, A. Armesilla, A. Silva, A. Porras, Apoptosis by cisplatin requires
612 p53 mediated p38alpha MAPK activation through ROS generation, Apoptosis, 2007, 12, 1733-1742,
613 b) J. Alexandre, Y. Hu, W. Lu, H. Pelicano, P. Huang, Novel action of paclitaxel against cancer cells:
614 bystander effect mediated by reactive oxygen species, Cancer research, 2007, 67, 3512-3517, c) M.
615 Jeanne, V. Lallemand-Breitenbach, O. Ferhi, M. Koken, M. Le Bras, S. Duffort, L. Peres, C. Berthier,
616 H. Soilihi, B. Raught, H. de The, PML/RARA oxidation and arsenic binding initiate the antileukemia
617 response of As2O3, Cancer Cell, 2010, 18, 88-98.
618 27. W.H. Chiu, S.J. Luo, C.L. Chen, J.H. Cheng, C.Y. Hsieh, C.Y. Wang, W.C. Huang, W.C. Su, C.F. Lin,
619 Vinca alkaloids cause aberrant ROS-mediated JNK activation, Mcl-1 downregulation, DNA damage,
620 mitochondrial dysfunction, and apoptosis in lung adenocarcinoma cells, Biochem Pharmacol, (2012,
621 83,1159-1171.
622 28. S. Fulda, L. Galluzzi, G. Kroemer, Targeting mitochondria for cancer therapy, Nat Rev Drug Discov,
623 2010, 9, 447-464.
624 29. Y.H. Ling, L. Liebes, Y. Zou, R. Perez-Soler, Reactive oxygen species generation and mitochondrial
625 dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460
626 non-small cell lung cancer cells, J Biol Chem, 2003, 278, 33714-33723.
627 30. T. Wada, J.M. Penninger, Mitogen-activated protein kinases in apoptosis regulation, Oncogene, 2004,
628 23, 2838-2849.
629 31. a) Ammann JU, Haag C, Kasperczyk H, Debatin KM, Fulda Sensitization of neuroblastoma cells for
630 TRAIL-induced apoptosis by NF-kappa B inhibition. Int J Cancer. 2009 Mar 15;124 (6):1301-1, b)
631 Tomasetti M, Rippo MR, Alleva R, Moretti S, Andera L, Neuzil J, Procopio A, α–Tocopheryl succinate
632 and TRAIL selectively synergize in apoptosis induction in human malignant mesothelioma cells. Br J
633 Cancer, 2004, 90: 1644–1653.
634 32. W.K. Leung, M.S. Wu, Y. Kakugawa, J.J. Kim, K.G. Yeoh, K.L. Goh, K.C. Wu, D.C. Wu, J. Sollano,
635 U. Kachintorn, T. Gotoda, J.T. Lin, W.C. You, E.K. Ng, J.J. Sung, C. Asia Pacific Working Group on
636 Gastric, Screening for gastric cancer in Asia: current evidence and practice, Lancet Oncol, 9, 2008, 9,

637 279-287.

638 33. L.W. Law, Origin of the resistance of leukaemic cells to folic acid antagonists, Nature, 1952, 169 628-
639 629.

640 34. L.W. Law, Effects of combinations of antileukemic agents on an acute lymphocytic leukemia of mice,
641 Cancer research, 1952, 12, 871-878.

642 35. H.E. Skipper, J.R. Thomson, M. Bell, Attempts at dual blocking of biochemical events in cancer
643 chemotherapy, Cancer research, 1954, 14, 503-507.

644 36. J.R. Pritchard, P.M. Bruno, L.A. Gilbert, K.L. Capron, D.A. Lauffenburger, M.T. Hemann, Defining
645 principles of combination drug mechanisms of action, Proceedings of the National Academy of
646 Sciences of the United States of America, 2013, 110, E170-179.

647 37. a) J. Neuzil, X.F. Wang, L.F. Dong, P. Low, S.J. Ralph, Molecular mechanism of 'mitocan'-induced
648 apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family
649 proteins, FEBS Lett, 580 (2006) 5125-5129, b) Veronese FM[1], Mero A. The impact of PEGylation on
650 biological therapies. BioDrugs. 2008; 22(5):315-29.

651 38. H. Mo, C.E. Elson, Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to
652 cancer chemotherapy and chemoprevention, Exp Biol Med (Maywood), 2004, 229, 567-585.

653 39. S.V. Bachawal, V.B. Wali, P.W. Sylvester, Enhanced antiproliferative and apoptotic response to
654 combined treatment of gamma-tocotrienol with erlotinib or gefitinib in mammary tumor cells, BMC
655 Cancer, 2010, 10, 84.

656 40. A.B. Shirode, P.W. Sylvester, Synergistic anticancer effects of combined gamma-tocotrienol and
657 celecoxib treatment are associated with suppression in Akt and NFkappaB signaling, Biomed
658 Pharmacother, 2010, 64, 327-332.

659 41. N.M. Ayoub, S.V. Bachawal, P.W. Sylvester, gamma-Tocotrienol inhibits HGF-dependent

660 mitogenesis and Met activation in highly malignant mammary tumour cells, Cell Prolif, 2011, 44, 516-
661 526.

662 42. A. Malaviya, P.W. Sylvester, Mechanisms Mediating the Effects of gamma-Tocotrienol When Used in
663 Combination with PPARgamma Agonists or Antagonists on MCF-7 and MDA-MB-231 Breast Cancer
664 Cells, Int J Breast Cancer, vol. 2013, Article ID 101705, 17 pages,
665 2013. https://doi.org/10.1155/2013/101705..

666 43. R.V. Tiwari, P. Parajuli, P.W. Sylvester, Synergistic anticancer effects of combined gamma-tocotrienol
667 and oridonin treatment is associated with the induction of autophagy, Mol Cell Biochem, 2015,
668 408,123-137.

669 44. S. Yamashita, K. Baba, A. Makio, M. Kumazoe, Y. Huang, I.C. Lin, J. Bae, M. Murata, S. Yamada, H.
670 Tachibana, gamma-Tocotrienol upregulates aryl hydrocarbon receptor expression and enhances the
671 anticancer effect of baicalein, Biochem Biophys Res Commun, 2016, 473, 801-807.

672 45. M. Stapelberg, N. Gellert, E. Swettenham, M. Tomasetti, P.K. Witting, A. Procopio, J. Neuzil, Alpha-
673 tocopheryl succinate inhibits malignant mesothelioma by disrupting the fibroblast growth factor
674 autocrine loop: mechanism and the role of oxidative stress, J Biol Chem, 2005, 280, 25369-25376.

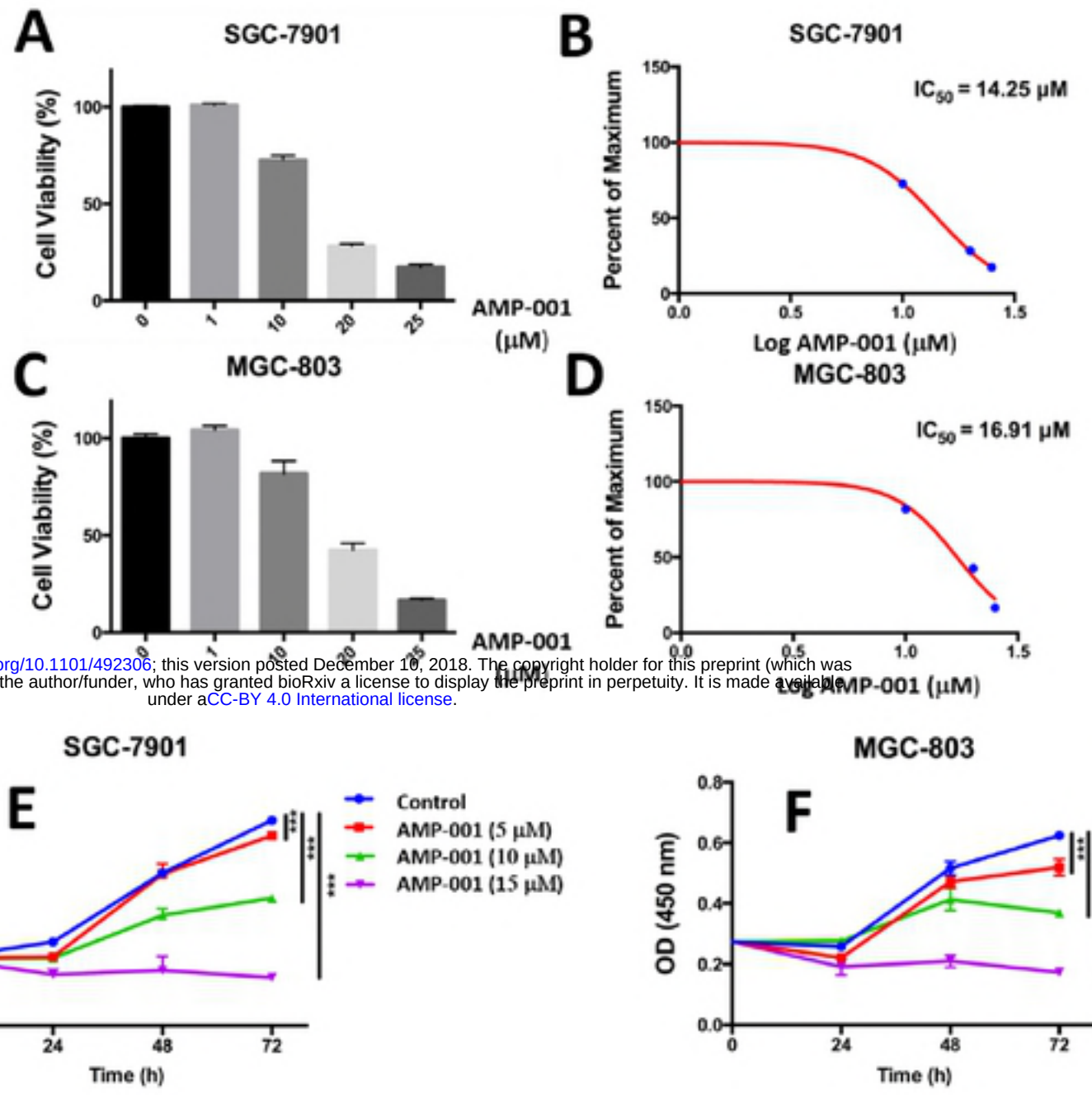
675 46. E. Swettenham, P.K. Witting, B.A. Salvatore, J. Neuzil, Alpha-tocopheryl succinate selectively induces
676 apoptosis in neuroblastoma cells: potential therapy of malignancies of the nervous system?, J
677 Neurochem, 2005, 94, 1448-1456.

678 47. A.M. James, M.S. Sharpley, A.R. Manas, F.E. Frerman, J. Hirst, R.A. Smith, M.P. Murphy, Interaction
679 of the mitochondria-targeted antioxidant MitoQ with phospholipid bilayers and ubiquinone
680 oxidoreductases, J Biol Chem, 2007, 282, 14708-14718.

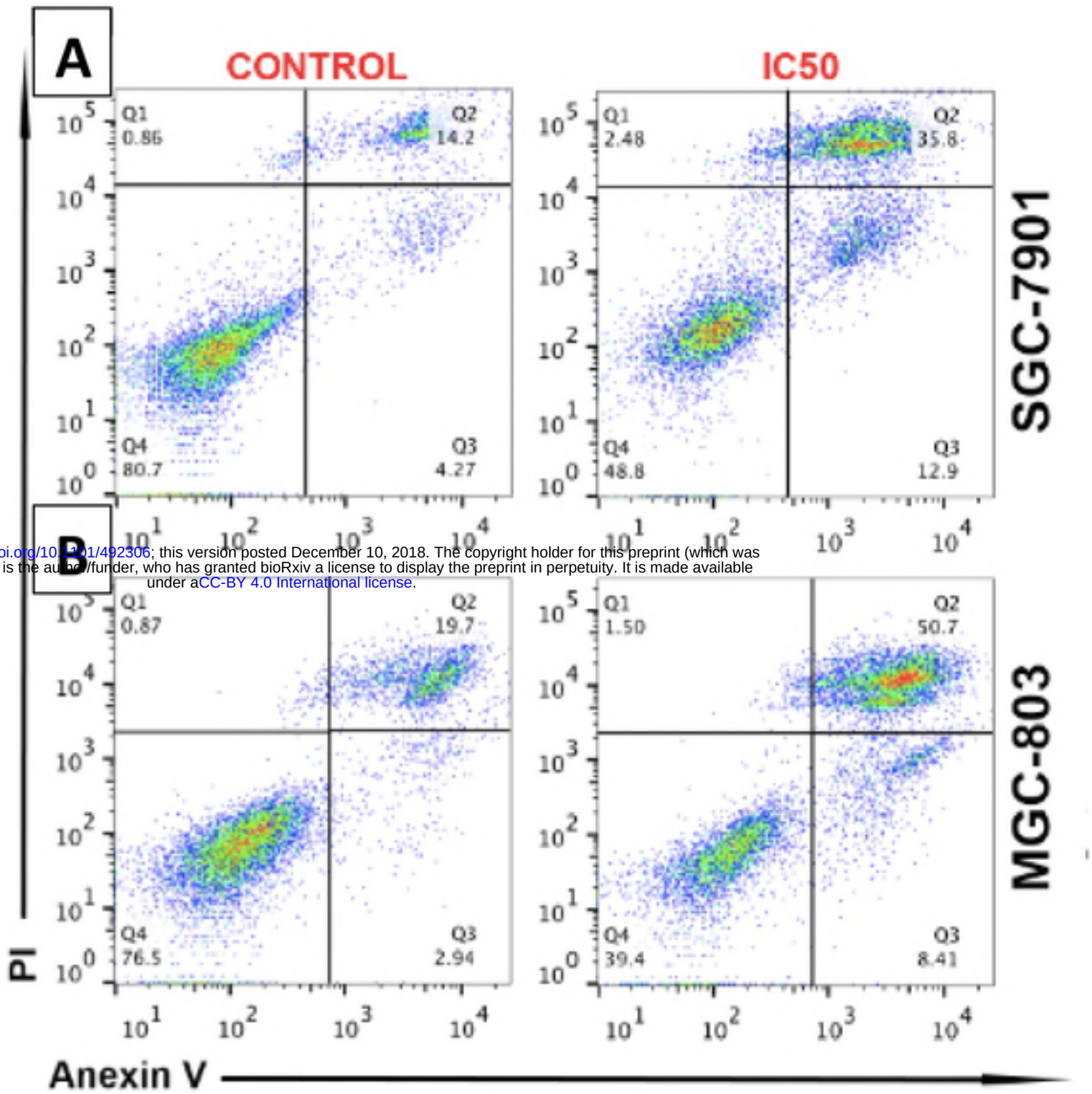
681 48. A.M. James, H.M. Cocheme, R.A. Smith, M.P. Murphy, Interactions of mitochondria-targeted and
682 untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species.

683 Implications for the use of exogenous ubiquinones as therapies and experimental tools, J Biol Chem,
684 2005, 280, 21295-21312.

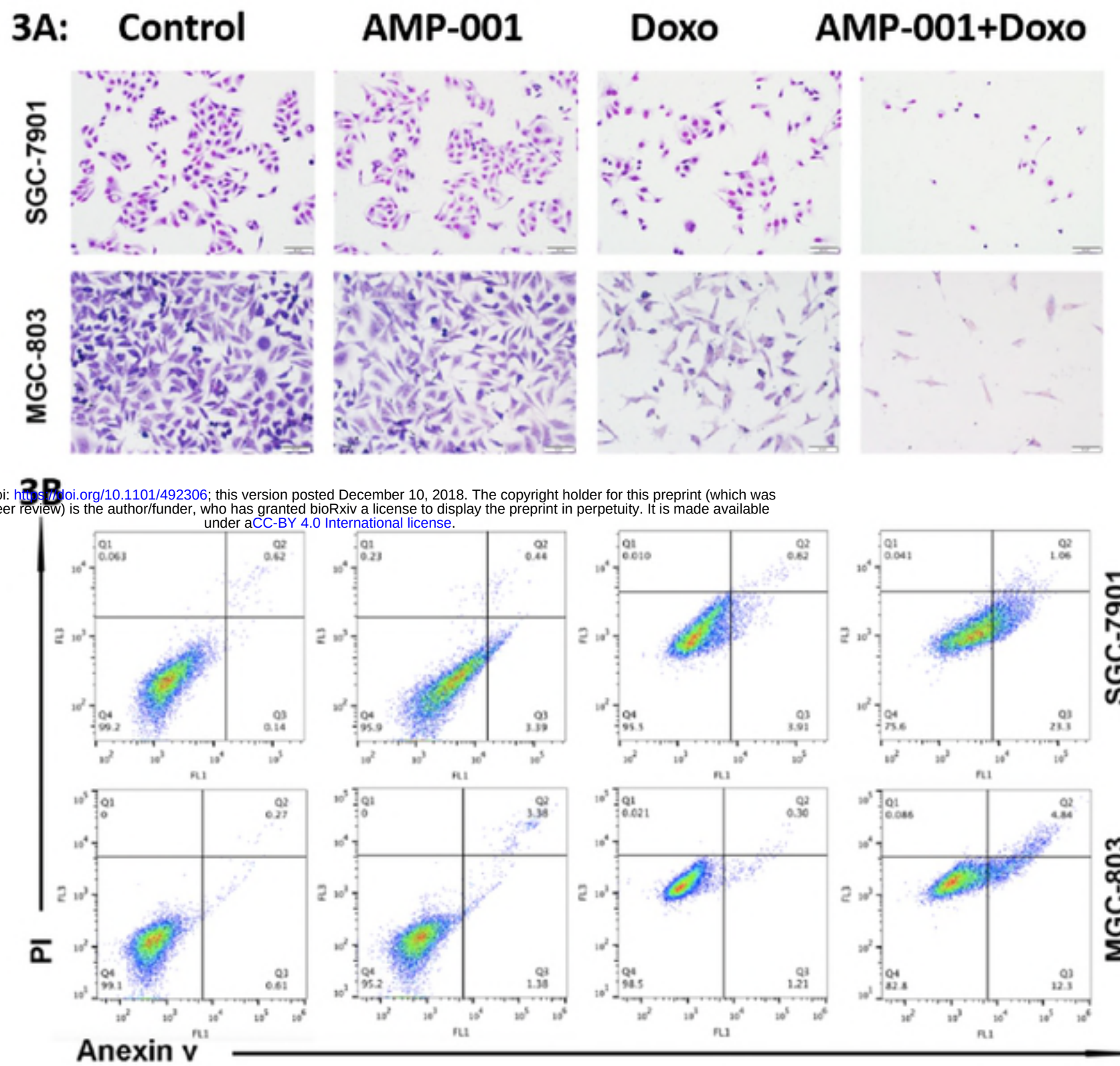
685 49. I. Tabas, D. Ron, Integrating the mechanisms of apoptosis induced by endoplasmic reticulum
686 stress, Nat Cell Biol, 2011, 13, 184-190.



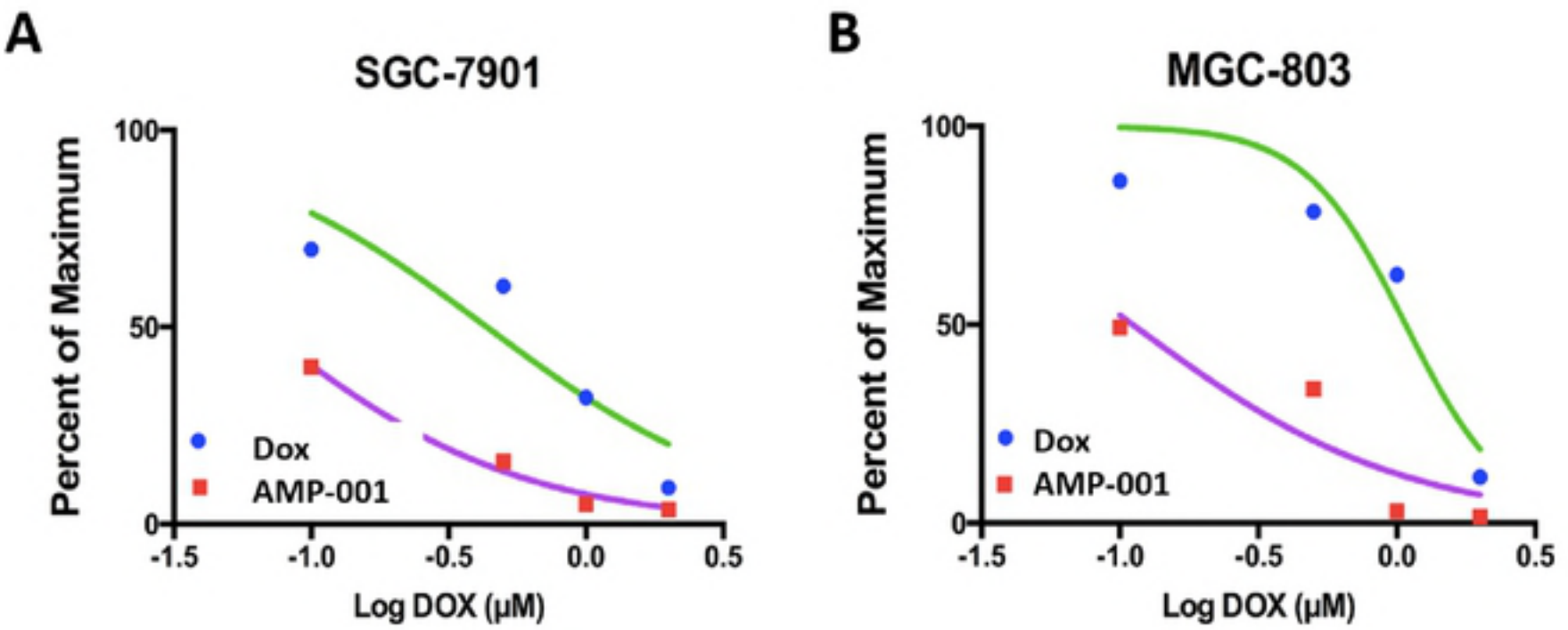
Figure



Figure

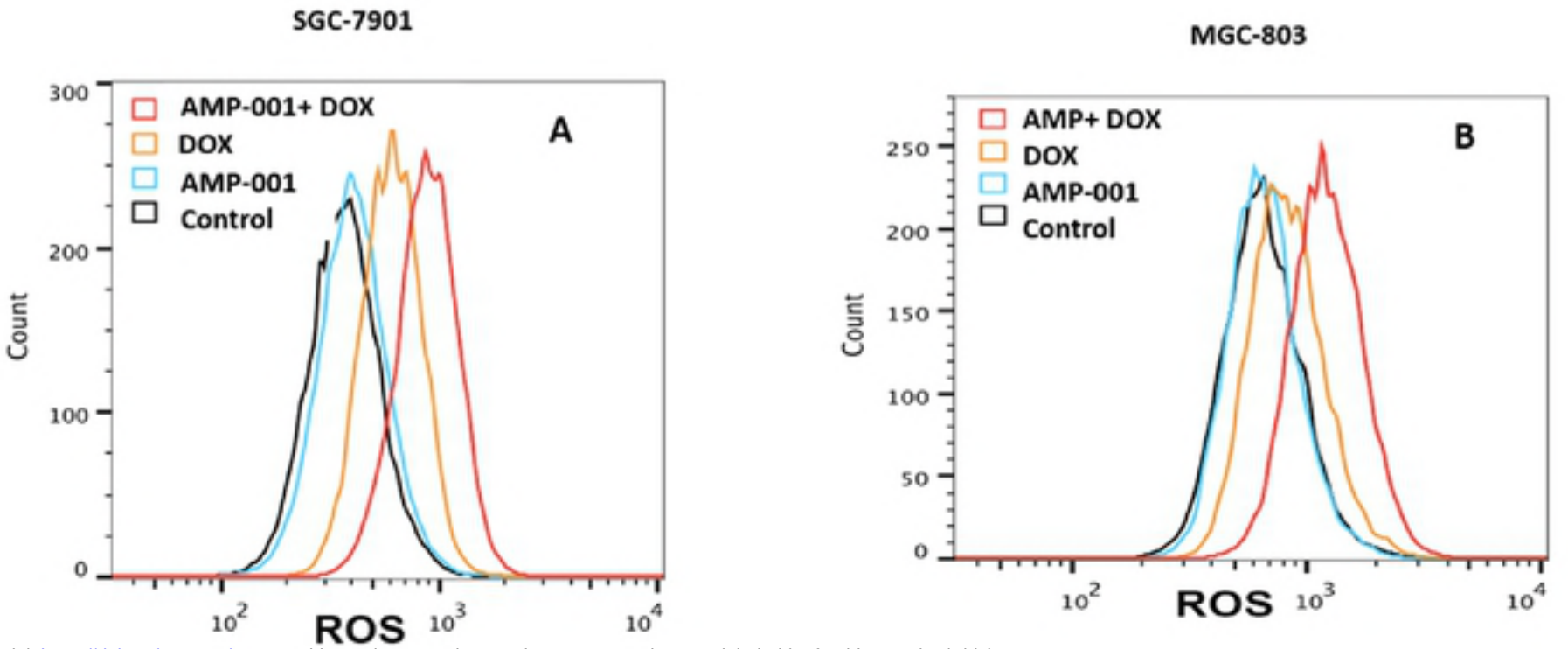


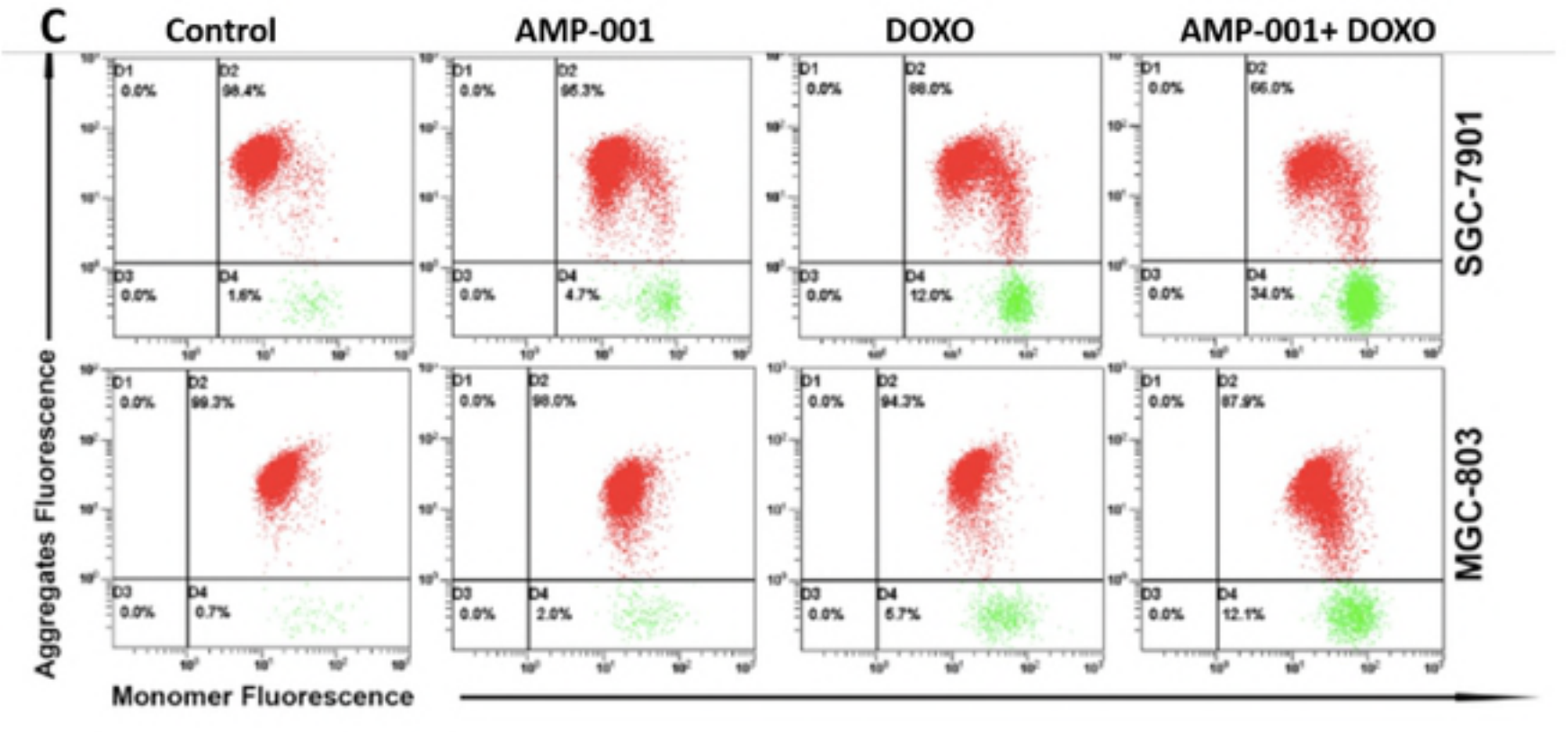
Figure



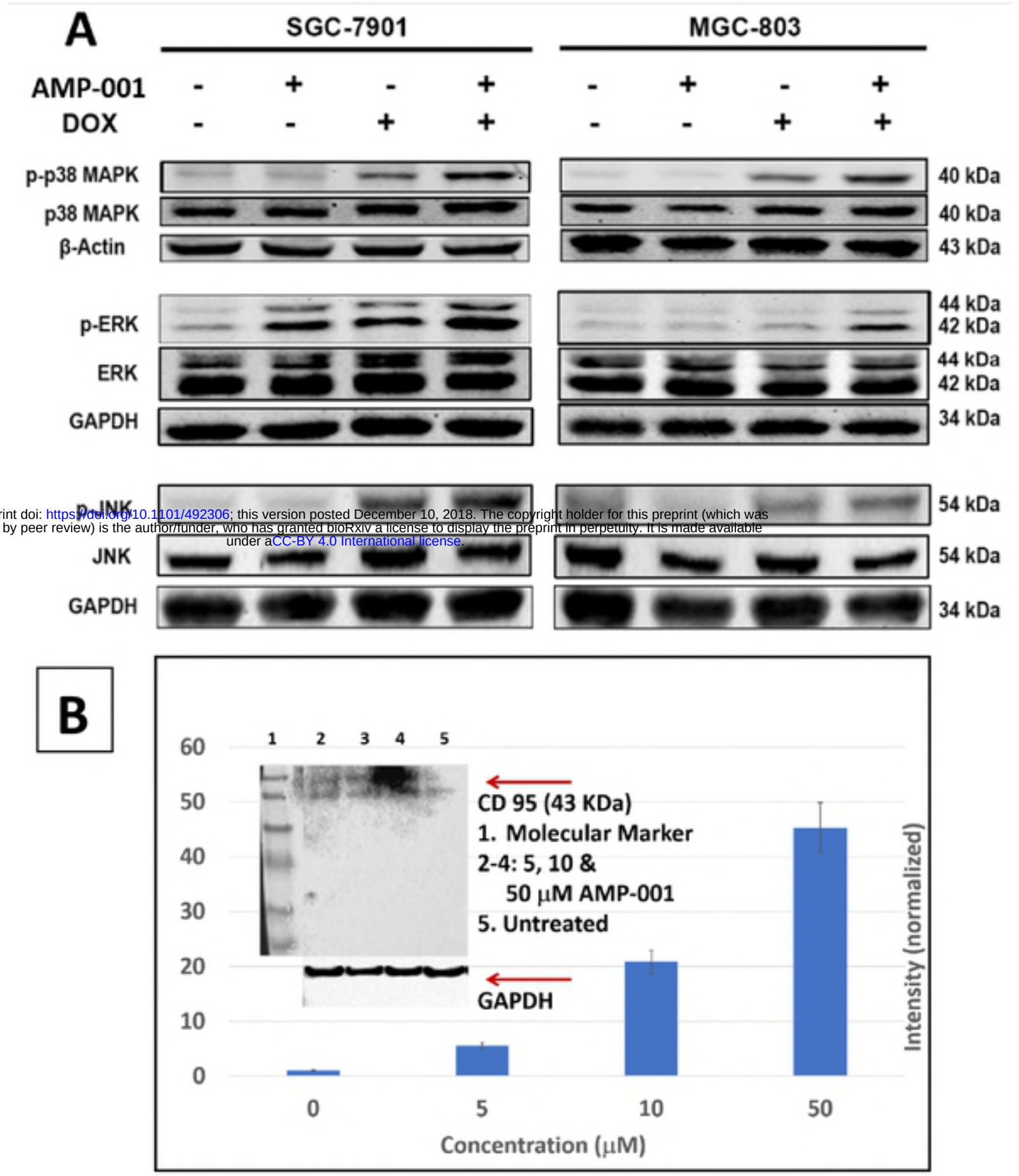
Drug Regimen (Table 1)	IC-50 (μM)	
Cancer Cell	SGC-7901	MGC-803
Dox Alone	0.436	1.076
Doxo+AMP-001	0.065	0.111

Figure

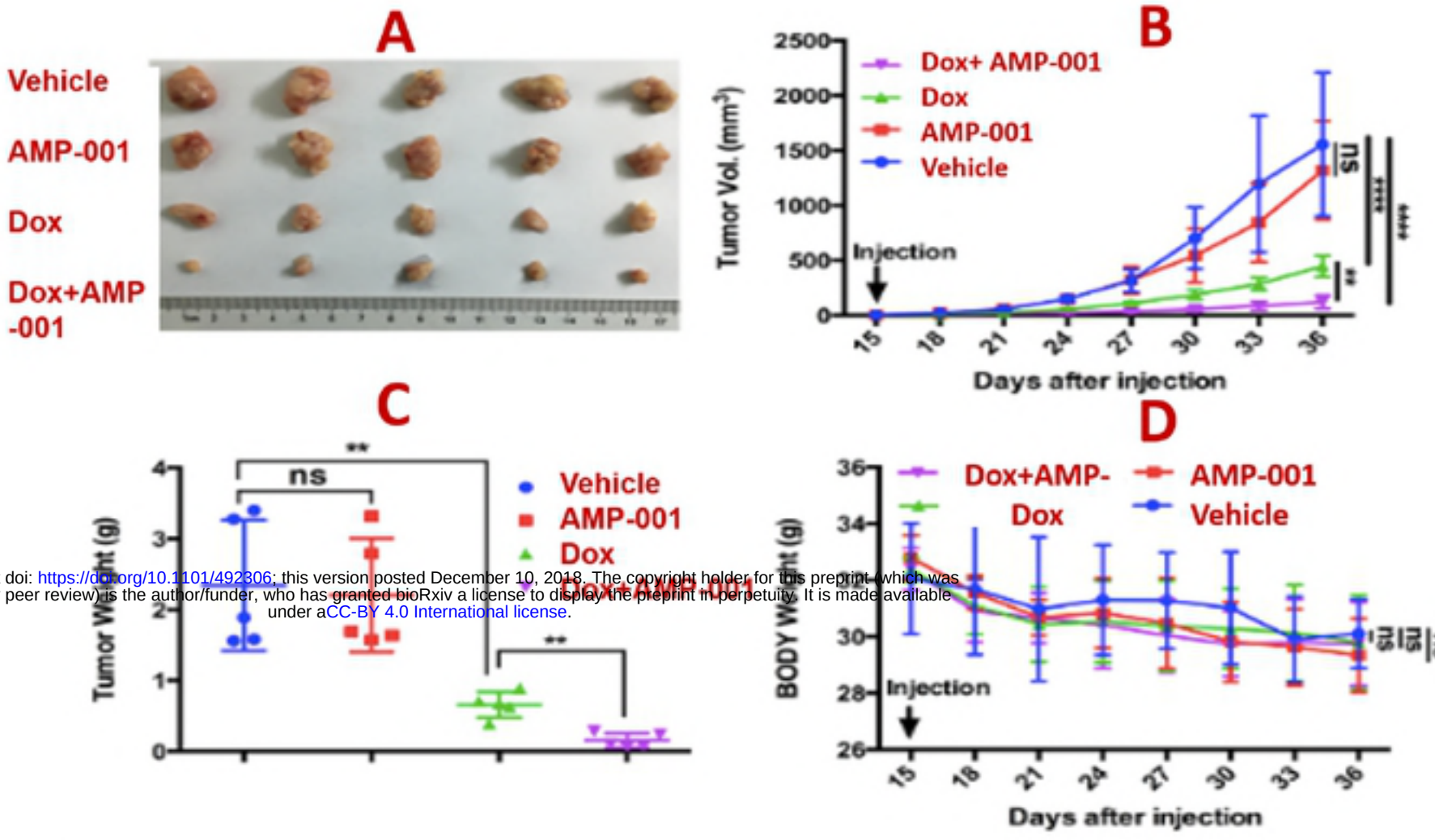




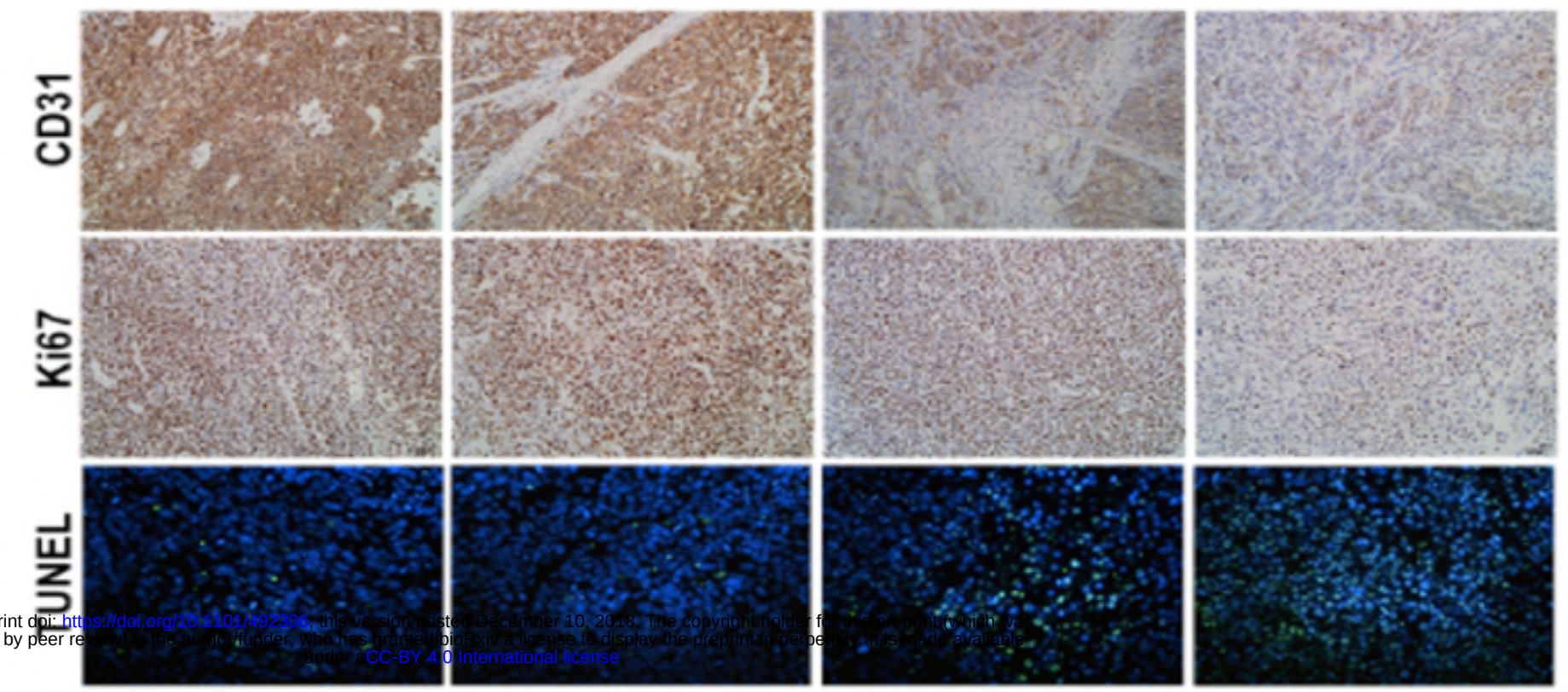
Figure



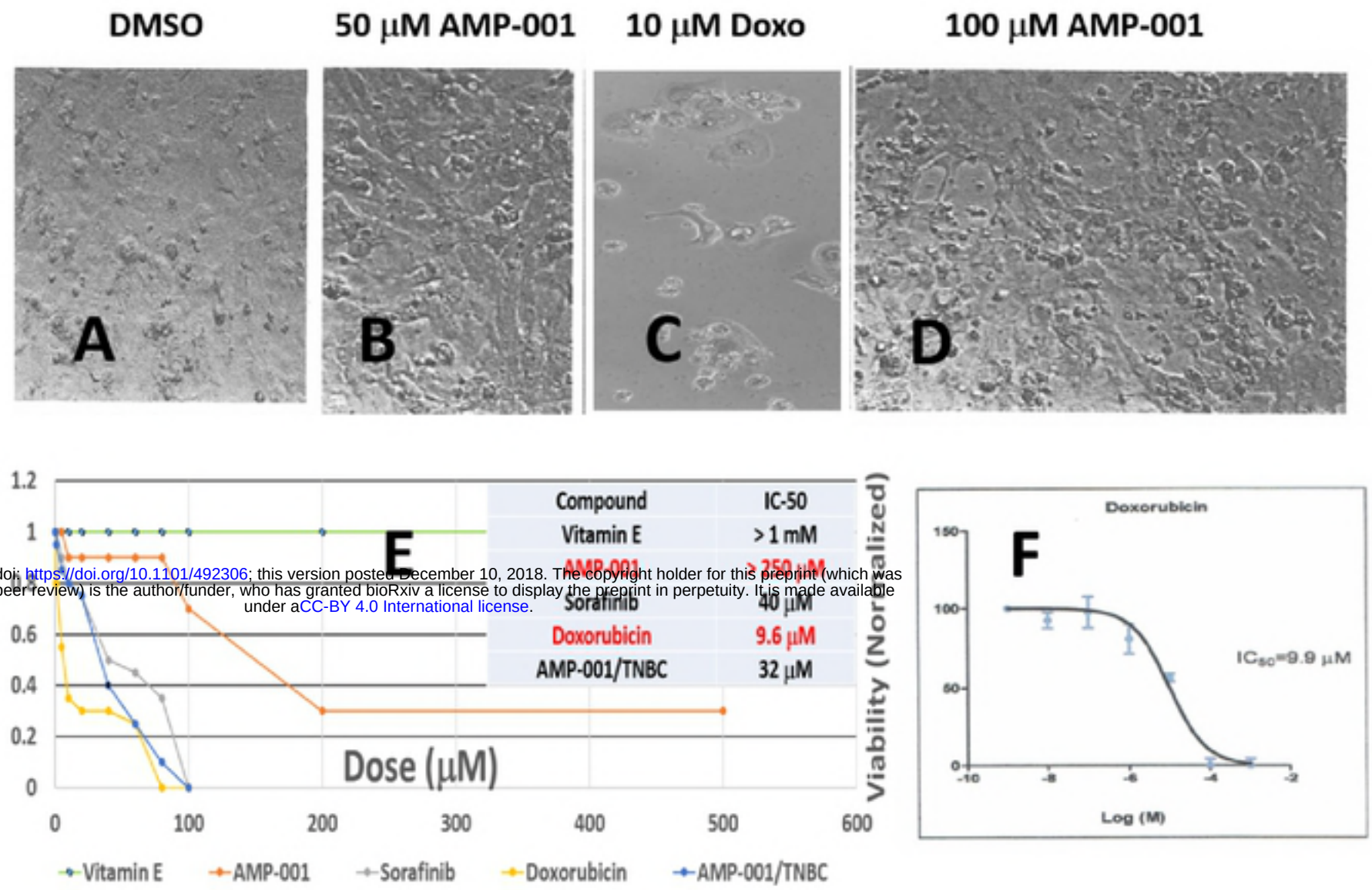
Figure



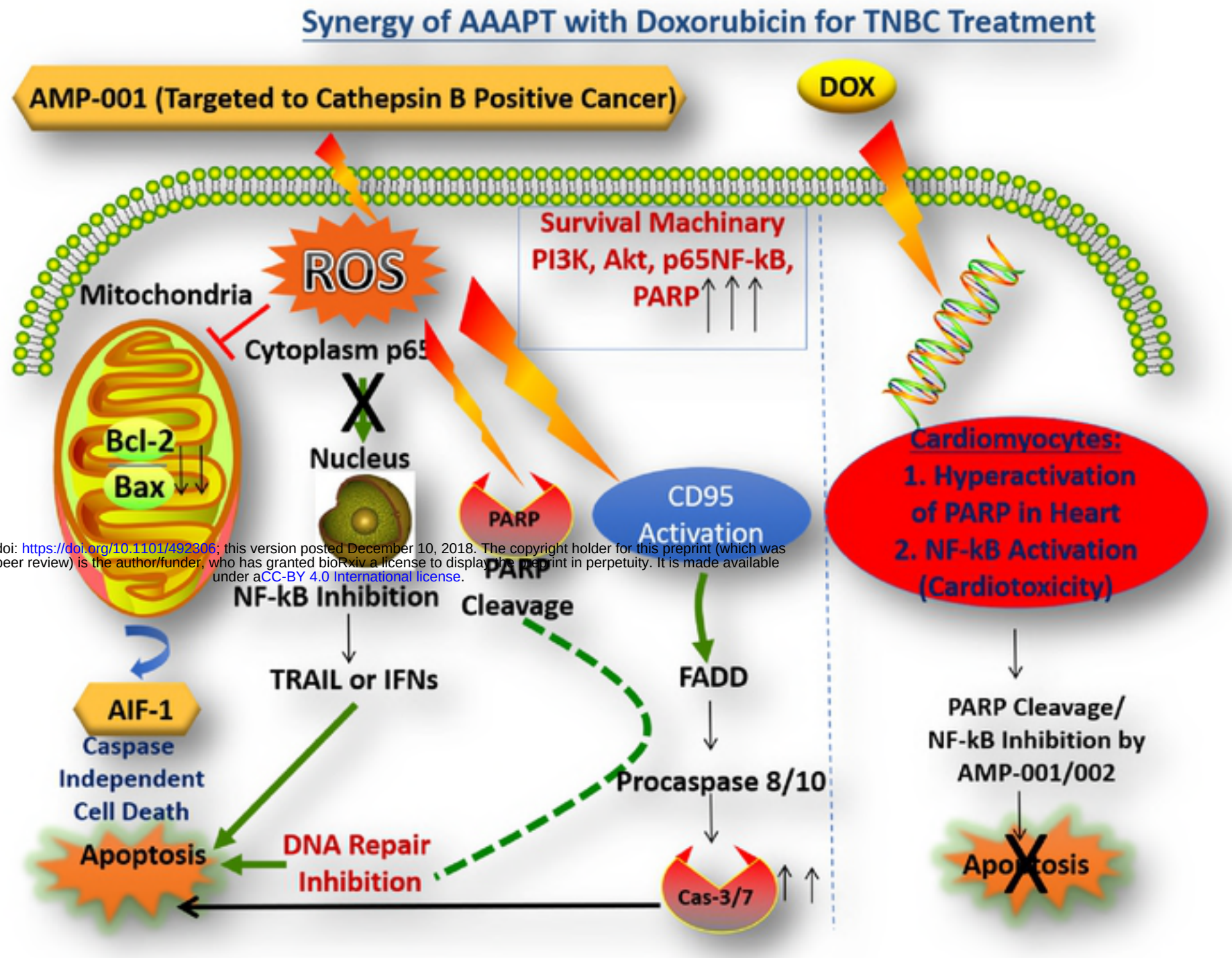
Figure



Figure



Figure



Figure