ANTICANCER RESEARCH *25*: 789-794 (2005)

Inhibition of mTOR Suppresses Experimental Liver Tumours

MAGNUS RIZELL and PER LINDNER

Department of Surgery, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden

Abstract. *Sirolimus, and its antiproliferative capacity, was studied in vivo in three different syngenic rat tumours in the liver. Sirolimus is an inhibitor of the cytosolic mTOR-kinase, associated with the phosphoinositide-3-kinase/Akt pathway. After one week of daily sirolimus treatment, initiated on the day of tumour-cell inoculation, a dose-response relationship was shown at doses between 0.01 mg/kg/day and 1 mg/kg/day, decreasing tumour weight from 0.5±0.1g in control rats (n=9) to 0.09±0.04g for sirolimus 1 mg/kg (n=9). Treating established liver adenocarcinoma (n=15), sirolimus halved the tumour weight (1.4±0.2g vs 0.7±01g, p=0.005). Trough concentration in blood was 6.4±0.2 ng/ml after five days of daily treatment with 1 mg/kg sirolimus intraperitoneally. At this dose, there was no decrease in food consumption or rat weight, but decrease in weight of spleen, and increase in weight of liver (p<0.01). The three tumours studied, an nitrosoguanidin-induced adenocarcinoma, a Leydic cell sarcoma and a hepatoma, all responded, establishing sirolimus as a promising anticancer drug.*

Sirolimus might be a potential cancer therapeutic, which is approved and used for allograft antirejection purposes due to its inhibition of T- and B-lymphocyte activation. It is a macrocyclic lactone bacterially produced by *Streptomyces hygroscopicus*. The target for sirolimus is the mammalian target of rapamycin (mTOR), which is a 290kDa evolutionary conserved cytosolic serine/threonine kinase that is inhibited (1). The coordination between cell growth and cell cycle progression is important for tumours to grow, and for this coordination mTOR is important (2). This makes it attractive to inhibit mTOR. Blocking a tyrosine kinase, and hence protein translation in cancer cells, is a novel anticancer strategy with clinical impact (3).

Correspondence to: Magnus Rizell, Transplantation and Liver Surgical Department, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden. Tel: +46 705259301, Fax: +46 31413892, e-mail: magnus.rizell@surgery.gu.se

Key Words: Sirolimus, liver, rat, adenocarcinoma, sarcoma, hepatoma.

The anticancer effects of sirolimus could depend on at least six different, but associated, mechanisms (reviewed in the discussion), which all relate to the effect on mTOR, which is an energy sensor and a vital enzyme in regulating cell growth.

The dose-response curve was studied *in vitro* in direct cell cytotoxicity tests. A broad sensitivity was shown, but with the finding that there are two populations of cell lines, differing in sensitivity and inhibited by 1nM or 1000 nM sirolimus. The sensitivity of mTOR seemed equal in both groups (4, 5). When used to inhibit lymphocytes in the clinical setting, the dose of sirolimus (MW 914 g/M) is adjusted to the trough level, aiming at 10-20 μg/ml, and administered once daily.

Based on these and earlier findings, mainly *in vitro* studies (6), we investigated the antitumour effect of sirolimus in relevant phamacological doses on three different experimental tumours in the rat, which have been characterised and are in use in our laboratory (7).

Materials and Methods

Animals. The rats were housed with three to four rats in each cage. They were fed pellets containing a balanced diet, as well as water *ad libitum*. The room temperature was controlled and the light/dark cycle was 12/12 hours. Food consumption/day and cage was monitored. Inbred Lister-Hooded (LH) rats were used for the hepatoma series (n=12). For the adenocarcinoma and the Leydig cell sarcoma, we used Wistar/Furth rats (n=90, B&K Universal, Sollentuna, Sweden). The ethical committee for animal experiments at Göteborg University, Sweden, approved the study.

Anaesthesia. Pentobarbital (Apoteksbolaget, Umea, Sweden), 36 mg/kg intraperitoneally, was used for anaesthesia during laparotomy.

Tumour. As hepatoma, a 3-methyl-diaminobenzidine-induced syngenic rat hepatoma from generation 484-525 was used. The Leydig cell sarcoma (LTW) is a syngenic sarcoma originally described in 1981 (8), and the adenocarcinoma is a nitrosoguanidin-induced syngenic colonic adenocarcinoma (NGW), that originates from the Wallenberg Laboratory in Lund, Sweden (9). The syngenic tumours were maintained by serial transplantation.

Treatment model. The viability of the cells were checked by the Nigrosin dye exclusion method. One million cells in suspension were injected into the centre of a liver lobe through a midline incision. If treated adjuvantly, the animals were initially randomly

allocated. Sirolimus was administered intraperitoneally (*i.p.*) once daily, in different doses (0.01mg/kg-10 mg/kg). The control rats received injections with saline.

Treatment was started on the inoculation day, and tumour weight and blood tests were studied on the final day after 5 or 7 days of treatment. When treating established tumours, treatment started after a laparotomy was done on day 5 or 7. After assessing the tumour volume, treatment was given daily for 5 or 7 days. Tumours were well circumscribed, and the registered tumour weight was wet-weight. Liver tumour volume was estimated by measuring the largest (a) and the smallest (b) perpendicular diameter. Volume was calculated as $V = (a + b^2) /2$.

Drug. Sirolimus (Rapamune®) 1 mg/ml, as a suspension, was purchased from Wyeth (Philadelphia, USA).

Laboratory. Haemoglobin, leukocyte count and thrombocytes were checked at the Chemical Laboratory at Sahlgrenska University Hospital, Sweden. AST, ALT and ALP were analysed with a Boehringer-Mannheim multichannel analyser. Sirolimus was analysed with a HPLC-method at the Chemical Laboratory, Huddinge University Hospital, Stockholm, Sweden.

Statistics. The results are given as mean ± SE. Group comparisons were made using nonparametric test as indicated or ANOVA when relevant. Differences were considered to be significant when $p<0.05$.

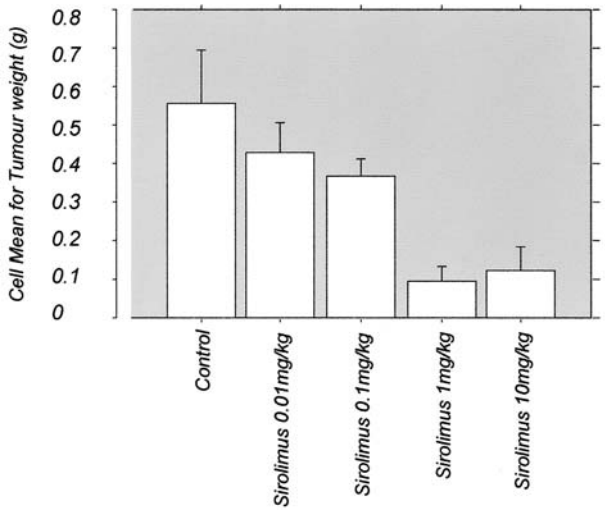

Figure 1. *Tumour weight (gram±SE) after seven days of treatment after one million tumour cells were inoculated in liver. Control (saline,n=9), sirolimus 0.01 mg/kg (n=10), sirolimus 0.1 mg/kg (n=10), sirolimus 1 mg/kg (n=9) and sirolimus 10 mg/kg (n=3). There were significant differences (p<0.01) between control and sirolimus doses higher than 0.01 mg/kg.*

Results

NGW adenocarcinoma. In animals treated with daily *i.p.* injections from the day of tumour cell inoculation in the liver, a dose-response relationship was established in 42 rats. On the seventh day after inoculating 1 million cells in the liver, the tumour weight showed an inverse relationship with the given sirolimus doses up to 1 mg/kg. The tumour weight of the control rats (0.55±0.14, n=9) decreased with increasing sirolimus concentrations (0.01 mg/kg: 0.42±0.08, 0.1 mg/kg: 0.36±0.05, 1 mg/kg: 0.09±0.04, 10 mg/kg: 0.12±0.06), Figure 1. The behaviour of the rats was normal up to 1 mg/kg. In the 10 mg/kg group, one animal died without known cause on day 5, and we did not explore this concentration any further. The weight of the animals did not differ between control rats (195±2), sirolimus 0.01 mg/kg (193±2), sirolimus 0.1 mg/kg (193±4) and sirolimus 1 mg/kg (189±2). The weight of the rats receiving 10 mg/kg was significantly higher (210±1, $p=0.02$).

Food consumption. Food consumption was measured as dry food/rat/day. Between the cages, it did not differ between control rats (10.9±0.5 g/day, n=23) and sirolimus 0.01 mg/kg (11.8±0.3 g/day, n=11), sirolimus 0.1 mg/kg (11.1±0.4 g/day, n=11), or sirolimus 1 mg/kg (10,6±0,6 g/day, n=23). Food consumption was, however, significantly lower in the 10 mg/kg sirolimus group (8.2±1.5 g/day, n=6, $p=0.04$ Kruskal-Wallis).

Weight of liver and spleen. Compared with control rats, the weight of liver was increased (> 8%) in all sirolimus groups ($p<0.01$). This increase was not due to accumulation of interstitial water, since the dry weight of the liver was also higher after sirolimus treatment (30.9±0.2,n=7 *versus* 28.7±0.3, n=8, $p= 0.002$, Mann-Whitney), whereas the weight of the spleen was significantly lower for all groups except for sirolimus 0.01 mg/kg, Table I.

To study the effect on established NGW adenocarcinoma (n=15), tumour volume was assessed after one week (Table I), and daily *i.p.* sirolimus treatment (1 mg/kg) was given. There was a significant reduction in tumour volume ($p=0.003$) and tumour weight ($p=0.005$) after 7 days of treatment (Table II).

LTW sarcoma. For LTW sarcoma, two doses (0.4 and 2 mg/kg) were compared with control. Treatment was given for one week after tumour cell inoculation in the liver. Animal weight did not differ between the groups ($p=0.7$), but tumour weight was half in the sirolimus groups ($p<0.01$). There was no statistical dose-response effect between the two sirolimus doses ($p=0.25$) (Table III).

In an additional series in 15 animals with established LTW tumours, the treatment effect of sirolimus was compared with the control. Tumour volume was assessed on day 5, before they were randomised to treatment, with no difference between groups. After 5 days of *i.p.* treatment with sirolimus 1mg/kg, there was a significant

Table I. *Weight of liver and spleen related to treatment in rats with adenocarcinoma.*

Treatment	Liver (g±SE)	Spleen (g±SE)	N=
Control	6.0±0.1	0.54±0.01	26
Sirolimus 0.01 mg/kg	6.7±0.2 *	0.54±0.2	10
Sirolimus 0.1 mg/kg	6.8±0.2 *	0.49±0.02 *	10
Sirolimus 1 mg/kg	6.6±0.1 *	0.44±0.01 *	27
Sirolimus 10 mg/kg	6.6±0.2 *	0.45±0.02 *	3

The asterisk (*) mark a statistical difference ($p<0.01$) between control animals and treatment groups

Table II. *Tumour volume and weight in established NGW adenocarcinoma in liver.*

Treatment	Tumour volume pre treatment (mm^3±SE)	Tumour volume day 7 (mm^3±SE)	Tumour weight day 7 (gram±SE)	Body weight day 7 (gram±SE)
Control (n=7)	93±13	880±144	1.4±0.2	201 ±3
Sirolimus (n=8)	109±14	361±44 *	0.7±0.1 #	204 ±3

Statistical differences between control and sirolimus group. Asterisk (*), $p=0.003$, or a cross (#), $p=0.005$.

reduction in tumour volume ($p=0.002$, Mann-Whitney) and tumour weight ($p=0.03$), as compared to control (Table IV).

Hepatoma. Twelve Lister-Hooded rats were inoculated with a hepatoma cell suspension and treated daily with *i.p.* injections of sirolimus (1 mg/kg, n=6) or saline (n=6). Tumour weight after 5-day treatment, starting at the day of tumour inoculation in the liver, was significantly lower after treatment with sirolimus (control 0.5±0.2 g, and sirolimus 0.08±0.03 g, $p<0.02$).

Sirolimus concentration and liver laboratory tests. Sirolimus trough concentration (±SE) was measured in 22 rats, 24 hours after the last dose. After five days of *i.p.* sirolimus 1 mg/kg, the concentration was 6.4±0.2 ng/ml.

In tumour-bearing animals (NGW, n=28), treatment with sirolimus halved the leukocyte count ($p<0.001$). A minor, but statistically significant, increase in haemoglobin and decreases in platelets, alanine aminotransferase (ALT) as well as alkaline phosphatase (ALP), were seen (Table V).

Table III. *LTW sarcoma weight, 7 days after inoculation and treatment.*

Treatment N=6 in each group	Tumour weight (gram±SE)	Rat weight (gram±SE)
Control	1.3±0.1	179±5
Sirolimus 0.4 mg/kg	0.6±0.05	185±5
Sirolimus 2 mg/kg	0.5±0.1	183±4

Discussion

An inhibitory effect of sirolimus on tumour growth was shown in these experiments with an NGW adenocarcinoma, a hepatoma and a LTW sarcoma. Sirolimus inhibited the establishment of tumour, studied by administering sirolimus from the day of tumour cell inoculation, as well as reducing the growth of an already established tumour, which add these tumours to the list of tumours responding to sirolimus *in vivo* (6, 10). The dose-response effect shown here is of importance, but the optimal administration dose, rate and route remain to be determined. Although the administration route in this experiment had been established earlier, conclusions regarding adequate tumour-inhibiting concentrations are not easily drawn. The *i.p.* route of administration compared with plasma could enhance drug exposure in the liver (measured as area under curve) (11). The tumour sirolimus concentration in the liver could hence be higher than indicated by the measured trough levels. To check administration routes other than the *i.p.*, a one-week treatment with sirolimus perorally (1 or 5 mg/kg, n=14, NGW adenocarcinoma) resulted in a significantly reduced tumour weight as well (unpublished results). Further studies of the tumour pharmacokinetics of sirolimus and the necessary tumour-inhibiting concentration in the liver parenchyma are needed (12). The measured trough level of sirolimus was adequate in relation to what is known about maintaining the mTOR- inhibiting effect in lymphocytes, used for immunosuppression. The leukocyte count was halved and the weight of spleen significantly reduced.

Interestingly, a novel observation is that there was a slight increase in the weight of the liver after treatment with sirolimus, and this effect was statistically significant after the 0.01 mg/kg daily dose, which was not effective in tumour weight reduction. Whether this relates to mTOR as a sensor for ATP and energy (13), or other mechanisms, remains to be elucidated.

The reduction in tumour weight and volume was not associated with cachexia, measured as weight of the rats. The tumour-inhibiting effects in these series do not seem to be mediated by the nutritive stimuli, and showed consistency both in inhibiting cells trying to establish a tumour, as well as inhibiting an already measurable tumour.

Table IV. *Established LTW sarcoma in liver treated for 5 days with sirolimus.*

Established LTW sarcoma Day10	Tumour volume (mm^3±SE)	Tumour weight (gram±SE)	Rat body weight (gram±SE)	Liver weight (gram±SE)	Spleen weight (gram±SE)
Control (n=7)	648±131	0.859±0.209	211±5	6.05±0.18	0.62±0.02
Sirolimus (n=8)	148±42	0.328±0.098	209±3	6.47±0.05	0.53±0.02

Table V. *Blood cells and liver enzymes in rats with adenocarcinoma treated with sirolimus compared with control.*

	Control	Sirolimus 1mg/kg *i.p.*	N=	*p*=
Leukocytes ($x10^9$/l)	4.9±0.4	2.4±0.3	28	< 0.001
Platelets ($x10^9$/l)	872±29	667±35	28	< 0.001
Haemoglobin (g/l)	128±4	143±2	7	< 0.01
AST (μkat/l)	4.4±0.8	3.2±0.5	8	0.23
ALT (μkat/l)	1±0.1	0.7±0.03	8	0.02
ALP (μkat/l)	4.8±0.5	3.5±0.1	8	0.04

One could speculate that different mechanisms in the action of rapamune could be of importance for the tumour-inhibiting effects: (i) *Growth factor inhibition.* Growth factors like IGF-1 stimulate phosphatidylinositol 3-kinase (PI3K)/Akt and its pathway. This pathway impacts cell survival and proliferation (14). mTOR, localised in the cytosol, mediates many of the signals regulated by PI3K/Akt, and senses the signals from growth hormones. (ii) *Blockage of nutrient stimuli.* Together with an evolutionary conserved protein, raptor, mTOR is also a nutrient sensor, independent of the PI3K pathway. When nutrients are deprived, mTOR is inhibited, which down-regulates cell size. Since mTOR also has a regulatory role in protein translation, this regulation makes sense (15). (iii) *Cell cycle (G1) arrest and apoptosis.* mTOR regulates cell cycle-regulating proteins. Sirolimus prevents phosphorylation of downstream targets such as the S6 kinase (with the ribosomal protein S6 as in important product) and inhibits the release of the translation initiation factor (eIF)-4E-binding protein (14). Both S6 kinase and 4EBP1 regulate translation of cell cycle-regulating proteins, such as cyclin D1 and c-myc. Inhibiting mTOR mediates a G1 arrest (16). By interaction with the initiation factor (eIF)-4E-binding protein, in cells lacking a functional p53, apoptosis is induced (17). By evading apoptosis, the Akt-mTOR pathway can promote oncogenesis and, in cells expressing Akt, sirolimus can restore the apoptotic program (18). (iv) *Anti-angiogenic.* Sirolimus decreases the VEGF levels. The anti-angiogenic effect has been studied in tumour cells resistant to sirolimus *in vitro*, where tumour angiogenesis was blocked (6). PI3K and mTOR are critical for VEGF-mediated angiogenesis. Angiogenesis is also stimulated by HIF, which is negatively regulated by mTOR inhibitors (19). (v) *Tumour invasion inhibition.* The level of metallomatrixprotein 2 decreases, which is of known importance for basement membrane degradation, tumour invasion and metastasis (20). In an *in vivo* mouse model, a decreased rate of pulmonary metastases was observed (10). (vi) *Anti-inflammatory.* COX-2 and iNOS mRNA (with their products NO and PGE2) levels were inhibited in macrophages in an *in vitro* model (21), and the production of radical oxygen species was also reduced in another one (22).

The different mechanisms of probable importance for the tumour inhibition makes it attractive to combine sirolimus with other drugs aiming at increasing, *e.g.,* the anti-angiogenetic or metallomatrixprotein-inhibiting effects. The optimal combination probably differs between tumour stage as well as tumour biology, and more mechanistic studies are needed (23, 24). It is now also evident that, in tumours expressing Akt, sirolimus might restore the apoptotic capacity and reverse sensitivity to cytostatic drugs (18). Although highly selective, targeting mTOR along the PI3K/Akt pathway, the broad effect on several tumour lines seems promising. The reported up-regulation of the PI3K pathway in hepatocellular and colon carcinoma offers hope that sirolimus can be used clinically. Sirolimus is on track to clinical studies. We are now conducting a phase II trial in hepatocellular carcinoma and have observed clinical reponse (unpublished results), which may offer promise for sirolimus use in the clinic.

Acknowledgements

The authors are grateful to Lilian Karlsson and Rigmor Söderberg for their excellent technical assistance. The work was supported by the Swedish Cancer Society, the Faculty of Medicine, University of Göteborg, and Sahlgrenska University Hospital (Sweden) funds.

References

1 Heitman J, Movva NR and Hall MN: Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*: 905-909, 1991.

2 Fingar DC and Blenis J: Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene *23*: 3151-3171, 2004.

3 Sawyers CL: Opportunities and challenges in the development of kinase inhibitor therapy for cancer. Genes Dev *17*: 2998-3010, 2003.

4 Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R, Frost P, Gibbons JJ, Wu H and Sawyers CL: Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci USA *98*: 10314-10319, 2001.

5 Douros J and Suffness M: New antitumor substances of natural origin. Cancer Treat Rev *8*: 63-87, 1981.

6 Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch KW and Geissler EK: Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med *8*: 128-135, 2002.

7 Rizell M: Histamine Treatment of Malignant Tumour. Sahlgrenska Academy, Department of Sugery. Göteborg: University of Göteborg, 2003.

8 Mordes JP and Rossini AA: Tumour-induced anorexia in the Wistar rat. Science *213*: 565-567, 1981.

9 Steele G Jr and Sjogren HO: Cross-reacting tumour-associated antigen (s) among chemically induced rat colon carcinomas. Cancer Res *34*: 1801-1807, 1974.

10 Boffa DJ, Luan F, Thomas D, Yang H, Sharma VK, Lagman M and Suthanthiran M: Rapamycin inhibits the growth and metastatic progression of non-small cell lung cancer. Clin Cancer Res *10*: 293-300, 2004.

11 Lindner P, Heath DD, Shalinsky DR, Howell SB, Naredi P and Hafstrom L: Regional lymphatic drug exposure following intraperitoneal administration of 5-fluorouracil, carboplatin, and etoposide. Surg Oncol *2*: 105-112, 1993.

12 Choe G, Horvath S, Cloughesy TF, Crosby K, Seligson D, Palotie A, Inge L, Smith BL, Sawyers CL and Mischel PS: Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients *in vivo*. Cancer Res *63*: 2742-2746, 2003.

13 Thevananther S, Sun H, Li D, Arjunan V, Awad SS, Wyllie S, Zimmerman TL, Goss JA and Karpen SJ: Extracellular ATP activates c-jun N-terminal kinase signaling and cell cycle progression in hepatocytes. Hepatology *39*: 393-402, 2004.

14 Shamji AF, Nghiem P and Schreiber SL: Integration of growth factor and nutrient signaling: implications for cancer biology. Mol Cell *12*: 271-280, 2003.

15 Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P and Sabatini DM: mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*: 163-175, 2002.

16. Panwalkar A, Verstovsek S and Giles FJ: Mammalian target of rapamycin inhibition as therapy for hematologic malignancies. Cancer *100*: 657-666, 2004.

17 Huang S, Shu L, Dilling MB, Easton J, Harwood FC, Ichijo H and Houghton PJ: Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21(Cip1). Mol Cell *11*: 1491-1501, 2003.

18 Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, Cordon-Cardo C, Pelletier J and Lowe SW: Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature *428*: 332-337, 2004.

19 Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ and Abraham RT: Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol Cell Biol *22*: 7004-7014, 2002.

20 Zhang D and Brodt P: Type 1 insulin-like growth factor regulates MT1-MMP synthesis and tumor invasion *via* PI 3-kinase/Akt signaling. Oncogene *22*: 974-982, 2003.

21 Attur MG, Patel R, Thakker G, Vyas P, Levartovsky D, Patel P, Naqvi S, Raza R, Patel K, Abramson D, Bruno G, Abramson SB and Amin AR: Differential anti-inflammatory effects of immunosuppressive drugs: cyclosporin, rapamycin and FK-506 on inducible nitric oxide synthase, nitric oxide, cyclooxygenase-2 and PGE2 production. Inflamm Res *49*: 20-26, 2000.

22 Tunon MJ, Sanchez-Campos S, Gutierrez B, Culebras B, Gonzalez-Gallego J: Effects of FK506 and rapamycin on generation of reactive oxygen species, nitric oxide production and nuclear factor kappa B activation in rat hepatocytes. Biochem Pharmacol *66(3)*: 439-45, 2003.

23 Bruns CJ, Koehl GE, Guba M, Yezhelyev M, Steinbauer M, Seeliger H, Schwend A, Hoehn A, Jauch KW and Geissler EK: Rapamycin-induced endothelial cell death and tumor vessel thrombosis potentiate cytotoxic therapy against pancreatic cancer. Clin Cancer Res *10*: 2109-2119, 2004.

24 Seeliger H, Guba M, Koehl GE, Doenecke A, Steinbauer M, Bruns CJ, Wagner C, Frank E, Jauch KW and Geissler EK: Blockage of 2-deoxy-D-ribose-induced angiogenesis with rapamycin counteracts a thymidine phosphorylase-based escape mechanism available for colon cancer under 5-fluorouracil therapy. Clin Cancer Res *10*: 1843-1852, 2004.

Received September 23, 2004
Revised December 27, 2004
Accepted February 4, 2005