mTOR Inhibition Sensitizes Gastric Cancer to Alkylating Chemotherapy *In Vivo*

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Abstract. *Background: Gastric cancer is a highly chemoresistant tumor. Previous studies suggest that cancer cells can be sensitized to standard chemotherapy, and especially alkylating agents, by inhibition of mammalian target of rapamycin (mTOR) signaling. The work presented here shows that the mTOR inhibitor everolimus, in combination with cyclophosphamide, exhibits synergistic antitumor activity in gastric cancer xenografts. Materials and Methods: Treatment with everolimus at the minimal effective dose was studied in combination with cyclophosphamide at maximum tolerated dose in a human gastric cancer severe combined immunodeficient (SCID) mouse xenograft model. Besides tumor size, biomarker expression for proliferation (Ki-67), hypoxia (HIF-1α), apoptosis (activated caspase 3), angiogenesis (microvascular density, MVD) and levels of circulating endothelial progenitors (CEPs) were assessed. Results: Everolimus single agent treatment significantly inhibited tumor growth relative to control and cyclophosphamide treatment (T/C 19%, p<0.01). This anti-tumor activity was linked to a significant decrease in tumor cell proliferation (p<0.01) and a trend towards lower tumor MVD, HIF-1α expression and levels of CEPs. Notably, the combination of everolimus with cyclophosphamide resulted in synergistic anti-tumor activity (T/C 9%, p<0.01). This anti-tumor activity coincided with a statistically significant decrease in MVD (p<0.01). Whereas treatment with everolimus was well tolerated, cyclophosphamide at maximum tolerated dose (MTD) showed significant toxicity both as monotherapy and in combination with everolimus. Conclusion: The antiangiogenic activity of everolimus combined with a high dose of cyclophosphamide shows synergistic antitumor activity against gastric cancer in vivo. In potential future clinical trials, the toxicity of cyclophosphamide in combination regimens with everolimus deserves careful evaluation.*

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The mammalian target of rapamycin (mTOR) pathway has become a major focus of preclinical and clinical cancer research (1). Rapamycin inhibits the kinase activity of mTOR, which has been shown to result in G1 arrest, apoptosis or autophagy, depending on cell type studied (2-4). In gastric cancer, mTOR activity and components of the mTOR signaling network including PTEN, 4E-BP1 and eIF-4 are deregulated and correlate with progression of disease, metastasis, and inferior survival (5-9). In addition to its direct effect on tumor cells, mTOR inhibitors show antiangiogenic activity by lowering VEGF secretion, impairing endothelial cell proliferation, inducing tumor vessel thrombosis and inhibiting circulating endothelial progenitor cell (CEP) activity and survival (10-13).

Expression of the transcription factor hypoxia-inducible factor 1α (HIF-1α) has been identified as a marker for poor prognosis in different solid tumors including gastric cancer (14). Studies on expression status of HIF-1α target genes such as *GLUT-1* or *VEGF* support the unfavorable role of HIF-1α in gastric cancer (15-17). Preclinical studies indicate that blocking HIF-1α activity leads to profound inhibition of tumor growth and angiogenesis in gastric cancer models *in vivo* (18, 19). Interestingly, translation of HIF-1α has been reported to be sensitive to mTOR inhibition (20-22). Recent evidence suggests that silencing of HIF-1α activity may be a major mechanism of action for the antitumor effect of mTOR inhibitors (23).

Since mTOR signaling plays a key role in cancer cell metabolism and growth factor signal transduction, many clinical trials are being performed in order to evaluate the therapeutic potential of the rapamycin derivatives everolimus, temsirolimus and deforolimus in solid tumors (24). The first FDA-approved mTOR inhibitor for cancer therapy was temsirolimus (25). As promising as these results

may be, monotherapy with mTOR inhibitors often shows only modest therapeutic activity. For instance, treatment with temsirolimus significantly prolongs median overall survival of patients with advanced renal cell cancer by about 3 months (25). Although encouraging, there is obviously still room for improvement of their therapeutic activity, for example by combining mTOR inhibitors with chemotherapeutics. In fact, preclinical data suggest that mTOR inhibition may synergize with alkylating cytotoxic chemotherapy (26). For instance, Wendel *et al*. demonstrated that resistance to cyclophosphamide could be reversed by rapamycin in an experimental murine model of lymphoma (27). To date, gastric cancer has been regarded to be resistant to cyclophosphamide in experimental studies (28).

In this study it is hypothesized that mTOR inhibition may synergize with cyclophosphamide, a prototypical alkylating cytotoxic agent. Thus, the combination of everolimus at minimal effective dose (MED), defined as lowest dose leading to maximum antitumor effect when used as single agent, with cyclophosphamide at maximum tolerated dose (MTD) was assessed for antitumor activity in a gastric cancer xenograft model. Furthermore, biomarkers for cancer cell proliferation, apoptosis, hypoxia, tumor vascularization and levels of circulating endothelial progenitor cells were studied to elucidate the mechanism of action for the applied treatment regimens.

Materials and Methods

Gastric cancer cells. NCI-N87 human gastric cancer cells (intestinal type) were purchased from ATCC. Cells were cultured in RPMI-1640 medium supplemented with 10% FCS, L-glutamine and an antibiotic mixture containing penicillin, streptomycin and amphotericin B (all Gibco Invitrogen, Lofer, Austria) in a fully humidified 5% CO_2, 95% ambient air atmosphere at 37˚C.

Compounds. Everolimus microemulsion was kindly provided by The Novartis Institutes for BioMedical Research Basel, Oncology, Switzerland. For *in vivo* experiments, formulated everolimus was supplied as oral microemulsion. Aliquots were stored at –20˚C and diluted with 5% glucose solution before administration. Microemulsion without everolimus was given as carrier control. Cyclophosphamide (Ebewe, Unterach, Austria) was diluted in normal saline (0.9% w/v).

Western blot. Snap-frozen tumor xenografts were homogenized with a MM 200 mixer mill (Retsch, Germany) and protein was extracted with a cell lysis buffer (adjusted to pH 7.4, containing 1% Nonidet P40, 0.1% SDS, 100 mM NaCl, 50 nM Tris and 10 mM EDTA, 10 mM *p*-Nitrophenolphosphate, 250 U/ml aprotinin, 40 μg/ml leupeptin, 1 mM PMSF, 10 mM NaF and 40 mM β-glycerolphosphate). Debris was removed by centrifugation and supernatant was stored at –80˚C until further analysis.

Protein extracts (10 μg protein per lane) were electrophoretically separated by SDS-PAGE and blotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with I-Block (Tropix, Bedford, MA, USA) in PBS with 0.1% Tween blocking solution at room temperature for 1 h followed by incubation with primary antibodies diluted in blocking solution at 4˚C overnight. Polyclonal antibodies directed against phospho-S6 kinase Thr421/Ser424 (dilution 1:5,000) of rabbit origin were obtained from Cell Signaling Technology (Danvers, MA, USA). Actin antibody (purified from rabbit serum, dilution 1:10,000) was obtained form Sigma. Primary antibodies were detected by alkaline phosphatase-conjugated secondary anti-rabbit antibody (Tropix, 1:5,000) and visualized by chemiluminescence using CSPD (Tropix) substrate.

Flow cytometry. For *in vivo* detection of circulating endothelial cells, heparinized whole blood was stained with FITC-labeled anti-Sca-1 and PE-labeled anti-VEGFR2 antibodies (both BD Biosciences, Schwechat, Austria) followed by red cell lysis with FACS lysing solution (BD Biosciences). Respective isotype controls were purchased from Serotec (Raleigh, NC, USA). Cells stained $Sca\text{-}1^+$ /$VEGFR2^+$ in the lymphocyte gate were defined as circulating endothelial progenitors as described earlier (29).

Tumor xenograft model. Pathogen-free, 4- to 6-week-old, female CB-17 SCID mice (Charles River Laboratories, Sulzfeld, Germany) were housed under sterile conditions and treated according to the regulations imposed and approved by the local animal welfare committee. In this study, the combination of everolimus at the MED and chemotherapy with cyclophosphamide at MTD was assessed. MED of everolimus and MTD of cyclophosphamide in this model were established in previous studies (data not shown). NCI-N87 gastric cancer cells (10^7 cells) solved in 100 μl DPBS were inoculated into the lower left flank. When tumor volume reached approximately 200 mm^3, mice (n=8/group) were randomly assigned to one of the following four treatment groups: 1) Cyclophosphamide (Cy): cyclophosphamide at a dose of 170 mg/kg intraperitoneally (*i.p.*) on day 1 and day 6 + microemulsion placebo orally (*p.o.*) 5x/week; 2) Everolimus (E): everolimus (5 mg/kg *p.o.* 5x/week) + 200 μl of saline *i.p.* on day1 and day 6); 3) Everolimus + cyclophosphamide (E + Cy): everolimus (5 mg/kg 5x/week *p.o.*) + cyclophosphamide (170 mg/kg *i.p.* on day 1 and day 6); 4) Solvent control (Ctrl): 200 μl of microemulsion placebo *p.o.* 5x/week + 200 μl of saline *i.p.* on day 1 and day 6.

Tumor volume was assessed biweekly by caliper measurements and calculated according to the approximation formula: volume (mm^3) = 4/3 π x (long diameter^2 x short diameter)/2. All mice were sacrificed when the tumors in the control group exceeded 1,000 mm^3 as the predefined surrogate endpoint for terminal disease. For quantification of CEPs, mice were anesthetized and blood was drawn by cardiac puncture immediately prior to sacrifice.

Immunohistochemistry (IHC). Phosphate-buffered formaldehyde (4.5%) fixed specimens were paraffin-embedded and cut at a thickness of 3-5 μm. Sections were deparaffinized in xylene. Slides were heated in 0.01 M citrate buffer (pH 6.0) for 30 minutes for anti-Ki-67 immunostaining, 20 minutes for anti-caspase-3 immunostaining, or 10 minutes for anti-Factor-VIII immunostaining in a microwave oven at 600 W.

The following antibodies were used for immunostainings: anti-Ki-67 (monoclonal mouse, clone MIB-1; Dako, Glostrup, Denmark; dilution 1:50), anti-HIF-1α (monoclonal mouse, clone 54; Transduction Laboratories, Lexington, UK; dilution 1:30), anti-factor-VIII (polyclonal rabbit; Dako; dilution 1:1,000), anti-caspase-3

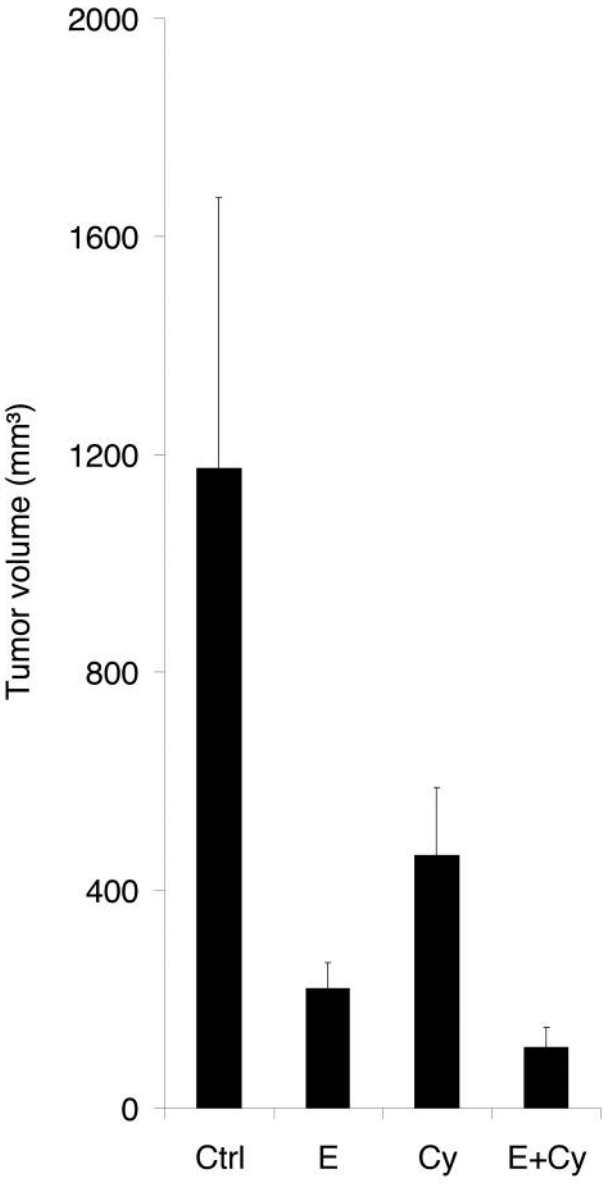


Figure 1. *Combination of everolimus with cyclophosphamide is superior to either monotherapy in vivo. Tumor volume of NCI-N87 gastric cancer xenografts in SCID mice. Animals were treated with placebo (Ctrl), everolimus (E), cyclophospamide (Cy), or a combination of both (E + Cy). Mice were sacrificed 25 days after initiation of treatment.*

(monoclonal rabbit, clone 5A1; Cell Signalling; dilution 1:100). Detection of immunostaining was performed using the Envision Kit (Dako) and DAB chromogen.

Results of IHC studies were interpreted by a trained pathologist blind with regard to the treatment groups. For all IHC stainings, control of antibody specificity included omission or substitution of the primary antibodies by non-specific, isotype-matched antibodies or substitution of the primary antiserum by normal rabbit serum.

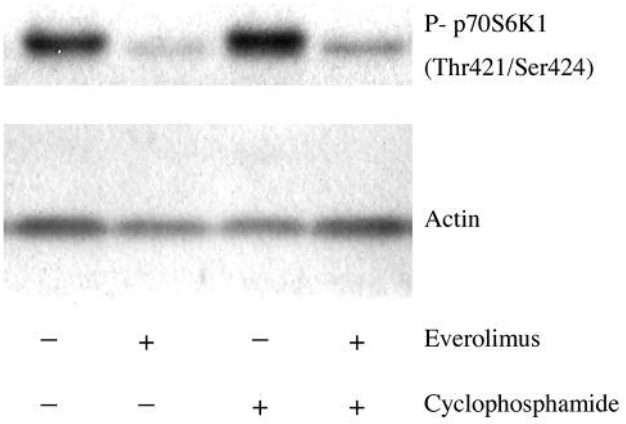


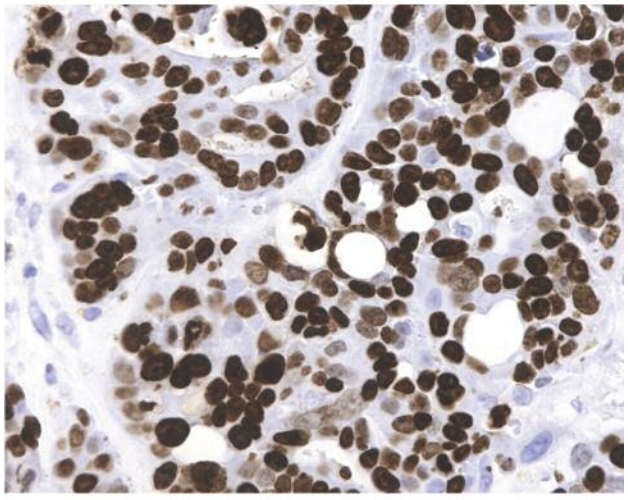
Figure 2. *Everolimus but not cyclophosphamide inhibits phosphorylation of S6K in vivo. Representative Western blots from tumor xenograft tissue showing marked inhibition of S6K1 (Thr421/Ser424) by everolimus. Tumors were excised 24 h after the last administration of everolimus. Mice received microemulsion placebo (Ctrl), everolimus (E), cyclophosphamide (Cy), or a combination of both (E+Cy) as indicated.*

Statistical analysis. Data is given as mean ± standard error of the mean (SEM) unless indicated otherwise. To test for statistically significant differences between groups, the Kruskal-Wallis test was followed by pairwise comparisons using the Mann-Whitney *U*-test and Bonferroni-Holm correction for multiple testing. *P*-values <0.05 were considered statistically significant. Boxplots show median values, interquartile ranges and 95% confidence intervals, respectively. Outliers are indicated as circles or asterisks. Statistical analysis was performed using SPSS 10.0 software (SPSS Inc., Chicago, Il, USA).

Results

SCID mice with established gastric cancer xenografts were treated with carrier control (Ctrl), everolimus (E), high-dose cyclophosphamide (Cy) or a combination of both therapeutic agents (E + Cy). Everolimus monotherapy for 25 days delayed xenograft growth remarkably, resulting in more than 80% smaller xenografts relative to the control group, with a treated tumor *versus* control tumor size (T/C) of 19% ($p<0.001$, Figure 1). In contrast, mice treated with cyclophosphamide monotherapy at MTD showed 60% reduced tumor growth (T/C 39% ; $p<0.01$). Notably, combination of everolimus with cyclophosphamide inhibited tumor growth by over 90% (T/C 9% , $p<0.01$ *vs*. all other groups).

Everolimus monotherapy was well tolerated as shown by the similar weight and clinical appearance of the treated animals as compared to the control animals. Cyclophosphamide at MTD resulted in severe toxicity resulting in weight loss, ruffled fur and anogenital staining in both cyclophosphamide-treated groups. Whereas mice in the cyclophosphamide monotherapy group recovered fully, mice



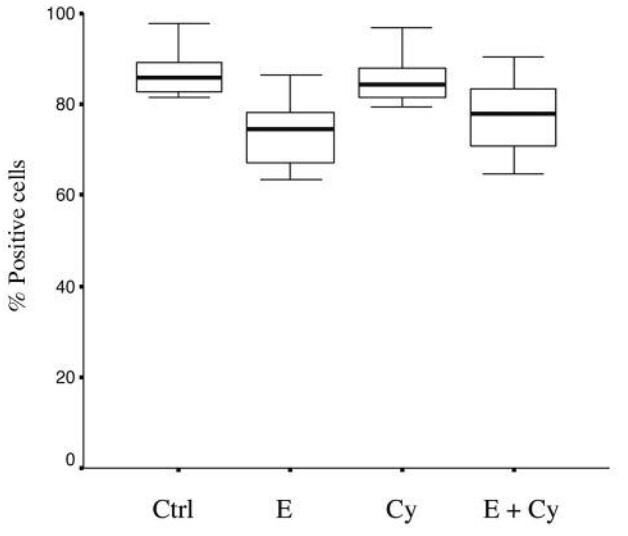
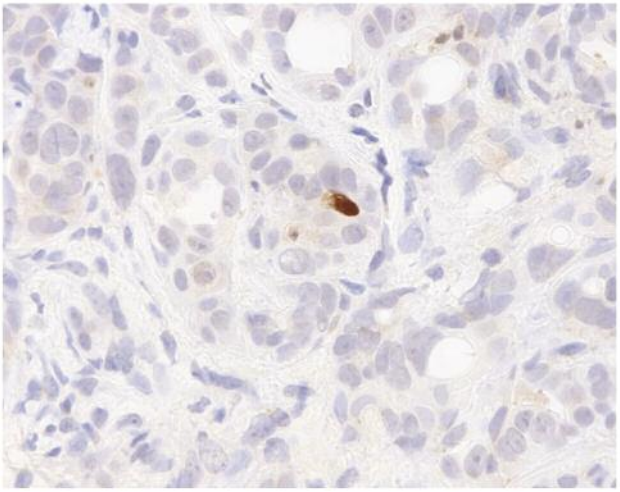


Figure 3. *Tumor cell proliferation. Ki-67 expression in gastric cancer xenografts decreased significantly by everolimus treatment. Mice received microemulsion placebo (Ctrl), everolimus (E), cyclophosphamide (Cy) or combination of both (E+Cy). Upper panel: Representative tissue section of Ki-67 immunostaining. Lower panel: Boxplots show median values, interquartile ranges and 95% confidence intervals, of Ki-67 expression.*



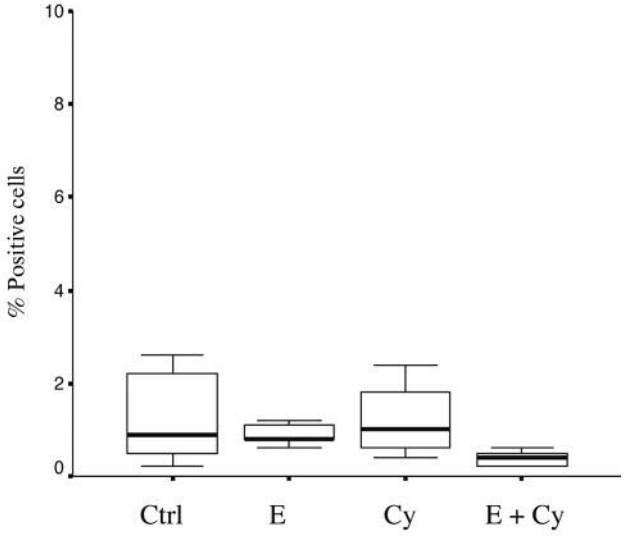
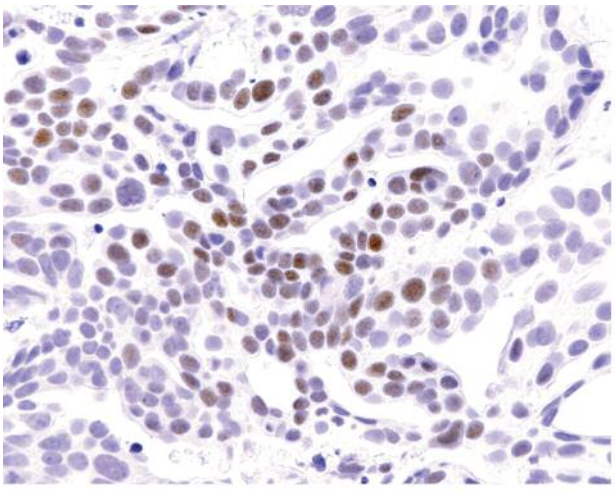


Figure 4. *Cancer cell apoptosis. No statistically significant differences in activation of caspase-3 among treatment groups were detected. Upper panel: Representative tissue section of activated caspase-3 immunostaining. Lower panel: Boxplots show median values interquartile ranges and 95% confidence intervals of caspase-3 expression.*

in the combination treatment group experienced significant toxicity and one animal died 4 days after the second administration of cyclophosphamide (day 10). The average mouse weight at sacrifice was 18.6±0.9 g for the control, 18.7±1.2 g for everolimus, 18.2±0.8 g for cyclophosphamide and 12.5±1.6 g for combination therapy.

To allow for cross-sectional analysis of biomarkers, all mice were sacrificed 25 days after initiation of therapy. Immunoblotting of phosphorylated p70S6K1 was evaluated as a biological marker for everolimus activity *in vivo* (Figure 2). Treatment with everolimus resulted in markedly decreased phosphorylation status of p70S6K1 at Thr421/Ser424 in tumor xenografts, whereas cyclophosphamide did not cause any changes in p70S6K1 phosphorylation.

In order to investigate the biology underlying the antitumor activity of treatments in more detail, immunohistochemical (IHC) studies of paraffin-embedded tumor tissue were performed. Enumeration of cells stained positive for Ki-67 and activated caspase-3 (Figures 3 and 4) revealed that everolimus treatment inhibited tumor cell proliferation (–12% relative to



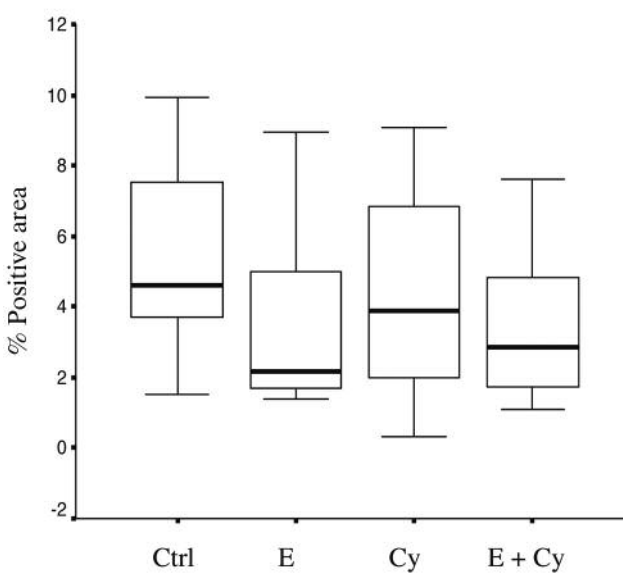
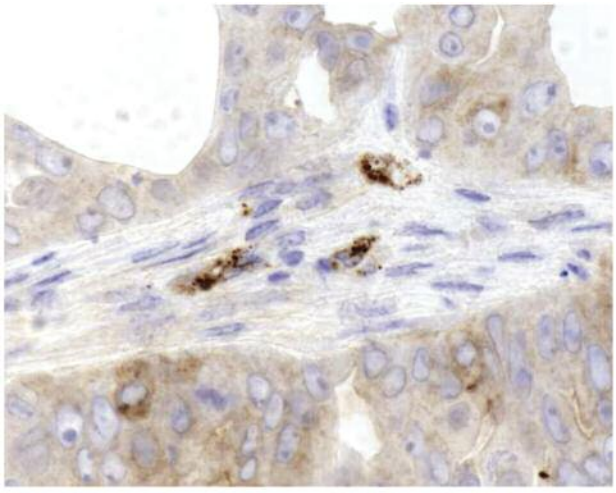


Figure 5. *Tumor hypoxia. Treatment groups including everolimus showed a non-significant tendency for reduced HIF-1α protein expression. Upper panel: Representative tissue section of HIF-1α immunostaining. Lower panel: Boxplots show median values interquartile range and 95% confidence intervals of HIF-1α expression.*



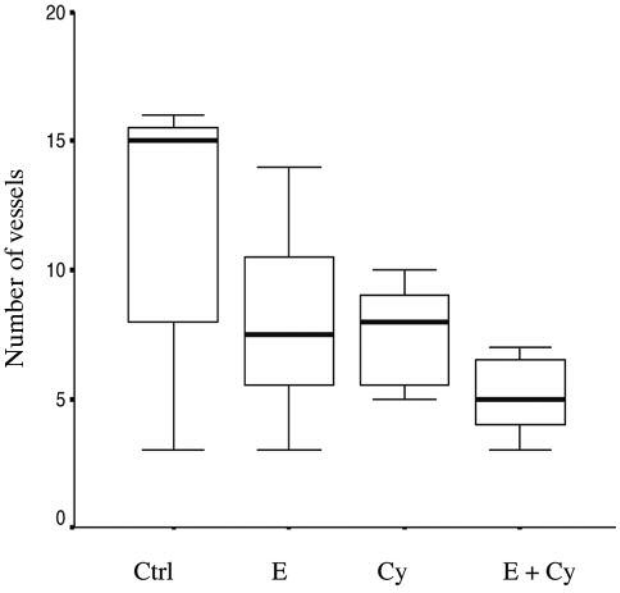


Figure 6. *Tumor vascularization. Both everolimus as well as anti-angiogenic cyclophosphamide were found to decrease MVD. Combination therapy reduced MVD of tumors even further. Upper panel: Representative tissue section of Factor-VIII immunostaining. Lower panel: Boxplots show median values, interquartile range and 95% confidence intervals of MVD.*

the control; $p<0.01$) without inducing cell death (p=n.s.). Both biomarkers were altered similarly by combination treatment as by everolimus monotherapy alone. In contrast, no changes in gastric cancer cell proliferation or apoptosis induction were detected by cyclophosphamide monotherapy (p=n.s.). With respect to HIF-1α, only everolimus-containing treatment regimens resulted in a 35% mean reduction of HIF-1α expression compared to the control (Figure 5). However, HIF-1α staining was inhomogeneous and differences between treatment groups did not reach statistical significance (p=n.s.).

In order to study possible anti-angiogenic effects, vascularization of tumor xenografts was assessed by MVD (Figure 6). Single agent treatment with everolimus or cyclophosphamide showed a trend for lower tumor MVD (–30% relative to the control, p=0.06). However, only the combination of everolimus and cyclophosphamide significantly reduced tumor vascularization compared to the control (–57% MVD; $p<0.01$).

Levels of circulating endothelial progenitor cells (CEPs) have been found to correlate with the antiangiogenic activity

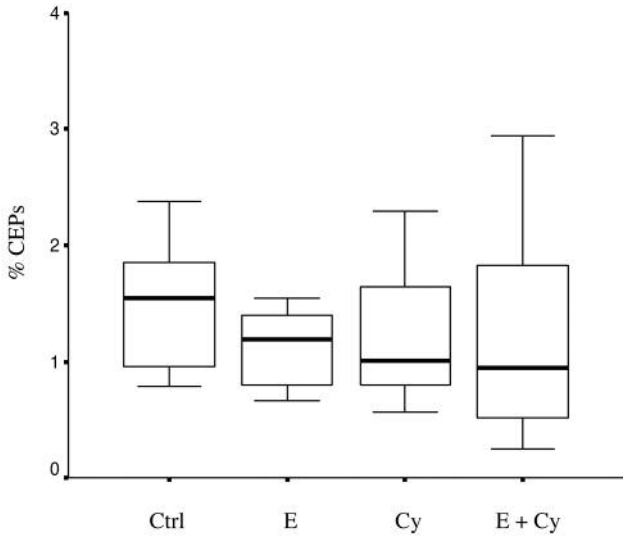


Figure 7. *Circulating endothelial progenitor cells (CEPs). CEPs defined as Sca-1$^+$/Flk-1$^+$ were detected by flow cytometry after erythrocyte lysis. Median, interquartile range and 95% confidence intervals percentages of CEPs in the lymphocyte gate are shown. Mice received microemulsion placebo (Ctrl), everolimus (E), cyclophosphamide (Cy) or combination of both (E + Cy).*

of therapeutics in experimental and clinical settings (30, 31). Hence, levels of CEPs were measured in peripheral blood as a biomarker for antiangiogenic activity, a surrogate also easily implementable in clinical trials (Figure 7). Although an obvious trend was observed for decreased CEP levels by –23% , –35% and –39% for everolimus, cyclophosphamide and the combination treatment, respectively, this trend did not reach statistical significance (p=n.s. for all).

Discussion

mTOR inhibition has become a clinically relevant treatment concept in oncology. Beyond monotherapy, optimization of therapy regimens with rapamycin and standard of care chemotherapeutics is currently a subject of intensive investigations. In the present study synergistic antitumor activity for mTOR-inhibition by everolimus in combination with high-dose cyclophosphamide in a mouse xenograft model of gastric cancer was investigated. It was observed, as with the majority of tumor entities, that mTOR inhibition did not induce cell death but slowed gastric cancer cell proliferation in the gastric cancer xenograft model studied. Besides lowering proliferation of tumor cells, everolimus monotherapy decreased MVD of gastric cancer xenografts, which was close to statistical significance (p=0.06). This decrease in vascularization could be the result of a combination of direct anti-endothelial effects and suppressed VEGF secretion of gastric cancer cells, as well as decreased tumor cell proliferation. As proposed by Hlatky *et al.*, attenuated tumor cell proliferation might result in decreased metabolic burden and lowered need for vascular supply, ultimately leading to lower MVD (32). Interestingly, although cyclophosphamide monotherapy significantly hampered xenograft growth, no changes in cancer cell death could be detected. This result might be explained by the time gap between the last administration of cyclophosphamide and tumor sampling, since chemotherapy induced apoptosis is expected to take place within hours to days after the pro-apoptotic stimulus (33). Alternatively, it has been proposed that the primary target of high-dose chemotherapy is not the cancer cell itself, but rather the tumor vasculature, hence leading to tumor shrinkage through antiangiogenic effects. Ferrara *et al.* proposed that conventional chemotherapy may have such anti-angiogenic properties (34). The mechanism of action for anti-angiogenic properties of chemotherapeutics appears to be pleiotropic. For instance, dividing endothelial cells may be particularly sensitive to cytotoxics. Furthermore, myelosuppression induced by high-dose chemotherapy may lead to reduced levels of pro-angiogenic monocytes, pericyte precursors and circulating endothelial progenitor cells. This concept is supported by experimental evidence as cyclophophamide at MTD decreases CEP levels within days after therapy in tumor-bearing mice (35).

Intriguingly, tumors treated with cyclophosphamide monotherapy showed a trend towards lower tumor vascularization in the presented study. Moreover, the combination of everolimus and cyclophosphamide caused a statistically significant decrease in MVD. Based on this biomarker finding, it is tempting to speculate that the synergistic antitumor activity of both agents is, at least in part, mediated by a synergistic antiangiogenic mode of action. On the other hand, this finding has to be discussed cautiously. Immunohistochemical studies of MVD are inherently hampered by considerable heterogeneity of tumor architecture and the invasive nature of tumor biopsies limiting their (pre-)clinical use (32). The use of alternative biomarkers detectable in blood such as CEPs to monitor anti-angiogenic therapies would be clearly advantageous for pharmacodynamic studies. Although there was a high variability in CEP measurements within treatment groups, median values of CEPs were reduced in the everolimus single agent treatment group, reflecting MVD and antitumor activity. Therefore measurements of CEP levels might have the potential to guide dose regulation of mTOR inhibitors to optimize antiangiogenic application of rapamycin derivatives. However, the relationship between tumor vascular density and CEPs necessitates further studies.

HIF-1α was found to be heterogeneously distributed and tumors in all therapy groups were focally positive for HIF-1α.

Additionally, intra-group variability of HIF-1α expression was high, a finding which also has been previously reported for HIF-1α immunostaining of surgical specimens in clinical studies (36). Accordingly, differences in HIF-1α expression between treatment groups did not reach statistical significance. However, mean HIF-1α expression levels in everolimus-treated tumors were lowered by 35%, supporting the hypothesis that everolimus may act as an HIF-1α-lowering agent *in vivo*.

Despite the promising synergistic antitumor activity of everolimus and cyclophosphamide, the combination of everolimus at MED with cyclophosphamide at MTD was found to be toxic in the SCID mouse model studied. This definitely raises questions about the feasibility of clinical use of this combination regimen in patients. In potential future clinical trials of combination regimens with everolimus and cyclophosphamide, toxicity should be closely monitored. However, given the poor prognosis of gastric cancer patients at advanced stage of disease, experimental treatment concepts with molecular targeting compounds and chemotherapy may provide a possibility to overcome treatment resistance of gastric cancer.

In conclusion, our data suggest that potent antitumor effects may be achieved in gastric cancer using a combination of mTOR inhibition with the alkylating chemotherapeutic agent cyclophosphamide.

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