COMPOSITION AND METHOD

FIELD

This invention relates to methods of treatment and diagnosis of disease, and molecules and compositions for use in such methods.

BACKGROUND

Carcinoma of the liver (hepatocellular carcinoma, HCC) is the fifth most common malignancy worldwide and third highest cause of global cancer mortality (Ferley J, Bray F. Pisani P, Parkin DM. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. IARC CancerBase No5 version 20. IARCPress, Lyon, France; 2004).

Hepatocellular carcinoma (HCC) has replaced stomach cancer as the 3rd highest ranking cancer among Singaporean males, accounting for 8.1% of all cancers diagnosed in 1998-2002 (Seow A, K. W., Chia KS, Shi LM, Lee HP, Shanmugaratnam K (2004). "Trends in Cancer Incidence in Singapore 1968-2002." Singapore Cancer Registry (Report no. 6)). In Asia, where about 250,000 new cases per year are reported in China alone, HCC is endemic. Worldwide, it is the leading cause of cancer mortality (Bosch, F. X., J. Ribes, et al. (2005). "Epidemiology of hepatocellular carcinoma." Clin Liver Dis **9**(2): 191-211, v.). Even in countries like the USA and Europe where the prevelance of HCC is lower, the incidence of HCC is on the rise (El-Serag, H. B. and A. C. Mason (1999). "Rising incidence of hepatocellular carcinoma in the United States." N Engl J Med **340**(10): 745-50).

There are substantial challenges towards the clinical management and treatment of HCC.

More than 90% of HCCs are diagnosed at an advanced stage and are often associated with liver cirrhosis. HCCs typically exhibit highly aggressive clinical behaviour with the majority of patients dying within 12 months of diagnosis (El-Serag HB.

Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 2002; 35(5 Suppl 2):S72-8).

Often, patients present at such advanced disease stages that surgery is not an option; in cases where surgical resections are performed the two-year recurrence rate is still as high as 50% (Nagasue N, Kohno H, Chang YC, Taniura H, Yamanoi A, Uchida M, et al. Liver resection for hepatocellular carcinoma. Results of 229 consecutive patients during 11 years. Ann Surg 1993; 217:375-84; Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S, Ozaki H, et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996; 83:1219-22.).

HCC is a relatively chemotherapy refractory cancer. There is no single agent or multi-agent chemotherapy that is particularly effective. Doxorubicin is the most commonly used chemotherapy agent in metastatic HCC with response rates of less than 20% (Johnson, P. J., R. Williams, et al. (1978). "Induction of remission in hepatocellular carcinoma with doxorubicin." Lancet **1** (8072): 1006-9) and statistically insignificant survival advantage. More recent results of a trial using 3-drug combination chemotherapy and interferon showed response rates of 20.9% and median survival of 8.67 months (Yeo, W., T. S. Mok, et al. (2005). "A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma." J Natl Cancer Inst **97**(20): 1532-8). This regimen did not show better survival compared to doxorubicin alone and was associated with more toxicities.

Recent results from randomized clinical trials have shown that standard chemotherapeutic regimens are minimally effective in prolonging HCC patient survival (Yeo W, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, et al. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. J Natl Cancer Inst 2005; 97:1532-38).

Besides tumor recurrence and metastasis, peritoneal ascites is another significant cause of morbidity in advanced stage HCC patients, often arising as a result of compromised liver function, portal vein blockage, and increased endothelial cell permeability.

Thus, an outstanding challenge facing the cancer research community lies in identifying novel molecularly-targeted therapies to treat HCC and its associated disease comorbidities. There is great need for more innovative therapies with new drugs or novel regimens of existing drugs in metastatic HCC.

SUMMARY

Surprisingly, it has now been found that molecules, in particular, angiogenesis inhibitors, may be used in conjunction with antagonists or inhibitors of mTOR activity, to achieve an improvement in cancer treatment of individuals. In particular, the combinations disclosed here may be used to treat individuals suffering from hepatocellular carcinoma (HCC) or to prevent incidence of such cancer.

According to a 1st aspect of the present invention, we provide a combination of a first agent comprising rapamycin (Sirolismus) together with a second agent comprising bevacizumab (Avastin) for use in the treatment or prevention of hepatocellular carcinoma (HCC) in an individual.

One or both of the first agent comprising rapamycin and the second agent comprising bevacizumab may be in the form of a pharmaceutical composition comprising the agent or agents, together with a pharmaceutically acceptable carrier, excipient or diluent.

The first agent comprising rapamycin may be provided in a form suitable for oral administration, such as a tablet, and the second agent comprising bevacizumab may be provided in a form suitable for intravenous administration, or both.

The method of treatment or prevention of hepatocellular carcinoma (HCC) may comprise administering the first agent comprising rapamycin simultaneously or sequentially with a second agent comprising bevacizumab to an individual.

There is provided, according to a 2nd aspect of the present invention, rapamycin for use in a method of treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the method comprises administering rapamycin simultaneously or sequentially with bevacizumab.

We provide, according to a 3rd aspect of the present invention, bevacizumab for use in a method of treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the method comprises administering bevacizumab simultaneously or sequentially with rapamycin.

As a 4th aspect of the present invention, there is provided use of rapamycin for the preparation of a combination for treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the combination comprises bevacizumab .

We provide, according to a 5th aspect of the present invention, use of bevacizumab for the preparation of a combination for treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the combination comprises rapamycin .

The combination, rapamycin for a use as specified therein or bevacizumab for a use as specified therein, may further comprise instructions for administration of the agents to an individual to treat or prevent hepatocellular carcinoma (HCC) in an individual.

The present invention, in a 6th aspect, provides a method of preparing a combination as set out above, the method comprising bringing together a first agent comprising rapamycin with a second agent comprising bevacizumab .

The rapamycin may be present in an amount to provide a dosage of between about 1 mg/day to about 10 mg/day.

The bevacizumab may be present in an amount to provide a dosage of between about 5 mg/kg/2 weeks to about 10 mg/kg/2 weeks.

In a 7th aspect of the present invention, there is provided an *ex-vivo* method of preventing the growth or proliferation, or both, of a cell or tissue, the cell or tissue being a liver cancer cell or liver cancer tissue, the method comprising exposing the cell or tissue to a first agent comprising rapamycin and a second agent comprising bevacizumab .

The first agent may have the features as set out above, and the second agent may have the features as set out above, or both.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practic*e; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; Using Antibodies : A Laboratory Manual : Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7); Antibodies : A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-314-2), 1855, Lars-Inge Larsson "*Immunocytochemistry: Theory and Practice*", CRC Press inc., Baca Raton, Florida, 1988, ISBN 0-8493-6078-1, John D. Pound (ed); "*Immunochemical Protocols, vol 80*", in the series: "Methods in Molecular Biology", Humana Press, Totowa, New Jersey, 1998, ISBN 0-89603-493-3, Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9); Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3; and The Merck Manual of Diagnosis and Therapy (17th Edition, Beers, M. H., and Berkow, R, Eds, ISBN: 0911910107, John Wiley & Sons). Each of these general texts is herein incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the effects of bevacizumab on growth rate of subcutaneous HCC xenografts. 2-1318, 26-1004, 5-1318 and 2006 xenograft lines were s.c. implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were IP administered either PBS or 5mg/kg bevacizumab every two weeks for 21 days. Treatment started on day seven after tumor cell injection. Tumor growth was measured and calculated as described in Materials and Methods. Tumor volume at a given time for PBS- and bevacizumab-treated 2-1318 (**A**), 26-1004 (**B**), 5-1318 (**C**), and 2006 (**D**) xenografts is plotted and shown. Differences in tumor weight and tumor volume among the treatment groups were statistically significant at $p<0.01$, as analyzed by ANOVA. Experiments were repeated at least three times with similar results.

Figure 2 shows the effects of bevacizumab on the expression of cell cycle regulators in HCC xenografts. 2-1318 xenografts were s.c. implanted in SCID mice as described in Materials and Methods. Mice bearing HCC xenografts were IP administered either PBS or 5mg/kg bevacizumab every two weeks for 21 days. Treatment started on day seven after tumor cell injection. Lysates from vehicle- and treated tumors were subjected to western blot analysis described in Materials and Methods. Blots were incubated with the indicated antibodies. Representative blots are shown. Densitometric data (Fold-changed) were shown below each group. Similar results were obtained for 26-1004, and 30-1004 xenografts. Experiments were repeated at least three times with similar results.

Figure 3 shows the therapeutic effects of bevacizumab on IP tumor burden, tumor cell dissemination to the liver, and survival rate. Male SCID mice were injected with 5 x 10^6 of 26-1004(Met) cells in 200 μl PBS in the peritoneal cavity. Mice bearing IP tumors were randomized and were treated with either 200 μl of PBS (n=14) or bevacizumab (5 mg/kg) (n=14) once every two weeks for indicated time. Survival was monitored three times weekly. The mice were sacrificed and necropsied when they became moribund. Representative PBS- and bevacizumab-treated mice (**A**) and the omental tumors in peritoneal cavity of PBS- and bevacizumab-treated mice (**B**), dissemination of tumor mass

in the liver (**C**) on day 30-36 after inoculation of 26-1004 (Met) cells are shown. Survival was evaluated by the Kaplan-Meier method and shown (**D**). Note that bevacizumab significantly prolongs survival of IP mice ($p<0.01$) by inhibiting ascites formation, tumor dissemination and reducing IP tumor burden. Arrows indicate tumors. Experiments were repeated at least three times with similar results.

Figure 4 shows the effects of Avastin, rapamycin, and Avastin plus rapamycin on tumor growth of 5-1318, 2006, 2-1318, and 26-1004 HCC xenografts. The indicated xenografts were SC implanted on the right side of male SCID mice as described in Materials and Methods. They were randomized to one of the four treatment groups (n=14) and treated with vehicle, Avastin (5 mg/kg), rapamycin (1 mg/kg), or combined rapamycin (1 mg/kg) plus Avastin (5 mg/kg) for 21 days as described in Materials and Methods. Representative tumor samples for indicated lines of HCC xenografts are shown.

Figure 5 shows the ffects of Avastin, rapamycin and Avastin plus rapamycin on growth rate of HCC xenografts. 2-1318 and 26-1004 xenograft lines were SC implanted on the right side of male *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were treated with vehicle, Avastin (5 mg/kg), rapamycin (1 mg/kg), or rapamycin (1 mg/kg) plus Avastin (5 mg/kg) for 21 days. Treatment started on day 3 after tumor cell injection. Tumor growth was measured and calculated as described in Materials and Methods. Tumor volume at a given time for PBS-, Avastin, rapamycin, or combined Avastin-rapamycin of the 26-1004 (**A**), and 2-1318 (**B**) xenografts is plotted and shown. Differences in tumor volume among the treatment groups were statistically significant ($p<0.01$) as analyzed by ANOVA. Experiments were repeated at least three times with similar results.

Figure 6 shows the effects of Avastin, rapamycin and Avastin plus rapamycin on the phosphorylation of mTOR, p70S6 kinase, S6R, and 4E-BP1(A) and the levels of cyclin D1, cyclin B1, Cdk-2, Cdk-4, p27, and pRb (B) in HCC xenografts. 2006 xenografts were SC implanted on the right side of male *SCID* mice, as described in Materials and Methods. Mice bearing 2006 xenografts were treated with vehicle, Avastin (5 mg/kg), rapamycin (1 mg/kg), or combined rapamycin (1 mg/kg) plus Avastin (5 mg/kg)

as described in Materials and Methods. Lysates from vehicle- and treated tumors were subjected to western blot analysis described in Materials and Methods. Blots were incubated with the indicated antibodies. Representative blots are shown. Similar results were obtained for 2-1318, 5-1318, 26-1004, and 30-1004 xenografts. Experiments were repeated at least three times with similar results.

Figure 7 shows the therapeutic effects of Avastin, rapamycin and Avastin plus rapamycin on IP tumor burden, tumor dissemination to the liver, and survival rate. Male SCID mice were injected with 5 x 10^6 26-1004(Met) cells in 200 μl PBS in the peritoneal cavity. Mice were treated with vehicle, Avastin (5 mg/kg), rapamycin (1 mg/kg), or combined rapamycin (1 mg/kg) plus Avastin (5 mg/kg) for 4-6 weeks as described in Materials and Methods. The mice were sacrificed and necropsied when they became moribund. Representative PBS- and treated mice, and the omental tumors in the peritoneal cavity of PBS- and treated mice are shown (**A**), and dissemination of tumor mass in the liver (**B**) on day 30-36 after inoculation of 26-1004 (Met) cells are shown. Survival was evaluated by the Kaplan-Meier method and is shown (**C**). Note that while all mice in the control, Avastin, and rapamycin groups were moribund at day 48, 120, and 118, respectively. Avastin plus rapamycin significantly prolonged the survival of IP mice ($p<0.01$) and all were still alive at the day 125.

Figure 8 shows the reversal of ascites accumulation in IP mice by Avastin plus rapamycin therapy. Mice were injected with 26-1004 (Met) HCC xenografts as described in Materials and Methods. For therapeutic experiments, the ascites was partially drained from the control 26-1004(Met) IP mice. They were divided into vehicle-treated (n=14) and combined rapamycin (1 mg/kg) plus Avastin (5 mg/kg) treated (n=14) group. Note that the control IP mice rapidly developed ascites and became cachectic. The Avastin-rapamycin group showed no sign of ascites formation after seven days of treatment and completely recovered by day fourteen after treatment.

Figure 9 shows Histological Sections of Hepatocellular carcinoma (HCC) from Patient Tumors, Primary Xenografts, and Cell Lines

All sections were stained with haematoxylin and eosin (H&E) and evaluated by two qualified pathologists (MST and TPH). (a) and (d) Line 2-1318, from the established xenograft (a) and primary patient tumor (d), at 200x magnification. Both tumors show a common sinusoidal pattern.(b) and (e) Line 26-1004, from the established xenograft (b) and primary patient tumor (e), at 200x magnification. Both tumors comprise sheets of tumor cells with a focal sinusoidal pattern. Necrosis is observed in (e). (c) and (f) Line 2006, from the established xenograft (c) and primary patient tumor (f), at 200x magnification. Both tumors comprise tumor cells of relative low histologic grade (grade 1), with an associated sinusoidal pattern. (g) Line 30-1004, from the established xenograft, at 200x magnification. The tumor cells show a characteristic hepatoid cytology and moderate histologic grade (grade 2). (h) Cell line PLC/PRF/5. Shown is the xenografted tumor at 200x magnification, comprising tumor cells in solid sheets with fine compressed fibrovascular cores and areas of necrosis. No distinct tumor vasculature is observed. (i) Cell line HepG2. Shown is the xenografted tumor at 200x magnification, comprising nests and acini of tumor cells separated by delicate sinusoidal vessels, some of which are ectatic containing red blood cells.

Figure 10 shows the activation of the mTOR signaling pathway in HCC.

(A) Five independent normal (N) and tumor (T) HCC pairs were analyzed by Western blotting using both total and phosphorylation specific antibodies against components of the mTOR signaling pathway (mTOR, p70 S6K, RPS6, and 4EBP1). Compared to adjacent non-malignant normal tissues, HCC tumors exhibit elevated expression levels of phosphorylated p70 S6K (Thr421/Ser424), phosphorylated RPS6 (Ser235/236 and Ser 240/244), and total 4EBP1 (panels with * symbols).

(B) Immunohistochemical analysis of PTEN and phosphorylated RPS6 (Ser 235/236) in HCC. The top panels depict non-malignant background liver samples from four separate HCC patients, stained with antibodies to PTEN (leftmost two panels) and phosphorylated RPS6 (rightmost two panels). The bottom panels depict the corresponding HCC tumors.

(C) Western blot analysis of HCC xenografts using both total and phosphorylation specific antibodies against components of the mTOR signaling pathway. Similar to primary tumors, the majority of xenografts show detectible expression of phosphorylated p70 S6K (Thr421/Ser424), phosphorylated RPS6 (Ser235/236 and Ser 240/244), and total 4EBP1 (panels with * symbols). Line 5-1318(1) appears to show decreased RPS6 phosphorylation compared to other lines, and is slightly less sensitive to RAPA than 2-1318, 26-1004, and 2006 (data not shown).

Figure 11 shows the phenotypic effects of RAPA, BEV, and RAPA/BEV on HCC xenografts.

(a) Effects on gross tumor morphology and size. Shown are representative dissected tumors for HCC xenograft lines 2-1318, 5-1318(3), 2006, and 26-1004. Xenografts were randomized to one of the four treatment groups (n=14 per group) and treated with vehicle (control), RAPA (1 mg/kg), BEV (5 mg/kg), or combined RAPA/BEV. All treatments were initiated after day 3 following tumor cell injection.

(b) Effects on growth rate. Shown are tumor growth rates for xenograft lines 2-1318 and 26-1004 during treatment with vehicle (control), RAPA (1 mg/kg), BEV (5 mg/kg), or RAPA/BEV for 21 days. Differences in tumor volume among the treatment groups were statistically significant ($p<0.01$) as analyzed by ANOVA. Experiments were repeated at least three times with similar results.

Figure 12 shows the molecular effects of RAPA, BEV, and RAPA/BEV in HCC xenografts.

(a) Western blot analysis of line 2006 using both total and phosphorylation-specific antibodies to mTOR pathway components (mTOR, p70S6 kinase, S6R, and 4E-BP1) and cell-cycle components (cyclin D1, cyclin B1, Cdk-2, Cdk-4). The combined RAPA/BEV treatment induced enhanced reductions compared to the control and single treatment arms in the levels of phosphorylated p70 S6K (Thr421/Ser424), and phosphorylated RPS6 (Ser235/236 and Ser 240/244). Downregulation of cyclin D1 was

only observed in the combined treatment arm, and not the single treatment arms. Similar results were obtained for xenograft lines 2-1318, 5-1318(3), 26-1004, and 30-1004 (data not shown). Experiments were repeated at least three times with similar results.

(b) Immunohistochemical analysis of VEGF (left panel) and CD31 (right panel) expression. For VEGF, maximal downregulation of VEGF is observed in the combined treatment arm. For CD31, maximal reductions in the numbers of CD31 positive staining vessels is observed in the combined treatment arm. Similar results were obtained for xenograft lines 2-1318, 5-1318(3), 26-1004, and 30-1004 (data not shown). Experiments were repeated at least three times with similar results.

Figure 13 shows the therapeutic effects of RAPA, BEV, and RAPA/BEV in orthotopic intra-liver tumors, peritoneal metastases, and ascites. The peritoneal cavities of male SCID mice were injected with 26-1004 cells and subsequently treated with vehicle (control), RAPA(1 mg/kg), BEV (5 mg/kg), or RAPA/BEV for 4-6 weeks.

(A) Gross dissections reveal omental tumors (arrows) in the peritoneal cavity of control and treated mice, and dissemination of tumor cells to the mouse livers in control treated animals. Untreated mice exhibit swollen abdomens and accumulation of ascites.

(B) Intra-liver tumor growth. Tumors were detected by immunohistochemical staining with human specific EGFR antibodies. Representative samples are shown. Intra-liver tumors were observed in 14 out of 14 (100%) control mock-treated mice, two out of 14 (14.2%) mice treated with BEV or RAPA, and 0 out of 14 (0%) RAPA/BEV treated mice. Figures in A and B are taken on day 30-36 after inoculation of 26-1004 cells.

(C) Kaplan Meier survival analysis. While all mice in the control, RAPA, and BEV -treated and groups were moribund at day 48, 120, and 118, respectively, mice treated with the RAPA/BEV combination exhibited significantly prolonged overall survival ($p<0.01$, log-rank test) and were all still alive at day 125.

Figure 14 shows the Genomic Profiles of Primary HCC Xenografts

(a) Array-CGH genome copy number analysis : Genomic DNA isolated from 5 pairs of HCC xenografts and their associated primary patient tumors (2-1318, 5-1318(1), 2006, 26-1004(cirr), and 30-1004) were profiled on Agilent 185K microarrays to determine genomic regions of copy number gain or loss. Shown are the genome wide copy number profiles for all chromosomes, with regions of copy number gain represented by increases along the y-axis, and regions of copy number loss represented by decreases along the y-axis. Xenograft profiles are indicated in red, while primary tumor profiles are depicted in blue. A few regions of striking similarity are indicated by the black arrows (eg Chr 1 in line 5-1318(1). A detailed study of the specific amplifications and deletions found in the xenografts and primary HCCs will be reported elsewhere.

(b) Gene expression profiles of HCC xenografts (red), primary HCC tumors (blue), and HCC cell lines (green), clustered by an unsupervised average-linkage hierarchical clustering algorithm using the top 800 most highly varying array probes. Colors within the heat-map are red (high expression) and green (low expression). The HCC xenografts intermingle with the primary tumors, and in several cases a xenograft was most closely associated with its cognate primary tumor (eg 26-1004(cirr), 30-1004).

Figure 15 shows Gene Expression Profiles of RAPA, BEV and RAPA/BEV Treatment

a) RAPA/BEV induced gene expression alterations. 3 independent xenograft lines (2-1318, 5-1318(3), 26-1004) were treated with RAPA/BEV and subsequently expression profiled. Genes that were commonly regulated in all three lines between control and RAPA/BEV treated tumors were identified using a paired t-test with BH correction for multiple hypotheses. The 148 significant genes ($p<0.05$) were visualized in a heat-map diagram where red indicates high expression and green indicates low expression (scale bar for a)-c) is the same). Genes were grouped into two categories : I – regulated in RAPA-only, II – regulation is specific to RAPA/BEV.

b) and c) RAPA and BEV induced gene expression alterations. The 148 RAPA/BEV regulated genes were compared in the RAPA (b) and BEV (c) only

treatments. About 70% of genes (gene set I) were also regulated in RAPA only, and also in BEV to a lesser extent.

d) Comparison of RAPA/BEV and RAPA regulated genes. Expression levels of the 148 RAPA/BEV-regulated genes were compared between the RAPA/BEV and RAPA only treatments. Several genes (>20) in set I are significantly different between RAPA/BEV and RAPA ($P<0.05$, Y.K. data not shown). Genes in set II show a clear difference in expression levels between the RAPA/BEV and RAPA-only treatments. Note that the scale bar for d) is different from a) – c).

Figure 16 shows the Effects of BEV, RAPA, and BEV plus RAPA on Tumors at Sacrifice. 5-1318 xenograft line was subcutaneously implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were daily IP administered with 200 µl saline (vehicle/control), 0.8 mg BEV/kg, 1 mg RAPA/kg, or 200 µl of BEV/RAPA cocktail (This provides 0.8 mg BEV and 1 mg RAPA per kg body weight per day). Treatment commenced after day seven of tumor implantation when the tumors were approximately 100 mg, and continued for two weeks. Tumors (A) and Tumor weight at sacrifice (**B**) for vehicle, BEV, RAPA and BEV plus RAPA of 5-1318 xenografts is shown. Experiments were repeated at least three times with similar results.

Figure 17 shows the Effects of RAPA, BEV, and RAPA/BEV on downstream targets of mTOR. 5-1318 xenograft line was subcutaneously implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were daily IP administered with 200 µl saline (vehicle/control), 0.8 mg BEV/kg, 1 mg RAPA/kg, or 200 µl of BEV/RAPA cocktail (This provides 0.8 mg BEV and 1 mg RAPA per kg body weight per day). Western blot analysis of line 5-1315 using phosphorylation-specific antibodies to mTOR pathway components (p70S6 kinase, S6R, and 4E-BP1). RAPA downregulates phosphorylated 4EBP1 at Ser70, p70S6 at Thr421/424 and S6R at Ser 235/236. The combined RAPA/BEV treatment induced enhanced reductions compared to the control and single treatment arms in the levels of phosphorylated 4EBP1 at Ser37/46.

Figure 18 shows the Effects of RAPA, BEV, and RAPA/BEV on cell cycle regulators. 5-1318 xenograft line was subcutaneously implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were daily IP administered with 200 µl saline (vehicle/control), 0.8 mg BEV/kg, 1 mg RAPA/kg, or 200 µl of BEV/RAPA cocktail (This provides 0.8 mg BEV and 1 mg RAPA per kg body weight per day). Western blot analysis of line 5-1315 using cell-cycle components (p21, p27, cdc-2, survivin, cyclin D1, cyclin B1, Cdk-2, Cdk-4, and p130/Rb2). Downregulation of p21, cdk-2 and upregulation of p130/Rb2 was observed in the combined treatment arm.

DETAILED DESCRIPTION

This invention is based on the surprising discovery that it is possible to treat or prevent cancer, in particular hepatocellular carcinoma (HCC), by inhibiting mTOR activity together with angiogenesis in an individual. We have found that use of an agent capable of antagonising mTOR, in combination with an angiogenesis inhibitor, is effective for treating cancer, neoplasms and tumours in individuals.

We have established that growth of tumour tissue is inhibited by a combination of rapamycin, an mTOR inhibitor, and bevacizumab, an angiogenesis inhibitor, to a significantly greater degree than rapamycin or bevacizumab monotherapy.

We establish that reductions in tumor growth by the combination are associated with alterations in mTOR pathway components, reductions in VEGF levels, and tumor microvessel density (MVD). Furthermore, in an orthotopic setting, a rapamycin / bevacizumab combination potently inhibited both intra-liver and intra-peritoneal tumor growth, reduced ascites levels, and significantly prolonged mouse survival.

Accordingly, the results establish that a combination of an agent capable of antagonising mTOR together with an angiogenesis inhibitor, as represented by a rapamycin / bevacizumab combination, is effective for treating cancer, neoplasms and tumours associated with in particular hepatocellular carcinoma (HCC).

We also find that the combination of an agent capable of antagonising mTOR activity, in combination with an angiogenesis inhibitor, is capable of preventing or slowing down cell proliferation.

We describe methods of treatment of individuals suffering from cancer, particularly hepatocellular carcinoma (HCC), comprising administration (simultaneously or sequentially, in any order), pharmaceutically effective amounts of an agent capable of antagonising mTOR activity and an angiogenesis inhibitor.

A pharmaceutically or therapeutically effective amount is an amount of a composition which achieves the desired effect in an animal, human or individual. The actual amount will vary on a number of factors, as known to those skilled in the art. Using the guidance given herein and knowledge of the art, the determination of a pharmaceutically effective amount is within the ordinary skill of a physician. Pharmaceutically effective amounts designed for particular applications may be packaged as unit doses to facilitate administration.

The term "treating" refers to any indicia of success in the treatment or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the injury, pathology or condition more tolerable to the patient; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; improving a patient's physical or mental well-being; or, in some situations, preventing the onset of cancer.

The treatment or amelioration of symptoms can be based on objective or subjective parameters including the results of a physical examination, laboratory tests, biopsy results, biochemical profiles, etc. For example, the methods and compositions described here may be used to treat cancer, a tumour or a neoplasm in a patient by improving his health and/or slowing or preventing the rate of, or extent of, decline.

"Expression", as in gene expression, is used herein to refer to the process of transcription and translation of a gene to produce a gene product, be it RNA or protein.

Thus, inhibition of expression may occur at any one or more of many levels, including transcription, post-transcriptional processing, translation, post-translational modification, and the like. Agents which modulate gene expression, including transcription or translation, include for example agents which downregulate or knock out endogenous genes; including agents which knock out genes in pluripotent cells which give rise to all or part of an animal.

Inhibition of mTOR or VEGF "synthesis or activity" refers to the inhibition of mTOR or VEGF, as the case may be, at the protein level, to prevent or downregulate the production of the protein, or at least one biological activity of the protein once produced.

COMBINATION

The first agent which is an antagonist of mTOR activity, and the second agent which comprises an angiogenesis inhibitor, may be administered simultaneously, that is to say, at the same time. For this purpose, a mixture of both agents may be administered, or a separate first agent may be administered together with a separate second agent to the individual at the same time.

A composition comprising both agents may be administered to achieve simultaneous administration, or separate compositions, one containing the first agent, and the other containing the second agent, may be administered to the individual at the same time.

The first agent and the second agent may be administered sequentially, that is to say, not at the same time. One agent may be administered, followed by the other. Subsequent administrations of the or each agent may follow. The agents may be alternated, or there may be two or more consecutive administrations of the same agent, at the same or different dosages. Therefore, we envisage regimes such as A1-A2, A2-A1, A1-A2-A1, A2-A1-A2, A1-A2-A1-A2, A2-A1-A2-A1, etc, where A1 is the first agent, and A2 the second agent. Other combinations are of course also possible.

In all cases, the administration of the agents may be by the same route, or a different route. For example, the first agent may be administered by an oral route. The second agent may be administered by a intravenous route.

TREATMENT OF CANCER

The methods and compositions described here suitably enable an improvement in a measurable criterion in an individual to whom the treatment is applied, compared to one who has not received the treatment.

For this purpose, a number of criteria may be designated, which reflect the progress of cancer or the well-being of the patient. Useful criteria may include tumour size, tumour dimension, largest dimension of tumour, tumour number, presence of tumour markers (such as alpha-feto protein), degree or number of metastates, etc.

Thus, as an example, a treated individual may show a decrease in tumour size or number as measured by an appropriate assay or test. A treated individual may for example show a 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or more decrease in tumour size of a particular tumour, or decrease in tumour number, or both, compared to an individual who has not been treated.

In some embodiments, the effect of the treatment is suitably quantified using standard tests, such as the international criteria proposed by the Response Evaluation Criteria in Solid Tumours (RECIST) Committee, as described in detail in Therasse, P., S. G. Arbuck, et al. (2000). "New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada." J Natl Cancer Inst **92**(3): 205-16.

In other embodiments, the effect of the treatment may be quantified by following the administration and testing protocols described in the Clinical Trial (Examples E1 to E8). Thus, assessment of the effect of the treatment may be carried out using one or more

of the protocols, preferably all, as set out in Example E8: Measurement of Effect. Where this is the case, the treatment may result in a Partial Response (PR) or a Complete Response (CR).

Although the controls described above have been described as individuals who have not received treatment, in some cases, a more suitable control may be the patient himself, prior to receiving treatment.

For the purposes of this document, the term "cancer" can comprise any one or more of the following: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), adrenocortical cancer, anal cancer, bladder cancer, blood cancer, bone cancer, brain tumor, breast cancer, cancer of the female genital system, cancer of the male genital system, central nervous system lymphoma, cervical cancer, childhood rhabdomyosarcoma, childhood sarcoma, chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), colon and rectal cancer, colon cancer, endometrial cancer, endometrial sarcoma, esophageal cancer, eye cancer, gallbladder cancer, gastric cancer, gastrointestinal tract cancer, hairy cell leukemia, head and neck cancer, hepatocellular cancer, Hodgkin's disease, hypopharyngeal cancer, Kaposi's sarcoma, kidney cancer, laryngeal cancer, leukemia, leukemia, liver cancer, lung cancer, malignant fibrous histiocytoma, malignant thymoma, melanoma, mesothelioma, multiple myeloma, myeloma, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, nervous system cancer, neuroblastoma, non-Hodgkin's lymphoma, oral cavity cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, parathyroid cancer, penile cancer, pharyngeal cancer, pituitary tumor, plasma cell neoplasm, primary CNS lymphoma, prostate cancer, rectal cancer, respiratory system, retinoblastoma, salivary gland cancer, skin cancer, small intestine cancer, soft tissue sarcoma, stomach cancer, stomach cancer, testicular cancer, thyroid cancer, urinary system cancer, uterine sarcoma, vaginal cancer, vascular system, Waldenstrom's macroglobulinemia and Wilms' tumor.

mTOR

Where the term mTOR is used in this document, it should be taken to refer a polypeptide sequence having the accession number NM_004958.2, P42345 or NP_004949, more particularly NM_004958.2.

Preferably, mTOR refers to a human sequence. Thus, particular homologues encompassed by this term include human homologues, for example, accession numbers NM_004958.2, NP_004949, Hs.509145. However, the term also covers alternative peptides homologous to mTOR, such as polypeptides derived from other species, including other mammalian species. For example, mouse homologues of mTOR having accession number NM_020009.1, NP_064393, Mm .21158 , Q9JLN9, AAF73196 and AF152838 are included. Bovine and rat homologues of mTOR are also known (accession numbers NM_174319 and NM_019906 respectively).

mTOR is also known as FKBP12-Rapamycin Complex-Associated Protein 1, FRAP1, FK506-Binding Protein 12-Rapamycin Complex-Associated Protein 1, FRAP, FRAP2, Mammalian Target of Rapamycin and RAFT1.

Preferably, mTOR includes fragments, homologues, variants and derivatives of such a nucleotide sequence. The terms "variant", "homologue", "derivative" or "fragment" as used here include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acids from or to the sequence of a mTOR nucleotide sequence. Unless the context admits otherwise, references to "mTOR" include references to such variants, homologues, derivatives and fragments of mTOR. These are described in more detail below.

Preferably, the resultant nucleotide sequence encodes a polypeptide having mTOR activity, preferably having at least the same activity of the human mTOR referred to above. Preferably, the term "homologue" is intended to cover identity with respect to structure and/or function such that the resultant nucleotide sequence encodes a polypeptide which has mTOR activity. With respect to sequence identity (i.e. similarity), preferably there is

at least 70%, more preferably at least 75%, more preferably at least 85%, more preferably at least 90% sequence identity. More preferably there is at least 95%, more preferably at least 98%, sequence identity. These terms also encompass allelic variations of the sequences.

The following description of mTOR, referred to as FRAP, is provided from the Online Mendelian Inheritance in Man website (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=601231)

FKBP12-rapamycin associated protein (FRAP) is one of a family of proteins involved in cell cycle progression, DNA recombination, and DNA damage detection. In rat, it is a 245-kD protein (symbolized RAFT1) with significant homology to the Saccharomyces cerevisiae protein TOR1 and has been shown to associate with the immunophilin FKBP12 (186945) in a rapamycin-dependent fashion (Sabatini et al., 1994). Brown et al. (1994) noted that the FKBP12-rapamycin complex was known to inhibit progression through the G1 cell cycle stage by interfering with mitogenic signaling pathways involved in G1 progression in several cell types, as well as in yeast. The authors stated that the binding of FRAP to FKBP12-rapamycin correlated with the ability of these ligands to inhibit cell cycle progression.

Rapamycin is an efficacious anticancer agent against solid tumors. In a hypoxic environment, the increase in mass of solid tumors is dependent on the recruitment of mitogens and nutrients. When nutrient concentrations change, particularly those of essential amino acids, the mammalian target of rapamycin (mTOR/FRAP) functions in regulatory pathways that control ribosome biogenesis and cell growth. In bacteria, ribosome biogenesis is independently regulated by amino acids and ATP. Dennis et al. (2001) demonstrated that the human mTOR pathway is influenced by the intracellular concentration of ATP, independent of the abundance of amino acids, and that mTOR/FRAP itself is an ATP sensor.

Castedo et al. (2001) delineated the apoptotic pathway resulting from human immunodeficiency virus (HIV)-1 envelope glycoprotein (Env)-induced syncytia formation

in vitro and in vivo. Immunohistochemical analysis demonstrated the presence of phosphorylated ser15 of p53 (191170) as well as the preapoptotic marker tissue transglutaminase (TGM2; 190196) in syncytium in the apical light zone (T-cell area) of lymph nodes, as well as in peripheral blood mononuclear cells, from HIV-1-positive but not HIV-1-negative donors. The presence of these markers correlated with viral load (HIV-1 RNA levels). Quantitative immunoblot analysis showed that phosphorylation of ser15 of p53 in response to HIV-1 Env is mediated by FRAP and not by other phosphatidylinositol kinase-related kinases, and it is accompanied by downregulation of protein phosphatase 2A (see 176915). The phosphorylation is significantly inhibited by rapamycin. Immunofluorescence microscopy indicated that FRAP is enriched in syncytial nuclei and that the nuclear accumulation precedes the phosphorylation of ser15 of p53. Castedo et al. (2001) concluded that HIV-1 Env-induced syncytium formation leads to apoptosis via a pathway that involves phosphorylation of ser15 of p53 by FRAP, followed by activation of BAX (600040), mitochondrial membrane permeabilization, release of cytochrome C, and caspase activation.

Fang et al. (2001) identified phosphatidic acid as a critical component of mTOR signaling. In their study, mitogenic stimulation of mammalian cells led to a phospholipase D-dependent accumulation of cellular phosphatidic acid, which was required for activation of mTOR downstream effectors. Phosphatidic acid directly interacted with the domain in mTOR that is targeted by rapamycin, and this interaction was positively correlated with mTOR's ability to activate downstream effectors. The involvement of phosphatidic acid in mTOR signaling reveals an important function of this lipid in signal transduction and protein synthesis, as well as a direct link between mTOR and mitogens. Fang et al. (2001) concluded that their study suggested a potential mechanism for the in vivo actions of the immunosuppressant rapamycin.

Kim et al. (2002) and Hara et al. (2002) reported that mTOR binds with RAPTOR (607130), an evolutionarily conserved protein with at least 2 roles in the mTOR pathway. Kim et al. (2002) showed that RAPTOR has a positive role in nutrient-stimulated signaling to the downstream effector S6K1 (601684), maintenance of cell size, and mTOR protein expression. The association of RAPTOR with mTOR also negatively regulates

mTOR kinase activity. Conditions that repress the pathway, such as nutrient deprivation and mitochondrial uncoupling, stabilize the mTOR-RAPTOR association and inhibit mTOR kinase activity. Kim et al. (2002) proposed that RAPTOR is a component of the mTOR pathway that, through its association with mTOR, regulates cell size in response to nutrient levels.

Hara et al. (2002) showed that the binding of RAPTOR to mTOR is necessary for the mTOR-catalyzed phosphorylation of 4EBP1 (602223) in vitro and that it strongly enhances the mTOR kinase activity toward p70-alpha (S6K1). Rapamycin or amino acid withdrawal increased, whereas insulin strongly inhibited, the recovery of 4EBP1 and RAPTOR on 7-methyl-GTP sepharose. Partial inhibition of RAPTOR expression by RNA interference reduced mTOR-catalyzed 4EBP1 phosphorylation in vitro. RNA interference of C. elegans Raptor yielded an array of phenotypes that closely resembled those produced by inactivation of CE-Tor. Thus, the authors concluded that RAPTOR is an essential scaffold for the mTOR-catalyzed phosphorylation of 4EBP1 and mediates TOR action in vivo.

Vellai et al. (2003) demonstrated that TOR deficiency in *C. elegans* more than doubles its natural life span. The absence of Let363/TOR activity caused developmental arrest at the L3 larval stage. At 25.5 degrees C, the mean life span of Let363 mutants was 25 days compared with a life span of 10 days in wildtype worms.

Huntington disease (HD; 143100) is an inherited neurodegenerative disorder caused by a polyglutamine tract expansion in which expanded polyglutamine proteins accumulate abnormally in intracellular aggregates. Ravikumar et al. (2004) showed that mammalian target of rapamycin (mTOR) is sequestered in polyglutamine aggregates in cell models, transgenic mice, and human brains. Sequestration of mTOR impairs its kinase activity and induces autophagy, a key clearance pathway for mutant huntingtin fragments. This protects against polyglutamine toxicity, as the specific mTOR inhibitor rapamycin attenuates huntingtin accumulation and cell death in cell models of HD, and inhibition of autophagy has converse effects. Furthermore, rapamycin protects against neurodegeneration in a fly model of HD, and the rapamycin analog CCI-779 improved

performance on 4 different behavioral tasks and decreased aggregate formation in a mouse model of HD. The data provided proof of principle for the potential of inducing autophagy to treat HD.

Moore et al. (1996) assigned the FRAP gene to 1p36 by fluorescence in situ hybridization (FISH). Lench et al. (1997) mapped the FRAP gene to 1p36.2 by FISH following radiation-hybrid mapping to that general region. Chromosome 1p36.2 is the region most consistently deleted in neuroblastomas. Given the role of PIK-related kinase proteins in DNA repair, recombination, and cell cycle checkpoints, the authors suggested that the possible role of FRAP in solid tumors with deletions at 1p36 should be investigated. Onyango et al. (1998) established the order of genes in the 1p36 region, telomere to centromere, as CDC2L1 (176873)--PTPRZ2 (604008)--ENO1 (172430)--PGD (172200)--XBX1 (604007)--FRAP2 (FRAP1)--CD30 (153243).

mTOR is described in detail in Beugnet, et al. J. Biol. Chem. 278 (42), 40717-40722 (2003); Kristof, et al., J. Biol. Chem. 278 (36), 33637-33644 (2003); Chen,Y., et al., Oncogene 22 (25), 3937-3942 (2003); Garami, et al., Mol. Cell 11 (6), 1457-1466 (2003); Nojima, et al., J. Biol. Chem. 278 (18), 15461-15464 (2003); Kimura, et al., Genes Cells 8 (1), 65-79 (2003); McMahon, et al., Mol. Cell. Biol. 22 (21), 7428-7438 (2002); Tee, et al., Proc. Natl. Acad. Sci. U.S.A. 99 (21), 13571-13576 (2002); Hudson, et al., Mol. Cell. Biol. 22 (20), 7004-7014 (2002); Choi, et al., EMBO Rep. 3 (10), 988-994 (2002); Inoki, et al., Nat. Cell Biol. 4 (9), 648-657 (2002); Zhang, et al., J. Biol. Chem. 277 (31), 28127-28134 (2002); Castedo, et al., EMBO J. 21 (15), 4070-4080 (2002); Hara, et al., Cell 110 (2), 177-189 (2002); Kim, et al., Cell 110 (2), 163-175 (2002); Fingar, et al., Genes Dev. 16 (12), 1472-1487 (2002); Reynolds, et al., J. Biol. Chem. 277 (20), 17657-17662 (2002); Fang, et al., Science 294 (5548), 1942-1945 (2001); Dennis, et al., Science 294 (5544), 1102-1105 (2001); Onyango, et al., Genomics 50 (2), 187-198 (1998); Lench, et al., Hum. Genet. 99 (4), 547-549 (1997); Choi, et al., Science 273 (5272), 239-242 (1996); Moore, et al., Genomics 33 (2), 331-332 (1996); Chen, et al., Proc. Natl. Acad. Sci. U.S.A. 92 (11), 4947-4951 (1995); Chiu et al., Proc. Natl. Acad. Sci. U.S.A. 91 (26), 12574-12578 (1994); Brown, et al., Nature 369 (6483), 756-758 (1994).

INHIBITOR OF mTOR ACTIVITY

The methods and compositions described here rely, in some embodiments, on blocking, reducing, or decreasing the activity of mTOR protein. Such inhibition of mTOR activity may be used in conjunction with inhibition of angiogenesis to treat cancer or prevent cell or tissue growth or proliferation according to the methods and compositions described here.

While any means of doing so may be used, in general, the methods and compositions described here employ modulators of mTOR activity or expression. Agents which are capable of decreasing the activity of mTOR protein are referred to as inhibitors or antagonists of that activity. For the purpose of this document, the terms "inhibitor" and "antagonist" may be regarded as synonymous, where the context requires.

In preferred embodiments, antagonists of mTOR activity have the ability to decrease a relevant activity of mTOR, for example, kinase activity, by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more. Preferably, mTOR activity is assayed as described below in the section "Assays for mTOR Activity".

The term "antagonist", as used in the art, is generally taken to refer to a compound which binds to an enzyme and inhibits the activity of the enzyme. The term as used here, however, is intended to refer broadly to any agent which inhibits the activity of a molecule, not necessarily by binding to it. Accordingly, it includes agents which affect the expression of an mTOR protein, or the biosynthesis of a regulatory molecule, or the expression of modulators of the activity of mTOR. The specific activity which is inhibited may be any activity which is exhibited by, or characteristic of, the enzyme or molecule, for example, any activity of mTOR as the case may be, for example, a kinase activity. The kinase activity may comprise the ability to phosphorylate one or either of S6K1 and/or 4E-BP1.

The antagonist may bind to and compete for one or more sites on the relevant molecule preferably, the catalytic site of the enzyme. Preferably, such binding blocks the

interaction between the molecule and another entity (for example, the interaction between a enzyme and its substrate). However, the antagonist need not necessarily bind directly to a catalytic site, and may bind for example to an adjacent site, another protein (for example, a protein which is complexed with the enzyme) or other entity on or in the cell, so long as its binding reduces the activity of the enzyme or molecule.

Where antagonists of a enzyme such as mTOR are concerned, an antagonist may include a substrate of the enzyme, or a fragment of this which is capable of binding to the enzyme. In addition, whole or fragments of a substrate generated natively or by peptide synthesis may be used to compete with the substrate for binding sites on the enzyme. Alternatively, or in addition, an immunoglobulin (for example, a monoclonal or polyclonal antibody) capable of binding to the enzyme may be used. The antagonist may also include a peptide or other small molecule which is capable of interfering with the binding interaction. Other examples of antagonists are set forth in greater detail below, and will also be apparent to the skilled person.

Non-functional homologues of a mTOR may also be tested for inhibition of mTOR activity as they may compete with the wild type protein for binding to other components of the cell machinery whilst being incapable of the normal functions of the protein. Alternatively, they may block the function of the protein bound to the cell machinery. Such non-functional homologues may include naturally occurring mutants and modified sequences or fragments thereof.

Alternatively, instead of preventing the association of the components directly, the substance may suppress the biologically available amount of a mTOR. This may be by inhibiting expression of the component, for example at the level of transcription, transcript stability, translation or post-translational stability. An example of such a substance would be antisense RNA or double-stranded interfering RNA sequences which suppresses the amount of mRNA biosynthesis.

Blocking the activity of an inhibitor of the mTOR protein may therefore also be achieved by reducing the level of expression of the protein or an inhibitor in the cell. For

example, the cell may be treated with antisense compounds, for example oligonucleotides having sequences specific to the mTOR mRNA. The level of expression of pathogenic forms of adhesion proteins may also be regulated this way.

In general, agonists, antagonists of mTOR may comprise agents such as an atom or molecule, wherein a molecule may be inorganic or organic, a biological effector molecule and/or a nucleic acid encoding an agent such as a biological effector molecule, a protein, a polypeptide, a peptide, a nucleic acid, a peptide nucleic acid (PNA), a virus, a virus-like particle, a nucleotide, a ribonucleotide, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a modified amino acid analogue, a steroid, a proteoglycan, a lipid, a fatty acid and a carbohydrate. An agent may be in solution or in suspension (e.g., in crystalline, colloidal or other particulate form). The agent may be in the form of a monomer, dimer, oligomer, etc, or otherwise in a complex.

The terms "modulator", "antagonist" and "agent" are also intended to include, a protein, polypeptide or peptide including, but not limited to, a structural protein, an enzyme, a cytokine (such as an interferon and/or an interleukin) an antibiotic, a polyclonal or monoclonal antibody, or an effective part thereof, such as an Fv fragment, which antibody or part thereof may be natural, synthetic or humanised, a peptide hormone, a receptor, a signalling molecule or other protein; a nucleic acid, as defined below, including, but not limited to, an oligonucleotide or modified oligonucleotide, an antisense oligonucleotide or modified antisense oligonucleotide, cDNA, genomic DNA, an artificial or natural chromosome (e.g. a yeast artificial chromosome) or a part thereof, RNA, including mRNA, tRNA, rRNA or a ribozyme, or a peptide nucleic acid (PNA); a virus or virus-like particles; a nucleotide or ribonucleotide or synthetic analogue thereof, which may be modified or unmodified; an amino acid or analogue thereof, which may be modified or unmodified; a non-peptide (e.g., steroid) hormone; a proteoglycan; a lipid; or a carbohydrate. Small molecules, including inorganic and organic chemicals, which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented, are also

included. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

In a particular embodiment, the technique of RNA interference (RNAi) may be used to abolish or knock out or reduce gene activity, for example, mTOR activity. The overall strategy is to prepare double stranded RNA (dsRNA) specific to each gene of interest and to transfect this into a cell of interest to inhibit the expression of the particular gene.

The following protocol may be used: a sample of PCR product is analysed by horizontal gel electrophoresis and the DNA purified using a Qiagen QiaQuick PCR purification kit. 1μg of DNA is used as the template in the preparation of gene specific single stranded RNA using the Ambion T7 Megascript kit. Single stranded RNA is produced from both strands of the template and is purified and immediately annealed by heating to 90 degrees C for 15 mins followed by gradual cooling to room temperature overnight. A sample of the dsRNA is analysed by horizontal gel electrophoresis, and introduced into the relevant cell by conventional means.

ANTAGONISTS OF mTOR ACTIVITY

Any agent which is capable of reducing mTOR activity or expression, as described above, may be used as an antagonist of mTOR for the purposes of reducing its activity.

Butanol

1-Butanol is an inhibitor of mTOR activity, as described in Kam and Exton, FASEB J. 2004 Feb;18(2):311-9 and Fang et al., Science 294:1942-1945. Butanol may therefore be used in the methods and compositions described here as an agent capable of reducing mTOR activity.

Anti-Peptide mTOR Antibodies

Anti-peptide antibodies may be produced against mTOR peptide sequences. The sequences chosen may be based on the mouse sequences as follow from the following mTOR reference sequence:

```
   1 mlgtgpavat asaatssnvs vlqqfasglk srneetraka akelqhyvtm elremsqees
  61 trfydqlnhh ifelvsssda nerkggilai asligveggn strigrfany lrnllpssdp
 121 vvmemaskai grlamagdtf taeyvefevk ralewlgadr negrrhaavl vlrelaisvp
 181 tfffqqvqpf fdnifvavwd pkqairegav aalraclilt tqrepkemqk pqwyrhtfee
 241 aekgfdetla kekgmnrddr ihgallilne lvrissmege rlreemeeit qqqlvhdkyc
 301 kdlmgfgtkp rhitpftsfq avqpqqpnal vgllgysspq glmgfgtsps pakstlvesr
 361 ccrdlmeekf dqvcqwvlkc rssknsliqm tilnllprla afrpsaftdt qylqdtmnhv
 421 lscvkkeker taafqalgll svavrsefkv ylprvldiir aalppkdfah krqktvqvda
 481 tvftcismla ramgpgiqqd ikellepmla vglspaltav lydlsrqipq lkkdiqdgll
 541 kmlslvlmhk plrhpgmpkg lahqlaspgl ttlpeasdva sitlalrtlg sfefeghslt
 601 qfvrhcadhf lnsehkeirm eaartcscll tpsihlisgh ahvvsqtavq vvadvlskll
 661 vvgitdpdpd irycvlasld erfdahlaqa enlqalfval ndqvfeirel aictvgrlss
 721 mnpafvmpfl rkmliqilte lehsgigrik eqsarmlghl vsnaprlirp ymepilkali
 781 lklkdpdpdp npgvinnvla tigelaqvsg lemrkwvdel fiiimdmlqd ssllakrqva
 841 lwtlgqlvas tgyvvepyrk yptllevlln flkteqnqgt rreairvlgl lgaldpykhk
 901 vnigmidqsr dasavslses kssqdssdys tsemlvnmgn lpldefypav smvalmrifr
 961 dqslshhhtm vvqaitfifk slglkcvqfl pqvmptflnv irvcdgaire flfqqlgmlv
1021 sfvkshirpy mdeivtlmre fwvmntsiqs tiillieqiv valggefkly lpqliphmlr
1081 vfmhdnsqgr ivsikllaai qlfganlddy lhlllppivk lfdapevplp srkaaletvd
1141 rltesldftd yasriihpiv rtldqspelr stamdtlssl vfqlgkkyqi fipmvnkvlv
1201 rhrinhqryd vlicrivkgy tladeeedpl iyqhrmlrss qgdalasgpv etgpmkklhv
1261 stinlqkawg aarrvskddw lewlrrlsle llkdssspsl rscwalaqay npmardlfna
1321 afvscwseln edqqdelirs ielaltsqdi aevtqtllnl aefmehsdkg plplrddngi
1381 vllgeraakc rayakalhyk elefqkgptp aileslisin nklqqpeaas gvleyamkhf
1441 geleiqatwy eklhewedal vaydkkmdtn kedpelmlgr mrclealgew gqlhqqccek
1501 wtlvndetqa kmarmaaaaa wglgqwdsme eytcmiprdt hdgafyravl alhqdlfsla
1561 qqcidkardl ldaeltamag esysraygam vschmlsele eviqyklvpe rreiirqiww
1621 erlqgcqriv edwqkilmvr slvvsphedm rtwlkyaslc gksgrlalah ktlvlllgvd
1681 psrqldhplp tahpqvtyay mknmwksark idafqhmqhf vqtmqqqàqh aiatedqqhk
1741 qelhklmarc flklgewqln lqginestip kvlqyysaat ehdrswykaw hawavmnfea
1801 vlhykhqnqa rdekkklrha sganitnatt aattaasaaa atstegsnse seaesnensp
1861 tpsplqkkvt edlsktllly tvpavqgffr sislsrgnnl qdtlrvltlw fdyghwpdvn
1921 ealvegvkai qidtwlqvip qliaridtpr plvgrlihql ltdigryhpq aliypltvas
1981 kstttarhna ankilknmce hsntlvqqam mvseelirva ilwhemwheg leeasrlyfg
2041 ernvkgmfev leplhammer gpqtlketsf nqaygrdlme aqewcrkymk sgnvkdltqa
2101 wdlyyhvfrr iskqlpqlts lelqyvspkl lmcrdlelav pgtydpnqpi iriqsiapsl
2161 qvitskqrpr kltlmgsngh efvfllkghe dlrqdervmq lfglvntlla ndptslrknl
2221 siqryavipl stnsgligwv phcdtlhali rdyrekkkil lniehrimlr mapdydhltl
2281 mqkvevfeha vnntagddla kllwlkspss evwfdrrtny trslavmsmv gyilglgdrh
2341 psnlmldrls gkilhidfgd cfevamtrek fpekipfrlt rmltnamevt gldgnyrttc
2401 htvmevlreh kdsvmavlea fvydpllnwr lmdtntkgnk rsrtrtdsys agqsveildg
2461 velgepahkk agttvpesih sfigdglvkp ealnkkaiqi inrvrdkltg rdfshddtld
2521 vptqvellik qatshenlcq cyigwcpfw
```

Thus, preferred anti-peptide antibodies may be raised from any one or more of the following sequences: amino acids 22-139; amino acids 647-907; amino acids 937-1140; amino acids 1382-1982; amino acids 2019-2112; or amino acids 2181-2549.

Corresponding sequences from human mTOR may be chosen for use in eliciting anti-peptide antibodies from immunised animals. Antibodies may be produced by injection into rabbits, and other conventional means, as described in for example, Harlow and Lane (supra).

Antibodies are checked by Elisa assay and by Western blotting, and used for immunostaining as described in the Examples.

RAPAMYCIN

In some embodiments, an agent capable of reducing mTOR activity comprises rapamycin. As the term is used in this document, "rapamycin" includes the specific compound rapamycin (also known as Sirolimus, C_{51} H_{79} NO_{13}, which is described below) as well as any of its derivatives. Such derivatives are described in detail and include rapamycin prodrugs, rapamycin dialdehydes, structural analogues of rapamycin (rapalogs), etc.

Rapamycin, including its derivatives, etc, is therefore provided as a specific antagonist of mTOR activity.

Rapamycin and its derivatives may be employed at concentrations over 1nM, for example, 10nM, 20nM, 30nM, 40nM, 50 nM, 100nM, 500nM, 1μm, 10μm, 100μm, or more. In some embodiments, rapamycin and its derivatives are used at about 50nM. Rapamycin and its derivatives may be administered to human individuals at dosages of for example between about 1 mg/day and 10 mg/day.

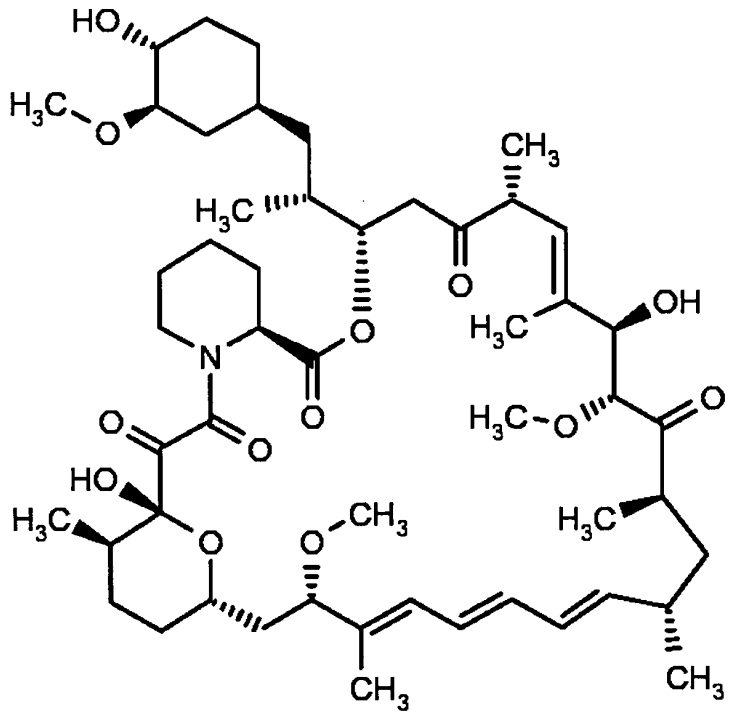
Rapamycin (Sirolimus)

Rapamycin ($C_{51}H_{79}NO_{13}$, molecular mass 914.172 g/mol.) is an antifungal antibiotic which is extractable from a streptomycete, e.g., *Streptomyces hygroscopicus*.

Rapamycin has an IUPAC name of (3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-

9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]-oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone.

Rapamycin is identified by its CAS number 53123-88-9, ATC code L04AA10, PubChem 6436030, DrugBank APRD00178. The structural formula of rapamycin is shown below:



Rapamycin is also known as Sirolimus.

Methods for the preparation of rapamycin are disclosed in Sehgal et al., U.S. Pat. Nos. 3,929,992, and 3,993,749. In addition, monoacyl and diacyl derivatives of rapamycin and methods for their preparation are disclosed by Rakhit, U.S. Pat. No. 4,316,885. Furthermore, Stella et al., U.S. Pat. No. 4,650,803 disclose water soluble prodrugs of rapamycin, i.e., rapamycin derivatives including the following rapamycin prodrugs: glycinate prodrugs, propionate prodrugs and the pyrrolidino butyrate prodrugs.

The methods and compositions described here include the use of natural and synthetic rapamycin, genetically engineered rapamycin and all derivatives and prodrugs of rapamycin, such as described in the aforementioned U.S. patents, U.S. Pat. Nos. 3,929,992; 3,993,749; 4,316,885; and 4,650,803, the contents of which are hereby incorporated by reference.

Rapamycin is a 31-membered macrolide lactone, C_{51} H_{79} NO_{13}, with a molecular mass of 913.6 Da. In solution, sirolimus forms two conformational trans-, cis-isomers with a ratio of 4:1 (chloroform) due to hindered rotation around the pipecolic acid amide bond. It is sparingly soluble in water, aliphatic hydrocarbons and diethyl ether, whereas it is soluble in alcohols, halogenated hydrocarbons and dimethyl sulfoxide. Rapamycin is unstable in solution and degrades in plasma and low-, and neuteral-pH buffers at 37 degrees C with half-life of <10 h. the structures of the degradation products have recently been characterized. Rapamycin is a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus, which was found to have antifungal activity, particularly against Candida albicans, both in vitro and in vivo [C. Vezina et al., J. Antibiot. 28, 721 (1975); S. N. Sehgal et al., J. Antibiot. 28, 727 (1975); H. A. Baker et al., J. Antibiot. 31, 539 (1978); U.S. Pat. No. 3,929,992; and U.S. Pat. No. 3,993,749].

Rapamycin alone (U.S. Pat. No. 4,885,171) or in combination with picibanil (U.S. Pat. No. 4,401,653) has been shown to have antitumor activity. R. Martel et al. [Can. J. Physiol. Pharmacol. 55, 48 (1977)] disclosed that rapamycin is effective in the experimental allergic encephalomyelitis model, a model for multiple sclerosis; in the adjuvant arthritis model, a model for rheumatoid arthritis; and effectively inhibited the formation of IgE-like antibodies.

The immunosuppressive effects of rapamycin have been disclosed in FASEB 3, 3411 (1989). Cyclosporin A and FK-506, other macrocyclic molecules, also have been shown to be effective as immunosuppressive agents, therefore useful in preventing transplant rejection [FASEB 3, 3411 (1989); FASEB 3, 5256 (1989); and R. Y. Calne et al., Lancet 1183 (1978)]. Although it shares structural homology with the immunosuppressant tacrolimus and binds to the same intracellular binding protein in

lymphocytes, rapamycin inhibits S6p70-kinase and therefore has a mechanism of immunosuppressive action distinct from that of tacrolimus. Rapamycin was found to prolong graft survival of different transplants in several species alone or in combination with other immunosupressants. In animal models its spectrum of toxic effects is different from that of cyclosporin or FK-506, comprising impairment of glucose homeostasis, stomach, ulceration, weight loss and thrombocytopenia, although no nephrotoxicity has been detected.

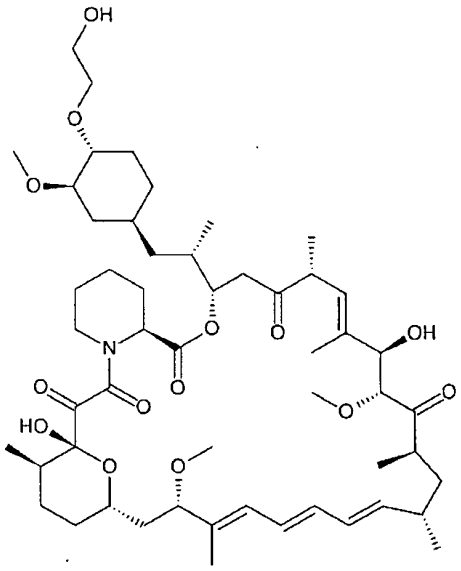
Rapamycin Derivatives

Rapamycin derivatives include rapamycin prodrugs, rapamycin dialdehydes, structural analogues of rapamycin (rapalogs), etc, and are described in detail below.

Specific derivatives of rapamycin which may be used in the methods and compositions described here include RAD001 (Everolimus) and CCI-779 (Wyeth).

RAD001 (Everolimus)

RAD001 ($C_{53}H_{83}NO_{14}$, molecular mass 958.224 g/mol) is a derivative of rapamycin. RAD001 is identified by its CAS number 159351-69-6, ATC code L04AA18 and PubChem 6442177. The structural formula of RAD001 is shown below:



RAD001 is also known as Everolimus and is manufactured by Novartis AG. It is currently used as an immunosuppressant to prevent rejection of organ transplants.

RAD001 is described in detail in O'Reilly TM,Wood JM, Littlewood-Evans A, et al. Differential anti-vascular effects of mTOR or VEGFR pathway inhibition: a rational basis for combining RAD001 and PTK787/ZK222584. Presented at: 96th Annual Meeting of the American Association for Cancer Research. Anaheim, Calif; April 16-20, 2005. Abstract 3038.

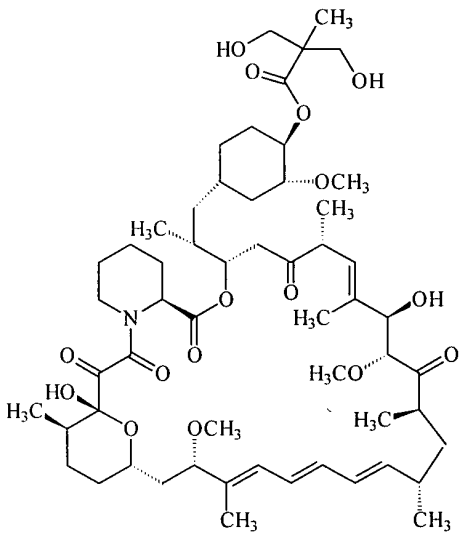
RAD001 is also described in 105. Van Oosterom AT, Dumez H, Desai J, et al. Combination signal transduction inhibition: a phase I/II trial of the oral mTOR-inhibitor everolimus (E, RAD001) and imatinib mesylate (IM) in patients (pts) with gastrointestinal stromal tumor (GIST) refractory to IM [abstract]. Proc Am Soc Clin Oncol. 2004;23:195. Abstract 3002.

CCI 779 (Temsirolimus)

CCI 779 (cell cycle inhibitor-779, $C_{56}H_{87}NO_{16,}$ molecular weight 1030.3) is an ester analogue of Rapamycin.

CCI 779 is also known as rapamcyin-28-N,N-dimethlyglycinate methanesulfonate salt, rapamycin, 42-[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate], (3*S*,6*R*,7*E*,9*R*,10*R*,12*R*,14*S*,15*E*,17*E*,19*E*,21*S*,23*S*,26*R*,27*R*,34a*S*)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1*R*)-2-[(1*S*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3*H*-pyrido[2,1-*c*][1,4]oxaazacyclohentriacontine-1,5,11,28,29(4*H*,6*H*,31*H*)-pentone 4′-[2,2-bis(hydroxymethyl)propionate] and rapamycin 42-[2,2-bis(hydroxymethyl)propionate].

CCI 779 is identified by its CAS registry number 162635-04-3. The structural formula of CCI 779 is shown below:



CCI 779 is also known as Temsirolimus and is manufactured by Wyeth. Temsirolimus binds to the cytosolic protein, FKBP, which subsequently inhibits mTOR (mammalian target of rapamycin).

In animal models of human cancers, temsirolimus has been found to inhibit the growth of a diverse range of cancer types even when an intermittent dosing schedule was used. The compound also appears to have potential for the blockade of inflammatory responses associated with autoimmune and rheumatic diseases by inhibiting T-cell proliferation.

CCI 779 is a water soluble ester (prodrug) of rapamycin that releases rapamycin *in vivo*. It is believed to be more tolerable than rapamycin when used clinically and is currently being studied for use in oncology patients in Phase II and III trials (including brain tumors).

CCI 779 is described in detail in Nat Genet. 2004;36:585-95 and J Clin Oncol. 2004;22:2336-47. Reference should also be made to K Yu, L Toral-Barza, C Discafani, WG Zhang, J Skotnicki, P Frost, and JJ Gibbons (2001). mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. Endocrine-Related Cancer 8 (3) 249-258 and Josep Maria Peralba, Linda deGraffenried,

William Friedrichs, Letitia Fulcher, Viktor Grünwald, Geoffrey Weiss and Manuel Hidalgo (2003. Pharmacodynamic Evaluation of CCI-779, an Inhibitor of mTOR, in Cancer Patients. Clinical Cancer Research Vol. 9, 2887-2892.

Rapamycin Prodrugs

The mTOR inhibitor, particularly rapamycin, may be provided in the form of a prodrug. A specific example of a rapamycin prodrug is CCI 779, described above.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" Biochemical Society Transactions, 14, pp. 375 382,615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," Directed Drug Delivery, Borchardt et al., (ed.), pp. 247 267, Humana Press (1985). The prodrugs described here include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, .beta.-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of drugs that can be derivatized into a prodrug form for use in the methods and compositions described here include, but are not limited to, those chemotherapeutic agents described above.

Rapamycin Dialdebydes

Rapamycin prodrugs such as rapamycin dialdehydes described in United States Patent 6,680,330 (Zhu, et al) may be employed in the methods and compositions described here.

Mono- and diacylated derivatives of rapamycin (esterified at the 28 and 43 positions) have been shown to be useful as antifungal agents (U.S. Pat. No. 4,316,885) and used to make water soluble prodrugs of rapamycin (U.S. Pat. No. 4,650,803). Recently, the numbering convention for rapamycin has been changed; therefore according to Chemical Abstracts nomenclature, the esters described above would be at the 31-and 42-positions. Carboxylic acid esters (PCT application No. WO 92/05179), carbamates (U.S. Pat. No. 5,118,678), amide esters (U.S. Pat. No. 5,118,678), (U.S. Pat. No. 5,118,678) fluorinated esters (U.S. Pat. No. 5,100,883), acetals (U.S. Pat. No. 5,151413), silyl ethers (U.S. Pat. No. 5,120,842), bicyclic derivatives (U.S. Pat. No. 5,120,725), rapamycin dimers (U.S. Pat. No. 5,120,727) and O-aryl, O-alkyl, O-alkyenyl and O-alkynyl derivatives (U.S. Pat. No. 5,258,389) have been described.

Rapamycin is metabolized by cytochrome P-450 3A to at least six metabolites. During incubation with human liver and small intestinal microsomes, sirolimus was hydroxylated and demethylated and the structure of 39-O-demethyl sirolimus was identified. In bile of sirolimus-treated rats >16 hydroxylated and demethylated metabolites were detected.

In rapamycin, demethylation of methoxy group at C-7 Carbon will lead to the change in the conformation of the Rapamycin due to the interaction of the released C-7 hydroxyl group with the neighbouring pyran ring system which is in equilibrium with the open form of the ring system. The C-7 hydroxyl group will also interact with the triene system and possibly alter the immunosupressive activity of rapamycin. This accounts for the degradation of rapamycin molecule and its altered activity.

Structural Analogues of Rapamycin (Rapalogs)

A large number of structural variants of rapamycin have been reported, typically arising as alternative fermentation products or from synthetic efforts to improve the compound's therapeutic index as an immunosuppressive agent. Each of these may be employed in the methods and compositions described here.

For example, the extensive literature on analogs, homologs, derivatives and other compounds related structurally to rapamycin ("rapalogs") include among others variants of rapamycin having one or more of the following modifications relative to rapamycin: demethylation, elimination or replacement of the methoxy at C7, C42 and/or C29; elimination, derivatization or replacement of the hydroxy at C13, C43 and/or C28; reduction, elimination or derivatization of the ketone at C14, C24 and/or C30; replacement of the 6-membered pipecolate ring with a 5-membered prolyl ring; and alternative substitution on the cyclohexyl ring or replacement of the cyclohexyl ring with a substituted cyclopentyl ring. In nearly all cases, potent immunosuppressive activity is reported to accompany antifungal activity of the rapalogs. Additional historical information is presented in the background sections of U.S. Pat. Nos. 5,525,610; 5,310,903 and 5,362,718.

Rapalogs

"Rapalogs" as that term is used herein denotes a class of compounds comprising the various analogs, homologs and derivatives of rapamycin and other compounds related structurally to rapamycin. "Rapalogs" include compounds other than rapamycin (or those rapamycin derivatives modified in comparison to rapamycin only with respect to saturation of one or more of the carbon--carbon double bonds at the 1, 2, 3, 4 or 5, 6 positions) which comprise the substructure shown in Formula I, bearing any number of a variety of substituents, and optionally unsaturated at one or more carbon--carbon bonds unless specified to the contrary herein.

Rapalogs include, among others, variants of rapamycin having one or more of the following modifications relative to rapamycin: demethylation, elimination or replacement of the methoxy at C7, C42 and/or C29; elimination, derivatization or replacement of the hydroxy at C13, C43 and/or C28; reduction, elimination or derivatization of the ketone at C14, C24 and/or C30; replacement of the 6-membered pipecolate ring with a 5-membered prolyl ring; and elimination, derivatization or replacement of one or more substituents of the cyclohexyl ring or replacement of the cyclohexyl ring with a substituted or unsubstituted cyclopentyl ring. Rapalogs, as that term is used herein, do not include rapamycin itself, and preferably do not contain an oxygen bridge between C1 and C30.

Illustrative examples of rapalogs are disclosed in the documents listed in Table I. Examples of rapalogs modified at C7 are shown in Table II.

TABLE I

WO9710502	WO9418207	WO9304680	US5527907	US5225403
WO9641807	WO9410843	WO9214737	US5484799	US5221625
WO9635423	WO9409010	WO9205179	US5457194	US5210030
WO9603430	WO9404540	US5604234	US5457182	US5208241
WO9600282	WO9402485	US5597715	US5362735	US5200411
WO9516691	WO9402137	US5583139	US5324644	US5198421
WO9515328	WO9402136	US5563172	US5318895	US5147877
WO9507468	WO9325533	US5561228	US5310903	US5140018
WO9504738	WO9318043	US5561137	US5310901	US5116756
WO9504060	WO9313663	US5541193	US5258389	US5109112
WO9425022	WO9311130	US5541189	US5252732	US5093338
WO9421644	WO9310122	US5534632	US5247076	US5091389

Anti-Peptide mTOR Antibodies

Anti-peptide antibodies may be produced against mTOR peptide sequences. The sequences chosen may be based on the mouse sequences as follow from the following mTOR reference sequence:

```
   1 mlgtgpavat asaatssnvs vlqqfasglk srneetraka akelqhyvtm elremsqees
  61 trfydqlnhh ifelvsssda nerkggilai asligveggn strigrfany lrnllpssdp
 121 vvmemaskai grlamagdtf taeyvefevk ralewlgadr negrrhaavl vlrelaisvp
 181 tfffqqvqpf fdnifvavwd pkqairegav aalraclilt tqrepkemqk pqwyrhtfee
 241 aekgfdetla kekgmnrddr ihgallilne lvrissmege rlreemeeit qqqlvhdkyc
 301 kdlmgfgtkp rhitpftsfq avqpqqpnal vgllgysspq glmgfgtsps pakstlvesr
 361 ccrdlmeekf dqvcqwvlkc rssknsliqm tilnllprla afrpsaftdt qylqdtmnhv
 421 lscvkkeker taafqalgll svavrsefkv ylprvldiir aalppkdfah krqktvqvda
 481 tvftcismla ramgpgiqqd ikellepmla vglspaltav lydlsrqipq lkkdiqdgll
 541 kmlslvlmhk plrhpgmpkg lahqlaspgl ttlpeasdva sitlalrtlg sfefeghslt
 601 qfvrhcadhf lnsehkeirm eaartcscll tpsihlisgh ahvvsqtavq vvadvlskll
 661 vvgitdpdpd irycvlasld erfdahlaqa enlqalfval ndqvfeirel aictvgrlss
 721 mnpafvmpfl rkmliqilte lehsgigrik eqsarmlghl vsnaprlirp ymepilkali
 781 lklkdpdpdp npgvinnvla tigelaqvsg lemrkwvdel fiiimdmlqd ssllakrqva
 841 lwtlgqlvas tgyvvepyrk yptllevlln flkteqnqgt rreairvlgl lgaldpykhk
 901 vnigmidqsr dasavslses kssqdssdys tsemlvnmgn lpldefypav smvalmrifr
 961 dqslshhhtm vvqaitfifk slglkcvqfl pqvmptflnv irvcdgaire flfqqlgmlv
1021 sfvkshirpy mdeivtlmre fwvmntsiqs tiillieqiv valggefkly lpqliphmlr
1081 vfmhdnsqgr ivsikllaai qlfganlddy lhlllppivk lfdapevplp srkaaletvd
1141 rltesldftd yasriihpiv rtldqspelr stamdtlssl vfqlgkkyqi fipmvnkvlv
1201 rhrinhqryd vlicrivkgy tladeeedpl iyqhrmlrss qgdalasgpv etgpmkklhv
1261 stinlqkawg aarrvskddw lewlrrlsle llkdssspsl rscwalaqay npmardlfna
1321 afvscwseln edqqdelirs ielaltsqdi aevtqtllnl aefmehsdkg plplrddngi
1381 vllgeraakc rayakalhyk elefqkgptp aileslisin nklqqpeaas gvleyamkhf
1441 geleiqatwy eklhewedal vaydkkmdtn kedpelmlgr mrclealgew gqlhqqccek
1501 wtlvndetqa kmarmaaaaa wglgqwdsme eytcmiprdt hdgafyravl alhqdlfsla
1561 qqcidkardl ldaeltamag esysraygam vschmlsele eviqyklvpe rreiirqiww
1621 erlqgcqriv edwqkilmvr slvvsphedm rtwlkyaslc gksgrlalah ktlvlllgvd
1681 psrqldhplp tahpqvtyay mknmwksark idafqhmqhf vqtmqqqaqh aiatedqqhk
1741 qelhklmarc flklgewqln lqginestip kvlqyysaat ehdrswykaw hawavmnfea
```

```
1801 vlhykhqnqa rdekkklrha sganitnatt aattaasaaa atstegsnse seaesnensp
1861 tpsplqkkvt edlsktllly tvpavqgffr sislsrgnnl qdtlrvltlw fdyghwpdvn
1921 ealvegvkai qidtwlqvip qliaridtpr plvgrlihql ltdigryhpq aliypltvas
1981 kstttarhna ankilknmce hsntlvqqam mvseelirva ilwhemwheg leeasrlyfg
2041 ernvkgmfev leplhammer gpqtlketsf nqaygrdlme aqewcrkymk sgnvkdltqa
2101 wdlyyhvfrr iskqlpqlts lelqyvspkl lmcrdlelav pgtydpnqpi iriqsiapsl
2161 qvitskqrpr kltlmgsngh efvfllkghe dlrqdervmq lfglvntlla ndptslrknl
2221 siqryavipl stnsgligwv phcdtlhali rdyrekkkil lniehrimlr mapdydhltl
2281 mqkvevfeha vnntagddla kllwlkspss evwfdrrtny trslavmsmv gyilglgdrh
2341 psnlmldrls gkilhidfgd cfevamtrek fpekipfrlt rmltnamevt gldgnyrttc
2401 htvmevlreh kdsvmavlea fvydpllnwr lmdtntkgnk rsrtrtdsys agqsveildg
2461 velgepahkk agttvpesih sfigdglvkp ealnkkaiqi inrvrdkltg rdfshddtld
2521 vptqvellik qatshenlcq cyigwcpfw
```

Thus, preferred anti-peptide antibodies may be raised from any one or more of the following sequences: amino acids 22-139; amino acids 647-907; amino acids 937-1140; amino acids 1382-1982; amino acids 2019-2112; or amino acids 2181-2549.

Corresponding sequences from human mTOR may be chosen for use in eliciting anti-peptide antibodies from immunised animals. Antibodies may be produced by injection into rabbits, and other conventional means, as described in for example, Harlow and Lane (supra).

Antibodies are checked by Elisa assay and by Western blotting, and used for immunostaining as described in the Examples.

ANGIOGENESIS

The angiogenic process requires the proliferation and migration of a normally quiescent endothelium, the controlled proteolysis of the pericellular matrix, and the synthesis of new extracellular matrix components by developing capillaries. The establishment of new intra- and intercellular contacts and the morphological differentiation of endothelial cells to capillary-like tubular networks provide support for their subsequent maturation, branching, remodeling and selective regression to form a highly organized, functional microvascular network. The autocrine, paracrine and amphicrine interactions of the vascular endothelium with its surrounding stromal components, as well as with the pro-angiogenic and angiostatic cytokines and growth

factors orchestrating physiologic angiogenesis, are normally tightly regulated both spatially and temporally.

Angiogenesis is crucial to the growth of neoplastic tissues. Several experimental studies have suggested that both primary tumor growth and metastasis require neovascularization. In contrast to the well orchestrated process described above for normal tissue growth, the pathologic angiogenesis necessary for active tumor growth is generally sustained and persistent, with the initial acquisition of the angiogenic phenotype being a common mechanism for the development of a variety of solid and hematopoietic tumor types. Tumors that are unable to recruit and sustain a vascular network typically remain dormant as asymptomatic lesions in situ. Metastasis is also angiogenesis-dependent: for a tumor cell to metastasize successfully, it generally must gain access to the vasculature in the primary tumor, survive the circulation, arrest in the microvasculature of the target organ, exit from this vasculature, grow in the target organ, and induce angiogenesis at the target site. Thus, angiogenesis appears to be necessary at the beginning as well as the completion of the metastatic cascade.

Appropriate anti-angiogenic agents may therefore act directly or indirectly to influence tumor-associated angiogenesis either by delaying its onset (i.e., blocking an "angiogenic switch") or by blocking the sustained and focal neovascularization that is characteristic of many tumor types. Anti-angiogenesis therapies may also be directed against the tumor-associated endothelium and the multiple molecular and cellular processes and targets implicated in sustained pathologic angiogenesis.

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman et al. J. Biol Chem. 267:10931 10934 (1992); Klagsbrun et al Annu. Rev. Physiol. 53:217 239 (1991); and Garner A, Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach. Garner A, Klintworth G K, Eds. 2nd Edition Marcel Dekker, NY, pp 1625 1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative

autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (Weidner et al. N Engl J Med 324:1 6 (1991); Horak et al. Lancet 340:1120 1124 (1992); and Macchiarini et al. Lancet 340:145 146 (1992)).

ANGIOGENESIS INHIBITORS

Positive regulators of angiogenesis, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc. (Folkman et al. and Klagsbrun et al) are known in the art. An angiogenesis inhibitor, as used in the document, includes generally any molecule capable of decreasing the activity, by any means, of any of these molecules, and specifically includes any inhibitor or antagonist of any of these molecules.

The negative regulators so far identified include thrombospondin (Good et al. Proc. Natl. Acad. Sci. USA. 87:6624 6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp et al. Endocrinology, 133:1292 1299 (1993)), angiostatin (O'Reilly et al. Cell, 79:315 328 (1994)) and endostatin (O'Reilly et al. Cell, 88:277 285 (1996)).

An angiogenesis inhibitor may therefore comprise any of the following: angiostatin, endostatin and thrombospondin.

Angiogenesis inhibitors furthermore may include generally any molecule capable of increasing the activity, by any means, of any of these molecules, and specifically includes any activator or agonist of any of these molecules.

An angiogenesis inhibitor may also comprise any of the following: an interferon, platelet factor 4, prolactin 16Kd fragment, TIMP-1 (tissue inhibitor of metalloproteinase-1), TIMP-2 (tissue inhibitor of metalloproteinase-2), TIMP-3 (tissue inhibitor of metalloproteinase-3) or TIMP-4 (tissue inhibitor of metalloproteinase-4).

An angiogenesis inhibitor may comprise (*Z,E*)-3-(Imidazol-4-ylmethylene)indolin-2-one. This compound is a cell-permeable indolinone compound that displays anti-

angiogenesis properties (30% inhibition of control at 10 μM in an *in vitro* rat aortic ring model) with a potency that is comparable to that of SU5416 (3-[(2,4-Dimethylpyrrol-5-yl)methylidene]-indolin-2-one; 22% inhibition of control at 10 μM). It acts as a moderate ATP-competitive inhibitor of hEGF-R tyrosine kinase activity (54% inhibition at 10 μM). (*Z,E*)-3-(Imidazol-4-ylmethylene)indolin-2-one is described in Braud, E., et al. 2003. *J. Enzyme Inhib. Med. Chem.* **18,** 243.

An angiogenesis inhibitor may comprise 3-(2,4-dimethylpyrrol-5-yl)methylidene-inodolin-2-one, or (Z)-3-(2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol- -3-yl)-propionic acid

An angiogenesis inhibitor may also comprise a 1,2-dithiol-3-thione derivative or metabolite thereof, 5-(2-pyrazinyl)-1,2-diothiol-3-thione (ADT), 5-(2-pyrazinyl)-4-methyl-1,2-diothiol-3-thione (Oltipraz) or a metabolite thereof, as described in US 7,199,122 (Ruggeri).

The angiogenesis inhibitor may be provided in the form of a prodrug, as described above.

Endothelial Cell Growth Inhibitors

An angiogenesis inhibitors may comprise a molecule which directly inhibits the growth of endothelial cells. Included in this category is endostatin, a naturally occurring protein known to inhibit tumor growth in animals. Another drug, combretastatin A4, causes growing endothelial cells to commit suicide (apoptosis).

Other drugs, which interact with a molecule called integrin, also can promote the destruction of proliferating endothelial cells.

Endothelial cell growth inhibitors further include EMD121974, TNP470, Squalamine, combretastatin A4, Thalidomide and BMS-582664.

Thalidomide is a drug which is a sedative used in the 1950s that was subsequently taken off the market because it caused birth defects when taken by pregnant women. Although this drug clearly would not be suitable for pregnant women, its ability to prevent endothelial cells from forming new blood vessels make it suitable for use in the methods and compositions described here.

Thalidomide is described in detail in the references Urologic Oncology (2006) 24:260-268 and Cancer Research (2006) 66: 11520-11530. Each of these molecules may be employed as an angiogenesis inhibitor in the methods and compositions described here.

BMS-582664 (brivanib alaninate) is an oral dual inhibitor of VEGFR and FGFR tyrosine kinases (IC50 34, 10, 145, 125 nM for VEGFR2, VEGFR3, FGFR1 and FGFR2 respectively). BMS-582664 is the alaninate salt of Brivanib, a vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor with potential antineoplastic activity. Brivanib strongly binds to and inhibits VEGFR2, a tyrosine kinase receptor expressed almost exclusively on vascular endothelial cells; inhibition of VEGFR2 may result in inhibition of tumor angiogenesis, inhibition of tumor cell growth, and tumor regression.

Brivanib is a substituted 4-(4-fluoro-1*H*-indol-5-yloxy)pyrrolo[2,1-*f*][1,2,4]triazine and is an inhibitor of vascular endothelial growth factor receptor-2 kinase. Brivanib and its L-alaninate prodrug BMS-582664 are described in Bhide, et al. (2006). *Discovery and Preclinical Studies of (R)-1-(4-(4-Fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan- 2-ol (BMS-540215), an In Vivo Active Potent VEGFR-2 Inhibitor.* J. Med. Chem., 49 (7), 2143 -2146, 2006.

Each of these molecules may be employed as an angiogenesis inhibitor in the methods and compositions described here.

Extracellular Matrix Breakdown Inhibitors

The activation of endothelial cells by VEGF or bFGF sets in motion a series of steps toward the creation of new blood vessels. First, the activated endothelial cells produce matrix metalloproteinases (MMPs), a class of degradative enzymes. These

enzymes are then released from the endothelial cells into the surrounding tissue. The MMPs break down the extracellular matrix--support material that fills the spaces between cells and is made of proteins and polysaccharides. Breakdown of this matrix permits the migration of endothelial cells.

Accordingly, an angiogenesis inhibitor may comprise generally any molecule capable of decreasing breakdown of the extracellular matrix, an extracellular matrix breakdown inhibitor. It may decrease the activity, by any means, of a matrix metalloproteinases, and specifically includes any inhibitor or antagonist of any of these molecules, i.e., a Matrix Metalloprotease Protein Inhibitor.

For example, Marimistat, AG3340, COL-3, Neovastat or BMS-275291 may be employed as an angiogenesis inhibitor.

Angiogenesis Signaling Cascade Inhibitors

An angiogenesis inhibitor may specifically include any molecule which interferes with or inhibits any of the steps in the angiogenesis signaling cascade.

For example, it may comprise an inhibitor of VEGF activity. The angiogenesis signalling cascade inhibitor may comprise a molecule that is capable of blocking the VEGF receptor from binding growth factor. Such molecules may comprise immunoglobulins, in particular an anti-VEGF antibody.

The anti-VEGF antibody Bevacizumab (Avastin) has been proven to delay tumor growth and more importantly, to extend the lives of patients. It is described in more detail later.

Other examples of Angiogenesis Signalling Cascade Inhibitors are interferon-alpha, SU5416, SU6668 and PTK787/ZK 22584.

Interferon-alpha is a naturally occurring protein that inhibits the production of bFGF and VEGF, preventing these growth factors from starting the signaling cascade.

Interferon-alpha, SU5416, SU6668 and PTK787/ZK 22584 are described in detail in Cancer Research (2006) 66: 11520-11530.

VEGF Activity

Angiogenesis inhibitors which may be used in the methods and compositions described here include specifically VEGF inhibitors.

Vascular endothelial growth factor (VEGF) is an important signaling protein involved in both vasculogenesis (the de novo formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature). As its name implies, VEGF activity is restricted mainly to cells of the vascular endothelium, although it does have effects on a limited number of other cell types (e.g. stimulation monocyte/macrophage migration). In vitro, VEGF has been shown to stimulate endothelial cell mitogenesis and cell migration. VEGF also enhances microvascular permeability and is sometimes referred to as vascular permeability factor.

The term 'VEGF' is intended to encompass each of a number of proteins that result from alternate splicing of mRNA from a single, 8 exon, *VEGF* gene. The different VEGF splice variants are referred to by the number of amino acids they contain (in humans: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$; the rodent orthologs of these proteins contain one fewer amino acid). These proteins differ by the presence or absence of short C-terminal domains encoded by exons 6a, 6b and 7 of the *VEGF* gene. These domains have important functional consequences for the VEGF splice variants as they mediate interactions with heparan sulfate proteoglycans (HSPGs) and neuropilin co-receptors on the cell surface, enhancing their ability to bind and activate the VEGF signaling receptors (VEGFRs).

The VEGF splice variants are released from cells as glycosylated disulfide-bonded homodimers. Structurally VEGF belongs to the PDGF family of cystine-knot growth factors. Subsequently, several closely-related proteins were discovered (Placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D) which together comprise the VEGF sub-family of growth factors. The term VEGF is intended to include such subfamily members.

VEGF is sometimes referred to as VEGF-A to differentiate it from these related growth factors. A number of VEGF-related proteins have also been discovered encoded by viruses (VEGF-E) and in the venom of some snakes (VEGF-F). The term VEGF also includes these VEGF related proteins.

All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGFRs) on the cell surface, causing them to dimerize and become activated through transphosphorylation. The VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain. VEGF-A binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF. The function of VEGFR-1 is less well defined, although it is thought to modulate VEGFR-2 signaling. Another function of VEGFR-1 may be to act as a dummy/decoy receptor, sequestering VEGF from VEGFR-2 binding (this appears to be particularly important during vasculogenesis in the embryo). A third receptor has been discovered (VEGFR-3), however, VEGF-A is not a ligand for this receptor. VEGFR-3 mediates lymphangiogenesis in response to VEGF-C and VEGF-D.

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara et al. Endocr. Rev. 18:4 25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara et al.).

Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara et al.). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman et al. J Clin Invest 91:153 159 (1993); Brown et al. Human Pathol. 26:86 91 (1995); Brown et al. Cancer Res. 53:4727 4735 (1993); Mattern et al. Brit. J. Cancer. 73:931 934 (1996); and Dvorak et al. Am J. Pathol. 146:1029 1039 (1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with

diabetic and other ischemia-related retinopathies (Aiello et al. N. Engl. J. Med. 331:1480 1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez et al. Invest. Ophtalmo. Vis. Sci. 37:855 868 (1996)).

Therefore, inhibitors of VEGF activity may be used as angiogenesis inhibitors according to the methods and compositions described here.

VEGF Inhibitors

An angiogenesis inhibitor may comprise a VEGF inhibitor.

The term "VEGF inhibitor" should be taken to include any molecule which is capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. A VEGF inhibitor may include an antagonist of VEGF, and may act for example by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the fms-like tyrosine kinase (flt), a transmembrane receptor in the tyrosine kinase family. DeVries et al., Science 255:989 (1992); Shibuya et al., Oncogene 5:519 (1990). The flt receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the flk-1 receptor (also referred to as KDR). Matthews et al., Proc. Nat. Acad. Sci. 88:9026 (1991); Terman et al., Oncogene 6:1677 (1991); Terman et al., Biochem. Biophys. Res. Commun. 187:1579 (1992). Binding of VEGF to the fit receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex

is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

In some embodiments, a VEGF inhibitor may include a molecule capable of inhibiting a biological activity of a human VEGF. The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung et al., Science 246:1306 (1989), and Houck et al., Mol. Endocrin. 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

Anti-VEGF Antibodies

Inhibitors of VEGF activity include anti-VEGF antibodies such as anti-VEGF monoclonal antibodies.

Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim et al. Nature 362:841 844 (1993); Warren et al. J. Clin. Invest. 95:1789 1797 (1995); Borgstrom et al. Cancer Res. 56:4032 4039 (1996); and Melnyk et al. Cancer Res. 56:921 924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis et al. Arch. Ophthalmol. 114:66 71 (1996)).

Anti-VEGF antibodies, such as those described in detail in United States Patent 7,169,901 (Baca), may also be employed in the methods and compositions described here.

Any of the antibodies described in these references may be employed as angiogenesis inhibitors according to the methods and compositions described here.

BEVACIZUMAB (AVASTIN)

In some embodiments, the angiogenesis inhibitor may comprise Bevacizumab. Bevacizumab, also known as Avastin, is a monoclonal antibody, and is the first of the anti-VEGF antibodies to be FDA-approved.

Bevacizumab is described in United States Patent 6,054,297 (Carter).

Bevacizumab is a recombinant humanised monoclonal antibody against VEGF. Bevacizumab inhibits binding to the VEGF receptor and activation of downstream signaling. Bevacizumab is composed of IgG1 framework regions and antigen-binding complementary determining regions from a murine monoclonal antibody that block the binding of human VEGF to its receptors (Presta, L. G., H. Chen, et al. (1997). "Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders." Cancer Res **57**(20): 4593-9). Bevacizumab has a molecular weight of approximately 149000 Dalton and is glycosylated.

Bevacizumab is also known as Avastin and may be obtained commercially under that name from Genentech, Inc, (South San Francisco, USA).

Avastin is the first angiogenesis inhibitor that provides therapeutic benefit in the treatment of patients with cancer, improving overall response rates, time to progression, and survival [reviewed in Rhee J and Hoff PM. Angiogenesis inhibitors in the treatment of cancer. Expert Opin Pharmacother 2005;6:1701-1711]. Recent data for Avastin in HCC indicates that this agent can be administered safely in carefully selected HCC patients and has demonstrated additive clinical activity of Avastin to chemotherapy (Zhu AX, Sahani D, Norden-Zfoni N, et al. A phase I1 study of gemcitabine, Oxaliplatin in combination with bevacizumb (GEMOX-B) in patients with Hepatocellular Carcinoma. 2005. Presented at 2005 American Society of Clinical Oncology Annual Meeting, Orlando, FL; Schwartz JD, Schwartz M, Leher D, et al. Bevacizumab in hepatocellular carcinoma (HCC) inpatients without metastasis and without invasion of the portal vein. 2005.).

Its pharmacokinetics are characterized by dose linearity within the dose range 1-10mg/kg in a 2-compartment model, a low clearance and a volume of distribution consistent with limited extravascular distribution. Population PK analysis indicated an initial half-life of 1.4 days and a terminal half-life of 19-20 days (range 11-50 days) and the steady state is reached at roughly 100 days. To date the pharmacokinetics of bevacizumab have not been evaluated in HCC nor in combination with rapamycin.

Bevacizumab has been tested in phase 2 and 3 studies in a variety of solid tumours. It is licensed for use in combination with 5-fluorouracil as first line treatment for metastatic colorectal cancer. Results from trials show that bevacizumab can be safely given to patients with unresectable HCC at doses of 5mg/kg or 10mg/kg, even when combined with chemotherapy. See A. X. Zhu, D. S., A. Norden-Zfoni, N. S. Holalkere, L. Blaszkowsky, D. P. Ryan, J. Clark, K. Taylor, J. V. Heymach, K. Stuart (2005). "A Phase II Study of Gemcitabine, Oxaliplatin in Combination with Bevacizumab (GEMOX-B) in Patients with Hepatocellular Carcinoma." ASCO Proceedings; J. D. Schwartz, M. S., D. Lehrer, D. Coll, M. Kinkhabwala, M. Sung, S. B. Holloway, S. Wadler (2005). "Bevacizumab in hepatocellular carcinoma (HCC) in patients without metastasis and without invasion of the portal vein." ASCO Proceedings; Zhu, A. X., L. S. Blaszkowsky, et al. (2006). "Phase II study of gemcitabine and oxaliplatin in combination with bevacizumab in patients with advanced hepatocellular carcinoma." J Clin Oncol **24**(12): 1898-903.

In a phase 2 trial, 20% of patients had disease response and 27% had stable disease after use of bevacizumab combined with gemcitabine and oxaliplatin.

Other Molecules

Angiogenesis inhibitors may further comprise any of the following: CAI – an inhibitor of calcium uptake, Interleukin-12 – up-regulator of interferon-gamma and IP-10 and IM862 – unknown function.

IDENTIFYING mTOR ANTAGONISTS AND ANGIOGENESIS INHIBITORS

Antagonists, in particular, small molecules may be used to specifically inhibit mTOR. Similarly, they may be used to specifically inhibit angiogenesis activity.

We therefore disclose small molecule mTOR inhibitors, as well as assays for screening for these. Antagonists of mTOR kinase may be screened by detecting modulation, preferably down regulation, of binding or other activity. Any mTOR antagonists identified may be employed in the methods and compositions described here.

We also disclose small molecule inhibitors of angiogenesis, as well as assays for screening for these. Inhibitors of angiogenesis are screened by detecting modulation, preferably down regulation, of angiogenesis itself, or any activity associated with angiogenesis, for example, endothelial cell growth, extracellular matrix breakdown, angiogenesis cascade signalling, including VEGF activity, etc.

By "down-regulation" we include any negative effect on the behaviour being studied; this may be total, or partial. Thus, where binding is being detected, candidate antagonists are capable of reducing, ameliorating, or abolishing the binding between two entities. Preferably, the down-regulation of binding (or any other activity) achieved by the candidate molecule is at least 10%, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90%, or more compared to binding (or which ever activity) in the absence of the candidate molecule. Thus, a candidate molecule suitable for use as an antagonist is one which is capable of reducing by 10% more the binding or other activity.

Polypeptide Binding Assays

Modulators and antagonists of mTOR activity or expression may be identified by any means known in the art. Putative such molecules may be identified by their binding to mTOR, in an assay which detects binding between mTOR and the putative molecule.

Similarly, modulators and antagonists of angiogenesis activity (including VEGF activity) or expression may be identified by any means known in the art. Putative such molecules may be identified by their binding to VEGF, in an assay which detects binding between VEGF and the putative molecule.

One type of assay for identifying substances that bind to a polypeptide involves contacting a polypeptide, which is immobilised on a solid support, with a non-immobilised candidate substance determining whether and/or to what extent the polypeptide and candidate substance bind to each other. Alternatively, the candidate substance may be immobilised and the polypeptide non-immobilised. This may be used to detect substances

capable of binding to mTOR polypeptides or VEGF as the case may be, or fragments, homologues, variants or derivatives thereof.

In a preferred assay method, the polypeptide is immobilised on beads such as agarose beads. Typically this is achieved by expressing the mTOR polypeptide or VEGF as the case may be, or a fragment, homologue, variant or derivative thereof as a GST-fusion protein in bacteria, yeast or higher eukaryotic cell lines and purifying the GST-fusion protein from crude cell extracts using glutathione-agarose beads (Smith and Johnson, 1988). As a control, binding of the candidate substance, which is not a GST-fusion protein, to the immobilised polypeptide is determined in the absence of the polypeptide. The binding of the candidate substance to the immobilised polypeptide is then determined. This type of assay is known in the art as a GST pulldown assay. Again, the candidate substance may be immobilised and the polypeptide non-immobilised.

It is also possible to perform this type of assay using different affinity purification systems for immobilising one of the components, for example Ni-NTA agarose and histidine-tagged components.

Binding of the mTOR polypeptide or VEGF polypeptide, or a fragment, homologue, variant or derivative thereof to the candidate substance may be determined by a variety of methods well-known in the art. For example, the non-immobilised component may be labeled (with for example, a radioactive label, an epitope tag or an enzyme-antibody conjugate). Alternatively, binding may be determined by immunological detection techniques. For example, the reaction mixture can be Western blotted and the blot probed with an antibody that detects the non-immobilised component. ELISA techniques may also be used.

Candidate substances are typically added to a final concentration of from 1 to 1000 nmol/ml, more preferably from 1 to 100 nmol/ml. In the case of antibodies, the final concentration used is typically from 100 to 500 μg/ml, more preferably from 200 to 300 μg/ml.

Modulators and antagonists of mTOR and VEGF may also be identified by detecting modulation of binding between mTOR and VEGF and any molecule to which these bind, such as (in the case of VEGF) a VEGF receptor.

Activity Assays

Assays to detect modulators or antagonists typically involve detecting modulation of any activity of mTOR, preferably kinase activity, or VEGF activity, in the presence, optionally together with detection of modulation of activity in the absence, of a candidate molecule.

The assays involve contacting a candidate molecule (e.g., in the form of a library) with mTOR or VEGF whether in the form of a polypeptide, a nucleic acid encoding the polypeptide, or a cell, organelle, extract, or other material comprising such, with a candidate modulator. The relevant activity of mTOR or VEGF (as described below) may be detected, to establish whether the presence of the candidate modulator has any effect. Promoter binding assays to detect candidate modulators which bind to and/or affect the transcription or expression of mTOR or VEGF may also be used. Candidate modulators may then be chosen for further study, or isolated for use. Details of such screening procedures are well known in the art, and are for example described in, Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9).

The screening methods described here preferably employ *in vivo* assays, although they may be configured for *in vitro* use. *In vivo* assays generally involve exposing a cell comprising mTOR or VEGF to the candidate molecule. In *in vitro* assays, mTOR or VEGF is exposed to the candidate molecule, optionally in the presence of other components, such as crude or semi-purified cell extract, or purified proteins. Where *in vitro* assays are conducted, these preferably employ arrays of candidate molecules (for example, an arrayed library). *In vivo* assays are preferred. Preferably, therefore, the mTOR or VEGF is comprised in a cell, preferably heterologously. Such a cell is preferably a transgenic cell, which has been engineered to express mTOR or VEGF as described above.

Where an extract is employed, it may comprise a cytoplasmic extract or a nuclear extract, methods of preparation of which are well known in the art.

It will be appreciated that any component of a cell comprising mTOR or VEGF may be employed, such as an organelle. A preferred embodiment utilises a cytoplasmic or nuclear preparation, e.g., comprising a cell nucleus which comprises mTOR as described. See Zhang, et al, Predominant Nuclear Localization of Mammalian Target of Rapamycin in Normal and Malignant Cells in Culture. J. Biol. Chem., Jul.2002; 277: 28127 – 28134. The nuclear preparation may comprise one or more nuclei, which may be permeabilised or semi-permeabilised, by detergent treatment, for example.

Thus, in a specific embodiment, an assay format may include the following: a multiwell microtitre plate is set up to include one or more cells expressing mTOR or VEGF in each well; individual candidate molecules, or pools of candidate molecules, derived for example from a library, may be added to individual wells and modulation of mTOR or VEGF activity measured. Where pools are used, these may be subdivided in to further pools and tested in the same manner. mTOR or VEGF activity, for example, kinase activity, is then assayed.

Alternatively or in addition to the assay methods described above, "subtractive" procedures may also be used to identify modulators or antagonists of mTOR or VEGF. Under such "subtractive" procedures, a plurality of molecules is provided, which comprises one or more candidate molecules capable of functioning as a modulator (e.g., cell extract, nuclear extract, library of molecules, etc), and one or more components is removed, depleted or subtracted from the plurality of molecules. The "subtracted" extract, etc, is then assayed for activity, by exposure to a cell comprising mTOR or VEGF (or a component thereof) as described.

Thus, for example, an 'immunodepletion' assay may be conducted to identify such modulators as follows. A cytoplasmic or nuclear extract may be prepared from a pluripotent cell, for example, a pluripotent EG/ES cell. The extract may be depleted or fractionated to remove putative modulators, such as by use of immunodepletion with

appropriate antibodies. If the extract is depleted of a modulator, it will lose the ability to affect mTOR or VEGF function or activity or expression. A series of subtractions and/or depletions may be required to identify the modulators or antagonists.

It will also be appreciated that the above "depletion" or "subtraction" assay may be used as a preliminary step to identify putative modulatory factors for further screening. Furthermore, or alternatively, the "depletion" or "subtraction" assay may be used to confirm the modulatory activity of a molecule identified by other means (for example, a "positive" screen as described elsewhere in this document) as a putative modulator.

Candidate molecules subjected to the assay and which are found to be of interest may be isolated and further studied. Methods of isolation of molecules of interest will depend on the type of molecule employed, whether it is in the form of a library, how many candidate molecules are being tested at any one time, whether a batch procedure is being followed, etc.

The candidate molecules may be provided in the form of a library. In a preferred embodiment, more than one candidate molecule is screened simultaneously. A library of candidate molecules may be generated, for example, a small molecule library, a polypeptide library, a nucleic acid library, a library of compounds (such as a combinatorial library), a library of antisense molecules such as antisense DNA or antisense RNA, an antibody library etc, by means known in the art. Such libraries are suitable for high-throughput screening. Different cells comprising mTOR or VEGF may be exposed to individual members of the library, and effect on the mTOR activity (or activity of a component of an angiogenesis pathway, for example VEGF) determined. Array technology may be employed for this purpose. The cells may be spatially separated, for example, in wells of a microtitre plate.

In a preferred embodiment, a small molecule library is employed. By a "small molecule", we refer to a molecule whose molecular weight is preferably less than about 50 kDa. In particular embodiments, a small molecule has a molecular weight preferably less than about 30 kDa, more preferably less than about 15 kDa, most preferably less than 10

kDa or so. Libraries of such small molecules, here referred to as "small molecule libraries" may contain polypeptides, small peptides, for example, peptides of 20 amino acids or fewer, for example, 15, 10 or 5 amino acids, simple compounds, etc.

Alternatively or in addition, a combinatorial library, as described in further detail below, may be screened for modulators or antagonists of mTOR or VEGF.

Assays for mTOR Activity

Any of the activities of mTOR may be used as the basis of the assay.

In particular, cellular activities mediated by mTOR may be assayed to identify antagonists. For example, mTOR is responsible for phosphorylating substrates including eukaryotic initiation factor 4E (eIF4E) and ribosomal S6 kinase 1 (S6K1), RNA polymerase I and eEF2 kinase. Accordingly, the effects of the putative antagonist or agonist on kinase activity mediated by mTOR one one or more of these substrates (or peptides derived from their sequences) may be assayed using for example kinase assays as known in the art.

Such assays may employ 4E-BP1 and/or S6K1 as substrates, or use peptides from these polypeptides as substrates. mTOR is known to phosphorylate 4E-BP1 at Thr37 and Thr46 and S6K1 at Thr389 (Schalm SS, Fingar DC, Sabatini DM, Blenis J. Curr Biol. 2003 May 13;13(10):797-806; Schalm SS, Blenis J. Curr Biol. 2002 Apr 16;12(8):632-9.), and accordingly peptide substrates containing these positions may be generated using known peptide synthesis methods.

An exemplary assay for kinase activity of mTOR is described in Gary G. Chiang, Robert T. Abraham. *Determination of the Catalytic Activities of mTOR and Other Members of the Phosphoinositide-3-Kinase-Related Kinase Family*. Checkpoint Controls and Cancer: Volume 2: Activation and Regulation Protocols, July 2004, pps. 125-142 (ISBN: 1-59259-811-0), Volume #: 281, Series: Methods in Molecular Biology.

mTOR Kinase Assay

A further mTOR assay is disclosed in Molecular Mechanism of mTOR Downstream Signalling (PhD Thesis, S. Schalm, 17th September 2003, Fachbereich Biologie, Chemie, Pharmazie, Freie Universität Berlin, http://www.diss.fu-berlin.de/2003/249/index.html).

Cells are grown for 48 hours in DMEM containing 10% FBS, and lysed in lysis buffer B (40 mM HEPES, 120 mM NaCl, 50 mM NaF, 1 mM EDTA, 50 mM β-glycerophosphate, 0.2% CHAPS, 1 mM Na3 VO4, 40 mg/ml PMSF, 5 µg/ml pepstatin, 10 µg/ml leupeptin, 1 mM DTT, ddH2, O, pH 7.5). One third of total cell lysate from a 150-mm plate is incubated with an anti mTOR-antibody (e.g., Bethyl, Inc, Texas USA) for 2 h, followed by another hour of incubation with protein-G-Sepharose beads. Immunopreciptates are washed twice with 1 ml mTOR wash buffer A (20 mM Tris, 500 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 40 mg/ml PMSF, 10 µg/ml leupeptin, 5 µg/ml pepstatin, in ddH_2O, pH 7.4), once with mTOR wash buffer B (10 mM HEPES, 50 mM β-glycerophosphate, 50 mM NaCl, 1 mM DTT, 1 mM Na_3VO_4, 40 mg/ml PMSF, 10 µg/ml leupeptin, 5 µg/ml pepstatin, in ddH_2O, pH 7.4), and once with ST (50 mM Tris-HCl, 5 mM Tris base, 150 mM NaCl, ddH_2O, pH 7.28).

Kinase assays towards recombinant GST-4E-BP1 WT or GST-4E-BP1 F114A (i.e., human 4E-BP1 subcloned into pGEX-2T/GST, Pharmacia) in washed immunoprecipitates is assayed in mTOR kinase assay buffer (10 mM HEPES, 50 mM NaCl, 50 mM β-glycerophosphate, 10 mM $MnCl_2$, 100 µM ATP unlabeled, 10 µCi [γ-^{32}P] ATP (New England Nuclear), pH 7.4) for 30 min at 30°C. The reaction is separated by 12% SDS-PAGE and ^{32}P incorporated into GST-4E-BP1 is assessed by autoradiography and quantified by phosphoimaging (BioRad). One kinase unit is defined by the amount of kinase ie protein required to catalyze the transfer of 1 pmol of phosphate to the substrate per reaction volume in one minute at 30°C.

mTOR Reporter Assay

Molecules and agents which activate or promote mTOR activity may be identified as follow: To screen for mTOR activating molecules, a hybrid gene encoding for a mRNA with a 5'UTR derived from a TOP mRNA e.g. L5 ribosomal protein mRNA and coding region from a reporter gene e.g. GFP or luciferase is transfected into mammalian cells. The cells are either serum starved or rapamycin-treated to shut off translation of the reporter. Cells are exposed to a candidate molecule or a member of a library. Addition of an mTOR activating molecule will upregulate translation of the reporter (see Figure 8A and Example 8)

Molecules and agents which inhibit mTOR activity are identified as follow: To screen for mTOR inhibiting molecules, a hybrid gene encoding a mRNA with a 5'UTR derived from mRNAs whose translation is upregulated when cap-mediated translation is inhibited e.g. p27Kip1 mRNA and coding region from a reporter gene e.g. GFP or luciferase is transfected into mammalian cells . The cells are either serum starved or rapamycin-treated to turn on translation of the reporter. Then serum will be added or rapamycin removed to activate mTOR and turn off translation of reporter. Cells are exposed to a candidate molecule or a member of a library. When the reporter is off, mTOR inhibiting molecule will be added to upregulate translation of the reporter (see Figure 8B and Example 9).

Cell Cycle Assay

Furthermore, we show that mTOR activity is capable of lengthening cell cycle times; accordingly, the cell cycle period may be assayed in the presence and absence of a candidate molecule to identify antagonists or agonists of mTOR activity.

Assays for VEGF Activity

Any of the various biochemical other activities of VEGF may be measured in order to assay VEGF activity. Accordingly, the effects of a putative antagonist or agonist on VEGF activity may be assayed by any one or more of the following methods as known in the art.

An assay for VEGF activity may involve detection of binding to a VEGF receptor. The assay may detect dimerisation of the receptor caused by VEGF binding. The assay may also detect activation through transphorylation of the VEGF receptor.

In particular, cellular activities mediated by VEGF may be assayed to identify antagonists. Thus, VEGF activity may be assayed by determining stimulation of any one or more of endothelial cell mitogenesis, cell migration and microvascular permeability, through methods known in the art.

In particular, VEGF activity may also be assayed by detecting and quantitating vasculogenesis and/or angiogenesis. Thus, in one embodiment, VEGF activity is assayed by detecting its anti-angiogenic activity in a chotioallantic membrane assay (CAM). Such a CAM assay may be conducted by using 4.5 day-old chick embryos as described by Kim et al (2000) Int. J. Cancer 87:269-275 or Deoanne at al., (2002) Oncogene 21:427-436.

Libraries

Libraries of candidate molecules, such as libraries of polypeptides or nucleic acids, may be employed in the screens for mTOR antagonists and angiogenesis inhibitors described here. Such libraries are exposed to mTOR protein, and their effect, if any, on the activity of the protein determined. Similarly, the libraries may be exposed to an experimental system (including a component of an angiogenesis pathway, for example VEGF, as the case may be) and their effect, if any, on angiogenesis determined.

Selection protocols for isolating desired members of large libraries are known in the art, as typified by phage display techniques. Such systems, in which diverse peptide sequences are displayed on the surface of filamentous bacteriophage (Scott and Smith (1990 *supra*), have proven useful for creating libraries of antibody fragments (and the nucleotide sequences that encoding them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen. The nucleotide sequences encoding the V_H and V_L regions are linked to gene fragments which encode leader signals that direct them to the periplasmic space of *E. coli* and as a result the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage

coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids (phagebodies). An advantage of phage-based display systems is that, because they are biological systems, selected library members can be amplified simply by growing the phage containing the selected library member in bacterial cells. Furthermore, since the nucleotide sequence that encodes the polypeptide library member is contained on a phage or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

Methods for the construction of bacteriophage antibody display libraries and lambda phage expression libraries are well known in the art (McCafferty *et al.* (1990) **supra**; Kang *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.,* 88: 4363; Clackson *et al.* (1991) *Nature*, 352: 624; Lowman *et al.* (1991) *Biochemistry*, 30: 10832; Burton *et al.* (1991) *Proc. Natl. Acad. Sci U.S.A.*, 88: 10134; Hoogenboom *et al.* (1991) Nucleic Acids Res., 19: 4133; Chang *et al.* (1991) *J. Immunol.*, 147: 3610; Breitling *et al.* (1991) *Gene*, 104: 147; Marks *et al.* (1991) supra; Barbas *et al.* (1992) supra; Hawkins and Winter (1992) *J. Immunol.*, 22: 867; Marks *et al.*, 1992, *J. Biol. Chem.*, 267: 16007; Lerner *et al.* (1992) *Science*, 258: 1313, incorporated herein by reference). Such techniques may be modified if necessary for the expression generally of polypeptide libraries.

One particularly advantageous approach has been the use of scFv phage-libraries (Bird, R.E., *et al.* (1988) *Science* **242**: 423-6, Huston *et al.*, 1988, Proc. Natl. Acad. Sci U.S.A., 85: 5879-5883; Chaudhary *et al.* (1990) Proc. Natl. Acad. Sci U.S.A., 87: 1066-1070; McCafferty *et al.* (1990) supra; Clackson *et al.* (1991) supra; Marks *et al.* (1991) supra; Chiswell *et al.* (1992) Trends Biotech., 10: 80; Marks *et al.* (1992) supra). Various embodiments of scFv libraries displayed on bacteriophage coat proteins have been described. Refinements of phage display approaches are also known, for example as described in WO96/06213 and WO92/01047 (Medical Research Council *et al.*) and WO97/08320 (Morphosys, supra), which are incorporated herein by reference.

Alternative library selection technologies include bacteriophage lambda expression systems, which may be screened directly as bacteriophage plaques or as colonies of lysogens, both as previously described (Huse *et al.* (1989*) Science*, 246: 1275; Caton and

Koprowski (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87; Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87: 8095; Persson *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, 88: 2432) and are of use in the methods and compositions described here. These expression systems may be used to screen a large number of different members of a library, in the order of about 10^6 or even more. Other screening systems rely, for example, on direct chemical synthesis of library members. One early method involves the synthesis of peptides on a set of pins or rods, such as described in WO84/03564. A similar method involving peptide synthesis on beads, which forms a peptide library in which each bead is an individual library member, is described in U.S. Patent No. 4,631,211 and a related method is described in WO92/00091. A significant improvement of the bead-based methods involves tagging each bead with a unique identifier tag, such as an oligonucleotide, so as to facilitate identification of the amino acid sequence of each library member. These improved bead-based methods are described in WO93/06121.

Another chemical synthesis method involves the synthesis of arrays of peptides (or peptidomimetics) on a surface in a manner that places each distinct library member (e.g., unique peptide sequence) at a discrete, predefined location in the array. The identity of each library member is determined by its spatial location in the array. The locations in the array where binding interactions between a predetermined molecule (e.g., a receptor) and reactive library members occur is determined, thereby identifying the sequences of the reactive library members on the basis of spatial location. These methods are described in U.S. Patent No. 5,143,854; WO90/15070 and WO92/10092; Fodor *et al.* (1991) *Science*, 251: 767; Dower and Fodor (1991) *Ann. Rep. Med. Chem.*, 26: 271.

Other systems for generating libraries of polypeptides or nucleotides involve the use of cell-free enzymatic machinery for the *in vitro* synthesis of the library members. In one method, RNA molecules are selected by alternate rounds of selection against a target ligand and PCR amplification (Tuerk and Gold (1990) *Science*, 249: 505; Ellington and Szostak (1990) *Nature*, 346: 818). A similar technique may be used to identify DNA sequences which bind a predetermined human transcription factor (Thiesen and Bach (1990) *Nucleic Acids Res.*, 18: 3203; Beaudry and Joyce (1992) *Science*, 257: 635; WO92/05258 and WO92/14843). In a similar way, *in vitro* translation can be used to

synthesise polypeptides as a method for generating large libraries. These methods which generally comprise stabilised polysome complexes, are described further in WO88/08453, WO90/05785, WO90/07003, WO91/02076, WO91/05058, and WO92/02536. Alternative display systems which are not phage-based, such as those disclosed in WO95/22625 and WO95/11922 (Affymax) use the polysomes to display polypeptides for selection. These and all the foregoing documents also are incorporated herein by reference.

The library may in particular comprise a library of zinc fingers; zinc fingers are known in the art and act as transcription factors. Suitable zinc finger libraries are disclosed in, for example, WO 96/06166 and WO 98/53057. Construction of zinc finger libraries may utilise rules for determining interaction with specific DNA sequences, as disclosed in for example WO 98/53058 and WO 98/53060. Zinc fingers capable of interacting specifically with methylated DNA are disclosed in WO 99/47656. The above zinc finger libraries may be immobilised in the form of an array, for example as disclosed in WO 01/25417. Accordingly, preferred molecules capable of altering the potency of a cell include zinc fingers.

Combinatorial Libraries

Libraries, in particular, libraries of candidate molecules, may suitably be in the form of combinatorial libraries (also known as combinatorial chemical libraries).

A "combinatorial library", as the term is used in this document, is a collection of multiple species of chemical compounds that consist of randomly selected subunits. Combinatorial libraries may be screened for molecules which are capable of inhibiting mTOR or angiogenesis.

Various combinatorial libraries of chemical compounds are currently available, including libraries active against proteolytic and non-proteolytic enzymes, libraries of agonists and antagonists of G-protein coupled receptors (GPCRs), libraries active against non-GPCR targets (e.g., integrins, ion channels, domain interactions, nuclear receptors, and transcription factors) and libraries of whole-cell oncology and anti-infective targets, among others. A comprehensive review of combinatorial libraries, in particular their

construction and uses is provided in Dolle and Nelson (1999), *Journal of Combinatorial Chemistry*, Vol 1 No 4, 235-282. Reference is also made to *Combinatorial peptide library protocols* (edited by Shmuel Cabilly, Totowa, N.J. : Humana Press, c1998. *Methods in Molecular Biology* ; v. 87). Specific combinatorial libraries and methods for their construction are disclosed in United States Patent 6,168,914 (Campbell, et al), as well as in Baldwin et al. (1995), "Synthesis of a ***Small Molecule Library*** Encoded with Molecular Tags," J. Am. Chem. Soc. 117:5588-5589, and in the references mentioned in those documents.

In a preferred embodiment, the combinatorial library which is screened is one which is designed to potentially include molecules which interact with a component of the cell to influence gene expression. For example, combinatorial libraries against chromatin structural proteins may be screened. Other libraries which are useful for this embodiment include combinatorial libraries against histone modification enzymes (e.g., histone acetylation or histone metylation enzymes), or DNA modification, for example, DNA methylation or demethylation.

Further references describing chemical combinatorial libraries, their production and use include those available from the URL http://www.netsci.org/Science/Combichem/, including The Chemical Generation of Molecular Diversity. Michael R. Pavia, Sphinx Pharmaceuticals, A Division of Eli Lilly (Published July, 1995); Combinatorial Chemistry: A Strategy for the Future - MDL Information Systems discusses the role its Project Library plays in managing diversity libraries (Published July, 1995); Solid Support Combinatorial Chemistry in Lead Discovery and SAR Optimization, Adnan M. M. Mjalli and Barry E. Toyonaga, Ontogen Corporation (Published July, 1995); Non-Peptidic Bradykinin Receptor Antagonists From a Structurally Directed Non-Peptide Library. Sarvajit Chakravarty, Babu J. Mavunkel, Robin Andy, Donald J. Kyle*, Scios Nova Inc. (Published July, 1995); Combinatorial Chemistry Library Design using Pharmacophore Diversity Keith Davies and Clive Briant, Chemical Design Ltd. (Published July, 1995); A Database System for Combinatorial Synthesis Experiments - Craig James and David Weininger, Daylight Chemical Information Systems, Inc. (Published July, 1995); An Information Management Architecture for Combinatorial Chemistry, Keith Davies and

Catherine White, Chemical Design Ltd. (Published July, 1995); Novel Software Tools for Addressing Chemical Diversity, R. S. Pearlman, Laboratory for Molecular Graphics and Theoretical Modeling, College of Pharmacy, University of Texas (Published June/July, 1996); Opportunities for Computational Chemists Afforded by the New Strategies in Drug Discovery: An Opinion, Yvonne Connolly Martin, Computer Assisted Molecular Design Project, Abbott Laboratories (Published June/July, 1996); Combinatorial Chemistry and Molecular Diversity Course at the University of Louisville: A Description, Arno F. Spatola, Department of Chemistry, University of Louisville (Published June/July, 1996); Chemically Generated Screening Libraries: Present and Future. Michael R. Pavia, Sphinx Pharmaceuticals, A Division of Eli Lilly (Published June/July, 1996); Chemical Strategies For Introducing Carbohydrate Molecular Diversity Into The Drug Discovery Process.. Michael J. Sofia, Transcell Technologies Inc. (Published June/July, 1996); Data Management for Combinatorial Chemistry. Maryjo Zaborowski, Chiron Corporation and Sheila H. DeWitt, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company (Published November, 1995); and The Impact of High Throughput Organic Synthesis on R&D in Bio-Based Industries, John P. Devlin (Published March, 1996).

Techniques in combinatorial chemistry are gaining wide acceptance among modern methods for the generation of new pharmaceutical leads (Gallop, M. A. et al., 1994, J. Med. Chem. 37:1233-1251; Gordon, E. M. et al., 1994, J. Med. Chem. 37:1385-1401.). One combinatorial approach in use is based on a strategy involving the synthesis of libraries containing a different structure on each particle of the solid phase support, interaction of the library with a soluble receptor, identification of the `bead` which interacts with the macromolecular target, and determination of the structure carried by the identified `bead` (Lam, K. S. et al., 1991, Nature 354:82-84). An alternative to this approach is the sequential release of defined aliquots of the compounds from the solid support, with subsequent determination of activity in solution, identification of the particle from which the active compound was released, and elucidation of its structure by direct sequencing (Salmon, S. E. et al., 1993, Proc.Natl.Acad.Sci.USA 90:11708-11712), or by reading its code (Kerr, J. M. et al., 1993, J.Am.Chem.Soc. 115:2529-2531; Nikolaiev, V. et al., 1993, Pept. Res. 6:161-170; Ohlmeyer, M. H. J. et al., 1993, Proc.Natl.Acad.Sci.USA 90:10922-10926).

Soluble random combinatorial libraries may be synthesized using a simple principle for the generation of equimolar mixtures of peptides which was first described by Furka (Furka, A. et al., 1988, Xth International Symposium on Medicinal Chemistry, Budapest 1988; Furka, A. et al., 1988, 14th International Congress of Biochemistry, Prague 1988; Furka, A. et al., 1991, Int. J. Peptide Protein Res. 37:487-493). The construction of soluble libraries for iterative screening has also been described (Houghten, R. A. et al.1991, Nature 354:84-86). K. S. Lam disclosed the novel and unexpectedly powerful technique of using insoluble random combinatorial libraries. Lam synthesized random combinatorial libraries on solid phase supports, so that each support had a test compound of uniform molecular structure, and screened the libraries without prior removal of the test compounds from the support by solid phase binding protocols (Lam, K. S. et al., 1991, Nature 354:82-84).

Thus, a library of candidate molecules may be a synthetic combinatorial library (e.g., a combinatorial chemical library), a cellular extract, a bodily fluid (e.g., urine, blood, tears, sweat, or saliva), or other mixture of synthetic or natural products (e.g., a library of small molecules or a fermentation mixture).

A library of molecules may include, for example, amino acids, oligopeptides, polypeptides, proteins, or fragments of peptides or proteins; nucleic acids (e.g., antisense; DNA; RNA; or peptide nucleic acids, PNA); aptamers; or carbohydrates or polysaccharides. Each member of the library can be singular or can be a part of a mixture (e.g., a compressed library). The library may contain purified compounds or can be "dirty" (i.e., containing a significant quantity of impurities).

Commercially available libraries (e.g., from Affymetrix, ArQule, Neose Technologies, Sarco, Ciddco, Oxford Asymmetry, Maybridge, Aldrich, Panlabs, Pharmacopoeia, Sigma, or Tripose) may also be used with the methods described here.

In addition to libraries as described above, special libraries called diversity files can be used to assess the specificity, reliability, or reproducibility of the new methods. Diversity files contain a large number of compounds (e.g., 1000 or more small molecules)

representative of many classes of compounds that could potentially result in nonspecific detection in an assay. Diversity files are commercially available or can also be assembled from individual compounds commercially available from the vendors listed above.

ANTIBODIES

Specific antagonists of mTOR, which may be used to regulate the activity of these proteins (for example, for methods of treating or preventing diseases such as cancer) may include antibodies against the mTOR protein.

Similarly, angiogenesis inhibitors may include antibodies against any molecule involved in the angiogenesis process, for example, VEGF.

Antibodies, as used herein, refers to complete antibodies or antibody fragments capable of binding to a selected target, and including Fv, ScFv, Fab' and $F(ab')_2$, monoclonal and polyclonal antibodies, engineered antibodies including chimeric, CDR-grafted and humanised antibodies, and artificially selected antibodies produced using phage display or alternative techniques. Small fragments, such as Fv and ScFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution.

The antibodies described here may be altered antibodies comprising an effector protein such as a label. Especially preferred are labels which allow the imaging of the distribution of the antibody *in vivo* or *in vitro*. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within an embryo or a cell mass. Moreover, they may be fluorescent labels or other labels which are visualisable on tissue samples.

Recombinant DNA technology may be used to improve the antibodies as described here. Thus, chimeric antibodies may be constructed in order to decrease the immunogenicity thereof in diagnostic or therapeutic applications. Moreover, immunogenicity may be minimised by humanising the antibodies by CDR grafting [see

European Patent Application 0 239 400 (Winter)] and, optionally, framework modification [EP 0 239 400].

Antibodies may be obtained from animal serum, or, in the case of monoclonal antibodies or fragments thereof, produced in cell culture. Recombinant DNA technology may be used to produce the antibodies according to established procedure, in bacterial or preferably mammalian cell culture. The selected cell culture system preferably secretes the antibody product.

Therefore, we disclose a process for the production of an antibody comprising culturing a host, e.g. E. coli or a mammalian cell, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said antibody protein, and isolating said protein.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. foetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

In vitro production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilised or

entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells *in vivo*. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumours. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The foregoing, and other, techniques are discussed in, for example, Kohler and Milstein, (1975) Nature 256:495-497; US 4,376,110; Harlow and Lane, Antibodies: a Laboratory Manual, (1988) Cold Spring Harbor, incorporated herein by reference. Techniques for the preparation of recombinant antibody molecules is described in the above references and also in, for example, EP 0623679; EP 0368684 and EP 0436597, which are incorporated herein by reference.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of PGCs or other pluripotent cells, such as ES or EG cells, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange

chromatography, chromatography over DEAE-cellulose and/or (immuno-) affinity chromatography, e.g. affinity chromatography with mTOR, or fragments thereof, or with Protein-A.

Hybridoma cells secreting the monoclonal antibodies are also provided. Preferred hybridoma cells are genetically stable, secrete monoclonal antibodies of the desired specificity and can be activated from deep-frozen cultures by thawing and recloning.

Also included is a process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to mTOR, characterised in that a suitable mammal, for example a Balb/c mouse, is immunised with a one or more mTOR polypeptides, or antigenic fragments thereof; antibody-producing cells of the immunised mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example spleen cells of Balb/c mice immunised with mTOR are fused with cells of the myeloma cell line PAI or the myeloma cell line Sp2/0-Ag14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma cell line, characterised in that Balb/c mice are immunised by injecting subcutaneously and/or intraperitoneally between 10 and 10^7 and 10^8 cells expressing mTOR and a suitable adjuvant several times, e.g. four to six times, over several months, e.g. between two and four months, and spleen cells from the immunised mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PAI in the presence of a fusion promoter, preferably polyethylene glycol. Preferably the myeloma cells are fused with a three- to twentyfold excess of spleen cells from the immunised mice in a solution containing about 30 % to about 50 % polyethylene glycol of a molecular weight around 4000. After the fusion the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

Recombinant DNAs comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of antibodies directed to mTOR as described hereinbefore are also disclosed. By definition such DNAs comprise coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain variable domain and/or for a light chain variable domain of antibodies directed to mTOR can be enzymatically or chemically synthesised DNA having the authentic DNA sequence coding for a heavy chain variable domain and/or for the light chain variable domain, or a mutant thereof. A mutant of the authentic DNA is a DNA encoding a heavy chain variable domain and/or a light chain variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain variable domain and/or of the light chain variable domain of the antibody. Such a mutant DNA is also intended to be a silent mutant wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly *E. coli*, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term mutant is intended to include a DNA mutant obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and

light chain constant domains, then transferred into appropriate host cells, for example after incorporation into hybrid vectors.

Also disclosed are recombinant DNAs comprising an insert coding for a heavy chain murine variable domain of an antibody directed to mTOR fused to a human constant domain g, for example γ1, γ2, γ3 or γ4, preferably γ1 or γ4. Likewise recombinant DNAs comprising an insert coding for a light chain murine variable domain of an antibody directed to mTOR fused to a human constant domain κ or λ, preferably κ are also disclosed.

In another embodiment, we disclose recombinant DNAs coding for a recombinant polypeptide wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a cleavage site and/or a peptide spacer and/or an effector molecule.

The DNA coding for an effector molecule is intended to be a DNA coding for the effector molecules useful in diagnostic or therapeutic applications. Thus, effector molecules which are toxins or enzymes, especially enzymes capable of catalysing the activation of prodrugs, are particularly indicated. The DNA encoding such an effector molecule has the sequence of a naturally occurring enzyme or toxin encoding DNA, or a mutant thereof, and can be prepared by methods well known in the art.

Antibody Delivery

The antibodies against the mTOR protein or antibodies against any molecule involved in the angiogenesis process, for example, anti-VEGF antibodies, may be delivered into a cell by means of techniques known in the art, for example by the use of liposomes, polymers, (e.g., polyethylene glycol (PEG), N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers, polyamidoamine (PAMAM) dendrimers, HEMA, linear polyamidoamine polymers etc) etc. The immunoglobulins and/or antibodies may also be delivered into cells as protein fusions or conjugates with a protein capable of

crossing the plasma membrane and/or the nuclear membrane. For example, the immunoglobulin and/or target may be fused or conjugated to a domain or sequence from such a protein responsible for the translocational activity. Preferred translocation domains and sequences include domains and sequences from the HIV-1-trans-activating protein (Tat), Drosophila Antennapedia homeodomain protein and the herpes simplex-1 virus VP22 protein.

ADMINISTRATION

The first and/or second agents, or a composition comprising them, may be delivered by conventional medicinal approaches, in the form of a pharmaceutical composition. A pharmaceutical composition in the context of the present document is a composition of matter comprising at least an inhibitor or antagonist of mTOR, together with a second agent which comprises an angiogenesis inhibitor, as an active ingredient.

The active ingredient(s) of a pharmaceutical composition is contemplated to exhibit excellent therapeutic activity, for example, in the alleviation of cancer, tumours, neoplasms and other related diseases. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (e.g. using slow release molecules). Depending on the route of administration, the active ingredient may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredient.

In some embodiments, the inhibitor of mTOR activity is provided as an oral composition and administered accordingly. The dosage of the inhibitor of mTOR activity may be between about 1 mg /day to about 10 mg /day.

In some embodiments, the angiogenesis inhibitor is provided as an injectable or intravenenous composition and administered accordingly. The dosage of the angiogenesis inhibitor may be between about 5 mg/kg/2 weeks to about 10 mg/kg/2 weeks, for example where Avastin is being administered. For other drugs, the angiogenesis inhibitor may for example be provided in a dosage of between 10-300 mg/day, preferably at least 30 mg/day, preferably less than 200 mg/day, preferably between 30mg/day to 200 mg/day.

In order to administer the combination by other than parenteral administration, it will be coated by, or administered with, a material to prevent its inactivation. For example, the combination may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin.

Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes.

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. In some embodiments, the dispersions may be prepared in 30% Capsitol (CyDex, Inc., Lenexa, Kansas, USA). Capsitol is a polyanionic ß-cyclodextrin derivative with a sodium sulfonate salt separated from the lipophilic cavity by a butyl ether spacer group, or sulfobutylether (SBE). The cyclodextrin may be SBE7-ß-CD.

Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under

the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene gloycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the combination of polypeptides is suitably protected as described above, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs,

suspensions, syrups, wafers, and the like. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to

produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such as active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredients are compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In some embodiments, the first agent which is an antagonist of mTOR activity, and the second agent which comprises an angiogenesis inhibitor (as described in detail elsewhere) may be provided in the form of a pharmaceutical composition.

While it is possible for the composition comprising the first and second agents to be administered alone, it is preferable to formulate the active ingredient or ingredients as a pharmaceutical formulation. We therefore also disclose pharmaceutical compositions comprising a first agent which is an antagonist of mTOR activity, together with a second agent which comprises an angiogenesis inhibitor. We also disclose a pharmaceutical composition comprising a first agent which is an antagonist of mTOR activity, suitable for administration in conjunction with a second agent which comprises a angiogenesis inhibitor. We furthermore disclose a pharmaceutical composition comprising a second agent which comprises a angiogenesis inhibitor, suitable for administration in conjunction with a first agent as described.

Such pharmaceutical compositions are useful for delivery of the first or second agents, or both, preferably in the form of a composition as described, to an individual for the treatment or alleviation of symptoms as described.

The composition may include the first agent which is an antagonist of mTOR activity, optionally together with a second agent which comprises a angiogenesis inhibitor, or a fragment, homologue, variant or derivative thereof, a structurally related compound, or an acidic salt of either. The pharmaceutical formulations comprise an effective amount of the first and/or second agent, fragment, homologue, variant or derivative thereof, together with one or more pharmaceutically-acceptable carriers. An "effective amount" is the amount sufficient to alleviate at least one symptom of a disease as described, for example, cancer, a tumour, neoplasm, including hepatocellular carcinoma (HCC).

The effective amount will vary depending upon the particular disease or syndrome to be treated or alleviated, as well as other factors including the age and weight of the patient, how advanced the disease etc state is, the general health of the patient, the severity of the symptoms, and whether the first and/or second agent or variant or derivative thereof is being administered alone or in combination with other therapies.

Suitable pharmaceutically acceptable carriers are well known in the art and vary with the desired form and mode of administration of the pharmaceutical formulation. For example, they can include diluents or excipients such as fillers, binders, wetting agents, disintegrators, surface-active agents, lubricants and the like. Typically, the carrier is a solid, a liquid or a vaporizable carrier, or a combination thereof. Each carrier should be "acceptable" in the sense of being compatible with the other ingredients in the formulation and not injurious to the patient. The carrier should be biologically acceptable without eliciting an adverse reaction (e.g. immune response) when administered to the host.

The pharmaceutical compositions disclosed here include those suitable for topical and oral administration, with topical formulations being preferred where the tissue affected is primarily the skin or epidermis (for example, psoriasis, eczema and other epidermal diseases). The topical formulations include those pharmaceutical forms in which the composition is applied externally by direct contact with the skin surface to be treated. A conventional pharmaceutical form for topical application includes a soak, an ointment, a cream, a lotion, a paste, a gel, a stick, a spray, an aerosol, a bath oil, a solution and the like. Topical therapy is delivered by various vehicles, the choice of vehicle can be

important and generally is related to whether an acute or chronic disease is to be treated. As an example, an acute skin proliferation disease generally is treated with aqueous drying preparations, whereas chronic skin proliferation disease is treated with hydrating preparations. Soaks are the easiest method of drying acute moist eruptions. Lotions (powder in water suspension) and solutions (medications dissolved in a solvent) are ideal for hairy and intertriginous areas. Ointments or water-in-oil emulsions, are the most effective hydrating agents, appropriate for dry scaly eruptions, but are greasy and depending upon the site of the lesion sometimes undesirable. As appropriate, they can be applied in combination with a bandage, particularly when it is desirable to increase penetration of the agent composition into a lesion. Creams or oil-in-water emulsions and gels are absorbable and are the most cosmetically acceptable to the patient. (Guzzo et al, in Goodman & Gilman's Pharmacological Basis of Therapeutics, 9th Ed., p. 1593-15950 (1996)). Cream formulations generally include components such as petroleum, lanolin, polyethylene glycols, mineral oil, glycerin, isopropyl palmitate, glyceryl stearate, cetearyl alcohol, tocopheryl acetate, isopropyl myristate, lanolin alcohol, simethicone, carbomen, methylchlorisothiazolinone, methylisothiazolinone, cyclomethicone and hydroxypropyl methylcellulose, as well as mixtures thereof.

Other formulations for topical application include shampoos, soaps, shake lotions, and the like, particularly those formulated to leave a residue on the underlying skin, such as the scalp (Arndt et al, in Dermatology In General Medicine 2:2838 (1993)).

In general, the concentration of the composition in the topical formulation is in an amount of about 0.5 to 50% by weight of the composition, preferably about 1 to 30%, more preferably about 2-20%, and most preferably about 5-10%. The concentration used can be in the upper portion of the range initially, as treatment continues, the concentration can be lowered or the application of the formulation may be less frequent. Topical applications are often applied twice daily. However, once-daily application of a larger dose or more frequent applications of a smaller dose may be effective. The stratum corneum may act as a reservoir and allow gradual penetration of a drug into the viable skin layers over a prolonged period of time.

In a topical application, a sufficient amount of active ingredient must penetrate a patient's skin in order to obtain a desired pharmacological effect. It is generally understood that the absorption of drug into the skin is a function of the nature of the drug, the behaviour of the vehicle, and the skin. Three major variables account for differences in the rate of absorption or flux of different topical drugs or the same drug in different vehicles; the concentration of drug in the vehicle, the partition coefficient of drug between the stratum corneum and the vehicle and the diffusion coefficient of drug in the stratum corneum. To be effective for treatment, a drug must cross the stratum corneum which is responsible for the barrier function of the skin. In general, a topical formulation which exerts a high *in vitro* skin penetration is effective *in vivo*. Ostrenga et al (J. Pharm. Sci., 60:1175-1179 (1971) demonstrated that *in vivo* efficacy of topically applied steroids was proportional to the steroid penetration rate into dermatomed human skin *in vitro*.

A skin penetration enhancer which is dermatologically acceptable and compatible with the agent can be incorporated into the formulation to increase the penetration of the active compound(s) from the skin surface into epidermal keratinocytes. A skin enhancer which increases the absorption of the active compound(s) into the skin reduces the amount of agent needed for an effective treatment and provides for a longer lasting effect of the formulation. Skin penetration enhancers are well known in the art. For example, dimethyl sulfoxide (U.S. Pat. No. 3,711,602); oleic acid, 1,2-butanediol surfactant (Cooper, J. Pharm. Sci., 73:1153-1156 (1984)); a combination of ethanol and oleic acid or oleyl alcohol (EP 267,617), 2-ethyl-1,3-hexanediol (WO 87/03490); decyl methyl sulphoxide and Azone.RTM. (Hadgraft, Eur. J. Drug. Metab. Pharmacokinet, 21:165-173 (1996)); alcohols, sulphoxides, fatty acids, esters, Azone.RTM., pyrrolidones, urea and polyoles (Kalbitz et al, Pharmazie, 51:619-637 (1996));

Terpenes such as 1,8-cineole, menthone, limonene and nerolidol (Yamane, J. Pharmacy & Pharmocology, 47:978-989 (1995)); Azone.RTM. and Transcutol (Harrison et al, Pharmaceutical Res. 13:542-546 (1996)); and oleic acid, polyethylene glycol and propylene glycol (Singh et al, Pharmazie, 51:741-744 (1996)) are known to improve skin penetration of an active ingredient.

Levels of penetration of an agent or composition can be determined by techniques known to those of skill in the art. For example, radiolabeling of the active compound, followed by measurement of the amount of radiolabeled compound absorbed by the skin enables one of skill in the art to determine levels of the composition absorbed using any of several methods of determining skin penetration of the test compound. Publications relating to skin penetration studies include Reinfenrath, W G and G S Hawkins. The Weaning Yorkshire Pig as an Animal Model for Measuring Percutaneous Penetration. In:Swine in Biomedical Research (M. E. Tumbleson, Ed.) Plenum, New York, 1986, and Hawkins, G. S. Methodology for the Execution of *In Vitro* Skin Penetration Determinations. In: Methods for Skin Absorption, B W Kemppainen and W G Reifenrath, Eds., CRC Press, Boca Raton, 1990, pp.67-80; and W. G. Reifenrath, Cosmetics & Toiletries, 110:3-9 (1995).

For some applications, it is preferable to administer a long acting form of agent or composition using formulations known in the arts, such as polymers. The agent can be incorporated into a dermal patch (Junginger, H. E., in Acta Pharmaceutica Nordica 4:117 (1992); Thacharodi et al, in Biomaterials 16:145-148 (1995); Niedner R., in Hautarzt 39:761-766 (1988)) or a bandage according to methods known in the arts, to increase the efficiency of delivery of the drug to the areas to be treated.

Optionally, the topical formulations described here can have additional excipients for example; preservatives such as methylparaben, benzyl alcohol, sorbic acid or quaternary ammonium compound; stabilizers such as EDTA, antioxidants such as butylated hydroxytoluene or butylated hydroxanisole, and buffers such as citrate and phosphate.

The pharmaceutical composition can be administered in an oral formulation in the form of tablets, capsules or solutions. An effective amount of the oral formulation is administered to patients 1 to 3 times daily until the symptoms of the disease alleviated. The effective amount of agent depends on the age, weight and condition of a patient. In general, the daily oral dose of agent is less than 1200 mg, and more than 100 mg. The preferred daily oral dose is about 300-600 mg. Oral formulations are conveniently

presented in a unit dosage form and may be prepared by any method known in the art of pharmacy. The composition may be formulated together with a suitable pharmaceutically acceptable carrier into any desired dosage form. Typical unit dosage forms include tablets, pills, powders, solutions, suspensions, emulsions, granules, capsules, suppositories. In general, the formulations are prepared by uniformly and intimately bringing into association the agent composition with liquid carriers or finely divided solid carriers or both, and as necessary, shaping the product. The active ingredient can be incorporated into a variety of basic materials in the form of a liquid, powder, tablets or capsules to give an effective amount of active ingredient to treat the disease.

Other therapeutic agents suitable for use herein are any compatible drugs that are effective for the intended purpose, or drugs that are complementary to the agent formulation. The formulation utilized in a combination therapy may be administered simultaneously, or sequentially with other treatment, such that a combined effect is achieved.

EXAMPLES

Examples A1 to A7 demonstrate that Bevacizumab inhibits xenograft growth and prolongs survival in a peritoneal implant model of human hepatocellular carcinoma.

Example A1. Materials and Methods for Examples A2 to A7

Reagents

Antibodies against cdc-2, Cdk-4, Cdk-2, Cdk-6, Cyclin A, Cyclin B1, Cyclin D1, and α-tubulin are from Santa Cruz Biotechnology Inc, Santa Cruz, CA. CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1), VEGF, $p16^{INK4a}$, $p21^{WAF1}$, $p27^{Kip1}$, and Ki-67 antibodies are from Lab Vision, Fremont, CA. Conjugated secondary antibodies are supplied by Pierce, Rockford, IL. The chemiluminescent detection system is supplied by Amersham, Pharmacia Biotech, Arlington Heights, IL.

Effects of bevacizumab on the growth of subcutaneous HCC xenografts

The study received ethics board approval at the National Cancer Centre of Singapore and Singapore General Hospital. All mice are maintained according to the "Guide for the Care and Use of Laboratory Animals" published by National Institute of Health, USA. They are provided with sterilised food and water *ad libitum*, and housed in negative pressure isolators with 12 h light/dark cycles.

HCC xenografts are carried out with male *SCID* mice of 9-10 weeks age (Animal Resources Centre, Canning Vale, West Australia). Six established HCC xenograft lines, 2-1318, 5-1318, 26-1004, 30-1004, 26-1004(Cirr) and 2006, are implanted in *SCID* mice as described previously (ref A27). The creation and characterization of these xenografts are reported elsewhere (ref A27).

To investigate the effects of VEGF on the growth of HCC xenografts, bevacizumab is diluted in saline solution at an appropriate concentration. Mice bearing HCC xenografts are IP injected with either 100 µl of saline (n=12) or 5 mg/kg bevacizumab (n=12) every 14 days starting from the day 7 after tumor implantation. By this time, the HCC xenografts reached the size of approximately 100 mg. Growth of established tumor xenografts is monitored at least twice weekly by Vernier calliper measurement of the Length and Width of tumor. Tumor volume is calculated as follows: tumor volume = [(Length) x ($Width^2$) x ($\pi/6$)]. At the end of the experiments, animals are sacrificed, body weight and tumor weight are recorded, and tumors are harvested for analysis.

Effects of bevacizumab on the peritoneal implant model of HCC

Male SCID mice are intraperitoneally (IP) injected with 5 x 10^6 of 26-1004(Met) HCC cells in 200 µl phosphate buffer saline (PBS) into the peritoneal cavity. Cells could disseminate in the abdominal cavity of mice and form ascitic fluid four weeks after IP injection.

Two weeks after inoculation of tumor cells, mice bearing IP tumors are randomized (n=14) to one of the two treatment groups as follows: (a) control mice: IP

injection of 200 μl PBS every two weeks, (b) IP injection of 5 mg/kg bevacizumab every two weeks for indicated time. Survival and ascites formation are monitored three times weekly. To monitor the extent of the development of peritoneal carcinomatosis, the body weights are routinely measured. The mice are sacrificed and necropsied when they became moribund. The presence of ascitic fluid is recorded for each mouse. We also examined the macroscopic peritoneal tumor dissemination and the size and number of tumors in the abdomen. Survival is evaluated by the Kaplan-Meier method. The study is repeated at least twice.

Immunohistochemistry

Five μM sections are dewaxed, rehydrated and subjected to antigen retrieval. After blocking endogenous peroxidase activity and blocking non-specific staining, the sections are incubated with the primary antibodies against PECAM-1, VEGF, Ki-67 (Lab Vision), and cleaved caspase-3 (Cell Signaling Technology) (overnight at 4 ^{0}C). Immunohistochemistry is performed using the streptavidin-biotin peroxidase complex method, according to the manufacturer's instructions (Lab Vision) using DAB as the chromogen. Sections known to stain positively are incubated in each batch and negative controls are also prepared by replacing the primary antibody with preimmune sera. For Ki-67, only nuclear immunoreactivity is considered positive. The number of labeled cells among at least 500 cells per region is counted and then expressed as percentage values. For the quantification of mean vessel density in sections stained for CD31, 10 random 0.159 mm^2 fields at 100x magnification are captured for each tumor and microvessels are quantified. For VEGF expression, immunolocalization is scored by two independent observers as follows: positive staining, pathchy staining, and negative staining, specific staining is detectable in 60 to 100%, 20 to 60%, and 0 to 20% of hepatoma cells, respectively.

Western blot analysis

Tissue lysates are prepared and subjected to Western blot analysis as previously described (ref A27). All primary antibodies are used at a final concentration of 1 μg/ml.

Statistical analysis

Body weight, ascites formation, peritoneal tumor burden, tumor weight, Ki-67, VEGF expression, mean vessel density, and cleaved caspase-3-positive cells are compared using ANOVA tests. Survival analysis is computed by the Kaplan-Meier method and compared by Log-rank test.

Example A2. Suppression of Growth of Xenografts with Bevacizumab

To examine the role of tumor-derived VEGF in HCC growth, mice bearing xenografts 5-1318, 2-1318, 30-1004, 2006, 26-1004(Cirr) and 26-1004 are treated with bevacizumab, a recombinant humanized monoclonal antibody to VEGF. Both animal toxicity and the ability of bevacizumab to inhibit tumor formation and progression are determined. In preliminary studies, we found that treatment with a nonspecific antibody of the same IgG isotype had no effect on tumor growth and is essentially equivalent to vehicle alone (data not shown).

For dose response experiments, mice bearing the 2-1318 xenografts are weekly IP injected with 2.5, 5 and 10 mg of bevacizumab per kg body weight starting on day seven after tumor implantation.

Tumor formation is 100% in both the control and three bevacizumab-treated groups. 2-1318 xenograft growth rate is inhibited by 15%, 75% and 80% upon treating mice with 2.5, 5 and 10 mg of bevacizumab, respectively ($p<0.01$). Since the dose of 5 mg/kg gave maximal growth inhibition, we selected this dose of bevacizumab for our subsequent studies. Bevacizumab when given at the dose of 5 mg/kg also suppressed the growth of the 26-1004 (Figure 1B), 5-1318 (Figure 1C) and 2006 (Figure 1D) xenografts ($p<0.01$).

Table A1 shows that bevacizumab significantly inhibited the growth of 5 out of 6 subcutaneous HCC tumors. Bevacizumab had very little effect on the growth of 26-1004(Cirr) xenografts. The growth suppression is seen approximately one week after

treatment. Reduction of tumor growth rates without causing regressions is observed in all lines of xenografts treated with bevacizumab (Figure 1). No significant effects of bevacizumab on body weight (Table A1), animal behavior, or severe toxicity are observed during the course of treatment (data not shown).

Table A1 shows the effects of bevacizumab on tumor weight at harvest, microvessel density, cell proliferation, VEGF expression, and apoptosis of six HCC xenografts. Six indicated lines of HCC xenografts s.c. implanted on the right side of male *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts are IP administered either vehicle or 5 mg/kg bevacizumab once every two weeks for 21 days starting on day seven after tumor cell injection. By this time, the HCC xenografts reached the size of approximately 100 mg. Mean vessel density in the tumors, VEGF expression, Ki-67 index, and apoptosis are determined by immunohistochemical staining with antibodies against CD31, VEGF, Ki-67 and cleaved caspase-3, respectively. Differences in tumor weight at harvest, microvessel density, number of VEGF positive cells, Ki-67 index, and cleaved caspase-3 positive cells between vehicle- and bevacizumab-treated groups are analysed by ANOVA. The symbol * indicates $p<0.01$.

Table A1: Effects of bevacizumab on tumor weight at harvest, cell proliferation, apoptosis, VEGF expression and microvessel density of 6 Human HCC xenografts

Lines of Xenografts	Tumour Weight (mg)		Microvessel Density [1]		Ki-67 index (%)		Cleaved caspase-3 (%)		VEGF expression (%)	
			Vehicle	Bevacizumab		Bevacizumab	Vehicle	Bevacizumab	Vehicle	Bevacizumab
2-1318	2870±289	983±144*	38±8	8±4*	19.7±5.6	6±3.4*	9.9±2.4	8.4±2.0	79 ± 9	73 ± 8
5-1318	1630±192	634±95*	27±9	9±5*	9.2±2.9	3.2±2.4*	4.9±2.5	3.8±1.7	75 ± 7	73 ± 7
26-1004	3182±390	1285±149*	26±7	6±5*	19.4±8.3	7.6±3.5*	10.6±3.1	8.8±2.9	81 ± 8	76 ± 9
26-1004 (Cirr)	1657±198	1542±187	17±8	13±6	8.5±4.2	6.8±2.9	5.6±2.2	5.1±1.8	24 ± 5	22 ± 7
30-1004	1580±171	704±89*	18±6	7±5*	13.2±5.4	7.4±2.8*	6.1±1.7	5.0±1.5	65 ± 7.4	61 ± 6
2006	1669±183	854±90*	24±7	8±5*	14.1±5.0	6.3±2.5*	6.8±2.3	6.0±1.8	68 ± 8.3	65 ± 7

[1]Mean microvessel density of 10 random 0.159 mm^2 fields at 100 magnification

Example A3. Inhibition of Blood Vessel Formation in Xenografts by Bevacizumab

We next examined the association between anti-tumor activity of bevacizumab and its ability to inhibit blood vessel formation in these xenografts. Tumors harvested 21 days after administration of either bevacizumab or vehicle. Mean vessel density in the tumors is determined by immunohistochemical staining with an antibody against CD31. The median number of CD-31-positive tumor cells from vehicle- and bevacizumab-treated mice is shown in Table A1.

Treatment with bevacizumab significantly decreased mean blood vessel density in 2-1318, 5-1318, 26-1004, 30-1004 and 2006 ($p<0.01$). Table A1 shows that VEGF expression is detected at various degree in all xenograft lines and bevacizumab had no effects on its expression. The 26-1004(Cirr) xenografts that are resistant to bevacizumab had lower levels of VEGF expression than other lines of HCC xenografts (Table A1).

Example A4. Anti-Proliferative and Apoptotic Effects of Bevacizumab *in vivo*

To examine the anti-proliferative and apoptotic effects of bevacizumab *in vivo*, sections from vehicle- and bevacizumab-treated HCC tumors are stained with Ki-67 and cleaved caspase-3 antibodies respectively.

Immunohistochemical analysis revealed that the Ki-67 labelling index in bevacizumab-treated 2-1318, 5-1318, 26-1004, 30-1004 and 2006 but not 26-1004(Cirr) xenografts is significantly decreased ($p<0.01$) (Table A1). The percentage of cells stained for cleaved caspase-3 is not significant different between vehicle- and bevacizumab-treated xenografts suggesting that bevacizumab did not cause apoptosis of HCC cells (Tables A1 and A2).

These results support the view that the reduction in HCC growth by bevacizumab is associated with the inhibition of cell proliferation.

Example A5. Status of Cell Cycle Regulators in Bevacizumab-treated Tumors

Cell cycle regulators play an important role in the development and progression of HCC. Of the positive cell cycle regulators, alterations in cyclin A and cyclin B1 expression relative to normal tissue have been associated with increased cellular proliferation and clinical outcome (reviewed in ref A18).

To understand the potential mechanisms of bevacizumab action, we investigated the status of the cell cycle regulators in bevacizumab-treated tumors. No significant alterations in the levels of pRB and cyclin D1, $p16^{INK4a}$, $p21^{WAF1}$, and $p27^{Kip1}$ are observed in bevacizumab-treated tumors (data not shown). However, the levels of cdc-2, Cdk-2, Cdk-4, cyclin A and cyclin B1 are significantly reduced ($p<0.01$, Figure 2), suggesting that addition to inhibition of neovascularization, bevacizumab also inhibits cell cycle.

Example A6. Effect of Bevacizumab on IP Tumor Growth, Tumor Dissemination and Ascites Formation

VEGF is now considered to play an important role in malignant ascites formation by increase endothelial cell permeability (ref A28). Upon IP inoculation of *SCID* mice, 26-1004(Met) cells efficiently formed tumor in the peritoneal cavity, disseminated to the liver, and induced ascites. We took the advantage of this model and tested the effect of bevacizumab on the IP tumor growth, tumor dissemination and ascites formation.

However, IP tumor growth could not be monitored directly and, because of its spread within the abdomen, could not be quantified accurately. Therefore, IP tumor burden is

assessed qualitatively at postmortem examination. In all animals, treatment is initiated 14 days after inoculation of 26-1004(Met) cells and repeated every two weeks.

Figure 2A shows that all IP-injected mice receiving PBS treatment developed a swollen abdomen, indicative of ascites formation within 4-6 weeks of 26-1004(Met) tumor injection (Table A2 & Figure 3A). Soon after (within 6-10 days) the appearance of abdominal swelling, vehicle treated mice became cachectic and as a consequence are euthanized in accordance with the animal care protocol. None of the bevacizumab-treated mice showed prominent swelling of the abdomen until the end of experiment (Figure 3A). This is confirmed during autopsy, where six to eight of ascites could be collected from vehicle-treated mice. None of the bevacizumab-treated IP animals showed signs of ascites formation or cachexia at the time of postmortem examination.

Table A2 shows the effects of bevacizumab on intraperitoneal (IP) tumor burden, tumor cell dissemination to the liver, cell proliferation, VEGF expression, and ascites formation in a peritoneal model of HCC in SCID mice. Male SCID mice are IP injected with 5×10^6 of 26-1004(Met) cells in 200 µl PBS. Mice bearing IP tumors are randomized (n=14) to one of two treatment groups as follows: (a) control mice: IP injection of 200 µl PBS, or (b) IP injection of bevacizumab (5 mg/kg) once every two weeks for 42 days. Survival and ascites formation are monitored three times weekly. The mice are sacrificed and necropsied when they became moribund. The presence of ascitic fluid, macroscopic peritoneal tumor dissemination to the liver, and IP tumor burden are recorded. Mean vessel density in the tumors, VEGF expression, Ki-67 index, and apoptosis are determined by immunohistochemical staining with antibodies against CD31, VEGF, Ki-67 and cleaved caspase-3, respectively. Differences in IP tumor burden, Ki-67 index, percent of VEGF positive cells, cleaved caspase-3 positive cells, tumor cell dissemination to the liver and incidence of ascites formation between PBS- and bevacizumab-treated groups are analysed by ANOVA. The symbol * indicates $p<0.01$.

Table A2: Effects of bevacizumab on IP tumor burden, tumor dissemination to the liver, cell proliferation, microvessel density, apoptosis, and ascites formation in peritoneal model of HCC in SCID mice.

Treatment	Body weight (g)	IP tumor burden (mg)	Tumor to the liver dissemination (%)	Ascites formation (%)	Ki-67 Index (%)	Microvessel[1] Density	Cleaved-Caspase-3 (%)	VEGF expression (%)
Vehicle (n=14)	22.4 ±2.1	4230 ± 1204	14/14 (100)	12/14 (85.7)	16.4 ± 3.4	27.6 ± 5.9	6.4 ± 3.7	72 ± 8
Bevacizumab (n=14) 5mg/kg	26.4 ± 3.2	206 ± 48*	2/14 (14.2)*	0/14 (0)*	8.1 ± 2.6 *	10.1 ± 4.2*	8.1 ± 3.6 *	67 ± 7

[1]Mean microvessel density of 10 random 0.159mm^2 fields at 100x magnification

Widespread dissemination of tumor mass in the peritoneal cavity of control mice is observed (Figure 3B; Table A2). Tumors are found on the surface of the liver, diaphragm, intestines, and stomach. However, a substantial decrease in the amount of tumor mass is visible in bevacizumab-treated groups compared with the control (Figure 3B & Table A2, $p<0.01$).

Bevacizumab also inhibited the growth of small subcutaneous tumors at the site of 26-1004(Met) injection that developed in some IP animals (data not shown). Tumor dissemination is detected on the liver of all vehicle-treated mice (Figure 3C & Table A2). There are no dose-dependent effects of bevacizumab on IP tumor burden, ascites volume, and tumor dissemination when the antibody concentration is increased to 10 mg/kg (data not shown).

Figure 3D shows that while all mice in the control group are moribund at the day 42, all bevacizumab-treated mice are still alive at the day 62. These results suggest that bevacizumab can prolong survival of IP mice by inhibiting ascites formation, tumor dissemination and reducing IP tumor burden.

Example A7. Discussion of Examples A1 to A7

HCC is the second most fatal cancer worldwide. Most patients present at clinics with late stage HCC and some of them already have widespread tumor dissemination and ascites. All established non-surgical therapies reveal a poor efficiency and although therapies have been further optimized in the last decade, the mortality due to HCC remains unchanged. Therefore, new therapeutic strategies for HCC treatment are urgently needed. The role of VEGF in promoting tumor angiogenesis and metastasis, together with its negative prognostic significance in HCC, makes it an appropriate target for therapy. In the present study, we specifically ablate tumor-derived VEGF activity in subcutaneous and IP mice using the function-blocking antibody, bevacizumab. This neutral antibody, which blocks access of

VEGF to VEGF receptors, inhibits the activity of human, but not mouse, VEGF and therefore specifically blocks the activity of tumor-derived VEGF. We show that bevacizumab inhibits the growth of human HCC xenografts (5 out of 6 lines). This observation has clinical implications, because it is known that patients with known cirrhosis, hepatitis B or C viral infection, or other primary risk factors, and HCC patients who have undergone liver transplantation, resection, or liver-directed therapy have the greatest likelihood of developing primary or recurrent HCC. Therefore, targeted inhibition of angiogenesis with bevacizumab or other anti-angiogenic agents (refs A26;A29) may represent a high value, alternative approach for the treatment of HCC. Blocking angiogenesis may also be useful in maintaining dormancy of micrometastasis and preventing the development of overt recurrence or metastasis after surgical resection of a primary tumor.

In the present study, we show that bevacizumab induced a significant decrease in the number of proliferating cells, and a decreased mean vessel density compared with controls. The exact mechanisms by which bevacizumab inhibits the growth of subcutaneous HCC tumors are not known. One chief function of bevacizumab is to prevent the tumor mass from expanding by cutting off the supply of fresh nutrients and growth factors to the tumor cells. It has been reported that tumor associated endothelial cells are targets of bevacizumab *in vivo*. These cells express VEGF-R and require VEGF for proliferation and survival (ref A22). With the inhibition of VEGF activity by bevacizumab, tumor-associated endothelial cells, whose proliferating frequency is 20 to 2000 times higher than that of endothelial cells in normal organs would be more sensitive to bevacizumab therapy (refs A30;A31). It has been proposed that aberrant expression of both CDKs and CDK inhibitors is important in the development of HCC (reviewed in ref A18). It is possible that down-regulation of positive cell cycle regulators such as cyclin B1, cdc-2, Cdk-4, Cdk-2, and cyclin A by bevacizumab as reported in the present study contributes to its anti-proliferative activity.

The peritoneal cavity offers a markedly different environment for tumor growth and spread than the subcutaneous space. Within the peritoneum, 26-1004(Met) cells are not confined as they are when administered as a subcutaneous bolus. Consequently, subcutaneous

tumors grow only as a spherical mass under skin, whereas in the peritoneum, tumors grow as solid tumor foci extending into the peritoneal cavity and disseminate to various internal organs. In all bevacizumab-treated mice, the extent of IP tumor burden is significantly less than that of PBS-treated animals. These small tumors can probably survive by diffusion of nutrients from underlying host vasculature and the surrounding peritoneal fluid. Interestingly, tumor dissemination to the liver is observed only in two out of fourteen bevacizumab-treated mice, suggesting that tumor derived VEGF is obligatory for IP tumor growth and dissemination. In IP-injected mice, inhibition of tumor-derived VEGF activity by bevacizumab significantly prolongs life and completely inhibits ascites formation. The mechanisms by which bevacizumab inhibits 26-1004 (Met) dissemination to the liver are still not well understood. It is possible that bevacizumab blocks this process by interrupting the interaction between VEGF, CXCR4, and CXCL12 as described previously (refs A32;A33). Our data suggest that tumor-derived VEGF plays a pivotal role in malignant ascites formation in the IP model of HCC, possibly by increasing vascular permeability. Although this model is convenient for investigating the role of tumor-derived VEGF in ascites formation, it is not particularly analogous to the course of human HCC. In human HCC patients, the overwhelming cause of ascites is liver dysfunction and portal hypertension. Thus, it remains to be determined whether bevacizumab is able to prevent ascites accumulation caused by these conditions.

In the present study we have shown that bevacizumab inhibits the growth of five out of six lines of human HCC xenografts when given alone. Analysis of VEGF expression in this line reveals that xenografts expressed high levels of VEGF are more sensitive to bevacizumab than those had low levels of VEGF. Supporting to this view, we observe that lesser than 30% of cells in the bevacizumab-insensitive 26-1004(Cirr) xenograft stained with VEGF antibody compared to other xenograft lines (Table A1). Hence, a positive correlation between the VEGF expression and bevacizumab-induced growth inhibition exists. The data suggest that at least some patients may be amenable to single agent therapy. Designing clinical trials that accrue patients based on the level of VEGF expression in their tumors is therefore a special interest. Analysis of tumor samples for this specific biomarker is important for determining

the likely *in vivo* effects of bevacizumab. As for all potential anticancer drugs, inhibitors of the VEGF pathway may not be very effective as individual therapeutic agents because HCC tumors possess more than one genetic defect (ref A34). Since bevacizumab lowers vascular permeability by normalization of vascular architecture and function (ref A35), bevacizumab treatment may help to increase delivery of chemotherapeutic drugs to cancer cells. For maximal therapeutic benefit, it may be necessary to combine bevacizumab with another signal transduction inhibitor or conventional chemotherapeutic drugs such as doxorubicin or 5-FU. In the meantime, a multitude of other targeted agents have become available that should also tested in combination with bevacizumab. The optimal combinations can be elucidated over time.

Examples B1 to B7 demonstrate simultaneous inhibition of the mTOR pathway and angiogenesis with rapamycin and Avastin in the treatment of hepatocellular carcinoma.

Example B1. Materials and Methods for Examples B2 to B7

Reagents

Anti-p70S6 kinase, anti-cleaved caspase-3, anti-mTOR, anti-S6R, anti-4E-BP1 antibodies, and phosphorylation-specific antibodies against mTOR (Ser2448), p70S6 kinase (Thr421/Ser424), p70S6 kinase (Thr389), S6R (Ser235/236), S6R (Ser240/242), 4E-BP1 (Ser37/46), 4E-BP1 (Thr70), and 4E-BP1 (Ser65) are obtained from Cell Signaling Technology, Beverly, MA. The antibody against α-tubulin is from Santa Cruz Biotechnology Inc, Santa Cruz, CA. CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1), VEGF, and Ki-67 antibodies are from Lab Vision, Fremont, CA. Conjugated secondary antibodies are supplied by Pierce, Rockford, IL. The chemiluminescent detection system is supplied by Amersham, Pharmacia Biotech, Arlington Heights, IL.

Effects of Avastin, Rapamycin and the combination of Avastin plus Rapamycin on the growth of subcutaneous HCC xenografts

The study received ethics board approval at the National Cancer Centre of Singapore and Singapore General Hospital. All mice are maintained according to the "Guide for the Care and Use of Laboratory Animals" published by National Institute of Health, USA. They are provided with sterilised food and water *ad libitum*, and housed in negative pressure isolators with 12 h light/dark cycles.

HCC xenografts are carried out with male *SCID* mice of 9-10 weeks age (Animal Resources Centre, Canning Vale, West Australia). Seven lines of HCC xenografts (2-1318, 5-1318, 6-1205, 26-1004, 30-1004, 26-1004(Cirr) and 2006) are minced under sterile conditions. Fragments that passed through an 18-gauge needle are mixed with RPMI-1640 for transplantation in *SCID* mice. The creation and characterization of these xenograft lines are reported elsewhere (ref B49).

To investigate the effects of Avastin and rapamycin (Rapamune, Wyeth Pharmaceuticals Company, Guayama) on the growth of HCC xenografts, mice bearing HCC xenografts are weekly IP injected with either 100 μl of saline (n=14) or 5 mg/kg Avastin (n=14), or orally administered 1 mg (n=14) of rapamycin per kg of body weight (BW) daily, or the combination of VEGF antibody weekly and rapamycin (n=14) daily for three weeks starting from the day seven after tumor implantation. By this time, the HCC xenografts had reached the size of approximately 100 mg. Growth of established xenografts is monitored at least twice weekly, and tumor volume is calculated as described (ref B49). At the end of the experiments, animals are sacrificed, body weight and tumor weight are recorded, and tumors are harvested for analysis.

Effects of Avastin, rapamycin, and combined Avastin-rapamycin treatments on peritoneal carcinomatosis of HCC

Male SCID mice are IP injected with 5 x 10^6 26-1004(Met) HCC cells in 200 μl PBS into the peritoneal cavity. Cells could disseminate in the abdominal cavity of mice and form ascitic fluid 4-6 weeks after IP injection. Two weeks after inoculation of tumor cells, mice bearing IP tumors (n=14) are randomized and treated with saline, or Avastin, or rapamycin, or Avastin plus rapamycin for the indicated times as described above. Survival and ascites formation are monitored three times weekly. To monitor the extent of the development of peritoneal carcinomatosis, the body weights are routinely measured. The mice are sacrificed and necropsied when they became moribund. The presence of ascitic fluid is recorded for each mouse. We also examined the macroscopic peritoneal tumor dissemination, and the size and number of tumors in the abdomen. Survival is evaluated by the Kaplan-Meier method.

Immunohistochemistry

Five μM sections are dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity, and blocking non-specific staining, the sections are incubated with the primary antibodies against CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1), VEGF, Ki-67 (Lab Vision, Fremont, CA) and cleaved caspase-3 (Cell Signaling Technology) (overnight at 4 ^{0}C). Immunohistochemistry is performed as previously described (ref B49). For Ki-67, only nuclear immunoreactivity is considered positive. The number of labeled cells among at least 500 cells per region is counted, and then expressed as percent values. For the quantification of mean vessel density in sections stained for CD31, ten random 0.159 mm^2 fields at 100x magnification are captured for each tumor, and microvessels are quantified. For VEGF expression, immunolocalization is scored by two independent observers as follows: positive staining, patchy staining, and negative staining, specific staining is detectable in 60 to 100%, 20 to 60%, and 0 to 20% of hepatoma cells, respectively.

Statistical analysis

Body weight, ascites formation, peritoneal tumor burden, subcutaneous tumor weight, mean vessel density, Ki-67 index, VEGF expression, and percentage of cleaved caspase-3-positive cells are compared using ANOVA. Survival analysis is computed by the Kaplan-Meier method and compared by Log-rank test.

Example B2. Suppression of Growth of Xenografts with Avastin and Rapamycin

To directly examine the role of tumor-derived VEGF and the mTOR pathway in HCC growth, we established an *in vivo* model in which HCC tumor xenografts are grown in the flanks of SCID mice. Well-defined subcutaneous tumors developed within seven days of HCC tumor implantation and are sufficient size to permit accurate measurement. In preliminary studies, we found that treatment with a nonspecific antibody of the same IgG isotype had no effect on tumor growth and is essentially equivalent to vehicle alone (data not shown).

In addition, we showed that Avastin and rapamycin, when given at the dose of 5 mg/kg and 1 mg/kg to various lines of HCC xenografts, inhibited tumor growth by 58.6% ± 7.5% and 60% ± 9.6%, respectively. Therefore, we selected these doses for our combined studies.

Mice bearing 5-1318, 2-1318, 6-1205, 30-1004, 2006, 26-1004(Cirr) and 26-1004 xenografts are treated with Avastin, rapamycin, and Avastin plus rapamycin. Both animal toxicity, and the ability of these treatments to inhibit tumor formation, and progression are determined. Avastin and rapamycin, when given at the dose of 5 mg/kg and 1 mg/kg, respectively, suppressed the growth of six (6-1205, 2-1318, 5-1318, 26-1004, 30-1004, and 2006) out of seven [6-1205, 2-1318, 5-1318, 26-1004, 30-1004, 26-1004(Cirr), and 2006] xenografts examined (Table B1).

Table B1 shows the effects of Avastin, rapamycin and combined Avastin-rapamycin therapies on intraperitoneal tumor burden, VEGF expression, microvessel density, cell proliferation, and apoptosis of seven HCC xenografts. Indicated xenografts are SC implanted on the right side of male SCID mice as described in Materials and Methods. Mice bearing HCC xenografts are randomized to one of the 4 treatment groups (n=14) and treated with vehicle, Avastin (5 mg/kg), rapamycin (1 mg/kg), or combined rapamycin (1 mg/kg) and Avastin (5 mg/kg) as described in Materials and Methods. Mean vessel density, VEGF expression, Ki-67 index, and apoptosis in the tumors are determined by immunohistochemical staining with antibodies against CD31, VEGF, Ki-67, and cleaved caspase-3, respectively. Differences in tumor weight, microvessel density, VEGF expression, Ki-67 index, and cleaved caspase-3 between vehicle-, Avastin-, rapamycin, and Avastin-rapamycin-treated groups are significant ($p<0.01$) as analysed by ANOVA. The different letter indicates $p<0.01$.

Table B1: Effects of rapamycin, Avastin, and Avastin plus rapamycin on cell proliferation, apoptosis, and microvessel density, and VEGF expression of seven lines of HCC xenografts.

Lines of xenografts	Treatments	Tumour weight (mg)	Microvessel*[1] density	Ki-67 index (%)	Cleaved caspase-3 (%)	VEGF expression
2-1318	Vehicle	1961±241[a]	31±7[a]	20±5.9a	12±3.5	87±7[a]
	Avastin	896±171[b]	7±4[b]	7.8±4.1[b]	10±2.6	83±9[a]
	Rapamycin	968±178[b]	12±6[c]	9±4.3[b]	9.7±3.1	17±5[b]
	Avastin+ Rapamycin	187±39[c]	3±2.4[d]	3.6±1.7[a]	6.9±4.1	14±4[b]
5-1318	Vehicle	1971±132[a]	21.8±8[a]	10.8±2.9[a]	6.3±2.1	74±9[a]
	Avastin	1032±149[b]	9.3±6[b]	5.1±1.8[b]	5.9±1.6	71±8[b]
	Rapamycin	1040±118[b]	13.1±5[b]	6±2.4[b]	5.7±1.4	16±5[b]
	Avastin+ Rapamycin	121±39[c]	4.2±3[c]	2.3±1.6[c]	5.1±1.5	12±3.5[b]
26-1004	Vehicle	1889±209[a]	29±7.3[a]	16.8±5.1[a]	10.7±3	74±8[a]
	Avastin	930±140[b]	9.8±4.7[b]	8.6±3.1[b]	9.8±2.7	72±9[a]
	Rapamycin	911±136[b]	10.8±6.1[b]	9.8±2.1[b]	9.1±1.9	21±5[b]
	Avastin+ Rapamycin	141±29[c]	3.7±2.4[c]	4±1.2[c]	8.1±1.7	14.6±4[b]
26-1004 (Cirr)	Vehicle	1430±107[a]	14±5[a]	8.3±3.2[a]	5.9±2.1	38±5[a]
	Avastin	1218±84[a]	12±3[a]	6.9±2.4[a]	5.0±1.9	34±5[a]
	Rapamycin	1301±102[a]	12±3.4[a]	7.1±2.9[a]	5.1±1.7	31±4[a]
	Avastin+ Rapamycin	560±88[b]	5±3[b]	4.1±1.6[b]	4.8±1.4	14.7±5[b]
30-1004	Vehicle	1580±171[a]	18.1±6[a]	15.2±4.4[a]	7.1±2.4	68±7[a]
	Avastin	752±88[b]	8.5±4[b]	8.8±2.1[b]	6.1±1.8	64±5[a]
	Rapamycin	940±170[b]	10.6±5[b]	9.2±2.4[b]	6.3±1.5	18±3[b]
	Avastin+ Rapamycin	214±36[c]	3.4±1.8[c]	3±1.6[c]	5.8±1.2	14±4[b]
2006	Vehicle	1357±168[a]	21.3±6[a]	12.3±3.2[a]	6.8±1.8	64±6[a]
	Avastin	795±93[b]	10.1±4[b]	5.8±1.6[b]	7.4±1.5	61±5[a]
	Rapamycin	690±84[b]	9.6±4[b]	6.9±1.8[b]	6.4±1.2	15±4[b]
	Avastin+ Rapamycin	146±32[c]	4±2[c]	2.8±1.7[c]	5.7±1.4	11±3[b]
6-1205	Vehicle	1457±171[a]	20±5[a]	15.1±4[a]	5.1±1.7	60±7[a]
	Avastin	731±86[b]	9±3[b]	6.3±2.0[b]	4.8±1.5	57±6[a]
	Rapamycin	859±90[b]	11±4[b]	7.9±1.8[b]	4.3±1.1	22±5[b]
	Avastin+ Rapamycin	260±54[c]	5±2.5[c]	3.1±1.3[c]	4.0±0.8	17±4[b]

The tumor weight at day 21 during treatment with rapamycin, Avastin, and Avastin plus rapamycin treatments is 39.6% ± 5.4%, 39.4% ± 4.6%, and 10.8% ± 7% of the control, respectively (Figure 4 and Table B1). While reduction of tumor growth rates without causing regressions is observed in six out of seven xenograft lines treated with either Avastin or rapamycin (Figure 5), growth suppression is observed in seven of seven lines of xenografts treated with Avastin plus rapamycin (Table B1).

While Avastin and rapamycin had very little effect on the growth of 26-1004(Cirr) xenografts, its growth rate is inhibited by 60% when Avastin is given in combination with rapamycin (Table B1). No significant effects of Avastin, rapamycin, and the combined therapies on body weight or severe toxicity are observed during the course of treatment.

Example B3. Inhibition of Blood Vessel Formation in Xenografts by Avastin and Rapamycin

We next examined the association between anti-tumor activity of Avastin, rapamycin, and Avastin-rapamycin treatments and its ability to inhibit blood vessel formation in these xenografts. Tumors are harvested 21 days after administration of Avastin, rapamycin, and Avastin plus rapamycin. Immunohistochemical staining with an antibody against CD31 determined mean vessel density in the tumors. The median number of CD31-positive tumor cells from vehicle-, rapamycin, Avastin- and Avastin-rapamycin-treated 2-1318 tumors is shown in Table B1.

Treatment of mice bearing tumor xenografts with Avastin or rapamycin significantly decreased mean blood vessel density (Table B1). The number of blood vessel is further decreased when Avastin is administered together with rapamycin, and is approximately 10-15% of that seen in vehicle-treated tumors (Table B1). The mean of blood vessels in the 26-1004 (Cirr) tumors is significantly reduced only in Avastin-rapamycin treatment ($p<0.01$).

Example B4. Anti-Proliferative and Apoptotic Effects of Avastin and Rapamycin *in vivo*

To examine the anti-proliferative and apoptotic effects of Avastin, rapamycin, and combined Avastin-rapamycin treatments *in vivo*, sections from vehicle-, rapamycin-, Avastin-, and Avastin-rapamycin-treated HCC tumors are stained with Ki-67 and cleaved caspase-3 antibodies.

Immunohistochemical analysis revealed that the Ki-67 labelling index in Avastin, rapamycin-, and Avastin-rapamycin-treated 2-1318, 5-1318, 26-1004, 6-1205, 30-1004 and 5-1318, but not 26-1004(Cirr) xenografts, is significantly decreased compared to vehicle-treated tumors ($p<0.01$) (Table B1). Further decrease in the number of cells stained with Ki-67 antibody is observed in all xenografts treated with Avastin plus rapamycin (Table B1). The percentage of cells stained for cleaved caspase-3 is not significantly different among the treatments, suggesting that none of the treatments caused apoptosis (Table B1).

These results support the view that the anti-tumor effects of Avastin, rapamycin, and Avastin plus rapamycin are associated with inhibition of cell proliferation.

Example B5. Levels of Phosphorylated p70S6 kinase, S6R, and 4E-BP1 in Avastin and Rapamycin-treated Tumors

Since downstream targets of mTOR play an important role in regulating cell proliferation and angiogenesis [reviewed in 50], the levels of phosphorylated p70S6 kinase, S6R, and 4E-BP1 are investigated.

Figure 6A shows that the levels of phospho-p70S6 kinase at Thr421/Ser424, and phospho-S6R at Ser235/236 and Ser240/244 in tumors derived from mice treated with rapamycin, but not Avastin, are significantly inhibited ($p<0.01$). Both Avastin and rapamycin caused a significant decrease in total 4E-BP1 and its phosphorylation at Ser37/46 and Ser70.

Example B6. Status of Cell Cycle Regulators in Avastin and Rapamycin-treated Tumors

In addition to complete inactivation of p70S6 kinase, S6R, and 4E-BP1, the combined Avastin-rapamycin treatment also inhibited the expression of cyclin D1, Cdk-2, and cyclin B1 ($p<0.01$, Figure 6B).

The levels of phosphorylated mTOR at Ser2448 (Figure 6A), and the expression of p27, pRb and Cdk-4 (Figure 6B) are not significantly altered by any treatment. The levels of phosphorylated ERK1/2, phosphorylated STAT-3 at Tyr705, phosphorylated cdc-2 at Tyr15, phosphorylated Akt at Ser473, and cyclin A are not affected by any treatment (data not shown).

Example B7. Effect of Avastin and Rapamycin on the Development of Experimental Peritoneal Carcinomatosis in Mice

VEGF is considered to play an important role in malignant ascites formation by increasing endothelial cell permeability. Upon IP inoculation of SCID mice, the 26-1004(Met) xenograft efficiently produced peritoneal carcinomatosis. We took advantage of this model and tested the effect of Avastin, rapamycin, and Avastin plus rapamycin on the development of experimental peritoneal carcinomatosis in mice.

However, IP tumor growth could not be monitored directly and, because of its spread within the abdomen, could not be quantified accurately. Therefore, IP tumor burden is assessed qualitatively at postmortem examination. In all animals, treatment is initiated 14 days after inoculation with 26-1004(Met) cells.

Figure 7A shows that all IP-injected mice receiving PBS treatment developed a swollen abdomen, indicative of ascites formation and peritoneal carcinomatosis within 4-6 weeks of 26-1004(Met) injection. Soon after (within 6-10 days) the appearance of abdominal

swelling, vehicle-treated mice became cachetic and as a consequence are euthanized in accordance with the animal care protocol. None of the Avastin or Avastin-rapamycin-treated mice showed prominent swelling of the abdomen until the end of the experiment (Figure 7A). This is confirmed during autopsy, where large volumes of ascites could be collected from vehicle-treated (6-8 ml) mice. One out of 14 rapamycin-treated IP mice developed mild ascites (Table B2). None of the Avastin or Avastin-rapamycin-treated IP animals showed signs of ascites formation or cachexia at the time of postmortem examination.

Table B2 shows the effects of Avastin, rapamycin, and combined Avastin-rapamycin on intraperitoneal (IP) tumor burden, tumor cell dissemination to the liver, cell proliferation, ascites formation, and apoptosis in a peritoneal model of HCC in SCID mice. Male SCID mice are IP injected with 5 x 10^6 26-1004(Met) cells in 200 μl PBS. Mice bearing IP tumors are randomized to one of four treatment groups (n=14) and treated with vehicle, Avastin (5 mg/kg), rapamycin (1 mg/kg), or combined rapamycin (1 mg/kg) and Avastin (5 mg/kg) as described in Materials and Methods. Survival and ascites formation are monitored three times weekly. The mice are sacrificed and necropsied when they became moribund. The presence of ascitic fluid, macroscopic peritoneal tumor dissemination to the liver, and IP tumor burden are recorded. Mean vessel density, Ki-67 index, and apoptosis in the tumors are determined by immunohistochemical staining with antibodies against CD31, Ki-67, and cleaved caspase-3, respectively. Differences in IP tumor burden, microvessel density, Ki-67 index, ascites formation, and cleaved caspase-3 between vehicle-, Avastin-, rapamycin, and Avastin-rapamycin-treated groups are significant ($p<0.01$) as analysed by ANOVA. The different letter indicates $p<0.01$.

Table 2: Effect of Rapamycin, Avastin and Avastin plus rapamycin on intraperitoneal tumor burden, tumor dissemination to the liver, ascites formation, cell proliferation, apoptosis, and microvessel density of 26-1004(Met) HCC xenografts

Treatment	Body weight (g)	IP tumor burden (mg)	Tumor dissemination to the liver (%)	Ascites formation (%)	Ki-67 index (%)	Micro-vessel Density	Cleaved caspase-3 (%)
Vehicle (n=14)	23.7 ± 2.4	4744 ± 630[a]	14/14 (100)[a]	14/14 (100)[a]	20.6 ± 5.2[a]	32.4 ± 6.1[a]	8.6 ± 4
Avastin (n=14) 5mg/kg	24.4 ± 1.7	840 ± 150[b]	2/14 (14.2)[b]	0/14 (0[)b]	8.6 ± 3.5[b]	12.1 ± 5.4[b]	7.5 ± 3.6
Rapamycin (n=14) 1mg/kg	26.35 ± 1.8	1231.6 ± 235[c]	2/14 (14.2)[b]	1/14 (7.1)[b]	9.4 ± 4[b]	19.2 ± 4.8[b]	7.8 ± 5.0
Rapamycin + Avastin (n=14)	24.8 ± 1.5	341 ± 62[d]	0/14 (0)[c]	0/14 (0)[b]	3.1 ± 2.7[c]	3.4 ± 2.7[c]	7 ± 4.1

Widespread dissemination of tumor mass in the peritoneal cavity of vehicle-treated mice is observed (Figure 7B). Tumors are found on the surface of the liver, diaphragm, intestines, and stomach. However, a substantial decrease in the amount of tumor mass is visible in rapamycin-, and Avastin-treated groups compared with the control (Figure 7A and Table B2, $p<0.01$). Peritoneal tumor burden is further decreased in the combined Avastin-rapamycin treatment (Table B2). Tumor dissemination is detected on the liver of 14 out of 14 (100%) vehicle-treated mice. Only two out of 14 (14,2%) mice treated with Avastin or rapamycin had tumor dissemination to the liver. No tumor dissemination to the liver is observed in mice treated with the combination of Avastin-rapamycin (Table B2). Figure 7C shows that while all mice in the vehicle-, Avastin-, and rapamycin-treated groups are moribund at the day 48, 120, and 118, respectively, all Avastin-treated mice are still alive at the day 125. These results suggest that the Avastin-rapamycin treatment is effective to prolong the survival of IP mice by effectively inhibiting ascites formation, tumor dissemination, and by reducing IP tumor burden.

To determine if the combined Avastin-rapamycin treatment is able to reverse ascites accumulation in IP mice, the ascites is partially drained from the control 26-1004(Met) IP mice. They are divided into two groups: vehicle-treated and Avastin-rapamycin-treated. As expected, the control IP mice rapidly developed ascites and became cachectic within one week (Figure 8A). The Avastin-rapamycin-treated group showed no sign of ascites after seven days of treatment (Figure 8B), and almost completely recovered by day 14 after treatment (Figure 8C). They lived until sacrifice (8 weeks after treatment) and intraperitoneal tumor growth is significantly regressed (data not shown). These data suggest that in HCC, both tumor-derived VEGF and downstream targets of mTOR are absolutely required for ascites formation, tumor dissemination, and IP tumor growth.

Example B8. Discussion of Examples B1 to B7

HCC is the second most fatal cancer worldwide and is endemic in Asia because of hepatitis B and C prevalence. Most patients present at clinics with late stage HCC and some of them already have widespread tumor dissemination and ascites. Established non-surgical therapies reveal a modest efficiency inspite of the availability of new anti-cancer agents. The mortality due to HCC remains unchanged. Therefore, new therapeutic strategies for HCC treatment are urgently needed. The role of VEGF and downstream targets of the mTOR pathway in promoting tumor angiogenesis, ascites formation and metastasis, together with their negative prognostic significance in HCC, makes them the appropriate targets for therapy. In the present study, we show that Avastin or rapamycin, when given as a single agent, inhibits the growth of six out of seven HCC xenografts. The growth of HCC xenografts is further suppressed when both agents are given simultaneously. So far, all the HCC xenografts tested are sensitive to this combined therapy. These observations have clinical implications, because this non-cytotoxic combination can be studied as adjuvant therapy in resected HCC if proven effective in metastatic disease. Therefore, targeted inhibition of angiogenesis with Avastin, and the mTOR pathway with rapamycin may represent an attractive and well-tolerated approach for the treatment of this fatal disease. Beside inhibition of protein synthesis and cell cycle regulatory proteins, inhibition of angiogenesis by the combined Avastin-rapamycin therapy may prove to be useful in maintaining dormancy of micrometastasis and preventing the development of overt recurrence or metastasis after surgical resection of a primary tumor.

In the present study, we observed that phosphorylation of mTOR at Ser2448 is not decreased by rapamycin or Avastin plus rapamycin. It remains to be determined whether phosphorylation of that site is a good indication of mTOR activity. Although both Avastin and rapamycin causes a significant decrease in total 4E-BP1 and its phosphorylation at Ser37/46 and Ser70, complete inactivation of p70S6 kinase, S6R, and 4E-BP1 can only be seen in the combined Avastin-rapamycin treatment. This combined

treatment also reduced the expression of cyclin D1, Cdk-2, and cyclin B1 (Figure 6B). Despite the significant growth inhibition observed in the Avastin, rapamycin, and Avastin plus rapamycin treatments, none of them causes apoptosis as determined by cleaved caspase-3 immunostaining. The precise mechanisms responsible for potent anti-tumor activity of combined Avastin-rapamycin treatment in HCC xenografts remain to be elucidated. It is possible that the anti-tumor activity of this combination is a cumulative effect of various pathways including cell cycle, protein synthesis, and angiogenesis.

In this study, we have shown that rapamycin, but not Avastin, significantly reduced the number of VEGF-positive hepatoma cells. Both Avastin and rapamycin decreased the number of proliferating cells, and mean vessel density compared with vehicle alone. These are further decreased by the combined Avastin-rapamycin treatment. The exact mechanisms by which the combined Avastin-rapamycin treatment inhibits the growth of HCC tumors are not well understood. It is possible that this combined therapy prevents the tumor mass from expanding by blocking the supply of fresh nutrients and growth factors to the tumor cells. It has been reported that tumor associated endothelial cells are targets of both Avastin and rapamycin *in vivo*. These cells express VEGF-R and require VEGF for proliferation and survival (ref B20). With the inhibition of VEGF activity by Avastin and VEGF production by rapamycin, tumor-associated endothelial cells, whose proliferating frequency is 20 to 2000 times higher than that of endothelial cells in normal organs (refs B51,B52) would be more sensitive to the combined Avastin-rapamycin treatment.

However, HCC in human is not a naturally subcutaneous disease. Metastases of HCC is usually limited to the peritoneal cavity. Therefore, we performed similar experiments using the intraperitoneal (IP) model. In IP-injected mice, inhibition of tumor-derived VEGF activity by Avastin or VEGF production by rapamycin improves survival, and prevents ascites formation. In most Avastin-, and rapamycin-treated IP mice, the extent of IP tumor burden is significantly less than that of PBS-treated animals, suggesting that tumor derived VEGF is obligatory for IP tumor growth. In Avastin-

rapamycin-IP-treated mice, very few small tumors are still seen in the peritoneal cavity (Figure 7A). These small tumors can probably survive by diffusion of nutrients from underlying host vasculature and the surrounding peritoneal fluid.

The formation of malignant ascites, as part of the continuum of the malignant process, represents a poor prognosis in advanced HCC. With the progression of cancer to the stage of ascites formation, quality of life and survival become limited (ref B53). The management of malignant ascites is an important clinical problem. One approach to the control of malignant ascites is to limit the formation of ascites by affecting the causative malignant cells in the peritoneal cavity. Various chemotherapeutic agents, such as carboplatin, cisplatin, 5-FU, and leucovarin have had limited success in reducing malignant ascites when used systematically or intraperitoneally (ref B54). In the present study, Avastin, rapamycin, and combined Avastin-rapamycin treatments can effectively inhibit the development of ascites in an HCC model of peritoneal carcinomatosis. For long-term therapy, combined Avastin-rapamycin treatment is far more effective in suppressing ascites formation than either Avastin or rapamycin alone. The inhibition of experimental peritoneal carcinomatosis by Avastin-rapamycin treatment is clearly shown by the inhibition of ascitic fluids and the inhibition of growth of disseminated tumors in Avastin-rapamycin-treated mice in comparison with either Avastin- or rapamycin-treated animals. Furthermore, Avastin-rapamycin treatment is capable of reversing ascites accumulation. This observation suggests that combined Avastin-rapamycin therapy may be useful for the treatment of peritoneal carcinomatosis, an incurable complication of HCC, and especially beneficial for patients with IP-free cancer cells without macroscopic peritoneal metastasis.

Because VEGF is important for the development, progression, and metastasis of HCC and the mTOR is essential for VEGF production, inhibiting VEGF activity by Avastin and VEGF production by rapamycin, therefore, would be a logical combination for treating HCC.

Examples C1 to C7 show effective inhibition of tumor growth in patient-derived xenografts of hepatocellular carcinoma by rapamycin and bevacizumab

Examples C1 to C7 demonstrate effective inhibition of tumor growth in patient-derived xenografts of hepatocellular carcinoma by rapamycin and bevacizumab

Example C1. Materials and Methods for Examples C2 to C7

Reagents

Antibodies to total p70S6K, cleaved caspase-3, mTOR, RPS6, 4EBP1, and phospho-mTOR (Ser2448), p70S6K (Thr421/Ser424, Thr389), RPS6 (Ser235/236, Ser240/242), 4E-BP1 (Ser37/46, Thr70, Ser65) are obtained from Cell Signaling Technology, Beverly, MA. α-tubulin, cyclin D1, Cdk-2, Cdk-4, cyclin B1, and p27 antibodies are from Santa Cruz Biotechnology Inc, Santa Cruz, CA. CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1), VEGF, PTEN, and Ki-67 antibodies are from Lab Vision, Fremont, CA. Conjugated secondary antibodies are from Pierce, Rockford, IL. Chemiluminescent detection kits are from Amersham, Pharmacia Biotech, Arlington Heights, IL.

Generation and Maintenance of Patient-derived HCC Xenografts

The Institutional Review and Ethics Boards of the National Cancer Centre of Singapore and Singapore General Hospital approved this study. Mice are maintained following "Guide for the Care and Use of Laboratory Animals" protocols (National Institutes of Health, USA) and Institutional Animal Care and Use Committee (IACUC) guidelines. The establishment of the primary HCC xenografts has been previously described (ref C18). Briefly, primary HCCs obtained during liver resection are minced into fine fragments capable of passing through an 18-gauge needle, mixed in a 1:1 (v/v) ratio with Matrigel (Collaborative Research, Bedford, MA) and injected in the subcutaneous flanks of 8-week-old male severe combined immune deficient (SCID/SCID,

The Jackson Laboratory, Harbor, ME) mice. Six to eight mice are injected for each primary tumor. Growth of established tumor xenografts is monitored twice weekly by vernier caliper measurement of tumor length (a) and width (b), and tumor volume is calculated as $(a \times b^2) / 2$. Serial passages of xenograft lines are obtained by dissecting tumors from sacrificed animals and reinjecting the dissociated tumor cells into successive generations of *SCID* mice as described above. Seven xenograft lines are studied in this report (2-1318, 5-1318(1), 5-1318(3), 2006, 26-1004, 26-1004(cirr), and 30-1004). Xenografts 2-1318 and 5-1318(1) are derived from Hep B-positive patients.

Histopathology and Immunohistochemistry of HCC Xenografts and Primary Tumors

Haematoxylin and eosin (H&E) stained sections of HCC patient tumors are obtained from hospital pathology archives. Xenograft tumors are paraffin-embedded and sectioned (5 μM), dewaxed, rehydrated, and subjected to antigen retrieval and staining. Fixed sections are incubated with primary antibodies against CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1) to assess microvessel density, VEGF, Ki-67, cleaved caspase-3 (overnight at 4C), PTEN, and RPS6 (total and phosphorylated (S240/242)). For Ki-67, only nuclear immunoreactivity is considered positive, and the number of labeled cells among at least 500 cells per region are counted and expressed as percent values. For CD31, the mean microvessel density (MVD) is quantified for each tumor by counting microvessels in ten random 0.159 mm^2 fields at 100x magnification. For VEGF, expression is scored by two independent observers according to the categories: positive staining (60-100% tumor cells), patchy staining (20-60%), and negative staining (0-20%).

Array-based Comparative Genomic Hybridization

Genomic DNA is isolated from primary and xenograft tumors using a Qiagen DNA extraction kit, and processed for hybridization on Agilent 185K microarrays according to the manufacturer's instructions (Agilent, USA). Normal male human

genomic DNA is used as reference. After hybridization and washing, the arrays are scanned on an Agilent 2565BA microarray scanner. Images are analyzed using Feature Extraction software (version 9.1, Agilent Technologies) and CGHAnalytics software (version 3.4, Agilent Technologies), using the ADM-2 (Aberration Detection Module -2) algorithm, which identifies all aberrant intervals in a given sample with consistently high or low log ratios based on a statistical score. The PROGENETIX CGH (www.progenetix.com) database is used to compare the aCGH data with known amplifications and deletions in HCC.

Gene Expression Profiling

RNA is extracted from primary and xenograted tumors using Trizol (Invitrogen, Carlsbad, CA) reagent and processed for Affymetrix Genechip (Affymetrix Inc., Santa Clara, CA) hybridizations using U133plus Genechips according to the manufacturer's instructions. Hybridization signals on the chip is scanned and processed by GeneSuite software (Affymetrix Inc., Santa Clara, CA). Raw Genechip scans are quality controlled using GeneDataTM Refiner (Genedata, Basel, Switzerland). Paired t-tests with Benjamini and Hochberg correction (for multiple hypotheses correction) are used to identify differentially regulated genes between untreated and treated samples (Table C2). Gene Ontology analysis is performed using the GOSTAT tool (http://gostat.wehi.edu.au/). The microarray data is accessible under GEO accession number GSE6465.

Mutation Genotyping

Xenograft samples are analyzed for mutations in *p53*, *PTEN*, *PIK3CA*, *TSC1*, *TSC2*, and *HIF1A*. *p53* mutations (exons 5-9) are determined using a combined DHPLC/sequencing approach (ref C19), and revealed the xenograft lines to carry the following *p53* mutations : 2-1318 (R249S), 5-1318(1) (R249S), 5-1318(3) (R249S), 2006 (P177L), and 30-1004 (H214R). Mutations in mTOR-related genes (*PTEN* (exons 1-9), *PIK3CA* (exons 9 and 20), *TSC1* (exons 3-23) and *TSC2* (exons 1-41)) are determined by

direct sequencing of genomic DNA. All PCR products are sequenced in both directions. Potential mutations are confirmed by two independent rounds of PCR sequencing.

Drug Treatments (Subcutaneous Model)

HCC xenograft lines are treated according to the following categories: 1) IP injection with 100 µl saline (vehicle/control), 2) Oral administration of 1mg/kg rapamycin (RAPA, Rapamune, Wyeth Pharmaceuticals Company, Guayama), 3) IP injection with 5 mg/kg bevacizumab (BEV, Avastin, Genentech, Inc., South San Francisco, CA), and 4) combined oral RAPA and injected BEV (BEV/RAPA). Control and BEV injections are performed on a weekly basis, while oral RAPA is administered daily. Treatment commenced after day seven of tumor implantation when the tumors are approximately 100 mg, and continued for three subsequent weeks. Each treatment arm involved fourteen independent tumor-bearing mice representing the same xenograft line. Tumor growth is monitored as described above. At the end of the treatment regimen, animals are sacrificed, body and tumor weights are recorded, and tumors are harvested for molecular analysis.

Drug Treatments (Orthotopic Model)

Orthotopic models are established by injecting the peritoneal cavity of male SCID mice with 5 x 10^6 HCC cells from the 26-1004 xenograft line. Gross ascites is typically detected 4-6 weeks after intra-peritoneal (IP) injection. Two weeks after tumor cell inoculation, mice bearing IP tumours (n=14 per treatment arm) are randomized and treated with control saline, RAPA, BEV, or RAPA/BEV. Body weight, ascites formation, and overall survival are monitored three times weekly. Tumor-bearing mice are sacrificed when they became moribund, and the presence of ascitic fluid is recorded for each mouse. The extent of macroscopic peritoneal tumor dissemination, and the size and number of tumors in the abdomen is also recorded.

Pharmacokinetic Analysis

RAPA concentrations are determined in mice whole blood (0.6mLs) using a reverse-phase HPLC method as described (ref C20).

Statistical analysis

Body weight, ascites formation, peritoneal tumor burden, subcutaneous tumor weight, mean vessel density, Ki-67 index, VEGF expression, and percentage of cleaved caspase-3-positive cells are compared using ANOVA. Differences in survival are evaluated by the Kaplan-Meier method and compared by a log-rank test.

Example C2. Histologic and Molecular Profiling of Patient-Derived HCC Xenografts

We have previously generated patient-derived tumor xenografts of HCC to screen novel therapeutic combinations (ref C18). Primary HCC tumors obtained from patient surgery are directly implanted into the flanks of SCID mice and well-defined subcutaneous tumors developed within seven days of tumor implantation. Seven independent xenograft lines are tested in this study. Each xenograft grew at its own characteristic growth rate, which remained stable across subsequent passages (data not shown). At the histologic level, the xenograft tumors closely resembled the cellular architecture and tumor grade of the original patient tumors when evaluated by two independent clinical histopathologists (TPH and MST) - specifically, the solid, trabecular or tubular cellular arrangements are maintained along with the nuclear polymorphism of the tumor cells (Figure 9, A-G). All seven xenografts exhibited prominent vascularity, indicating that they are likely to express angiogenic factors. In comparison, xenografted tumors established from *in vitro* cultured cancer cell lines are associated with ectatic vascular structures (eg HepG2) or are devoid of discernible vasculature (eg PLC/PRF/5, Fig 1H and I) indicating the potential absence of angiogenic factor expression in the latter.

To extend our analysis beyond microscopy, we also performed genome-wide array-based comparative genomic hybridization and gene expression profiling on the patient-derived xenografts. When compared by array-based comparative genomic hybridization (aCGH) using Agilent 185K oligonucleotide microarrays, five out of seven lines exhibited strikingly similar patterns of chromosomal amplifications and deletions between the xenografts and primary tumors (Figure 14A), including alterations frequently observed in HCC such as chromosomal bands 1p32-36, 4q13.2, 6p21, and 17q21-22. A comparison of the major genomic aberrations between early passage (passage 1-2) and late passage lines (passage 9-10, representing the oldest available lines) confirmed the stablility of the xenograft karyotypes within this window of time (data not shown). We also compared the gene expression profiles of the xenografted tumors to primary tumors and HCC cell lines. Using an unsupervised hierarchical clustering algorithm, we found that the expression profiles of the xenografts intermingled with the profiles of the primary tumors, while HCC cell lines collectively migrated in a related but distinct branch (Figure 14B). These results suggest that despite being grown in another species (SCID mice) in a non-orthotopic location (flank vs liver), the patient-derived HCC xenografts nevertheless retain a reasonably similar histologic and molecular expression profile to clinical HCC.

Example C3. Activation of the mTOR Pathway in HCC Xenografts and Primary HCCs

To examine the role of the mTOR pathway in HCC, we used a panel of antibodies targeted to the mTOR pathway (mTOR, S6K1, RPS6, and 4EBP1) to investigate mTOR signaling in HCCs or non-malignant normal livers (Figure 10A). There is no observable difference between non-malignant livers and HCCs in either total or phosphorylated mTOR at serine 2448, a site that is phosphorylated by Akt (ref C21). However, the functional significance of S2448 phosphorylation with respect to mTOR pathway activation is unclear (ref C22). Indeed, we noted activation of multiple downstream components of the mTOR-signaling pathway specifically in tumors, including phosphorylated S6K1 and RPS6, a target of S6K1. When examined by

immunohistochemistry with antibodies to phosphorylated RPS6 and PTEN, a negative regulator of the mTOR pathway, non-malignant background liver exhibited strong PTEN protein expression and minimal phosphorylated RPS6, while a reciprocal pattern of minimal PTEN expression and abundant phosphorylated RPS6 is observed in HCC tumors (Figure 10B). Similar patterns of mTOR activation are also observed in the xenograft lines (Figure 10C). A mutational analysis of mTOR related genes (*PIK3CA*, *PTEN, TSC1, TSC2* and *HIF1A*) revealed that one xenograft line (26-1004) exhibited a 16 bp deletion in exon 8 of the *PTEN* gene. Taken collectively, our results suggest that the mTOR pathway is likely activated in a significant fraction of HCCs and xenograft lines.

Example C4. A RAPA/BEV Combination Protocol Inhibits HCC Xenograft Growth

Echoing the chemorefractory nature of clinical HCC, we have previously shown that tumor growth in the patient-derived HCC xenografts is resistant or only modestly sensitive to a variety of commonly used chemotherapeutic drugs (oxaliplatin, cisplatin, 5-FU, and doxorubicin), and small molecule inhibitors including the EGFR inhibitor gefitinib (ref C16). To examine if mTOR or VEGF inhibition might cause growth inhibition, we treated tumor-bearing mice with RAPA (1 mg/kg daily, administered orally), BEV (5 mg/kg weekly, injected), or RAPA/BEV (same dosing as single agent therapy). In humans, typical BEV intravenous dosings range from 5mg/kg to 10mg/kg every 2 weeks. For RAPA, we performed pharmacokinetic analysis and determined circulating RAPA levels in the xenografts to range from 20-80 ng/ml (average 33 ng/ml), similar to therapeutically achievable levels in human (5-20 nM) (ref C23). As single agents, both RAPA and BEV inhibited tumor growth by 58.6% ± 7.5% and 60% ± 9.6% respectively, with the weights of RAPA and BEV treated tumors being 39.6% ± 5.4% (RAPA) and 39.4% ± 4.6% (BEV) of controls after 21 days. Treatment of the xenografts with a nonspecific antibody of the same IgG isotype as BEV had minimal effects on tumor growth and is essentially equivalent to vehicle alone (data not shown). The

combination treatment RAPA/BEV, however, induced a significantly greater inhibition of growth than single agent RAPA or BEV. At day 21 post-treatment, the tumor weights of the treated xenografts are 39.6 ± 5.4% for RAPA, 39.4 ± 4.6% for BEV, and 10.8 ± 7% for RAPA/BEV compared to controls (Figure 11A and Table C1).

Table C1. Effects of RAPA, BEV and RAPA/BEV on tumor weight, VEGF expression, MVD, cell proliferation, and apoptosis in HCC xenografts. Data from four representative xenograft lines are shown. Differences in tumor weight, MVD, VEGF expression, Ki-67 index, and cleaved caspase-3 between control, RAPA, BEV and RAPA/BEV-treated groups are significant ($p<0.01$) as analysed by ANOVA (indicated by different letters - ^{b} indicates a significant difference to ^{a}, ^{c} a significant difference to ^{a} and ^{b})

Lines	Treatments	Tumour weight (mg)	Microvessel density	Ki-67 index (%)	Cleaved caspase-3 (%)	VEGF expression
2-1318	Vehicle	1961±241^{a}	31±7^{a}	20±5.9^{a}	12±3.5	87±7^{a}
	RAPA	968±178^{b}	12±6^{c}	9±4.3^{b}	9.7±3.1	17±5^{b}
	BEV	896±171^{b}	7±4^{b}	7.8±4.1^{b}	10±2.6	83±9^{a}
	RAPA/BEV	187±39^{c}	3±2.4^{d}	3.6±1.7^{a}	6.9±4.1	14±4^{b}
5-1318(3)	Vehicle	1971±132^{a}	21.8±8^{a}	10.8±2.9^{a}	6.3±2.1	74±9^{a}
	RAPA	1040±118^{b}	13.1±5^{b}	6±2.4^{b}	5.7±1.4	16±5^{b}
	BEV	1032±149^{b}	9.3±6^{b}	5.1±1.8^{b}	5.9±1.6	71±8^{b}
	RAPA/BEV	121±39^{c}	4.2±3^{c}	2.3±1.6^{c}	5.1±1.5	12±3.5^{b}
26-1004	Vehicle	1889±209^{a}	29±7.3^{a}	16.8±5.1^{a}	10.7±3	74±8^{a}
	RAPA	911±136^{b}	10.8±6.1^{b}	9.8±2.1^{b}	9.1±1.9	21±5^{b}

	BEV	930±140[b]	9.8±4.7[b]	8.6±3.1[b]	9.8±2.7	72±9[a]
	RAPA/BEV	141±29[c]	3.7±2.4[c]	4±1.2[c]	8.1±1.7	14.6±4[b]
	Vehicle	1430±107[a]	14±5[a]	8.3±3.2[a]	5.9±2.1	38±5[a]
26-1004	RAPA	1301±102[a]	12±3.4[a]	7.1±2.9[a]	5.1±1.7	31±4[a]
(cirr)	BEV	1218±84[a]	12±3[a]	6.9±2.4[a]	5.0±1.9	34±5[a]
	RAPA/BEV	560±88[b]	5±3[b]	4.1±1.6[b]	4.8±1.4	14.7±5[b]

When treatments are monitored over time, single-agent RAPA or BEV reduced but did not completely suppress the rate of xenograft tumor growth. In contrast, near-stable growth suppression over time is observed in all seven xenografts lines treated with RAPA/BEV (Figure 11B). Furthermore, while single-agent RAPA and BEV had little effect on the growth rate of 26-1004(Cirr) xenografts, RAPA/BEV inhibted the growth rate of this xenograft line by 60% (Table C1). No significant effects on body weight, morbidity or severe toxicities are observed in the RAPA, BEV, and RAPA/BEV treatment arms. Thus, the RAPA/BEV combination protocol appears to inhibit tumor growth to a significantly greater degree than standard chemotherpay or RAPA and BEV alone, and may show efficacy against tumors that are resistant to single-agent treatment.

Example C5. Target Modulation and Potential Synergy of RAPA/BEV

To better understand the potential molecular mechanisms of RAPA/BEV action, we investigated the status of the mTOR pathway in tumors after 21 days of treatment. No significant alterations in the levels of total or phoshorylated mTOR (S2448) are observed in all three treatments. However, RAPA and RAPA/BEV, but not BEV alone, induced significant reductions in the levels of phosphorylated S6K1 (Thr421/Ser424), and phosphorylated RPS6 (Ser235/236 and Ser240/244) ($p<0.01$, ANOVA), suggesting inhibition of the mTOR pathway in the treated xenografts (Figure 12A). Confirming the specificity of mTOR inhibition, the levels of phosphorylated ERK1/2, STAT-3(Tyr705), cdc-2(Tyr15), Akt(Ser473), and cyclin A are not affected by these treatments (data not shown). The levels of target reduction achieved by RAPA/BEV in the mTOR components are greater than RAPA alone, suggesting that the RAPA/BEV combination likely induces greater levels of mTOR pathway inhibition than RAPA single-agent treatment (compare RAPA and RAPA/BEV columns in Table C1). In contrast, BEV treatment did not induce similar alterations in mTOR pathway components, suggesting that at these *in vivo* concentrations BEV mono-therapy may not suppress mTOR pathway activity. Notably, all three treatments – RAPA, BEV, and RAPA/BEV induced significant decreases in the

levels of total and phosphorylated 4E-BP1 compared to controls. This result suggests that one mechanism by which RAPA/BEV inhibits tumor growth is by suppressing activity of the mTOR-signaling pathway.

We then investigated the tumor vasculature of the treated tumors for alterations in gross morphology, VEGF expression, and tumor microvessel density (MVD). RAPA, BEV, and RAPA/BEV-treated tumors all exhibited a gross reduction in tumor-associated vasculature compared to controls, with RAPA/BEV treated tumors being associated with the least numbers of visible blood vessels (Figure 11A). By immunohistochemisty, we found that VEGF expression is significantly suppressed in both the RAPA and RAPA/BEV treated tumors, but not in BEV treated tumors (Figure 12B), suggesting that VEGF expression from HCC tumor cells is likely to depend on mTOR pathway activity. To quantitate this finding at the microscopic level, we measured MVDs in the treated tumors using an endothelial cell specific anti-CD31 antibody. Both RAPA and BEV significantly reduced tumor MVD (Figure 12B), which is further decreased by RAPA/BEV to approximately 10-15% of control tumors (Table C1). Notably, significant reductions in tumor MVD in 26-1004 (Cirr) tumors, which are resistant to growth inhibition by RAPA or BEV alone, are only observed in the RAPA/BEV treatment arm ($p<0.01$, ANOVA). Thus, there is a good correlation between the ability of RAPA/BEV to suppress HCC growth and to inhibit tumor angiogenesis.

Besides angiogenesis, mTOR pathway signaling has also been associated with other oncogenic processes, including tumor proliferation and resistance to apoptosis. We investigated the potential anti-proliferative and apoptotic effects of RAPA, BEV and RAPA/BEV by performing IHC on treated tumor sections with antibodies to both Ki-67 (a marker of cell proliferation) and cleaved caspase-3 (a marker of apoptosis). Ki-67 labeling is significantly reduced in the RAPA, BEV, and RAPA/BEV treatment arms in all xenograft lines with the exception of 26-1004(Cirr) compared to untreated tumors ($p<0.01$, ANOVA) (Table C1), with the most extreme reductions observed in the RAPA/BEV arm. Surprisingly, the percentage of tumor cells positive for cleaved caspase-

3 is not significantly different between any of the treatment arms and mock-treated controls, suggesting that levels of apoptosis are unlikely to be altered by the treatments. Taken collectively, these results support a model where the anti-tumor effects of RAPA/BEV are likely to involve mTOR pathway inhibition and targeting of the tumor vasculature, which may then lead to a secondary inhibition of cell proliferation but not an increase in tumor apoptosis. In addition, our data does not exclude the possibility that RAPA/BEV may also exert an anti-proliferative effect directly on tumor cells (see Discussion).

The enhanced levels of tumor inhibition in the RAPA/BEV treatment arm are likely to involve both additive and synergistic effects. There is clearly an additive component, since both RAPA and BEV are able to induce growth inhibition when administered as single agents in the majority of xenograft lines (Figure 11). However, purely additive effects are unlikely to explain the occurrence of several molecular alterations specifically associated with RAPA/BEV treatment. For example, while RAPA/BEV inhibited the expression of several cell-cycle related components, including cyclin D1, Cdk-2, and cyclin B1 ($p<0.01$, ANOVA, Figure 12A), such effects are not observed when RAPA or BEV are used as monotherapy. We conducted a microarray analysis of three independently treated xenograft lines (2-1318, 5-1318(3), 26-1004) and identified 148 significantly regulated genes in RAPA/BEV treated tumors compared to controls after correcting for multiple hypotheses ($p<0.05$, paired t-test with Benjamini and Hochberg correction) (Figure 15). These RAPA/BEV regulated genes are enriched in gene ontologies related to amino acid metabolism ($p=7.3E-6$, corrected p-value), macromolecular metabolism ($p=0.0002$), and steroid biosynthesis ($p=0.058$). When compared against monotherapy, a significant proportion of the RAPA/BEV regulated genes (70%) are also regulated in the RAPA and to a lesser extent BEV-only treatments. However, the degree of regulation for many of these overlapping genes in the RAPA/BEV treatment is significantly more dramatic compared to the RAPA-only arm (corrected $p<0.05$) (Figure 15).

In addition to RAPA-regulated genes, we also identified a smaller but distinct set of genes that appeared to be distinctly regulated by RAPA/BEV, including genes involved in cell growth and protein translation such as *IRS1*, *CDC2*, and *EIF4A1* (Table C2).

Table C2. Genes specifically regulated by RAPA/BEV.

Affy probe ID	GENE NAME	Unigene
a) *upregulated genes upon RAPA/BEV treatment*		
211787_s_at	eukaryotic translation initiation factor 4A, isoform 1	Hs.356129
1554390_s_at	ARP2 actin-related protein 2 homolog (yeast)	Hs.393201
213397_x_at	ribonuclease, RNase A family, 4	Hs.283749
223244_s_at	13kDa differentiation-associated protein	Hs.44163
210759_s_at	proteasome (prosome, macropain) subunit, alpha type, 1	Hs.82159
226978_at	hypothetical protein MGC2452	Hs.275711
218890_x_at	Homo sapiens transcribed sequence with strong similarity to protein sp:O15532 (H.sapiens) SELW_HUMAN Selenoprotein W	Hs.433439
200767_s_at	Homo sapiens transcribed sequence with weak similarity to protein sp:Q9NZB2 (H.sapiens) CI10_HUMAN Protein C9orf10	Hs.446534
205538_at	coronin, actin binding protein, 2A	Hs.44396
213346_at	hypothetical protein BC015148	Hs.13413
1565717_s_at	Fusion (involved in t(12;16) in malignant liposarcoma)	Hs.513522
205633_s_at	aminolevulinate, delta-, synthase 1	Hs.78712
242121_at	Sapiens cDNA FLJ33139 fis, clone UTERU1000109.	Hs.349570
231968_at	similar to Gamma-glutamyltranspeptidase 1 precursor (Gamma-glutamyltransferase 1) (CD224 antigen)	Hs.124660
204686_at	Homo sapiens transcribed sequences	Hs.390242
225568_at	hypothetical protein MGC14141	Hs.356744
228006_at	Homo sapiens transcribed sequence	Hs.253309
224156_x_at	interleukin 17 receptor B	Hs.5470
200723_s_at	membrane component, chromosome 11, surface marker 1	Hs.278672
226104_at	hypothetical protein DKFZp564A022	Hs.170822
242110_at	Homo sapiens transcribed sequences	Hs.395468
239630_at	Homo sapiens transcribed sequences	Hs.270975
225909_at	hypothetical protein LOC155036	Hs.58582
202761_s_at	Homo sapiens transcribed sequences	Hs.444069
227354_at	hypothetical gene supported by AK095177	Hs.13256
226354_at	lactamase, beta	Hs.410388
203176_s_at	transcription factor A, mitochondrial	Hs.75133
200931_s_at	vinculin	Hs.75350
209226_s_at	Sapiens cDNA FLJ31107 fis, clone IMR322000152.	Hs.405954
203825_at	bromodomain containing 3	Hs.86896
227531_at	Sapiens cDNA FLJ39000 fis, clone NT2RI2022468.	Hs.40289
231896_s_at	density-regulated protein	Hs.22393
231968_at	similar to Gamma-glutamyltranspeptidase 1 precursor (Gamma-glutamyltransferase 1) (CD224 antigen)	Hs.124660
204686_at	Homo sapiens transcribed sequences	Hs.390242
225568_at	hypothetical protein MGC14141	Hs.356744
228006_at	Homo sapiens transcribed sequence	Hs.253309
224156_x_at	interleukin 17 receptor B	Hs.5470
200723_s_at	membrane component, chromosome 11, surface marker 1	Hs.278672
226104_at	hypothetical protein DKFZp564A022	Hs.170822
242110_at	Homo sapiens transcribed sequences	Hs.395468

239630_at	Homo sapiens transcribed sequences	Hs.270975
225909_at	hypothetical protein LOC155036	Hs.58582
202761_s_at	Homo sapiens transcribed sequences	Hs.444069
227354_at	hypothetical gene supported by AK095177	Hs.13256
226354_at	lactamase, beta	Hs.410388
203176_s_at	transcription factor A, mitochondrial	Hs.75133
200931_s_at	vinculin	Hs.75350
209226_s_at	Sapiens cDNA FLJ31107 fis, clone IMR322000152.	Hs.405954
203825_at	bromodomain containing 3	Hs.86896
227531_at	Sapiens cDNA FLJ39000 fis, clone NT2RI2022468.	Hs.40289
231896_s_at	density-regulated protein	Hs.22393

b) ***downregulated genes upon RAPA/BEV treatment***

220094_s_at	chromosome 6 open reading frame 79	Hs.261023
210559_s_at	cell division cycle 2, G1 to S and G2 to M	Hs.334562
218497_s_at	Homo sapiens transcribed sequence with weak similarity to protein ref:NP_060265.1 (H.sapiens) hypothetical protein FLJ20378 [Homo sapiens]	Hs.390499
201278_at	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	Hs.81988
201351_s_at	Homo sapiens transcribed sequences	Hs.439510
203214_x_at	cell division cycle 2, G1 to S and G2 to M	Hs.334562
221435_x_at	hypothetical protein HT036 [BLAST]	Hs.321669
212811_x_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Hs.323878
210757_x_at	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	Hs.81988
201176_s_at	archain 1	Hs.33642
235138_at	Homo sapiens transcribed sequences	Hs.444568
208447_s_at	phosphoribosyl pyrophosphate synthetase 1	Hs.56
222231_s_at	hypothetical protein PRO1855	Hs.283558
209080_x_at	thioredoxin-like 2	Hs.42644
225469_at	LOC341944	Hs.447488
224657_at	Gene 33/Mig-6	Hs.11169
203622_s_at	putatative 28 kDa protein	Hs.193384
204020_at	purine-rich element binding protein A	Hs.29117

These findings, coupled with the ability of RAPA/BEV but not RAPA or BEV monotherapy to inhibit 26-1004(Cirr) growth, suggest that the potent tumor inhibition ability of RAPA/BEV is likely to involve both additive and synergistic components. Further research is required to better understand the complexities of RAPA/BEV action – for example BEV activity may cause normalization of the vascular architecture to allow greater delivery of RAPA to the cancer cells, a mechanism that has been previously proposed (ref C24).

Example C6. RAPA/BEV Inhibits Peritoneal Metastasis, Malignant Ascites, and Prolongs Survival

Finally, we investigated the efficacy of RAPA/BEV treatment against tumors growing in the primary target organ (ie liver). To establish an orthotopic model of HCC, we performed intra-peritoneal (IP) injections of tumor cells from the 26-1004 xenograft line into the abdomens of SCID mice. As we are unable to directly monitor IP tumor growth and spread within the abdomen cavity over time, we assessed IP tumor burden at postmortem examination. Within 4-6 weeks of tumor introduction, injected mice developed a swollen abdomen, indicative of ascites formation, and became highly cachetic (Figure 13A). Upon autopsy, the abdomens of injected mice exhibited large volumes of ascites (6-8 ml), and widespread dissemination of tumors to peritoneal organs (diaphragm, intestines, and stomach) including the liver under the serosal surface (Figure 13B). We tested if RAPA, BEV, or RAPA/BEV might confer a therapeutic benefit in these animals, initiating treatment 14 days after IP inoculation. Unlike mock-treated controls, none of the BEV or RAPA/BEV treated mice exhibited abdominal swelling or cachexia (Figure 13A), and only one out of fourteen RAPA–treated mice developed mild ascites (Table C3).

Table C3 : Effect of RAPA, BEV and RAPA/BEV on intra-liver and intraperitoneal tumor burden, ascites formation, cell proliferation, apoptosis, and microvessel density of IP injected 26-1004 HCC xenografts

Treatment	Body weight (g)	IP tumor burden (mg)	Tumor dissemination to the liver (%)	Ascites formation (%)	Ki-67 index (%)	Micro-vessel Density	Cleaved caspase-3 (%)
Vehicle (n=14)	23.7 ± 2.4	4744 ± 630[a]	14/14 (100)[a]	14/14 (100)[a]	20.6 ± 5.2[a]	32.4 ± 6.1[a]	8.6 ± 4
BEV (n=14) 5mg/kg	24.4 ± 1.7	840 ± 150[b]	2/14 (14.2)[b]	0/14 (0)[b]	8.6 ± 3.5[b]	12.1 ± 5.4[b]	7.5 ± 3.6
RAPA (n=14) 1mg/kg	26.35 ± 1.8	1231.6 ± 235[c]	2/14 (14.2)[b]	1/14 (7.1)[b]	9.4 ± 4[b]	19.2 ± 4.8[b]	7.8 ± 5.0
RAPA/BEV (n=14)	24.8 ± 1.5	341 ± 62[d]	0/14 (0)[c]	0/14 (0)[b]	3.1 ± 2.7[c]	3.4 ± 2.7[c]	7 ± 4.1

Both the RAPA and BEV-treated animals exhibited significant decreases in liver and intra-peritoneal tumor size and burden compared to controls (Figure 13A and Table C3, $p<0.01$). However, histological examination of the RAPA and BEV treated tumors revealed the persistence of micro-sized tumors in the animal livers (Figure 13B). These small tumors may maintain their survival by passive diffusion of nutrients from the underlying host vasculature and the surrounding peritoneal fluid. In contrast, RAPA/BEV treatment elicited the strongest level of tumor size reduction, and micro-sized tumors are not detectable in treated livers (Figure 13B and Table C3). A Kaplan-Meier survival analysis confirmed that while all mice in the control, RAPA, and BEV-treated groups are moribund at days 48, 120, and 118, the RAPA/BEV treated mice had a significantly prolonged overall survival and are still alive at the day 125 ($p<0.01$, log-rank test, Figure 13C). These results suggest that RAPA/BEV may be effective in prolonging the survival of tumor-IP injected mice by effectively inhibiting ascites formation, tumor dissemination, and reducing IP tumor burden.

Example C7. Discussion of Examples C1 to C6

The dismal prognosis of advanced HCC patients, coupled with the current paucity of therapeutic options, makes the identification of targeted therapies for HCC an important goal. In this report, we used a panel of early-passage patient-derived xenografts to identify RAPA/BEV as a potential molecularly targeted combinational treatment for HCC. Such patient-derived xenografts may prove useful for pre-clinical studies evaluating novel combination therapies, due to the close histological and molecular similarities between the xenografts and cognate primary tumors, and the ability to test drug compounds in an *in vivo* setting (ref C25). Interestingly, preliminary experiments suggest that when tested *in vitro*, RAPA/BEV has a minimal effect on cellular proliferation against HCC cell lines (unpublished observations). Nevertheless, the ultimate efficacy RAPA/BEV therapy for HCC will only be known after a carefully conducted clinical trial – such a trial is currently underway at our centre.

In our study, both RAPA and BEV, when administered as single agents, reduced but did not fully suppress the growth of six out of seven HCC xenografts. However, RAPA/BEV significantly suppressed HCC growth to a greater degree than single-agent therapy, including 26-1004(Cirr) tumors, whose growth is not significantly affected by RAPA or BEV alone. Thus, all the HCC xenografts tested in our study appear to be are sensitive to this combined therapy. The mechanism of action of RAPA/BEV is likely to be multifactorial. First, RAPA and BEV likely inhibit both VEGF expression and VEGF protein activity to potently reduce tumor angiogenesis. Depriving growing tumors of circulating nutrients and growth factors may result in a secondary reduction in tumor proliferation and cell growth. Second, BEV may act directly on tumor-associated endothelial cells to increase vascular permeability (ref C24), thereby enhancing delivery of RAPA to cancer cells and facilitating the direct inhibition by RAPA of tumor proliferation and growth. Third, as described above, synergistic effects of the RAPA/BEV combination which are not observed in the single-agent treatments, such as reductions in cyclinD1, may also contribute to tumor inhibition. The precise mechanisms responsible for the potent anti-tumor activity of RAPA/BEV treatment in HCC xenografts should be further investigated.

Example D1. Drug Treatments (for IP delivery of RAPA and BEV)

Pure rapamycin (RAPA, Nacalai Tesque Inc. Kyoto, Japan) is dissolved in dimethylsulfoxide (DMSO) to obtain the stock solution of 12.5 mg/ml. Bevacizumab (BEV, Avastin) is obtained from Genentech, Inc., South San Francisco, CA. To make a BEV/RAPA cocktail for IP injection, BEV and RAPA are dissolved in saline to obtain a final concentration of 100 μg BEV and 125 μg RAPA.

5-1318 xenograft line is daily IP administered with 200 μl saline (vehicle/control), 0.8 mg BEV/kg, 1 mg RAPA/kg, or 200 μl of BEV/RAPA cocktail (This provides 0.8 mg BEV and 1 mg RAPA per kg body weight per day). Treatment commenced after day seven of tumor implantation when the tumors are approximately 100 mg, and continued for two weeks. Each treatment arm involved fourteen independent tumor-bearing mice representing the same xenograft line. At the end of the treatment regimen, animals are

sacrificed, body and tumor weights were recorded, and tumors are harvested for molecular analysis.

Results

In vivo IP administration of BEV, RAPA, or the combination of BEV and RAPA in mice bearing 5-1318 HCC xenografts results in approximately 32% ± 12%, 44% ± 11%, and 66% ± 9% growth inhibition, respectively (Figure 16). RAPA-inhibited tumor growth is associated with inhibition of phosphorylation of p70S6, 4EBP1, and S6R (Figure 17), upregulation of p27, p130/Rb2 and down-regulation of cell cycle regulators, including cyclin D1, cdc-2, Cdk-2, Cdk-4, cyclin B1, p21 and survivin (Figure 18). The BEV-RAPA combined protocol also induces an additive effect, including decreased expression of phospho-4EBP1 at Ser37/46, cdk-2, p21 increased expression of the p130/Rb2 tumor suppressor gene (Figures 16 & 17).

Example E1: A Phase I Study of Rapamycin in combination with Bevacizumab in Patients with Unresectable Hepatocellular Carcinoma

Based on the results obtained from *in vivo* studies above, we propose a phase I clinical and pharmacokinetic study of rapamycin to answer the following hypotheses: Rapamycin is safe for use when combined with bevacizumab in the treatment of hepatocellular carcinoma. A therapeutic plasma level needs to be reached for anti-tumor activity. Targeting of the activated mTOR pathway and angiogenic pathway in solid tumors will result in tumor regression. Sensitivity to rapamycin and bevacizumab correlates with 4EBP-1, S6K, CD31 and VEGF expression. DCE CT can assess anti-angiogenic effect of rapamycin and bevacizumab and this correlates with tumor response and drug levels.

The objectives are as follow:

Primary endpoints

Determine the optimal dose of rapamycin combined with bevacizumab in patients with unresectable hepatocellular carcinoma and determine the toxicity profile

Secondary endpoints

Assess the pharmacokinetics of rapamycin and determine the biologically active dose range of rapamycin. Describe the clinical activity of this drug combination. Examine the relationship between phosphorylated p70S6K activity in peripheral blood mononuclear cells (PBMCs) and clinical response to study drugs. Examine the relationship between PTEN, 4EBP-1, phosphorylated p70S6K, CD31 and VEGF expression in tumour tissue and clinical response to study drugs. Examine the relationship between degree of angiogenesis measured by Dynamic Contrast-Enhanced Computed Tomography (DCE CT) with drug levels and clinical response.

A schema for the clinical trial is shown below:

SCHEMA

Unresectable hepatocellular carcinoma
ECOG 0-2
↓
Phase I study of rapamycin and bevacizumab
↓
Determine biologically relevant dose and DLT
↓
Expression of S6-kinase, 4EBP-1, CD31, PTEN and VEGFR on immunohistochemistry of tumour tissue and p70S6K activity in peripheral blood mononuclear cells
DCE CT to assess change in degree of angiogenesis

Dose Escalation Schedule		
Dose Level	Dose of Rapamycin (oral tablets)	Dose of Bevacizumab (intravenous)
Level 1 (n=3)	1mg/day	5mg/kg every 2 weeks
Level 2 (n=3)	2mg/day	
Level 3 (n=3)	3mg/day	
Level 4 (n=3)	4mg/day	
Level 5 (n=3)	5mg/day	
Level 6 (n=3)	6mg/day	
Level 7 (n=3)	6mg/day	7.5mg/kg every 2 weeks
Level 8 (n=3)	6mg/day	10mg/kg every 2 weeks
	Loading oral dose of 3 times the maintenance dose will be given to each patient on day 1. Trough concentration of rapamycin taken 3-4 days after any change in dosing	First dose given by i.v. infusion over 90minutes. Subsequent doses given over 60 minutes if first dose tolerated

Establish MTD
Expanded cohort of 6 patients included at MTD

⇩

Proceed onto Phase II study

Example E2. Patient Selection for Clinical Trial

Eligibility Criteria

Patients must have histologically confirmed unresectable HCC. Age ≥18 years. ECOG performance status ≤2 (Karnofsky >60%) (see Appendix A). Life expectancy of greater than 3 months. Only patients with Child-Pugh score for liver cirrhosis of A and B (see Appendix A) will allowed into this trial. Patients must have normal organ and marrow function as defined below: leukocytes 3.0 x 10^9/L; absolute neutrophil count ≥ 1.5 x 10^9/L; platelets ≥ 100 x 10^9/L; total bilirubin ≤ 3 X institutional upper limit of normal; AST(SGOT)/ALT(SGPT) ≤ 5 X institutional upper limit of normal; creatinine within normal institutional limits; activated PTT less than 1.5 of institutional upper limit of normal.

Many patients with HCC are Hepatitis B carriers. As the effect of immunosuppression from rapamycin on these patients is not known, all Hep B carriers in this study must be on lamuvudine during the period of this study and continued on it for at least 6 months after the end of this study. Eligibility of patients receiving any medications or substances known to affect or with the potential to affect the activity or pharmacokinetics of rapamycin will be determined following review of their case by the Principal Investigator. Efforts should be made to switch patients who are taking enzyme-inducing anticonvulsant agents to other medications.

The effects of rapamycin on the developing human fetus are unknown. For this reason, women of child-bearing potential and men must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation. Should a woman become pregnant or suspect she is pregnant while participating in this study, she should inform her treating physician immediately.

Ability to understand and the willingness to sign a written informed consent document.

Patients must have measurable disease, defined as at least one lesion that can be accurately measured in at least one dimension (longest diameter to be recorded) as ≥20 mm with conventional techniques or as ≥10 mm with spiral CT scan.

Patients must have fasting serum cholesterol ≤ 9 mmol/L (350mg/dL) and triglycerides ≤ 3.39 mmol/L (300mg/dL)

Patient's surgery must be more than 28 days before start of study drug and any surgical wounds must be completely healed

Exclusion Criteria

Patients with bone metastases without any other measurable disease present. Patients are excluded if they are receiving any other investigational agents. Patients with known brain metastases should be excluded from this clinical trial because of their poor prognosis and because they often develop progressive neurologic dysfunction that would confound the evaluation of neurologic and other adverse events. History of allergic reactions attributed to compounds of similar chemical or biologic composition to rapamycin or bevacizumab. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements. Pregnant women or breastfeeding mothers are excluded from this study because of the potential risks to the fetus or baby.

Because patients with immune deficiency are at increased risk of lethal infections when treated with marrow-suppressive therapy, HIV-positive patients are excluded from this study. Because of the concerns regarding bleeding or clotting problems related to the use of bevacizumab, patients with active oesophageal varices, bleeding disorders, deep vein thromboses or other thromboembolic disease (except portal vein thrombosis) are excluded. Patients with a clinical history of haemetemesis or haemoptysis are excluded.

Due to the risks of bleeding, patients who need long term anticoagulation with heparin or warfarin are excluded from this study. Patients who have failed 2 or more lines of chemotherapy. Patients who are unable to take orally will be excluded. Patients who have baseline urine dipstick proteinuria $\geq$ 2+ and 24 hr urine protein is more than 1 g.

Normal Controls

Five healthy volunteer subjects will be included in this study. Ten mls of whole blood will be drawn from each subject as a control for pharmacodynamic studies.

Concomittant medication and treatment

All concomitant medications must be reported in the case report form (CRF). There is a potential for interaction of *Rapamycin* with other concomitantly administered drugs through the cytochrome P450 system.

Patients on the study should avoid systemic antifungal agents, clarithromycin, cyclosporine and CYP3A4 inducers (carbamezepine, rifampicin, phenytoin, phenobarbital, nafcillin, aminoglutethimide), CYP3A4 inhibitors (diclofenac, doxycycline, erythromycin, imatinib, isoniazid, nefazodone, micardipine, propofol, protease inhibitors, quinidine and verapamil), diltiazem, voriconazole and vaccines. All these agents may increase serum concentrations of rapamycin. (Appendix C)

Patients should also avoid St John's wort, Echinacea and cat's claw.

The Principal Investigator should be alerted if the patient is taking any of these agents.

In a combined analysis of trials using bevacizumab combined with chemotherapy, the incidence of bleeding among low-dose aspirin users and those who did not use aspirin was not significantly different (Hambleton J 2005). Thus, prophylactic low-dose aspirin used for patients at risk of an arterial thromboembolic event is allowed in this study.

Because of the unknown interactions, patients are not allowed to take traditional Chinese medicine during the study.

Example E3. Treatment Plan for Clinical Trial

Agent Administration

Treatment will be administered on an outpatient basis. Reported adverse events and potential risks for *Rapamycin* and *bevacizumab* are described below. Appropriate dose modifications *Rapamycin* and *bevacizumab* are described below. No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the patient's malignancy.

Dose Schedule

Phase 1 dose schedule

The dose of rapamycin will be escalated according to the schedule shown below while bevacizumab will be given at a fixed dose of 5mg/kg. Bevacizumab dose will be escalated only if there is no tumour response at the maximum tolerated dose of rapamycin. (see table)

Rapamycin (available as 1mg per tablet; Wyeth) will be given orally once in the morning before meal. The starting dose of rapamycin will be 1mg administered once daily. All doses of rapamycin will be preceded by an oral loading dose three times the maintenance dose on day 1. The dose of rapamycin will be increased at each dose level.

Bevacizumab (100mg/4ml; Roche) will start concurrently with rapamycin. It will be diluted in a total of 100ml of 0.9% sodium chloride given via intravenous injection. The first dose will be infused over 90 minutes. If the first infusion is tolerated without any adverse infusion-related events (fever and/or chills), the second infusion may be delivered over 60 minutes. If the 60- minute infusion is well tolerated, the subsequent doses may be delivered over 30 minutes.

During administration, patient should be monitored for signs/symptoms of abdominal pain, constipation or vomiting. Also close monitoring for hypertension. In event of a suspected anaphylactic reaction during drug infusion, the drug will be stopped and antihistamines, epinephrine and other medication administered as required and necessary medical resuscitation instituted.

Dose Escalation Schedule

Dose Level	**Dose of Rapamycin (oral tablets)**	**Dose of Bevacizumab (intravenous)**
Level 1 (n=3)	**1mg/day**	**5mg/kg every 2 weeks**
Level 2 (n=3)	**2mg/day**	
Level 3 (n=3)	**3mg/day**	
Level 4 (n=3)	**4mg/day**	
Level 5 (n=3)	**5mg/day**	
Level 6 (n=3)	**6mg/day**	
Level 4 (n=3)	**6mg/day**	**7.5mg/kg every 2 weeks**
Level 5 (n=3)	**6mg/day**	**10mg/kg every 2 weeks**
	Loading oral dose of 3 times the maintenance dose will be given to each patient on day 1. Trough concentration of rapamycin taken 3-4 days after any change in dosing	**First dose given by i.v. infusion over 90minutes. Subsequent doses given over 60 minutes if first dose tolerated**

The standard "3+3" rule will be employed for dose escalation. Three patients will be accrued at the starting dose level of 2 mg/day. If no dose limiting toxicities greater than grade 2 were observed, 3 patients would be entered at the next dose level. If, at any dose level, one of the first 3 patients experiences a DLT, 3 additional patients will be entered at that dose level. If 2 out of 6 patients experience dose limiting toxicities at this dose level, dose escalation will cease.

The maximally tolerated dose (MTD) will be defined as one dose level below that at which 2 or more patients experienced DLT.When the MTD is determined, an additional 6 patients will be accrued at this dose level (expanded cohort).

If DLT is observed at initial level of 1 mg/day, the trial will be terminated..

A patient who experiences any DLT will be allowed to continue treatment with a one dose level reduction if the toxicity resolves within 14 days. If this patient experiences a DLT at the lowered dose level, study treatment will be stopped for this patient.

No intra-patient dose escalation will be allowed. Re-entry of a patient accrued at a lower dose into a higher dose cohort will not be allowed.

Accrual rate is expected to be 3-4 patients per month.

Registration Guidelines

Patients can only be registered after all pretreatment evaluation is completed and all the eligibility criteria are met.

Issues that would cause treatment delays should be discussed with the Principal Investigator. If a patient does not receive protocol therapy following registration, the patient's registration on the study may be canceled. The Study Coordinator should be notified of cancellations as soon as possible.

To complete the registration process, the Coordinator will assign a patient study number, assign the patient a dose level and register the patient on the study.

Clinical and Safety Assessments

Clinical Assessments

The following tumour and safety related assessments will be completed according to the schedule (see Study Calendar): Physical examination; Vital signs, ECOG status and

physical measurements (blood pressure, pulse rate, body temperature and weight); Tumour assessment (CT or MRI etc) will be performed at baseline (not more than 4 weeks before start of study), week 8, 16, 24, 32, 40 and then every 8 weeks thereafter until confirmed evidence of disease progression. If treatment has been stopped for any reason other than progression of disease, tumour assessment will be performed every 12 weeks till disease progression; Chinese University Prognostic Index (CUPI) will be calculated at baseline (see Appendix A)

Assessments will also be performed at occurence of any adverse events

Laboratory Assessment

The following parameters will be measured according to the schedule (see Study Calendar) and at baseline, within 2 weeks of day 1 of cycle 1: Full blood count; PT / aPTT; Serum Chemistry; Liver function test; Alfa-feto protein at baseline, day 1 of every cycle and at end of study visit; Fasting lipids (including TG, LDL, total cholesterol) at baseline and every 4 weeks; Urine dipstick for proteinuria at baseline and before every bevacizumab administration. If baseline dipstick positive i.e $\geq$ 2+ , 24 hr urine protein will be collected; Hepatitis B surface and e antigen and HBV DNA load at baseline if not done before; ECG; Serum pregnancy test (women of child bearing potential).

Additional tests may be performed at the discretion of the investigator

Correlative studies

Pharmacokinetic studies

3ml of blood will be taken for rapamycin trough level on day 4 of starting treatment. Whenever there is a change in dose, blood rapamycin trough level will be obtained on day 4.

Pharmacokinetic studies will be done on day 8 of the first cycle after attainment of steady state. 3ml of whole blood will be drawn at pre-treatment, then 1h, 2h, 3h, 5h, 24 h and 72h after the first dose. Subsequent predose rapamycin whole blood levels will be

done on days 8, 29 and and every 2 weeks till end of treatment. Blood samples will be collected in EDTA tubes and stored at -20°C until analysis. Samples are analyzed within 3 months of collection to avoid degradation. Whole blood is used for analysis as 95% of the drug is sequestered in red blood cells. Peak drug concentrations will be determined by high-performance liquid chromatography with detection by tandem mass spectrophotometry.

Blood draw schedule

0 h	1h	2h	3h	5h (day 8)	24h (day 9)	72h (day11)	day 29	end of cycle 2

Pharmacodynamic Studies

The objective of the pharmacodynamic study is to identify suitable molecular biomarkers that can serve as useful markers of response. As $p70^{s6}$ is a direct downstream target of mTOR and is also constitutively activated in peripheral blood mononuclear cells (PBMCs), quantifying $p70^{s6}$ activity following treatment with rapamycin will serve as a useful surrogate biomarker.

$p70^{s6}$ kinase activity will be quantified in PBMCs obtained from healthy individuals (N=5) on days 0 (day of first collection), day 4 (3 days after day 0 collection), and 8 (7 days after day 0 collection). The purpose of these serial measurements will be to define the inter- as well as intra-patient variabilities in $p70^{s6}$ activity.

Fifteen mls of blood for PBMCs will be collected from the cancer patients at the following time points for quantification of $p70^{s6}$ activity: 0h, 72h (day 4), days 8 and 29.

Tumour Pharmacodynamics

Tumour tissue (archival or obtained before enrolling in the study) will be examined for PTEN, , 4E-BP1, VEGF, p70S6K and CD31 by immunohistochemistry, to determine if expression of one or a combination of these biomarkers predict for response to rapamycin and bevacizumab..

DCE CT Assessment of Angiogenesis

The hypothesis that will be examined is that DCE-CT will be a useful biomarker of angiogenesis, and would correlate with drug levels and clinical response.

DCE CT will be performed at Baseline (within 1 week of drug administration), and at Day 29

One target lesion is selected per patient for use in the DCE-CT assessment and should meet the following criteria: Lesion should be more than 3 cm in longest diameter and appear solid; Calcified lesions or lesions with non enhancing centers should be avoided; Lesions in relatively fixed locations such as cervical adenopathy, mediastinal adenopathy, pleural masses, retroperitoneal adenopathy and peritoneal nodules are preferred over lesions in organs that move with respiration (liver or pulmonary metastases); Lesions in the pelvis will be excluded.

A 64-detector multislice CT scanner (General Electric, Milwaukee) will be used. A 20 G venula will be set at the upper extremity . The patient is positioned in the scanner. After a pilot scan, a 4cm slab is placed over the selected lesion with the following detector settings: 16 slices at 2.5mm per slice(120kVp 70 mA for chest and abdomen, 80 kVp 200mA for neck). A precontrast slab is acquired. The patient is instructed to employ quiet breathing. Subsequently, 70 ml of non ionic iodinated contrast (Omnipaque 300) is administered at 3 to 4 ml per second with a power injector followed by 30ml of saline and a maximum total of 30 consecutive acquisitions over the same table position after a scan delay of 5 to 20 seconds. The patient is instructed to breathhold for 20 seconds and thereafter asked to breath out and then breath in again to breathhold for consecutive blocks of 20 seconds.

Data analysis will be performed by software developed by our group. Image registration is performed to correct for respiratory motion. A region of interest (ROI) will be drawn to include the lesion. The arterial input function is obtained from the aorta or a major artery. A pixel map will calculated for the ROI and the following parameters

analyzed: Median Ktrans; Mean IAUC90; Median Flow, Permeability-Surface Area Product, fractional extracellular extravascular volume, fractional intravascular volume obtained by a Distributed Parameters model.

Changes in the above parameters will be analyzed and correlated to drug exposure and RECIST response.

Potential correlation with other biomarkers examined in this study will also be explored.

Risks of DCE CT

Risk of CT contrast

The risk of life-threatening allergy to CT contrast ranges from 1 in 40 000 to 1:168 000. The risk of severe reaction ranges from 1 in 2215 to 1 in 6056. (Katayama, Yamaguchi et al. 1990) This risk is generally low. Patients who have had previous contrast allergy are at increased risks and are not examined.

Risk of Extravasation

Extravasation of contrast can be seen in up to 0.4% (4 per 1000). Of the patients who have had this infrequent occurrence, severe injury (such as skin necrosis) can be seen in up to 1 in 10 (or 1 in 200 extravasations)(Cohan, Ellis et al. 1996). Hence the chance of severe reaction from a single extravasation occurrence is very low (approximately 4 per 10 000 examinations). A high rate of injection (7ml/sec) was used in other IRB approved studies (Van Beers, Leconte et al. 2001).

Risk of Radiation from CT

We measured the radiation dose of our protocol on a anthropomorphic phantom and found that the radiation burden is 69 mSv (which is 1.9 x that of a routine multiphasic liver scan). The chance of fatal cancer is approximately 0.51% for one DCE CT scan compared to 0.25% for a routine multiphasic liver scan. The patients are expected to have up to 2 scans and hence their maximum risk of induced fatal cancer is 1.02%.

However this increased risk is small when compared considering the natural risk of fatal cancer in humans is approximately 1:3. (2001).

Moreover, the latent period required for an induced cancer to manifest is generally thought to be long (1991). The increased radiation dose is small and the patients may not have time to manifest the risks of an induced cancer in view of their advanced malignancy and poor prognosis.

The protocols and risks are similar to that which has been submitted to the NCC Institutional Review Board under a platform grant and was previously approved. (NCC IRB Ref No: 06-15-OTH)

Duration of Therapy

In the absence of treatment delays due to adverse events, treatment may continue for 6 cycles or until one of the following criteria applies: Disease progression; Intercurrent illness that prevents further administration of treatment; Unacceptable adverse event(s); Patient decides to withdraw from the study, or General or specific changes in the patient's condition render the patient unacceptable for further treatment in the judgment of the investigator.

Duration of Follow Up

Patients will be followed for 52 weeks after removal from study or until death, whichever occurs first. Patients removed from study for unacceptable adverse events will be followed until resolution or stabilization of the adverse event.

Example E4. Dosing Delays or Dose Modifications

Bevacizumab-specific dose delays or modifications

The bevacizumab dose (5mg/kg) will not be reduced or modified. Missed doses of bevacizumab should not be administered later.

However, patients should not continue on bevacizumab if the following events occur: Gastrointestinal perforation; Arterial thromboembolic events; Grade3/4 haemorrhagic events; Symptomatic grade 4 thrombosis; Grade 4 hypertension (hypertensive crisis); Grade 4 proteinuria

Dose Adjustments for hypertension

NCI-CTC grade	Hypertension pattern	Action taken
1	Asymptomatic, transient (>24h) DBP increase by >20mmHg or to >150/100mmHg if previously within normal limits	Continue with bevacizumab
2	Recurrent or persistent (>24h) or symptomatic increase by DBP>20mmHg or to >150/100 mmHg if previously within normal limits	Hold bevacizumab. Start anti-hypertensive therapy. Continue bevacizumab when BP <150/100
3	Requires more than 1 ant-hypertensive drug or more intensive therapy than previously	Hold bevacizumab if persistent or symptomatic hypertension and discontinue permanently if hypertension not controlled
4	Hypertensive crisis	Permanently discontinue bevacizumab

Dose adjustments for proteinuria

All patients will have dipstick for proteinuria done within 3 days prior to starting bevacizumab. <2+ proteinuria, administer bevacizumab therapy. At 1st occurrence of proteinuria > or = 2, administer bevacizumab as planned and collect 24h urinary protein within 3 days before the next bevacizumab administration: If 24h proteinuria 2g or less, continue with bevacizumab; If 24h urine protein more than 2g, omit next scheduled bevacizumab till repeat 24h urine protein is less than or equal to 2g at the next cycles. Permanently discontinue bevacizumab if Grade 4 proteinuria (Nephrotic syndrome)

Rapamycin-specific dose delays or modifications

Therapeutic drug monitoring (TDM) will be conducted every 2 weeks during this study. Rapamycin levels will not determine drug dose used as patients will be monitored

for toxicities and Dose Limiting Toxicities will determine doses used as explained above. Concerns about immunosuppressive effects of rapamycin will be dealt with by full blood count monitoring every 2 weeks during period of treatment (see study calendar). Because of the risk of exacerbation of hepatic toxicity, study treatment should be withheld if Bilirubin is >3 times and AST is more than 5 times the upper limit of normal. Rapamycin should also be delayed if absolute neutrophil counts are less than 1.5×10^9/L and platelets are less than 100×10^9/L

Definition of Dose-Limiting Toxicity and Maximal Tolerated Dose

All toxicities will be graded according to the National Cancer Institute Common Toxicity Criteria (NCI CTC version 3). In particular, the following toxicities which tend to be more common with a) rapamycin use will be recorded in detail: mucositis, diarrhea, constipation, arthralgia, rash, haematologic toxicities (neutropenia, thrombocytopenia, anemia), fasting lipid elevations, hyperglycemia, hypertension, oedema, neutropenic infections, infections including pneumonia, urinary tract infection, bacteremia, hepatobiliary sepsis; pneumonitis, proteinuria, elevated bilirubin/ALT/AST, raised creatinine, thrombosis, fatigue, weight loss, nausea and vomiting.

Hematological Dose Limiting Toxicity (DLT) is defined as follows: grade 4 neutropenia of >7 days duration, neutropenic fever, grade 4 anemia or grade 3-4 thrombocytopenia that occurs during the first month of treatment.

Non-hematologic DLT is defined as any grade 3 or grade 4 non-hematologic toxicity that occurs during the first month of treatment.

Any toxicity causing a total of 14 days delay will also be considered dose limiting.

Toxicities will be classified as related to the study drug unless they were attributable to either underlying tumour progression, concurrent medical condition or a concomitant medication.

Any unusual toxicities must be reported to the Principal Investigator

Example E5. Adverse Events: List and Reporting Requirements

Potential Adverse Effects of Rapamycin (see Appendix C for list)

Incidence of many adverse effects is dose related.

Rapamycin is associated with a number of possible adverse effects, including leukopenia, thrombocytopenia, anemia, hypercholesterolemia, hypertriglyceridemia, diarrhea, and others.

Hematologic effects — Anemia, thrombocytopenia, and leukopenia can be observed(Augustine, Knauss et al. 2004). In clinical trials, anemia has been reported in 27 to 57 percent of patients, which variability based in part on time post-transplant. Thrombocytopenia has been observed in 13 to 30 percent of subjects. Reductions in platelet count are dose-related and usually occur 9 to 10 days after initiation of treatment. Normalization of platelet counts is seen within two weeks of discontinuation. Leukopenia, which does not appear to be dose-related, is evident within two weeks of initiation of therapy and is reversible upon discontinuation

HUS/Thrombotic microangiopathy — Hemolytic uremic syndrome (HUS)/Thrombotic microangiopathy has been reported with the combination cyclosporine/rapamycin immunosuppressive regimen.(Fortin, Raymond et al. 2004) and discontinuation of these agents resulted in reversal of HUS in most cases.

An increased rate of hepatic artery thrombosis, graft loss, and death has also been reported in liver transplant recipients. In two multicentre, randomized trials in de novo liver transplant recipients, the use of rapamycin in combination with either cyclosporine or tacrolimus was associated with an increased rate of hepatic artery thrombosis. Furthermore, in one phase II study, the use of rapamycin and tacrolimus was associated with an increased rate of death and graft loss.

Metabolic effects — Hyperlipidemia (38 to 57 percent) and hypercholesterolemia (38 to 46 percent) are dose-related effects of rapamycin therapy that occur via the inhibition of lipoprotein lipase(Kraemer, Takeda et al. 1998). In one study of 26 patients, triglyceride levels exceeding 5 mmol/L (454 mg/dL) were seen in 75 percent of patients receiving various doses of rapamycin in addition to cyclosporine-based regimens, with mean peak levels of 14.5 mmol/L (1272 mg/dL) occurring after a mean of seven weeks(Brattstrom, Wilczek et al. 1998). Parallel increases in total cholesterol were observed, but were less pronounced (mean peak of 12 mmol/L [469 mg/dL]). In patients receiving trough level-controlled rapamycin, a reduction in target trough levels from 30 to 15 ng/mL after two months led to a decrease in triglyceride levels. Triglyceride levels returned to pretreatment status in all patients receiving rapamycin after six months. Despite these effects, rapamycin has been demonstrated to help prevent atherosclerosis in murine models and in heart transplant recipients.

Gastrointestinal system — Common gastrointestinal adverse events including constipation (28 to 36 percent), diarrhea (25 to 42 percent), dyspepsia (17 to 25 percent), nausea (25 to 36 percent), and vomiting (19 to 25 percent. Mouth sores, not related to herpes simplex virus, have been reported in some patients taking rapamycin oral solution. This is probably dose-related.

Respiratory system — Progressive interstitial pneumonitis has been observed in a number of transplant recipients).(Morelon, Stern et al. 2001). Clinical symptoms consist of dyspnea, dry cough, fever, and fatigue. In one report of 15 patients, clinical and radiologic improvement was observed in all patients within three weeks of drug discontinuation or dose reduction.

Kidney function — As shown in animals, rapamycin is minimally nephrotoxic when used alone, although there are no definitive data in humans. Rapamycin has also been associated with glomerulonephropathy associated with proteinuria (Izzedine, Brocheriou et al. 2005). In one retrospective study of 68 renal transplant recipients in whom rapamycin was substituted for a calcineurin-inhibitor, proteinuria was assessed prior to and at 3, 6, 12, and 24 months after the substitution. Compared with baseline

levels (mean of 0.36 grams/day), proteinuria markedly increased at 3, 6, 12, and 24 months (1.35, 1.67, 1.27, and 1.14 grams/day, respectively). Proteinuria was reversible among the 19 patients in whom rapamycin was withdrawn (1.95 to 0.9 grams/day). (Letavernier, Pe'raldi et al. 2005)

Teratogenicity/effects in pregnancy – Rapamycin is contraindicated in pregnancy and its use should also be discontinued at least 12 weeks prior to attempted conception.

Others- In two case reports, rapamycin has been associated with the development of leukocytoclastic vasculitis. Rapamycin is also associated with post-operative wound complications. (Hardinger, Cornelius et al. 2002). Tongue edema was also reported in five patients being administered high doses of rapamycin and angiotensin-converting enzyme (ACE) inhibitors. The symptom resolved after ACE inhibitors were withdrawn, and did not recur after reintroduction of lower doses of rapamycin and ACE inhibitors. A large number of cutaneous adverse events can be observed with rapamycin. In one study from France, skin disorders were reported in 79 of 80 renal transplant patients; the most frequent were acne-like eruptions (46 percent), scalp folliculitis (26 percent), hidradenitis suppurativa (12 percent), edema (55 percent), angioedema (15 percent), aphthous ulceration (60 percent), and epistaxis (60 percent).

Potential adverse effects of bevacizumab

Gastrointestinal perforation, intra-abdominal abscess, and wound dehiscence have been reported in patients receiving bevacizumab (not related to treatment duration); monitor patients for signs/symptoms of abdominal pain, constipation or vomiting. Permanently discontinue in patients who develop these complications. The appropriate interval between administration of bevacizumab and surgical procedures to avoid impairment in wound healing has not been established. Do not initiate therapy within 28 days of major surgery and only following complete healing of the incision. Bevacizumab should be discontinued prior to elective surgery and the estimated half-life (20 days) should be considered.

Avoid use in patients with recent hemoptysis; significant pulmonary bleeding has been reported in patients receiving bevacizumab (primarily in patients with non-small cell

lung cancer). Avoid use in patients with CNS metastases; patients with CNS metastases were excluded from clinical trials due to concerns for bleeding. Other serious bleeding events may occur, but with a lower frequency; discontinuation of treatment is recommended in all patients with serious hemorrhage.

Use with caution in patients with cardiovascular disease; patients with significant recent cardiovascular disease were excluded from clinical trials. An increased risk for arterial thromboembolic events (e.g, stroke, MI, TIA, angina) is associated with bevacizumab use in combination with chemotherapy. History of arterial thromboembolism or ≥65 years of age may present an even greater risk; permanently discontinue if serious arterial thromboembolic events occur.

May cause CHF and/or potentiate cardiotoxic effects of anthracyclines. Bevacizumab may cause and/or worsen hypertension significantly; use caution in patients with pre-existing hypertension and monitor BP closely in all patients. Permanent discontinuation is recommended in patients who experience a hypertensive crisis. Temporarily discontinue in patients who develop uncontrolled hypertension. Interrupt therapy in patients experiencing severe infusion reactions; there are no data to address reinstitution of therapy in patients who experience CHF and/or severe infusion reactions. Proteinuria and/or nephrotic syndrome has been associated with bevacizumab; discontinuation of therapy is recommended in patients with nephrotic syndrome. Safety and efficacy in pediatric patients have not been established

Adverse Event Reporting

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 3.0. A copy of the CTCAE version 3.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov/reporting/ctc.html).

Expedited Adverse Event Reporting

All AEs reported **must** be copied to the Study Coordinator *(e-mail)*. The Study Coordinator will submit AE reports to the Principal Investigator for timely review.

Expedited Reporting Guidelines – Phase 1 studies with an investigational agent:

UNEXPECTED EVENT		EXPECTED EVENT	
GRADES 2 – 3 **Attribution of Possible, Probable or Definite**	**GRADES 4 and 5** **Regardless of Attribution**	**GRADES 1 - 3**	**GRADES 4 and 5** **Regardless of Attribution**
Grade 2 - Expedited report within 10 calendar days. **Grade 3**. Fully detailed expedited report must follow within 4 calendar days. (**Grade 1** – Adverse Event Expedited Reporting NOT required.)	Report within 24 hrs. Fully detailed expedited report must follow within 4 calendar days. **Death** Expedited reports must be submitted within 10 calendar days after knowledge of a death following treatment with an investigational agent: • Event occurred within 30 days of the last dose of treatment regardless of attribution, or • Event occurred greater than 30 days with attribution of possible, probable, or definite.	Expedited reporting required only if associated with hospitalization or prolongation of hospitalization. Adverse Event Expedited Reporting NOT required.	Report within 24 hrs. Expedited report to follow within 10 calendar days. **Death** Expedited reports must be submitted within 10 calendar days after knowledge of a death following treatment with an investigational agent: • Event occurred within 30 days of the last dose of treatment regardless of attribution, or • Event occurred greater than 30 days with attribution of possible, probable, or definite.

Note: All deaths on study must be reported using expedited reporting regardless of causality. Attribution to treatment or other cause should be provided.

Any medical event equivalent to CTCAE grade 3, 4, or 5 that precipitates hospitalization (or prolongation of existing hospitalization) must be reported regardless of designation as expected or unexpected).

Use the NCI protocol number on all reports.

Those AEs that do not require expedited reporting **must** be reported in routine study data submissions

Example E6. Pharmaceutical Information

Rapamycin and Bevacizumab

Adverse Events and Potential Risks

See Appendix B

Availability

Rapamycin is an investigational agent supplied to investigators by Wyeth International. Bevacizumab is a FDA-approved agent by Roche.

Agent Ordering

Drugs may be requested by the Principal Investigator (or their authorized designees)

Agent Accountability

The Investigator, or a responsible party designated by the Investigator, must maintain a careful record of the inventory and disposition of all agents received from the respective drug companies

Example E7. Study Calendar

Baseline evaluations are to be conducted within 2 weeks prior to start of protocol therapy. Scans and x-rays must be done 4 weeks prior to the start of therapy. In the event that the patient's condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next cycle of therapy. (See Appendix D).

Example E8. Measurement of Effect

Although response is not the primary endpoint of this trial, patients with measurable disease will be assessed by standard criteria. For the purposes of this study, patients should be reevaluated every 8 weeks. In addition to a baseline scan, confirmatory

scans will also be obtained 4 weeks following initial documentation of an objective response.

Definitions

Response and progression will be evaluated in this study using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) Committee (Therasse, Arbuck et al. 2000)]. Changes in only the largest diameter (uni-dimensional measurement) of the tumor lesions are used in the RECIST criteria. Note: Lesions are either measurable or non-measurable using the criteria provided below. The term "evaluable" in reference to measurability will not be used because it does not provide additional meaning or accuracy.

Evaluation of Measurable Disease

All measurements should be taken and recorded in metric notation using a ruler or calipers. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging-based evaluation is preferred to evaluation by clinical examination when both methods have been used to assess the antitumour effect of a treatment.

Clinical lesions. Clinical lesions will only be considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes). In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

Conventional CT. These techniques should be performed with cuts of 10 mm or less in slice thickness contiguously. This is the preferred mode of measurement

Alfa-Feto Protein (AFP) Tumour markers alone will not be used to assess response.

Example E9. Response Criteria

Evaluation of Target Lesions

Complete Response (CR): Disappearance of all target lesions

Partial Response (PR): At least a 30% decrease in the sum of the longest diameter (LD) of target lesions, taking as reference the baseline sum LD

Progressive Disease (PD): At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started

Evaluation of Best Overall Response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best response assignment will depend on the achievement of both measurement and confirmation criteria

Target Lesions	Non-target Lesions	New Lesions	Overall Response
CR	CR	No	CR
CR	Incomplete response/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Note:

Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be classified as having "symptomatic deterioration." Every effort should be made to document the objective progression, even after discontinuation of treatment.

In some circumstances, it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends on this determination, it is recommended that the residual lesion be investigated (fine needle aspirate/biopsy) before confirming the complete response status.

Confirmatory Measurement/Duration of Response

Confirmation

To be assigned a status of PR or CR, changes in tumor measurements must be confirmed by repeat assessments that should be performed 4 weeks after the criteria for response are first met. In the case of SD, follow-up measurements must have met the SD criteria at least once after study entry at a minimum interval of 6-8 weeks.

Duration of Overall Response

The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started).

The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that recurrent disease is objectively documented.

Duration of Stable Disease

Stable disease is measured from the start of the treatment until the criteria for progression are met, taking as reference the smallest measurements recorded since the treatment started.

Progression-free survival

Progression-free survival is defined as the duration of time from start of treatment (Day 1) to time of disease progression. Patients who do not progress would be censored at the date of last follow-up.

Overall survival

wOverall survival is defined as the duration of time from start of treatment (Day 1) to time of death. Survivors are censored at the date of last follow-up.

Example E10. Data Reporting / Regulatory Considerations

Method

No interim analysis is planned. A monitor from the Clinical Trials Compliance Unit will make periodic checks on the collected data and the investigator's files. All monitoring activities will be documented in monitoring reports.

Responsibility for Submission

The data manager will be responsible for compiling and submitting data to for all participants and for providing the data to the Principal Investigator for review.

Example E10. Statistical Considerations

This is a phase I dose-finding study. All patients enrolled in this study will be evaluated for toxicity and response.

The primary aim of this study is to determine the dose limiting toxicities of rapamycin combined with bevacizumab and the maximal tolerated dose (MTD) of this drug combination in patients with hepatocellular carcinoma. The toxicity profile will be graded as described above and reported with the corresponding dose level.

The MTD will be dose at which less than 2 DLTs occur. Steady state plasma drug concentrations, area under the curve values, volume of distribution and clearance of rapamycin in each patient will be calculated and correlated with dose. These quantities will be summarized for all patients. Serial blood rapamycin levels will be described in relation to toxicities.

Response rates (Complete response, partial response, stable disease) to study treatment will be described based on RECIST criteria and summarized by dose.

Progression free survival and overall survival will be measured from day 1 of starting treatment and analyzed by the Kaplan-Meier method.

Repeated measure ANOVA will be used to compare distribution of p70S6K activity in PBMCs (on days 1, 4 and 8) between normal controls (n=5) and study patients. Patients will be separated by dose for this analysis

p70S6K activity in PBMCs will be correlated with tumour response using repeated measure ANOVA, if a sufficient number of responses are seen

Expression of tumour tissue biomarkers (PTEN, 4EBP-1, CD31, pS6K and VEGF) will be described by dose

To correlate tumour data with response dose, a Wilcoxon rank sum test to compare tumour markers between responders and non-responders to rapamycin will be performed. All doses will be pooled for this analysis

Best overall response would be categorized into three groups: CR/PR, SD and progressive disease (PD). An exploratory analysis of the changes of DCE CT assessment of angiogenesis between baseline and day 8 in each group of best overall response (as described) would be performed.

Changes of DCE CT assessment of angiogenesis will also be correlated with drug level obtained on day 8.

Sample size considerations: Between 3-6 patients will be observed on each of 3 dose levels and an expanded cohort of 6 patients will be observed on the MTD. The anticipated accrual to this study is between 6 (if there are unacceptable DLT at dose level 1) to 36 patients (if dose level 5 is reached and the expanded cohort is included) and 5 healthy controls.

Example E11. Appendix A

Childs-Pugh Classification			
Parameter*	Score		
	1	2	3
Ascites	None	Moderate or easily treated	Severe or intractable
Encephalopathy	None	Mild	Severe
Prothrombin time	<4	4-6	>6
Serum bilirubin (mg/dL)	<2.0	2.0-3.0	>3.0
Serum albumin (g/dL)	>3.5	2.8-3.5	<2.8
Classification	Sum of Scores for Five Parameters		
Childs-Pugh A	5-6		
Childs-Pugh B	7-9		
Childs-Pugh C	10-15		
* The sum of the score of the five parameters determines the classification.			

CUPI Score

Variable	Score
TNM stage	
I and II	-3
IIa and IIIb	-1
IVa and IVb	0
Asymptomatic disease	-4
Ascites	3
AFP > 500 ng/dL	2
Bilirubin	
<34 µmol/L	0
34-51 µmol/L	3
≥ 52 µmol/L	4
Alkaline phosphatase ≥ 200 IU/L	3

Score ranges between -7 and 12.
AFP, α-fetoprotein; CUPI, Chinese University Prognostic Index; TNM, tumor, nodes, and metastasis.

Karnofsky Performance status score

The Karnofsky score is another method which measures patient performance of activities of daily living. The score has proven useful not only to follow the course of the illness (usually progressive deficit and ultimately death), but also a prognosticator: patients with the highest (best) Karnofsky scores at the time of tumor diagnosis have the best survival and quality of life over the course of their illness.

SCORE FUNCTION

100 Normal, no evidence of disease. 90 Able to perform normal activity with only minor symptoms. 80 Normal activity with effort, some symptoms. 70 Able to care for self but unable to do normal activities. 60 Requires occasional assistance, cares for most needs. 50 Requires considerable assistance. 40 Disabled, requires special assistance. 30 Severely disabled. 20 Very sick, requires active supportive treatment.10 Moribund

ECOG/Zubrod Score Performance Status

The Zubrod Score is similar to the 'performance status' table:

0 Asymptomatic. 1 Symptomatic, fully ambulatory. 2 Symptomatic,in bed < 50% of the day. 3 Symptomatic, in bed > 50% of the day but not bedridden. 4 Bedridden

Example E12. Appendix B. Drug information

Rapamycin Pharmacodynamics/pharmacokinetics

Absorption: Rapid

Distribution: 12 L/kg (range: 4-20 L/kg)

Protein binding: 92%, primarily to albumin

Metabolism: Extensively hepatic via CYP3A4; P-glycoprotein-mediated efflux into gut lumen

Bioavailability: Oral solution: 14%; Oral tablet: 18%

Half-life elimination: Mean: 62 hours

Time to peak: 1-2hours

Excretion: Feces (91%); urine (2%)

Adverse effects of Rapamycin:

>20%:

Cardiovascular: Hypertension (39% to 49%), peripheral edema (54% to 64%), edema (16% to 24%), chest pain (16% to 24%)

Central nervous system: Fever (23% to 34%), headache (23% to 34%), pain (24% to 33%), insomnia (13% to 22%)

Dermatologic: Acne (20% to 31%)

Endocrine & metabolic: Hypercholesterolemia (38% to 46%), hypophosphatemia (15% to 23%), hyperlipidemia (38% to 57%), hypokalemia (11% to 21%)

Gastrointestinal: Abdominal pain (28% to 36%), nausea (25% to 36%), vomiting (19% to 25%), diarrhea (25% to 42%), constipation (28% to 38%), dyspepsia (17% to 25%), weight gain (8% to 21%)

Genitourinary: Urinary tract infection (20% to 33%)

Hematologic: Anemia (23% to 37%), thrombocytopenia (13% to 40%)

Neuromuscular & skeletal: Arthralgia (25% to 31%), weakness (22% to 40%), back pain (16% to 26%), tremor (21% to 31%)

Renal: Increased serum creatinine (35% to 40%)

Respiratory: Dyspnea (22% to 30%), upper respiratory infection (20% to 26%), pharyngitis (16% to 21%)

3% to 20% (Limited to important or life-threatening):

Cardiovascular: Atrial fibrillation, CHF, postural hypotension, syncope, thrombosis, venous thromboembolism

Central nervous system: Anxiety, confusion, depression, emotional lability, neuropathy, somnolence

Dermatologic: Hirsutism, pruritus, skin hypertrophy, rash (10% to 20%)

Endocrine & metabolic: Cushing's syndrome, diabetes mellitus, hypercalcemia, hyperglycemia, hyperphosphatemia, hypocalcemia, hypoglycemia, hypomagnesemia, hyponatremia, hyperkalemia (12% to 17%)

Gastrointestinal: Esophagitis, gastritis, gingival hyperplasia, ileus

Genitourinary: Impotence

Hematologic: TTP, hemolytic-uremic syndrome, hemorrhage, leukopenia (9% to 15%)

Hepatic: Transaminases increased, ascites

Neuromuscular & skeletal: Increased CPK, bone necrosis, tetany, paresthesia

Otic: Deafness

Renal: Acute tubular necrosis, nephropathy (toxic), urinary retention

Respiratory: Asthma, pulmonary edema, pleural effusion

Miscellaneous: Flu-like syndrome, infection, peritonitis, sepsis

Postmarketing and/or case reports: Anaphylactoid reaction, anaphylaxis, anastomotic disruption, angioedema, fascial dehiscence, hepatic necrosis, hypersensitivity vasculitis; interstitial lung disease (pneumonitis, pulmonary fibrosis, and bronchiolitis obliterans organizing pneumonia) with no identified infectious etiology, lymphedema, neutropenia, pancytopenia. In liver transplant patients (not an approved use), an increase in hepatic artery thrombosis and graft failure were noted in clinical trials. In lung transplant patients (not an approved use), bronchial anastomotic dehiscence has been reported

Pharmacodynamics and pharmacokinetics of Bevacizumab

Distribution: Vd: 46 mL/kg

Half-life elimination: 20 days (range: 11-50 days)

Excretion: Clearance: 2.75-5 mL/kg/day

Adverse effects of Bevacizumab:

>10%:

Cardiovascular: Hypertension (23% to 34% vs 14%, severe/life-threatening 12% vs 2%); hypotension (7% to 15% vs 7%); thromboembolism (18% vs 15%)

Central nervous system: Pain (61% to 62% vs 55%, severe 8% vs 5%); headache (26% vs 19%); dizziness (19% to 26% vs 20%)

Dermatologic: Alopecia (6% to 32% vs 26%), dry skin (7% to 20% vs 7%), exfoliative dermatitis (3% to 19% vs 3%), skin discoloration (2% to 16% vs 3%)

Endocrine & metabolic: Weight loss (15% to 16% vs 10%), hypokalemia (12% to 16% vs 11%)

Gastrointestinal: Abdominal pain (50% to 61% vs 55%, severe/life-threatening 8% vs 5%); diarrhea (severe/life-threatening 34% vs 25%); vomiting (47% to 52% vs 47%); anorexia (35% to 43% vs 30%); constipation (29% to 40% vs 29%, severe/life-threatening 4% vs 2%); stomatitis (30% to 32% vs 18%); dyspepsia (17% to 24% vs 15%); flatulence (11% to 19% vs 10%); taste disorder (14% to 21% vs 9%)

Hematologic: Leukopenia (severe/life-threatening 37% vs 31%), gastrointestinal hemorrhage (19% to 24% vs 6%), neutropenia (severe/life-threatening 21% vs 14%)

Neuromuscular & skeletal: Weakness (73% to 74% vs 70%, severe/life-threatening 10% vs 7%); myalgia (8% to 15% vs 7%)

Ocular: Tearing increased (6% to 18% vs 2%)

Renal: Proteinuria includes nephrotic syndrome in some patients (36% vs 24%)

Respiratory: Upper respiratory infection (40% to 47% vs 39%), epistaxis (32% to 35% vs 10%), dyspnea (25% to 26% vs 15%)

1% to 10%:

Cardiovascular: DVT (6% to 9% vs 3%; severe/life-threatening 9% vs 5%); intra-arterial thrombosis (severe/life-threatening 4% vs 2%), syncope (severe/life-threatening 3% vs 1%), cardio-/cerebrovascular arterial thrombotic event (2% vs 1%)

Central nervous system: Confusion (1% to 6% vs 1%), abnormal gait (1% to 5% vs 0%)
Dermatologic: Skin ulcer (6% vs 1%), nail disorders (2% to 8% vs 3%)
Endocrine & metabolic: Infusion reactions (<3%)
Gastrointestinal: Dry mouth (4% to 7% vs 2%), colitis (1% to 6% vs 1%)
Hematologic: Thrombocytopenia (5% vs 0%)
Hepatic: Bilirubinemia (1% to 6% vs 0%)
Renal: Urinary frequency/urgency (3% to 6% vs 1%)
Respiratory: Voice alteration (6% to 9% vs 2%)

<1% (Limited to important or life-threatening): Anastomotic ulceration, hypertensive encephalopathy, hyponatremia, intestinal necrosis, intestinal obstruction, mesenteric venous occlusion, pancytopenia, polyserositis, subarachnoid hemorrhage, ureteral stricture

REFERENCES

A1. Ferlay J BF, Pisani P, Parkin DM. Globocan 2002: Cancer incidence, Mortality and Prevalence Worldwide. 5 ed. Lyon: IARC Press, 2004.

A2. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A et al. Cancer statistics, 2005. CA Cancer J Clin 2005; 55(1):10-30.

A3. Smith RA, Cokkinides V, Eyre HJ. American Cancer Society Guidelines for the Early Detection of Cancer, 2005. CA Cancer J Clin 2005; 55(1):31-44.

A4. Schafer DF, Sorrell MF. Hepatocellular carcinoma. Lancet 1999; 353(9160):1253-1257.

A5. Ince N, Wands JR. The increasing incidence of hepatocellular carcinoma. N Engl J Med 1999; 340(10):798-799.

A6. Nagasue N, Kohno H, Chang YC, Taniura H, Yamanoi A, Uchida M et al. Liver resection for hepatocellular carcinoma. Results of 229 consecutive patients during 11 years. Ann Surg 1993; 217(4):375-384.

A7. Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S, Ozaki H et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996; 83(9):1219-1222.

A8. Huguet CSFaGA. Primary hepatocellular cancer: Western experience. In: L.Blumgart, editor. Surgery of the Liver and Billary Tract. London: Churchill Livingstone, 2000: 1365-1369.

A9. Lai EaWJ. Hepatocellular carcinoma: the Asian experience. In: L.Blumgart, editor. Surgery of the Liver and the Biliary Tract. London: Churchill Livingstone, 1994: 1349-1363.

A10. Colombo M. Hepatocellular carcinoma. J Hepatol 1992; 15(1-2):225-236.

A11. Lai EC, Fan ST, Lo CM, Chu KM, Liu CL, Wong J. Hepatic resection for hepatocellular carcinoma. An audit of 343 patients. Ann Surg 1995; 221(3):291-298.

A12. Takenaka K, Kawahara N, Yamamoto K, Kajiyama K, Maeda T, Itasaka H et al. Results of 280 liver resections for hepatocellular carcinoma. Arch Surg 1996; 131(1):71-76.

A13. Simonetti RG, Liberati A, Angiolini C, Pagliaro L. Treatment of hepatocellular carcinoma: a systematic review of randomized controlled trials. Ann Oncol 1997; 8(2):117-136.

A14. Yoshiji H, Kuriyama S, Yoshii J, et al. Vascular endothelial growth factor tightly regulates in vivo development of murine hepatocellular carcinoma cells. Hepatology [28], 1498-1496. 1998.

A15. Miura H, Miyazaki T, Kuroda M, et al. Increased expression of vascular endothelial growth factor in himan hepatocellular carcinoma. J Hepatol. [27], 854-861. 1997.

A16. Torimura T, Sata M, Ueno T, et al. Increased expression of vascular endothelial growth factor is associated with tumor progression in hepatocellular carcinoma. Hum Pathol [29], 986-991. 1998.

A17. Poon RT, Ng IO, Lau C, et al. Correlation of serum basic fibroblast growth factor levels with clinicopathological features and postoperative recurrence in hepatocellular carcinoma. Am J Surg [182], 298-304. 2001.

A18. Thomas MB, Abbruzzese JL. Opportunities for targeted therapies in hepatocellular carcinoma. J Clin Oncol 2005; 23(31):8093-8108.

A19. Li X, Tang Z, Zhou G. Expression if vascular endothelial growth factor correlates with invasion and metastasis of hepatocellular carcinoma. Chung-Hua Chung Liu Tsa Chih (Chinese Journal of Oncology) 1998;(20):12-14.

A20. Moon WS, Rhyu KH, Kang MJ, Lee DG, Yu HC, Yeum JH et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma?. Mod Pathol 2003; 16(6):552-557.

A21. Jinno K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Doi T et al. Circulating vascular endothelial growth factor (VEGF) is a possible tumor marker for metastasis in human hepatocellular carcinoma. J Gastroenterol 1998; 33(3):376-382.

A22. Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG et al. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Commun 1999; 264(3):781-788.

A23. Li XM, Tang ZY, Qin LX, et al. Serum vascular endothelial growth factor in normal liver and hepatocellular carcinoma. J Exp Clin Cancer Res 1999;(18):511-517.

A24. Hurwitz HI, Fehrenbacher L, Hainsworth JD, Heim W, Berlin J, Holmgren E et al. Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. J Clin Oncol 2005; 23(15):3502-3508.

A25. Bevacizumab in unresectable hepatocellular carcinoma (HCC) for patients without metastasis and without invasion of the portal vein. San Francisco, CA: American Society pf Clinical Oncology Gastrointestinal Cancers Symposium, 2006.

A26. Zhu AX, Blaszkowsky LS, Ryan DP, Clark JW, Muzikansky A, Horgan K et al. Phase II study of gemcitabine and oxaliplatin in combination with bevacizumab in patients with advanced hepatocellular carcinoma. J Clin Oncol 2006; 24(12):1898-1903.

A27. Huynh H, Soo KC, Chow PK, Panasci L, Tran E. Xenografts of Human Hepatocellular Carcinoma: A Useful Model for Testing Drugs. Clin Cancer Res 2006; 12(14):4306-4314.

A28. Mesiano S, Ferrara N, Jaffe RB. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am J Pathol 1998; 153(4):1249-1256.

A29. Yokoi K, Sasaki T, Bucana CD, Fan D, Baker CH, Kitadai Y et al. Simultaneous inhibition of EGFR, VEGFR, and platelet-derived growth factor receptor signaling combined with gemcitabine produces therapy of human pancreatic carcinoma and prolongs survival in an orthotopic nude mouse model. Cancer Res 2005; 65(22):10371-10380.

A30. Hobson B, Denekamp J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. Br J Cancer 1984; 49(4):405-413.

A31. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res 2000; 60(5):1388-1393.

A32. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. Cancer Res 2002; 62(24):7203-7206.

A33. Kryczek I, Lange A, Mottram P, Alvarez X, Cheng P, Hogan M et al. CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. Cancer Res 2005; 65(2):465-472.

A34. Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 2004; 4(12):937-947.

A35. Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 2004; 64(11):3731-3736.

(B1) Ferlay J BF, Pisani P and Parkin DM. Globocan 2002: Cancer incidence, Mortality and Prevalence Worldwide. 5 ed.Lyon: IARC Press, 2004.

(B2) Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55:10-30.

(B3) Smith RA, Cokkinides V and Eyre HJ. American Cancer Society Guidelines for the Early Detection of Cancer, 2005. CA Cancer J Clin 2005;55:31-44.

(B4) Chedid A, Ryan LM, Dayal Y, Wolf BC and Falkson G. Morphology and other prognostic factors of hepatocellular carcinoma. Arch Pathol Lab Med 1999;123: 524-528.

(B5) Nagasue N, Kohno H, Chang YC, Taniura H, Yamanoi A, Uchida M, et al. Liver resection for hepatocellular carcinoma. Results of 229 consecutive patients during 11 years. Ann Surg 1993;217:375-384.

(B6) Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S, Ozaki H, et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996;83:1219-1222.

(B7) Huguet CSF. Primary hepatocellular cancer: Western experience. In L.Blumgart (ed.), Surgery of the Liver and Billary Tract. London: Churchill Livingstone, 2000. p. 1365-1369.

(B8) Lai EWJ. Hepatocellular carcinoma: the Asian experience. In L.Blumgart (ed.), Surgery of the Liver and the Biliary Tract. London: Churchill Livingstone, 1994. p, 1349-1363.

(B9) Colombo M. Hepatocellular carcinoma. J Hepatol 1992;15:225-236.

(B10) Lai EC, Fan ST, Lo CM, Chu KM, Liu CL and Wong J. Hepatic resection for hepatocellular carcinoma. An audit of 343 patients. Ann Surg 1995;221:291-298.

(B11) Takenaka K, Kawahara N, Yamamoto K, Kajiyama K, Maeda T, Itasaka H, et al. Results of 280 liver resections for hepatocellular carcinoma. Arch Surg 1996;131: 71-76.

(B12) Simonetti RG, Liberati A, Angiolini C and Pagliaro L. Treatment of hepatocellular carcinoma: a systematic review of randomized controlled trials. Ann Oncol 1997;8: 117-136.

(B13) Miura H, Miyazaki T, Kuroda M, et al. Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. J Hepatol 1997;27:854-861.

(B14) Torimura T, Sata M, Ueno T, et al. Increased expression of vascular endothelial growth factor is associated with tumor progression in hepatocellular carcinoma. Hum Pathol 1998;29:986-991.

(B15) Poon RT, Ng IO, Lau C, et al. Correlation of serum basic fibroblast growth factor levels with clinicopathological features and postoperative recurrence in hepatocellular carcinoma. Am J Surg 2001;182;298-304.

(B16) Li X, Tang Z and Zhou G Expression if vascular endothelial growth factor correlates with invasion and metastasis of hepatocellular carcinoma. Chung-Hua Chung Liu Tsa Chih (Chinese Journal of Oncology);1998;12-14.

(B17) Jinno K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Doi T, et al. Circulating vascular endothelial growth factor (VEGF) is a possible tumor marker for metastasis in human hepatocellular carcinoma. J Gastroenterol 1998;33:376-382.

(B18) Moon WS, Rhyu KH, Kang MJ, Lee DG, Yu HC, Yeum JH, et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? Mod Pathol 2003;16:552-557.

(B19) Thomas MB and Abbruzzese JL. Opportunities for targeted therapies in hepatocellular carcinoma. J Clin Oncol 2005;23:8093-8108.

(B20) Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG, et al. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Commun 1999;264: 781-788.

(B21) Schmelzle T and Hall MN. TOR, a central controller of cell growth. Cell 2000;103: 253-262.

(B22) Gingras AC, Raught B and Sonenberg N. Regulation of translation initiation by FRAP/mTOR. Genes Dev 2001;15:807-826.

(B23) Scott PH, Brunn GJ, Kohn AD, Roth RA and Lawrence JC Jr. Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. Proc Natl Acad Sci USA 1998: 95: 7772-7777.

(B24) Sekulic A, Hudson CC, Homme JL, Yin P, Otterness DM, Karnitz LM, et al. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res 2000;60:3504-3513.

(B25) Fang Y, Vilella-Bach M, Bachmann R, Flanigan A and Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 2001;294:1942-1945.

(B26) Dufner A and Thomas G. Ribosomal S6 kinase signaling and the control of translation. Exp Cell Res 1999;253:100-109.

(B27) Sonenberg N and Gingras AC. The mRNA 5' cap-binding protein eIF4E and control of cell growth. Curr Opin Cell Biol 1998;10:268-275.

(B28) Jefferies HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB and Thomas G. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. EMBO J 1997;16:3693-3704.

(B29) Sahin F, Kannangai R, Adegbola O, Wang J, Su G and Torbenson M. mTOR and P70 S6 kinase expression in primary liver neoplasms. Clin.Cancer Res 2004;10: 8421-8425.

(B30) Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL and Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 2002;277: 27975-27981.

(B31) Rhee J and Hoff PM. Angiogenesis inhibitors in the treatment of cancer. Expert Opin Pharmacother 2005;6:1701-1711.

(B32) Zhu AX, Sahani D, Norden-Zfoni N, et al. A phase I1 study of gemcitabine, Oxaliplatin in combination with bevacizumb (GEMOX-B) in patients with Hepatocellular Carcinoma. 2005. Presented at 2005 American Society of Clinical Oncology Annual Meeting, Orlando, FL.

(B33) Schwartz JD, Schwartz M, Leher D, et al. Bevacizumab in hepatocellular carcinoma (HCC) inpatients without metastasis and without invasion of the portal vein. 2005. Presented at 2005 American Society of Clinical Oncology Annual Meeting, Orlando, FL.

(B34) Wiederrecht G J, Sabers CJ, Brunn GJ, Martin MM, Dumont FJ and Abraham RT. Mechanism of action of rapamycin: new insights into the regulation of G1-phase progression in eukaryotic cells. Prog Cell Cycle Res 1995;1:53-71.

(B35) Luan FL, Hojo M, Maluccio M, Yamaji K and Suthanthiran M. Rapamycin blocks tumor progression: unlinking immunosuppression from antitumor efficacy. Transplantation 2002;73:1565-1572.

(B36) Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 1999;397: 530-534.

(B37) Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128-135.

(B38) Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128-135.

(B39) Luan FL, Ding R, Sharma VK, Chon WJ, Lagman M and Suthanthiran M. Rapamycin is an effective inhibitor of human renal cancer metastasis. Kidney Int 2003;63:917-926.

(B40) Elsharkawi M, Staib L, Henne-Bruns D and Mayer J. Complete remission of postransplant lung metastases from hepatocellular carcinoma under therapy with sirolimus and mycophenolate mofetil. Transplantation 2005:79: 855-857.

(B41) Stippel DL, Kasper HU, Schleimer K, Tox U, Bangard C, Holscher AH, et al. Successful use of sirolimus in a patient with bulky ovarian metastasis of hepatocellular carcinoma after liver transplantation. Transplant Proc 2005;37: 2185-2187.

(B42) Mathew T, Kreis H and Friend P. Two-year incidence of malignancy in sirolimus-treated renal transplant recipients: results from five multicenter studies. Clin Transplant 2004;18:446-449.

(B43) Guba M, Graeb C, Jauch KW and Geissler EK. Pro- and anti-cancer effects of immunosuppressive agents used in organ transplantation. Transplantation 2004;77: 1777-1782.

(B44) 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 2004;23:3151-3171.

(B45) Elit L. CCI-779 Wyeth. Curr Opin Investig Drugs 2002;3:1249-1253.

(B46) Vivanco I and Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489-501.

(B47) Huang S and Houghton PJ. Targeting mTOR signaling for cancer therapy. Curr Opin Pharmacol 2003;3:371-377.

(B48) Luo J, Manning BD and Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 2003;4:257-262,.

(B49) Huynh H, Soo KC, Chow PK, Panasci L, and Tran E. Xenografts of Human Hepatocellular Carcinoma: A Useful Model for Testing Drugs. Clin Cancer Res 2006;12:4306-4314.

(B50) Hay N and Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926-1945.

(B51) Hobson, B. and Denekamp, J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. Br J Cancer 1984;49:405-413.

(B52) Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH and Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res 2000;60:1388-1393.

(B53) Garrison RN, Kaelin LD, Galloway RH and Heuser LS. Malignant ascites. Clinical and experimental observations. Ann Surg 1986;203:644-651.

(B54) Marincola FM and Schwartzentruber DJ. Malignant ascites. In De-Vita VT, Hellman J, and Rosenberg SA (eds.), Cancer: Priciples and Practice of Oncology, 5th ed. Philadelphia: Lippincott-Raven, 1997. p. 2598-2606.

C1. Ferley J, Bray F. Pisani P, Parkin DM. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. IARC CancerBase No5 version 20. IARCPress, Lyon, France; 2004.

C2. Nagasue N, Kohno H, Chang YC, Taniura H, Yamanoi A, Uchida M, et al. Liver resection for hepatocellular carcinoma. Results of 229 consecutive patients during 11 years. Ann Surg 1993; 217:375-84.

C3. Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S, Ozaki H, et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996; 83:1219-22.

C4. El-Serag HB. Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 2002; 35(5 Suppl 2):S72-8.

C5. Yeo W, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, et al. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. J Natl Cancer Inst 2005; 97:1532-38

C6. Moon WS, Rhyu KH, Kang MJ, Lee DG, Yu HC, Yeum JH, et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? Mod Pathol 2003;16:552-7

C7. Dhar DK, Naora H, Yamanoi A, Ono T, Kohno H, Otani H, et al. Requisite role of VEGF receptors in angiogenesis of hepatocellular carcinoma: a comparison with angiopoietin/Tie pathway. Anticancer Res 2002; 22:379-86

C8. Poon RT, Ho JW, Tong CS, Lau C, Ng IO, Fan ST. Prognostic significance of serum vascular endothelial growth factor and endostatin in patients with hepatocellular carcinoma. Br J Surg 2004; 91:1354-60.

C9. Chao Y, Li CP, Chau GY, Chen CP, King KL, Lui WY, et al. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. Ann Surg Oncol 2003; 10:355-62.

C10. Claudio PP, Russo G, Kumar CA, Minimo C, Farina A, Tutton S, et al. pRb2/p130, vascular endothelial growth factor, p27(KIP1), and proliferating cell nuclear antigen expression in hepatocellular carcinoma: their clinical significance. Clin Cancer Res 2004; 10:3509-17

C11. Zhu AX, Blaszkowsky LS, Ryan DP, Clark JW, Muzikansky A, Horgan K, et al. Phase II study of gemcitabine and oxaliplatin in combination with bevacizumab in patients with advanced hepatocellular carcinoma. J Clin Oncol 2006;24:1898-903

C12. Sabatini,D.M. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer 2006; 6:729-34

C13. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489-01

C14. Elit, L. CCI-779 Wyeth. Curr Opin Investig Drugs 2002; 3:1249-53

C15. Huang S, Houghton PJ. Targeting mTOR signaling for cancer therapy. Curr Opin Pharmacol 2003;3:371-77

C16. Luo J, Manning BD, Cantley, LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 2003; 4:257-62

C17. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen, E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 2002; 277:27975-81

C18. Huynh H, Soo KC, Chow PK, Panasci L, Tran E. Xenografts of Human Hepatocellular Carcinoma: A Useful Model for Testing Drugs. Clin Cancer Res 2006;12: 4306-14

C19. Chin TM, Anuar D, Soo R, Salto-Tellez M, Li WQ, Ahmad B, et al. Detection of Epidermal Growth Factor Receptor Variations by Partially Denaturing HPLC. Clin Chem 2007; 53:62-70

C20. French DC, Saltzgueber M, Hicks DR, Cowper AL, Holt DW. HPLC assay with ultraviolet detection for therapeutic drug monitoring of sirolimus. Clin Chem 2001;47:1316-19

C21. Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J 1999 ; 344:427-31

C22. Sekulic A, Hudson CC, Homme JL, Yin P, Otterness DM, Karnitz LM, et al. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Research 2000; 60:3504-13

C23. Stromberg T, Dimberg A, Hammarberg A, Carlson K, Osterborg A, Nilsson K, et al. Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. Blood 2004;103:3138-47

C24. Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 2001;64:3731-36

C25. Sausville EA, Burger AM. Contributions of Human Tumor Xenografts to Anticancer Drug Development. Cancer Research 2006; 66:3351-54.

(1991). "ICRP 1990 Recommendations of the International Commission on Radiation Protection." Ann ICRP 21(60): 1-201.

(2001). "Radiation and your patient: a guide for medical practitioners." Ann ICRP 31(4): 5-31.

A. X. Zhu, D. S., A. Norden-Zfoni, N. S. Holalkere, L. Blaszkowsky, D. P. Ryan, J. Clark, K. Taylor, J. V. Heymach, K. Stuart (2005). "A Phase II Study of Gemcitabine, Oxaliplatin in Combination with Bevacizumab (GEMOX-B) in Patients with Hepatocellular Carcinoma." ASCO Proceedings.

Abraham, R. T. and G. J. Wiederrecht (1996). "Immunopharmacology of rapamycin." Annu Rev Immunol 14: 483-510.

Atkins, M. B., M. Hidalgo, et al. (2004). "Randomized phase II study of multiple dose levels of CCI-779, a novel mammalian target of rapamycin kinase inhibitor, in patients with advanced refractory renal cell carcinoma." J Clin Oncol 22(5): 909-18.

Augustine, J. J., T. C. Knauss, et al. (2004). "Comparative effects of rapamycin and mycophenolate mofetil on erythropoiesis in kidney transplant patients." Am J Transplant 4(12): 2001-6.

Bjornsti, M. A. and P. J. Houghton (2004). "The TOR pathway: a target for cancer therapy." Nat Rev Cancer 4(5): 335-48.

Bosch, F. X., J. Ribes, et al. (2005). "Epidemiology of hepatocellular carcinoma." Clin Liver Dis 9(2): 191-211, v.

Brattstrom, C., H. E. Wilczek, et al. (1998). "Hypertriglyceridemia in renal transplant recipients treated with rapamycin." Transplant Proc 30(8): 3950-1.

Chan, S., M. E. Scheulen, et al. (2005). "Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer." J Clin Oncol 23(23): 5314-22.

Chang, S. M., P. Wen, et al. (2005). "Phase II study of CCI-779 in patients with recurrent glioblastoma multiforme." Invest New Drugs 23(4): 357-61.

Chow, N. H., P. I. Hsu, et al. (1997). "Expression of vascular endothelial growth factor in normal liver and hepatocellular carcinoma: an immunohistochemical study." Hum Pathol 28(6): 698-703.

Chung, T. W., Y. C. Lee, et al. (2003). "Hepatitis B Virus X protein modulates the expression of PTEN by inhibiting the function of p53, a transcriptional activator in liver cells." Cancer Res 63(13): 3453-8.

Cohan, R. H., J. H. Ellis, et al. (1996). "Extravasation of radiographic contrast material: recognition, prevention, and treatment." Radiology 200(3): 593-604.

DeGraffenried, L. A., L. Fulcher, et al. (2004). "Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway." Ann Oncol 15(10): 1510-6.

Dong-Dong, L., Z. Xi-Ran, et al. (2003). "Expression and significance of new tumor suppressor gene PTEN in primary liver cancer." J Cell Mol Med 7(1): 67-71.

Edinger, A. L., C. M. Linardic, et al. (2003). "Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells." Cancer Res 63(23): 8451-60.

El-Assal, O. N., A. Yamanoi, et al. (1998). "Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver." Hepatology 27(6): 1554-62.

El-Serag, H. B. and A. C. Mason (1999). "Rising incidence of hepatocellular carcinoma in the United States." N Engl J Med 340(10): 745-50.

Elsharkawi, M., L. Staib, et al. (2005). "Complete remission of postransplant lung metastases from hepatocellular carcinoma under therapy with rapamycin and mycophenolate mofetil." Transplantation 79(7): 855-7.

Eng, C. P., S. N. Sehgal, et al. (1984). "Activity of rapamycin (AY-22,989) against transplanted tumors." J Antibiot (Tokyo) 37(10): 1231-7.

Ferrara, N. (2004). "Vascular endothelial growth factor as a target for anticancer therapy." Oncologist 9 Suppl 1: 2-10.

Fortin, M. C., M. A. Raymond, et al. (2004). "Increased risk of thrombotic microangiopathy in patients receiving a cyclosporin-sirolimus combination." Am J Transplant 4(6): 946-52.

Galanis, E., J. C. Buckner, et al. (2005). "Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study." J Clin Oncol 23(23): 5294-304.

Gehan, E. A. and M. C. Tefft (2000). "Will there be resistance to the RECIST (Response Evaluation Criteria in Solid Tumors)?" J Natl Cancer Inst 92(3): 179-81.

Gingras, A. C., S. G. Kennedy, et al. (1998). "4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway." Genes Dev 12(4): 502-13.

Grewe, M., F. Gansauge, et al. (1999). "Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6K pathway in human pancreatic cancer cells." Cancer Res 59(15): 3581-7.

Grunwald, V., L. DeGraffenried, et al. (2002). "Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells." Cancer Res 62(21): 6141-5.

Guba, M., C. Graeb, et al. (2004). "Pro- and anti-cancer effects of immunosuppressive agents used in organ transplantation." Transplantation 77(12): 1777-82.

Guba, M., G. E. Koehl, et al. (2005). "Dosing of rapamycin is critical to achieve an optimal antiangiogenic effect against cancer." Transpl Int 18(1): 89-94.

Guba, M., P. von Breitenbuch, et al. (2002). "Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor." Nat Med 8(2): 128-35.

Hambleton J, S. J., Kabbinavar F, Bergsland E, Holmgren E, Holden SN, Hurwitz H, Scappaticci FA. (2005). "Safety of low-dose aspirin in pooled analysis of 3 randomized, controlled trials of bevacizumab with chemotherapy in patients with metastatic colorectal cancer." ASCO Proceedings(3554).

Hara, K., Y. Maruki, et al. (2002). "Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action." Cell 110(2): 177-89.

Hardinger, K. L., L. A. Cornelius, et al. (2002). "Induced-induced leukocytoclastic vasculitis." Transplantation 74(5): 739-43.

Heesom, K. J. and R. M. Denton (1999). "Dissociation of the eukaryotic initiation factor-4E/4E-BP1 complex involves phosphorylation of 4E-BP1 by an mTOR-associated kinase." FEBS Lett 457(3): 489-93.

Hosoi, H., M. B. Dilling, et al. (1999). "Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells." Cancer Res 59(4): 886-94.

Humar, R., F. N. Kiefer, et al. (2002). "Hypoxia enhances vascular cell proliferation and angiogenesis in vitro via rapamycin (mTOR)-dependent signaling." Faseb J 16(8): 771-80.

Izzedine, H., I. Brocheriou, et al. (2005). "Post-transplantation proteinuria and rapamycin." N Engl J Med 353(19): 2088-9.

J. D. Schwartz, M. S., D. Lehrer, D. Coll, M. Kinkhabwala, M. Sung, S. B. Holloway, S. Wadler (2005). "Bevacizumab in hepatocellular carcinoma (HCC) in patients without metastasis and without invasion of the portal vein." ASCO Proceedings.

Jeng, K. S., I. S. Sheen, et al. (2004). "Prognostic significance of preoperative circulating vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma: a prospective study." World J Gastroenterol 10(5): 643-8.

Johnson, P. J., R. Williams, et al. (1978). "Induction of remission in hepatocellular carcinoma with doxorubicin." Lancet 1(8072): 1006-9.

Katayama, H., K. Yamaguchi, et al. (1990). "Adverse reactions to ionic and nonionic contrast media. A report from the Japanese Committee on the Safety of Contrast Media." Radiology 175(3): 621-8.

Kraemer, F. B., D. Takeda, et al. (1998). "Insulin regulates lipoprotein lipase activity in rat adipose cells via wortmannin- and rapamycin-sensitive pathways." Metabolism 47(5): 555-9.

Letavernier, E., M. N. Pe'raldi, et al. (2005). "Proteinuria following a switch from calcineurin inhibitors to rapamycin." Transplantation 80(9): 1198-203.

Li, B., S. A. Desai, et al. (2002). "A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis." Gene Ther 9(4): 233-44.

Ma, D. Z., Z. Xu, et al. (2005). "Down-regulation of PTEN expression due to loss of promoter activity in human hepatocellular carcinoma cell lines." World J Gastroenterol 11(29): 4472-7.

Mathew, T., H. Kreis, et al. (2004). "Two-year incidence of malignancy in treated-treated renal transplant recipients: results from five multicenter studies." Clin Transplant 18(4): 446-9.

McLeod, L. E. and C. G. Proud (2002). "ATP depletion increases phosphorylation of elongation factor eEF2 in adult cardiomyocytes independently of inhibition of mTOR signalling." FEBS Lett 531(3): 448-52.

Metcalfe, S. M., C. E. Canman, et al. (1997). "Rapamycin and p53 act on different pathways to induce G1 arrest in mammalian cells." Oncogene 15(14): 1635-42.

Miller, J. C., H. H. Pien, et al. (2005). "Imaging angiogenesis: applications and potential for drug development." J Natl Cancer Inst 97(3): 172-87.

Miura, H., T. Miyazaki, et al. (1997). "Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma." J Hepatol 27(5): 854-61.

Morelon, E., M. Stern, et al. (2001). "Characteristics of associated-associated interstitial pneumonitis in renal transplant patients." Transplantation 72(5): 787-90.

Morgan, B., A. L. Thomas, et al. (2003). "Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies." J Clin Oncol 21(21): 3955-64.

Neshat, M. S., I. K. Mellinghoff, et al. (2001). "Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR." Proc Natl Acad Sci U S A 98(18): 10314-9.

Peralba, J. M., L. DeGraffenried, et al. (2003). "Pharmacodynamic Evaluation of CCI-779, an Inhibitor of mTOR, in Cancer Patients." Clin Cancer Res 9(8): 2887-92.

Podsypanina, K., R. T. Lee, et al. (2001). "An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice." Proc Natl Acad Sci U S A 98(18): 10320-5.

Presta, L. G., H. Chen, et al. (1997). "Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders." Cancer Res 57(20): 4593-9.

Reardon, D. A., J. A. Quinn, et al. (2006). "Phase 1 trial of gefitinib plus rapamycin in adults with recurrent malignant glioma." Clin Cancer Res 12(3 Pt 1): 860-8.

Rubio-Viqueira, B. and M. Hidalgo (2006). "Targeting mTOR for cancer treatment." Curr Opin Investig Drugs 7(6): 501-12.

Sahin, F., R. Kannangai, et al. (2004). "mTOR and P70 S6 kinase expression in primary liver neoplasms." Clin Cancer Res 10(24): 8421-5.

Schumacher, G., M. Oidtmann, et al. (2005). "Rapamycin inhibits growth of human hepatoma cells alone or combined with tacrolimus, while tacrolimus promotes cell growth." World J Gastroenterol 11(10): 1420-5.

Seow A, K. W., Chia KS, Shi LM, Lee HP, Shanmugaratnam K (2004). "Trends in Cancer Incidence in Singapore 1968-2002." Singapore Cancer Registry(Report no. 6).

Shi, Y., A. Frankel, et al. (1995). "Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro." Cancer Res 55(9): 1982-8.

Shimoda, K., M. Mori, et al. (1999). "Vascular endothelial growth factor/vascular permeability factor mRNA expression in patients with chronic hepatitis C and hepatocellular carcinoma." Int J Oncol 14(2): 353-9.

Stallone, G., A. Schena, et al. (2005). "Rapamycin for Kaposi's sarcoma in renal-transplant recipients." N Engl J Med 352(13): 1317-23.

Stippel, D. L., H. U. Kasper, et al. (2005). "Successful use of rapamycin in a patient with bulky ovarian metastasis of hepatocellular carcinoma after liver transplantation." Transplant Proc 37(5): 2185-7.

Therasse, P., S. G. Arbuck, et al. (2000). "New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada." J Natl Cancer Inst 92(3): 205-16.

Torimura, T., M. Sata, et al. (1998). "Increased expression of vascular endothelial growth factor is associated with tumor progression in hepatocellular carcinoma." Hum Pathol 29(9): 986-91.

Van Beers, B. E., I. Leconte, et al. (2001). "Hepatic perfusion parameters in chronic liver disease: dynamic CT measurements correlated with disease severity." AJR Am J Roentgenol 176(3): 667-73.

Venook, A. P. (2004). "Key research issues in the management of hepatocellular carcinoma." Cancer Chemother Pharmacol 54 Suppl 1: S87-90.

Vermeulen, P. B., G. Gasparini, et al. (2002). "Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours." Eur J Cancer 38(12): 1564-79.

Watanabe, S., Y. Horie, et al. (2005). "Hepatocyte-specific Pten-deficient mice as a novel model for nonalcoholic steatohepatitis and hepatocellular carcinoma." Hepatol Res.

Witzig, T. E., S. M. Geyer, et al. (2005). "Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma." J Clin Oncol 23(23): 5347-56.

Yeo, W., T. S. Mok, et al. (2005). "A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma." J Natl Cancer Inst 97(20): 1532-8.

Yu, K., L. Toral-Barza, et al. (2001). "mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer." Endocr Relat Cancer 8(3): 249-58.

Zhang, L., Q. Yu, et al. (2004). "Study of the PTEN gene expression and FAK phosphorylation in human hepatocarcinoma tissues and cell lines." Mol Cell Biochem 262(1-2): 25-33.

Zhu, A. X., L. S. Blaszkowsky, et al. (2006). "Phase II study of gemcitabine and oxaliplatin in combination with bevacizumab in patients with advanced hepatocellular carcinoma." J Clin Oncol 24(12): 1898-903.

Zimmerman, J. J., K. C. Lasseter, et al. (2005). "Pharmacokinetics of rapamycin (rapamycin) in subjects with mild to moderate hepatic impairment." J Clin Pharmacol 45(12): 1368-72.

Each of the applications and patents mentioned in this document, and each document cited or referenced in each of the above applications and patents, including during the prosecution of each of the applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited or mentioned in each of the applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments and that many modifications and additions thereto may be made within the scope of the invention. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the claims. Furthermore, various combinations of the features of the following dependent claims can be made with the features of the independent claims without departing from the scope of the present invention.

CLAIMS

1. A combination of a first agent comprising rapamycin (Sirolismus) together with a second agent comprising bevacizumab (Avastin) for use in the treatment or prevention of hepatocellular carcinoma (HCC) in an individual.

2. A combination according to Claim 1, in which one or both of the first agent comprising rapamycin and the second agent comprising bevacizumab is in the form of a pharmaceutical composition comprising the agent or agents, together with a pharmaceutically acceptable carrier, excipient or diluent.

3. A combination according Claim 1 or 2, in which the first agent comprising rapamycin is provided in a form suitable for oral administration, such as a tablet, or in which the second agent comprising bevacizumab is provided in a form suitable for intravenous administration, or both.

4. A combination according to Claim 1, 2 or 3, in which the method of treatment or prevention of hepatocellular carcinoma (HCC) comprises administering the first agent comprising rapamycin simultaneously or sequentially with a second agent comprising bevacizumab to an individual.

5. Rapamycin for use in a method of treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the method comprises administering rapamycin simultaneously or sequentially with bevacizumab.

6. Bevacizumab for use in a method of treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the method comprises administering bevacizumab simultaneously or sequentially with rapamycin.

7. Use of rapamycin for the preparation of a combination for treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the combination comprises bevacizumab .

8. Use of bevacizumab for the preparation of a combination for treatment or prevention of hepatocellular carcinoma (HCC) in an individual, in which the combination comprises rapamycin .

9. A combination according to any of Claims 1 to 4, rapamycin according to Claim 5 for a use as specified therein or bevacizumab according to Claim 6 for a use as specified therein, further comprising instructions for administration of the agents to an individual to treat or prevent hepatocellular carcinoma (HCC) in an individual.

109. A method of preparing a combination according to any of Claims 1 to 4 or 9, the method comprising bringing together a first agent comprising rapamycin with a second agent comprising bevacizumab .

11. A combination according to any of Claims 1 to 4 or 9, rapamycin according to Claim 5 or 9 for a use as specified therein or bevacizumab according to Claim 6 or 9 for a use as specified therein, use of rapamycin according to Claim 7, use of bevacizumab according to Claim 8 or a method according to Claim 10, in which the rapamycin is present in an amount to provide a dosage of between about 1 mg/day to about 10 mg/day.

12. A combination according to any of Claims 1 to 4 or 9, rapamycin according to Claim 5 or 9 for a use as specified therein or bevacizumab according to Claim 6 or 9 for a use as specified therein, use of rapamycin according to Claim 7, use of bevacizumab according to Claim 8 or a method according to Claim 10, in which the bevacizumab is present in an amount to provide a dosage of between about 5 mg/kg/2 weeks to about 10 mg/kg/2 weeks.

13. An *ex-vivo* method of preventing the growth or proliferation, or both, of a cell or tissue, the cell or tissue being a liver cancer cell or liver cancer tissue, the method comprising exposing the cell or tissue to a first agent comprising rapamycin and a second agent comprising bevacizumab .

14. A method according to Claim 12 or 13, in which the first agent has the features as set out in any preceding claim, or in which the second agent has the features as set out in any preceding claim, or both.