Marked Activity of Irinotecan and Rapamycin Combination toward Colon Cancer Cells In vivo and In vitro Is Mediated through Cooperative Modulation of the Mammalian Target of Rapamycin/Hypoxia-Inducible Factor-1α Axis

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Abstract

Purpose: Despite recent progress, colon cancer is often resistant to combination chemotherapy, highlighting the need for development of novel therapeutic approaches. An attractive target is hypoxia-inducible factor-1α (HIF-1α), a key transcription factor with a pivotal role in tumor cell metabolism. One potential class of therapeutic agents targeting HIF-1α are mammalian target of rapamycin inhibitors such as rapamycin. A second class are topoisomerase I inhibitors, such as irinotecan, which are able to inhibit the accumulation of HIF-1α. We here investigated whether combination of rapamycin and irinotecan was active in human colon cancer models.

Experimental Design: Human metastatic tumors were xenografted in nude mice and treated with low doses of irinotecan alone, rapamycin alone, or combination of both drugs. The cellular effects of irinotecan and rapamycin were further characterized for HT-29 and HCT-116 colon cancer cells in vitro.

Results: In contrast to single-agent therapy, xenografted tumors treated with combination of irinotecan and rapamycin showed potent inhibition of the mammalian target of rapamycin/HIF-1α axis, which was accompanied by a dramatic reduction in tumor volume. In vitro experiments showed that exposure to low concentrations of the two drugs resulted in massive HT-29 cell death under hypoxic, but not normoxic, conditions, in full agreement with a cytotoxic effect mediated through HIF-1α rather than through induction of genotoxic lesions. HCT-116 cells were less sensitive to the combined treatment due to constitutive activation of phosphatidylinositol 3-kinase/Akt and Ras/mitogen-activated protein kinase pathways.

Conclusion: These results identify HIF-1α as a promising target and provide a rationale for clinical trials of low-dose irinotecan and rapamycin combination toward metastatic colon cancer.

A glycolytic shift and high levels of lactate and pyruvate are hallmarks of most cancer cells, and this phenomenon, first described more than half a century ago, is known as the Warburg effect (1). The discovery of the hypoxia-inducible factor-1α (HIF-1α) transcription factor and the finding that it directly controls the transcription of nearly every enzyme needed for glycolysis provided an alternative approach to cancer treatment (2). Indeed, targeting HIF-1α represents a unique mechanism for cancer therapy because tumor cell survival depends on the ability of the glycolytic pathway to produce sufficient ATP. Moreover, increased HIF-1α levels are frequently observed in human tumors due to a variety of mechanisms including (a) oncogene signaling (3), (b) loss of VHL and/or increased hypoxia (4, 5), (c) hypoxia (6), and (d) increased reactive oxygen species generation (7). The mTOR pathway is a major positive regulator of HIF-1α expression (8), and rapamycin, a well-tolerated macrocyclic lactone, is a potent mTOR inhibitor/immunosuppressive agent with activity toward a wide variety of tumors (9–12). Both in vitro and in vivo studies have shown the ability of mTOR inhibitors to prevent angiogenesis through inhibition of the HIF-1α/vascular endothelial growth factor (VEGF) pathway (13–15), and rapamycin is currently undergoing clinical trials in patients with different types of malignancies (16).

HIF-1α is frequently overexpressed in colorectal cancers at very high levels, including both primary and metastatic tumors
Translational Relevance

HIF-1 is a key transcription factor that regulates vascularization, tumor cell metabolism, invasion, and metastasis. HIF-1 overexpression is frequently observed in colon cancer and correlates with treatment failure, thus validating HIF-1-targeted therapy as an exciting approach for cancer treatment. Among drugs validated for metastatic colon cancer, topoisomerase I poison irinotecan is a potent inhibitor of HIF-1α, protein accumulation. Our data clearly establish that irinotecan has major antiangiogenic properties and we show that targeting of the mTOR/HIF-1α axis by the mTOR inhibitor rapamycin sensitizes colon cancer cells to irinotecan in vitro and in vivo in a model of xenografted metastases of human colorectal tumors. Combined treatment at low doses resulted in major tumor shrinkage even in specimens resistant to irinotecan alone. We propose use of rapamycin in combination with irinotecan as an alternative therapeutic strategy for metastatic colon cancer patients who develop resistance to classic regimens.

(17). Despite major progress in the treatment options, advanced colorectal cancer is or becomes resistant to current therapeutic regimens. Several studies have assessed the therapeutic value of irinotecan (CPT-11), a camptothecin derivative, administered in combination with bolus or infusion 5-fluorouracil and folinic acid regimens for first-line treatment of patients with advanced metastatic colorectal cancer (18, 19). Interestingly, camptothecin derivatives have recently been identified as potent inhibitors of HIF-1α protein accumulation and transcriptional activity (20, 21). This effect requires the presence of catalytic active topoisomerase I, but not DNA replication, strongly suggesting that the antitumor activity of irinotecan is not limited to the induction of genotoxic lesions.

We hypothesized that rapamycin might cooperate with irinotecan and tested this combination, with both drugs at low doses, in vivo and in vitro. Our results showed that rapamycin potentiates irinotecan activity in a model of ectopically xenografted human colorectal cancer liver metastasis. We also characterized the in vitro effect of the rapamycin/irinotecan combination toward HT-29 and HCT-116 colon cancer cells and observed that combination therapy induced massive cell death after short-term (24 h) exposure to low concentrations of the two drugs under hypoxic, but not normoxic, conditions, in full agreement with a cytotoxic effect mediated through HIF-1α rather than through induction of genotoxic lesions. The effect was dependent on the degree of activation of the upstream phosphatidylinositol 3-kinase (PI3K)/Akt axis and correlated with treated cell transcriptional activity (20, 21). This effect requires the presence of catalytic active topoisomerase I, but not DNA replication, strongly suggesting that the antitumor activity of irinotecan is not limited to the induction of genotoxic lesions.

Together, our study suggests that rapamycin is likely to increase the efficiency of irinotecan-based regimens through the mTOR/HIF-1α axis, thereby improving patient outcome with limited genotoxic side effects.

Materials and Methods

Chemicals. The pharmacy of the University Hospital Strasbourg provided CPT-11 (Campto, irinotecan chloride, 3H2O). Rapamycin was obtained from LC Laboratories, LY294002 was obtained from Calbiochem, and salirasib (farnesyl thiosalicylic acid) was purchased from Sigma.

Animals and human tumor xenografts. Male athymic nude mice (nu/nu), 6 to 8 weeks old, were purchased from Charles River and maintained under pathogen-free conditions in facilities approved by the French Ethical Committee and under the supervision of authorized investigators. Human tumor tissue fragments were obtained in accordance with the ethical standards of the institutional committee from an untreated patient undergoing colectomy and synchronous liver metastasis resection before treatment. Cancer tissues were intensively washed in DMEM containing antibiotics, minced on ice, and injected subcutaneously in the right and left flanks. Mice were observed daily for tumor appearance and randomized into four groups, control (untreated), irinotecan alone at 10 mg/kg given intraperitoneally every 5 days, rapamycin alone at 3 mg/kg given intraperitoneally every 5 days, and a combination treatment (irinotecan plus rapamycin). Tumors derived from human colon metastasis were grown subcutaneously in athymic nude mice and the treatment was initiated when the tumors reached a mean volume of 150 to 300 mm3 and continued for 3 weeks. Each group consisted of four tumor-bearing mice.

Histologic studies. Tumors were excised after 20 days of treatment; one part of the tumor tissue was fixed in formalin and embedded in paraffin, whereas another part was embedded in OCT/Tissue-Tek (Sakura, Labonord), rapidly frozen on dry ice, and stored at -80°C. For conventional histology, 6 μm paraffin-embedded sections were stained with H&E.

Immunohistochemical determination of CD31/PECAM-1. Frozen tissues were used for immunohistochemical identification of CD31/PECAM-1. Sections were mounted on positively charged Superfast Plus slides (Kindler), fixed in acetone for 5 min, and postfixed in 10% formalin. Endogenous peroxidase activity was blocked by 0.3% H2O2. After blocking with 5% bovine serum albumin and 10% normal goat serum, sections were incubated for 1 h at room temperature with primary antibody (FITC-conjugated rat anti-mouse CD31 monoclonal antibody 1:1,000; BD Biosciences) and washed in PBS. Slides were then incubated for 30 min with secondary antibody (horseradish peroxidase-conjugated anti-FITC 1:500; Roche Molecular Biochemicals) and washed in PBS. Immunostaining was developed with a liquid DAB substrate kit (Roche) according to the manufacturer’s instructions.

Immunofluorescence staining for CD31/PECAM-1. Frozen sections of xenografted human tumors were mounted on slides and fixed. Immunohistochemical procedures were carried out as described above, except for the dilution of primary antibody (1:50). Cells were washed three times in 1× PBS and mounted using Vectashield mounting medium (Vector Laboratories). Fluorescence images were captured using an Olympus AX60 epifluorescent microscope equipped with a CCD camera and analyzed with AnalySIS software (Soft Imaging System). All images were obtained using the same variables (exposure time, contrast, and brightness). Signals were further processed and quantified with Adobe Photoshop software (Adobe Systems).

Cell culture. Human colon carcinoma HCT-116, HT-29, SW480, and Caco2 cells were maintained at 37°C in normoxic (20% O2) and/or hypoxic (94% N2, 5% CO2, 1% O2; Sanyo) conditions in DMEM (1 g/L glucose) supplemented with 10% fetal bovine serum. Cells were treated during exponential growth conditions (30% confluence).

Cell viability assay. To determine the effect of irinotecan (0.05-500 μmol/L), rapamycin (0.1-1,000 nmol/L), and the combination of both drugs on tumor cell lines, cells were seeded at 3 × 104 per well (500 μL) in 24-well flat-bottomed plates and viability was determined after 24 or 48 h exposure to the drug(s) by cell counting and trypan blue dye exclusion. To evaluate the effect of drug combination, cells were maintained at 1 μmol/L irinotecan and exposed to increasing concentrations of rapamycin (0.1-1,000 nmol/L). Cells collected by trypsinization were stained with trypan blue and the viable cells in each
well were counted. The viability of the untreated cells was regarded as 100%.

**Cell cycle analysis.** One million cells per condition were washed in cold PBS and cell cycle distribution was characterized by propidium iodide incorporation after treatment with 5 μg/mL RNase A. Fluorescence was measured on a FACScan (BD Biosciences). Data were analyzed using the CellQuest program (BD Biosciences).

**Small interfering RNA transient transfection.** Validated stealth small interfering RNAs (siRNA) targeting HIF-1α were obtained from Invitrogen. HCT-116 cells were transfected using Lipofectamine RNAiMAX according to the manufacturer’s instructions and two different siRNAs were tested alone or in combination. HCT-116 cells were seeded in hypoxic conditions (1% O₂) at 3 x 10⁴ per well in 24-well flat-bottomed plates and siRNA complexes were added to subconfluent cells. Trypan blue viability assay was done after 24 and 48 h, cell cycle analysis and total protein extraction were done after 48 h.

**VEGF measurement.** VEGF antigen levels were determined by a sandwich enzyme immunoassay using Quantikine kit (R&D Systems) according to the manufacturer’s instructions. The total protein concentration of samples was measured by the Bradford method to standardize VEGF antigen levels.

**Relative quantitative PCR.** mRNA expression of the human VEGF, GLUT-1, PKG-1, ALDOC, p21, and WEE1 genes was evaluated by relative quantitative real-time PCR (QRT-PCR) analysis using the LightCycler system (Roche Molecular Biochemicals) and FastStart DNA Master Mix SYBR Green I (Roche Diagnostics). RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription of 2 μg RNA was done using reverse transcriptase (Finnzyme) and oligo(dT) primers. PCR was done as follows: denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 20 s and 62°C for 20 s and elongation at 72°C for 20 s using the maximum temperature transition rate of 20°C/s. Fluorescence measurements were taken at the end of the elongation phase. The specificity of the PCR products was assessed by generating a melting curve. All quantifications were done in duplicate for three independent experiments and normalized with respect to the endogenous cytokeratin 19 mRNA levels for each reaction. Target cDNA expression was quantified according to the manufacturer’s instructions. The total protein concentration of samples was measured by the Bradford method to standardize VEGF antigen levels.

**Fig. 1.** Rapamycin and irinotecan combination are effective in vivo in human colon tumors xenograft models. A, tumor growth was measured before and during intraperitoneal administration of rapamycin (3 mg/kg), irinotecan (10 mg/kg), or combination of the two, administered every 5 d. Data represent RTV plotted as a function of time, which was calculated on four tumor-bearing mice per condition. B, rapamycin and irinotecan combination caused significant histologic alterations of xenografted tumors compared with controls. H&E staining showed massive necrosis and reactive fibrosis, which correlated with tumor volume reduction.
using the ΔΔCt method. Validated primers were obtained from Qiagen.

**Immunoblotting.** Tumor tissues were crushed on ice, whereas HCT-116 and HT-29 cells were scraped and pelleted by centrifugation at 4°C at 1,000 rpm for 10 min. The pellets were washed twice in ice-cold PBS and resuspended in lysis buffer [10 mmol/L Tris (pH 7.5), 5 mmol/L MgCl2, 10 mmol/L NaCl, 0.5% NP-40, protease and phosphatase inhibitors]. Lysate supernatants were subjected to a Bradford assay for protein concentration measurement. Aliquots (20 μg) of total proteins were denatured in Laemmli buffer (95°C, 5 min), resolved on 10% SDS-PAGE gels, transferred onto a nitrocellulose membrane (Schleicher & Schuell), and incubated with primary antibodies overnight at 4°C after blocking the membranes with PBS-0.1% Tween 20 containing 5% fat-free milk powder. Protein expression was detected by using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Amersham) with enhanced chemiluminescence reagents (Amersham). The following primary antibodies were used: monoclonal antibody anti-HIF-1α (BD Biosciences), anti-γH2AX and H2AX (Upstate Biotechnology), polyclonal anti-α/β-tubulin, anti-Akt, phospho-Akt, and anti-phospho-S6 ribosomal protein (S6RP; Cell Signaling).

**Mutation analysis.** Genomic DNA from xenografted tumors and colon cancer cell lines (HCT-116 and HT-29) was isolated by the phenol/chloroform method. The regions to be sequenced in the PIK3CA and KRAS genes were amplified by PCR and bidirectional sequencing reactions were done with BigDye Terminator version 1.1 chemistry (Applied Biosystems) according to the manufacturer's instructions. The sequences of the oligonucleotides used for PCR are available in Supplementary Table S1. Sequencing was done on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and sequences were analyzed with SeqScape version 2.5 software (Applied Biosystems).

## Results

**Rapamycin sensitizes colorectal tumors to irinotecan in a xenograft model.** We first determined the in vivo sensitivity of two xenografted human colorectal cancer liver metastases to irinotecan in the presence or absence of rapamycin. Four tumor-bearing mice in each group were treated every 5 days with low drug concentrations of rapamycin alone (3 mg/kg given intraperitoneally), irinotecan alone (10 mg/kg given intraperitoneally), or a combination of both drugs. Experiments were terminated after 3 weeks and mice were ethically sacrificed when control/untreated tumors reached a mean tumor volume (MTV) of 2,000 mm³ and before sensitive tumors were undetectable. We hypothesized that the effect of both rapamycin and irinotecan was principally mediated through HIF-1α inhibition and tested this hypothesis in xenografted samples. The results showed that irinotecan alone is able to inhibit the accumulation of HIF-1α protein in vivo (Fig. 2A). This effect was more important for tumor 2, where the influence of irinotecan on tumor growth was more pronounced. Interestingly, the inhibition of HIF-1α protein accumulation seems to occurred through mTOR-independent mechanisms, because no modulation of the phosphorylation levels of S6RP (Ser235/Ser236) was observed when the mice were treated with irinotecan alone. In contrast, Western blot analysis indicated that reduced HIF-1α expression was mediated via inhibition of the mTOR pathway in rapamycin-treated animals. Strikingly, combination therapy resulted in complete inhibition of HIF-1α protein accumulation. The relative inhibition of HIF-1α protein levels in tumors 1 and 2 corresponded to 50% for rapamycin, 20% and 60%, respectively, for irinotecan, and complete (100%) inhibition for combination of the two drugs.

**Combination of rapamycin and irinotecan shows strong antiglycolytic and antiangiogenic properties.** HIF-1α is a transcriptional regulator of genes needed for the glycolytic shift of cancer cells as well as for hypoxia-induced angiogenesis. To establish if HIF-1α suppression correlated with reduced expression of glycolytic enzymes and impaired tumor vascularity, we first analyzed the expression of human VEGF-A, glucose transporter GLUT-1, and two glycolytic enzymes, ALDOC and PGK-1, at the mRNA level by QRT-PCR. The results showed that exposure to irinotecan or rapamycin 1 was less sensitive than tumor 2 to both irinotecan and rapamycin alone, because ΔMTV (variation of MTV) was 156% (versus 33%) and 385% (versus 89%) and TGI was 67% (versus 81%) and 37% (versus 69%), respectively, for the two drugs (Fig. 1A; Table 1). In contrast, combination therapy resulted in a significant and comparable reduction of tumor volume (ΔMTV -59% and -53% and TGI 95% and 93%, respectively), which was independent of the median tumor volume at the start of the treatment. These results were fully coherent with H&E-stained features of frozen tissue sections, indicating major remission with massive necrosis and reactive fibrosis as well as minor residual tumor masses (Fig. 1B). In contrast, tumors treated with rapamycin alone showed similar histologic structure as the untreated controls, except for a slight, diffuse increase of fibrotic areas. Irinotecan-treated tumors presented an intermediate pattern with both necrotic/fibrotic areas and intact tumor tissue.

**Rapamycin and irinotecan combination therapy suppresses HIF-1α expression.** We hypothesized that the effect of both rapamycin and irinotecan was principally mediated through HIF-1α inhibition and tested this hypothesis in xenografted samples. The results showed that irinotecan alone is able to inhibit the accumulation of HIF-1α protein in vivo (Fig. 2A). This effect was more important for tumor 2, where the influence of irinotecan on tumor growth was more pronounced. Interestingly, the inhibition of HIF-1α protein accumulation seems to occurred through mTOR-independent mechanisms, because no modulation of the phosphorylation levels of S6RP (Ser235/Ser236) was observed when the mice were treated with irinotecan alone. In contrast, Western blot analysis indicated that reduced HIF-1α expression was mediated via inhibition of the mTOR pathway in rapamycin-treated animals. Strikingly, combination therapy resulted in complete inhibition of HIF-1α protein accumulation. The relative inhibition of HIF-1α protein levels in tumors 1 and 2 corresponded to 50% for rapamycin, 20% and 60%, respectively, for irinotecan, and complete (100%) inhibition for combination of the two drugs.

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### Table 1. Combination of irinotecan and rapamycin reduces xenografted tumor volume at low doses

<table>
<thead>
<tr>
<th>Tumor 1</th>
<th>Tumor 2</th>
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</thead>
<tbody>
<tr>
<td>MTV D0 (mm³)</td>
<td>MTV D21 (mm³)</td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>153 ± 8</td>
</tr>
<tr>
<td>Irinotecan (10 mg/kg)</td>
<td>285 ± 71</td>
</tr>
<tr>
<td>Rapamycin (3 mg/kg)</td>
<td>286 ± 34</td>
</tr>
<tr>
<td>Irinotecan + rapamycin</td>
<td>370 ± 93</td>
</tr>
</tbody>
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**NOTE:** Two human liver metastasis were ectopically xenografted to nude mice and tested for sensitivity to low doses of irinotecan alone (10 mg/kg), rapamycin alone (3 mg/kg), or a combination of both drugs. MTV was calculated and compared between day 0 (before the initiation of the treatment) and the end of the treatment.
Fig. 2. Rapamycin, irinotecan, and their combination suppress HIF-1α protein accumulation in vivo. A, Western blot analysis of the mTOR pathway activation and HIF-1α protein accumulation in xenografted tumors. Analysis of phosphorylated S6RP shows that the irinotecan-mediated inhibition of HIF-1α protein accumulation in vivo is independent of the mTOR pathway. Low doses of rapamycin partially suppress the mTOR/S6/HIF-1α axis in xenografted tumors, whereas combination of rapamycin and irinotecan dramatically reduce HIF-1α levels. B, QRT-PCR analysis of the expression of HIF-1α target genes was consistent with a potent decrease of HIF-1α protein levels. VEGF, glucose transporter GLUT-1, ALDOC, and PGK-1 mRNA levels are expressed in relative ratios (mean ± SD). Cytokeratin 19 mRNA levels were used as internal standard. C, immunohistochemical analysis of CD31 expression in human colon cancer xenografts. Rapamycin and irinotecan treatments were associated with reduced CD31 labeling and disorganized tumor vascular architecture and potentiated when both molecules were combined. Frozen sections of two xenografted tumor tissues were fixed and stained as described in Materials and Methods. CD31 expression was analyzed by peroxidase staining and quantified by immunofluorescence. Six frozen sections of two xenografted samples were tested for CD31 expression for each treatment condition.
reduced the expression of HIF-1α target genes (Fig. 2B). This effect was at least additive when the two agents were administered together, similar to the effect on HIF-1α protein levels (Fig. 2A). Because VEGF plays a key role in the induction of tumor angiogenesis, the vascular architecture of tumor xenografts was characterized by CD31 immunostaining (Fig. 2C). The results showed that both irinotecan and rapamycin treatment resulted in decreased CD31 labeling and a clear disorganization of the vascular architecture within the tumor. The antiangiogenic effect was dramatically increased when irinotecan and rapamycin were combined, because no CD31 labeling was observed on frozen tissue sections by neither immunohistochemical nor immunofluorescent labeling.

**In vitro sensitivity to irinotecan and rapamycin combination is dependent on oxygen pressure.** To further characterize the mechanisms underlying the effect of irinotecan and rapamycin combination and evaluate the effect of the genetic context on the response, the proliferation of HCT-116 and HT-29 colorectal cancer cells was examined under both normoxic (20% O2) and hypoxic (1% O2) conditions. These cell lines were chosen for two main reasons: (a) HT-29 was obtained from a CIN tumor and HCT-116 was obtained from a MIN tumor and (b) they differ in the status of p53 (HT-29 cells have a R273H mutation and HCT-116 cells express a wild-type protein). Cells were exposed to 1 μmol/L irinotecan (corresponding to an IC50 in HCT-116 and an IC10 in HT-29 cell line; Supplementary Fig. S1), 20 nmol/L rapamycin (corresponding to an IC10 in HCT-116 and an IC20 in HT-29 cell line; Supplementary Fig. S1), or a combination of both drugs and counted after trypan blue exclusion at 0, 24, and 48 h (Fig. 3A). Under normoxic conditions, only a minor antiproliferative effect was observed, even when cells were exposed to both drugs. In clear contrast, when cells were incubated under hypoxic conditions, a major antiproliferative effect was observed for HT-29 at 24 and 48 h, which was associated with a drastic reduction of viable cells. Accordingly, similar results were observed in SW480 and Caco2 cell lines in vitro in hypoxic conditions (Supplementary Fig. S1). These results are in full agreement with a cytotoxic effect mediated through HIF-1α.

Unexpectedly, irinotecan and rapamycin did not have the same effects on HCT-116 under similar conditions. These results were confirmed by morphologic analysis and 4’,6-diamidino-2-phenylindole staining of drug-treated HCT-116 and HT-29 cells (Fig. 3B). In addition, an increased sub-G1 fraction (37%) was observed for HT-29 cells by flow cytometry analysis, whereas no significant modification of the cell cycle distribution was observed for HCT-116 (Fig. 3C).

Efficient inhibition of the mTOR/HIF-1α axis is required for major antitumor effect under hypoxic conditions. Next, we wanted to establish if the differences in the sensitivity to the irinotecan and rapamycin combination correlated with differences in the inhibition of the mTOR/HIF-1α axis. The expression of the phosphorylated form of the S6RP (Ser235/236) and the levels of HIF-1α protein were determined by Western blot analysis of cells exposed to rapamycin under hypoxic conditions (Fig. 4A). For HCT-116, neither rapamycin nor irinotecan influenced the cellular levels of phospho-S6RP. Irinotecan, but not rapamycin, reduced the cellular levels of HIF-1α. However, no additional effect was observed when cells were incubated with both irinotecan and rapamycin. In marked contrast, HT-29 cells were highly sensitive to rapamycin alone.
and showed complete loss of HIF-1α after 24 h exposure to combination of rapamycin and irinotecan through mTOR-dependent and mTOR-independent mechanisms, respectively.

To further analyze the effects of HIF-1α suppression on proangiogenic signaling and glycolysis, the expression of VEGF-A and glycolytic enzymes were evaluated by QRT-PCR. As shown in Fig. 4B and C, under hypoxic conditions, efficient mTOR and HIF-1α inhibition in HT-29 cells by both rapamycin and irinotecan was accompanied by an important inhibition of HIF-1α targets, which was further pronounced in the presence of both drugs. In contrast, no difference between irinotecan alone and combined treatment with rapamycin was observed for HCT-116 cells, consistent with the absence of mTOR inhibition.

To validate the involvement of HIF-1α on cell survival in hypoxic conditions, we next performed transient transfection of HCT-116 cells with two different siRNAs targeting HIF-1α, alone or in combination. As shown in Fig. 4D, our results clearly show that efficient inhibition of HIF-1α by RNA interference results in major antiproliferative effects and massive cell death at 1% O2. Altogether, these data confirm that HIF-1α is a crucial factor for cancer cell survival and a promising target in colon cancer treatment.

Reduced sensitivity of HCT-116 cell line to mTOR inhibition by rapamycin correlates with constitutive activation of PI3K/Akt and Ras/mitogen-activated protein kinase pathways. The PI3K pathway is frequently activated in human cancers and ~30% of colorectal tumors exhibit activating mutations of the PI3Kα-catalytic subunit (22, 23). PI3K activation may modulate the response to mTOR inhibitors depending on the genetic context. Interestingly, it has recently been shown that Ras also regulates the mTOR pathway and that Ras-induced cell survival is accompanied by up-regulation of p70 S6 kinase activity (24). To further explore the mechanisms underlying the reduced sensitivity of HCT-116 cells to rapamycin, the hotspot mutation sites of the PI3Kα catalytic subunit (exons 9 and 20) and K-Ras (exon 1) in HCT-116 and HT-29 cells were sequenced (Fig. 5A). Our data confirmed that HCT-116 cells had a H1047R
activating mutation of PI3KCA and a G13D activating mutation of K-Ras, whereas HT-29 was wild-type for both genes. Comparison of hypoxic HT-29 and HCT-116 cells showed a clear activation of the mTOR/HIF-1α axis in HCT-116 cells (Fig. 5B). This observation supports mounting evidence that oncogenic signaling mediated by PI3K and Ras may promote the HIF-1α-dependent glycolytic shift of tumor cells.

Because mTOR inhibitors have a low therapeutic index and may induce severe adverse effects, escalating doses in vivo are limited and may not be sufficient to reach a major antitumor activity. Therefore, we next determined if other therapeutic approaches such as the modulation of the upstream targets K-Ras or PI3K could restore the sensitivity of HCT-116 cells to mTOR/HIF-1α inhibition in combination with low concentrations of irinotecan under hypoxic conditions (Fig. 5C). HCT-116 cells were maintained at 1% O2 and treated with either 10 μmol/L LY294002, a PI3K inhibitor, or 50 μmol/L farnesyl thiosalicylic acid, a competitive inhibitor of Ras signaling, alone or in combination with irinotecan. The cellular effects of the combination were determined by Western blot analysis of the mTOR/HIF-1α axis and by cell cycle analysis after 24 h treatment (Fig. 5D). Both PI3K and Ras inhibition resulted in potent inhibition of mTOR signaling with a significant reduction of S6RP phosphorylation and of HIF-1α protein levels. Importantly, combination of low-dose irinotecan and LY294002 or farnesyl thiosalicylic acid were accompanied by HIF-1α suppression and increased sub-G1 cell population (29% and 35%, respectively) that confirmed massive cell death.

**Fig. 5.** Constitutive activation of the Akt/mTOR/HIF-1α pathway in tumor cells influences the sensitivity to rapamycin and combination of rapamycin and irinotecan. A, sequence analysis of the PI3K catalytic subunit α exon 20 and K-Ras exon 1 in HT-29 and HCT-116 cells. B, comparison of the Akt/mTOR/HIF-1α pathway activation by Western blot analysis in HT-29 and HCT-116 cells. Serum-starved cells were grown under hypoxic conditions (1% O2) and exposed to fresh culture medium with 10% fetal bovine serum for 10 min. C, schematic representation of the Akt/mTOR/HIF-1α pathway and pharmacologic inhibitors of Ras (farnesyl thiosalicylic acid), PI3K (LY294002), and mTOR (rapamycin) used to suppress HIF-1α expression in combination with irinotecan. D, targeting of Ras or PI3K in HCT-116 cells inhibits the mTOR pathway as well as HIF-1α expression. HCT-116 cells were grown under hypoxic conditions and treated with the Ras inhibitor (farnesyl thiosalicylic acid) or the PI3K inhibitor (LY294002) in combination with irinotecan during 24 h and then subjected to cell cycle analysis and Western blot analysis of proteins involved in the mTOR/HIF-1α pathway.
genes p21 and WEE1 (Fig. 6B). These results suggest that the cytotoxic effects of camptothecin derivatives at low concentrations are, at least partly, independent of the formation of genotoxic lesions, coherent with an alternative activity mediated through HIF-1α.

**Discussion**

We here show that combination of rapamycin and irinotecan has major antitumor effect in vivo and induces massive cell death in vitro under hypoxic conditions after short-term exposure to low drug concentrations. These effects are correlated with a potent inhibition of HIF-1α accumulation in tumor cells.

**Oxygen pressure modulates the cellular sensitivity to rapamycin-based chemotherapy.** HIF-1α is the master transcriptional regulator of genes needed for energy metabolism (glycolysis) and angiogenesis during hypoxia. HIF-1α is frequently overexpressed in response to the hypoxic tumor environment and this overexpression has been associated with resistance to chemotherapy and poor outcome in a wide range of tumors (25–29). Hence, inhibition of the HIF-1 pathway is a promising approach for the treatment of colorectal cancer through antiglycolytic and antiangiogenic effects.

Importantly, our data show that cell culture conditions have major influence on the cytotoxic effects of irinotecan. It should be noted that nearly all published studies of the mechanism of camptothecin action have been carried out under normoxic conditions (20% O2), where HIF-1α has little or no influence on tumor cell metabolism. Whereas low doses of rapamycin and irinotecan had modest effect under normoxic conditions, the same treatment showed major antiproliferative effects under hypoxic conditions (1% O2), which more closely mimics the in vivo tumor environment. This was particularly pronounced for HT-29 cells, which usually are considered more resistant to irinotecan, than for HCT-116 cells. These findings clearly indicate that, at least for topoisomerase 1-directed agents, oxygen pressure is a critical component of the cytotoxic response, which needs to be taken into account.

**Antiglycolytic and antiangiogenic effects of the rapamycin/irinotecan combination.** Addition of rapamycin to irinotecan-based therapy was accompanied by complete inhibition of HIF-1α accumulation in vivo as well as in vitro as well as a dramatic reduction of tumor volume and massive tumor cell death. This was observed even at low doses of rapamycin and irinotecan due to a cooperative effect of the two agents on HIF-1α expression. The pronounced effect on tumor growth is most likely mediated through transcriptional inhibition of genes needed for glycolysis. Indeed, the combined treatment was associated with reduced (≈50%) expression of the glucose transporter (GLUT-1) as well as all glycolytic enzymes examined including PGK-1 or ALDOC. Generally, glycolysis is not an efficient metabolic way for ATP production, and production of the first energy-rich phosphate bound only occurs after the fifth step, where the aldehyde group of glyceraldehyde 3-phosphate is oxidized into a carboxylic acid. As a consequence, one can hypothesize that even a modest reduction in the expression of every single glycolytic enzyme may result in a dramatic decrease of ATP synthesis leading to rapid cell death.

HIF-1α is also a key regulator of factors needed for tumor angiogenesis, such as VEGF (30, 31), or invasion, such as SDF-1/CXCL12 and CXCR4 (32, 33). In agreement, even low doses of irinotecan (10 mg/kg) had a clear antiangiogenic effect on xenografted colorectal tumors as shown by reduced CD31 labeling and aberrant vascular architecture. This effect was greatly increased when rapamycin was added to the irinotecan treatment, because combination therapy was accompanied by nearly undetectable levels of vascular markers. Irinotecan alone or in combination with rapamycin showed similar effects on VEGF production in vitro when HT-29 or HCT-116 cells were cultured under hypoxic conditions.

Taken together, our data show that efficient inhibition of the mTOR/HIF-1α axis by combination of irinotecan and rapamycin results in major inhibitory effects on glycolytic and angiogenic pathways and massive tumor cell death.

**Determinants of cellular sensitivity to combination of rapamycin and irinotecan.** The PI3K-mTOR axis is a pivotal pathway downstream of several growth factor receptors including the epidermal growth factor, VEGF, and insulin-like growth factor receptors, which govern tumor growth and metabolism (34–36). A plethora of distinct mechanisms can lead to constitutive activation of the PI3K/Akt/mTOR pathway in cancer cells. Different processes leading to mTOR activation includes loss of PTEN function (37, 38), mutation or amplification of the PI3K p110 catalytic unit (39), amplification of one of the Akt isoforms (40), and inactivation of mTOR-regulatory proteins such as tuberous sclerosis 1 or 2 (41). More recently, it has been shown that the mTOR pathway
can also be activated by oncogenes including Ras, which may act through both PI3K-dependent and PI3K-independent mechanisms (24, 42).

In contrast to HT-29 cells, low concentrations of rapamycin had no effect on HIF-1α expression in HCT-116 cells under hypoxic conditions, suggesting that, in this cell line, rapamycin is less potent. Because PI3K and K-Ras mutations are frequent events in colon cancer and colon cancer cell lines, we sequenced these two genes in both HT-29 and HCT-116 cells. The results confirmed that both K-Ras and PI3K is wild-type in HT-29 cells, whereas HCT-116 displays two distinct activating mutations, one in the PI3K catalytic subunit (exon 20) and the other in K-Ras (exon 1). These mutations were accompanied by increased HIF-1α expression under hypoxic conditions when compared with the HT-29 wild-type cells. Interestingly, the specific small-molecule inhibitors LY294002 and farnesyl thiosalicylic acid, which target the PI3K/Akt and Ras pathways, respectively, were able to restore an efficient down-regulation of mTOR/HIF-1α axis in HCT-116 cells and this was accompanied by induction of massive cell death. These data show the interest of a systematic approach to determine the influence of frequent tumor-associated mutations on the response to irinotecan and rapamycin combination. With this respect, we tested both xenografted tumors for PI3Kα catalytic subunit and KRAS genes and found no mutation in these two sensitive tumors (data not shown).

Another important finding is that p53 deficiency did not hamper the response of HT-29 cells, which harbors the frequent R273H mutation, to combination therapy. Allelotype analysis of tumors 1 and 2 revealed loss of heterozygosity of the p53 gene locus (data not shown), further showing that p53 deficiency does not, by itself, interfere with the response to HIF-1α-targeted therapy. This is an important finding because loss of p53 function is frequent in colorectal tumors, especially in metastatic disease (43, 44). In agreement, it has been shown previously that wild-type p53 and HIF-1α may have opposite effects on glucose metabolism and mitochondrial respiration. For example, p53 is a negative regulator of glycolysis through TP53-induced glycolysis and apoptosis regulator induction (45) and a positive regulator of mitochondrial respiration through the expression of synthesis of cytochrome c oxidase 2 (46). Therefore, loss of p53 function may actually contribute to the glycolytic shift of cancer cells, thereby making cells more dependent on HIF-1α.

Irinotecan is a topoisomerase I poison that is approved for the treatment of metastatic colorectal cancer. The antitumor activity of irinotecan is generally believed to result from stabilization of covalent topoisomerase I-DNA complexes, which are converted into DNA double-strand breaks on collision with the replication fork (47). Double-strand breaks are an extremely toxic type of DNA lesion associated with the induction of a p53-mediated DNA damage response and cell cycle arrest in G2-M (48, 49). Importantly, our results suggest that the antitumor activity of irinotecan is, at least in part, independent of the genotoxic lesions, because low doses of irinotecan administered in combination with rapamycin were highly cytotoxic in the absence of major genotoxic stress.

Recent results suggest that inhibition of mTOR signaling is able to improve the response to conventional therapy in advanced poor prognosis renal cell carcinoma, and combination of mTOR inhibitors and classic chemotherapeutic agents is currently under active clinical development. The present study shows that addition of rapamycin greatly enhances the antitumor effect of irinotecan in colorectal cancer models through HIF-α inhibition. We further show that PI3K/Akt and Ras/mitogen-activated protein kinase activity have a major effect on the cellular response to combination of rapamycin and irinotecan, which might be exploited for patient stratification to identify patients most likely to benefit from this novel strategic approach.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Rapamycin Sensitizes Colon Cancer Cells to Irinotecan


Marked Activity of Irinotecan and Rapamycin Combination toward Colon Cancer Cells \textit{In vivo} and \textit{In vitro} Is Mediated through Cooperative Modulation of the Mammalian Target of Rapamycin/Hypoxia-Inducible Factor-1\(\alpha\) Axis

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