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Authors

Brand, Nathan

Yang, Yi-Wei

Ding, Vivianne

et al.

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Original Article
Novel dual action PARP and microtubule polymerization inhibitor AMXI-5001 powerfully inhibits growth of esophageal carcinoma both alone and in combination with radiotherapy

Nathan R Brand[1*], Yi-Wei Yang[2*], Vivianne Ding[2], Hannah Dutta[2], Csaba J Peto[2], Hassan Lemjabbar-Alaoui[3], David M Jablons[1]

[1]Department of Surgery, University of California, San Francisco, California, USA; [2]Thoracic Oncology Laboratory, University of California, San Francisco, California, USA; [3]AtlasMedx, Inc., San Ramon, California, USA. *Equal contributors.

Abstract: Esophageal cancer is one of the leading causes of cancer deaths globally with an incidence that is concentrated in specific hot spots in Eastern Asia, the Middle East, Eastern Africa, and South America. 10-year overall survival for patients treated with standard of care chemoradiation followed by surgical resection is below 40% highlighting the need for novel therapeutics to treat this disease. We assessed the effect of AMXI-5001, a novel small molecule poly ADP-Ribose polymerase (PARP) inhibitor and microtubule polymerization inhibitor on tumor growth inhibition in both *in-vitro* and *in-vivo* murine models. We found that AMXI-5001 was the most potent growth inhibitor of 8 out of 9 different esophageal carcinoma cell lines compared to other clinically available PARP inhibitors, Olaparib, Niraparib, Rucaparib, and Talazoparib. We then confirmed the previously described mechanism of action of AMXI-5001 as a PARP-inhibitor and microtubule polymerization inhibitor using both a PARP trapping assay and immunofluorescence. To further assess AMXI-5001's potential as a therapeutic for esophageal carcinoma we evaluated the effect of AMXI-5001 in combination with standard chemotherapy agents, Cisplatin and 5 Fluorouracil. We showed that AMXI-5001 synergistically inhibits growth in KYSE-70, a squamous esophageal cell line in combination with these drugs. In addition, we found that AMXI-5001 was an effective radiosensitizer, and squamous esophageal carcinoma cell lines treated 24 hours prior to external beam radiation showed significantly more growth inhibition compared to controls. Finally, we assessed the effect of AMXI-5001 monotherapy and in combination with radiotherapy in a xenograft mouse model implanted with subcutaneous KYSE-70 cells. Compared to vehicle control, and those treated with either AMXI-5001 alone or radiation alone, mice treated with both AMXI-5001 and radiation had significant tumor response. In conclusion, AMXI-5001 is an orally bioavailable dual-action PARP and microtubule polymerization inhibitor that holds promise in the treatment of esophageal carcinoma.

Keywords: Esophageal carcinoma, PARP inhibitors, microtubule polymerization inhibitors, radiotherapy, xenograft model

Introduction

Esophageal cancer is one of the leading causes of cancer deaths globally, with an age-standardized mortality rate of 5.5 per 100,000 people [1]. Interestingly, the incidence of this disease is not evenly distributed across the globe but rather concentrated in specific hot spots in Eastern Asia, the Middle East, Eastern Africa, and South America [2]. Histologically, the disease is primarily either adenocarcinoma (AC) or squamous cell carcinoma (SCC). Globally, squamous cell carcinoma is the most common form of esophageal cancer, accounting for almost the entire disease burden among the global hot spots [3]. In high income countries (HIC) such as Europe and the United States, the incidence of SCC has been declining, likely due to public health campaigns targeting tobacco smoking and alcohol use. This decline, however, has been matched with an increase in the incidence of AC thought to be caused by the worsening

obesity epidemic and the increasing prevalence of gastroesophageal reflux disease [2]. In the United States, while the incidence of SCC is declining among all races, Black Americans are still 7-fold more likely to be diagnosed with esophageal SCC than AC [4].

Chemoradiation followed by esophagectomy is the mainstay of treatment for medically patients fit with surgically resectable tumors. This treatment regimen is based on the results of the CROSS trial, which showed a survival benefit among those treated with neoadjuvant chemoradiation versus surgery alone [5]. However, a recent analysis assessing the 10-year overall survival of patients enrolled in the CROSS trial reported that the overall survival of patients treated with neoadjuvant chemotherapy versus surgery alone was 38% and 25%, respectively [6]. While these results show the effectiveness of neoadjuvant chemoradiation, the poor survival in both arms highlights the need for novel therapeutic regimens to improve the survival of patients with esophageal cancer.

One potential therapeutic opportunity is the inhibition of poly ADP-Ribose polymerase (PARP). PARPs are a class of proteins involved in base excision repair of single-stranded breaks in DNA. Cells have multiple methods of DNA repair, including base excision repair which addresses single-stranded breaks in DNA, and homologous recombination (HR) which repairs double-stranded breaks. BRCA mutations, linked to multiple cancers, are genetic alterations in the HR repair pathway. Patients with germline BRCA mutations are heterozygous and are therefore susceptible to somatic "second hit" mutations to the BRCA genes, leading to a loss of the HR repair pathway. This complete loss leads to neoplastic development and an increased reliance of these neoplastic cells on base excision repair and PARP to address DNA damage. This observation has led to the development of multiple PARP inhibitors for the treatment of triple negative breast cancers and other neoplasms which have been shown to have a loss of the HR pathway [7, 8].

In esophageal SCC, mutations to the BRCA gene have been found to be associated with an increased incidence of disease, suggesting that loss of HR may be an important step in the development of esophageal SCC [9]. *In vivo*

studies of esophageal SCC have shown that PARP inhibitors increase sensitivity to radiation and chemotherapy suggesting these novel therapeutics may have a role in the treatment of esophageal SCC [10, 11]. As well as promising *in vivo* studies, gene expression results from The Cancer Genome Atlas Program also show significantly increased expression of PARP in esophageal SCC compared to normal esophageal squamous cells, further suggesting the role of PARP inhibition in treating esophageal carcinoma. Recently, we developed a novel small molecule PARP and microtubule polymerization inhibitor called AMXI-5001.

This novel therapeutic elicited a robust antitumor effect against various cancer cell lines and is orally bioavailable [12]. Here, we report the effect of AMXI-5001 on various esophageal cancer cell lines, both alone and in combination with chemotherapy and radiation therapy. We show that AMXI-5001 exhibits more potent antitumor effects than all clinically approved PARP inhibitors against both squamous cell and adeno esophageal carcinoma and show promising results of the combination of AMXI-5001 and radiation therapy in esophageal cancer cell lines and in a murine xenograft model.

Material and methods

Cell culture

Cell lines were purchased from Sigma Aldrich (St. Louis, MO) and DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). KYSE-70, KYSE-30, Colo-680 N, KYSE-140, KYSE-520, KYSE-180, OE21, OE33 and OE19 were cultured in RPMI-1640 medium with 10% fetal bovine serum. KYSE-30 was cultured in RPMI-1640/F-12 (1:1) medium with 10% fetal bovine serum.

Chemicals and antibodies

5-FU and Cisplatin were purchased from Selleck Chemicals (Houston, TX). Olaparib was purchased from LC Laboratories (Woburn, MA). BMN-673, Rucaparib and Niraparib were purchased from MedChemExpress (Monmouth Junction, NJ). Cisplatin was dissolved in water and the other drugs were dissolved in DMSO. Anti-PAR (#83732), anti-PARP1 (#9531), anti-GAPDH (#5174), anti-Histone 3 (#4499), anti-phospho-Histone 3 (#3377) were purchased

from Cell Signaling Technology (Danvers, MA). Anti-tubulin antibody (#ATN02) was purchased from Cytoskeleton Inc. (Denver, CO). Anti-DNA topoisomerase I antibody (clone C-21.2, BD) was purchase from Fisher Scientific (Thermo-Fisher).

AMXI 5001

AMXI-5001 is an orally bioavailable dual PARP and microtubule polymerization inhibitor with favorable metabolic stability, oral bioavailability, and pharmacokinetic properties. AMXI-5001 has been given orally twice daily to mice, rats, and dogs at various dose levels in IND, enabling 29-day Repeated-Dose GLP tox studies, and has demonstrated a good safety profile (ATLASMEDX, INC IND confidential Files).

In addition, AMXI-5001 is currently progressing through a Phase I/II multi-center dose escalation and dose expansion study in patients with refractory, impossible-to-treat malignancies. In the clinic, AMXI-5001 is orally administered twice daily (BID) with a continuous (7 days/week) dosing regimen. The drug is well tolerated with no significant or dose-limiting drug-related toxicity or issues. Results of the AMXI-5001 IND and Phase I/II clinical trials will be reported in future manuscripts by ATLASMEDX.

Cell viability assay

2,000 cells were seeded to 96-well white wall plates in 100 µl of culture medium with 2% fetal bovine serum and cultured for 16-24 hours. 100 µl of 2× drug medium was added to wells on the next day and cells were cultured for 7 days. Cell viability was measured using CellTiter-Glo reagent (Promega, Madison, Wisconsin). Cell growth curves were plotted and IC50s were calculated using Prism GraphPad (San Diego, CA).

Combination therapy

2,000 cells were seeded to 96-well white wall plates in 100 µl of culture medium with 2% fetal bovine serum and cultured for 16-24 hours. For drug-drug combinations, 100 µl of 2× drug medium containing single drugs or both drugs were added to wells and cells were cultured for 7 days. For drug-radiation combinations, 2× drug medium containing single drugs was added to wells the day before radiation.

Radiation was performed using a SARRP irradiator (Xstrahl, Suwanee, GA) and the cells were cultured for 6 more days. Cell viability was measured using Promega CellTiter-Glo reagent.

Microtubule immunofluorescence staining

Cells were seeded to 8-well chambered slides (Corning, Corning, NY) in complete medium and cultured until attached. Drug stock solution was diluted in medium with 5% fetal bovine serum and added to cells. 24 hours post-treatment, cells were washed in PBS three times before fixed in 100% methanol. Cells were washed in PBS three times and permeabilized in 0.25% Triton X-100 in PBS. After three PBS washes, cells were blocked in 5% normal rabbit serum in PBS at room temperature for one hour, followed by incubating in anti-tubulin antibody in PBS with 1% normal rabbit serum at 4°C overnight. On the next day, cells were washed in PBS four times, 5 minutes each and then incubated in Dylight-594 conjugated rabbit anti-sheep IgG (H+L) antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in PBS with 1% normal rabbit serum at room temperature for one hour. After 4 times of 5-minute PBS washes, chamber was removed and antifade mounting media with DAPI (Vector Laboratories, Inc., Newark, CA) was added to cells. Images was taken using an AxioImager2 fluorescence microscopy (Zeiss, Jena, Germany).

Western blot

Cells were seeded to 6-well plates or 100 mm dishes in complete medium and cultured overnight, followed by starving in serum-free medium for 16-24 hours prior to drug treatment. 48 hours post-treatment, cells were washed in PBS and lyzed in M-PER lysis buffer (ThermoFisher, Waltham, MA). Cleared lysate was subjected to BCA quantification (ThermoFisher) to determine the protein concentration. Proteins were separated by electrophoresis (Bio-Rad, Hercules, CA), transferred to PVDF membrane and blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature for one hour. Blots were incubated with primary antibody diluted in 5% bovine serum albumin in TBST at 4°C overnight. After three 10-minute TBST washes, blots were incubated with secondary antibody diluted in blocking buffer at room temperature for one

hour. Blots were washed three time with TBST, 10 minutes each, and SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo-Fisher) was added to blots. Images was taken using a FluorChem Western Blot Imaging System (Bio-Techne, Minneapolis, MN) and processed using Image J software (NIH, Bethesda, MD).

Subcellular protein fractionation

Cells were seeded to 6-well plates in complete medium and cultured overnight, followed by starving in serum-free medium for 16-24 hours prior to drug treatment. Cells were treated with PARP inhibitors in the presence of 0.01% of DNA alkylating agent methyl methanesulfonate (MMS) in medium with 1% fetal bovine serum. 3 hours post-treatment, cells were harvested, washed with PBS once, and subjected to fractionation using Subcellular Protein Fractionation Kit (ThermoFisher) according to manufacturer instruction. Soluble nuclear extract and chromatin-bound nuclear extract were subject to Western Blot analysis.

Animal study

All animal experiments were approved by the Institutional Animal Care and Use Committee at UCSF (IACUC) and conducted by the UCSF Preclinical Therapeutics Core. In Brief, KYSE-70 cells in serum-free RPMI-1640 was mixed 1:1 with Matrigel (Corning) and 10 million of cells were injected subcutaneously into the left flank of female nude mice. When tumors reached 80 mm³ volume size, mice were randomized and assigned into four groups: (1) vehicle control, (2) 50 mg/kg AMXI-5001 only, (3) 6 Gy localized radiation only, and (4) 50 mg/kg AMXI-5001 + 6 Gy localized radiation combo. AMXI-5001 was first resuspended in 1-Methyl-2-pyrrolidinone (NMP) and further diluted with 0.5% carboxylmethyl cellulose (CMC) to final 50 mg/mL. AMXI-5001 was administrated twice per day via gavage with 5-day on and 2-day off schedule for 4 weeks. Mice that assigned to group 4 were radiated with a single dose of localized radiation (6 Gy) on day 3 after the first dose of AMXI-5001 was administrated using a XRAD 320 irradiator. Mouse body weight and tumor volumes were measured twice weekly and tumor samples were collected after the final dose of AMXI-5001 was administrated on day 29. Tumor tissue was formalin fixed,

parafilm embedded (FFPE) and cut into sections for H&E staining and IHC staining.

Immunohistochemistry

Tumor sections were deparaffinized, hydrated and under antigen retrieval in 1× SignalStain Citrate Unmasking solution (Cell Signaling Technology) at sub-boiled temperature for 10 minutes using a microwave. Sections were washed in PBS three times and incubated in 3% hydrogen peroxide for 10 minutes. After washed in PBS twice and once in TBST, sections were blocked in 5% normal goat serum in TBST at room temperature for one hour, followed by incubated with primary antibody at 4°C overnight. Sections were washed in TBST and SignalStain Boost Detection Reagent (rabbit, Cell Signaling Technology) was added to sections. After incubating at room temperature for 30 minutes, sections were washed in TBST before incubated in SignalStain DAB substrate (Cell Signaling Technology). Sections were counterstained with hematoxylin and mounted in DPX mounting medium (Sigma).

Results

AMXI-5001 is a more potent growth inhibitor of esophageal carcinoma than other clinically available PARPi

AMXI-5001 has been showed to be a potent cell growth inhibitor in a wide range of cancer cell lines [12]. We first assess the potency of AMXI-5001 and other clinically available PARP inhibitors, Olaparib, Rucaparib, Niraparib, and Talazoparib, on 9 different esophageal carcinoma cell lines, including two adenocarcinoma and seven squamous cell lines using a cell viability assay, in 7-day cell viability assays (**Figure 1**). Overall, PARP inhibitors were an effective treatment against all cell lines evaluated, and AMXI-5001 was shown to be the most potent inhibitor with a mean IC50 of 0.094 µM compared to a mean IC50 of 26.9 µM, 14.6 µM, 81.1 µM, and 0.8 µM for Olaparib, Niraparib, Rucaparib, and Talazoparib respectively. A full list of if the IC 50 of all the cell lines assessed is summarized in **Table 1**.

AMXI-5001 inhibits both PARP and microtubule polymerization

AMXI-5001 has been shown to be an effective inhibitor of parp1/2 and microtubule polymer-
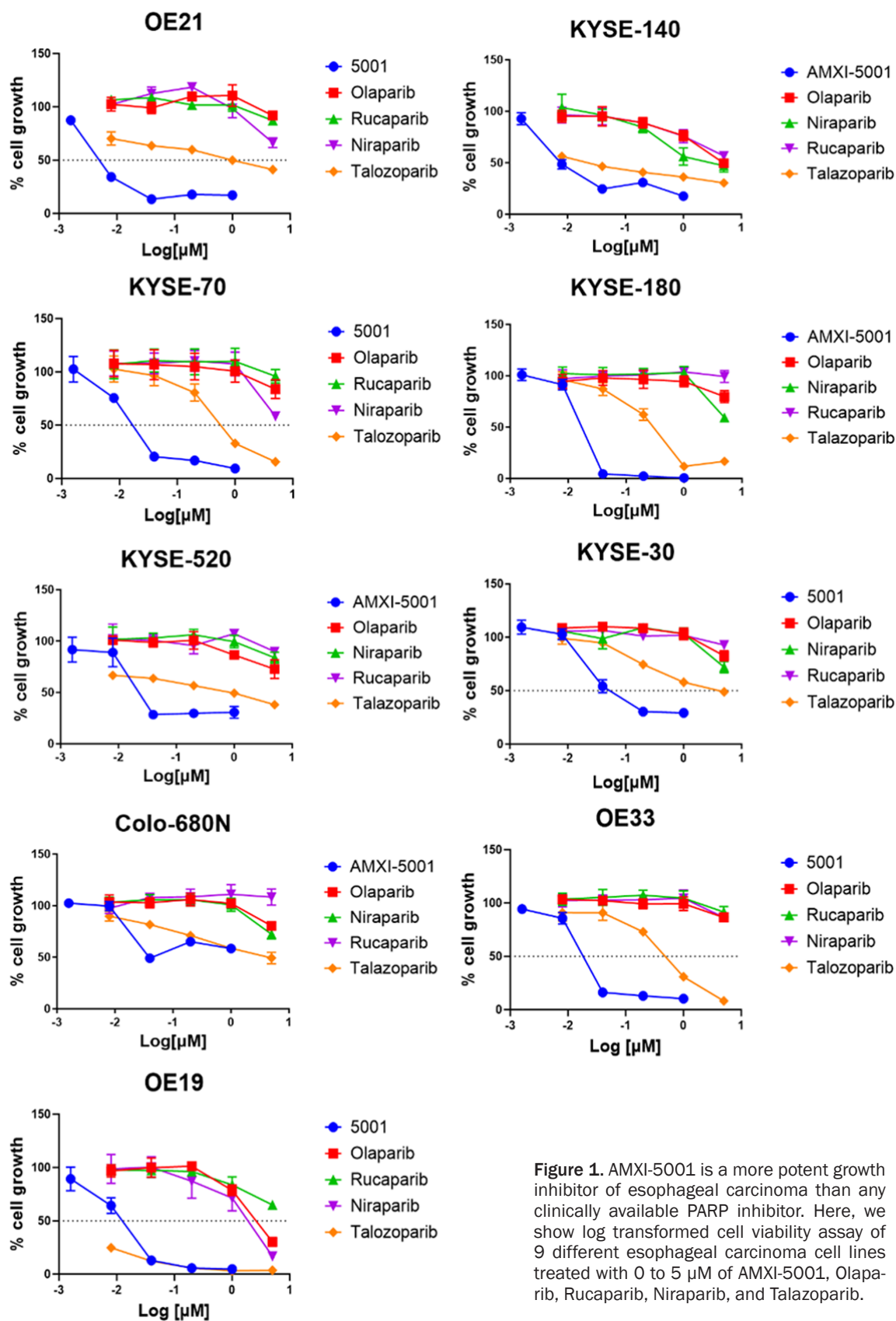
Figure 1. AMXI-5001 is a more potent growth inhibitor of esophageal carcinoma than any clinically available PARP inhibitor. Here, we show log transformed cell viability assay of 9 different esophageal carcinoma cell lines treated with 0 to 5 μM of AMXI-5001, Olaparib, Rucaparib, Niraparib, and Talozoparib.

Table 1. IC50 of AMXI-5001 and all clinically available PARP inhibitors as determined by cell viability assay for 9 esophageal carcinoma cell lines

Cell Line	Esophageal Carcinoma Subtype	IC50 AMXI-5001	IC50 Olaparib	IC50 Niraparib	IC50 Rucaparib	IC50 Talazoparib
OE21	Squamous	0.006 µM	90 µM	12 µM	38 µM	0.74 µM
KYSE-140	Squamous	0.01 µM	4.2 µM	2.3 µM	5.2 µM	0.04 µM
KYSE-70	Squamous	0.02 µM	29 µM	9.7 µM	317 µM	0.64 µM
KYSE-180	Squamous	0.02 µM	19 µM	9.1 µM	NC	0.27 µM
KYSE-520	Squamous	0.03 µM	12 µM	28 µM	55 µM	0.57 µM
KYSE-30	Squamous	0.08 µM	28 µM	16 µM	74 µM	2.3 µM
COLO-680N	Squamous	0.65 µM	24 µM	15 µM	NC	2.2 µM
OE33	Adeno	0.02 µM	33 µM	38 µM	70 µM	0.47 µM
OE19	Adeno	0.01 µM	2.8 µM	1.7 µM	8.3 µM	0.003 µM

ization [12]. To confirm the mechanism of action of AMXI-5001 in esophageal cancer cells, we treated KYSE-70, an esophageal squamous cell carcinoma cell line sensitive to all four commercially available PARP inhibitors, with of AMXI-5001, Olaparib, and Talazoparib. Cells were treated for 24 hours, and the expression levels of PAR, Tubulin, and phospho-histone 3 (Ser10) in the lysate were analyzed using a Western blot analysis. KYSE-70 cells treated with Olaparib and Talazoparib showed significant inhibition of PARP and no effect on tubulin as expected. However, cells treated with AMXI-5001 had significant inhibition of both tubulin and PARP (**Figure 2A**). This confirmed the mechanism of action previously described as an inhibitor of both PARP and tubulin polymerization [12].

We further studied the effect of AMXI-5001 on microtubule polymerization in cells using immunofluorescence. KYSE-70 cells grown on chambered slides were treated with DMSO control, AMXI-5001, Olaparib, Talazoparib, paclitaxel, or vinblastine for 24 hours, fixed and stained with anti-tubulin antibody. As shown in **Figure 2C** and **2D**, microtubule networks in AMXI-5001 treated KYSE-70 cells were disrupted comparing to DMSO. The disruption of microtubule networks is dose-dependent as microtubule networks were more severely unorganized at higher concentrations of AMXI-5001. On the contrary, PARP inhibitors Olaparib and Talazoparib did not affect microtubule networks compared to DMSO. Vinblastine, a known microtubule polymerization inhibitor, served as a positive control and caused microtubule disruption in KYSE-70 cells. Paclitaxel, a known microtubule polymerization enhancer, induced more microtubule polymerization in KYSE-70 cells. In con-

clusion, AMXI-5001 is an effective microtubule polymerization inhibitor in KYSE-70 cells.

AMXI-5001 effectively traps PARP1

Another important mechanism for PARP inhibitors is to induce DNA-trapping of PARP protein, generating toxic DNA adducts [12]. These toxic DNA adducts interfere with replication, causing cell death preferentially in cancer cells, which grow faster than non-cancerous cells. Using a PARP trapping assay, we confirmed AMXI-5001's mechanism for inhibiting PARP by trapping PARP DNA complexes at sites of DNA damage (**Figure 2B**). KYSE-70 cells were treated with an alkylating agent MMS to elicit DNA damage in the presence of PARP inhibitor AMXI-5001, Olaparib, or Talazoparib. Cell lysates were collected and subjected to fractionation. The soluble nuclear extracts and the chromatin-bound nuclear extracts were collected, and the PARP1 levels were analyzed using Western blot analysis. Topoisomerase I was used as a soluble nuclear marker, and histone 3 was used as a chromatin-bound marker. As shown in **Figure 2B**, PARP1 was trapped on the chromatin-bound fractions, which has been previously shown in the literature [12]. In cells treated with AMXI-5001, PARP1 was trapped in the chromatin-bound fractions in a dose-dependent manner. This result indicates that AMXI-5001 effectively induces DNA-trapping of PARP1 in KYSE-70 cells, which contributes to the inhibition of KYSE-70 cell growth.

AMXI-5001 synergistically inhibits growth in combination with Cisplatin and 5FU

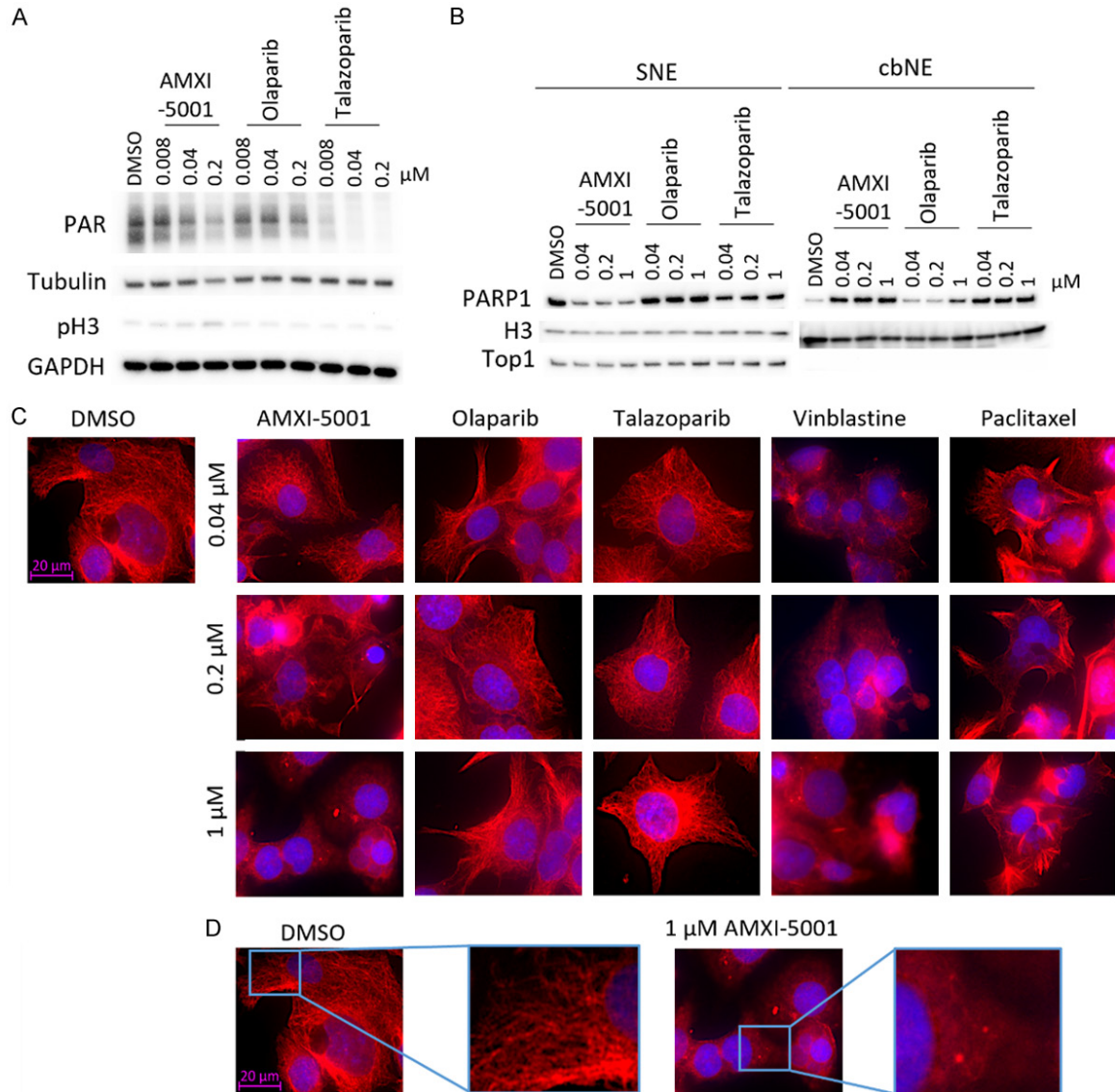Cisplatin and 5FU are two chemotherapeutic agents frequently used to treat esophageal car-

Figure 2. AMXI-5001 is a potent inhibitor of PARP and microtubule polymerization. A. Western blot analysis of KYSE-70 cells treated with DMSO, 0.008 μM, 0.04 μM, and 0.2 μM of AMXI-5001, Olaparib or Talazoparib for 48 hours. B. PARP trapping assay showing PARP1 levels in soluble nuclear extract (SNE), and chromatin-bound nuclear fraction (cbNE) of KYSE-70 cells treated with DMSO, 0.04 μM, 0.2 μM and 1 μM of AMXI-5001, Olaparib, and Talazoparib for 3 hours in the presence of DNA-damage agent, MMS. C. Immunofluorescence assay of KYSE-70 cells treated with AMXI-5001 for 24 hours showing depolymerization effect of the drug on the microtubule. Vinblastine is a tubulin polymerization inhibitor, and paclitaxel is a tubulin polymerization enhancer. D. Enlarged images (3-fold) from DMSO and 1 μM AMXI-5001 groups.

cinoma [11]. To assess the potential to improve clinical outcomes by adding AMXI-5001, we examined the effect of combining AMXI-5001 with Cisplatin and 5FU on the growth inhibition of esophageal carcinoma. Using a cell viability assay, we showed that AMXI-5001 is more potent in inhibiting KYSE-70 cell growth than Cisplatin or 5FU as monotherapy (Supplementary Figure 1A). We further investigated the potential of combining AMXI-5001 with a chemotherapeutic agent in treating esophageal cancer cells using cell viability assay. KYSE-70 cells were seeded to 96-well plates and treated with a combination of AMXI-5001 and either Cisplatin or 5FU on day 2, and the cell viability was measured on day 7 using Cell Titer-Glo reagent (Promega). The addition of AMXI-5001 synergistically inhibited the growth of cells
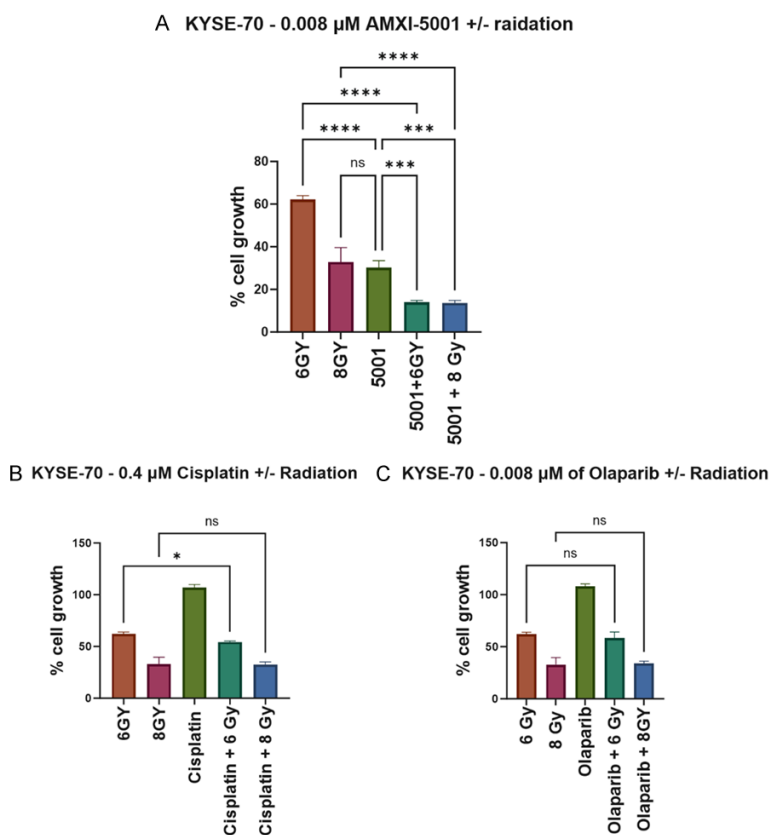
Figure 3. AMXI-5001 significantly inhibits the growth of squamous cell esophageal carcinoma in combination with radiation therapy. A. KYSE-70 was treated with 0.008 µM of AMXI-5001 with and without radiation. B. KYSE-70 was treated with 0.4 µM of Cisplatin with and without radiation. C. KYSE-70 treated with 0.008 µM of Olaparib with and without radiation. One-way ANOVA (Uncorrected Fisher's LSD test) was used for multiple comparisons (* = P < 0.1, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001).

compared to cells treated with either Cisplatin or 5FU alone (Supplementary Figure 1B and 1C).

AMXI-5001 is an effective radiosensitizer of esophageal carcinoma

A squamous cell esophageal cell line that was sensitive to AMXI-5001, KYSE-70, was plated in 96 well plates and treated with 0.008 µM of AMXI-5001, 0.008 µM Olaparib, or 0.4 µM of Cisplatin. Doses of each drug were chosen to be well below the IC50 to observe the combined effect of radiation and drug treatment. After 24 hours of treatment, the cells were irradiated with 0, 6, or 8 GY of radiation. Cell viability was measured on day 7 using CellTiter-Glo reagent (Promega). KYSE-70 treated with AMXI-5001 and radiation therapy had significantly more inhibition of cell growth than cells treated

with either drug or radiation alone (**Figure 3**). These findings were confirmed with two additional squamous esophageal cell lines sensitive to AMXI-5001, KYSE-80, and KYSE-140 (Supplementary Figure 2).

AMXI-5001 in combination with radiation shows anti-tumor effect in xenograft model

The efficacy of AMXI-5001 as monotherapy and in combination with radiation was further investigated in a KYSE-70 xenograft mouse model. KYSE-70 cells were implanted subcutaneously into the flank of nude mice and randomized into four groups when the tumor sizes reached 80 mm³. The mice were treated with vehicle control, AMXI-5001 (50 mg/kg) alone, irradiation alone, and a combination of AMXI-5001 and irradiation. AMXI-5001 was given twice daily (five days on, two days off) via oral gavage. Irradiation was given as a single dose of 6 GY radiation directed to the tumor after the first dose of AMXI-5001 on day 3. Treatment continued for a total of 28 days. Tumors treated with a combination of both AMXI-5001 and radiation showed a significant tumor response (**Figure 4A**) compared to vehicle control or mice treated with either radiation or AMXI-5001 alone. Throughout the entire treatment course, there was no difference in the body weight of the mice in any of the treatment arms (**Figure 4A**). H&E staining and cleaved caspase 3 immunohistochemistry of harvested tumors both showed significantly more fibrosis and increased apoptosis among tumors treated with combination of AMXI-5001 and radiation (**Figure 4B** and **4C**).

Discussion

This article describes the synergistic effect of AMXI-5001, a novel dual-action microtubule polymerization inhibitor and PARP inhibitor, in
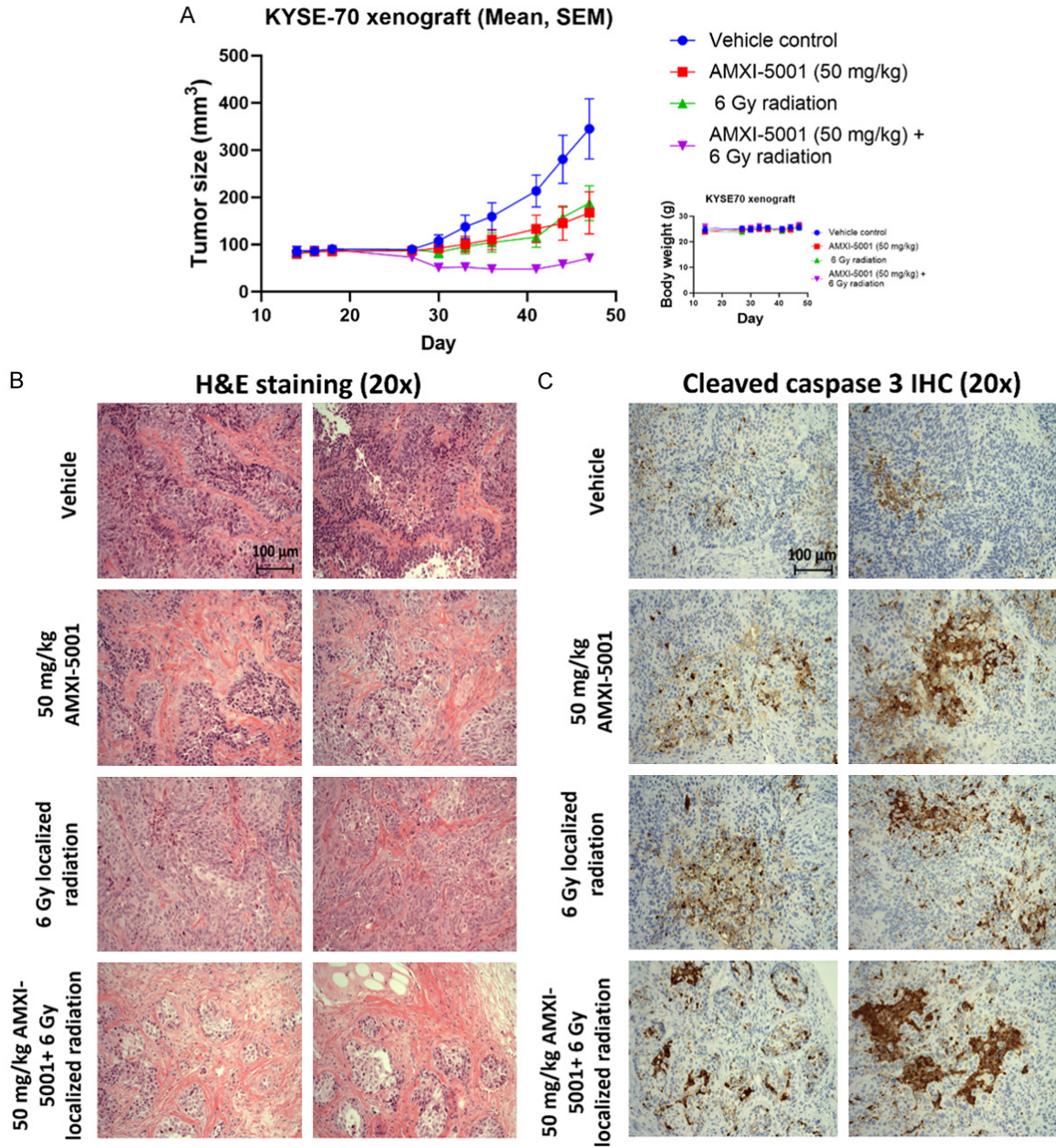
Figure 4. AMXI-5001 in combination with radiation shows an anti-tumor effect in the KYSE-70 xenograft model. A. KYSE-70 cells were implanted in nude mice and treated with vehicle only, 50 mg/kg AMXI-5001 alone, 6 Gy radiation alone, or a combination of AMXI-5001 and radiation when tumor size reached 80 mm^3. Tumor growth was inhibited by AMXI-5001 or radiation alone and further inhibited by the combination therapy. B. H&E staining of the KYSE-70 xenograft tumors shows more fibrosis in AMXI-5001 alone and in the combination groups. C. Cleaved caspase 3 Immunohistochemistry staining shows increased apoptosis in tumors from the combination therapy group. H&E and IHC images were taken using 20× objective.

treating esophageal carcinoma in combination with chemotherapeutic agents and radiation. Currently, esophageal carcinoma is the 8th most common cause of cancer-related death, and mortality remains stubbornly high [6, 13]. The current standard of care in the treatment of esophageal carcinoma is the combination of chemotherapy and radiation therapy, a known cause of single and double-stranded DNA breaks [14]. PARPs are a class of proteins integral in the repair of single-stranded DNA breaks, making PARP inhibitors an attractive potential

therapeutic to include in the treatment of esophageal cancer. Previous in-vitro experiments have shown that Olaparib, a clinically available PARP inhibitor, is effective at treating esophageal carcinoma both alone and in combination with radiation therapy, and DNA-damaging chemotherapeutic agents [10, 15, 16]. These studies, however, were limited to *in vitro* experiments and did not assess the efficacy in an *in vivo* model.

Studies assessing the effectiveness of other clinically available PARP inhibitors have shown that combining radiotherapy and PARP inhibitors is a safe treatment strategy that warrants further investigation [17]. A recently published phase 1 clinical trial of Olaparib and radiation therapy for the treatment of triple negative breast cancer showed that Olaparib was a well-tolerated radiosensitizer in the twenty-four women enrolled in the trial [18]. However, additional studies looking at combining radiotherapy with Olaparib and Cisplatin suggest that the combination of all three therapeutics may not be tolerated [19]. One phase 1 study assessed the tolerability of Olaparib in combination with radiation therapy in patients with esophageal cancer. Due to the study closure, a maximum tolerated dose could not be established. However, 100 mg twice a day in combination with 50 GY of radiation in 25 daily fractions was shown to be over the maximum tolerated dose, suggesting 50 mg twice a day may be a more suitable dose of Olaparib [20]. These results suggest that identifying a PARP inhibitor that is effective at very low dosages may be necessary for creating a tolerable and effective treatment strategy for esophageal carcinoma that includes a PARP inhibitor.

AMXI-5001's dual mechanism as both a microtubule polymerization inhibitor and PARP inhibitor is likely the reason for the effectiveness of the drug in the treatment of esophageal carcinoma, as seen by the low IC50s for AMXI-5001 compared to all the other clinically available PARP inhibitors. While previous studies have shown that other PARP inhibitors, such as Olaparib, can synergistically affect other chemotherapeutics and radiotherapy at a concentration of 5 µM [16, 21], we show that AMXI-5001 achieves these results at a concentration 625 times lower at 0.008 µM. The potency of the drug was also highlighted in our murine experiment, which showed effectiveness at a dose of 50 mg/kg, a treatment regimen that had no effect on body weight of the mice, suggesting that the dosage is well tolerated while still effective.

Finally, it is important to note that the mice treated with a single radiation dose combining AMXI-5001 started to re-grow. This is likely due to the design of our experiment, which treated the mice for a prolonged period after a single dose of radiation rather than a more prolonged course over multiple fractionations, as is the standard of care for the treatment of patients with esophageal carcinoma. The H&E staining of the tumors treated with the combination of AMXI-5001 and radiation therapy showed significant fibrosis and necrosis, suggesting the effectiveness of the therapy. Further studies are necessary to identify the ideal radiation schedule, AMXI-5001 dosage, and combination with other chemotherapeutic agents to obtain the best clinical response.

Despite these limitations, our results indicate that AMXI-5001 is a potential novel therapy for the treatment of esophageal carcinoma in combination with radiation and possibly other chemotherapeutic agents. As a potent, orally available small molecule PARP and microtubule polymerization inhibitor, AMXI-5001 offers great promise in treating this difficult disease. It may provide a much-needed advance in the treatment of esophageal carcinoma.

Acknowledgements

Disclosure of conflict of interest

AMXI-5001 is currently developed by AtlasMedx, Inc. Lemjabbar-Alaoui Hassan, Csaba Peto, and David Jablons may benefit financially from the development of AMXI-5001 through patents held jointly with University of California, San Francisco and AtlasMedx, Inc.

Address correspondence to: David M Jablons, Department of Surgery, Division of Cardiothoracic Surgery, University of California, 500 Parnassus Ave, San Francisco, California 94117, USA. Tel: 415-885-3882; E-mail: David.jablons@ucsf.edu

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A KYSE-70 - single drug cell viability

|	Cisplatin	5FU	AMXI-5001
IC50 (µM)	1.402	2.598	0.0254
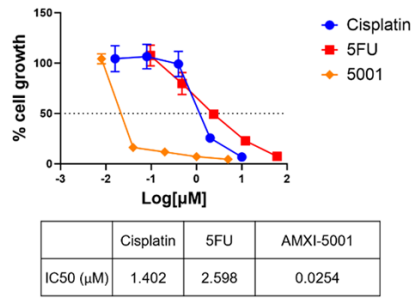
B KYSE-70 - combination therapy of 2µM of Cisplatin

C KYSE-70 - combination therapy of 12 µM of 5FU
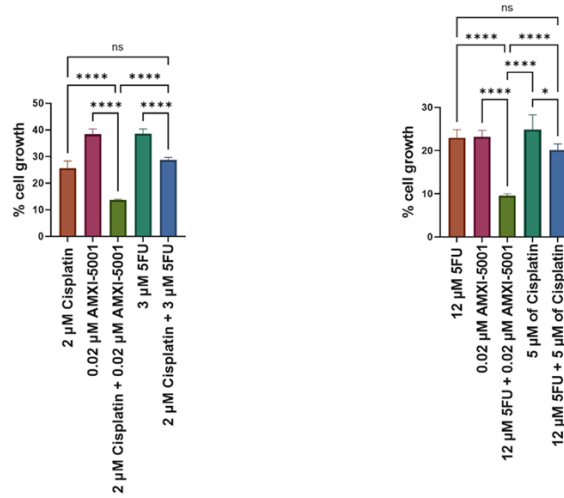
Supplementary Figure 1. AMXI-5001 in combination with Cisplatin, or 5 FU significantly inhibits the growth of esophageal carcinoma. A. Normalized response of KYSE-70 treated with Cisplatin, 5 FU, or AMXI-5001. B. Normalized response of KYSE-70 to 2 µM Cisplatin alone and in combination with AMXI-5001. C. Normalized response of KYSE-70 to 12 µM 5 FU both alone and in combination with AMXI-5001. One-way ANOVA (Uncorrected Fisher's LSD test) was used for multiple comparisons (* = $P < 0.1$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$).
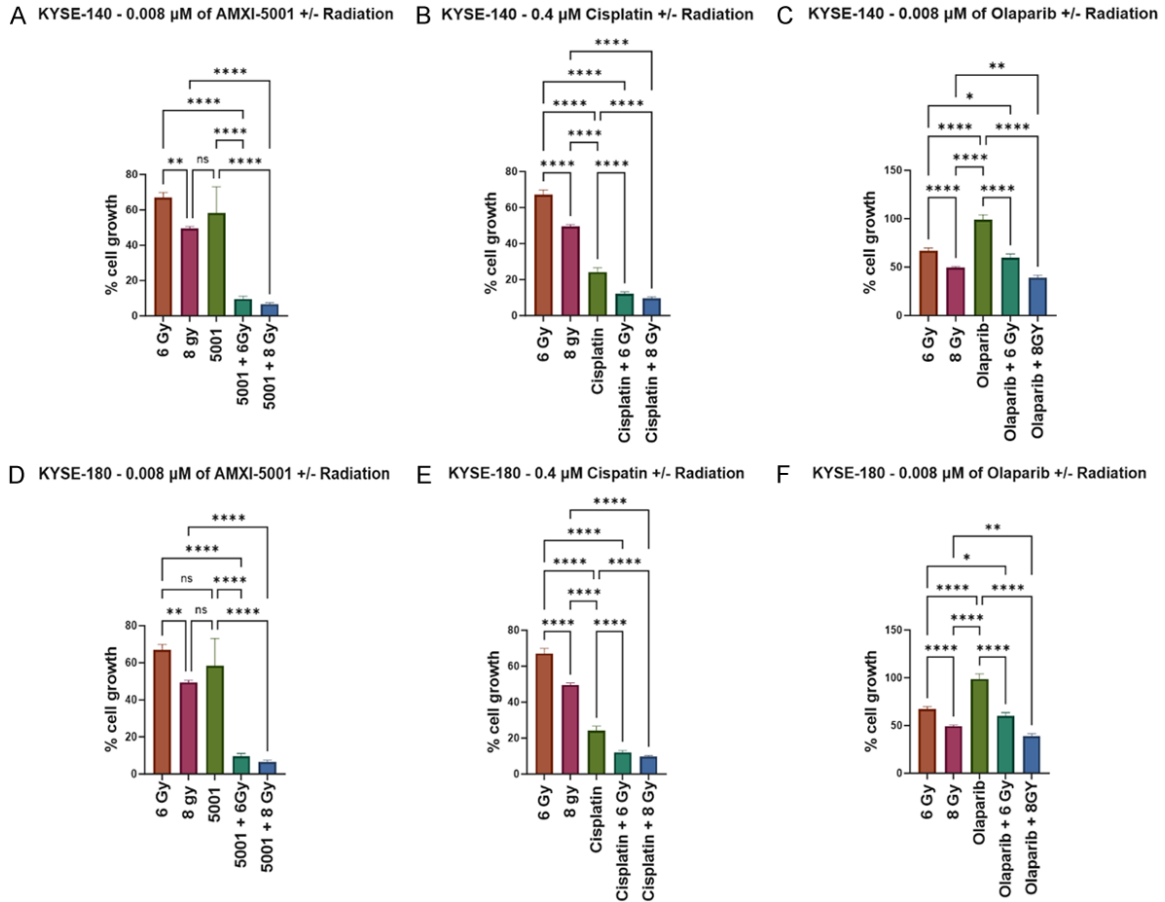
Supplementary Figure 2. AMXI-5001 significantly inhibits the growth of squamous cell esophageal carcinoma in combination with radiation therapy. A. KYSE-140 was treated with 0.008 µM of AMXI-5001 with and without radiation. B. KYSE-140 was treated with 0.4 µM of Cisplatin with and without radiation. C. KYSE-140 was treated with 0.008 µM of Olaparib with and without radiation. D. KYSE-180 was treated with 0.008 µM of AMXI-5001 with and without radiation. E. KYSE-180 was treated with 0.4 µM of Cisplatin with and without radiation. F. KYSE-180 was treated with 0.008 µM of Olaparib with and without radiation. One-way ANOVA (Uncorrected Fisher's LSD test) was used for multiple comparisons (* = P < 0.1, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001).