

• LIVER CANCER •

Sirolimus inhibits growth of human hepatoma cells alone or combined with tacrolimus, while tacrolimus promotes cell growth

Guido Schumacher, Marijke Oidtmann, Anne Rueggeberg, Dietmar Jacob, Sven Jonas, Jan M. Langrehr, Ruth Neuhaus, Marcus Bahra, Peter Neuhaus

Guido Schumacher, Marijke Oidtmann, Dietmar Jacob, Sven Jonas, Jan M. Langrehr, Ruth Neuhaus, Marcus Bahra, Peter Neuhaus, Departments of General, Visceral, and Transplantation Surgery, Charité Campus Virchow Klinikum, Berlin, Germany
Anne Rueggeberg, Department of Anesthesiology, Charité Virchow Klinikum, Berlin, Germany

Correspondence to: Guido Schumacher, M.D., Department of General, Visceral, and Transplantation Surgery, Charité Campus Virchow Klinikum, Humboldt University, Augustenburger Platz 1, Berlin 13353, Germany. guido.schumacher@charite.de

Telephone: +49-30-450-552001 Fax: +49-30-450-552900

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Abstract

AIM: Standard immunosuppression after organ transplantation stimulates tumor growth. Sirolimus has a strong antiproliferative and a tumor inhibiting effect. The purpose is to assess the effect on tumor growth of the immunosuppressive compounds sirolimus and tacrolimus alone and in combination on cells of human hepatocellular carcinoma.

METHODS: We used the human cell lines SK-Hep 1 and Hep 3B derived from hepatocellular carcinoma. Proliferation analyses after treatment with sirolimus, tacrolimus, or the combination of both were performed. FACS analyses were done to reveal cell cycle changes and apoptotic cell death. The expression of apoptosis-related proteins was estimated by Western blots.

RESULTS: Sirolimus alone or combined with tacrolimus inhibited the growth of both cell lines after 5 d by up to 35% in SK-Hep 1 cells, and by up to 68% in Hep 3B cells at 25 ng/mL. Tacrolimus alone stimulated the growth by 12% after 5 ng/mL and by 25% after 25 ng/mL in Hep 3B cells. We found an increase of apoptotic Hep 3B cells from 6 to 16%, and a G1-arrest in SK-Hep 1 cells with an increase of cells from 61 to 82%, when sirolimus and tacrolimus were combined. Bcl-2 was down-regulated in Hep 3B, but not in SK-Hep 1 cells after combined treatment.

CONCLUSION: Sirolimus appears to inhibit the growth of hepatocellular carcinoma cells alone and in combination with tacrolimus. Sirolimus seems to inhibit the growth stimulation of tacrolimus.

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Key words: Sirolimus; Tacrolimus; Hepatocellular carcinoma;

INTRODUCTION

The five-year survival rate of patients suffering from hepatocellular carcinoma (HCC) stands at about 50-75% considering all available treatment modalities^[1]. In addition to the tumor burden, patients who are considered as candidates for liver transplantation (LTX) usually suffer from progressive disease and advanced cirrhosis. In such cases, LTX can successfully treat both the tumor and the cirrhosis. LTX for hepatocellular carcinoma is the best treatment option when the tumor stage is limited. Multifocal growth, large tumors of more than 5 cm of diameter, high grading, and angioinvasion are factors which indicate a poor prognosis. The exact extent of the disease often becomes evident only after LTX through the pathological examination. When the result shows the presence of an advanced stage of the disease, a recurrence rate of up to 70% can be expected. No promising therapy is available for those patients, resulting in 100% fatality rate within months. New approaches to prevent tumor recurrence are of high interest for these patients.

Sirolimus, an immunosuppressive compound, has been successfully used for immunosuppression in kidney^[2,3] and liver^[4] transplant recipients. It has been successfully combined with other compounds such as cyclosporin^[5] and tacrolimus^[6]. In spite of the same receptor of sirolimus and tacrolimus, namely the FKBP-12, no clinically apparent competitive inhibition can be revealed. Thus, a combination of sirolimus and tacrolimus has achieved sufficient immunosuppression^[7,8]. The sirolimus-FKBP-12 complex acts differently from those including calcineurin inhibitors. This complex binds to a specific cell cycle regulatory protein, the mammalian target of rapamycin (mTOR), and inhibits its action. This inhibition causes growth inhibition of tumor cells which is achieved by different mechanisms^[9]. Briefly, the inhibition of mTOR inhibits the G1 to S phase transition in the cell cycle. It also inhibits the translation of an mRNA family, which encodes essential cell cycle regulatory proteins. Further mechanisms are an inhibition of the IL-2 induced transcription of the

proliferating cell nuclear antigen (PCNA), which is essential for DNA replication, and inhibition of the kinase activity of cdk4/cyclin D and cdk2/cyclin E complexes, causing decreased synthesis of the cell cycle proteins cdc2 and Cyclin A for cell cycle progression^[10]. The inhibition of the kinase activity is caused by the prolonged half-life of the tumor suppressor protein p27^{KIP1}, which is overexpressed in this situation, causing cell cycle inhibition in the G1-Phase^[11]. Sirolimus also reduces intimal proliferation following vascular injury in pigs^[12]. A strong antiangiogenic effect by a decrease in production of the vascular endothelial growth factor (VEGF) was observed in experiments using colon cancer cells^[13]. The growth of different cancer types could be inhibited by sirolimus, namely rhabdomyosarcoma cells^[14], osteosarcoma cells^[15], hepatoma cells^[16,17], lung cancer cells^[18], B lymphoma cells^[19], and renal cancer metastases^[20]. Conversely, the calcineurin inhibitors tacrolimus and cyclosporine promote cell cycle progression by a cell-autonomous mechanism^[21] such as an increase in cdk4 kinase activity^[22]. The purpose of our study was to examine the effect on tumor cell proliferation of hepatoma cells after treatment with sirolimus and tacrolimus alone or in combination of both, since this combination is regularly used in the clinic. We present an *in vitro* study that shows growth inhibition after incubation with sirolimus alone or in combination with tacrolimus in human hepatocellular carcinoma cells. We also show some of the possible mechanisms of growth inhibition.

MATERIALS AND METHODS

Cell lines and tissue culture

Two cell lines derived from human hepatocellular carcinoma were purchased from the American Type Tissue Collection (ATCC) harboring wild-type p53 (SK-Hep 1) or mutated p53 (Hep 3B). The cells were grown in Modified Eagle Medium (MEM) supplemented with 10% fetal calf serum, antibiotics, antimycotics, sodium pyruvate, non-essential aminoacids, and glutamine.

Drugs

Sirolimus and tacrolimus were generously provided by Wyeth-Pharma GmbH Münster, Germany and Fujisawa Healthcare, Inc., Munich, Germany respectively. Both were pure drugs, which were dissolved in absolute alcohol. The final concentrations were achieved by diluting the stock solution in culture medium.

Proliferation assays

Cells were set up from a 70–80% confluent T-flask in 24-well plates in MEM medium as described above at a density of 1 000 cells/well. Three wells per treatment group were used. Two days later, cells were incubated in serum-free medium with different concentrations of sirolimus and tacrolimus alone and also combined with each other. Serum-free medium was used to avoid interactions of proteins with sirolimus or tacrolimus. Doses were 5 or 25 ng/mL for each group. Additional groups treated with PBS or absolute alcohol at the same concentrations served as control groups. The medium was replaced by fresh medium

containing 10% serum 24 h after treatment. Cell counts were done on day five after incubation with the drugs. Average cell numbers were calculated from three counts per treatment group.

Western blot analysis

A standardized protocol to measure the quantities of cell proteins was used^[23]. Briefly, cells were set-up and treated in 5-cm dishes at the same doses as done for the proliferation assays. After 2 d, cell lysates were harvested and the analysis was performed. The detection of different protein expression patterns was performed using Western blot analysis. The antibodies used were p53 (p53 Ab-3, NeoMarkers) with a 1:500 dilution, p21^{WAF1} (Ab-1, Oncogene Research Products Calbiochem) with a dilution of 1:300, bcl-2 (PharMingen) with a dilution of 1:200, and β -actin (monoclonal anti- β -actin, Sigma-Aldrich) with a dilution of 1:700. As a secondary antibody we used the HRP-conjugated antibody (ImmunoPure, anti-goat, mouse IgG, Pierce) with a dilution of 1:5.000. The loading quantity of the proteins was 50 μ g/well for p53, 100 μ g/well for p21^{WAF1}, 100 μ g/well for bcl-2, and 15 μ g/well for β -actin detection. Western Blot analyses for these protein expressions were repeated at least thrice.

FACS analysis

To examine the presence or absence of apoptotic cell death, FACS analyses were performed. Furthermore, a possible G1-arrest after treatment with sirolimus alone or in combination with tacrolimus should be proved. Cells were seeded in 100-mm diameter dishes at 1×10^6 cells per dish and incubated with sirolimus, tacrolimus, or the combination of both at 25 ng/mL each compound. After one day of incubation, cells were trypsinized, washed in PBS, and fixed in 70% ice-cold ethanol for 60 min and stored at 4 °C until used. The procedure for FACS analysis was performed according to a protocol described previously^[24]. Briefly, fixed cells were incubated with 1 mg/mL of RNase (Sigma Chemical Co., Deidenhofen, Germany) for 15 min at room temperature. Thereafter, 0.5 mL PI solution (Sigma; 100 ng/mL PBS) was added for 15 min at room temperature in the dark. Cells were washed once in PBS and kept at 4 °C in the dark until measurement. We analyzed 10 000 cells using a FACS scan flow cytometer (Becton-Dickinson). These experiments were repeated twice.

Statistical analyses

A one-way ANOVA was used for statistical analyses for proliferation assays.

RESULTS

Sirolimus inhibits growth of hepatocellular carcinoma cells

Growth inhibition after treatment with sirolimus was dose-dependent in both cell lines after five days (Figure 1). When treated with 5 or 25 ng/mL sirolimus alone, we found an inhibition of 20–30% in SK-Hep 1 cells ($P = 0.0105$), and a growth inhibition of 55–65% in Hep 3B cells ($P < 0.0001$). Conversely, an increased cell proliferation was observed in the tacrolimus-treated group to up to 46% in SK-Hep 1 cells ($P = 0.0156$) and 15% in Hep 3B cells ($P = 0.0654$)

compared to non-treated control cells. The combination of sirolimus and tacrolimus showed a similar degree of cell growth inhibition as the groups treated with sirolimus alone. In Hep 3B cells, we found a highly significant inhibition of cells as compared to control cells of 55-61% when treated with 5 and 25 ng/mg ($P = 0.0002$). The growth inhibition in the combination group of both compounds was 18% and did not reach statistical significance in SK-Hep 1 cells. When the level of growth inhibition of the combination treatment with sirolimus and tacrolimus was compared to the tacrolimus alone group, there was a significant difference of the cell numbers in Hep 3B cells ($P < 0.0001$) and SK-Hep 1 cells ($P = 0.0005$).

G1-arrest and induction of apoptosis

To understand more about mechanisms of growth inhibition, cell cycle analyses were performed as shown in Figure 2 and Table 1. In Hep 3B cells, we found only a slight increase of cells in the G1-phase from 69 to 73% after treatment with sirolimus alone when compared to control non-treated cells. After treatment with tacrolimus alone, the amount of cells in the G1-phase decreased slightly from 69 to 65%, but increased in the S-phase from 10 to 17% indicating an increased DNA synthesis. As sirolimus and tacrolimus were combined at a dose of 25 ng/mL each, an increase of apoptotic cells from 6 to 16% was observed as compared to control non-treated cells. A decrease of cells in G2/M

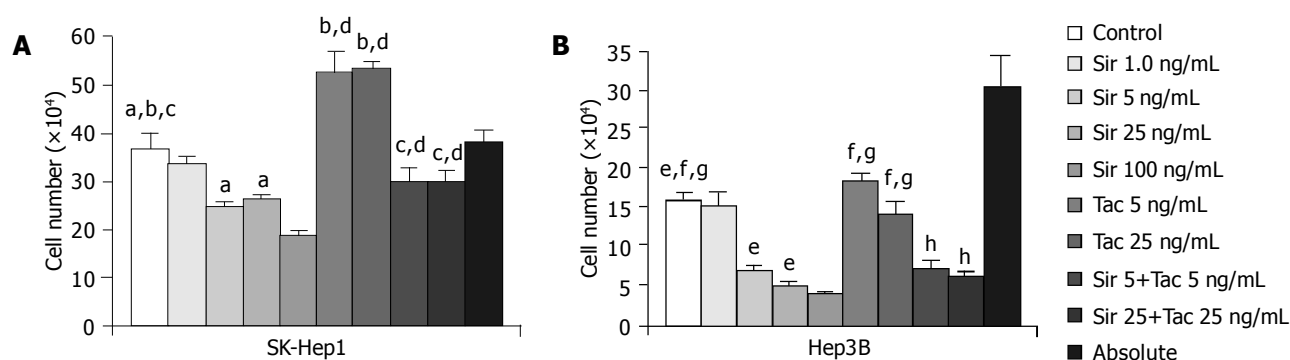


Figure 1 Proliferation assays showing cell numbers from mean counts of three experiments. Cells were treated as described in Materials and Methods with sirolimus, tacrolimus, or the combination of both. Sir = sirolimus; Tac = tacrolimus. Data are expressed as mean±SE. (A: SK-Hep 1: ^a $P = 0.0105$; ^b $P = 0.0156$; ^c $P = 0.254$; ^d $P < 0.0001$; B: Hep 3B: ^e $P < 0.0001$; ^f $P = 0.0654$; ^g $P = 0.0002$; ^h $P < 0.0001$).

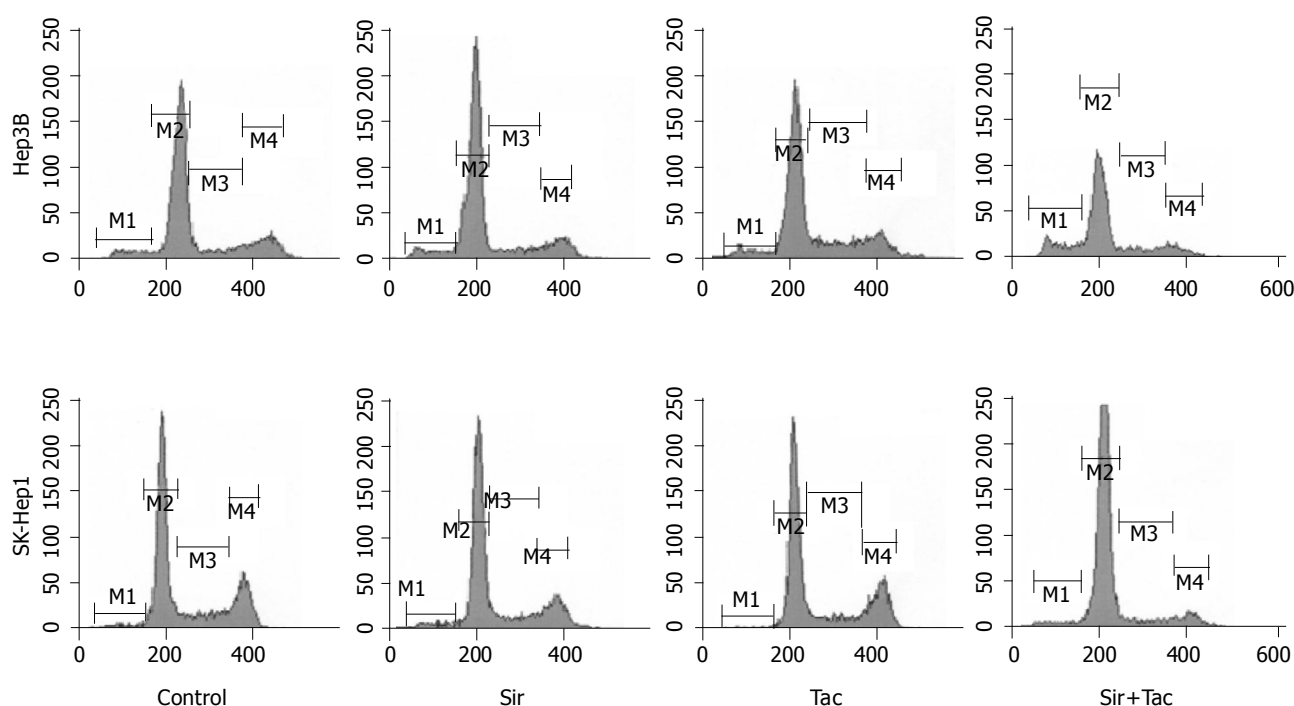


Figure 2 Cell-cycle analysis of both cell lines after treatment for 24 h with sirolimus, tacrolimus, or the combination of both at a dose of 25 ng/mL each compound. M1 = Sub-G1 region, indicating cells with small DNA fragments, a typical feature of apoptosis; M2 = G1-Phase; M3 = S-Phase; M4 = G2/M-Phase. The different cell cycle phases (M1-M4) were set in a reference analysis and kept constant throughout the measurements.

phase from 14 to 9% suggests an inhibition of mitosis in this treatment group. In contrast to Hep 3B cells, we found more changes of cells in the G1-phase in SK-Hep 1 cells with an increase from 61 to 69% after sirolimus alone. Tacrolimus resulted in a decrease of cells in the G1-phase from 61 to 54%. The combination of sirolimus and tacrolimus at a dose of 25 ng/mL resulted in a G1-arrest with an increase of cells from 61 to 82%. No induction of apoptosis was observed after treatment with sirolimus alone or in combination with tacrolimus in SK-Hep 1 cells.

Table 1 Relative cell numbers in different cell-cycle phases after treatment with sirolimus, tacrolimus and the combination of both. The doses used in these experiments were 25 ng/mL for each compound. Results of one representative experiment are shown

Cell cycle	Sub-G1	G1	S	G2/M
Hep3B				
Control	6	69	10	14
Sir	6	73	10	10
Tac	4	65	17	13
Sir + Tac	16	63	10	9
SK-Hep1				
Control	1	61	16	22
Sir	2	69	12	16
Tac	2	54	13	20
Sir + Tac	2	82	8	7

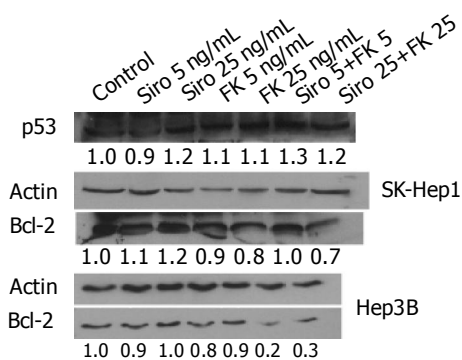


Figure 3 Western blot analysis of p53 and bcl-2. SK-Hep 1 cells express wild-type p53. Hep 3B cells harbor a deletion of the p53 gene and express no p53 protein. Expression levels were analyzed by densitometry referring to levels of actin. Relative values to controls are shown.

Expression of apoptosis-related proteins

In Figure 3, Western blot analysis of apoptosis-related proteins are shown to explain the induction of apoptosis in Hep 3B cells. SK-Hep 1 cells express a wild-type p53 gene. Since changes of p53 protein expression can be responsible for a p53-dependent induction of apoptosis, we measured the expression levels after treatment with sirolimus and tacrolimus alone or in combination with each other. There was no change of the expression levels of the p53 protein in all treatment groups. Bcl-2, a strong antiapoptotic gene, also showed no change in the expression levels in all treatment groups in SK-Hep 1 cells. Hep 3B cells expressed a deletion of the p53 gene. The expression of the bcl-2 protein in Hep 3B cells did not change after treatment with sirolimus or tacrolimus alone. When sirolimus and tacrolimus were

combined, we found a decrease of the bcl-2 protein expression by 55% in Hep 3B cells as measured by densitometry.

DISCUSSION

We hereby show that sirolimus is able to inhibit cell growth of human hepatocellular carcinoma *in vitro* by 50% in a concentration of 5 ng/mL. This low concentration may correspond to clinical serum levels considering a daily dose of 5 mg per patient. A dose-dependent growth inhibition was observed when cells were treated with doses ranging from 1 to 100 ng/mL. Control groups using absolute alcohol as the solvent at the same doses were not inhibited in growth. As shown previously, sirolimus is able to inhibit cell growth of different tumor cells^[13,14]. However, tacrolimus has been shown to promote cell growth^[21] through induction of cell cycle proteins such as cdk-4^[22]. For patient treatment after organ transplantation, a combination of sirolimus and tacrolimus has been shown to be effective for immunosuppression. No episodes of organ rejection were observed^[25]. Our experiments show that the combination of sirolimus and tacrolimus inhibits growth of hepatocellular carcinoma cells to a similar degree as sirolimus alone, while growth was stimulated after tacrolimus alone. In SK-Hep 1 cells, the combination of sirolimus and tacrolimus inhibited growth to 18% compared to control cells, which was not statistically significant. However, in this cell line, there was a large increase in cell numbers after treatment with tacrolimus alone ($P = 0.0156$). The cell numbers of combination treatment compared to treatment with tacrolimus alone were significantly lower in SK-Hep 1 cells ($P = 0.0005$). In Hep 3B, we found a significant decrease in cell numbers in the combination treatment group compared to control ($P = 0.0002$) and to the tacrolimus alone group ($P < 0.0001$). Thus, sirolimus inhibits the growth of HCC cells alone and in combination with tacrolimus. In SK-Hep 1 cells, which are strongly stimulated in growth by tacrolimus, sirolimus inhibits this proliferation significantly ($P = 0.0005$). According to these results, a combination of sirolimus and tacrolimus may prevent recurrence of HCC after LTX as much as treatment with sirolimus alone. The mechanisms of the observed G1-arrest in SK-Hep 1 and apoptosis in Hep 3B cells in the groups with combined treatment with sirolimus and tacrolimus are still not completely understood. Induction of apoptosis was observed in rhabdomyosarcoma cells^[14] and B lymphoma cells^[19] after incubation with sirolimus. We found a down-regulation of bcl-2 in Hep 3B cells as a possible mechanism of apoptosis. Since Hep 3B cells are deleted for the p53 gene, the induction of apoptosis appears to be p53-independent.

Cell cycle arrest after treatment with sirolimus has been described before^[11]. The mechanisms of an arrest in the G1-phase after the combined treatment with tacrolimus and sirolimus compared to sirolimus alone are not clear. The observed cell cycle arrest in SK-Hep 1 cells in our system may be p53-independent, because no change in the expression level of p53 was observed. In a different cell system, the observed induction of apoptosis in rhabdomyosarcoma cells was p53-independent^[14]. Also the G1 arrest, which is induced by both p53 and sirolimus appears to act through a different

mechanism^[26]. Another group showed that p53 and sirolimus cooperate in the induction of G1 arrest^[27]. Besides a decreased cell number in the G1-phase in Hep 3B cells after treatment with tacrolimus alone, an increase of cells in the S-Phase was observed, indicating cell proliferation. On the other hand, sirolimus caused a reduction of cells in the G2/M phase in SK-Hep 1 and Hep 3B cells, which corresponds to a reduced number of cells in mitosis with the subsequent reduced proliferation rate.

The growth inhibition of cells from hepatocellular carcinoma and their mechanisms in the present study are phenomena observed *in vitro*. Other mechanisms such as an antiangiogenic effect, which has been described in colon cancer^[13], could occur, which may increase the anti-tumor effect *in vivo*. The combination of sirolimus and tacrolimus resulted in a greater inhibition of intimal thickening in rat carotid arteries than sirolimus alone or the combination of sirolimus and cyclosporin^[28]. At low doses of tacrolimus, the growth inhibition of mesangial cell proliferation in kidneys observed after treatment with sirolimus could not be reversed. However, when cells were treated with higher doses of tacrolimus, such as 1 000 µmol/L, the cell inhibiting effect of sirolimus could be partially antagonized^[29]. Lymphocyte proliferation and IL-2 expression could be inhibited by sirolimus when combined with tacrolimus or cyclosporine. TGF-β was induced in this combination. These results show that combination treatments of sirolimus and calcineurin inhibitors can be used for immunosuppression^[30]. These studies demonstrate that the above-mentioned combinations of sirolimus and calcineurin inhibitors may be used for immunosuppression after organ transplantation, regardless of the transplanted organ or the disease which led to transplantation. The antiproliferative effect of sirolimus is a general phenomenon affecting both normal and tumor cells. A sirolimus-based immunosuppressive regimen in patients after liver transplantation due to HCC showed a beneficial effect on tumor recurrence and survival with an acceptable rate of rejection and toxicity^[31].

In conclusion, our data show that the two major mechanisms of sirolimus, namely immunosuppression and tumor inhibition, make this compound highly interesting for clinical application in patients who received a liver transplant for HCC. In the case of recurrence, no cure could be achieved so far. Thus, prevention of cancer recurrence is essential in the treatment of those patients. As we have shown, the combination of sirolimus and tacrolimus had a similar effect on cell growth inhibition as sirolimus alone *in vitro*. In the clinical situation, it has to be verified whether the recurrence rate of HCC correlates to the use of different immunosuppressive compounds, namely sirolimus and tacrolimus. In a long-term situation after liver transplantation, immunosuppression in these patients would be a sirolimus monotherapy or a combination of sirolimus and tacrolimus at low doses.

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