1    **A Priori Activation of Apoptosis Pathways of Tumor (AAAPT) Technology 1: Sensitization of**

2    **Tumor Cells Using Targeted and Cleavable Apoptosis Initiators in Gastric Cancer.**

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10                                                  **Abstract**

11          Cancer cells develop tactics to circumvent the interventions by desensitizing themselves to

12    interventions.  The principle route of desensitization includes activation of survival pathways (e.g. NF-kB,

13    PARP) and downregulation of cell death pathways (e.g. CD95, ASK1). As a result, it requires high dose of

14    therapy to induce cell death which, in turn damages normal cells through the collateral effects. Methods are

15    needed to sensitize the low and non-responsive resistant tumor cells in order to evoke a better response

16    from the current treatments. Current treatments including chemotherapy can induce cell death only in bulk

17    cancer cells sparing low-responsive and resistant tumor cells. Here we report a novel tumor sensitizer

18    derived from the natural Vitamin E analogue (AMP-001). The drug design is based on a novel "A priori

19    activation of apoptosis pathways of tumor technology (AAAPT) which is designed to activate cell death

20    pathways and inhibit survival pathways simultaneously. It involves an inbuilt targeting vector which targets

21    tumor specific Cathepsin B, overexpressed by many cancers including gastric cancer. Our results indicate

22    that AMP-001 sensitizes gastric cancer cells which resulted in expanding the therapeutic index of front-line

23    chemotherapy doxorubicin both *in vitro* and *in vivo* nude mouse model. The synergy between AMP-001

24    and doxorubicin could pave a new pathway to use AMP-001 as a neoadjuvant to chemotherapy to achieve

25    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

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51  bypass the intervention by reactivating specific dysregulated apoptosis pathways selectively in tumor

52  sparing normal cells. Since ubiquitous or systemic activation of apoptosis can induce undesirable

53  neurodegeneration and myelosupression[13], targeting is essential[14].

54  Gastric cancer remains the second leading cause of cancer-related deaths in the world[15a]. Almost two-

55  thirds of new diagnoses occur in developing countries with 42% in China alone, remaining high in

56  developed countries[15b]. Although, surgery is the main treatment for regressing gastric cancer, the majority

57  patients first diagnosed with gastric cancer have already presented local or distant metastasis. Thus,

58  adjuvant or perioperative chemotherapy and molecule-targeted chemotherapy have been prescribed for

59  gastric cancer, due to their proved benefits in controlling metastasis, reducing cancer recurrence and

60  increasing overall survival[16]. Gastric cancer cells are also known to downregulate CD95 pathway which

61  makes them insensitive to chemotherapy[17]. Hence, we investigate the tumor sensitizing potential of a

62  leading AAAPT molecule AMP-001 in gastric cancer to evoke a better response or synergistic effect with

63  a standard chemotherapy for example, doxorubicin. doxorubicin (dox) is a first-line anticancer agent which

64  is widely used in clinical therapeutic regimens for a variety of cancers including gastric cancer.

65  Nevertheless, the clinical application is limited due to the drug resistance and adverse effects, including

66  cardiomyopathy, typhilitis, and acute myelotoxicity[18]. To decrease dox-induced dose-dependent toxicity,

67  enhancing the effective therapeutic dox dose via combination with nontoxic or selective apoptotic inducing

68  ligands  has been investigated[19]. Here, we propose novel targeted tumor sensitizers which addresses how

69  the cancer cells circumvent interventions by reactivating cell death pathways. Since desensitization tactics

70  by cancer cells are successful in thwarting the efficacy of treatments, irrespective of nature of interventions

71  (targets), a fundamental reversal of cancer cells tactics may have high impact on several modes of

72  interventions (e.g. chemotherapy, radiation and immunotherapy). Here, we report to demonstrate the

73  synergistic effect of AMP-001with doxorubicin *in vitro* and *in vivo* in a gastric cancer animal model.

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



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

Cleavable Site

76    succinate (apoptogen) with a dipeptide linker valine-citrulline which is cleavable by tumor specific

77    Cathepsin B enzyme giving rise to a final molecule AMP-001(Scheme 1). This design provided impetus

78    for a) targeting apoptogen to cancer cells using tumor specific biomarker Cathepsin B sparing normal cells,

79    b) releasing the drug near tumor sites through the release of drug by cleaving at valine-citrulline link , c)

80    pegylation to make it water soluble, d) enhancing the bioavailability of AMP-001and e) to keep it intact as

81    a pro-drug in blood circulation for a long time to reach tumor sites. The rationale behind using cathepsin B

82    cleavable linkers is based on the better safety profile of Cathepsin B cleavable prodrugs doxorubicin[20a],

83    paclitaxel[20b] and antibodies[20c] (Seattle Genetics) compared to unmodified drugs. Cathepsin B is known to a)

84    be a tumor specific biomarker and b) cleave valine-citrulline substrate[21]. For example, high delineation of

85    tumor from the surrounding tissues by cathepsin B sensitive optical probes[22] shows that this enzyme is

86    restricted to invading cancers. Cathepsin B cleaves AMP-001 at the citrulline-OH bond to release pegylated

87    apoptogen at tumor sites.

88        Previous studies suggested that $\alpha$-tocopheryl succinate could destroy mitochondrial function and

89    cause the oxidative damage, resulting in over production of reactive oxygen species (ROS) and apoptosis.

90    Although, parent molecule $\alpha$-tocopheryl succinate ($\alpha$-TOS) showed promising anti-tumor properties *in*

91    *vitro*, studies in an immunocompetent mouse *in vivo* model showed that $\alpha$-TOS was not only ineffective at

92    the published doses, but resulted in severe side effects due to lack of targeting[23]. Elevated ROS levels can

93    render cancer cells more sensitive to agents that cause oxidative stress[24-25]. There are many anti-cancer

94    drugs such as daunorubicin[26a], cisplatin[26b], paclitaxel[26c] and trisenox[26d] that can augment ROS production

95    in cancer cells, despite their inability to target cancer cells leading to non-specific side effects.

96        Here, we examined the effect of combining AMP-001 and doxorubicin for a potential synergistic

97    effect in human gastric cancer both *in vitro* and *in vivo*. These results indicate that low dose pretreatment

98    of tumor *in vivo* prepares tumor to make it sensitive to doxorubicin by showing a cumulative and significant

99    tumor regression compared to individual drugs.  A potential mechanism of action is being suggested based

100   on the limited data.

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**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

123     Biotechnology, Nanjing, China) for 30min at 37℃, and then washed twice with PBS. Beckman flow

124     cytometer was used for final detection. Flow Jo vX.0.7 software was used to analyze the data.

### Measurement of intracellular ROS level

126     The intracellular ROS level was measured by flow cytometry as described previously[24]. Briefly, $2 \times 10^5$ cells

127     were placed in 12 well plates, allowed to attach overnight, and exposed to the treatment needed. Cells were

128     stained with 10μM DCFH-DA (Sigma) at 37℃ for 30 min with serum free culture. Cells were washed twice

129     with pre-cold PBS and photographed or collected for fluorescence analysis using Beckman flow cytometer.

130     In some experiments, cells were pretreated with 5mM NAC for 2h prior exposure to compounds and

131     analyzed for ROS generation.

### Cell Mitochondria Isolation

133     Fractionation of intracellular mitochondria was performed with Cell Mitochondria Isolation Kit

134     (Beyotime Institute of Biotechnology, Nanjing, China), according to the manufacturer's protocols. First,

135     $1 \times 10^7$ cells were collected and homogenized. Density gradient centrifugation at 600g for 10min was applied

136     to remove cellular debris, and supernatants were centrifuged at 11000g for 10min at 4℃ to separate

137     mitochondrial fractions. Then, supernatants containing cytoplasmic proteins were saved and the obtained

138     mitochondria pellet was lysated for further immunoblotting.

### Western Blotting

140     Cells were washed with cold PBS twice and prepared in RIPA lysis buffer, and western blot analysis

141     was performed as described previously[25]. Specific primary antibodies used were as follows: anti-Bcl-2

142     (50E3), Caspase-9 (C9), Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, Phospho-p44/42 (Erk1/2)

143     (Thr202/Tyr204), p44/42 MAPK (Erk1/2), Phospho-SAPK/JNK (Thr183/Tyr185), JNK2 antibodies were

144     purchased from Cell Signaling Technology (USA). Anti-Bax antibody was purchased from Abcam. Anti-

145     Cytochrome C and COX IV were purchased from Beyotime Biotechnology. Antibody against PARP1 was

146     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 $5 \times 10^6$ 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

171    peroxidase-conjugated secondary antibody (ABclonal) and diaminobenzidine colorimetric reagent solution

172    purchased from Dako (Carpinteria, CA) were used. The staining processes were according to standard

173    methods.

**TUNEL Assay**

175    The level of tumor tissue apoptosis in vivo was determined using an TdT-mediated dUTP nick end

176    labeling in situ apoptosis detection kit (Roche, USA) according to the manufacturer's protocol. The pictures

177    were photographed by fluorescence microscopy at a 200x magnification.

**Cardiotoxicity Assay**

179    The data were collected from Ionic Transport Assays Inc. In brief, the adult human heart cell

180    line was created by reprogramming an adult human fibroblast cell line by retroviral expression of the

181    reprogramming factors *sox7, oct4, nanog,* and *lin28* using MMLV viral constructs. Cardiomyocytes

182    were derived from this engineered stem cell clone line as follows. Stem cell aggregates were formed

183    from single cells and cultured in suspension in medium containing zebrafish bFGF (basic fibroblast

184    growth factor) and fetal bovine serum. Upon observation of beating cardiac aggregates, cultures were

185    subjected to blasticidin selection at 25 ug/ml to enrich the cardiomyocyte population. Cardiomyocyte

186    aggregate cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10%

187    fetal bovine serum during cardiomyocyte selection through the duration of the culture prior to

188    cryopreservation. At 30 to 32 days of culture the enriched, stem cell-derived cardiomyocytes were

189    subjected to enzymatic dissociation using 0.5% trypsin to obtain single cell suspensions of purified

190    cardiomyocytes, which were >98% cardiac troponin-T (cTNT) positive. These cells (iCell®

191    Cardiomyocytes) were cryopreserved and stored in liquid nitrogen before delivery to Ionic Transport

192    Assays from Cellular Dynamics International, Madison, WI.

193    Cells were plated into 6 well plates that percolated with 0.1% gelatin.  This was defined as culture day 1

194    for the purpose of this study. Cell plating media was changed at day 3 to cell maintenance media and cell

195     maintenance media subsequently was changed three times a week. Day 5-7 cells were re-suspended with

196     trypsin and re-plated as desired density (>10,000) at 96 well plate which percolated with 0.1% gelatin.

197     **Sample Preparation**: 3.75 mg AMP-001 was dissolved into 0.25 ml water to create a 10 mM stock

198     solution. This stock solution was added to maintenance medium for a final concentration 500 µM which

199     was then diluted to 200 µM, 100 µM, 10 µM and 1 µM AMP-001 solution. 3 mg Dox (Tocris, Cat No.

200     2252, Cas No.25316-40-9, MW=579.99) was dissolved in 0.5 ml water to create a 10 mM stock

201     solution. This stock was stored in desiccators at room temperature. This stock solution was added to

202     maintenance medium with 200 µM AMP-001 for a final concentration 20 µM which was then diluted

203     to, 10 µM, 1 µM, 0.1 µM and 0.01 µM Dox solution. The CCK-8 kit used in these experiments to

204     determine IC-50 values is a nontoxic, highly sensitive colorimetric assay for the determination of cell

205     viability in cell proliferation and cytotoxicity assays. Raw data were measured on a Spectra Max

206     micro-96 well plate reader and plotted using Prism 5. Transformation, normalization and nonlinear

207     regression were used to analyze data. According best-fit values were used to obtain IC-50. DMSO

208     concentrations less than 1% had no effect on cell viability

209

210     **Statistical Analysis**

211     All experiments were performed at least three times. Data are presented as means ± SD. All

212     statistical analyses were performed using GraphPad Prism 6.0 (GraphPad, SanDiego, CA). One-way

213     ANONVA and Student's t-test were applied to determine statistical significance. A value of $p < 0.05$ was

214     considered statistically significant.

215     **Results**

216     **1.0 Synthesis of AMP-001**

217     The general synthesis of cathepsin B cleavable peptide conjugation with pegylated apoptogen and/or

218     other apoptogens is accomplished through Fmoc chemistry to protect N end of peptide with Boc and then

9

219  couple it with tocopherol derivatives using DCC in DMF. This was further cleaved by TFA and purified

220  using HPLC method. Cathepsin cleavable compounds have been synthesized using the proprietary peptide

221  synthesis technology. In brief, dipeptide valine-citrulline was synthesized using a microwave peptide

222  synthesizer. The resin (containing 0.25 mmol of peptide anchors) was deprotected using piperidine resulting

223  in the formation of the primary amine. The carboxylic acids of the Fmoc protected amino acids (1 mmol)

224  were activated using COMU and conjugated to the primary amines of the growing peptide on the resin. The

225  process of deprotection, activation and conjugation was repeated until the desired peptide was synthesized.

226  Purification of the peptide linked pegylated apoptogen (AMP-001) was performed using a semi-preparative

227  Kromasil C18, 5u column with a flow rate of 5.0 mL/min. HPLC solvents were 0.1 % TFA acetonitrile

228  (solvent A) and 0.1% TFA in water (solvent B) to get off- white powder. The initial gradient A: B, t =0,

229  10: 90 and t = 30 100 % B. Analytical data: HPLC, $R_f$ = 14.3, single peak with a purity 96.62 %. The

230  compound is characterized using MS with a molecular ion peak at 1967 corresponding to M+H peak. The

231  compound was soluble in water (100mg/mL).

232

233  **2.0 Cytotoxicity of AMP-001 in Gastric Cancer Cell Lines**

234  To investigate the cytotoxicity of AMP-001 on human gastric cancer cells, cell lines SGC-7901 and

235  MGC-803 were treated with different concentration of AMP-001 *in vitro* for 48 hours and cell viability was

236  detected using CCK-8 assay. As shown in Fig. 1A-B, AMP-001 inhibited the growth of SGC-7901 and

237  MGC-803 cells respectively in a concentration dependent manner. The $IC_{50}$ values of AMP-001 against

238  SGC-7901 and MGC-803 were 14.25 and 16.91 μM, respectively (Fig 1C-D). Cell proliferation rates were

239  also decreased in a dose and time dependent manner as shown in Fig. 1E-F respectively.

240  Fig 1. A& C: Viability assay for AMP-001 in Gastric cancer cells SGC-7901 and MGC-803

241  respectively, B&D: IC50 curves, E&F: Concentration and time dependent cell proliferation assay for AMP-

242  001.

243 **3.0 AMP-001 Induces Apoptosis and Potentiates Doxorubicin-Induced Apoptosis in Gastric Cancer**

244 **Cells.**

245     The pro-apoptotic effect of AMP-001 was assessed using Annexin V/ staining assay. As shown in

246 Figure 2, two gastric cancer cell lines SGC-7901 and MGC-803 showed significant apoptosis after 48h

247 treatment with IC-50 dose of AMP-001. Although AMP-001 itself was able to induce cell apoptosis, the

248 aim is to use it as a tumor sensitizer in conjunction with chemotherapy (e.g. doxorubicin) to see the potential

249 synergy with it. This will enable us to extend the sensitizing potential of AMP-001 to other FDA approved

250 chemotherapeutics used to treat other kinds of cancers. We, thus investigated whether the low dose of AMP-

251 001 could be used in combination with doxorubicin, a widely used chemotherapeutic drug in the treatment

252 of gastric cancer. MGC-803 and SGC-7901 cells were pre-incubated with or without AMP-001 (10 μM)

253 for 8 hrs. and then treated with DOX (1 μM) for 48 hrs. before analyzed by FACS. Pre-incubation of gastric

254 cancer cells with AMP-001 for 8 hours rendered cancer cells much more susceptible to doxorubicin induced

255 cytotoxicity at a concentration at which AMP-001 itself did not cause substantial cell death. Fig 3A shows

256 pictures of gastric cancer cells by transmission electron microscopy before and after AMP-001 treatment

257 where the combination of doxorubicin and AMP-001 showed lower number of cancer cells compared to

258 individual AMP-001 (10 μM) or doxorubicin (1 μM). This was confirmed by the flow-cytometric analysis

259 of quantification of apoptosis, showing that AMP-001 enhanced early cellular damage induced by

260 doxorubicin in MGC-803 and SGC-7901 cells by several folds (Fig 3B). In addition, combination treatment

261 of cancer cells with 10μM AMP-001 significantly reduced the $IC_{50}$ of doxorubicin in SGC-7901 and MGC-

262 803 cells (~ 6.68 and 9.68 fold reduction, respectively) when compared with doxorubicin only treatment

263 (Fig 4 A-B), thus, establishing the synergy between doxorubicin and AMP-001. These results will have a

264 broader connotation that potentially, one can reduce the chemotherapeutic dose clinically without

265 compromising efficacy and in the process, could reduce potentially dose related off-target toxicity.  In other

266 words, it is possible to expand the therapeutic index of chemotherapeutics when AMP-001 is used as a

267 neoadjuvant to chemotherapeutics which are currently used for treating cancer.

268    Fig 2: A-B: Pro-apoptotic Effect of IC-50 dose of AMP-001 in SGC-7901(14 µM) and MGC-803 (17µM)

269    Gastric Cancer Cells Using Flow Cytometry with Annexin and PI Staining. AMP-001 increased cell death

270    significantly in gastric cancer cells.

271    Fig 3A: Synergy of AMP-001 (10 µM) with Doxorubicin (1 µM) in SGC-7901 and MGC-803 respectively,

272    assessed through transmission microscopy. Combination of AMP-001 and Doxorubicin yielded cumulative

273    cell death by an order of magnitude compared to individual drug treatments.  3B: Synergy of AMP-001 (10

274    µM) with Doxorubicin (1 µM) corroborated with FACS data. The combination of AMP-001 and

275    doxorubicin showed higher cell death compared to individual drugs.

276    Fig 4: Quantitative Drift of $IC_{50}$ value for the combination of AMP-001 and Doxorubicin in A)

277    SGC-7901 and B) in MGC-803 cancer cells respectively.

278

279    **4.0 AMP-001 Enhances DOX-induced Apoptosis through Mitochondrial Dysfunction**

280        As AMP-001 could induce human gastric cancer cells apoptosis at a decent dose, the potential

281    mechanism of cell death through reactive oxygen species (ROS) accumulation was investigated. As shown

282    in Fig. 5 A-B, pretreatment with AMP-001 for 24h before doxorubicin treatment in SGC-7901 and MGC-

283    803 cells caused a significant increase in DCF-reactive ROS than doxorubicin alone. It is well known that

284    the excessive ROS generation causes oxidative stress and impairs membrane potential, resulting in

285    mitochondrial dysfunction[28-29]. Hence, analysis of mitochondrial potential was carried out using

286    polychromatic dye JC-1 (5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidoazolylcarbocyanino iodide)

287    which forms red-orange clusters with high membrane potential mitochondria. while low membrane

288    potential mitochondria show green staining. The results revealed a significant decrease in mitochondrial

289    membrane potential through the enhanced monomer green fluorescence post combinational treatment

290    (12.1%) compared to individuals AMP-001 (2.1%) and doxorubicin (5.7%) (Fig 5C).

291

292    Fig 5: A-B: Enhancement of potentiation of reactive oxygen species (ROS) for the combination of AMP-

293    001 and doxorubicin compared to individual drugs, C: Decrease in mitochondrial potential (enhancement

294    of green fluorescence) using JC-1 dye in SGC-7 and MGC-803 cancer cells respectively.

295

296    **5.0 AMP-001 and Doxorubicin Synergistically Induce Sustained Mitogen Activated Protein Kinase**

297    **(MAPK) and CD 95 Pathways Activation**

298        The mitogen activated protein kinase (MAPK) signaling plays a critical role in the outcome and the

299    sensitivity to anticancer therapies. MAPK signaling is also associated with various cellular stress and

300    stimuli and has been shown to contribute to induction of apoptosis[30]. P38 MAPK, c-jun N-terminal kinase

301    (JNK) and extracellular-regulated kinase 1/2 (ERK1/2) are the three major kinases in MAPK family. We

302    thus examined whether AMP-001 could enhance the apoptosis inducing effects of doxorubicin through

303    MAPK signaling activation. The activated status of MAPKs, ERK and p-38MAPK was characterized using

304    MAPK antibodies that recognize the dually phosphorylated peptide sequence representing the catalytic core

305    of active MAPK enzymes. In all experiments, cells were preincubated with 10 μM of AMP-001for 8 hrs.

306    before adding doxorubicin and analyzed post 24 hrs. The results revealed clear increases in p-p38 MAPK,

307    p-JNK and p-ERK activity following the combination treatment compared with the control and with AMP-

308    001 alone (Fig 6A). It is to be noted that AMP-001 and doxorubicin have incremental effect on these

309    activities.  However, in combination with doxorubicin, there is a significant enhancement in the intensity

310    of the downstream phosphorylated bands. These results suggested that MAPK pathways could play

311    important role in doxorubicin/AMP-001-induced apoptosis. For CD 95 pathway activation assessment, Fas

312    resistant MDA-MB-231 TNBC cells were treated with different concentrations of AMP-001 (5, 10 and 50

313    μM) and assess the expression of CD95 (43 KDa) by Western blotting. Isolation of membrane proteins after

314    cell lysis showed concentration dependent CD95 expression compared to untreated control (Fig. 6B).

315    Translocation of CD95 from cytosol to the membrane is a common observation for CD95 Trail synergy[31]

316    which influences the cellular sensitivity to Fas death receptor pathway.

13

317 Fig 6A: Potential Mechanism of Action for AMP-0012. Activation of MAPKs signaling pathway during

318 AMP-001 and Doxorubicin combination treatment induced apoptosis, 6B: Expression of CD95 band in post

319 treated triple negative breast cancer cells MDA-MB-231 by AMP-001. CD95 pathways is associated with

320 the sensitization of tumor cells.

321 **6.0 AMP-001 Amplifies the Therapeutic Effect of Dox in a Xenograft Gastric Cancer Model in Nude Mice**

322 The sensitizing potential of AMP-001 has paved the way for evoking a better response from

323 chemotherapy, particularly doxorubicin *in vitro*. Hence, it is may be essential if the same thing is true in an

324 *in vivo* situation. Doxorubicin either alone or in combination with AMP-001 were injected i.p in a xenograft

325 gastric cancer model in nude mice. MGC-803 cells were implanted subcutaneously in the right flank of

326 nude mice. After a week, the mice were randomized into 4 groups and treated as described in the

327 experimental protocol. Animals were sacrificed after 3 weeks treatment. We found that low dose (10

328 mg/Kg) AMP-001 alone did not inhibit the growth of the tumor at that dose compared to control, while

329 doxorubicin alone (1mg/Kg) inhibited tumor growing to a certain degree.  However, the combination of

330 AMP-001 and doxorubicin was more effective in reducing tumor burden (Fig 7A). The tumor volume and

331 tumor weight in the combinational group was significantly lower than either doxorubicin or AMP-001. (Fig

332 7B, $p < 0.001$). Additionally, all mice did not undergo obvious body weight loss or abnormal symptoms in

333 drug treatments (Fig 7D). In alternate studies on AMP-001 in triple negative breast cancer model, we have

334 shown that high dose of AMP-001 (~200 mg/Kg) alone can have significant tumor regression with no

335 observable toxicity, despite higher dose[12]. Similarly, in the current studies also, there was no evident

336 histopathological abnormalities were observed in the vital organs such as heart, liver and kidney estimated

337 by HE staining (Fig 8). Ki67 (marker for cell proliferation) and CD31 (marker for micro vessel density)

338 were also examined by immunohistochemistry in the excised tumor sections. Although, doxorubicin also

339 downregulated the Ki67 expression, the combination group was most effective similar to the tumor

340 regression studies. The trend of CD31 expression was consistent with Ki67 (Fig 8). In order to determine

341 whether AMP-001/Dox combination treatment effectively promotes apoptosis in tumors, sections of

14

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.

365  Fig 9. Photomicrographs of treated with A) control DMSO, B) 50 ☐M AMP-001, C) 10 μM Dox, D) 100

366  μM AMP-001 and E) IC-50 measurements for Vitamin E, AMP-001, Sorafinib, Dox in induced pluripotent

367  stem cell cardiomyocytes (iPSc) compared with AMP-001 in MDA-MB-231 triple negative breast cancer

368  cells and F) IC-50 of Dox in iPSc cells.

369  **8.0 Discussion**

370  Due to the lack of early detection, human gastric cancer remains as the second most common cause of

371  death from cancer worldwide[32]. Chemotherapies have become inevitable despite high side effects.

372  However, the therapeutic efficacy decreases when cancer cells develop resistance to chemotherapeutics.

373  Combinational chemotherapies have been a mainstay in the treatment of disseminated malignancies for a

374  long time[33-35] and have been widely used for minimizing acquired resistance, enhancing cell-intrinsic drug

375  synergy with chemotherapy or for maximizing cumulative drug dose[36a]. One of the main reasons for tumor

376  cells to develop resistance is downregulation of cell death pathways by cancer cells to apoptosis. New

377  methods are required to activate these dysregulated cell death pathways and to sensitize the low-responsive

378  resistant tumor cells which are otherwise non-responsive to any therapy.

379  The present study proposes targeting the chemotherapeutics using a tumor specific biomarker

380  Cathespin B.  Cathepsin B is overexpressed in many cancers including gastric cancer making it a unique

381  biomarker of cancer to which drugs can be targeted[21]. Valine-citrulline dipeptide is one such linker which

382  is cleavable by Cathespsin B[20]. The drug design AMP-001 has to take into consideration the release of

383  apoptogen (apoptosis inducing ligand) at tumor sites in order to be selective to cancer cells. The pegylation

384  of the basic apoptogen core is expected to increase water solubility and make it stable in the circulatory

385  blood for a long time to reach the tumor[37]. The parent molecule α-tocopheryl succinate (α-TOS), although

386  showed high promise in both *in vitro* and *in vivo*, low solubility in water and a serious toxicity in

387  immunocompetent animal model limited its utility[23]. This observation could explain the lack of clinical

16

—

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

17

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.

20

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

490      **Conclusions**

491      Since cancer is a group of diseases, several targets and pathways are involved in the progression of

492      the disease. Although, conventional drugs apart from nonspecific chemotherapeutics target specific target

493      (s), each drug works with specific targets/pathways. The combination of AAAPT leading molecule with a

494      conventional chemotherapy brings multiple pathways involved in cancer cells desensitization process.

495

496      In summary, the effects of novel vitamin E analogue AMP-001 is as a preclinical candidate is

497      described. AMP-001 enhanced doxorubicin induced apoptosis in human gastric cancer cells via ROS-

498      dependent mitochondrial apoptotic pathway and MAPK pathway. The efficacy of doxorubicin antitumor

499      activity can be amplified by combining it with AMP-001, which permits lower dose use in patients without

500      affecting the positive clinical outcome. These effects are confirmed both *in vitro* and *in vivo*. Our current

501      studies indicated that AMP-001 might be a potential candidate in synergetic with the existing FDA

502      approved chemotherapeutic drugs for treatment of gastric cancer. Ultimately, the combination treatments

503      success depends on how low dose can be made effective in a clinical situation, reduced overall toxicity and

504      improved patient compliance. Further studies are planned to optimize the dose combination as to which one

505      has higher efficacy and low toxicity compared to the existing treatments.

506      **Acknowledgements**

508      **Conflict of interest**

509      The authors declare no competing financial interest.

510

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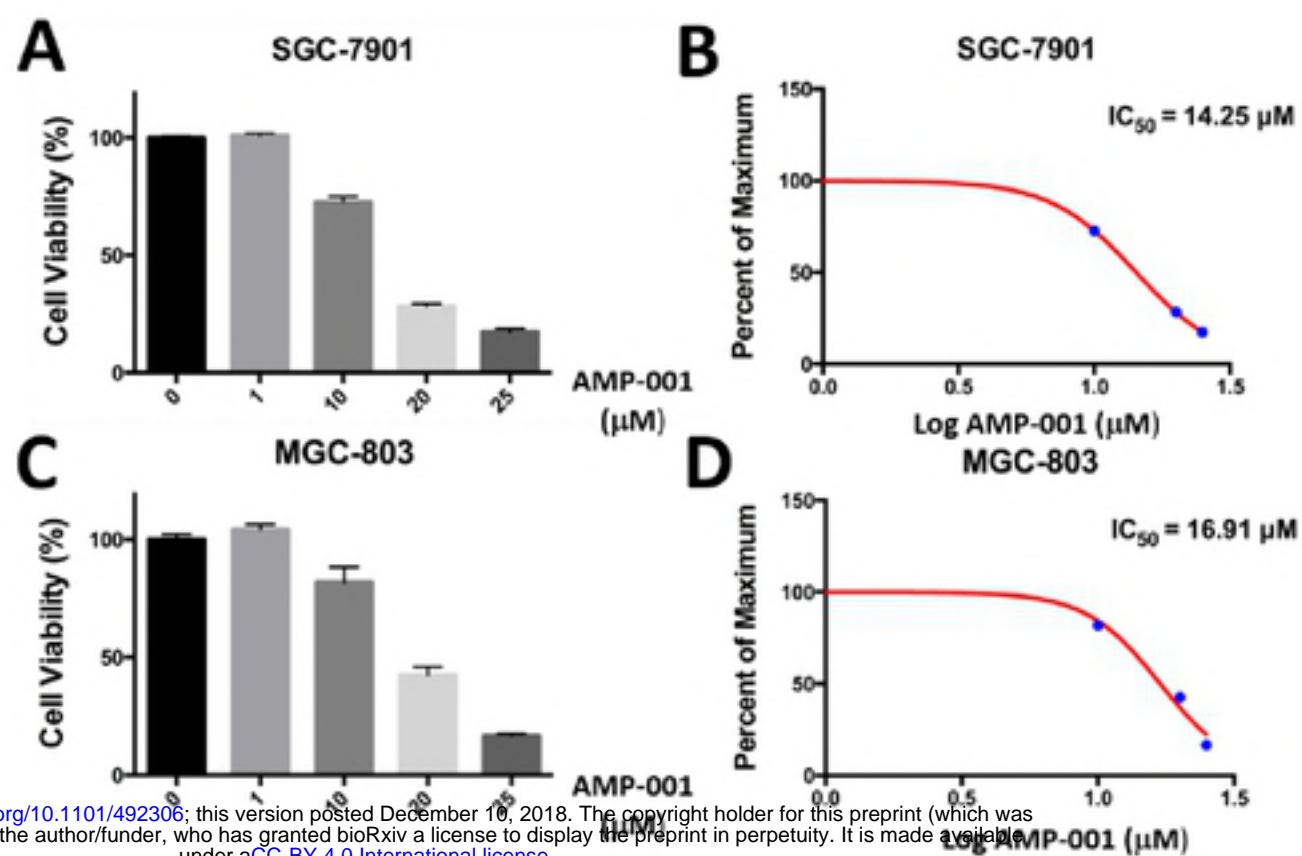
Figure

Figure
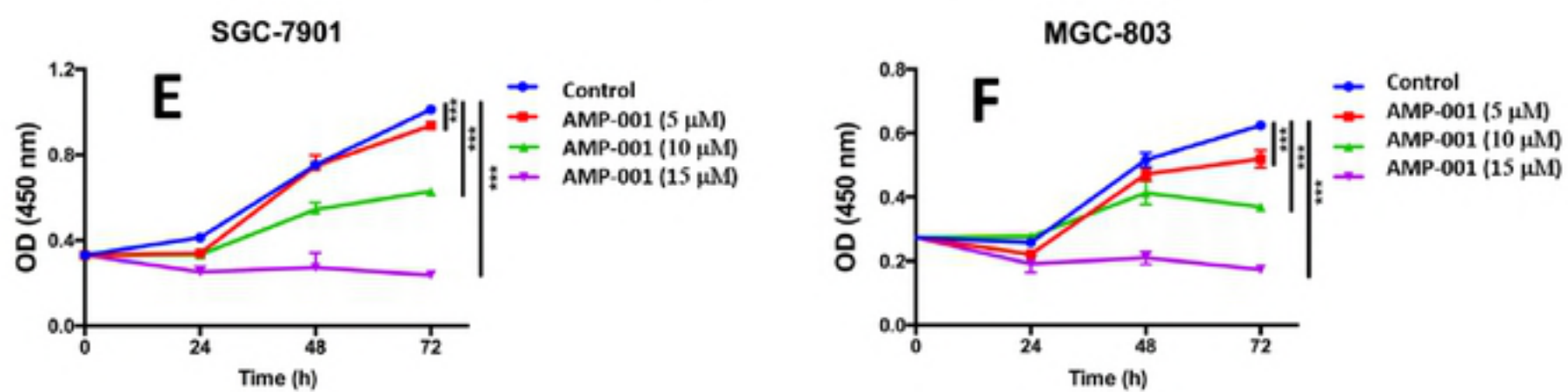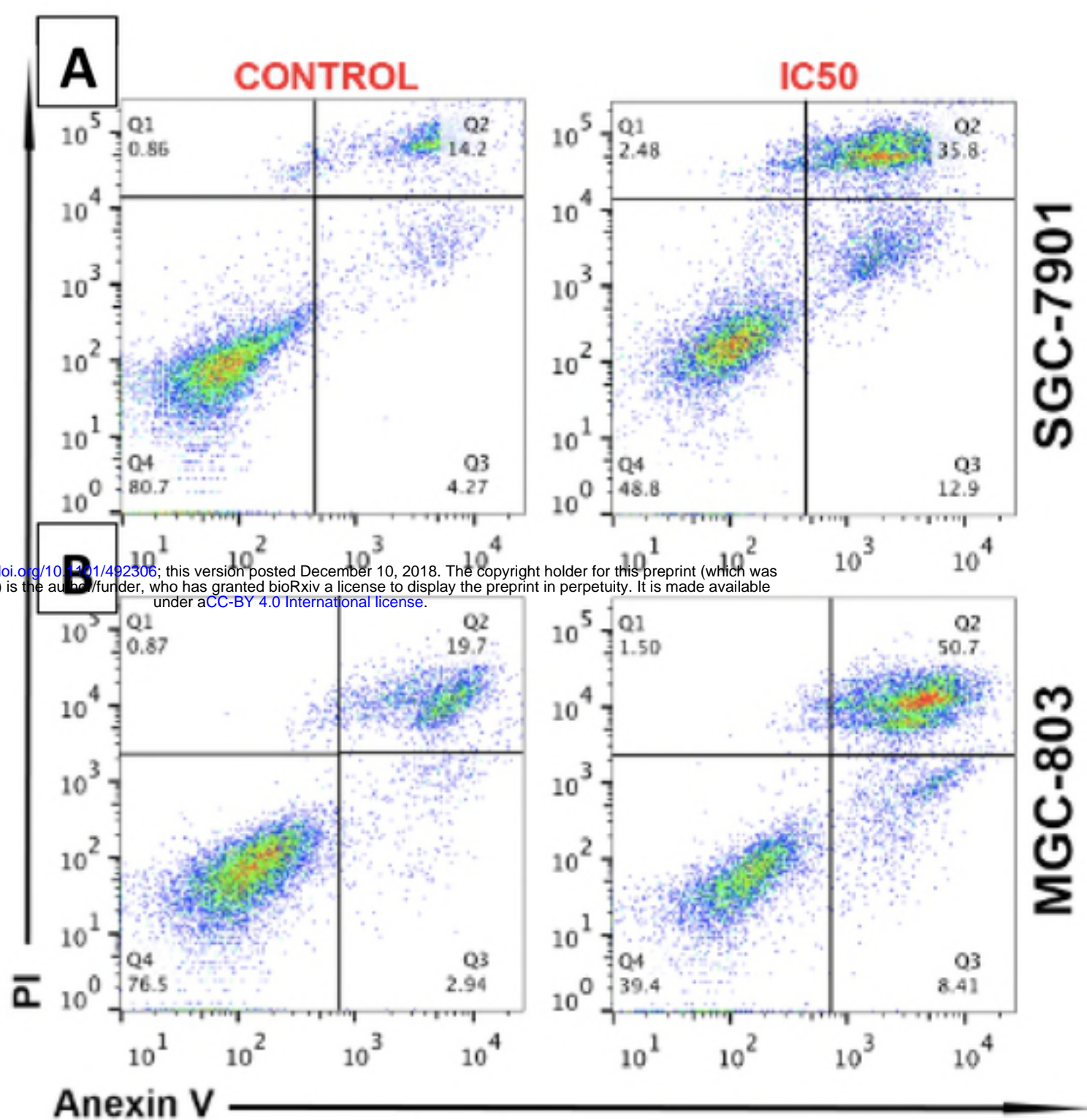
**3A:**

| | Control | AMP-001 | Doxo | AMP-001+Doxo |

SGC-7901

MGC-803

**3B**

SGC-7901

PI

MGC-803

**Anexin v**

Figure

**A** SGC-7901

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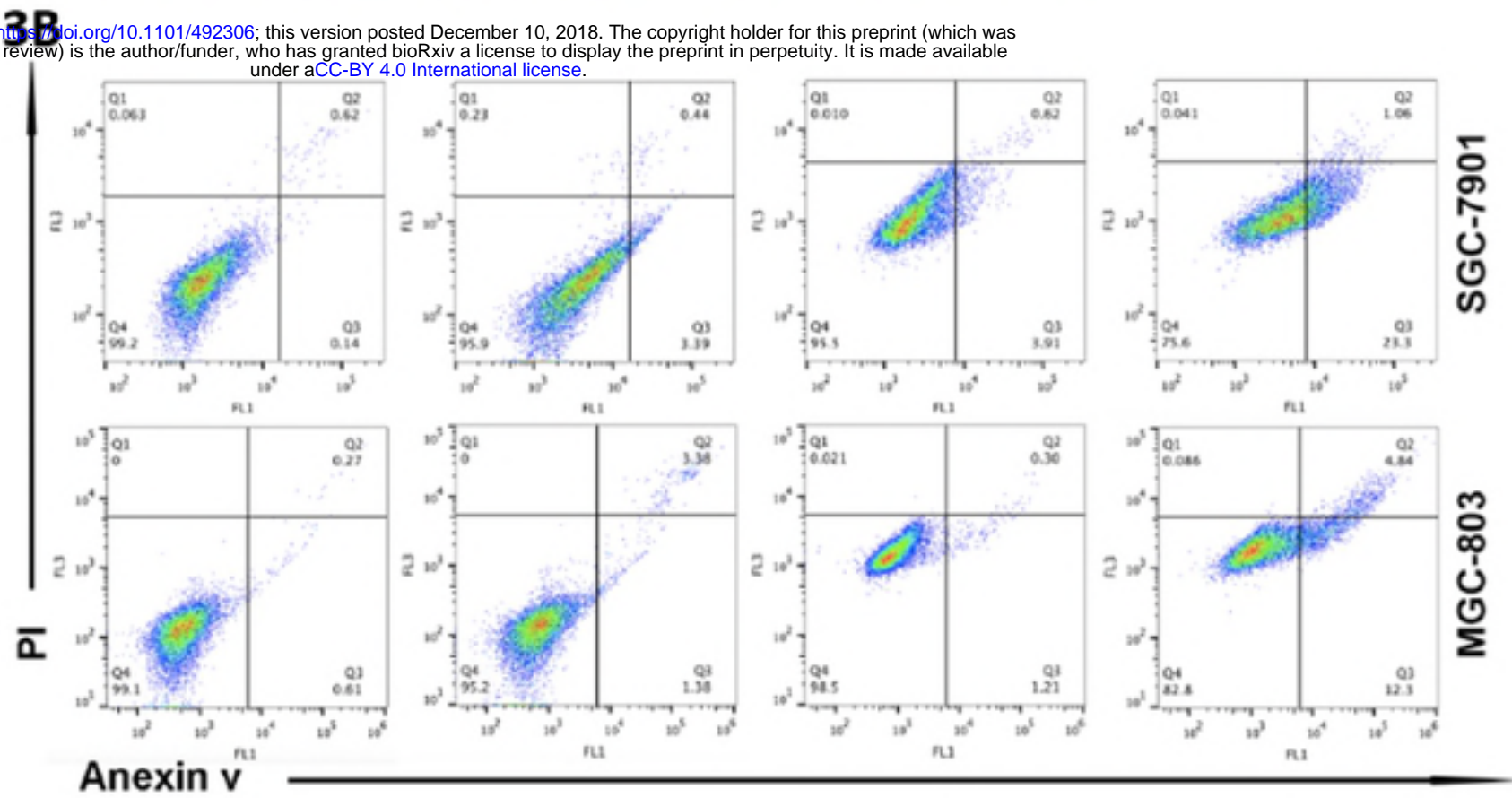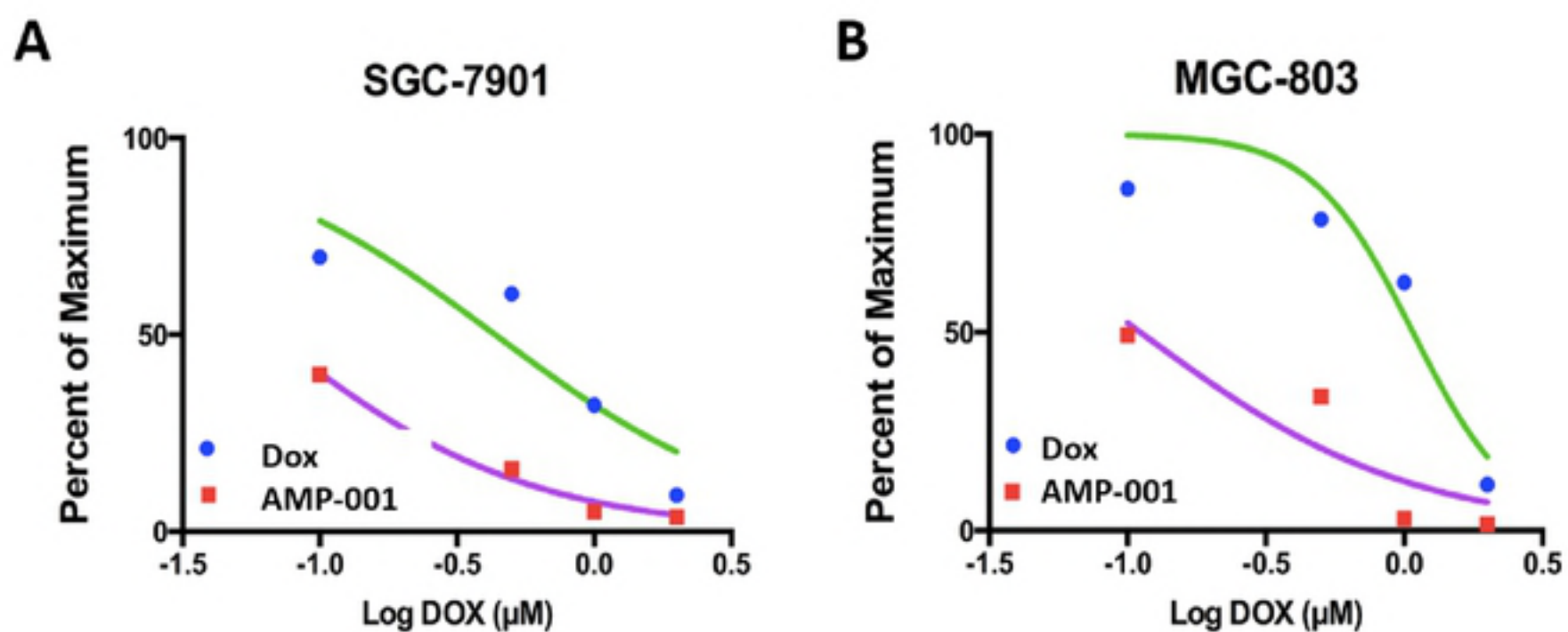
| Drug Regimen (Table 1) | IC-50 (μM) | |
|---|---|---|
| Cancer Cell | SGC-7901 | MGC-803 |
| Dox Alone | 0.436 | 1.076 |
| Doxo+AMP-001 | 0.065 | 0.111 |

Figure

## SGC-7901

## MGC-803

Figure

Figure

Figure

**Vehicle**  **AMP-001**  **Doxo**  **Doxo+AMP-001**
**(200x Magnifications, Scale Bar 50μm)**

Figure

DMSO          50 μM AMP-001      10 μM Doxo      100 μM AMP-001

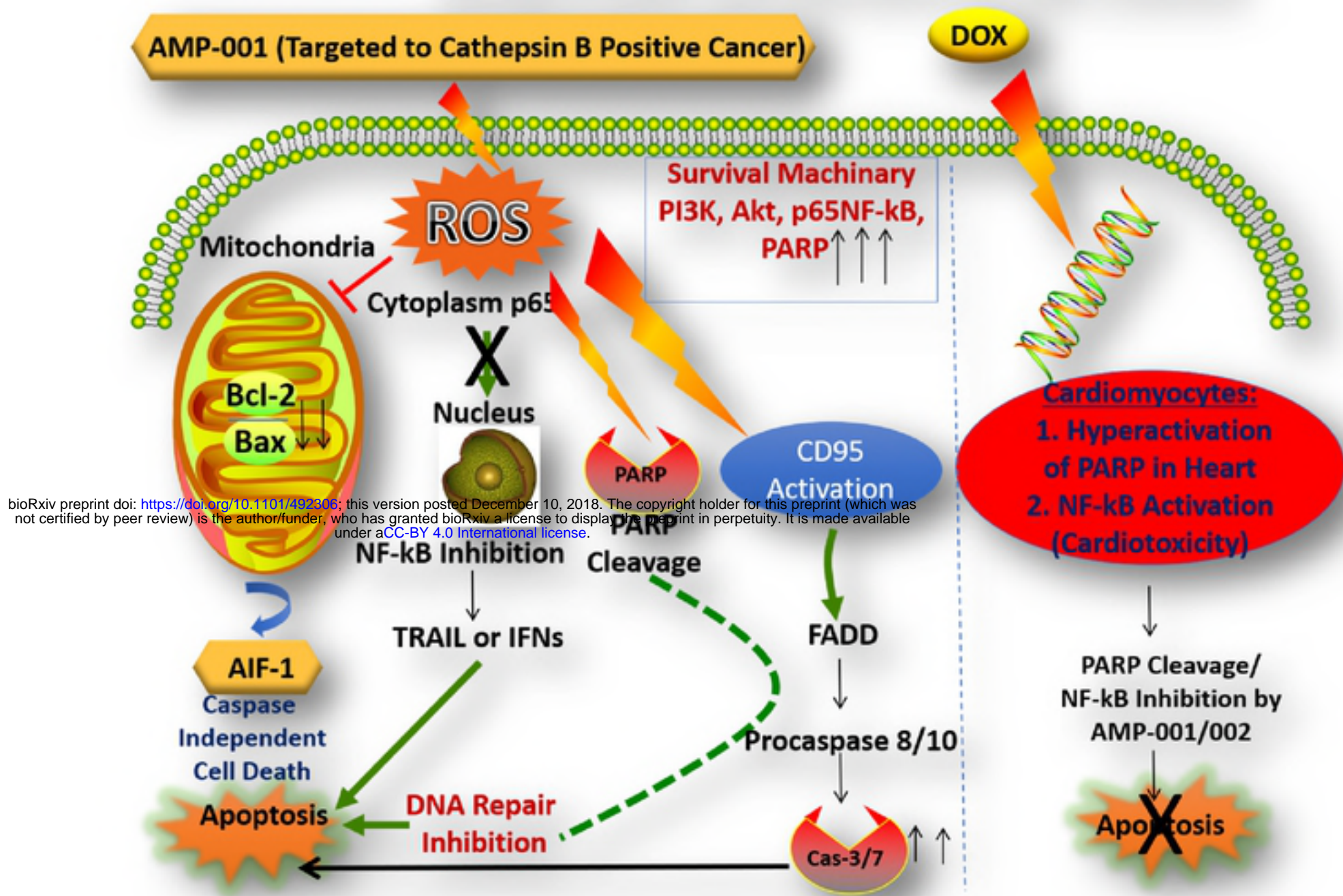| Compound | IC-50 |
|---|---|
| Vitamin E | > 1 mM |
| AMP-001 | > 250 μM |
| Sorafinib | 40 μM |
| Doxorubicin | 9.6 μM |
| AMP-001/TNBC | 32 μM |
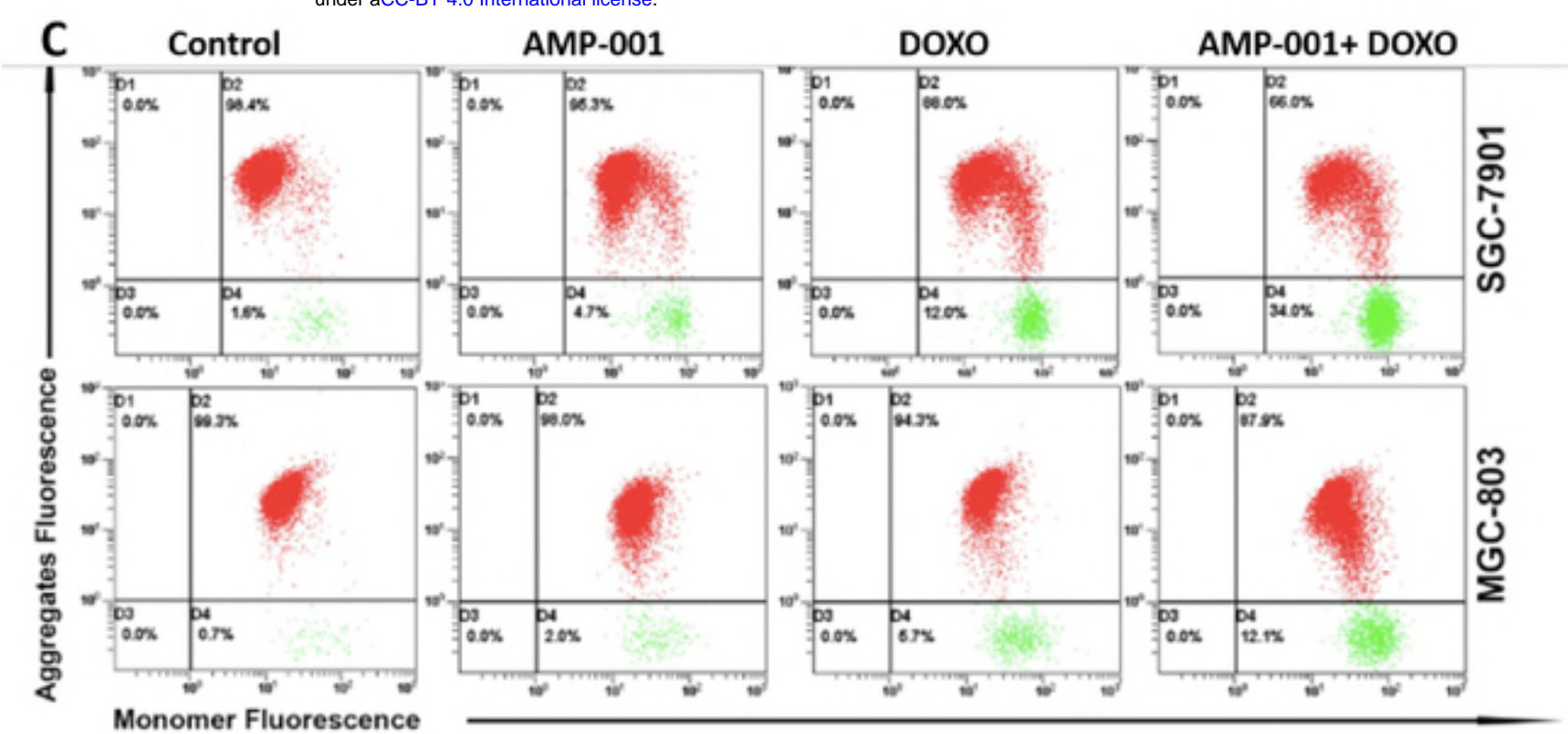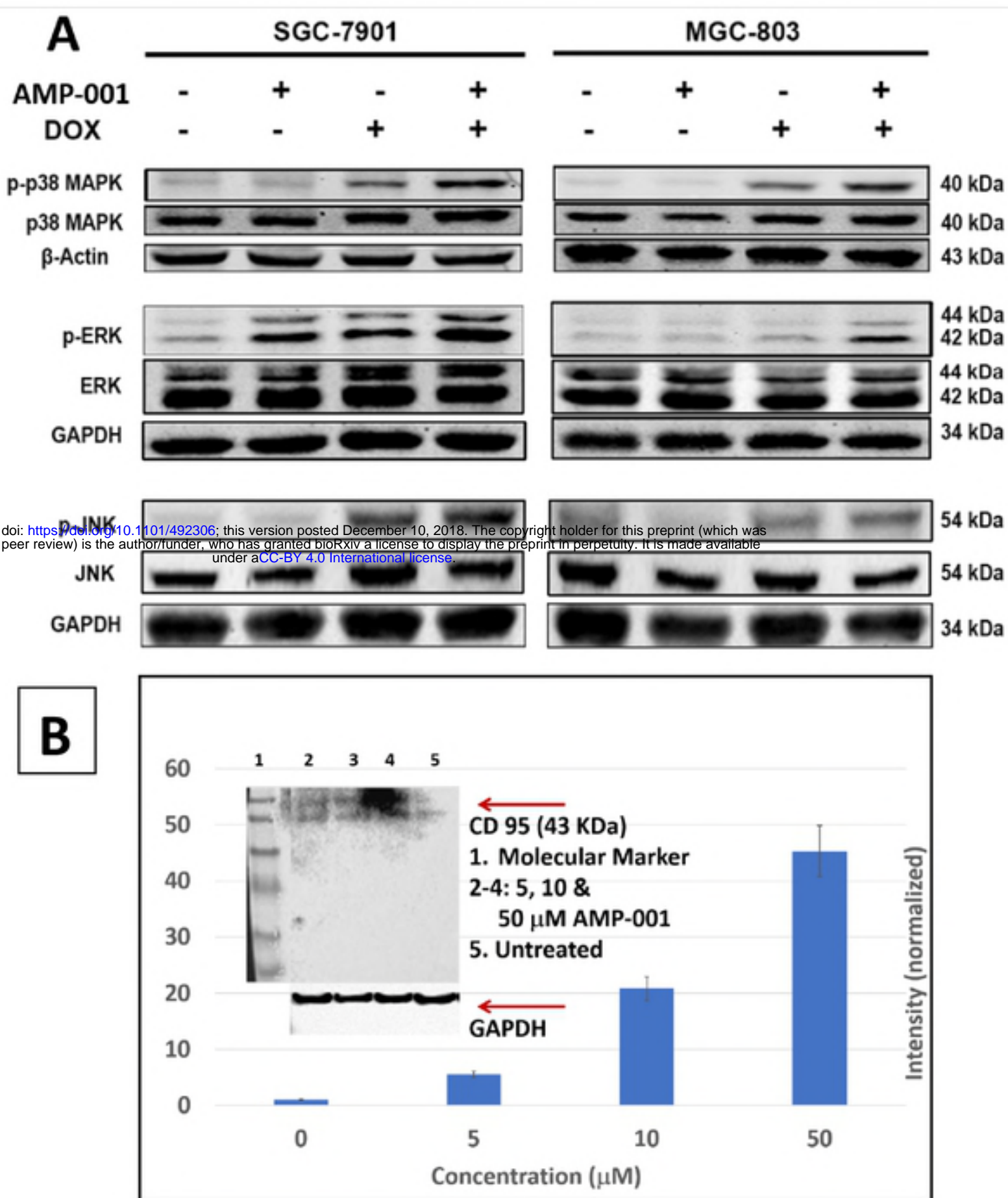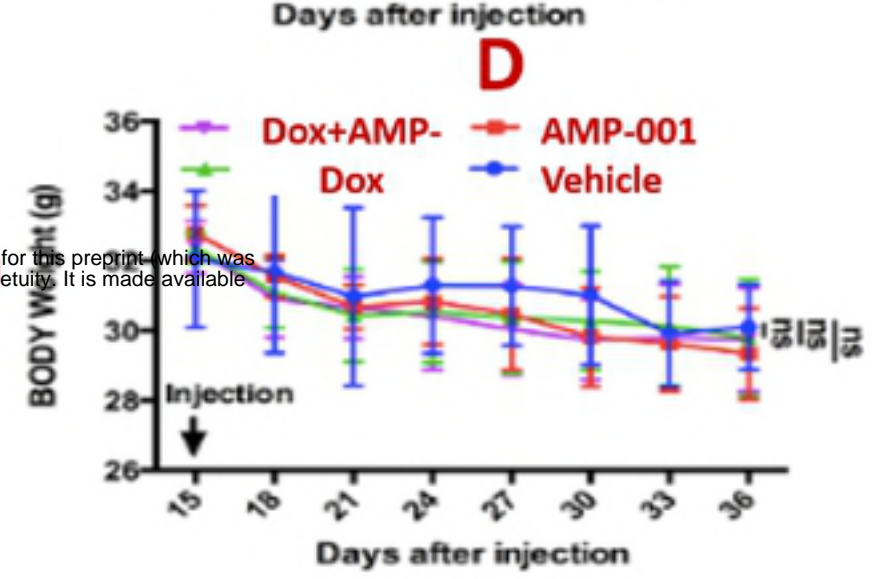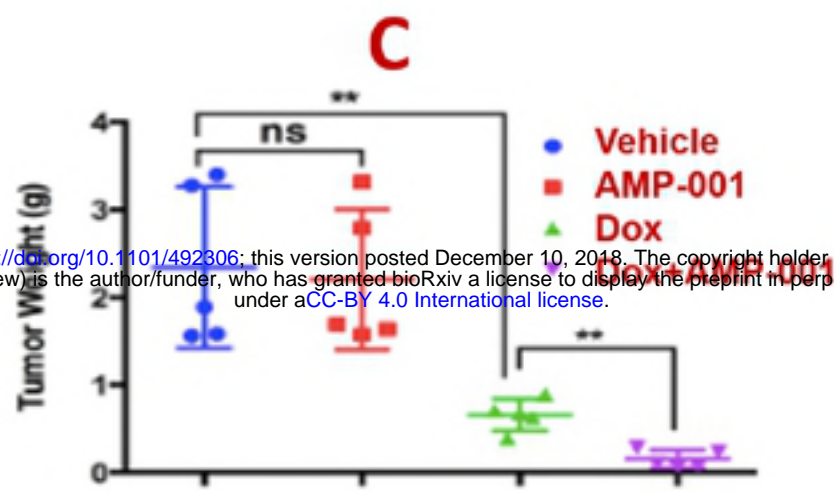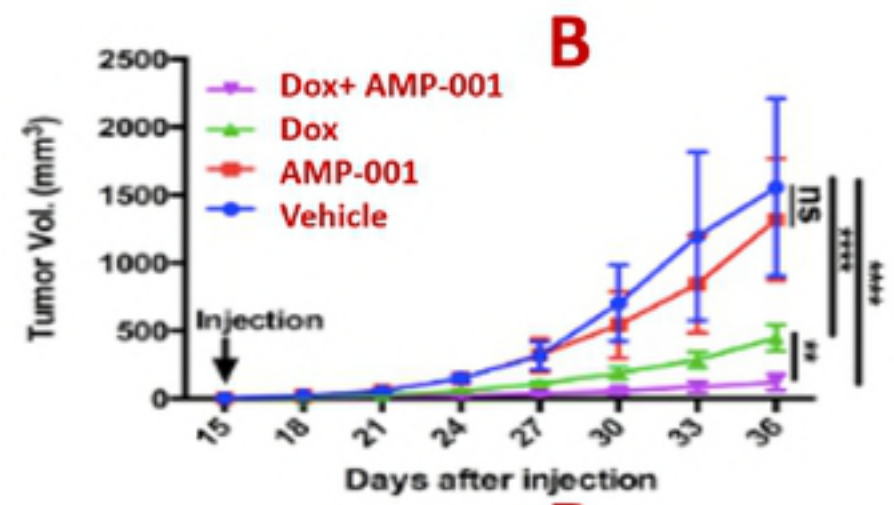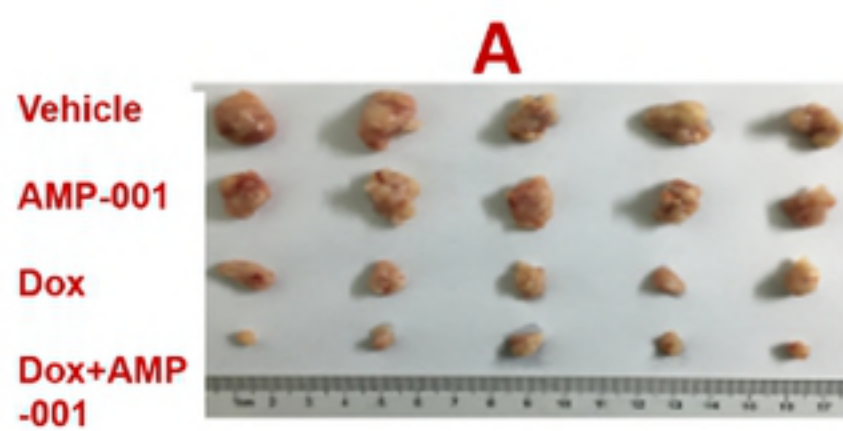
Figure

## Synergy of AAAPT with Doxorubicin for TNBC Treatment

Figure