Evaluation of the mTOR inhibitor, everolimus, in combination with cytotoxic antitumor agents using human tumor models *in vitro* and *in vivo*

Terry O'Reilly[a], Paul M.J. McSheehy[a], Markus Wartmann[a], Peter Lassota[c], Ralf Brandt[d] and Heidi A. Lane[a,b]

The aim was to determine the potential of the allosteric mammalian target of rapamycin inhibitor, everolimus, to act in combination with cytotoxic anticancer compounds *in vitro* and *in vivo*. A concomitant combination *in vitro* showed no evidence of antagonism, but enhanced the antiproliferative effects (additive to synergistic) with cisplatin, doxorubicin, 5-fluorouracil, gemcitabine, paclitaxel, and patupilone. Everolimus (1–5 mg/kg/d orally) was evaluated for antitumor activity *in vivo* alone or in combination with suboptimal cytotoxic doses using athymic nude mice bearing subcutaneous human H-596 lung, KB-31 cervical, or HCT-116 colon tumor xenografts. Everolimus monotherapy was very well tolerated and caused inhibition of tumor growth, rather than regression, and this was associated with a dose-dependent decline in tumor pS6 levels, a key downstream protein of mammalian target of rapamycin. At the doses used, the cytotoxics inhibited tumor growth and caused tolerable body-weight loss. Concomitant combinations of cisplatin, doxorubicin, paclitaxel, or patupilone with everolimus produced cooperative antitumor effects, in some cases producing regressions without clinically significant increases in toxicity. In contrast, combinations with gemcitabine and 5-fluorouracil were less well tolerated. Alternative administration schedules were tested for cisplatin, gemcitabine, or paclitaxel combined with everolimus: these did not dramatically affect cisplatin or gemcitabine activity or tolerability but were antagonistic for paclitaxel. Everolimus showed promising maintenance activity after treatment with doxorubicin or paclitaxel ceased. Overall, the results confirm that everolimus is an effective, well-tolerated suppressor of experimental human tumor growth, and although it did not show strong potentiation of efficacy, antitumor activity *in vivo* was increased without marked increases in toxicity, supporting clinical use of everolimus as a partner for conventional cytotoxics. *Anti-Cancer Drugs* 22:58–78 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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[a]Oncology Research, Novartis Institutes for Biomedical Research, [b]Basilea Pharmaceutica International AG, Postfach, Basel, Switzerland, [c]Oncology Research, Novartis Institutes for Biomedical Research, Cambridge, Massachusetts, USA and [d]VivoPharm PTY, Adelaide, Australia

Correspondence to Paul M.J. McSheehy, PhD, Oncology Research, Novartis Institutes for Biomedical Research, WKL 125.13.17, CH-4002 Basel, Switzerland
Tel: +41 69 61 68189; fax: +41 69 61 66242;
e-mail: paul_mj.mcsheehy@novartis.com

Present address: Peter Lassota, Caliper Life Sciences, Hopkington, MA 01478, USA

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Introduction

Everolimus (RAD001) is an orally active mammalian target of rapamycin (mTOR) inhibitor currently under clinical testing for its utility, alone and in combination, as an anticancer agent. The target of this class of agents is mTORC1, a multifunctional signal-transducing protein, which obtains signals from many upstream inputs, propagating the information through the regulation of multiple downstream pathways (reviewed in [1–4]). Inhibition of mTOR seems to be a broadly applicable anticancer strategy [1,2], perhaps because activity against both primary and metastatic cells is provided by direct inhibition of tumor cells and antivascular activity [4,5]. Inhibition of mTOR also seems to have some impact on reversal of drug resistance [3].

Everolimus has been extensively profiled in experimental in-vitro and in-vivo models as a single agent and in chemotherapy combinations, predominantly using cytotoxics. Everolimus in combination with cyclophosphamide produced enhanced, durable antitumor effects, including enhanced antiangiogenic activity in gastric tumor xenografts [6,7]. However, this combination was poorly tolerated, causing body weight (BW) losses approximately 34% greater than those in controls or monotherapies. A combination of everolimus with platinum compounds has been studied extensively. Thus, the enhancement of the apoptotic activity *in vitro* of oxaliplatin was observed with cholangiocarcinoma cell lines [8], and everolimus enhanced the activity of cisplatin against hepatomas both *in vitro* and *in vivo* [9], and also enhanced the effects of cisplatin against SKOV-3 tumor xenografts [10]. Everolimus was active against both cisplatin-sensitive and cisplatin-resistant nasopharyngeal

Paul M.J. McSheehy and Terry O'Reilly contributed equally to this article.
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carcinoma cell lines and increased activity in combination with cisplatin [11]. These results are in congruence with earlier reports of the positive effects of combining rapamycins and cisplatin [12,13]. Furthermore, everolimus is a potent inhibitor of mantle cell lymphoma cell lines *in vitro* and produces primarily positive interactions in combination with doxorubicin, vincristine, paclitaxel, vorinostat, bortezomib, and rituximab [14], and also with the latter antibody against diffuse large B-cell lines *in vitro* [15]. Everolimus combined with ara-C, danorubicin, idarubicin, fluvastatin, danorubicin and ara-C, or idarubicin and ara-C produced synergistic antiproliferative effects against KG-1 undifferentiated myeloid and AML-193 acute monocytic leukemia cell lines *in vitro* [16]. A combination of vincristine and everolimus is effective both *in vitro* and *in vivo* against B-cell progenitor, ALL [17]. Everolimus enhanced the antitumor effect of docetaxel and of zoledronate in an orthotopic bone cancer model using C4-2 cells [18]. However, everolimus failed to promote doxorubicin-induced apoptosis in phosphatase and tensin homolog positive and negative glioblastoma cell lines *in vitro* [19]. Combinations involving rapamycin have been shown to be more effective in reducing the cancer stem cell population of a pancreatic cancer model [20]. In melanoma xenografts, rapamycin positively interacted with dacarbazine *in vivo* but only with tumors displaying less sensitivity to dacarbazine [21]. Recently, a strong synergy was shown between the pan-vascular endothelial growth factor-R inhibitor, PTK/ZK, and everolimus in the metastatic B16/BL6 melanoma model *in vivo* [22].

Thus, a considerable number of combinations involving everolimus have already been described, although these are predominantly *in vitro*. However, quantification of the degree of the interaction between the combination partners *in vivo*, is more difficult and controversial. In the studies cited above, although the in-vitro assays used a formal method for determining the interaction between the drugs [12,16], not all studies carried out specific tests. With regard to the in-vivo evaluation of combinations, some used multiple assays specific for the determination of the combination effect [22,23], whereas the other studies used statistical analyses that were not specific for the assessment of the interaction. Furthermore, the increased toxicity of the combination is often ignored, challenging the utility of the combination. In our experience, total growth inhibition can nearly always be increased in any combination, but if toxicity is increased, perhaps also in an additive/synergistic manner, what does this really mean from the standpoint of clinical application? Consequently, the purpose of this study was to extend the preclinical evaluation of everolimus in combination with conventional cytotoxic anticancer agents to support future and ongoing clinical trials. The activity of everolimus in combination with paclitaxel, cisplatin, gemcitabine, doxorubicin, 5-fluorouracil (5-FU), or patupilone (epothilone B) was first evaluated in cell culture *in vitro* and then extensively *in vivo* using human tumor xenograft systems. Furthermore, in some cases the effect of the schedule of treatment administration (cisplatin, gemcitabine, and paclitaxel) and the effect of continuing everolimus treatment after its combination with paclitaxel and doxorubicin, that is, 'maintenance-therapy' were also evaluated. In particular, we have evaluated the degree of interaction by using different statistical and qualitative assessments on both tumor size and tolerability as assessed by mouse BW, and determining antitumor activity relative to tolerability by the use of a specific measure, the Efficacy Tolerability Quotient (ETQ).

Methods

Materials

Everolimus (dry powder) was obtained from Dr W. Schuler, Pharma Research, Novartis Pharma AG, Basel. Cell culture materials were from Integra BioSciences (Wallisellen, Switzerland). Liquid media, fetal bovine serum (FBS), and media additives were from Life Technologies (Basel, Switzerland). Microemulsion vehicle-formulated everolimus was obtained from Pharmaceutical Research and Development, Novartis Pharma AG, Basel. Paclitaxel (Taxol) and cisplatin (Platinol) were purchased from Bristol Meyers Squibb, Baar, Switzerland; doxorubicin (Adriblastin) from Pharmacia & Upjohn AG, Dübendorf, Switzerland; gemcitabine (Gemzar) from Eli Lilly, Vernier, Switzerland; and 5-FU from Roche, Basel, Switzerland.

In-vitro testing of everolimus as a combination partner for conventional anticancer agents

Cells and cell culture conditions

The human lung cell lines, A-549 (CCL-185) and NCI H-596 (HTB-178), and the human colon cell line, HCT-116 (CCL-247), were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Human cervical carcinoma KB-31 cells were obtained from Dr R.M. Baker, Rowel Park Memorial Institute (Buffalo, New York, USA) and have been described earlier [24]. The cells were maintained and cultured according to established techniques as recommended by the supplier. KB-31 cells were cultivated in alpha minimum essential medium, 5% v/v FBS, and 1% v/v penicillin-streptomycin; HCT-116 were cultivated in McCoy's 5A, 10% v/v FBS; and A-549 cells were cultivated in D-minimum essential medium (low glucose), 10% v/v FBS. All media were supplemented with 1% w/v glutamine.

In-vitro combination testing

The dry powder everolimus was dissolved in dimethyl sulfoxide and stored in aliquots at -20°C. The method of

determining the effect of everolimus in combination was based on cell mass quantification by methylene blue staining used for evaluating the antiproliferative activity of single agents [25]. For in-vitro testing, the recommendations of the CalcuSyn computer program documentation (Biosoft, Cambridge, UK) were followed. The IC_{50} values of all compounds as single agents were initially determined separately, but were also repeated in the same experiments testing for combination effects. For combination testing, 1500 cells/100 µl were added to each well of a 96-well plate and incubated overnight at 37°C under 5% v/v CO_2 and 80% relative humidity. The next day, the cells were exposed to the anticancer agents. Starting at 16 times the IC_{50} compounds were diluted in two-fold steps (nine in total) in a fixed 1 : 1 ratio. Compound dilutions were carried out in a separate 96-well plate. Each dilution (50 µl) was added to the appropriate well, and finally 50 µl of the fresh medium was added. Therefore, the starting concentration of each compound in combination was four times the IC_{50}. Calculation of the nonexclusive combination index (CI) [26] used the software provided by CalcuSyn. The CI was interpreted as follows: > 1.0 antagonistic, 0.85–1.0 additive, 0.7–0.84 moderate synergy, less than 0.7 synergy.

Determination of S6K (p70s6k) activity in tumors

The procedural details were analogs to those used earlier for the determination of pS6 in rat pancreatic tumors [27]. Briefly, nude mice bearing subcutaneous (s.c.) KB-31 tumors were treated orally with various daily doses of everolimus (0.5, 1.0, or 2.5 mg/kg) and the tumor and the skin were obtained from mice killed 1 h after the last dose, at the completion of a 35-day efficacy study. Tissue samples were rinsed with phosphate buffer and homogenized in a buffer containing protease inhibitors using a polytron. The samples were cleared by centrifugation and stored at −80°C in aliquots. S6K (p70s6k) activity was measured using 40S ribosomal subunits as an in-vitro substrate and phosphorylated S6 resolved by SDS-PAGE. The incorporation of [γ-^{32}P]phosphate into S6 was determined by phosphoimaging (Molecular Dynamics, Sunnyvale, California, USA).

In-vivo evaluation of everolimus alone or in combination with conventional agents

Preparation of compound solutions

A microemulsion of 2% w/v everolimus, or an everolimus-free vehicle was stored at −20°C as aliquots. Just before use, the aliquots were thawed and diluted with 5% w/v glucose in pyrogen-free water to administer the required dose in a volume of 10 ml/kg. Vehicle controls received the vehicle in 5% w/v glucose. Conventional anticancer agents were diluted from their clinical formulations using 0.9% w/v saline for injection to deliver the required dose in 10 ml/kg.

In-vivo antitumor activity against subcutaneously transplanted tumors

Female athymic BALB/c *nu/nu* (nude) or Harlan athymic mice were kept in a pathogen-controlled environment (10–12 mice/type III cage) with access to food and water *ad libitum*. Tumors were established by s.c. injection of cells (minimum 2×10^6 cells in 100 µl PBS) in carrier mice (4–8 mice per cell line). The resulting tumors were passaged once in carrier mice and experiments began on the second passage. Tumor fragments (approximately 25 mg) were implanted s.c. into the left flank of the animals with a 13-gauge trocar needle under Forene (Abbott, Switzerland) anesthesia. The tumors of the HCT-116 line were established by the s.c. injection of 10^6 cells.

Tumor volume (TVol) and BW were monitored twice weekly. All treatments were initiated when the mean TVol reached approximately 100 mm^3. TVol was determined according to the formula length $\times$ diameter$^2 \times \pi/6$, where length is the longest dimension and diameter the shortest. In addition to presenting changes in TVols over the course of the treatment, antitumor activity is expressed as the T/C ratio: difference (subtraction) in TVol of drug-treated animals divided by the difference in TVol of vehicle-treated controls. The number of tumors showing regression in response to treatment was also recorded: a 'transient regression' was where after an initial decrease, the tumor regrew, whereas a 'durable regression' was where no regrowth was visible during the experiment.

Everolimus was administered orally, once per day in a variety of well-tolerated doses, the highest being 10 mg/kg. The maximally tolerated dose (MTD) of everolimus in BALB/c nude mice was not reached, as even 60 mg/kg, orally, once per day was well tolerated (data not shown).

Determination of drug interactions

Drug interaction analyses

For the determination of the significance of the interaction (positive, negative, or indifferent) between compounds in combination therapy, a one-way analysis of variance [ANOVA (1WA)] was used to compare monotherapies with combination groups; a two-way ANOVA (2WA) in which each agent and dose represented a separate factor; and a three-way ANOVA (3WA) for an additional factor of treatment order (schedule). The interaction of the two drugs in combination was also assessed by the method described by Clarke [28] to provide the Clark CI (CCI), in which CCI values of approximately 0 indicate an additive effect, negative values would indicate a synergistic interaction, and positive values indicate antagonism. The 95% confidence limits (CL) are also provided based upon the principles of propagation of standard errors [29], in which CCI values with CLs that traverse 0 indicate that an additive effect is most likely to be statistically valid, as described earlier [22].

Determination of antitumor effect relative to tolerability

Drug combinations may provide increased antitumor effect, but potentially at the cost of increased tolerability problems. To approach this issue, we have applied a novel calculation called the ETQ, which quantifies efficacy and toxicity (using BW) and integrates the two parameters into a single value (ETQ), which provides a useful indicator of the therapeutic index of a particular drug or combination schedule in an animal tumor model. The ETQ was calculated for the whole time course of the experiment using area-under-the-curve (AUC) for TVol and BW. The calculation is as follows:

$$\mathrm{ETQ} = (\mathrm{T/C_{TVol}} + 1)/(\mathrm{T/C_{BW}} + 1)$$

where T/C values are calculated by taking the AUC of treated animals and dividing it by the AUC of controls. Lower ETQ values indicate increased antitumor effect relative to tolerability; details are provided in the Supplementary material (paul_mj.mcsheehy@novartis.com).

Statistical analyses

The results are presented as mean ± one standard error of the mean or one standard deviation. The differences in TVol (ΔTVol) and mouse BW (ΔBW) were statistically analyzed using a 1WA with post-hoc Dunnett's test to compare treatment groups with control groups, and the pair-wise Tukey test and Dunnett's test to compare the treatment groups versus controls being the primary post-hoc tests. In some cases, the data were normalized by taking $\log_{10}$ before statistical analyses. The Fisher's exact test was used to determine the differences in presumptive tumor cure rates, which was based on the appearance of a residual tissue material at the site, which, in our experience, does not increase in mass. For all tests, the level of significance was set at P less than 0.05. Statistical calculations were made using SigmaPlot 11.0 (Jandel Scientific, San Rafael, California, USA). ED_{50} values were estimated from dose–response curves using Prism Software (GraphPad Software Inc., La Jolla, California, USA).

Results

In-vitro activity of single agents

As described earlier [5], tumor cells can be described as sensitive ($IC_{50} < 10$ nmol/l) or insensitive ($IC_{50} > 1$ μmol/l) to everolimus based on the antiproliferative IC_{50} values *in vitro*. Table 1 shows two examples from both cell types and compares activity with the cytotoxics cisplatin, doxorubicin, 5-FU, gemcitabine, paclitaxel, and patupilone. For the everolimus-sensitive cells (A549, H-596), the IC_{50} value for everolimus activity was the median of seven different treatments, but for the everolimus-insensitive cells (KB-31, HCT-116), everolimus activity was markedly less compared with the cytotoxics.

In-vivo activity of everolimus as a single agent

Everolimus showed dose-dependent antitumor activity against all tumor models investigated and two examples are shown for KB-31 and H-596 xenografts in Table 2. Comparison of efficacy and tolerability of everolimus, with the anthracycline doxorubicin in the KB-31 model, showed that daily dosing of everolimus at 2.5 mg/kg provided similar antitumor activity to the cytotoxic agent without any BW loss (Fig. 1). Daily administration of everolimus seemed to have the best antitumor effect, even when the dose was raised for intermittent treatment (results not shown). In the KB-31 model, ED_{50} values for daily dosing were estimated to be 0.28 mg/kg, and 0.11, 1.2, and 2.4 mg/kg for A549, H-596, and HCT-116, respectively. Thus, as described earlier [26], everolimus showed significant activity *in vivo* against tumor models considered insensitive *in vitro*, probably largely reflecting the antiangiogenic/vascular activity of the drug.

In the KB-31 model, everolimus dose-dependently inhibited phosphorylation of the ribosomal S6 protein (which is downstream of mTOR) in both s.c. tumors and the skin (Fig. 2). The ED_{50} estimates for tumors were 0.81 and 0.78 mg/kg, and for the skin they were 0.94 and 0.90 (in two separate experiments each); results comparable to the ED_{50} values for growth inhibition.

In-vitro activity of everolimus in combination with cytotoxic anticancer agents

Everolimus seemed to be a good combination partner *in vitro* for the four cytotoxic agents tested (Table 3). In no case did concomitant exposure of cells to combinations involving everolimus result in antagonism. Rather, additive (everolimus and paclitaxel in KB-31 or everolimus and gemcitabine in HCT-116 cells) to synergistic interactions (most combinations) were observed.

In-vivo activity of everolimus in combination with cytotoxic agents

Tolerability

At the effective dose of 2.5 mg/kg/day of everolimus, only minor tolerability problems occurred in combination with the cytotoxic agents, although escalating the everolimus dose to 5 or 10 mg/kg/day tended to be less well tolerated and did not further augment efficacy (data not shown).

KB-31 cervical tumors

Results are summarized in Figs 3 and 4 and Table 4.

Cisplatin

In the first experiment, everolimus significantly inhibited tumor growth but cisplatin at a suboptimal regimen of 2.5 mg/kg/week (50% optimal dose) was inactive (T/C 1.06) (Fig. 3); however, increasing the cisplatin dose to 4 mg/kg/week still only produced weak activity (T/C 0.62) (Table 4). The antitumor activity of the combination was dominated by everolimus, with the combination

Table 1 ***In vitro*** **activity of everolimus as compared with cytotoxic anticancer agents**

	IC_{50} (nmol/l)							
Cell line	Everolimus	Cisplatin	Doxorubicin	5-Fluorouracil	Gemcitabine	Paclitaxel	Patupilone	Median
A549	23 ± 8	515 ± 25	13.5 ± 0.9	2290 ± 130	1356 ± 198	5.6 ± 1.6	0.18 ± 0.05	23.0
NCI H-596	1.5 ± 0.5	Nd	Nd	Nd	Nd	4.0 ± 0.9	0.22 ± 0.09	1.5
KB-31	4788 ± 1333	2545 ± 95	13.7 ± 1.8	3220 ± 513	12.0 ± 1.4	3.8 ± 0.9	0.32 ± 0.08	13.7
HCT-116	3662 ± 1128	654 ± 69	16.1 ± 2.1	2650 ± 100	3.5 ± 0.3	5.6 ± 1.4	0.50 ± 0.17	16.1

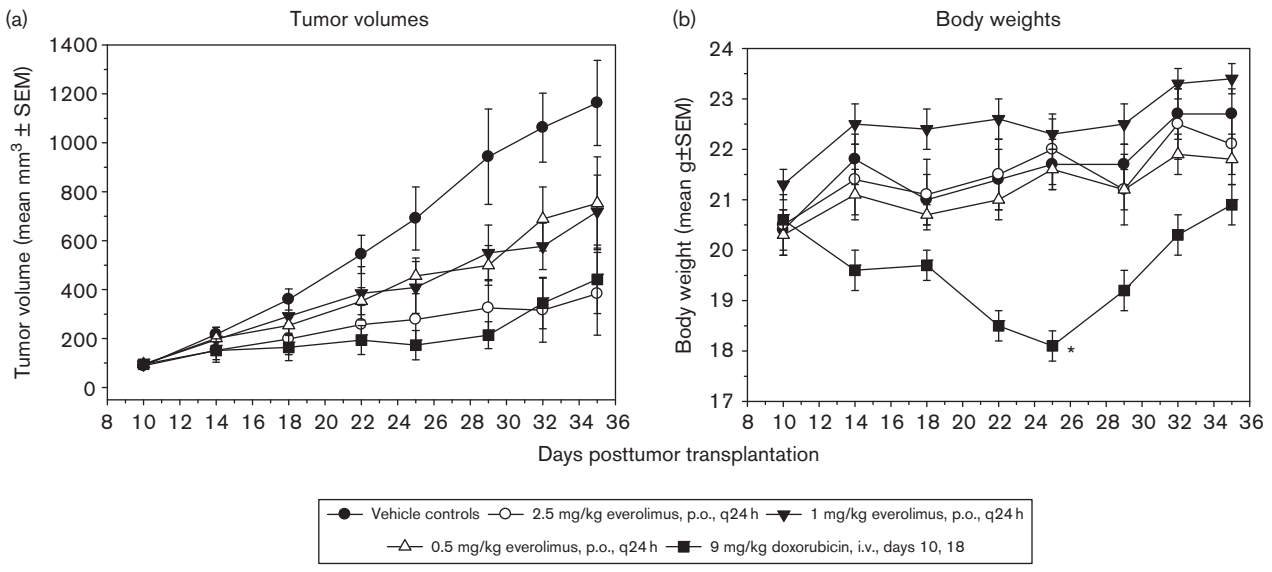
Cell lines were seeded in 96-well plates (1500 cells/well) and incubated overnight and then exposed to two-fold serial dilutions of each agent for 3–4 days, allowing for at least two population doublings. Cell numbers were estimated by quantification of protein content of fixed cells by methylene blue staining. IC_{50} values for net cell mass increase are shown as mean ± standard error of the mean from at least three independent experiments.
Nd, not done.

Table 2 **Antitumor effect of everolimus against NCI H-596 and KB-31 tumors in female BALB/c nude mice**

				Mean ± SEM	
Tumor	Compound	Dose	T/C (95% CL)	Delta tumor volume (mm^3)	Percentage delta body weight
NCI H-596 tumors	Vehicle	0 mg/kg	1.0 (0.12)	811 ± 101	11 ± 1 ($P<0.001$)
	Everolimus	5 mg/kg	0.11 (0.055)	87 ± 63 ($P<0.05$)	5 ± 1 ($P=0.009$)
	Everolimus	2.5 mg/kg	0.26 (0.053)	207 ± 56 ($P<0.05$)	8 ± 1 ($P<0.001$)
	Everolimus	0.5 mg/kg	0.79 (0.093)	641 ± 74 (NS)	10 ± 2 ($P=0.001$)
KB-31 tumors	Vehicle	0 mg/kg	1.0 (0.12)	1163 ± 174	11 ± 2 ($P=0.002$)
	Everolimus	2.5 mg/kg	0.25 (0.097)	295 ± 159 ($P<0.05$)	8 ± 1 ($P<0.001$)
	Everolimus	1 mg/kg	0.54 (0.097)	625 ± 143 (NS)	10 ± 1 ($P<0.001$)
	Everolimus	0.5 mg/kg	0.57 (0.12)	659 ± 186 (NS)	7 ± 1 ($P<0.001$)

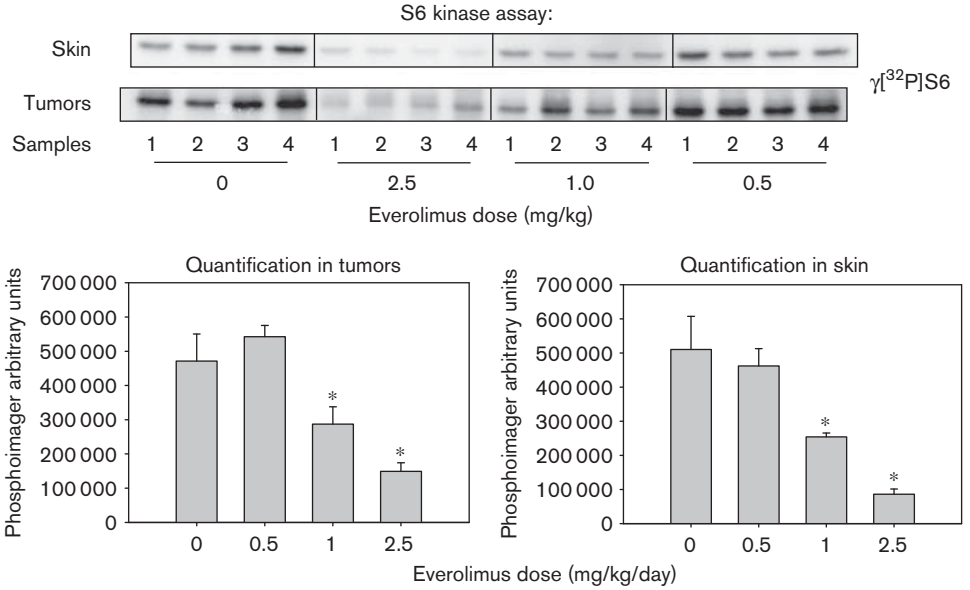
Fragments (approximately 25 mg) of NCI H-596 ($n=8$ per group) or KB-31 tumors ($n=6$ per group) were transplanted subcutaneously into the left flank of each female nude mouse. Daily treatments began when tumors were approximately 100 mm^3, everolimus was administered once per day at the doses indicated for 21 days (NCI H-596 tumors) or 35 days (KB-31 tumors). Data are from the final time-points and where applicable present means ± standard error of the mean. Antitumor activity is expressed as T/C and the 95% CL estimated based upon error propagation from the original tumor volume data. Statistical significance of delta tumor volumes as compared with controls was determined using a one-way analysis of variance with the Dunnett's test. The statistical significance of body weights was determined using paired *t*-tests.
CL, confidence limits; NS, not significant.

Fig. 1



Antitumor effect of everolimus against KB-31 tumors in female BALB/c nude mice. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n=6$ per group). Treatments were started when the tumors reached approximately 100 mm^3 (day 10) after tumor transplantation. Everolimus was administered orally once per day (q24 h) at 2.5 mg/kg from day 8 to day 35. Doxorubicin was administered intravenously at 9 mg/kg once per week. Data presented are means ± standard error of the mean, where $*P<0.05$ versus controls by a 1WA with the Dunnett's test.

Fig. 2



Effects of everolimus on S6K activity in KB-31 tumors and skin derived from a KB-31 xenograft model 1 h after the last dose. KB-31 tumor and skin extracts from mice were prepared 1 h after the last dose, on completion of a 35-day efficacy study, where everolimus was given orally, daily (q24 h) at the indicated milligram/kilogram doses. S6K ($p70^{s6k}$) activity was measured using 40S ribosomal subunits as an *in vitro* substrate. Top panel: autoradiograph of [γ-^{32}P]phosphate incorporation into S6 protein ([γ-^{32}P] S6). The two graphs represent phosphoimager quantification of the top panel. Data are means ± standard deviation, where $*P<0.05$ versus controls (1WA with Dunnett's test).

Table 3 ***In vitro*** **nonexclusive CI values for everolimus combined with cytotoxic antitumor agents**

	Combination partner							
	Cisplatin		Paclitaxel		Gemcitabine		Doxorubicin	
Cell line	*n*	CI	*n*	CI	*n*	CI	*n*	CI
KB-31	6	0.74 ± 0.06 (moderate synergy)	6	0.9 ± 0.1 (additive)	6	0.74 ± 0.05 (moderate synergy)	6	0.7 ± 0.08 (moderate synergy)
A-549	4	0.47 ± 0.03 (synergy)	4	0.74 ± 0.03 (moderate synergy)	4	0.76 ± 0.07 (moderate synergy)	4	0.64 ± 0.03 (synergy)
HCT-116	4	0.49 ± 0.07 (synergy)	4	0.63 ± 0.06 (synergy)	4	0.9 ± 0.1 (additive)	4	0.52 ± 0.06 (synergy)

Each cell line was added to 96-well plates (1500 cells/well in 100 µl) and incubated for 24 h. Subsequently, a two-fold dilution series was made in separate tubes, starting at 8 × the IC_{50} of each compound either alone or in paired combinations, and the dilutions were added to the cell culture medium. The cells were then reincubated for 4 days. Methylene blue staining was performed on day 4 and the amount of bound dye determined. IC_{50}s were subsequently determined using the CalcuSyn program. CI, non-exclusive combination index, as described by Chou [26].

group having greater activity compared with both cisplatin and everolimus alone (1WA), but a 2WA failed to show an interaction between the two agents. There was an additive interaction as judged by CCI. With regard to the tolerability of the combination, there was a small but significant increase in BW loss compared with monotherapies, but overall no deleterious interaction and no mortalities were observed.

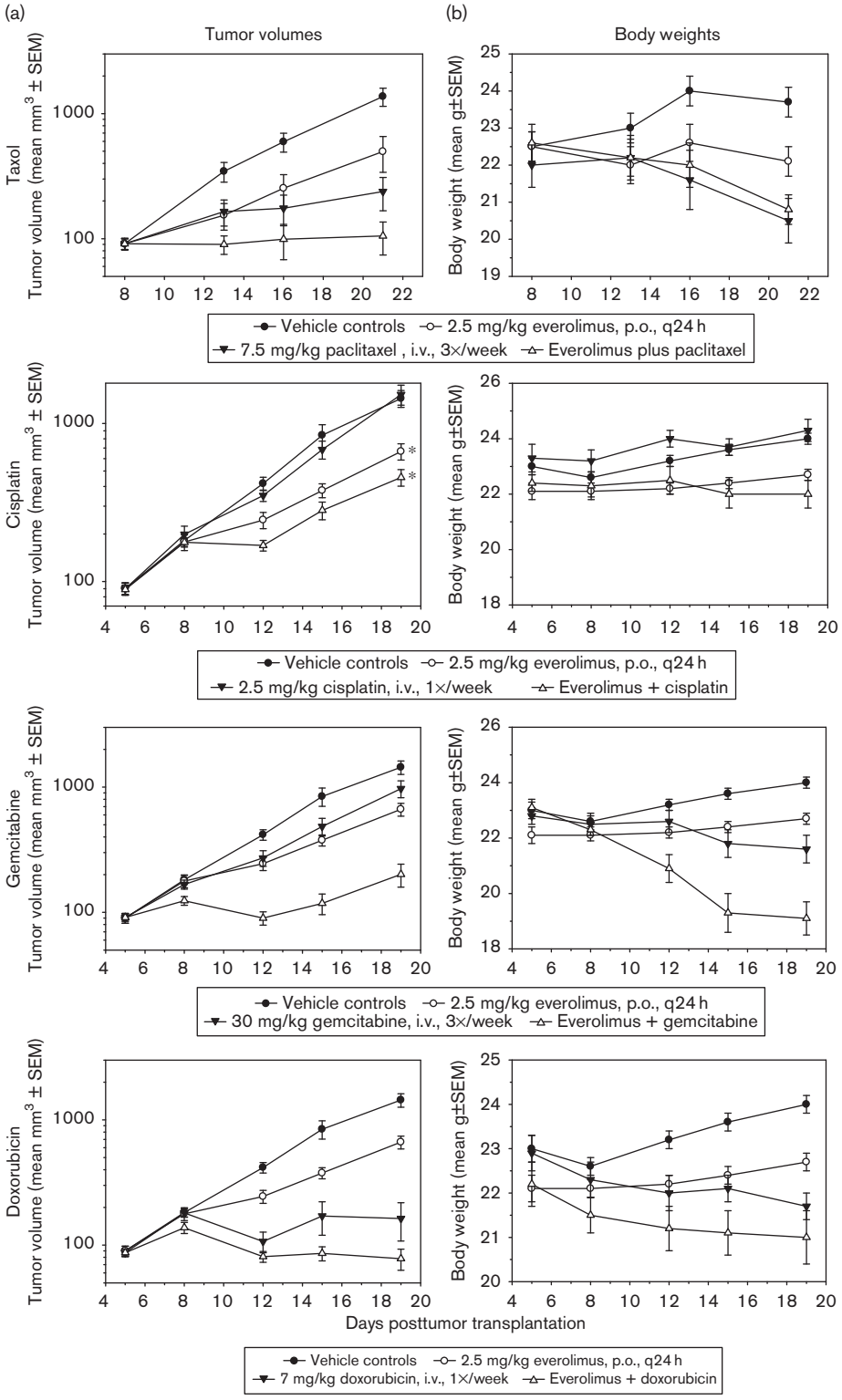
Doxorubicin

Doxorubicin (7 mg/kg/weekly) showed strong antitumor activity that was slightly increased by the combination to essentially produce a stable disease (Fig. 3), although this was not significantly different from doxorubicin monotherapy (Table 4). A 2WA showed a significant interaction and the CCI value suggested synergy. BW loss was dominated by doxorubicin and was not further increased in the combination. There were no mortalities in any treatment group. A repeat experiment using a lower dose of doxorubicin (5 mg/kg/week) showed similar results (Table 4). There was a statistically significant positive combination effect, as judged by a 1WA and the CCI value, which indicated synergy, although the 2WA did not confirm a significant cooperative interaction. BW loss was again dominated by doxorubicin and was significantly increased in the combination compared with either monotherapy, although this was an additive effect by CCI, and there was no interaction as judged by a 2WA.

Gemcitabine

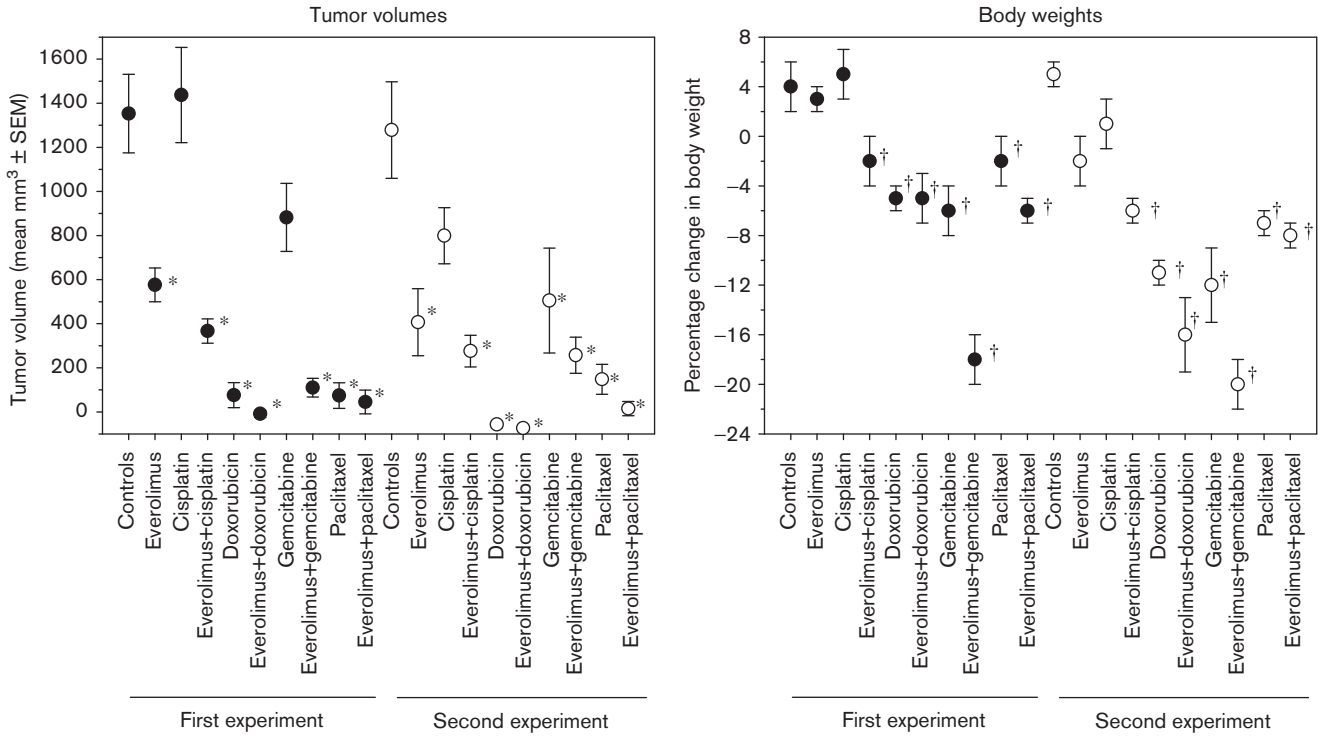
Gemcitabine (30 mg/kg thrice weekly) was weakly active as a single agent, but in combination the antitumor

Fig. 3



In vivo antitumor effect of everolimus in combination with paclitaxel, cisplatin, gemcitabine, or doxorubicin against KB-31 tumors. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n=8$ per group). Treatments were started on day 8 after tumor transplantation. The following drug administration regimens were used: everolimus was administered orally once per day at 2.5 mg/kg from day 8 to day 21. Doxorubicin was administered intravenously at 5 mg/kg once per week, gemcitabine at 30 mg/kg intraperitoneously, thrice per week, cisplatin 2.5 mg/kg intravenously once per week, and paclitaxel 7.5 mg/kg thrice per week. Data presented are means ± standard error of the mean, where $*P<0.05$ versus controls (1WA with Dunnett's test).

Fig. 4



Effects of everolimus in combination with cytotoxic anticancer agents against KB-31 tumors in female BALB/c nude mice. Fragments (approximately 25 mg) of KB-31 tumors ($n=8$ per group) were transplanted subcutaneously into the left flank of each female nude mouse. Treatments began when the tumors had reached approximately 100 mm^3. The administration regimens were as follows: first experiment: everolimus, 2.5 mg/kg, orally, q24 h, cisplatin 2.5 mg/kg, intravenously (i.v.), q7days; doxorubicin 7 mg/kg, i.v., q7days; gemcitabine 30 mg/kg, i.v., q7days; paclitaxel, 10 mg/kg, i.v., 3 × per week; second experiment: everolimus 2.5 mg/kg, orally, q24 h; cisplatin 4 mg/kg, i.v., q7days; doxorubicin 5 mg/kg, i.v., q7days; gemcitabine 25 mg/kg, i.v., 3 × per week; paclitaxel 7.5 mg/kg, i.v., 3 × per week. Data are from the final time-points and where applicable present means ± standard error of the mean in which * and † indicate a significant difference ($P<0.05$) to vehicle-treated tumors.

activity was greatly increased, producing near-stable disease with transient tumor regressions (Fig. 3), which was significantly better than gemcitabine monotherapy (Fig. 4, Table 4). Furthermore, a 2WA showed an interaction and the CCI indicated an additive response. However, the combination was poorly tolerated producing profound BW losses (mean of −18%). In the second experiment, a lower gemcitabine dose (25 mg/kg thrice weekly) was used, which retained significant antitumor activity, although this was only marginally increased by the combination and there was no evidence of a positive interaction (Table 4). However, the combination was again poorly tolerated, showing significantly greater BW loss (mean −20%) compared with the other groups. There were also two mortalities in the gemcitabine monotherapy group, and one in the combination group.

Paclitaxel

At 10 mg/kg, paclitaxel was very effective in producing near-stable disease and durable tumor regressions in three of eight mice. The combination possessed better activity when compared with everolimus monotherapy (1WA), but not with paclitaxel alone. However, the CCI indicated a synergistic response and a 2WA analysis also indicated a positive interaction (Table 4). Furthermore, there was no exacerbation of BW loss by the combination. In a second experiment using a lower dose of paclitaxel, the TVol of the combination group was consistently lower than that with paclitaxel monotherapy. However, as in the first experiment, a 1WA showed the combination to be superior to everolimus alone, but not to paclitaxel alone and a 2WA did not show statistical significance, although the CCI value suggested a synergistic response. BW losses were dominated by paclitaxel and were slightly increased in combination with everolimus and indeed both CCI and 2WA failed to show a significant negative interaction with regard to tolerability of the combination. No mortalities were observed.

Patupilone

Patupilone produced dose-dependent antitumor activity with 1 mg/kg/week impairing tumor growth and 2 mg/kg/week producing durable tumor regressions (Fig. 5). BW loss was also dose-dependent, particularly after the second patupilone administration. At the vehicle endpoint (day 14), all patupilone-containing groups had similar

Table 4 Statistical analyses of the effects of everolimus in combination with cytotoxic anticancer agents against KB-31 tumors in female BALB/c nude mice

	Tumor response				Host response			
Compound	T/C	1WA	CCI (CL)	2WA	Percentage delta BW	1WA	CCI (CL)	2WA
First experiment								
Vehicle controls	1.0				4 ± 2			
Everolimus	0.43*				3 ± 1			
Cisplatin	1.06				5 ± 2			
Everolimus plus cisplatin	0.27*	0.002 <0.001	−0.18 (1.0)	NS	−2 ± 2	0.028 0.001	−1.4 (4)	NS
Doxorubicin	0.06*				−5 ± 1			
Everolimus plus doxorubicin	−0.01*	<0.001 NS	−0.03 (0.008)*	0.003	−5 ± 2*	0.019 NS	−0.3 (1.6)	NS
Gemcitabine	0.65				−6 ± 2*			
Everolimus plus gemcitabine	0.08*[a]	NS 0.001	−0.20 (0.39)	0.009	−18 ± 2*	<0.001 0.002	−3.3 (32)	NS
Paclitaxel	0.05*[b]				−2 ± 2			
Everolimus plus paclitaxel	0.03*[c]	0.006 NS	−0.01 (0.006)*	<0.001	−6 ± 1*	0.046 NS	−1.1 (3.6)	NS
Second experiment								
Vehicle controls	1.00				5 ± 1			
Everolimus	0.32*				−2 ± 2			
Cisplatin	0.62				1 ± 2			
Everolimus plus cisplatin	0.22*	0.013 0.05	0.02 (0.6)	NS	−6 ± 1*	0.025 0.009	−1.1 (1.2)	NS
Doxorubicin	−0.003*				−11 ± 1*			
Everolimus plus doxorubicin	−0.005*	<0.001 0.043	−0.11 (0.08)*	NS	−16 ± 3*	<0.001 0.025	−4.1 (13)	NS
Gemcitabine	0.39*				−12 ± 3*			
Everolimus plus gemcitabine	0.20*	NS NS	−0.08 (0.32)	NS	−20 ± 2*	<0.001 0.042	−5.0 (16)	NS
Paclitaxel	0.12*				−7 ± 1*			
Everolimus plus paclitaxel	0.01*	<0.001 NS	−0.03 (0.03)*	NS	−8 ± 1*	0.025 0.05	−2.2 (6.3)	0.051

Fragments (approximately 25 mg) of KB-31 tumors ($n = 8$ per group) were transplanted subcutaneously into the left flank of each female nude mouse. Treatments began when the tumors had reached approximately 100 mm^3. The administration regimens were as follows: first experiment: everolimus, 2.5 mg/kg, orally, q24 h; cisplatin 2.5 mg/kg, intravenously (i.v.), q7 days; doxorubicin 7 mg/kg, i.v., q7 days; gemcitabine 30 mg/kg, i.v., q7 days; paclitaxel, 10 mg/kg, i.v., thrice per week; second experiment: everolimus 2.5 mg/kg, orally, q24 h; cisplatin 4 mg/kg, i.v., q7 days; doxorubicin 5 mg/kg, i.v., q7 days; gemcitabine 25 mg/kg, i.v., 3 × per week; paclitaxel 7.5 mg/kg, i.v., 3 × per week. Data are from the final time-points and where applicable present mean ± standard error of the mean. Antitumor activity is expressed as T/C determined from the original tumor volume data ('Methods' section). Statistical significance of delta tumor volumes as compared with controls was determined using a 1WA with the Dunnett's test. The statistical significance of body weights was determined using paired *t*-tests.

The CCI values were determined as described in the 'Methods' section with the 95% CL estimated based upon error propagation.

CCI, *in vivo* combination index; the value in parentheses is the 95% CL based upon propagation of errors. 2WA, the value shown is the significance of the interaction. 1WA, one way analysis of variance; 2WA, two way analysis of variance; BW, body weight; CCI, Clark Combination Index; CL, confidence limits; NS, not significant.

[a]Transient regressions.

[b]Three out of eight tumors displayed durable regression.

[c]Two out of eight tumors displayed durable regression.

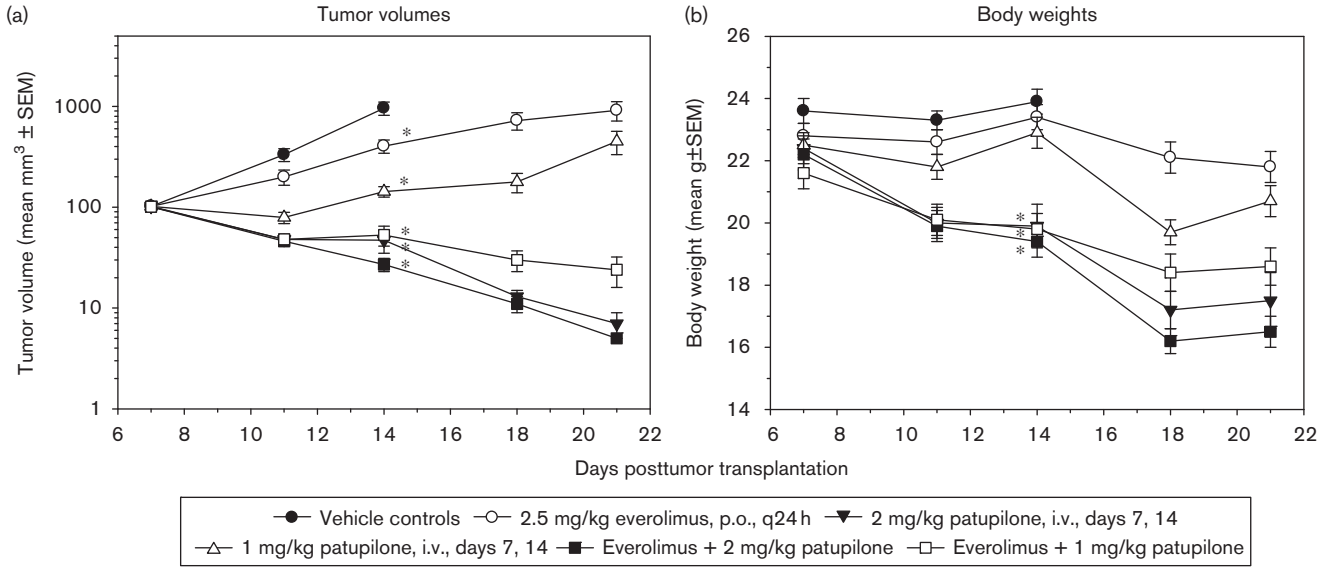
*$P < 0.05$ vs. controls (1WA with Dunnett's test vs. controls). 1WA with Tukey's pairwise comparison, upper number, combination versus everolimus alone, lower number, combination versus cytotoxic alone.

antitumor effects, being superior to everolimus monotherapy or vehicle controls. One week later, although there was no difference between the efficacy of everolimus and 2 mg/kg patupilone and the corresponding patupilone monotherapy, everolimus and 1 mg/kg patupilone were superior to either corresponding monotherapy, and indistinguishable from regimens containing 2 mg/kg patupilone as judged by 1WA. The 2WA did not indicate a significant positive interaction between the antitumor effects of everolimus and patupilone ($P = 0.22$). However, the CCI values −0.01 (0.02 95% CL) for everolimus combined with 1 mg/kg patupilone, and 0.01 (0.01) for the 2 mg/kg patupilone and everolimus group, suggested additive responses of the combination. Patupilone produced dose-dependent BW losses significantly greater than everolimus (or controls; all $P < 0.001$). Although a combination of everolimus and 2 mg/kg patupilone produced profound BW losses ($P < 0.001$ versus controls or everolimus), they were dominated by the patupilone component as the combination was not different from patupilone alone ($P = 0.9$). Furthermore, the CCI was −0.01 (0.07 95% CL) and a 2WA did not suggest an interaction. At lower dose patupilone combinations, the addition of everolimus exacerbated BW loss ($P < 0.001$ versus controls or everolimus, $P = 0.001$ versus patupilone alone) and the CCI was −0.11 (0.07 95% CL) suggesting an interaction, although the 2WA did not suggest an interaction.

H-596 non-small cell lung cancer tumors

Results are summarized in Fig. 6 and Table 5.

Fig. 5



In vivo antitumor effect of everolimus in combination with patupilone against KB-31 tumors. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n=8$ per group). Treatments were started on day 7 after tumor transplantation. Everolimus was administered at 2.5 mg/kg, orally, once per day and patupilone was administered at 1 or 2 mg/kg, intravenously, on days 7 and 14. Data presented are means ± standard error of the mean, where $*P<0.05$ versus controls (1WA with Dunnett's test).

Cisplatin

In two experiments, everolimus monotherapy showed strong activity and was well tolerated. Cisplatin (2.5 mg/kg/week) combined with everolimus showed significant antagonism by a 2WA. However, in the second experiment, in which a higher cisplatin dose (4 mg/kg/week) was used, a potent, regression-producing combination was observed and the 2WA indicated a positive interaction although the 1WA and the CCI only indicated additivity. In both experiments, all the treatments were well tolerated and no mortalities occurred.

Doxorubicin

In the two experiments, doxorubicin (7 and 5 mg/kg, respectively) had significant antitumor activity against NCI H-596 tumors but with marked BW loss. Combining doxorubicin (7 mg/kg) with everolimus tended to increase antitumor activity although both 1WA and 2WA failed to show this, and the CCI was indicative of only additivity. At the lower doxorubicin dose (5 mg/kg), the combination was more active than doxorubicin alone (1WA) and 2WA showed a positive interaction. However, the CCI again indicated an additive response. Most interestingly, the combination seemed to attenuate BW losses occurring because of doxorubicin therapy, although this did not reach significance.

Paclitaxel

Paclitaxel had strong activity in this model causing regression (experiment 1) or stable disease (experiment 2). With high-dose paclitaxel (10 mg/kg), there seemed to be a mild antagonism, which was significant by all three analytical methods. However, at a lower paclitaxel dose (7.5 mg/kg), a positive interaction was shown by a 2WA and the CCI. In both experiments there was a trend toward reduction in paclitaxel-produced BW loss, which was significant by the CCI but not by the 2WA. There were no mortalities in any group.

Gemcitabine

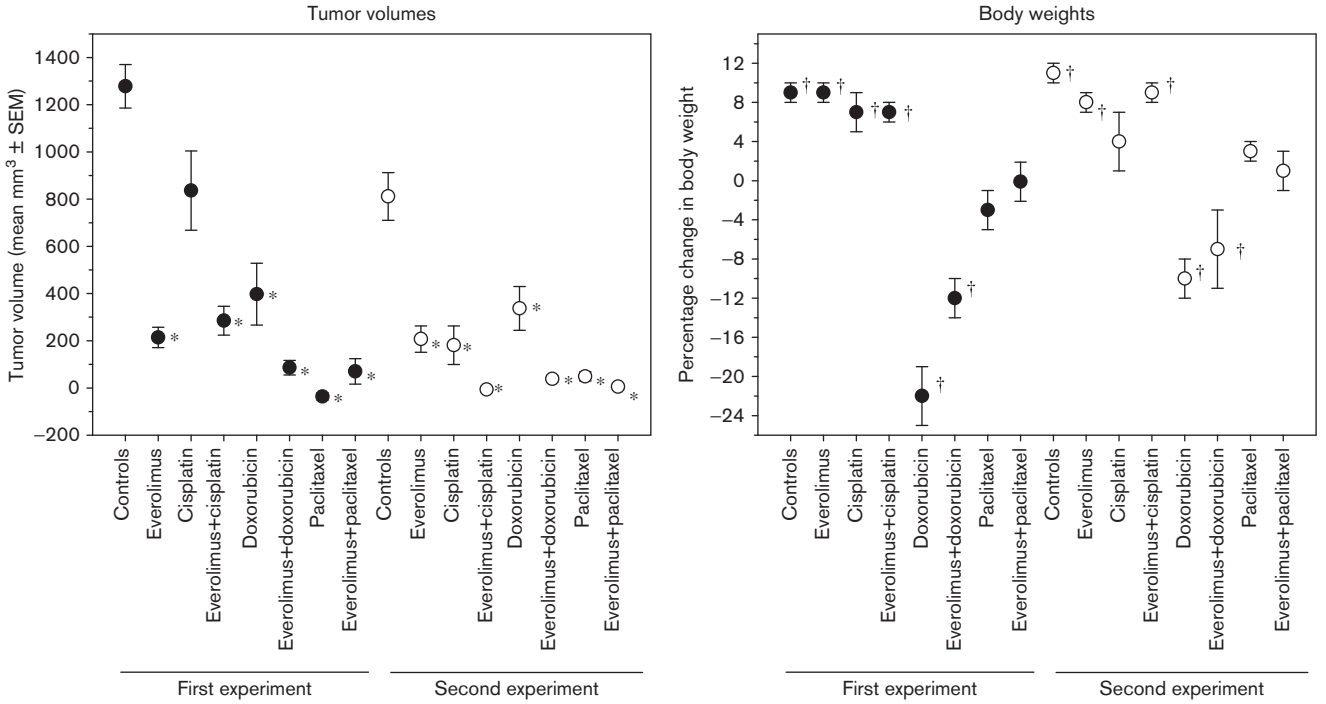
This combination was only explored in one experiment. Everolimus at 2.5 or 1 mg/kg/daily was administered alone or in combination with gemcitabine (25 mg/kg thrice weekly). As single agents, all treatments were effective (T/Cs = 0.07 and 0.18, 0.29, respectively) and the combination of gemcitabine with 2.5 mg/kg or 1 mg/kg everolimus gave lower T/C values of −0.05 and 0.04, respectively. However, the 1WA showed that the combination groups were not significantly better than everolimus therapy and 2WA confirmed no significant interaction ($P = 0.17$) and the CCI confirmed an additive interaction. BW losses were dominated by gemcitabine and did not increase with everolimus (data not shown).

HCT-116 colon tumors

Patupilone

Everolimus/patupilone combinations (Table 6) were evaluated in two independent experiments using 10 mg/kg/day and 1 mg/kg/week doses, respectively. In both experiments, everolimus and patupilone gave a similar significant efficacy as monotherapies and antitumor activity was further increased in the combination. The CCI

Fig. 6



Antitumor effect of everolimus in combination with cytotoxic anticancer agents against NCI H-596 tumors in female BALB/c nude mice. Fragments (approximately 25 mg) of NCI H-596 ($n=7$ or 8 per group) were transplanted subcutaneously into the left flank of each female nude mouse. Treatments began when the tumors had reached approximately 100 mm^3. Everolimus was administered once per day at the doses indicated alone or in combination with cytotoxic agents using the indicated regimens. On days where both agents were administered, everolimus was always administered 2 h before the cytotoxic agents. The administration regimens were as follows: first experiment: everolimus 2.5 mg/kg, orally, q24 h; cisplatin 2.5 mg/kg, intravenously, q7days; doxorubicin 7 mg/kg, intravenously (i.v.), q7days; paclitaxel, 10 mg/kg, i.v., 3 × per week; second experiment: everolimus 2.5 mg/kg, orally, q24 h; cisplatin 2.5 mg/kg, i.v., q7days; doxorubicin 5 mg/kg, i.v., q7days. Data are from the final time-points and where applicable present means ± standard error of the mean, in which * and † indicate a significant difference ($P<0.05$) to vehicle-treated tumors.

values indicated a trend toward an additive effect in each experiment, but neither 1WA nor 2WA showed a cooperative interaction, suggesting that only an additive response was occurring at best. The combination always caused the greatest BW loss and although this did not reach significance versus the monotherapies (1WA, 2WA), the CCI values suggested an additive interaction for tolerability. Mortalities were only observed in the patupilone monotherapy groups (12.5 and 25% in each experiment).

5-Fluorouracil

In one experiment the combination of 75 mg/kg/week 5-FU and 2.5 mg/kg/day everolimus was explored in three different schedules: everolimus before 5-FU, everolimus after 5-FU, or the two agents concomitantly (Table 7). The dose of everolimus was suboptimal dose for this model (see above, Table 6), whereas the 5-FU dose was near the MTD. Everolimus had marginal, albeit statistically significant activity, whereas 5-FU was strongly active (T/C = 0.23). Little increase in the antitumor effect over 5FU was observed with any schedule of the combination (T/Cs = 0.14–0.17), and although all combination groups were highly significantly different for vehicle or everolimus monotherapy, there was no improvement compared with 5-FU alone (1WA, 2WA), and CCI values indicated an additive effect. There was, however, an apparent schedule dependence for tolerability. Only in the group in which everolimus preceded 5-FU was there BW loss and the 2WA showed a significant negative interaction. Furthermore, almost uniquely for everolimus, there were eventually six out of eight (75%) mortalities in this particular schedule.

Compilation of analyses of combination efficacy

A compilation of the results from the different analytical methods used to determine drug interaction on antitumor effect are presented in Table 8. The results arising from each of the analyses (1WA with a positive interaction defined as the combination having significantly different activity compared with both agents as monotherapies, 2WA using analysis of interaction between each component, and CCI using the definitions of additivity, synergy, or antagonism as defined earlier [22,28]) are scored as + 1 for improved effect, 0 for no change, and − 1 for a decline in antitumor effect. A simple sum of these scores

Table 5 Antitumor effect of everolimus in combination with cytotoxic anticancer agents against NCI H-596 tumors in female BALB/c nude mice.

	Tumor response				Host response			
Compound	T/C	1WA	CCI (CL)	2WA	Percent change in BW	1WA	CCI (CL)	2WA
First experiment								
Vehicle controls	1.00				9 ± 1*			
Everolimus	0.17*				9 ± 1*			
Cisplatin	0.65				7 ± 2*			
Everolimus plus cisplatin	0.22*	<0.001 <0.001	0.11 (0.4)	0.03	7 ± 1*	NS NS	0 (0.6)	NS
Doxorubicin	0.31*				−22 ± 3*			
Everolimus plus doxorubicin	0.07*	NS NS	0.02 (0.12)	0.1	−12 ± 2*	0.001 0.007	1.1 (4.4)	<0.001
Paclitaxel	−0.03*				−3 ± 2			
Everolimus plus paclitaxel	5*	0.03 0.03	0.06 (0.007)*	<0.001	−0.1 ± 2	0.002 NS	0.3 (0.2)*	NS
Second experiment								
Vehicle controls	1.00				11 ± 1*			
Everolimus	0.26*				8 ± 1*			
Cisplatin	0.22*				4 ± 3			
Everolimus plus cisplatin	−0.08*	NS 0.09	−0.07 (0.09)	0.011	9 ± 1*	NS NS	0.55 (0.11)*	0.01
Doxorubicin	0.42*				−10 ± 2*			
Everolimus plus doxorubicin	0.05*	NS 0.01	−0.06 (0.2)	0.03	−7 ± 4*	<0.001 NS	−0.02 (2)	NS
Paclitaxel	0.06*				3 ± 1			
Everolimus plus paclitaxel	0.006*	0.04 NS	−0.04 (0.006)*	<0.001	1 ± 2	<0.001	−0.1 (0.07)*	NS

Fragments (approximately 25 mg) of NCI H-596 ($n=7$ or 8 per group) were transplanted subcutaneously into the left flank of each female nude mouse. Treatments began when the tumors had reached approximately 100 mm^3. Everolimus was administered once per day at the doses indicated alone or in combination with cytotoxic agents using the indicated regimens. On days where both agents were administered, everolimus was always administered 2 h before the cytotoxic agents. The administration regimens were as follows: first experiment: everolimus 2.5 mg/kg, orally, q24 h, cisplatin 2.5 mg/kg, intravenously (i.v.), q7 days, doxorubicin 7 mg/kg, i.v., q7 days, and paclitaxel, 10 mg/kg, i.v., 3 × per week; second experiment: everolimus 2.5 mg/kg, orally, q24 h, cisplatin 2.5 mg/kg, i.v., q7 days, doxorubicin 5 mg/kg, i.v., q7 days. Data are from the final time-points and where applicable present means ± standard error of the mean. Antitumor activity is expressed as T/C determined from the original tumor volume data ('Methods' section). Statistical significance of delta tumor volumes as compared with controls was determined using a 1WA with the Dunnett's test. The statistical significance of body weights was determined using paired *t*-tests. The CCI values were determined as described in the 'Methods' section with the 95% CL estimated based upon error propagation.

1WA with Tukey's pairwise comparison: upper number, combination versus everolimus alone, lower number, combination versus cytotoxic alone. CCI: *in vivo* combination index; the value in parentheses is the 95% CL based upon propagation of errors. 2WA, the value shown is the significance of the interaction.

1WA, one way analysis of variance; 2WA, two way analysis of variance; BW, body weight; CCI, Clark Combination Index; CL, confidence limits; NS, not significant.
*$P<0.05$ vs. controls (1WA with Dunnet's test versus controls).

Table 6 Efficacy and tolerability of patupilone and everolimus monotherapy and their combination against HCT-116 tumors 21 days after the initiation of treatment.

	Tumor response				Host response			
Compound	T/C	1WA	CCI (CL)	2WA	Percent change in BW	1WA	CCI (CL)	2WA
First experiment								
Vehicle controls	1.00				0.7 ± 1.8			
Everolimus	0.69	NS		0.03	4.8 ± 2.1	0.02		NS
Patupilone	0.60	NS		0.03	−0.6 ± 1.4	NS		0.001
Everolimus plus patupilone	0.25*		−0.01 (0.3)	NS	−9.3 ± 4.7		−7 (>100)	NS
Second experiment								
Vehicle controls	1.00				−9.5 ± 5.4			
Everolimus	0.39*	NS		<0.001	10.9 ± 2.4	NS		NS
Patupilone	0.54*	0.02		<0.001	−10.4 ± 4.0	NS		0.05
Everolimus plus patupilone	0.14*		−0.07 (0.4)	NS	−13.5 ± 3.0		−2.7 (1.8)*	NS

HCT-116 cells (1×10^6) were injected subcutaneously into the left flank of each female nude mouse ($n=7$ or 8 per group), and treatments were started 7 days later as follows: everolimus, 10 mg/kg, orally, once per day and patupilone 1 mg/kg, intravenously, once per week, controls treated with both orally and intravenously vehicles. Data are from the final time-points and where applicable present means ± standard error of the mean. Antitumor activity is expressed as T/C determined from the original tumor volume data ('Methods' section). Statistical significance of delta tumor volumes as compared with controls was determined using a 1WA with the Dunnett's test. The statistical significance of body weights was determined using paired *t*-tests. The CCI (*in vivo* combination index) values were determined as described in the 'Methods' section with the 95% CL estimated based upon error propagation in parentheses.

2WA: the value shown is the significance of the interaction.

1WA, one way analysis of variance; 2WA, two way analysis of variance; BW, body weight; CCI, Clark Combination Index; CL, confidence limits; NS, not significant.
*$P<0.05$ vs. controls (1WA with Dunnet's test vs. controls).

gave the overall score and resulting interpretation. In most cases the interaction should be considered additive, but the exact nature of the interaction is dependent on the tumor line and dose of the agents. Notably, paclitaxel–everolimus combinations tended to show additive effects but schedule-dependent antagonism.

Table 7 Sequence-dependent antitumor effect of everolimus and 5-FU combinations against HCT-116 human tumors in female BALB/c nude mice

	Tumor response				Host response			
Compound	T/C	1WA	CCI (CL)	2WA	Percent change in BW	1WA	CCI (CL)	2WA
Vehicle controls	1.00				0.7 ± 1.8			
Everolimus	0.70*	<0.006			1.8 ± 1.4	NS		
5FU	0.23*	<0.001			2.1 ± 1.7	NS		
Everolimus before 5FU	0.17*	<0.001 NS	0.01 (0.03)	0.07	−6.0 ± 4.2*	0.08	−16 (>100)	0.05
Everolimus after 5FU	0.14*	<0.001 NS	−0.03 (0.03)	0.1	2.7 ± 2.2	NS	−4 (67)	NS
Everolimus with 5FU	0.14*	<0.001 NS	−0.03 (0.03)	0.1	2.0 ± 1.0	NS	−5 (86)	NS

HCT-116 cells (1×10^6) were injected subcutaneously into the left flank of each female nude mouse ($n=8$ per group), and treatments were started 7 days later. The administration regimens were as follows: everolimus 2.5 mg/kg, orally, once per day; 5-FU, 75 mg/kg, intravenously, once per week; everolimus before 5-FU: everolimus on days 1, 3–7, 9–14; 5-FU on days 2, 8; everolimus after 5-FU: everolimus days 2–6, 8–14; 5-FU on days 1,7; everolimus with 5-FU: everolimus days 1-14, 5-FU on days 1, 7. On days when both agents were administered, everolimus was administered 2 h before 5-FU. Data are from the final time-points and where applicable present means ± standard error of the mean. Antitumor activity is expressed as T/C; statistical significance of delta tumor volumes as compared with controls was determined using a 1WA with the Dunnett's test. CCI values were determined as described in the 'Methods' section and shown in parentheses is the 95% CL estimated based upon error propagation from the original tumor volume data.

1WA, one way analysis of variance; 2WA, two way analysis of variance; 5-FU, 5-fluorouracil; BW, body weight; CCI, Clark Combination Index; CL, confidence limits; NS, not significant.

*$P<0.05$ vs. controls (1WA with Tukey's pairwise where upper number is combination versus everolimus monotherapy and lower number is versus cytotoxic monotherapy. 2WA value shown is the significance of the interaction. Percent change in body weight is where the statistical significance of body weights was determined using paired *t*-tests.

Table 8 Summary of antitumor efficacy *in vivo* of everolimus combinations

		Tumor volumes			
Partner agent	Tumor model	1WA	2WA	CCI	Score
Cisplatin	KB-31	1, 1	0, 0	0, 0	1, 1
	Interaction		Additive		
	NCI H-596	1, 1	1,1	0, 1	2, 3
	Interaction		Synergy, dose-dependent		
Gemcitabine	KB-31	0, 0	1, 1	0, 0	1, 1
	Interaction		Additive, dose-dependent		
	NCI H-596	0	0	−1	−1
	Interaction		Weak antagonism		
Doxorubicin	KB-31	0, 1	1, 0	1, 1	2, 2
	Interaction		Synergistic		
	NCI H-596	0, 0	0, −1	0, 0	0, −1
	Interaction		Additive		
Paclitaxel	KB-31	0, 0	1, 1	1, 0	2, 1
	Interaction		Synergistic to additive		
	NCI H-596	−1, 0	−1, −1	−1, 0	−3, −1
	Interaction		Synergy or schedule-dependent antagonism		
Patupilone	KB-31	0	1	0	1
	Interaction		Additive, dose-dependent		
	HCT-116	0, 0	0, 0	0, 0	0, 0
			Indifferent		

Combination-interactions are summarized as follows. +1, combination has a significantly greater antitumor effect or larger body-weight than the single agent; −1, combination has significantly less antitumor effect or lower body-weight than the single agents; 0, combination is not significantly different from single agents. These integers are based upon (a) statistical significance of the combination versus the most active partner (1WA), (b) significant interaction based upon 2WA, (c) CCI estimation of the interaction. The first and second numbers of each pair correspond to the results from the first and second experiments. The overall score is a sum of each of the preceding numbers shown, where values ≥ 2 are considered synergistic, 0–1 additive, and <0 antagonistic. An overall score of '0' is considered to show weak additivity, that is, indifferent.

1WA, one-way analysis of variance; 2WA, two-way analysis of variance; CCI, Clark Combination Index.

Relationship of efficacy and tolerability in everolimus drug combinations *in vivo*: the ETQ

With respect to the tolerability of the everolimus combinations, in most cases BW losses occurring with combination treatment could be characterized as showing a weak but significant interaction, as the cytotoxic agent was the major contributor based on BW losses after monotherapy. Indeed, only in the case of combinations with gemcitabine could this BW loss be considered prohibitive (see Table 4).

To determine more objectively the effects of combination treatment in relation to tolerability, the ETQ was derived (see above, Table 9, and Supplemental material). With this quotient, lower numbers represent a better antitumor effect relative to the tolerability of the therapy. Accordingly, when comparing the ETQ values of combination therapies with their corresponding single-agent effects, a reduction of the ETQ (i.e. a negative delta value, see Table 9) would suggest an improvement in the antitumor effect relative to any changes in tolerability because of the combination. In the studies shown in Table 9, in which only concomitant treatments are summarized, the ETQ values generally are reduced in the combinations suggesting overall a real benefit of combined therapy, albeit sometimes a small effect. Increases in ETQ, when they do occur, are small, except for those studies using everolimus–patupilone combinations against HCT-116 tumors. In this particular model, in which, notably, tumor growth itself causes BW loss [23], the ETQ value of the combination is increased relative to everolimus, but is decreased relative to patupilone because of the added antitumor effects of everolimus.

Effect of administration schedule on the activity of everolimus combined with cisplatin or paclitaxel in the KB-31 model

For each cytotoxic, one experiment compared schedules administering everolimus either 24 h before, 24 h after, or concomitantly with the combination partner.

Table 9 Analysis of combination effects using the ETQ value, an indicator of antitumor activity and tolerability

		Experiment 1 delta ETQ			Experiment 2 delta ETQ	
Tumor/group	ETQ	Cytotoxic	Everolimus	ETQ	Cytotoxic	Everolimus
KB-31						
Everolimus	0.78			0.72		
Paclitaxel	0.63			0.67		
Combination	0.61	−0.02	−0.17	0.60	−0.07	−0.12
Cisplatin	0.96			0.85		
Combination	0.71	−0.25	−0.07	0.71	−0.14	−0.01
Gemcitabine	0.85			0.79		
Combination	0.64	−0.21	−0.14	0.73	−0.06	+0.01
Doxorubicin	0.64			0.58		
Combination	0.61	−0.03	−0.17	0.58	0.00	−0.14
H-596						
Everolimus	0.63			0.71		
Paclitaxel	0.61			0.71		
Combination	0.60	−0.01	−0.03	0.63	−0.08	−0.08
Cisplatin	0.86			0.72		
Combination	0.63	−0.23	0.00	0.61	−0.11	−0.1
Doxorubicin	0.77			0.84		
Combination	0.61	−0.16	−0.02	0.69	−0.15	−0.02
KB-31[a]						
Everolimus	0.78			0.78		
Patupilone	0.62			0.64		
Combination	0.61	−0.01	−0.17	0.62	−0.02	−0.16
HCT-116						
Everolimus	0.82			0.65		
Patupilone	1.78			1.68		
Combination	1.23	−0.55	+0.41	0.98	−0.7	+0.33

The ETQ is calculated as $ETQ = (T/C_{TVol} + 1)/(T/C_{BW} + 1)$, see 'Methods' section. Lower ETQ values indicate a higher ratio of antitumor effect as compared with tolerability. The delta ETQ are calculated by substracting the $ETQ_{monotherapy}$ from the $ETQ_{combination}$: thus a negative delta-ETQ indicates a favorable interaction compared with the respective monotherapy.
ETQ, Efficacy Tolerability Quotient.
[a]Experiment 1 used patupilone at 2 mg/kg and experiment 2 at 1 mg/kg.

Cisplatin

Cisplatin was used at 5 mg/kg/week and everolimus at 2.5 or 5 mg/kg/day. The CCI value indicated that for all of the groups with everolimus, additive effects were obtained. For treatments involving 2.5 mg/kg everolimus, all combination treatment groups at the endpoint (day 21) had significantly lower TVols than controls (Fig. 7), whereas neither single agents had significant antitumor activity. A 1WA indicated that at 2.5 mg/kg everolimus, only concomitant combination treatment produced antitumor effects that were significantly greater than single-agent therapy although the three different schedule combination groups were not significantly different from each other (1WA). At 5 mg/kg everolimus, no combination group produced significantly greater antitumor activity than either single agent. A 3WA indicated a significant interaction between everolimus and cisplatin ($P = 0.041$), but no influence of treatment order on the activity of this combination. Similarly, no significant interactions could be detected for tolerability (Fig. 7b). In conclusion, there was a minor improvement in efficacy without a decrease in tolerability for concomitant treatment of everolimus and cisplatin.

Paclitaxel

Paclitaxel was used at 10 mg/kg every 3 days and everolimus at 1, 2.5, or 5 mg/kg/day. Although concomitant treatment with the combination produced positive effects (see Fig. 3, panels e and f), a 24 h separation in the administration time of the compounds, in either order, produced a profound reduction in antitumor effect, which was indicative of antagonism. Of note is that the administration of everolimus after paclitaxel resulted in antagonism that was dose-independent, at least over the range tested, but the administration of everolimus before paclitaxel produced an antagonism of the paclitaxel effect that was independent of the everolimus dose. These schedule effects did not, however, affect the tolerability of the combination (Fig. 8).

Effect of administration schedule on the activity of everolimus combined with gemcitabine in the H-596 model

The effect of schedule on the combination of everolimus and gemcitabine was evaluated by administering everolimus doses of 0.5 or 1 mg/kg everolimus combined with gemcitabine (15 mg/kg) in a 3-day cycle (Fig. 9 with schedule details). There was no clear effect of schedule on the interaction of gemcitabine and everolimus as judged by a 2WA (comparing groups within a treatment order), except for the BW loss associated with gemcitabine preceding everolimus (interaction $P = 0.007$), although all of the treatments could be considered well tolerated. A 3WA did show an interaction of everolimus and gemcitabine ($P = 0.004$) but no interaction with order. The CCI values indicated either a synergistic promotion of BW loss or no interaction, with the largest CCI values being for gemcitabine preceding everolimus (Fig. 9, panel c). Furthermore, although everolimus and gemcitabine were active as antitumor agents, their combination produced CCI values indicative of weak antagonism. A 2WA of groups within a treatment order indicated no interaction and a 3WA failed to show an effect of treatment order on outcome and no interaction of treatments with order.

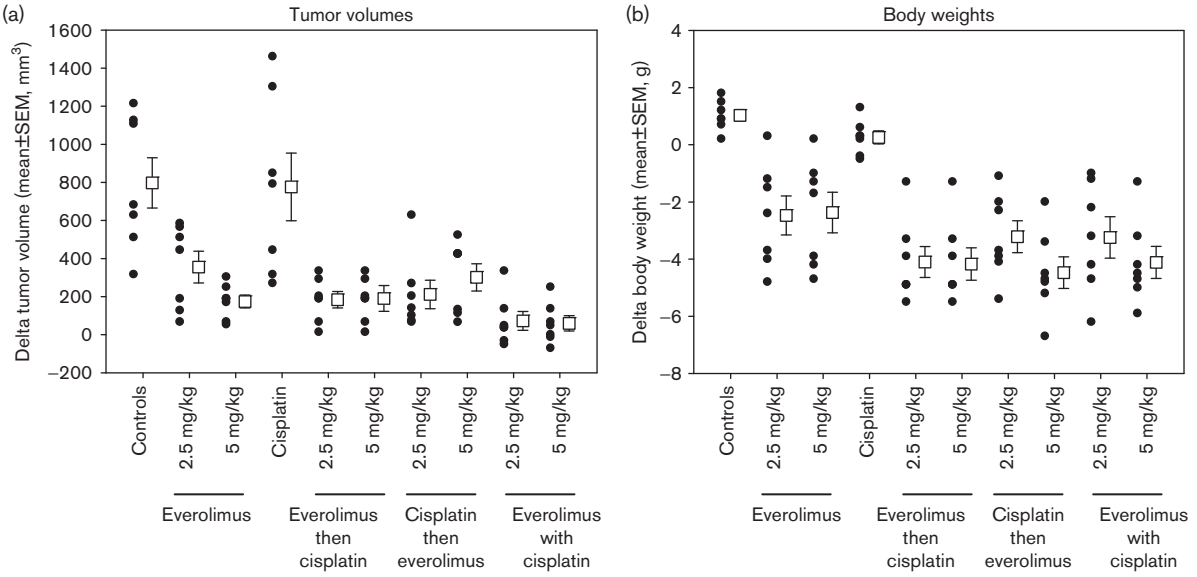
Everolimus maintenance treatment after combination with doxorubicin or paclitaxel in the KB-31 model

Cytotoxic antitumor therapy is normally given in cycles, with treatment pauses to allow the patient to recover from its deleterious effects. Everolimus, being of comparatively low toxicity compared with cytotoxic agents, may be useful in suppressing tumor outgrowth after cessation of cytotoxic treatment; a concept known as maintenance therapy. One experiment was done with each of doxorubicin and paclitaxel, in which the everolimus component of the combination therapy group was continued (2.5 mg/kg/day for 1 week) after cessation of the cytotoxic therapy and the effects compared with the cytotoxic monotherapy group. Vehicle controls and everolimus monotherapy groups were also included.

Doxorubicin

Doxorubicin (5 mg/kg/week for 2 weeks) induced regressions, but tumor regrowth began on day 24 after treatment

Fig. 7



Everolimus alone
Days 0 – 21
Cisplatin alone (5 mg/kg)
Days 0, 7, 14
Everolimus then cisplatin
Everolimus, days 0, 3 – 7, 10 – 14, 17–21; cisplatin, days 1, 8, 15
Cisplatin then everolimus
Cisplatin, days 0, 7, 14; everolimus, days 1 – 5, 8 – 12, 15 – 21
Everolimus with cisplatin
Everolimus, days 0–21; cisplatin, days 0, 7, 14

(c) Combination analyses

Everolimus (mg/kg)	Treatment order	Tumor volumes CCI	Tumor volumes 95% CL	Percentage delta body weights CCI	Percentage delta body weights 95% CL
2.5	Everolimus before cisplatin	−0.2	1.14	3.39	11.18
5	Everolimus before cisplatin	0.03	1.08	−3.48	11.7
2.5	Cisplatin before everolimus	−0.17	1.14	−2.52	7.6
5	Cisplatin before everolimus	0.16	1.08	−3.77	13
2.5	Everolimus with cisplatin	−0.34	1.14	−2.55	8.8
5	Everolimus with cisplatin	−0.14	1.08	−3.42	11.4

Effect of schedule on the antitumor activity of everolimus/cisplatin combinations against KB-31 tumors. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n = 7$ per group). After tumor transplantation, treatments were started when the tumors had reached approximately 100 mm^3 (day 7). The regimen included everolimus before, after, and concomitantly with everolimus, which was administered at 2.5 or 5 mg/kg orally and cisplatin at 5 mg/kg intravenously. On days where everolimus and cisplatin were both administered, everolimus was always used first at 2 h before cisplatin. Data shows individual tumor volumes and body weight and the associated mean ± standard error of the mean, from day 22, except controls which are from day 18, where $^*P < 0.05$ versus controls (1WA with Dunnett's test). CCI, Clark Combination Index; CL, confidence limits.

initiation or 9 days after the last administration (Fig. 10a). Everolimus combined with doxorubicin also produced tumor regressions, but continuing everolimus treatment from day 21 caused further regressions and prevented tumor regrowth. The TVols of the doxorubicin monotherapy group were significantly larger than those of the maintenance group ($P = 0.03$, t-test). Furthermore, by day 27, the number of tumor cures in the maintenance group was significantly greater than those in the doxorubicin alone group: eight out of eight versus three out of eight mice ($P = 0.026$ Fisher's exact test). BW loss was dominated by doxorubicin and not significantly affected by everolimus (Fig. 10b). There were no mortalities in any group.

Paclitaxel

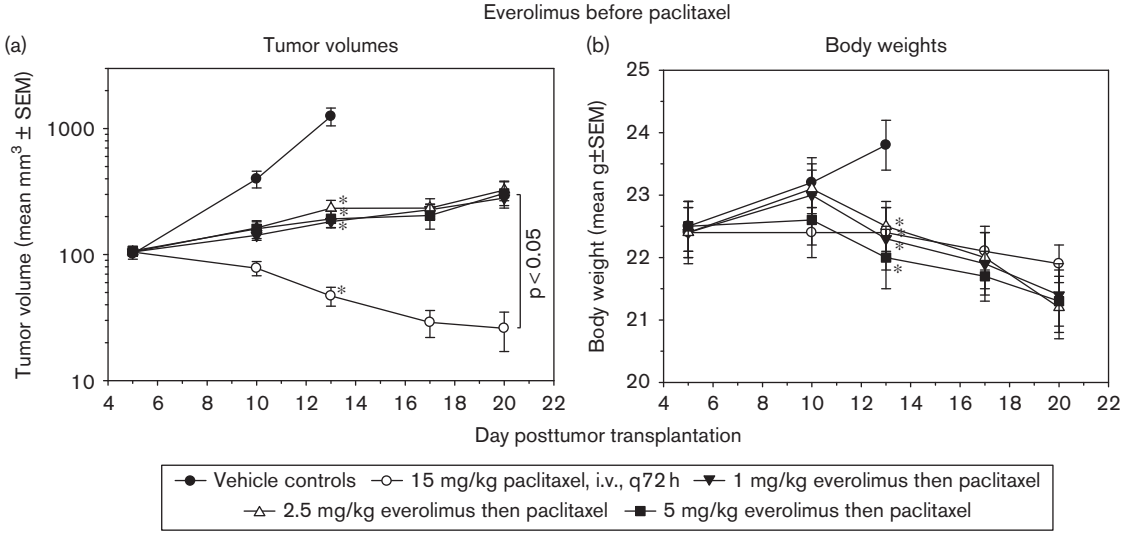
A similar pattern of events was observed when using paclitaxel (7.5 mg/kg 3 × per week for 2 weeks). Paclitaxel significantly inhibited tumor growth, but the tumors grew faster after ceasing paclitaxel treatment monotherapy compared with those receiving everolimus maintenance (Fig. 11a). Continuing everolimus treatment greatly slowed the growth rate of tumors after the cessation of the paclitaxel component of the combination therapy. BW loss was dominated by paclitaxel treatment, and the mice showed BW gain after cessation of paclitaxel treatment, which was not affected by everolimus maintenance (Fig. 11b). There were no mortalities in any group.

Overall, these results suggest that continuing the well-tolerated everolimus component of combinations may be useful in inhibiting the regrowth of tumors after the cessation of the cytotoxic compound.

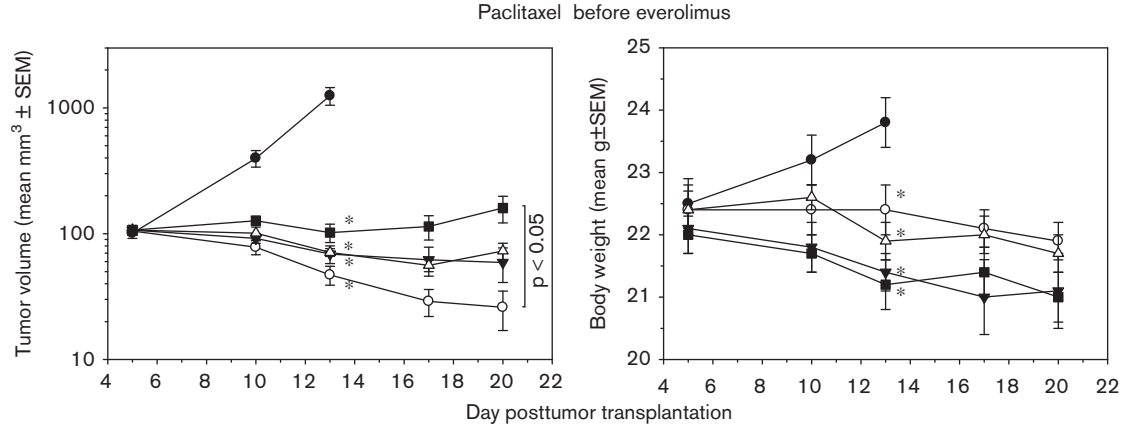
Discussion

Several reports have indicated potent combination effects between conventional cytotoxic agents and rapamycins in experimental settings. In this study, everolimus was

Fig. 8



Everolimus was administered p.o. on days 5, 8, 11, 14, 17, 20
Taxol was administered i.v. on days 6, 9, 12, 15, 18



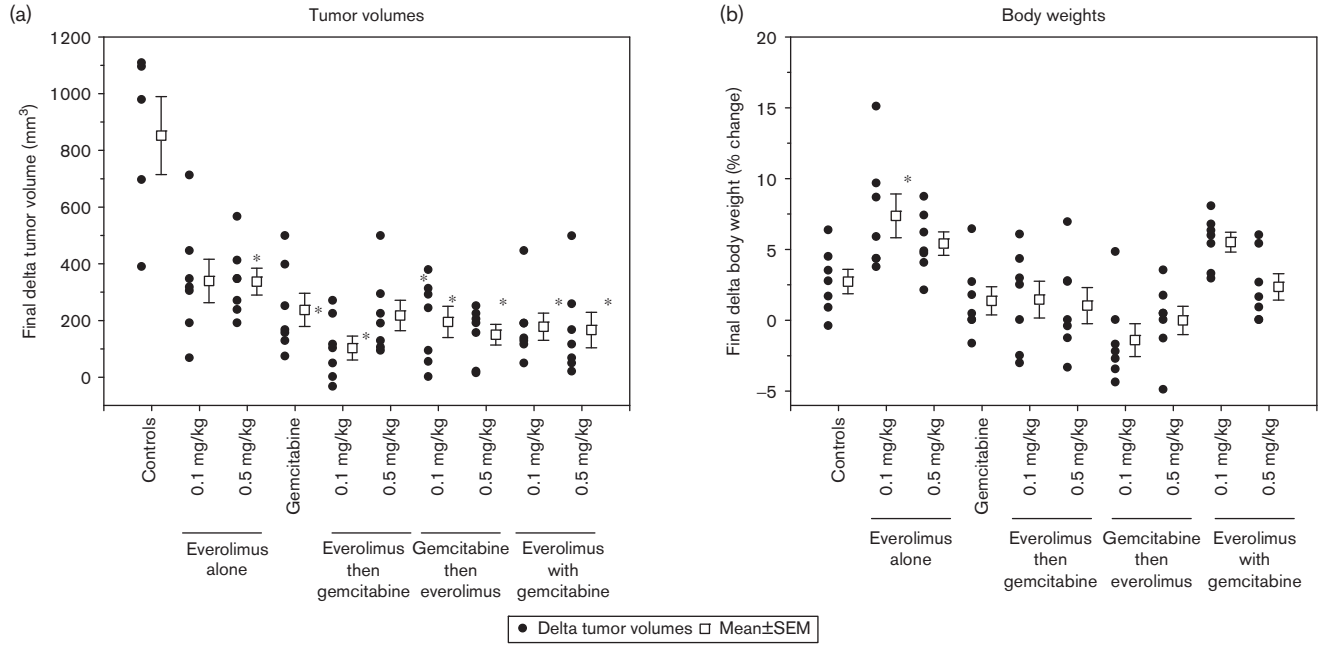
Taxol was administered i.v. on days 5, 8, 11, 14, 17, 20; everolimus was administered p.o. on days 6, 9, 12, 15, 18.

(c) Combination analyses

Everolimus (mg/kg)	Treatment order	Tumor volumes CCI	Tumor volumes 95% CL	Percentage delta body weights CCI	Percentage delta body weights 95% CL
1	Everolimus before paclitaxel	0.09	0.0027	–0.12	0.037
2.5		0.13	0.012	0.02	0.024
5		0.09	0.0054	–0.42	0.16
1	Paclitaxel before everolimus	–0.01	0.0018	–0.62	0.32
2.5		–0.02	0.0027	–0.4	0.18
5		0.01	0.0021	–0.64	0.37

Influence of administration order on the efficacy of everolimus/paclitaxel combinations against KB-31 tumors. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n=8$ per group). Treatments were started when the tumors reached approximately 100 mm^3 (day 5) after tumor transplantation. Everolimus was administered orally once per day at 1, 2.5, or 5 mg/kg and paclitaxel was administered intravenously at 15 mg/kg, each on a q72 h regimen. In the regimen where everolimus was before paclitaxel, everolimus was administered on days 5, 8, 11, 14, 17, and 20, and paclitaxel administered on days 6, 9, 12, 15, and 18. For the regimen where paclitaxel was first, the order of compound administration was reversed. Data presented are mean ± standard error of the mean, where $*P<0.05$ versus controls (1WA with Dunnett's test).

Fig. 9



Monotherapy:

Everolimus or gemcitabine alone, days 0, 3, 6, 9, 12, 15, 18, 21

Everolimus then gemcitabine:

Everolimus, days 0, 3, 6, 9, 12, 15, 18, 21; gemcitabine, days 1, 4, 7, 10, 13, 16, 19, 22

Gemcitabine then everolimus:

Gemcitabine, days 0, 3, 6, 9, 12, 15, 18, 21; everolimus, days 1, 4, 7, 10, 13, 16, 19, 22

Everolimus with gemcitabine:

Days 0, 3, 6, 9, 12, 15, 18, 21

(c) Combination analyses

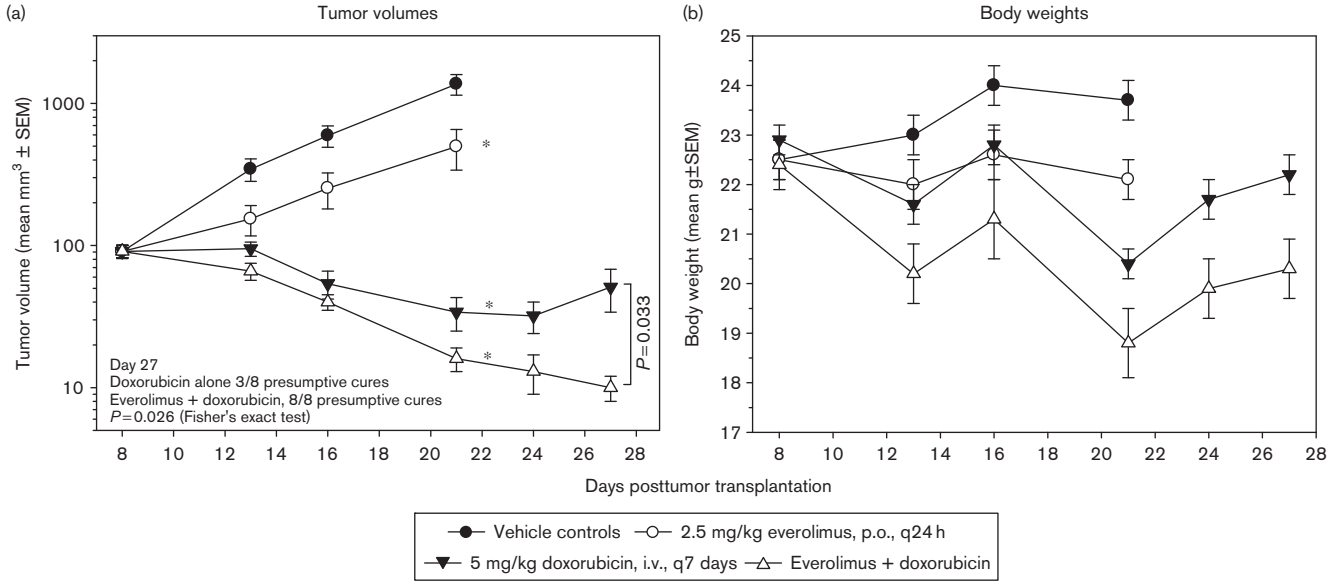
Everolimus (mg/kg)	Treatment order	Tumor volumes CCI	Tumor volumes 95% CL	Percentage delta body weights CCI	Percentage delta body weights 95% CL
0.5	Everolimus before gemcitabine	0.01	0.09	−0.82	0.48
1	Everolimus before gemcitabine	0.15	0.06	−0.62	0.4
0.5	Gemcitabine before everolimus	0.12	0.079	−0.87	0.21
1	Gemcitabine before everolimus	0.07	0.08	−1.0	0.65
0.5	Everolimus with gemcitabine	0.1	0.08	0.66	4.1
1	Everolimus with gemcitabine	0.09	0.07	−0.14	0.92

Effect of schedule on the antitumor activity of everolimus plus gemcitabine against NCI H-596 tumors. Fragments of NCI-H596 or A-549 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse (H-596, $n=8$ per group; A-549, $n=3$ to 4 per group). After tumor transplantation, treatments were started when the tumors had reached approximately 100 mm^3 (H-596, 7 days and A-549, 17 days). The regimen comprised everolimus and gemcitabine administration once in a 3-day cycle, with everolimus preceding, or following, gemcitabine by 1 day, or administered together with a 2-day pause. Everolimus was administered orally, and gemcitabine intraperitoneally at the indicated doses and regimens. Data presented are mean ± standard error of the mean, where $*P<0.05$ versus controls (1WA with Dunnett's test) after 4 weeks treatment.

shown to interact positively with cisplatin, paclitaxel, gemcitabine, and doxorubicin *in vitro*. The combinations produced additive to strongly synergistic interactions *in vitro* against tumor cell lines considered both sensitive (A-549) and insensitive (KB-31, HCT-116) to everolimus, and also displaying various sensitivities to the cytotoxic agents used. Importantly, no case of in-vitro antagonism was observed. However, once tested in mouse models *in vivo*, these promising effects *in vitro* were less readily observed.

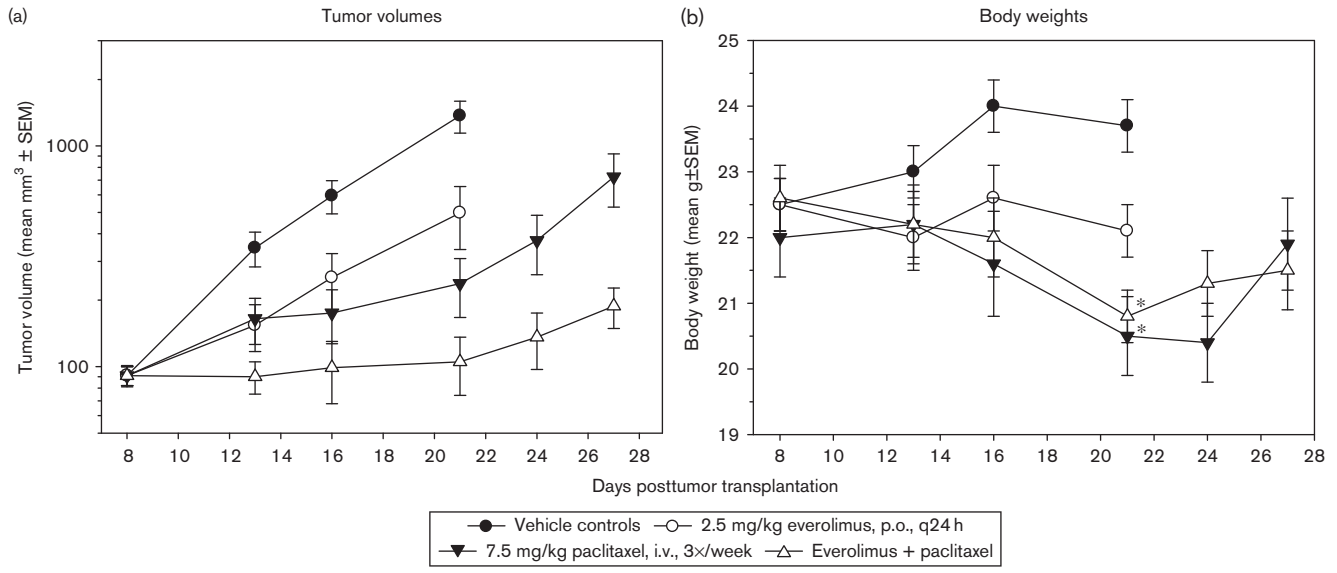
Monotherapy with everolimus administered orally, once per day at 2.5 mg/kg (< 5% of the > 60 mg/kg MTD), was effective in treating experimental human tumors growing as s.c. xenografts in immunosuppressed athymic nude mice, confirming earlier data [5,21,22,26]. This dose was chosen as optimal because it was effective against many tumor types and, in general, increasing the dose accrued little additional antitumor activity. The exception was for treating the HCT-116 model in which a dose of 10 mg/kg was used owing to the high-level insensitivity of HCT-116 cells to this compound *in vitro* [29]. The antitumor activity of everolimus monotherapy was one of the inhibition of tumor growth, rather than of inducing tumor regressions, and was well tolerated, with stable BWs occurring under treatment. Similar to the dose–responsiveness for the antitumor effect, dose–responsive inhibition of the target *in vivo* also occurred, as shown by the everolimus-dependent reduction of pS6 levels, a downstream effector of mTOR. The ED_{50} values for target inhibition *in vivo* were slightly higher than ED_{50} for tumor growth impairment, and may suggest that some accumulation of everolimus within the tumor is needed before effective inhibition of mTOR activity.

Fig. 10



Prolongation of everolimus treatment impairs regrowth of KB-31 tumors after doxorubicin/everolimus combination therapy. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n=8$ per group). Treatments were started when the tumors reached approximately 100 mm^3 (day 10) after tumor transplantation. Everolimus was administered orally once per day at 2.5 mg/kg from day 8 to day 21 or 27. Doxorubicin was administered intravenously at 5 mg/kg once per week. Data presented are mean ± standard error of the mean, where $*P<0.05$ versus controls (1WA with Dunnett's test). The comparison of doxorubicin to everolimus plus doxorubicin on day 27 used the *t*-test. The significance of treatment differences in the proportion of presumptive tumor cures was determined using the Fisher's exact test.

Fig. 11



Prolongation of everolimus treatment impairs regrowth of KB-31 tumors after paclitaxel/everolimus combination therapy. Fragments of KB-31 tumors (approximately 25 mg) were transplanted subcutaneously into the left flank of each female nude mouse ($n=8$ per group). Treatments were started when the tumors reached approximately 100 mm^3 (day 8) after tumor transplantation. Everolimus was administered orally, once per day at 2.5 mg/kg from day 8 to day 21 or 27. Paclitaxel was administered intravenously at 7.5 mg/kg 3 days per week with no further treatments after day 21. Data presented are mean ± standard error of the mean, where $*P<0.05$ versus controls (1WA with Dunnett's test). The comparison of paclitaxel to everolimus plus paclitaxel groups on day 27 used a two-tailed *t*-test.

For in-vivo cancer models, in which drug activity and drug interactions are subject to the influences of metabolism and clearance and possibly a modified target cell, the interaction of everolimus with cytotoxic agents seemed less clear than with the in-vitro systems. The poor correlation of in-vitro and in-vivo combination effects involving rapamycins has been shown earlier. In SKOV-3 cells, cisplatin-induced inhibition of in-vitro proliferation (as judged by an MTT assay) was only modestly enhanced by everolimus, although a clonogenic survival assay showed much greater combination effects. However, the combination showed dramatic effects *in vivo*, inhibiting the growth of SKOV-3 cells in the peritoneum or as s.c. xenografts [10]. In this study, less-than-optimal doses of paclitaxel, cisplatin, gemcitabine doxorubicin, and patupilone were used to attempt to reveal combination effects that may be masked by a maximal antitumor effect attained by optimal doses of one or the other partner compound. The exception was 5-FU, which was used at a near maximal dose given the relative insensitivity of HCT-116 tumors. Furthermore, earlier results had shown that the addition of supraoptimal everolimus doses to optimal doses of cytotoxic agents could increase the toxicity of the latter but in a manner that would be everolimus dose-dependent (data not shown). At doses of 50–80% of the optimum, cisplatin, doxorubicin, and paclitaxel all showed an increase in antitumor activity when individually combined with everolimus, albeit minor in some cases, with an acceptable decrease in tolerability to treatment as judged by BW losses. Although gemcitabine–everolimus combinations showed a marked increase in antitumor effect, the tolerability of this combination was poor. A similar pattern was seen with patupilone–everolimus combinations, although this combination was better tolerated than the one of gemcitabine–everolimus. Combinations with 5-FU at 100% of the optimal dose were not accompanied by an increased antitumor effect. In contrast, Wagner *et al.* [30] showed that combinations of rapamycin and 5-FU (or oxaliplatin) produced increased antitumor effects (without diminished tolerability) against peritoneal CT26 and SW620 tumors.

The different analytical methods that we have used gave rise to different indications of the extent of cytotoxic drug interaction with everolimus. Nevertheless, it is possible to conclude that with the exception of the combination with 5-FU, the concomitant combination of everolimus with five different cytotoxic agents tested could be considered at least additive in nature. Unfortunately, two of these everolimus combinations (gemcitabine and patupilone) also caused at least additive increases in BW loss. Thus, any gain in antitumor effect afforded by a drug combination must be judged with respect to the tolerability of the combination. Use of the 'efficacy–tolerability quotient' showed that with most of the combinations involving everolimus, the ETQ was reduced or stable with respect to the ETQ values of the monotherapies, indicating that the additive or synergistic increases in antitumor effects occurred without a corresponding effect on BW losses. In general, BW losses occurring with combination treatment could be characterized as weak but significant, with most of the BW loss contributed from the cytotoxic agent. However, the ETQ also needs to be considered in light of absolute BW loss. For example, despite the favorable change in ETQ values in combinations involving gemcitabine, the absolute BW losses warrant careful consideration of this combination clinically. Combination of everolimus and patupilone against HCT-116 tumors showed a lower ETQ compared with patupilone monotherapy, but an increased value compared with everolimus monotherapy, which was not observed with the KB-31 experiment. This may reflect the experimental differences (high dose of everolimus used against HCT-116 tumors, known to be toxic to the host), and should not detract interest in this combination. Despite these exceptions, the ETQ values generally are reduced in the combinations suggesting a benefit of combined therapy, albeit sometimes a small one.

Different treatment regimens were also used to evaluate the possible effects of schedule on the activity of everolimus. Although schedule had little effect on the tolerability or efficacy of cisplatin or gemcitabine combinations, schedule may play a role in the toxicity of 5-FU in combination with everolimus, as administering everolimus before 5-FU seemed to increase toxicity compared with concomitant therapy or administering everolimus after 5-FU. In contrast, antitumor efficacy was unaffected by schedule in this combination. Interestingly, clinical data showed that the rapalog CCI-779 (temsirolimus) was shown to have poor tolerability when combined with 5-FU in advanced cancer patients (including treatment-related deaths) [31]. For paclitaxel, there was a striking effect of schedule when combined with everolimus. Concomitant administration of everolimus yielded either a weakly additive interaction or only a mild antagonism of paclitaxel activity; but the administration of everolimus either 24 h before or 24 h after a paclitaxel administration produced a dose-dependent antagonism of paclitaxel activity against KB-31 tumors.

Reconciling the in-vitro combination effects with those seen *in vivo* is difficult but important. Particularly in the case of KB-31 and HCT-116 tumors, which are 'everolimus-insensitive', the penetration of everolimus into s.c. tumor xenografts in mice maybe insufficient in extent (C_{max}) and duration (time above the IC_{50}) to exert the same direct antitumor cell activity afforded by continuous exposure *in vitro* [23]. Therefore, any direct activity of everolimus against the tumor cell maybe diminished *in vivo* and thus account, at least in part for the less

synergistic responses predicted by in-vitro tests. In contrast, everolimus [5] and, perhaps, the cytotoxic agents can also display antivascular activities, which should therefore increase activity *in vivo*. This suggests that certain survival mechanisms may operate *in vivo* reducing the effectiveness of the particular combinations investigated here.

Creative use of everolimus in combination with other agents may facilitate improved antitumor outcome and one example of this is the so-called maintenance therapy. In the case of paclitaxel and doxorubicin, everolimus treatment was continued in the combination groups after the cytotoxic agent was stopped to determine if everolimus can suppress the outgrowth of tumors that had responded to the cytotoxics agents. In both cases, continuing everolimus treatment after ceasing the cytotoxic, inhibited tumor outgrowth. These results may indicate a new therapeutic modality for everolimus in the clinic, although further studies of this concept are required; for example, examination of a second round of combined chemotherapy and evaluation of the overall tolerability of this treatment regimen.

In conclusion, everolimus showed positive interactions with cytotoxic agents *in vitro*, which were not so readily produced *in vivo*, although in nearly all cases tumor cell kill was increased without markedly increasing toxicity. Scheduling of the different regimens *in vivo* was shown to play a role in some combinations, and, in particular maintenance therapy seemed to be a promising approach. In general, these data support the clinical investigation of combinations of everolimus with cytotoxics in various different cancer indications. Although true synergy may not be seen, our results suggest that increased antitumor effects can be achieved at doses that remain tolerable.

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