Moguntinones – new selective inhibitors for the treatment of human colorectal cancer

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Abstract

3-Indolyl and 3-Azaindolyl-4-Aryl Maleimide derivatives, called Moguntinones (MOGs), have been selected for their ability to inhibit protein kinases associated to angiogenesis and induce apoptosis. Here we characterize their mode of action and their potential clinical value in human colorectal cancer \textit{in vitro} and \textit{in vivo}.

MOG 19 and MOG 13 were characterized \textit{in vitro} using kinase, viability and apoptosis assays in different human colon cancer (HT-29, HCT-116, Caco-2, SW480) and normal colon cell lines (CCD-18Co, FHC, HCoEpiC) alone or in combination with topoisomerase I inhibitors. Intracellular signaling pathways were analyzed by Western blot. To determine their potential to inhibit tumor growth \textit{in vivo}, human HT-29 tumor xenograft model was used.

Moguntinones prominently inhibit several protein kinases associated with tumor growth and metastasis. Specific signaling pathways such as GSK3β and mTOR downstream targets were inhibited with IC$_{50}$ values in nanomolar range. GSK3β signaling inhibition was independent of KRAS, BRAF and PI3KCA mutation status. While MOGs alone induced apoptosis only in concentrations >10µM, MOG 19 combination with topoisomerase I inhibitors induced apoptosis synergistically at lower concentrations. Consistent with \textit{in vitro} data, MOG 19 significantly reduced tumor volume and weight in combination with a topoisomerase I inhibitor \textit{in vivo}.

Our \textit{in vitro} and \textit{in vivo} data present significant pro-apoptotic, anti-angiogenic and anti-proliferative effects of MOG 19 in different human colon cancer cells. Combination with clinically relevant topoisomerase I inhibitors \textit{in vitro} and xenograft mouse model demonstrate a high potency of Moguntinones to complement and improve standard chemotherapy options in human colorectal cancer.
Introduction

Targeting of tyrosine kinases in angiogenesis and other cellular signaling pathways has shown effective in advanced or metastatic human cancer (1). Inhibition of VEGFR1-3 and its ligands in the angiogenic pathway by different classes of inhibitors is an integral part of the clinical armamentarium (2). Vascular endothelial growth factors (VEGFs) induce angiogenesis by binding to its cognate receptors VEGFR1-3. Intracellular signal transduction results in endothelial cell proliferation, migration and new vessel formation (3).

Recently, we have developed and characterized a novel class of selective VEGFR-2/3 inhibitors (4-6). These novel indolyl- and azaindolyl-aryl-maleimides, called Moguntinones, are synthetically designed small molecules, including structural features of 3 natural compounds (combretastatin, rebeccamycin, staurosporin). They showed inhibition of the protein kinases VEGFR-2 and -3, PDGFRβ, FLT3 and GSK3β, anti-angiogenic efficacy and anti-proliferative activity. In addition they induce apoptosis in FLT3-ITD dependent AML cell lines and patient blasts at low micromolar concentrations (7). While key function of VEGF and PDGF receptors may be clearly attributed to angiogenesis, GSK3β regulates proliferation in a cell type specific manner. One of the important functions of GSK3β is the regulation of transcription factor β-catenin. Phosphorylation of β-catenin by a GSK3β protein complex leads to its ubiquitination and proteosomal degradation thereby abolishing its active role in cell proliferation. GSK3β can be inactivated by protein Disheveled if the Wnt signaling pathway is activated resulting in cell proliferation (8). But until now the role of GSK3β inhibition (9) is still controversial in a multiplicity of indications and under investigation (10, 11). FLT3, also known as CD135, binds FLT3 ligands and in turn activates signal transduction molecules regulating cell survival and proliferation in a cell type specific manner. While function of FLT3 in colorectal cancer remains unclear clinical interest for treatment is based on its immunological function (12). Injection of FLT-ligands was demonstrated to mobilize dendritic cells resulting in anti-tumor activity of the immune system in patients with colon cancer.
We have selected MOGs for their potential to effectively serve a dual role: inhibition of VEGFR-2/3 as well as cell signaling downstream of GSK3β. Based on our pre-selection process of more than a hundred substances (4-6), two MOGs (MOG 13 and MOG 19), were chosen for further characterization in vitro and in vivo with respect to their potential to treat human colorectal cancer.

Other tested small molecules tyrosine kinase inhibitors to VEGFR and PDGFR like Sunitinib or Sorafenib, though being effective in some indications, cause side effects as hand-foot syndrome and general fatigue (13). Such side effects were also observed with for instance the novel multikinase inhibitor Regorafinib (VEGFR, TIE-2) in colorectal cancer (14-16).

Especially in this indication effective treatment of metastatic disease has also been shown by blockage of intracellular signalling pathways promoting cell growth e. g. by inhibiting EGFR, KRAS and PI3K/mTOR dependent signalling cascades (17). However, EGFR blocking by monoclonal antibody e. g. Cetuximab or Panitumumab is limited in clinical use for KRAS wild type patients and also by severe side effects (18). The third approved targeted therapy for colorectal cancer is Bevacizumab, a VEGF binding antibody. Recently, continuation of Bevacizumab after first progression plus standard second-line chemotherapy proved the clinical efficiency of continuous angiogenesis inhibition (19). With respect to the work presented here it is of special interest that several studies on combination of chemotherapy and different TKI therapy have confirmed a lack of efficacy or increased toxicity. Taken together this demonstrates the need of novel TKI inhibitors with different specificity and the possibility of combination with chemotherapy (20).

In this manuscript we describe the effects of MOGs on cell signaling and apoptosis in different human colon cancer cell lines versus normal human epithelial cells, as well as in vivo significant anti-angiogenic properties in subcutaneously grown human colon tumors. For summary of key features see Figure 1 and Table 1.
Material and Methods

Cell culture and authentication of cell lines

The human colorectal cancer cell lines HT-29, HCT-116, Caco-2 and SW480 were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany, in 2006-2009). Caco-2 was cultured in McCoy’s 5A medium (Invitrogen, Karlsruhe, Germany) supplemented with 20% heat inactivated fetal calf serum (FCS; PAA, Cölbe, Germany), 100 units/mL penicillin, and 100 µg/mL streptomycin (1%, Cambrex, Germany). The other cell lines were cultured in RPMI 1640 (Invitrogen, Germany) supplemented with 10% FCS and 1% penicillin/streptomycin. The normal human epithelial colon cell lines FHC and HCoEpiC were obtained from ATCC (CRL-1831) and ScienCell Research Laboratories (2950). FHC was cultured in DMEM:F12 Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 mM HEPES, 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone, 10% FCS and 1% penicillin/streptomycin. HCoEpiC was cultured in Colonic Epithelial Cell Medium (CECM, Cat. No. 2951) and 1% penicillin/streptomycin. The human fibroblast cell line CCD-18Co was obtained from ATCC (CRL-1459) and cultured in EMEM (Invitrogen, Karlsruhe, Germany), 10% FCS and 1% penicillin/streptomycin.

Cultures were maintained in a 5% CO₂ humidified incubator at 37°C. All experiments were performed with exponentially growing cells. IdentiCell, Denmark, confirmed by short tandem repeat analysis the authenticity of the cell lines in 2011. The four cell lines were free of any cross-contamination.

Characterization of putative targets (VEGFR2/3, PDGFRβ, FLT3 and GSK3β) in these cell lines were done by RT-PCR as well as FACS (21) and mutational analyses were realized by high resolution melting PCR as described earlier (22).

Inhibitors

3,4-Diarylmaleimides (MOG19 and 13) were synthesized at the Department of Pharmacy and Biochemistry (4-6) (Johannes-Gutenberg-University of Mainz). They were dissolved and diluted in DMSO (Sigma, Munich, Germany) to a stock concentration of 10 mM and stored at
-80°C. The chemotherapeutic agents 5FU, Oxaliplatin, Irinotecan and Topotecan were
diluted in water by the Pharmacy of the University Medical Centre. Vandetanib and Sunitinib
were obtained from LC Laboratories, USA. Untreated control cells were incubated with the
equivalent doses of DMSO in all experiments, to ensure that there were no possible side
effects of DMSO.

**In vitro cytotoxicity and apoptosis assay**
The MTT assay was used to measure the effects of the test drugs on cancer tumor cell
proliferation (22). The cells were plated in 96 well plates in different densities, depending on
their doubling time, to enable log linear growth for 72 h. Cells were allowed to attach
overnight before drugs were added. MOGs were added in final concentrations ranging
between 0.01 and 20 µM. Growth inhibition was assessed as inhibitory concentration 50%
(IC50) after 72 h drug exposure to the cells compared with vehicle-treated controls and
relative to cell growth at the time of drug addition. Each experiment was performed in
triplicate and three independent experiments were performed.

Apoptosis detection was done by nuclear staining with propidium iodide (PI) (22) to measure
the sub G1 cells. Cells were incubated with MOG 19 or MOG 13 at the required dose alone
and in combination with cytostatic drugs such as Irinotecan (0.8 µg/mL) and Topotecan
(20 nM) for seven days. Stained cells were measured by flow cytometric analysis (FACS) by
a BD FACS Calibure. Each experiment was performed in triplicate.

**Caspase assay**
Assays were performed in white 96 well microtitre plates (Nunc, Germany) with the Caspase-
Glo® 3/7 Assay of Promega, Germany; according to the manufacturer’s protocol. Cells were
treated for 6 h with placebo, MOG 19 (5 or 8 µM) or Irinotecan (0.8 µg/mL) as well as in
combination of each other. Caspase 3/7 levels were detected by luminescence using a plate
reader (Appliscan, Thermo Fisher, Germany). Analyses were performed in triplicate.

**Western Blot analysis**
2 x 10^6 HT-29, HCT-116, SW480 and Caco-2 cells were harvested after 4 h exposure to placebo, MOG 19 and MOG 13 at different concentrations between 1 and 30 µM. Cells were washed twice with PBS and lysed in 2xRIPA solution. The xenograft materials were unfreezed on ice, covered with 2x RIPA buffer supplemented with Protease Inhibitor (Roche, USA) and lysed with Precellyse ceramic beads 2.8 (Peqlab, Germany). For Western blot analysis, 60 µg of protein were loaded on 10% SDS-PAGE gels. After separation, the gel was transferred to nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany). Proteins were detected with specific primary mouse and rabbit antibodies (all Cell Signaling, USA, used in indicated concentrations, 4°C, overnight) in 5% BSA. The specific secondary antibodies (anti-rabbit, anti-mouse from Santa Cruz, USA) diluted 1:2000 in 5% non-fat dried milk were exposed for 1 h at room temperature. The ECL chemiluminescence detection kit (Perkin Elmer, Waltham, USA) was used for visualization.

**Animals**

Female NOD/SCID gc -/- mice were purchased from the central animal facility (ZVTE, University of Mainz, Germany). The mice were maintained in a laminar airflow cabinet under pathogen-free conditions and used at 7–10 weeks of age. Mice were housed in micro-isolator cages with free access to laboratory chow and tap water. All animal experiments were performed in accordance with the UKCCCR regulations for the Welfare of Animals (23) and of the German Animal protection Law and approved by the local responsible authorities.

**Treatment of subcutaneous human colorectal carcinoma xenografts**

HT-29 colon carcinoma tumors were established as xenografts by injecting 1 x 10^7 cells, mixed in PBS medium (1:1), subcutaneously into the left flank of female NOD/SCID gc mice. Ten days after cell injection, all mice bore a tumor with a minimum diameter of 4 mm. At this time the mice were randomized into four groups of five animals and treated with either saline, MOG 19 at 25 mg/kg or 50 mg/kg three times a week, Irinotecan at 25 mg/kg twice a week or both in combination (MOG 19 25 mg/kg and Irinotecan 25 mg/kg). All substances were administered intraperitoneally at a volume of 0.2 mL/injection. The dose and schedule of
MOG 19 and Irinotecan were based on data from previous studies. The health of the treated animals was checked daily. Body weight and tumor diameters were measured two times weekly with a caliper. Tumor volumes were calculated according to $V = \frac{\pi}{6} \times \text{shorter diameter}^2 \times \text{longer diameter}$. At each measurement day the mean tumor volumes were calculated. After 36 days of treatment, mice were sacrificed by cervical dislocation, inspected for organ changes and tumors were dissected and weighted.

**RNA isolation and quantitative RT-PCR**

RNA isolation was performed using the RNeasy Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany) from the ectopic HT-29 xenograft tumors (24). Transcription of the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH), VEGF-A, CXCR4, Hif1α and EpCAM were analyzed by a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) using a LightCycler 480 system (Roche). RT-PCR was performed with 0.1 µg of RNA in a 35 cycle reaction (20 µL total volume; QuantiTect SYBRGreen RT-PCR, Qiagen) according to the recommendations of the manufacturer. All RT-PCR reactions were done in 2 replicates. All PCRs were established with an exponential phase efficiency of 2 to guarantee that the data were comparable. The evaluation of the expression of the target genes was performed relative to the expression of GAPDH. Control and test samples of the xenograft experiments were analyzed with the software of the LC480 based on the $\Delta\Delta$Ct approach. RT-PCR primers and used programs for all targets could be requested by the authors.

**Immunohistochemical staining**

Frozen tissue samples of the HT-29 xenografts were cut and immunohistochemically stained for Ki67 (DAKO, Germany, 1:100), CD31 (abcam, UK, 1:200, (25)), LYVE-1 (abcam, UK, 1:100) and VEGF-A (Santa Cruz, USA, 1:100). The acetone fixed tissues were peroxidase blocked in 3% H$_2$O$_2$ in methanol for 10 min, blocked with 5% BSA and subsequently incubated with the respective primary antibody overnight. The secondary antibody (anti-rabbit-mouse-goat-antibody) was incubated for 20 min at room temperature, followed by
incubation with strepavidin-POD (DAKO, Germany) for 20 min. Antibody binding was visualized using DAB-solution (DAKO, Germany). Finally, the tissues were counterstained by haemalaun solution (DAKO, Germany).

**Statistical analysis**

Significant effects between treatment groups or between treatment groups and control were accomplished by using the two-sample Student’s t-test using SPSS statistical analysis software. For more than two independent samples, the total significance level $a = 0.05$ was Bonferroni adjusted for each pairwise test. Results are expressed as mean standard deviation (SD). $p$ values $< 0.05$ were considered to indicate significant differences.
Results and discussion

Out of over a hundred different derivatives MOG 13 and 19 were selected as potent inhibitors of vessel growth in HET-CAM assay as well as having sub-nanomolar IC$_{50}$ values for the inhibition of VEGFR-2/-3, PDGFR$\beta$ and FLT3 in a kinase screen (see Figure 1 and Table 1, Ref. (5) and (4) for details). MOG 19 in comparison to MOG 13 is also able to inhibit the intracellular signaling by GSK3$\beta$ with an IC$_{50}$ value of 71 nM. In this investigation the in vitro and in vivo antitumor activity of MOG 13 and 19 were investigated as well as their effects on signal transduction pathways in four human colorectal cancer cell lines: Caco-2, HT-29, HCT-116, SW480 and the normal human colon cell lines FHC, CCD-18Co and HCoEpiC.

First, the presence of putative angiogenesis targets (VEGFR2/3, PDGFR$\beta$, FLT3) in these colorectal cancer cell lines was determined by RT-PCR (supplementary Figure 1 A). VEGFR-2 was only weak expressed in HCT-116 and detectable in HT-29 and SW480 cells. VEGFR-3 was expressed in all investigated cell lines. PDGFR$\beta$ was expressed in all but HCT-116 cells. The translation into the respective protein was measured (data not shown) and corresponded with the RNA expression. While strong RNA expression of FLT3 was detected in Caco-2, HCT-116 and SW480, no receptor protein was verifiable. Taken together these selected human cell lines display a differential expression of tyrosine kinase receptors involved in angiogenesis mediation and exhibit putative targets of Moguntinones.

To demonstrate MOG action downstream of key players KRAS, BRAF and PIK3CA in colon cancer, the mutational background of human KRAS (exons 12 and 13), BRAF (V600E) and PIK3CA (exons 9 and 20) for the cell lines was examined. Caco-2 did not show hot spot mutations in clinical relevant amino acids of any of the molecules, while we detected BRAF and PI3K hot spot mutations in HT-29, KRAS and PI3K mutations in HCT-116 and a KRAS mutation in SW480 (supplementary Figure 1 B, for a more detailed mutation status see http://www.sanger.ac.uk/genetics/CGP). The diversity of mutational hits in KRAS and PI3K signaling cascades in the colorectal cancer cell lines selected represents a reasonable basis for the analysis of MOG downstream activity.
The cytotoxic activity and apoptosis induction of MOG 19 and 13 was assayed using MTT and propidium iodide assays (see Fig.2 and supplementary Fig. 2 C). MOG 19 did not show cytotoxic activity in the dose range tested up to 20 µM after three days drug exposure in all cancer and two normal human colon cell lines. Only the human epithelial cell line HCoEpiC indicated a concentration-dependent reduction of viability starting up 1 µM. In contrast MOG 13 showed a concentration-dependent cytotoxicity, which was also cell line dependent. SW480 was the most sensitive with a reduction of viable tumor cells to 40% at 1 µM MOG 13 compared to the vehicle control (Fig. 2, IC₅₀: SW480 0.8 µM, HCT-116 14.7 µM, HT-29 28.3 µM, HCoEpiC 9 µM, FHC, CCD-18Co and Caco-2 not reached IC₅₀ up to 30µM). Interestingly, MOG 13 had no cytotoxic activity on the non-mutated KRAS, BRAF and PIK3CA Caco-2 cell line. MOGs alone induced apoptosis only in higher concentrations (>10 µM) or after a longer incubation period of >5 days. In spite of the very low IC₅₀ values of the kinase scan only partial reduction in viability or induction of apoptosis could be detected in these colorectal cancer and normal human cell lines. The same MOGs were, however, able to induce significant cytotoxicity and apoptosis in nM range in a FLT3-ITD mutated AML leukemia cell model and in low µM range in human AML blasts (7).

Moreover, we investigated in vitro the effect of MOGs in combination with cytotoxic drugs approved for treatment of colorectal cancer. No additional effect was observed when MOGs were combined with 5-FU and Oxaliplatin. Furthermore, no supportive effect was detected when cancer and normal human cells were treated with MOG 13 in combination with Irinotecan or Topotecan (data not shown). However, MOG 19 significantly raised the fraction of cells in sub G1 phase in the four colorectal cancer cell lines in the presence of 0.8 µg/mL Irinotecan or 20 nM Topotecan (Figure 3 A and B, supplementary Fig. 3). The most impressive effect was observed in HT-29 cells. MOG 19 induced a 6- and 4-fold elevated apoptosis level in combination with topoisomerase I inhibitors Topotecan or Irinotecan. The induced apoptosis was synergistically, means the combination clearly exceed the monotherapy with the topoisomerase I inhibitor or MOG 19 as well as the addition of these single treatments. This synergistic induction of apoptosis was not achieved in normal human...
colon cells (Figure 3 C). MOG 19 induced apoptosis in the human fibroblast and epithelial cell lines in only 25-35% of the cells after seven days of treatment. The combination with Irinotecan again showed only minimal apoptosis inducing effects in these cells.

Moguntinones showed especially during the first days of treatment a typical G1 arrest (supplementary Fig. 2 A) which is associated with a proliferation inhibition (4). In addition to the G2 shift caused by the topoisomerase I inhibitors the cells balanced between the cell cycle phases and consequently induced apoptosis. Strikingly MOG 19 in combination with Topoisomerase I did not induce apoptosis in HUVEC and normal human colon cells, indicating that this combination is predominantly effective in oncogenic cells (4) and reduces the potential of adverse events.

MOG 19 showed comparable suppressive effects independent of the KRAS, BRAF and PI3KCA mutation panel of the different human colorectal cancer cells. Having in mind that these mutations may lead to the loss of important therapeutic options in the treatment of metastatic colorectal cancer this clearly points towards a promising treatment strategy (26).

In contrast to the synergistic effect of MOG 19 with the two topoisomerase I inhibitors, combination treatment of Irinotecan with the tyrosine kinase inhibitors Vandetanib or Sunitinib induced less apoptotic cells (Figure 3 D). These benchmarking experiments favor the treatment with MOG 19 as combination and improvement of standard chemotherapy.

Using identical concentrations we next investigated whether this effect was caspase 3/7 mediated in HT-29 cells. As shown in Figure 3 E, MOG 19 increased apoptosis via a doubling of caspase 3/7 in combination with Irinotecan only. In this setting we suspect that checkpoint kinases (Chk) could be further possible direct or indirect targets of Moguntinones.

Similar effects could be seen in a breast cancer model where the combination therapy of Irinotecan and a Chk1 inhibitor induced checkpoint bypass and as consequence apoptosis in some cell lines. In this connection cells with specific mutational background respond to this combination therapies that combine DNA damage with Chk1 inhibition (27).

As MOGs used in this work were selected for their ability to act downstream of key players in cell signaling known to be highly mutated in colorectal cancer we next examined the
intracellular signaling pathways using Western blot analysis. Both MOGs reduce phosphorylated GSK3β in all human colon cancer cell lines in a concentration dependent manner (Figure 4). In line with the fact that phosphorylated GSK3β is inactive and unable to target β-catenin to proteasomal degradation an increase of β-catenin was observed in HT-29, HCT-116 as well as Caco-2 cells. MOG 13 only weak reduced the phosphorylation of GSK3β in SW480. Overall, this clearly indicates targeting of GSK3β pathway by MOGs. Thereby β-catenin is only one possible indirect target of the Moguntinones by GSK3β. To target the Wnt pathway is one therapy objective not only for colon cancer (28). Further functions of this complex modulator could be found in regulation of inflammation (29) and cyclins for cell cycle (30). Their activation resulted in the observed G1 arrest and in the combination treatment the induced apoptosis. Other GSK3β inhibitors e. g. Luteolin leads also to de-phosphorylation and activation of GSK3β (31). The activated protein then targeted cyclin D1 and induces G1 arrest. GSK3β, as a multi-player in cell metabolism, represents a promising therapeutic target. It has been shown to play an important role in the determination of cyclin D1 expression level by regulating mRNA transcription and protein degradation (30). In lung cancer, the modulation of GSK3β/β-catenin pathway inhibited angiogenesis and proliferation (32). Also in HUVEC, the inhibition of Akt/GSK3β and mTOR signaling led to a suppression of angiogenesis in vitro and in vivo (33). Furthermore in silencing experiments, Zhou et al. demonstrated that the elimination of GSK3β inhibited tumor growth and angiogenesis in a pancreatic cancer model (34). Concordantly, we demonstrated that both MOGs reduce the mTOR signaling in HT-29 (Figure 4). This cell line reacted most sensitive to the treatment with MOGs in all in vitro analyses. Therefore we used it in HT-29 tumor xenografts.

To check the suitability for clinical applications in vivo we analyzed the effect of MOGs on vessel formation, tumor volume and tumor weight of female NOD/SCID gc -/- mice. Mice were treated with either saline, MOG 19, Irinotecan or both in combination (Figure 5). Development of HT-29 tumors with respect to tumor volume measured was reduced to 74% (524 mm³) using Irinotecan after 36 days of treatment in comparison to control group (725
mm$^3$). However, further inhibition could be obtained by adding MOG 19 resulting in additional 30% (312 mm$^3$) reduction of tumor volume (Figure 5 A). After 36 days of treatment the weight of isolated tumors was compared. Treatment with MOG 19 (1.3 g) alone caused tumor masses comparable to control (1.25 g), while treatment with Irinotecan resulted in a reduction to 64% (0.8 g). The combination of Irinotecan and MOG 19 resulted in drastic reduction of the tumor mass to 15% (0.18 g) compared to controls (Figure 5 B). None of the treated animals showed pathological changes to internal organs such as the lung, liver or spleen. MOG 19 was well tolerated in mice, no significant weight loss, diarrhea or abnormal deaths were observed. MOG 19 alone showed in this concentration no effect, but after doubling to 50 mg/kg (Figure 5 C) the tumor volume was reduced to a third of the control group. In this setting MOG 19 is similar to the new oral multikinase inhibitor Regorafenib (35). This small molecule exhibits comparable MOG targets (36) and is the first multikinase inhibitor with survival benefits for metastatic colorectal cancer that will be approved by the FDA (37) after a positive monotherapy phase III study (CORRECT) (16). The used MOG concentration is similar to other TKIs such as Regorafenib in human colon cancer (38). On the contrary, MOG 19 may be used in a more non-toxic concentration of 25mg/kg to support the chemotherapy and make it more effective.

To further characterize MOG properties on the tumor environment in vivo, RNA and protein levels of proliferation and angiogenesis regulating proteins were measured. The mRNA levels of angiogenesis-associated proteins were significantly reduced in the combination group of Irinotecan and MOG 19 (Figure 6 A). Hif1$\alpha$, which is activated under hypoxic conditions, was halved in the MOG 19 group compared to the control and Irinotecan treated animals. The combination of a cytotoxic agent and the MOG 19 led to further reduction in Hif1$\alpha$ mRNA levels. VEGF-A, the ligand of VEGFR-1, also inducing vessel angiogenesis, was reduced for ~40% only in the combination whereas the other control groups were unaffected. mRNA of the cancer associated antigen EpCAM and the chemokine receptor CXCR4 showed a similar reduction in the double treated animals in comparison to the other control groups. The additional inhibition of the chemokine receptor CXCR4 support the anti-
tumoral and anti-angiogenic properties of MOGs as CXCR4 and CXCR7 regulate angiogenesis (39). Again, expression of EpCAM, just like CXCR4, is linked to the frequency of metastasis and tumor progression (40). Thus, the reduction of these characterized pathways clearly demonstrates the high activity of combinations of Moguntinones with chemotherapeutic agents against tumor growth and progression, particularly in human colorectal cancer. The Western blot analyses of the same targets are in accordance with the mRNA data (Figure 6 B). MOG 19 as GSK3β inhibitor in vitro again reduced the phosphorylation of GSK3β in vivo, especially in combination with Irinotecan. As a consequence, an associated accumulation of β-Catenin was confirmed in the same tumors. Double treatment of Irinotecan and MOG 19 resulted in protein level reductions of Hif1α, VEGF-A, EpCAM and CXCR4. Hif1α, induced by Irinotecan was considerably reduced by MOG 19. Concordantly, this kind of Hif1α reduction was also demonstrated under treatment with Irinotecan and Rapamycin in colon cancer (41). Even more, the reduction of VEGF-A levels again reflects the anti-angiogenic potential of MOGs, which was also seen in mRNA analyses and in immunohistochemical stainings.

As β-Catenin was shown to be an inducer of EpCAM in colon (42) and hepatocellular carcinoma (43), EpCAM activation may have been promoted in the MOG19 group by increasing β-Catenin levels induced by MOG alone. This effect was abrogated by the addition of Irinotecan combined with MOG19.

Comparison of vessel formation of isolated tumors by CD31 and LYVE-1 staining showed a visible decrease in large vessel formation in the double treated group compared to MOG 19 alone and, to a lesser extent, with Irinotecan alone (Figure 6 C). Again, a reduced smaller vessel density in the combination group was observed. Furthermore, in this group Ki67, a well-established proliferation marker, was reduced and not found in the tumor cell nuclei. In contrast, the active form of Ki67 could be measured in a higher intensity, in the tumor cell nuclei in the control and monotherapy groups. In addition VEGF-A mRNA-levels were reduced and clearly weaker expressed in MOG 19/Irinotecan animals than in the single controls.
Taken together in vivo experiments demonstrate that MOG 19 significantly reduced tumor volume and weight as well as vessel density (CD31, LYVE-1) in combination with Irinotecan. Concordantly, angiogenesis associated proteins like VEGF-A or Hif1α were clearly reduced in the combination group. These changes in vessel formation are most likely attributed to the composition of the MOGs, as MOGs include structural features of 3 natural anti-angiogenic and anti-proliferative compounds. One backbone of the MOGs is combretastatin, which binds the colchicine site of tubulin and prevents the formation of microtubules. It has also shown to induce vascular disruption in tumors (44). Furthermore, combretastatin A-4 and other derivates indicated typical anti-tumor effects by irreversible stoppage of tumor tissue blood flow for tumors growing in various tissues, organs and for metastases (45-47). Compared to combretastatin the MOGs lost the effect of tubulin depolymerisation (6).

Thus, these in vitro and in vivo experiments argue for a high potency of this new class of compounds complementing the current therapeutic options in human colorectal cancer. Currently, our focus is to further characterize the pharmacodynamic and pharmacokinetic aspects in larger animal species to clearly establish the scientific role of Moguntinones for an upcoming clinical phase I trial.

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<tr>
<th>Target</th>
<th>Moguntinon 19</th>
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**signaling cascade**

| GSK3β           | 71            | >10,000       |

**Table 1. IC$_{50}$ values of MOG 19 and 13**

IC$_{50}$ values of MOG 19 and 13 were carried out at Millipore (http://www.millipore.com/life_sciences/flx4/lk_inhases) by a kinase assay (in nM). MOG 19 is known as a potent VEGFR-2/3 inhibitor (IC$_{50}$: 70/5 nM) as well as FLT3 and GSK3β inhibitor and a moderate PDGFRβ inhibitor (IC$_{50}$: 680 nM). With the exception of GSK3β the same is true for MOG 13.
Figure Legends

Figure 1. Characterization of Moguntinon 19 and 13.
A, chemical structures of MOG19 and MOG 13. B, HET-CAM assay showed a significant inhibition of vessel growth for MOG 19 and 13 in comparison to the control.

Figure 2. Effects of Moguntinon 19 and 13 on cell viability
A, MOG 19 did not reduce cell viability of human colon cancer cells (HT-29, SW480, HCT-116, Caco-2) after 3 days of treatment, B, In the normal human colon epithelial cell line (FHC) as well as in the human fibroblast colon cell line (CCD-18Co) MOG19 did not influence cell viability. The second normal human epithelial colon cell line HCoEpiC showed a concentration-dependent reduction of cell viability. C, With the exception of Caco-2, MOG 13 decreased concentration-dependently viability in all cancer cell lines during the same incubation period. D, MOG13 also induced a concentration-dependent decrease of viability in the normal epithelial and fibroblast colon cell line (CCD-18Co< FHC<HCoEpiC).

Figure 3. Apoptosis and caspase 3/7 induction by combination Moguntinon 19 with topoisomerase inhibitors Irinotecan or Topotecan as well as benchmarking experiments with Sunitinib and Vandetanib
Cells were treated with 8 µM of MOG 19 and combined with 0.8 µg/mL Irinotecan (Iri) or 20 nM Topotecan (Topo) for seven days. Sub-G1 phase was detected by flow cytometry.
A, combination of Irinotecan with MOG 19 led to a synergistic induction of apoptosis in all human colon cancer cells. B, treatment of Topotecan combined with MOG 19 also intensified synergistic pro-apoptotic effects in all cancer cells. C, MOG 19 (8µM) induced apoptosis in 25-35% of normal human colon cells. The combination of Irinotecan with MOG 19 led to no additional induction of apoptosis. D, combination treatment of Irinotecan with Vandetanib or Sunitinib in comparison to treatment with MOG 19 induced less apoptotic cells. The MOG showed to be more effective in adding to standard chemotherapy. E, HT-29 cells were incubated with MOG 19 (5 or 8 µM) and combined with 0.8 µg/mL Irinotecan for one day. Caspase 3/7 levels were detected by luminescence assay. Combinations of Irinotecan with MOG 19 led to a significant induction of caspase 3/7.
Results are displayed as mean with standard deviations of three independent experiments. Statistically significance (p<0.05) is displayed with p=* . The synergistic effect could not show on HUVEC cells (normal tissue). MOG 13 induced no synergistic effects.
**Figure 4. Western blot analysis** of different human colon carcinoma cells were treated with increasing concentrations of MOG 19 and 13 for 4 hours. Western blot of total cell lysates shows phosphorylated and total GSK3β, β-Catenin, phosphorylated 4EBP1 and tubulin respectively. Inhibition of GSK3β led to an increase of β-Catenin.

**Figure 5. In vivo effects of Moguntinon 19 against subcutaneously grown human colon tumors.**

HT-29 tumor-bearing animals were treated with either MOG 19 alone or in combination with Irinotecan. After sacrifice of the animals, tumors were removed and analyzed as described. **A**, antitumour activity of MOG 19, Irinotecan and their combination in HT29. The maximum shrinkage of volume could be detected in the combination of MOG 19 and Irinotecan compared to monotherapy. **B**, tumor weight was measured at the end of the experiment. The lowest mean weight was found in the combination arm. **C**, application of MOG 19 in two different concentrations. 25 mg/kg showed only marginal tumor volume changes in comparison to control group, instead of 50 mg/kg which induced shrinkage up to third. Statistically significance (p<0.05) is displayed with p=*.

**Figure 6. RNA and protein analyses of angiogenic markers in HT-29 xenografts mice.**

**A**, Quantitative Hif1α, CXCR4, VEGF-A and EpCAM mRNA expression analysis of HT-29 xenografts. After expression levels were correlated with GAPDH, a reduction is seen particularly by the combination of Irinotecan and MOG 19. **B**, Western blot analysis of β-Catenin, pGSK3β, GSK3β, Hif1α, CXCR4, VEGF-A, EpCAM and Tubulin of subcutaneously grown HT-29 tumor xenografts. In the combination of MOG19 and Irinotecan, a decrease in protein levels of pGSK3β, Hif1α, CXCR4, VEGF-A and EpCAM were detected. **C**, Analysis of vessel formation (CD31, LYVE-1), angiogenic ligand (VEGF-A) and proliferation (Ki67) were done by IHC with subcutaneously grown tumor. IHC stainings were done on control (upper line), Irinotecan treated (second line), MOG 19 treated animals (third line) and on the combination of Irinotecan and MOG 19 group (lower line). (200x magnification)

The combined treatment with Irinotecan and MOG 19 showed a clear decrease in all presented markers compared to the controls and single agents.
Figure 1.

A

Moguntinon 19  Moguntinon 13

B

control  MOG 19 (82%)  MOG 13 (61%)
Figure 2.
Figure 3.
Figure 4.

<table>
<thead>
<tr>
<th>MOG 19 in µM</th>
<th>MOG 13 in µM</th>
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<tr>
<td>0 1 5 10 20 30</td>
<td>0 1 5 10 20 30</td>
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**HT-29**
- β-Catenin
- pGSK3β
- GSK3β
- p4EBP1
- Tubulin

**Caco-2**
- β-Catenin
- pGSK3β
- GSK3β
- p4EBP1
- Tubulin

**Hct-116**
- β-Catenin
- pGSK3β
- GSK3β
- p4EBP1
- Tubulin

**SW 480**
- β-Catenin
- pGSK3β
- GSK3β
- p4EBP1
- Tubulin
Figure 5.
Figure 6.

(A) Relative expression of Hif1α, CXCR4, VEGF-A, and EpCAM in the IRI and MOG19 conditions compared to control (untreated). Error bars represent standard deviation.

(B) Western blot analysis for β-Catenin, pGSK3β, GSK3β, Hif1α, CXCR4, VEGF-A, EpCAM, and Tubulin in the control, Iriotecan, MOG19, and Iriotecan+MOG19 conditions.

(C) Immunohistochemical staining for Ki67, CD31, LYVE 1, and VEGF-A in the untreated (untr.), Iriotecan, MOG19, and IRI+MOG19 conditions.
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