HHS Public Access

Author manuscript

Mol Cancer Ther. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Mol Cancer Ther. 2018 November ; 17(11): 2353–2364. doi:10.1158/1535-7163.MCT-18-0489.

Augmentation of *nab*-paclitaxel chemotherapy response by mechanistically diverse antiangiogenic agents in preclinical gastric cancer models

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Abstract

Gastric adenocarcinoma (GAC) remains the third most common cause of cancer deaths worldwide. Systemic chemotherapy is commonly recommended as a fundamental treatment for metastatic GAC, however, standard treatment has not been established yet. Angiogenesis plays a crucial role in the progression and metastasis of GAC. We evaluated therapeutic benefits of mechanistically diverse antiangiogenic agents in combination with *nab*-paclitaxel, a next-generation taxane, in preclinical models of GAC. Murine survival studies were performed in peritoneal dissemination models, while tumor growth studies were performed in subcutaneous GAC cell-derived or patient-derived xenografts. The mechanistic evaluation involved IHC and Immunoblot analysis in tumor samples. *Nab*-paclitaxel increased animal survival that was further improved by the addition of antiangiogenic agents ramucirumab (or its murine version DC101), cabozantinib and nintedanib. *Nab*-paclitaxel combination with nintedanib was most effective in improving animal survival, always greater than 300% over control. In cell-derived subcutaneous xenografts, *nab*-paclitaxel reduced tumor growth while all three antiangiogenic agents enhanced this effect, with nintedanib demonstrating the greatest inhibition. Furthermore, in GAC patient-derived xenografts the combination of *nab*-paclitaxel and nintedanib reduced tumor growth over single agents alone. Tumor tissue analysis revealed that ramucirumab and cabozantinib only reduced tumor vasculature, while nintedanib in addition significantly reduced tumor cell proliferation and increased apoptosis. Effects of *nab*-paclitaxel, a promising chemotherapeutic agent for GAC, can be enhanced by new-generation antiangiogenic agents, especially nintedanib.

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Disclosure of Potential Conflicts of Interest: The authors N. Awasthi and R.E. Schwarz received funding support from Celgene Corporation to perform this research. The authors M.A. Schwarz and C. Zhang declare no conflict of interest.

The data suggest that *nab*-paclitaxel combinations with multitargeted antiangiogenic agents carry promising potential for improving clinical GAC therapy.

Keywords

gastric cancer; *nab*-paclitaxel; ramucirumab; cabozantinib; nintedanib

Introduction:

Gastric adenocarcinoma (GAC) remains the fifth most frequent malignant tumor and the third leading cause of cancer-associated death in the world, despite its incidence and mortality having declined in the past few decades (1). The prognosis of metastatic GAC patients is generally very poor, mostly due to the limited efficacy of available treatment options. Surgical resection provides the best chance for long-term survival of locoregional disease stages, however, many GAC patients with resectable disease at the time of diagnosis have micrometastatic disease. Noncurative chemotherapy is commonly recommended as a treatment for advanced GAC that usually leads to a modest improvement, resulting in a median survival of 6 to 11 months. The chemotherapy regimen of epirubicin, cisplatin, and 5-fluorouracil (5-FU) or its modifications by substituting cisplatin and 5-FU with oxaliplatin and capecitabine, have been widely used as the standard treatment for GAC patients (2,3). Further, docetaxel, a semisynthetic taxane, based chemotherapy regimen including cisplatin and 5-FU (DCF) has shown promising clinical benefits in treating advanced and metastatic GAC (4). Recently, the docetaxel-based triplet chemotherapy regimen FLOT (5-FU/leucovorin, oxaliplatin and docetaxel) became standard therapy for gastric cancer patients based on its clinical benefits in a phase 2/3 trial (5). Although systemic chemotherapy treatment improves survival and quality of life in the treatment of GAC, the standard regimen remains to be established. Furthermore, these conventional chemotherapy regimens show a clinical response in not more than 50% of patients suggesting that a relatively large proportion of patients do not benefit from these intensive treatments (6). In addition, development of chemoresistance is a common clinical phenomenon even in patients who initially respond to these therapies (7). Therefore, novel and effective therapeutic approaches are desperately needed to improve clinical outcome for GAC patients.

Nanoparticle albumin-bound paclitaxel (*nab*-paclitaxel, NPT) is a next-generation taxane with an antimitotic activity that has shown greater antitumor efficacy than solvent-based paclitaxel in several preclinical and clinical studies. Up to now, *nab*-paclitaxel is an FDA-approved treatment for three indications, breast cancer, non-small cell lung cancer (NSCLC) and pancreatic cancer (8). We have previously demonstrated that *nab*-paclitaxel has higher anti-tumor efficacy than other commonly used chemotherapy drugs in preclinical models of GAC (9). Platinum-based chemotherapy has been widely used for advanced GAC treatment (10,11). We used oxaliplatin (Oxa), a third-generation platinum compound, as a reference agent to compare its antitumor response with *nab*-paclitaxel as single-agent chemotherapy.

Inhibiting tumor angiogenesis is a well-established anticancer strategy for the treatment of several solid tumors including GAC. Vascular endothelial growth factor (VEGF) and its

receptor VEGFR2 mediated angiogenesis is believed to play a crucial role in the pathogenesis of GAC, and higher circulating and intratumoral VEGF levels have been shown to associate with increased tumor aggressiveness and poor survival (12). Several clinical trials evaluated two categories of VEGF pathway inhibitors in GAC patients, antibodies or small molecule tyrosine kinase inhibitors (TKIs) (13–15). Ramucirumab (Ram), a human monoclonal antibody against VEGFR2, has shown significant clinical response as monotherapy and in combination with paclitaxel in advanced GAC patients who progressed after first-line therapy (13,14). DC101 is a murine version of ramucirumab and binds specifically to mouse VEGFR2. Hepatocyte growth factor (HGF) and its receptor HGFR (c-Met) signaling is implicated in cell proliferation, survival, metastasis, and angiogenesis. Activation of this pathway has been reported in 10–50% of GAC patients (16,17). Cabozantinib (Cab) is a potent, small molecule TKI that mainly targets c-Met, VEGFR2, Axl, Ret, and Kit signaling. Cabozantinib is an FDA-approved treatment of patients with medullary thyroid cancer and renal cell carcinoma (18,19). Other than VEGF and HGF, several growth factors and their receptors are overexpressed and have all been correlated with poor prognosis in GAC, including PDGFR, FGFR and IGFR (20). In addition, aberrant signaling of these growth factors has been implicated in resistance and escape mechanisms from anti-VEGF therapy (21). These findings provide a strong rationale for exploiting the therapeutic potential of multitarget antiangiogenic agent combinations together with effective cytotoxic regimens in GAC. Nintedanib (Nin) is a potent triple angiokinase inhibitor for VEGF receptor 1–3, FGF receptor 1–3 and PDGF receptor α/β. Nintedanib also inhibits the Src family kinases, RET, and FLT-3. Nintedanib is a next-generation TKI with high specificity towards its therapeutic targets and favorable toxicity profile (22). Nintedanib demonstrated significant antitumor activity in preclinical models of several tumor types (23). In the EU, nintedanib combination with docetaxel is an approved treatment of patients with advanced and metastatic NSCLC (24).

In this report, we aimed to evaluate the antitumor response of *nab*-paclitaxel, in combination with the mechanistically diverse antiangiogenic agents ramucirumab, cabozantinib or nintedanib, in several different preclinical models of GAC.

Materials and Methods:

Reagents

Nab-paclitaxel was obtained from Celgene Corporation (Summit, NJ). Ramucirumab (Eli Lilly, Indianapolis, IN) was obtained from the pharmacy at the Goshen Center for Cancer Care (Goshen, IN). Oxaliplatin, cabozantinib and nintedanib were purchased from LC Laboratories (Woburn, MA). Cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation (Indianapolis, IN).

Cell culture

The human GAC cell lines AGS, SNU-1, SNU-5, SNU-16, and KATO-III were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Human GAC cell line MKN-45 was purchased from Creative Bioarray (Shirley, NY). The characteristics of these GAC cell lines are presented in Supplementary Table S1 indicating that MKN-45 cells

express c-met and E-cadherin oncogenes, while KATO-III cells express c-met and uniquely overexpress FGFR2 oncogenes. The human umbilical vein endothelial cells HUVEC, and the human fibroblast cell line WI-38 were also purchased from ATCC. All these cell lines were tested and authenticated by ATCC. All GAC cells were grown in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10% or 20% FBS and maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. HUVECs were grown in EndoGRO-LS medium containing endothelial cell growth supplements (Millipore Corp., Billerica, MA). WI-38 cells were grown in DMEM supplemented with 10% FBS.

Cell viability assay

Cell viability experiments were performed using the colorimetric WST-1 reagent. Briefly, 4000–5000 cells were plated in each well of a 96-well plate in the regular growth medium. The medium was replaced after 16 hours with 2% FBS containing medium and the cells were treated with *nab*-paclitaxel, ramucirumab, cabozantinib or nintedanib. WST-1 reagent (10 μl) was added to each well after 72-hour incubation followed by additional incubation for 2 hours. The absorbance was measured at 450 nm using a microplate reader.

Western blot analysis

Cell monolayers were treated with *nab*-paclitaxel, ramucirumab, cabozantinib or nintedanib, incubated for 16 hours and whole cell lysates were prepared. Tumor tissue lysates were prepared as previously described (25). Briefly, tumor tissues from subcutaneous cell-derived and patient-derived xenografts were snap-frozen in liquid nitrogen and stored at −80°C. These tumor tissues were suspended in lysis buffer and homogenized using the Bullet Blender Homogenizer (Next Generation, Averill Park, NY), and extracts were sonicated. Proteins in supernatants were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C with the following antibodies: total ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), phospho-stathmin, total stathmin, cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), β-actin and GAPDH (both from Sigma). The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA) for 1 to 2 hours. Specific bands were visualized using the enhanced chemiluminescence reagent (ECL, Cell Signaling) in an Image360 system and quantitated by densitometry.

In vivo studies

Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the Indiana University School of Medicine (South Bend, IN). Animals were housed in a pathogen-free facility with access to food and water *ad libitum*. Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (4 to 6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA).

Cell-derived subcutaneous xenograft model: Human GAC cells MKN-45 (7.5×10^6) or SNU-5 (10×10^6) were implanted subcutaneously into the right flank region of NOD/SCID mice. Ten days after tumor cell injection, when all mice had a measurable tumor, mice were randomized (n=5) to receive PBS (control), *nab*-paclitaxel (10 mg/kg, twice a week), oxaliplatin (5 mg/kg, twice a week), ramucirumab (2 mg/kg, twice a week), cabozantinib (30

mg/kg, 5 times a week) or nintedanib (25 mg/kg, 5 times a week) via intraperitoneal injection for 2 weeks. The tumor size was measured twice weekly, and tumor volume (V) was calculated using the formula V = ½ (Length × Width^2). After the completion of the experiment, mice were euthanized; tumors dissected and processed for histological, immunohistochemical and Western blot analysis.

Patient-derived subcutaneous xenograft (PDX) model: Gastric cancer patient-originating tumor tissue, derived from a poorly differentiated diffuse-type GAC, was purchased from Celprogen (Torrance, CA). Tumor samples were sectioned into small pieces (1 cm × 1 cm) and subcutaneously implanted into the right flanks of NOD/SCID mice under anesthesia with isoflurane. Tumor growth was monitored twice weekly using a caliper. At about 1000 mm^3, tumors were extracted for serial transplantation into NOD/SCID mice and used for the experiment when tumor volume reached about 100–200 mm^3. PDX-bearing mice were randomized and divided into groups to receive PBS (control), *nab*-paclitaxel (10 mg/kg, twice a week) and nintedanib (25 mg/kg, 5 times a week) via intraperitoneal injection for 2 weeks. The tumor size was measured twice weekly, and tumor volume (V) was calculated using the formula V = ½ (Length × Width^2). After the completion of the experiment, mice were euthanized, followed by tumor dissection and processing for histological, immunohistochemical and Western blot analyses.

Immunohistochemical analysis

Subcutaneous xenograft tumors were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Tumor sections (5 μm) were deparaffinized and rehydrated followed by heat-mediated antigen retrieval in citrate buffer. The tumor sections were then incubated with CAS blocking buffer for 20 minutes. Tumor cell proliferation was measured by overnight incubation with anti-Ki67 antibody (Abcam, Cambridge, MA) at 4°C and 40 minutes incubation at room temperature with Cy3 secondary antibody. Slides were mounted with a mounting medium containing DAPI (Invitrogen, Carlsbad, CA) and imaged using a fluorescence microscope. Proliferative activity was evaluated by calculating Ki67-positive cells from five different high-power fields (HPF) in a blinded manner. Tumor cell apoptosis was determined by TUNEL assay using “Apoptag Apoptosis Detection Kit” according to the manufacturer’s (Millipore) instructions. Microvessel density (MVD) was evaluated by overnight incubation with anti-endomucin antibody (Millipore; MAB2624) at 4°C followed by Cy3 secondary antibody incubation at room temperature for 40 minutes. Tissues were then washed, mounted using a medium containing DAPI and imaged using a fluorescence microscope. Endomucin positive vessels were calculated within a microscopic HPF in a blinded manner. Fluorescence microscopy was performed using IX81 Olympus microscope and images were captured with a Hamamatsu Orca digital camera (Hamamatsu Corporation, Bridgewater, NJ) with a DSU spinning confocal unit using cellSens Dimension software (Olympus, Center Valley, PA).

Animal survival analysis

Animal survival studies were performed in a peritoneal dissemination xenograft model using 4–6-week-old female NOD/SCID mice. Gastric cancer cells MKN-45 (10×10^6) or KATO-III (10×10^6) were injected into the abdominal cavity of mice. Ten days after tumor cell

injection, mice were randomized (6–8 mice per group) to receive PBS (control), *nab*-paclitaxel and antiangiogenic agents for 2 weeks as described in the subcutaneous model experiment. Mice were euthanized when moribund according to predefined criteria, including rapid weight loss or gain (>15%), tumor size, lethargy, inability to remain upright and lack of strength. Animal survival was evaluated from the first day of treatment until death (26).

Statistical analysis

In vitro cell proliferation data are expressed as the mean ± standard deviation. Statistical significance was analyzed by the two-tailed Student's t-test using GraphPad Prism 6.0 Software (GraphPad Software, San Diego, CA) for the individual group comparison. Statistical analysis for *in vivo* tumor growth studies was performed by one-way ANOVA for multiple group comparisons and Student's t-test for the individual group comparisons. Survival study statistics included nonparametric testing with log-rank group comparisons (GraphPad Prism 6.0). Values of $p<0.05$ were considered to represent statistically significant group differences.

Results:

Antiangiogenic agents improve animal survival benefits of *nab*-paclitaxel

In a peritoneal dissemination model using MKN-45 GAC cells, median survival in the control group mice (PBS treated) was 21 days after the start of therapy. At the time of death, control mice carried tumor in all parts of the stomach including gastro-esophageal junction and post-pyloric duodenum, and metastases were found in liver, spleen and gallbladder. In comparison with control animals, there was no increase in median survival in the oxaliplatin (21 days), ramucirumab (21 days) or cabozantinib (21 days) therapy groups, but survival was significantly longer in the nintedanib group (26 days, a 24% increase). Animal survival was markedly increased by single-agent *nab*-paclitaxel treatment (40 days, a 90% increase), and was further increased by addition of antiangiogenic agents: NPT+Ram (48 days, a 129% increase), NPT+Cab (69 days, a 229% increase) and NPT+Nin (100 days, a 376% increase) (Figure 1A).

In another peritoneal dissemination model of gastric cancer using KATO-III cells, the median survival of mice in the control group (PBS treated) was 19 days after the start of therapy. Similar to the MKN-45 xenograft study, there was no significant change in median survival in animals receiving oxaliplatin or ramucirumab monotherapy. However, this model showed stronger effects of single-agent treatment with *nab*-paclitaxel (178 days), cabozantinib (40 days) or nintedanib (246 days). Again, animal survival was further increased by the addition of antiangiogenic agents to *nab*-paclitaxel (Figure 1B).

Antiangiogenic agents augment tumor growth inhibition response of *nab*-paclitaxel

In MKN-45 GAC cell-derived subcutaneous xenografts, PBS treated control mice displayed rapid tumor growth. Ramucirumab and oxaliplatin monotherapy resulted in a small inhibition in tumor growth (<39%), while a greater reduction was observed with *nab*-paclitaxel, cabozantinib and nintedanib monotherapy (range 67–85%). Combinations of *nab*-

paclitaxel with these antiangiogenic agents demonstrated an additive effect on tumor growth inhibition (>89%), with NPT+Nin being the most effective group showing a regression in tumor size (Figure 2A, 2B). Tumor weight, measured at the end of the experiment, correlated with the tumor volume data in different therapy groups and corroborated the antitumor benefits of *nab*-paclitaxel combination with antiangiogenic agents (Figure 2C).

In another GAC subcutaneous xenograft experiment using mutationally different SNU-5 cells, ramucirumab and oxaliplatin monotherapy showed no significant effect on tumor growth, while a marked reduction in tumor growth was observed with *nab*-paclitaxel, cabozantinib or nintedanib monotherapy (range 65–93%). Tumor growth inhibition response in NPT+Cab or NPT+Nin was significantly higher than monotherapy groups showing a regression in tumor size (Figure 2D, 2E). Again, harvested tumor weights in different therapy group mice at the end of the experiment demonstrated a good correlation with the tumor volume data (Figure 2F). There was no considerable change in mouse body weight in different therapy groups during the 2-week therapy period in both of these subcutaneous xenograft studies (Supplementary Figure S1).

DC101 enhances *nab*-paclitaxel antitumor activity

Determination of antitumor effects of DC101, a murine version of ramucirumab, in MKN-45 GAC cell-derived xenografts, revealed that DC101 and its combination with *nab*-paclitaxel significantly reduced the tumor growth (Figure 3A). In subcutaneous tumors, the net increase in tumor size was 851 mm^3 in controls, 116 mm^3 after NPT, 181 mm^3 after DC101 and −84 mm^3 (tumor regression) after NPT+DC101 (Figure 3B). Tumor weight at the end of the experiment corresponded with the tumor volume data (Figure 3C). In peritoneal dissemination xenografts, animal survival in the control group was 18 days and after DC101 monotherapy 19 days. The *nab*-paclitaxel monotherapy effect (39 days) was enhanced by the addition of DC101 (62 days) (Figure 3D). Again, there was no significant change in mouse body weight in different therapy groups during the 2-week therapy period (Supplementary Figure S2 A).

Nintedanib enhances *nab*-paclitaxel antitumor activity in PDX model

In GAC patient-derived xenografts, *nab*-paclitaxel, nintedanib or their combination also delayed tumor growth (Figure 3E). The net increase in tumor size was 599 mm^3 in controls, 204 mm^3 in NPT, 211 mm^3 in Nin and −24 mm^3 (tumor regression) in NPT+Nin (Figure 3F). Consistent with prior experiments, therapies appeared to be well tolerated as reflected by weight stability (Supplementary Figure S2 B).

Antiangiogenic agents and *nab*-paclitaxel: effects on tumor cell proliferation and apoptosis

Examination of tumor cell proliferation within tumor tissues obtained from MKN-45 cell-derived subcutaneous xenografts demonstrated that *nab*-paclitaxel was most effective in reducing intratumoral proliferation (by 64.5%) followed by oxaliplatin (40.5%), nintedanib (32.8%), and cabozantinib (21.7%). Ramucirumab had no significant effect on intratumoral proliferation in this setting. Combinations of *nab*-paclitaxel with cabozantinib (75%

reduction) or nintedanib (82% reduction) were more effective than single-agent therapies (Figure 4A).

Evaluation of tumor cell apoptosis in tumor tissues obtained from MKN-45 cell-derived subcutaneous xenografts revealed that compared with control (apoptosis index: 0.02), *nab*-paclitaxel (0.11) was more effective in inducing apoptosis followed by nintedanib (0.06) and oxaliplatin (0.04) monotherapy. Combinations of *nab*-paclitaxel with ramucirumab and cabozantinib were not significantly different than *nab*-paclitaxel alone, in contrast to NPT +Nin (0.15) (Figure 4B).

Antiangiogenic agents and *nab*-paclitaxel: effects on microvessel density and marker proteins

Nab-paclitaxel and oxaliplatin had no specific effect on tumor vasculature in MKN-45 cell-derived subcutaneous xenografts (<8%), in contrast to ramucirumab (22.6%), cabozantinib (79.8%) or nintedanib (64.4%). Reduction in microvessel counts in combinations of *nab*-paclitaxel with antiangiogenic agents was not different than antiangiogenic monotherapy groups (Figure 5A).

Nab-paclitaxel increased phospho-stathmin and cleaved caspase-3 expression in MKN-45 cells but had no significant effect on α-tubulin and phospho-ERK expression. Cabozantinib and nintedanib had no effect on α-tubulin, minimal effect on phospho-stathmin and phospho-ERK but significant increase in cleaved caspase-3 expression. Ramucirumab treatment had no effect on phospho-stathmin, α-tubulin, or cleaved caspase-3 protein expression in MKN-45 GAC cells (Figure 5B).

Antiangiogenic agents and *nab*-paclitaxel: effects on *in vitro* cell proliferation

Single-agent *nab*-paclitaxel had a dose-dependent *in vitro* growth inhibitory effect on several GAC cell lines tested. Inhibition in cell proliferation at 100 nM and 10 μM concentrations were 79.5% and 86.2% (AGS); 56.3% and 70.5% (SNU-1); 84.3% and 88.3% (SNU-5); 94% and 97% (SNU-16); 59.6% and 80.9% (KATO III); 28% and 63% (MKN-45) (Figure 6). As a single agent, all three antiangiogenic agents had very limited effects at low doses but led to a significant inhibition in cell viability at the higher dose level (Figure 6). Combination of *nab*-paclitaxel with anti-angiogenic agents demonstrated additive effects in higher dose combination groups (Figure 6). *Nab*-paclitaxel and all three antiangiogenic agents had similar effects on representative endothelial cells HUVECs and fibroblasts WI-38 cells (Supplementary Figure S3).

Discussion:

Combination chemotherapy is currently the standard approach for treating most patients with advanced GAC as it can prolong median patient survival by approximately 10 months. Advanced disease burden, tumor heterogeneity and aggressiveness, as well as innate and acquired drug resistance are the major factors responsible for the dismal response of GAC to chemotherapy treatment. The search for more effective combination cytotoxic regimens is ongoing, as all approved regimens have limited survival benefits and carry risks for clinical toxicity (2–5). More recently, targeted therapy approaches, either alone or in combination

with chemotherapy, have started to impact GAC care, primarily through biologic agents directed against vascular mechanisms (13–15,27). Tumor-induced angiogenesis is a well-established mediator of GAC growth, progression, and metastasis. GAC angiogenesis is multifactorial, and several growth factors have been shown to play a crucial role in this process including VEGF, FGF, PDGF, HGF, and EGF (12,20). Given these insights into susceptibility of GAC to various mechanisms of systemic therapies, it seems sensible to explore combination approaches of promising cytotoxic and antiangiogenic components to achieve greater disease control benefits. In the present study, the therapeutic efficacy of the next-generation chemotherapy agent *nab*-paclitaxel was evaluated in combination with the new class of antiangiogenic agents in several preclinical GAC models for this reason, and to examine if antiangiogenic mechanisms are capable of enhancing antitumor effects of a nanoparticle-formulated cytotoxic agent.

Peritoneal dissemination is one of the most frequent characteristics of extra-regional metastatic progression of GAC and is a decisive factor in its poor prognosis (28). In this study, *nab*-paclitaxel displayed marked survival advantage in the two GAC peritoneal dissemination animal survival models that closely resemble the clinical progression pattern of the disease. Despite generating a comparable survival in untreated animals, KATO-III xenografts were more sensitive to *nab*-paclitaxel therapy than MKN-45 xenografts probably due to the differences in their molecular characteristics, genetic and epigenetic alterations, and growth factor expression (29). Additionally, *nab*-paclitaxel also demonstrated significant antitumor activity in other models tested including subcutaneous cell-derived xenografts and patient-derived xenografts. Some advantages of *nab*-paclitaxel over other cytotoxic agents, as highlighted in the current study for oxaliplatin, have been reported before (9,30,31), and can likely be attributed to its drug distribution, tumor penetration and higher retention leading to enhanced anti-mitotic responses in epithelial tumor cells and stroma (32,33). Combining *nab*-paclitaxel with other biologic targeting agents has so far been feasible but not highly effective (34). However, the combination with antiangiogenic agents was of particular interested and relevance in this context as mentioned above.

Antiangiogenic treatment remains a highly promising cancer-therapeutic avenue, as this process is critical for the local and metastatic progression of solid tumors. Overall, the clinical benefits of single-agent antiangiogenic therapy are limited, favoring a combination therapy approach, e.g. combining antiangiogenic agents with standard chemotherapy regimens (35). In our studies, antiangiogenic agent monotherapy did not show much survival benefit except in case of nintedanib. Significantly higher animal survival benefit by nintedanib in KATO-III xenografts can be attributed to the fact that KATO-III cells carry *FGFR2* gene amplification (36) rendering it susceptible to nintedanib's unique targeting profile that includes the FGF-FGFR signaling axis (23). These findings indicate that nintedanib would be specifically promising for the well-known but small subset of GAC with FGFR amplification (37,38). Aside from this specific characteristic, the general observation is that in all tested models, the addition of antiangiogenic agents noticeably improved *nab*-paclitaxel response. A higher additive response of cabozantinib or nintedanib with *nab*-paclitaxel indicates the advantages of multitargeting approach compared with single-target VEGFR-2 inhibition by ramucirumab. Among the three-antiangiogenic agents tested, our studies repeatedly demonstrated a discernible advantage of nintedanib, either

alone or in combination with *nab*-paclitaxel. These findings are consistent with some clinical studies in ovarian or lung cancer showing that nintedanib combinations with chemotherapy exhibited promising antitumor activity where other antiangiogenic agents failed to show a response (39,40). The significant advantage of nintedanib can be accredited to its simultaneous inhibition of three prominent proangiogenic pathways with a high degree of specificity including VRGFR1/2/3 (IC_{50} 13–34 nmol/L), FGFR1/2/3 (IC_{50} 37–108 nmol/L), and PDGFRα/β (IC_{50} 59–65 nmol/L) (23). Furthermore, the observed antitumor response of nintedanib as monotherapy and in combination with *nab*-paclitaxel suggests that its direct antitumor effects extend beyond single target VEGF antibody effects. While targeting the VEGF signaling pathway in these systems appears to work, a possible reason for the limited effects of ramucirumab in a xenograft setting is that it is a humanized antibody and therefore would only block VEGFR2 produced from transplanted human xenografts; it would not block VEGFR2 produced from murine host tumor microenvironment cells. We confirmed this by examining the antitumor activity of DC101, a murine version of ramucirumab, and observed marked antitumor benefits of DC101 alone or in combination with *nab*-paclitaxel. These findings indicate a significant role of VEGF production and signaling in murine host tumor microenvironment cells, beyond those expected to reside within the epithelial GAC cells themselves. Previous studies have shown that VEGF production is not limited to endothelial cells in tumor microenvironment but it is also produced in epithelial tumor cells (41) as well as in many cell types in murine tumor microenvironment including endothelial cells, fibroblasts, myofibroblasts, macrophages and immune cells (42). Also, there are reports suggesting the existence of both autocrine and paracrine VEGF loops within the tumor microenvironment (43). Therefore, due to the differences in the expression of VEGF and other angiogenic factors in epithelial and stromal tumor cells, anti-VEGF therapies with distinct target specificities may have differential effects in different cellular compartments within the tumor microenvironment.

It is likely that specific mechanisms of action of the different therapeutic agents used in this study determine the ability to generate combination benefits. Known anti-stromal and anti-mitotic effects of *nab*-paclitaxel can primarily be correlated with a reduction in tumor cell proliferation and induction in apoptosis (44,45). All three antiangiogenic agents significantly reduced tumor vasculature but had differential effects on tumor cell proliferation. Notably, induction in tumor cell apoptosis was distinctly correlated with nintedanib, hence supporting its superior efficacy. While the precise mechanisms for the augmentation of *nab*-paclitaxel antitumor activity by the addition of antiangiogenic agents remains unclear, some likely mechanisms that qualify include normalization of tumor vasculature, decreased interstitial pressure causing enhanced delivery of the chemotherapeutic drug into the tumor microenvironment, reduced density of desmoplastic stroma, and possibly direct augmentation of *nab*-paclitaxel cytotoxic effects (46,47). The differential antitumor activity of the three antiangiogenic agents in this study is thus most certainly related to their spectrum of target specificity, ability to block escape mechanisms of tumor progression and potential to exert direct antiepithelial effects.

In vitro analysis of mutationally disparate GAC cells revealed that *nab*-paclitaxel significantly but differentially decreased cell viability. Antiangiogenic agents have small or no effect at low drug concentrations but induced some effects at high concentration; the

differential sensitivity of GAC cell lines to these drugs is likely related to their differences in the expression of angiogenic growth factors (48,49). The main target receptors such as VEGFR1/2/3, FGFR1/2/3, PDGFRα/β and HGFR (c-met) are differentially expressed in GAC cell lines and have been detected at mRNA level (29,49). Low protein expression of these target receptors limits their detection at the protein level and makes it difficult to determine specific changes by antiangiogenic agents. However, the observed in vitro effects and measurable impact on the expression of downstream members from critical pathways including PI3K, MAPK and apoptosis still support an impact on “angiogenic” molecular targets within epithelial GAC cells.

Multiple mechanisms are involved in GAC growth and progression including an increase in tumor cell proliferation, differentiation, invasion, migration, angiogenesis and epithelial-to-mesenchymal transition. Therefore, a more effective therapeutic regimen would likely impact on most of these protumorigenic mechanisms with manageable toxicity. The fact that antiangiogenic treatments have shown significant clinical activity in advanced GAC but in very few other solid tumors suggests that gastric epithelial neoplasms have additional angiogenic signaling that can be targeted therapeutically (50). New generation multitarget antiangiogenic agents with more precise targeting and better toxicity profiles as used in this study have compelling potential to improve treatment strategies of advanced GAC. The present study indicates that *nab*-paclitaxel is an effective chemotherapy for advanced GAC and its response can be significantly improved by new-generation multitarget antiangiogenic agents, thereby providing a direction for options to improve clinical GAC therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was financially supported by Celgene Corporation to N. Awasthi and R.E. Schwarz, Indiana University School of Medicine funds to R.E. Schwarz and the NIH grant 5RO1HL114977 to M.A. Schwarz.

References:

1. Torre LA, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends--An Update. Cancer Epidemiol Biomarkers Prev 2016;25:16–27. [PubMed: 26667886]
2. Cunningham D, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, et al. Capecitabine and oxaliplatin for advanced esophagogastric cancer. N Engl J Med 2008;358:36–46. [PubMed: 18172173]
3. Webb A, Cunningham D, Scarffe JH, Harper P, Norman A, Joffe JK, et al. Randomized trial comparing epirubicin, cisplatin, and fluorouracil versus fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer. J Clin Oncol 1997;15:261–7. [PubMed: 8996151]
4. Park SR, Chun JH, Kim YW, Lee JH, Choi IJ, Kim CG, et al. Phase II study of low-dose docetaxel/fluorouracil/cisplatin in metastatic gastric carcinoma. Am J Clin Oncol 2005;28:433–8. [PubMed: 16199979]
5. Al-Batran SE, Hofheinz RD, Pauligk C, Kopp HG, Haag GM, Luley KB, et al. Histopathological regression after neoadjuvant docetaxel, oxaliplatin, fluorouracil, and leucovorin versus epirubicin, cisplatin, and fluorouracil or capecitabine in patients with resectable gastric or gastro-oesophageal junction adenocarcinoma (FLOT4-AIO): results from the phase 2 part of a multicentre, open-label, randomised phase 2/3 trial. Lancet Oncol 2016;17:1697–708. [PubMed: 27776843]

6. Boku N, Yamamoto S, Fukuda H, Shirao K, Doi T, Sawaki A, et al. Fluorouracil versus combination of irinotecan plus cisplatin versus S-1 in metastatic gastric cancer: a randomised phase 3 study. Lancet Oncol 2009;10:1063–9. [PubMed: 19818685]
7. Okada K, Fujiwara Y, Takahashi T, Nakamura Y, Takiguchi S, Nakajima K, et al. Overexpression of forkhead box M1 transcription factor (FOXM1) is a potential prognostic marker and enhances chemoresistance for docetaxel in gastric cancer. Ann Surg Oncol 2013;20:1035–43. [PubMed: 23054116]
8. Kundranda MN, Niu J. Albumin-bound paclitaxel in solid tumors: clinical development and future directions. Drug Des Devel Ther 2015;9:3767–77.
9. Zhang C, Awasthi N, Schwarz MA, Hinz S, Schwarz RE. Superior antitumor activity of nanoparticle albumin-bound paclitaxel in experimental gastric cancer. PLoS One 2013;8:e58037. [PubMed: 23460921]
10. Satake H, Yasui H, Kotake T, Okita Y, Hatachi Y, Kotaka M, et al. First-line chemotherapy with capecitabine/oxaliplatin for advanced gastric cancer: A phase I study. Mol Clin Oncol 2017;7:347–50. [PubMed: 28894576]
11. Sugiyama K, Narita Y, Kadowaki S, Ura T, Tajika M, Muro K. Platinum-based Doublet Chemotherapy for Advanced Gastric Cancer with Disseminated Intravascular Coagulation. Anticancer Res 2017;37:309–13. [PubMed: 28011507]
12. Lieto E, Ferraraccio F, Orditura M, Castellano P, Mura AL, Pinto M, et al. Expression of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) is an independent prognostic indicator of worse outcome in gastric cancer patients. Ann Surg Oncol 2008;15:69–79. [PubMed: 17896140]
13. Fuchs CS, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, et al. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. Lancet 2014;383:31–9. [PubMed: 24094768]
14. Wilke H, Muro K, Van Cutsem E, Oh SC, Bodoky G, Shimada Y, et al. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. Lancet Oncol 2014;15:1224–35. [PubMed: 25240821]
15. Li J, Qin S, Xu J, Xiong J, Wu C, Bai Y, et al. Randomized, Double-Blind, Placebo-Controlled Phase III Trial of Apatinib in Patients With Chemotherapy-Refractory Advanced or Metastatic Adenocarcinoma of the Stomach or Gastroesophageal Junction. J Clin Oncol 2016;34:1448–54. [PubMed: 26884585]
16. Carneiro F, Sobrinho-Simoes M. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer 2000;88:238–40. [PubMed: 10618628]
17. Janjigian YY, Tang LH, Coit DG, Kelsen DP, Francone TD, Weiser MR, et al. MET expression and amplification in patients with localized gastric cancer. Cancer Epidemiol Biomarkers Prev 2011;20:1021–7. [PubMed: 21393565]
18. Elisei R, Schlumberger MJ, Muller SP, Schoffski P, Brose MS, Shah MH, et al. Cabozantinib in progressive medullary thyroid cancer. J Clin Oncol 2013;31:3639–46. [PubMed: 24002501]
19. Tannir NM, Schwab G, Grunwald V. Cabozantinib: an Active Novel Multikinase Inhibitor in Renal Cell Carcinoma. Curr Oncol Rep 2017;19:14. [PubMed: 28247252]
20. Kitadai Y, Kodama M, Shinagawa K. Stroma-directed molecular targeted therapy in gastric cancer. Cancers (Basel) 2011;3:4245–57. [PubMed: 24213136]
21. Bottsford-Miller JN, Coleman RL, Sood AK. Resistance and escape from antiangiogenesis therapy: clinical implications and future strategies. J Clin Oncol 2012;30:4026–34. [PubMed: 23008289]
22. Awasthi N, Schwarz RE. Profile of nintedanib in the treatment of solid tumors: the evidence to date. Onco Targets Ther 2015;8:3691–701. [PubMed: 26677336]
23. Hilberg F, Roth GJ, Krssak M, Kautschitsch S, Sommergruber W, Tontsch-Grunt U, et al. BIBF 1120: triple angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy. Cancer Res 2008;68:4774–82. [PubMed: 18559524]

24. Syrios J, Nintos G, Georgoulias V. Nintedanib in combination with docetaxel for second-line treatment of advanced non-small-cell lung cancer. Expert Rev Anticancer Ther 2015;15:875–84. [PubMed: 26204906]
25. Awasthi N, Zhang C, Ruan W, Schwarz MA, Schwarz RE. BMS-754807, a small-molecule inhibitor of insulin-like growth factor-1 receptor/insulin receptor, enhances gemcitabine response in pancreatic cancer. Mol Cancer Ther 2012;11:2644–53. [PubMed: 23047891]
26. Awasthi N, Kirane A, Schwarz MA, Toombs JE, Brekken RA, Schwarz RE. Smac mimetic-derived augmentation of chemotherapeutic response in experimental pancreatic cancer. BMC Cancer 2011;11:15. [PubMed: 21226944]
27. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010;376:687–97. [PubMed: 20728210]
28. Kanda M, Kodera Y. Molecular mechanisms of peritoneal dissemination in gastric cancer. World J Gastroenterol 2016;22:6829–40. [PubMed: 27570420]
29. Yokozaki H Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int 2000;50:767–77. [PubMed: 11107048]
30. Awasthi N, Zhang C, Ruan W, Schwarz MA, Schwarz RE. Evaluation of poly-mechanistic antiangiogenic combinations to enhance cytotoxic therapy response in pancreatic cancer. PLoS One 2012;7:e38477. [PubMed: 22723862]
31. Awasthi N, Zhang C, Schwarz AM, Hinz S, Wang C, Williams NS, et al. Comparative benefits of Nab-paclitaxel over gemcitabine or polysorbate-based docetaxel in experimental pancreatic cancer. Carcinogenesis 2013;34:2361–9. [PubMed: 23803690]
32. Chen N, Brachmann C, Liu X, Pierce DW, Dey J, Kerwin WS, et al. Albumin-bound nanoparticle (nab) paclitaxel exhibits enhanced paclitaxel tissue distribution and tumor penetration. Cancer Chemother Pharmacol 2015;76:699–712. [PubMed: 26231955]
33. Yardley DA. nab-Paclitaxel mechanisms of action and delivery. J Control Release 2013;170:365–72. [PubMed: 23770008]
34. Zhang C, Awasthi N, Schwarz MA, Schwarz RE. The dual PI3K/mTOR inhibitor NVP-BEZ235 enhances nab-paclitaxel antitumor response in experimental gastric cancer. Int J Oncol 2013;43:1627–35. [PubMed: 24042258]
35. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. Mol Cancer Ther 2008;7:3670–84. [PubMed: 19074844]
36. Bai A, Meetze K, Vo NY, Kollipara S, Mazsa EK, Winston WM, et al. GP369, an FGFR2-IIIb-specific antibody, exhibits potent antitumor activity against human cancers driven by activated FGFR2 signaling. Cancer Res 2010;70:7630–9. [PubMed: 20709759]
37. Jung EJ, Jung EJ, Min SY, Kim MA, Kim WH. Fibroblast growth factor receptor 2 gene amplification status and its clinicopathologic significance in gastric carcinoma. Hum Pathol 2012;43:1559–66. [PubMed: 22440694]
38. Liu YJ, Shen D, Yin X, Gavine P, Zhang T, Su X, et al. HER2, MET and FGFR2 oncogenic driver alterations define distinct molecular segments for targeted therapies in gastric carcinoma. Br J Cancer 2014;110:1169–78. [PubMed: 24518603]
39. Ledermann JA, Hackshaw A, Kaye S, Jayson G, Gabra H, McNeish I, et al. Randomized phase II placebo-controlled trial of maintenance therapy using the oral triple angiokinase inhibitor BIBF 1120 after chemotherapy for relapsed ovarian cancer. J Clin Oncol 2011;29:3798–804. [PubMed: 21859991]
40. Reck M, Kaiser R, Mellemgaard A, Douillard JY, Orlov S, Krzakowski M, et al. Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): a phase 3, double-blind, randomised controlled trial. Lancet Oncol 2014;15:143–55. [PubMed: 24411639]
41. Itakura J, Ishiwata T, Shen B, Kornmann M, Korc M. Concomitant over-expression of vascular endothelial growth factor and its receptors in pancreatic cancer. Int J Cancer 2000;85:27–34. [PubMed: 10585578]

42. Stewart M, Turley H, Cook N, Pezzella F, Pillai G, Ogilvie D, et al. The angiogenic receptor KDR is widely distributed in human tissues and tumours and relocates intracellularly on phosphorylation. An immunohistochemical study. Histopathology 2003;43:33–9. [PubMed: 12823710]

43. Goel HL, Mercurio AM. VEGF targets the tumour cell. Nat Rev Cancer 2013;13:871–82. [PubMed: 24263190]

44. Awasthi N, Hinz S, Brekken RA, Schwarz MA, Schwarz RE. Nintedanib, a triple angiokinase inhibitor, enhances cytotoxic therapy response in pancreatic cancer. Cancer Lett 2015;358:59–66. [PubMed: 25527450]

45. Liu CY, Huang TT, Chu PY, Huang CT, Lee CH, Wang WL, et al. The tyrosine kinase inhibitor nintedanib activates SHP-1 and induces apoptosis in triple-negative breast cancer cells. Exp Mol Med 2017;49:e366. [PubMed: 28798401]

46. Awasthi N, Zhang C, Schwarz AM, Hinz S, Schwarz MA, Schwarz RE. Enhancement of nab-paclitaxel antitumor activity through addition of multitargeting antiangiogenic agents in experimental pancreatic cancer. Mol Cancer Ther 2014;13:1032–43. [PubMed: 24608575]

47. Kerbel RS. Antiangiogenic therapy: a universal chemosensitization strategy for cancer? Science 2006;312:1171–5. [PubMed: 16728631]

48. Ji J, Chen X, Leung SY, Chi JT, Chu KM, Yuen ST, et al. Comprehensive analysis of the gene expression profiles in human gastric cancer cell lines. Oncogene 2002;21:6549–56. [PubMed: 12226758]

49. Lyros O, Mueller A, Heidel F, Schimanski CC, Gockel I, Galle PR, et al. Analysis of anti-proliferative and chemosensitizing effects of sunitinib on human esophagogastric cancer cells: Synergistic interaction with vandetanib via inhibition of multi-receptor tyrosine kinase pathways. Int J Cancer 2010;127:1197–208. [PubMed: 20039326]

50. Yu J, Zhang Y, Leung LH, Liu L, Yang F, Yao X. Efficacy and safety of angiogenesis inhibitors in advanced gastric cancer: a systematic review and meta-analysis. J Hematol Oncol 2016;9:111. [PubMed: 27756337]

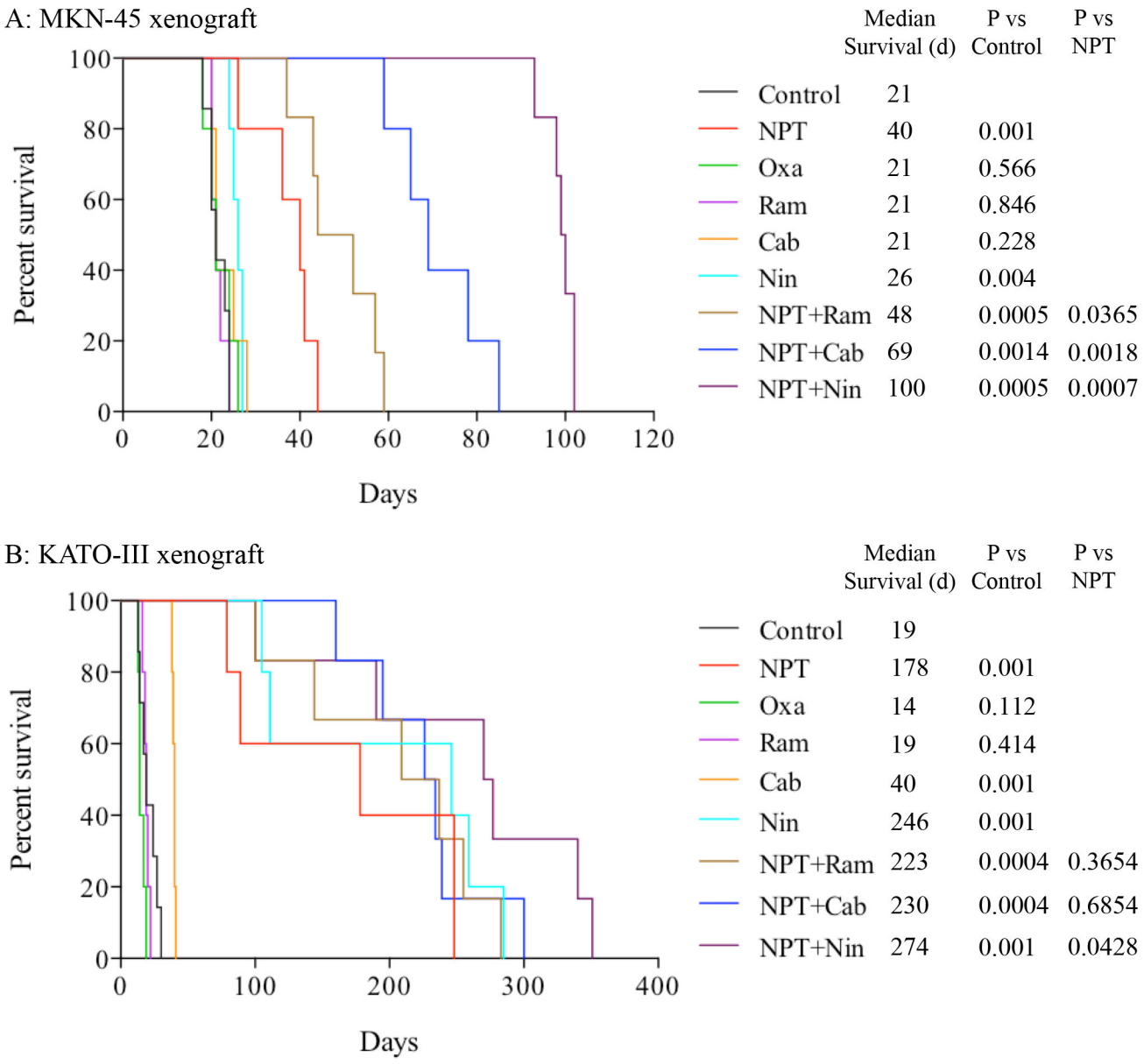

Figure 1: Antiangiogenic agents improve animal survival benefits of *nab*-paclitaxel.

A. Animal survival study in the MKN-45 GAC cell-derived peritoneal dissemination model. B. Animal survival study in the KATO-III GAC cell-derived peritoneal dissemination model. Ten days after tumor cell injection in NOD/SCID mice, treatment was started with *nab*-paclitaxel, ramucirumab, cabozantinib and nintedanib for 2 weeks. The curve represents the animal survival time from the beginning of therapy. Statistical group differences in survival time were calculated using log-rank testing.

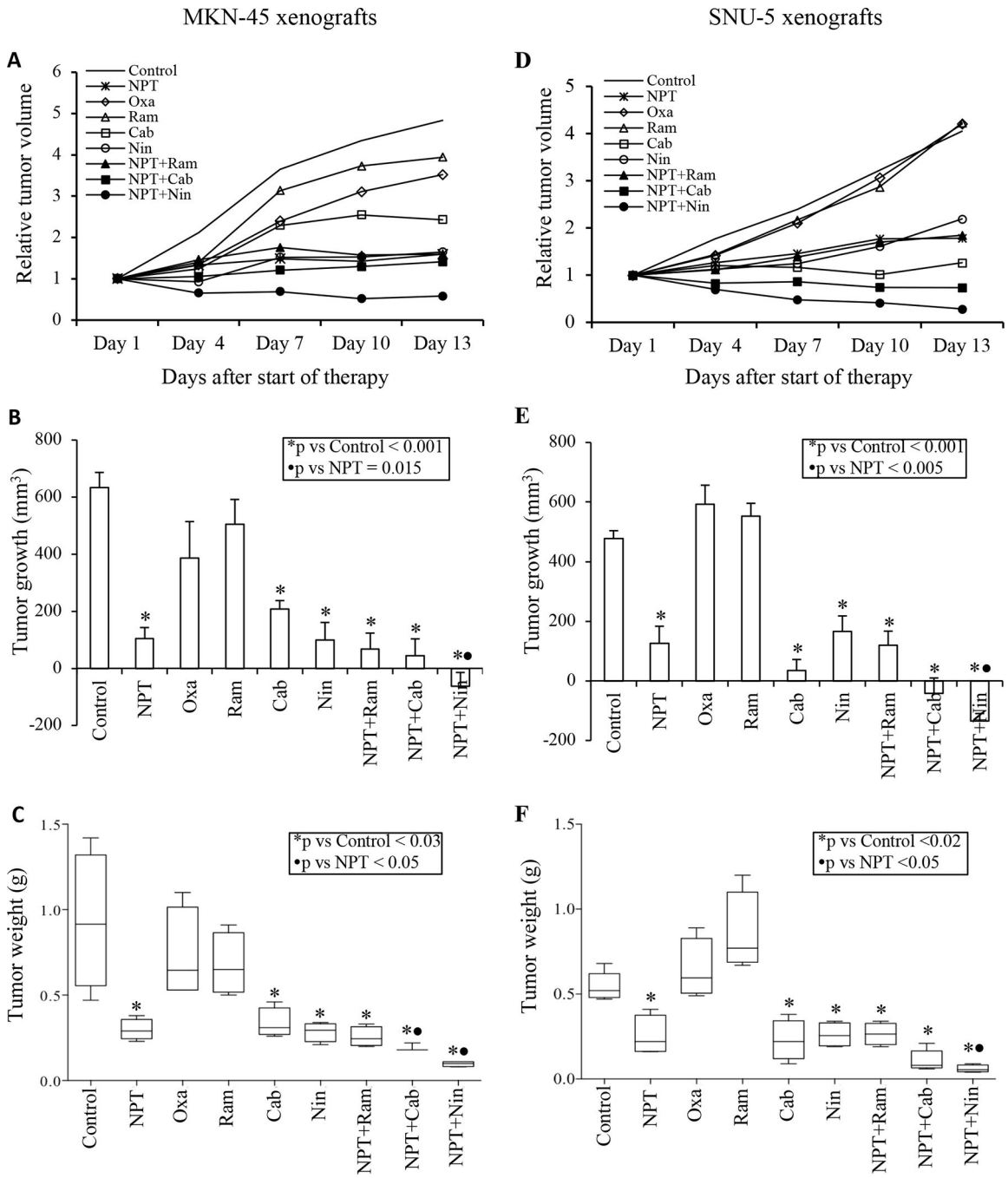

Figure 2: Antiangiogenic agents enhance *nab*-paclitaxel antitumor effects in GAC cell-derived xenografts.

Tumor growth inhibition in MKN-45 GAC cell-derived subcutaneous xenografts (A, B, C) or in SNU-5 GAC cell-derived subcutaneous xenografts (D, E, F). Ten days after tumor cell injection when all mice had a measurable tumor, mice were treated with *nab*-paclitaxel, ramucirumab, cabozantinib and nintedanib for 2 weeks. **A, D.** Tumor size was measured twice a week using calipers and plotted. **B, E.** Net growth in tumor size was calculated by subtracting tumor volume on the first treatment day from that on the final day. **C, F.** Mean

tumor weight was calculated from final day tumor weights in each group and presented as a Box plot. Data are representative of mean values ± standard deviation from 5 mice per group. Statistical analysis was performed by one-way ANOVA for multiple group comparison and Student's t-test for the individual group comparison.

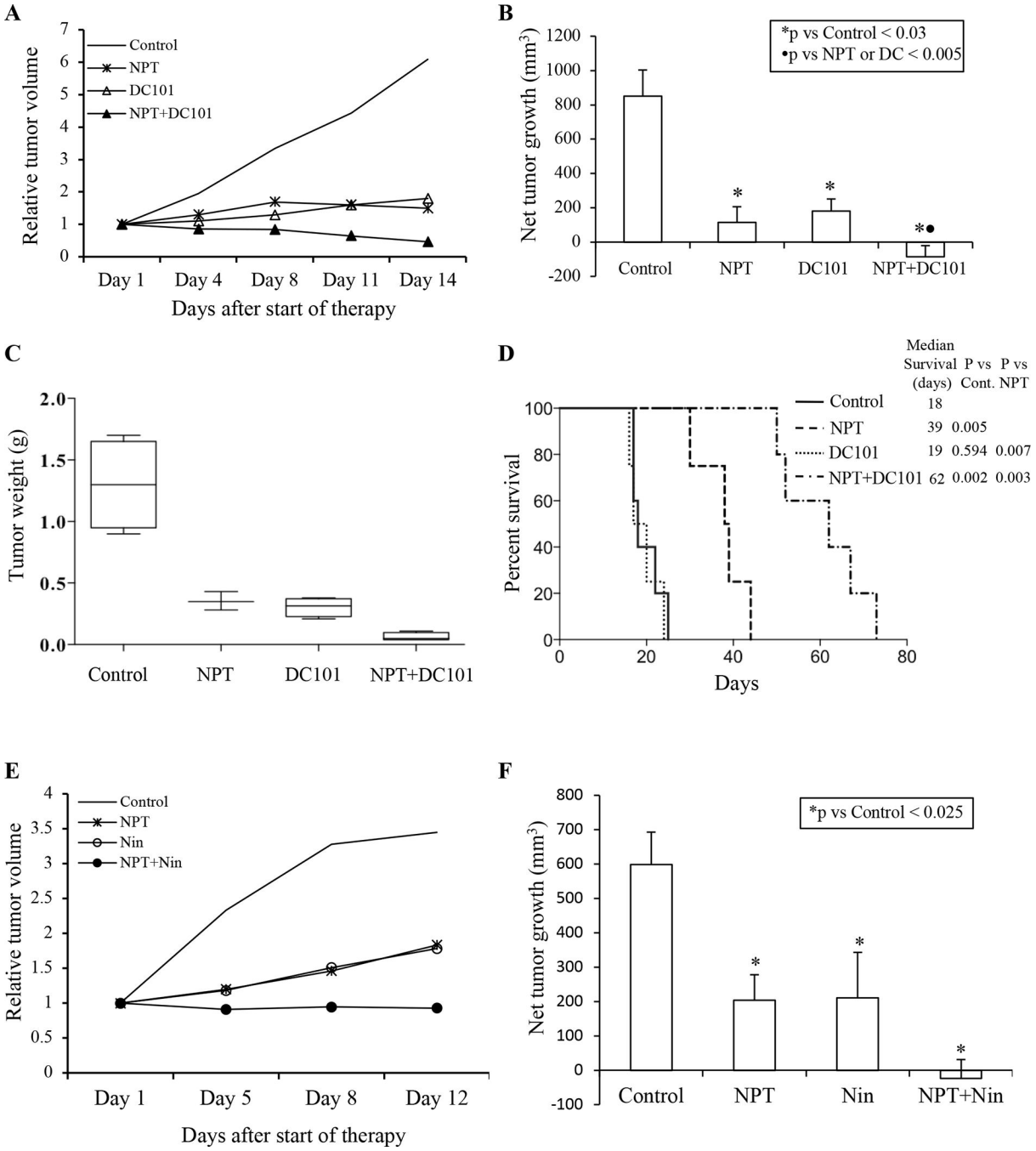

Figure 3: Antiangiogenic agents and *nab*-paclitaxel: Antitumor effects in MKN-45 cell-derived xenografts and in patient-derived xenografts.

Tumor-bearing NOD/SCID mice were treated with DC101 and *nab*-paclitaxel in MKN-45 GAC cell-derived subcutaneous xenografts, n=5 (A, B, C, D) or with nintedanib and *nab*-paclitaxel in GAC patient-derived xenografts, n=3 (E, F). When all mice had a measurable tumor, they were treated for 2 weeks. **A, E.** Tumor size was measured twice a week using calipers and plotted. **B, F.** Net growth in tumor size was calculated by subtracting tumor volume on the first treatment day from that on the final day. Data are representative of mean

values ± standard deviation. **C.** Mean tumor weight was calculated from final day tumor weights in each group and presented as a Box plot. D. The curve represents the animal survival time from the beginning of therapy. Statistical group differences in survival time were calculated using log-rank testing.

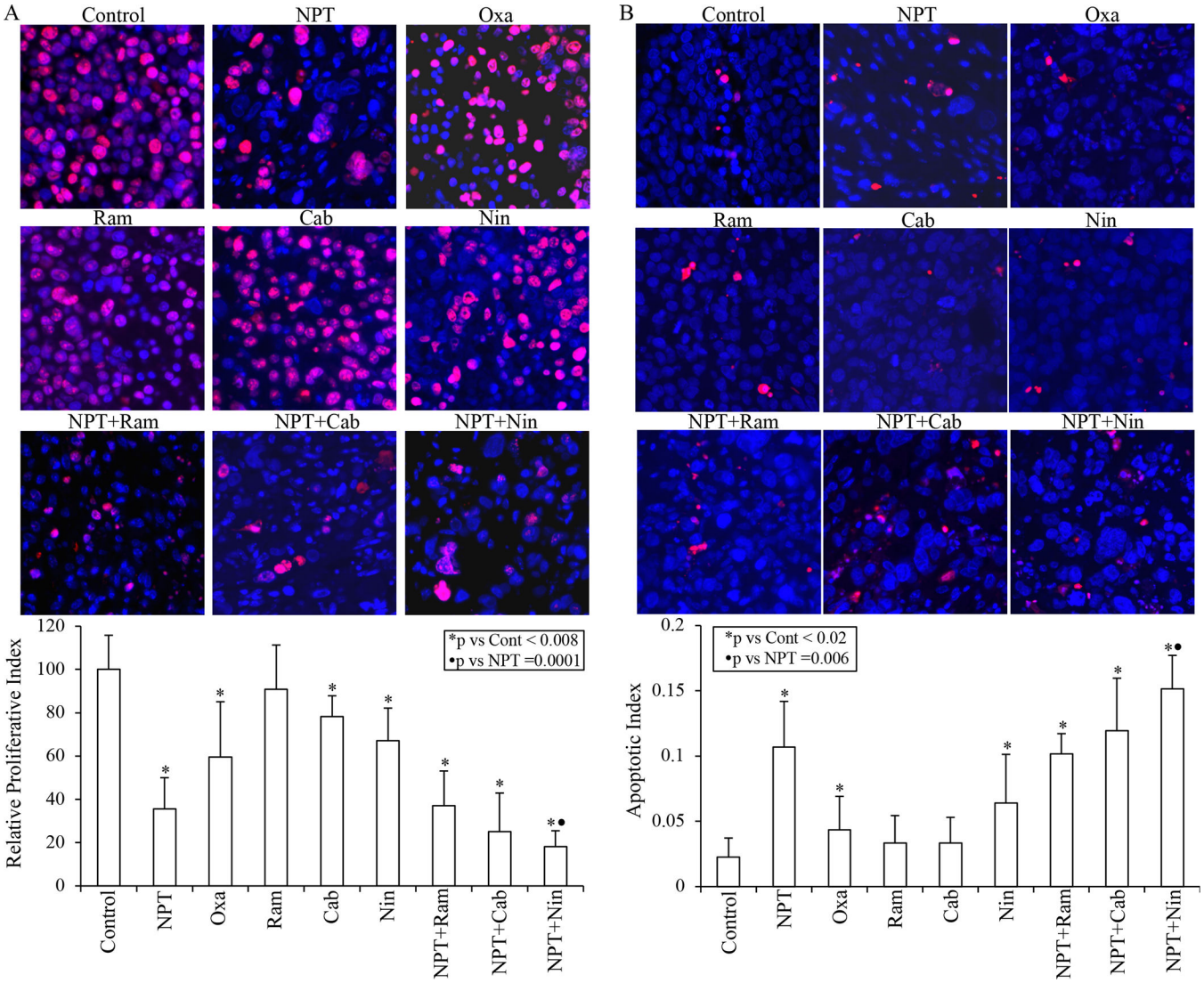

Figure 4: Antiangiogenic agents and *nab*-paclitaxel: effects on tumor cell proliferation and apoptosis.

Tumor tissue sections obtained from the MKN-45 subcutaneous xenograft study after 2-week treatment with *nab*-paclitaxel, ramucirumab, cabozantinib, and nintedanib, were used for the IHC analysis. **A.** Tissue sections were immunostained with Ki67 antibody to determine tumor cell proliferation. Ki67-positive cells were counted in five different high-power fields. **B.** Tumor cell apoptosis was measured by staining tumor tissue section with TUNEL procedure. TUNEL-positive apoptotic cells were counted in five different high-power fields. For both immunostaining experiments, slides were photographed under a fluorescent microscope and the data are expressed as the mean ± standard deviation.

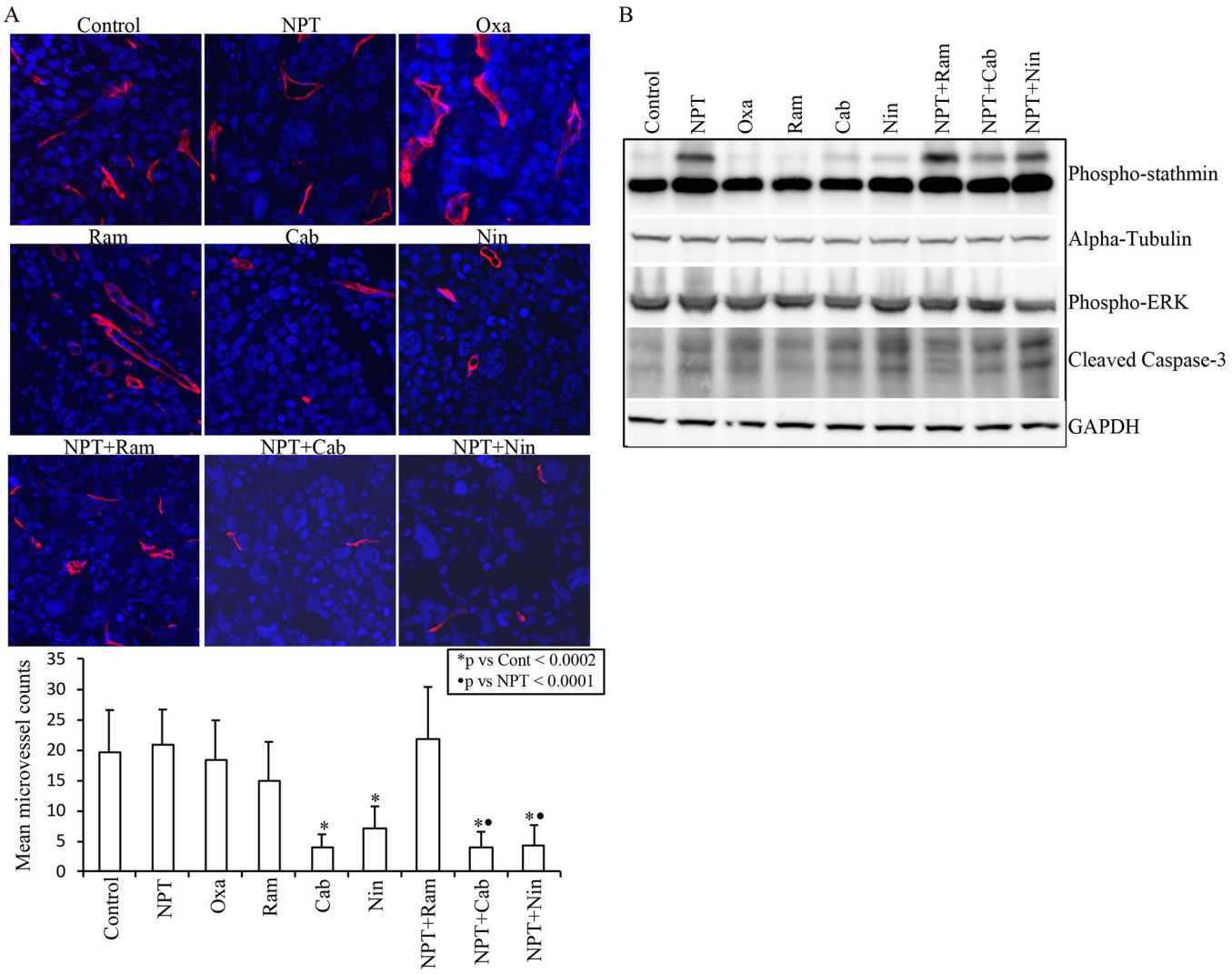

Figure 5: Antiangiogenic agents and *nab*-paclitaxel: effects on microvessel density and marker proteins. A.

Tumor tissue sections obtained from the MKN-45 subcutaneous xenograft study after 2-week treatment were used for evaluating intratumoral microvessel density. Tumor sections were incubated with anti-endomucin antibody and slides were photographed under a fluorescent microscope. Endomucin positive vessels were calculated within a microscopic HPF in a blinded manner and the data are expressed as the mean ± standard deviation. **B.** MKN-45 GAC cell monolayers were treatment with *nab*-paclitaxel, ramucirumab, cabozantinib or nintedanib, cell lysates were prepared after 16 hours incubation and analyzed by immunoblotting. The intensity of bands was quantitated by densitometry and is represented in the bar graph after normalizing values with corresponding total protein expression or GAPDH expression.

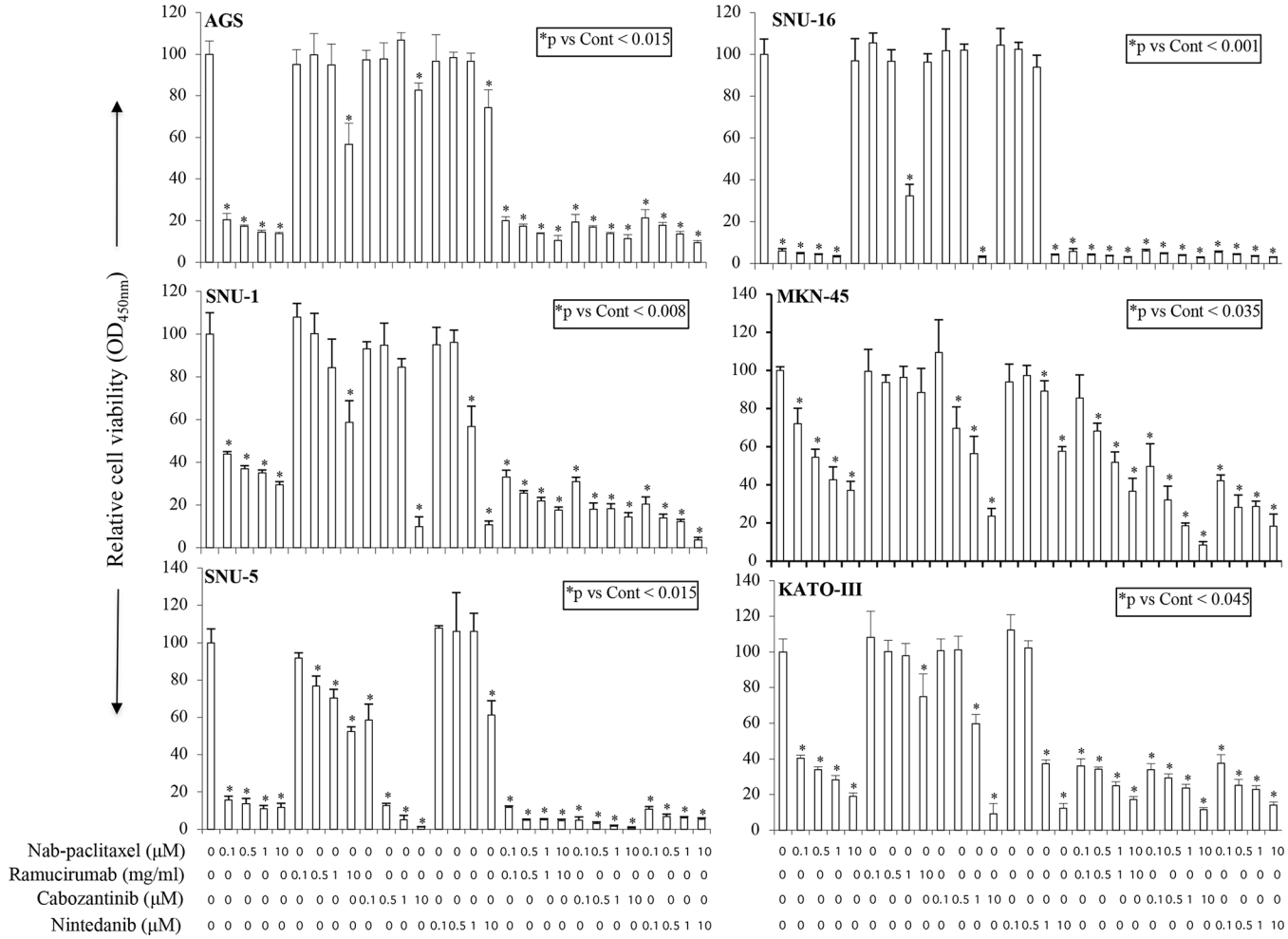

Figure 6: Antiangiogenic agents and *nab*-paclitaxel: *In vitro* GAC cell proliferation.

GAC cells (AGS, SNU-1, SNU-5, SNU-16, MKN-45, and KATO-III) were plated on 96-well plates and treated with *nab*-paclitaxel, ramucirumab, cabozantinib or nintedanib. WST-1 reagent (10 μl) was added to each well after 72-hour incubation followed by additional incubation for 2 hours. The absorbance at 450 nm was measured using a microplate reader. The resulting number of viable cells was calculated by measuring the absorbance of color produced in each well. Data are the mean ± SD of triplicate determinations.