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ORIGINAL ARTICLE

Everolimus and PTK/ZK show synergistic growth inhibition in the orthotopic BL16/BL6 murine melanoma model

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Abstract
Purpose Everolimus (RAD001, Afinitor) is an mTORC1 pathway inhibitor, and vatalanib (PTK/ZK) is a pan VEGF-R tyrosine kinase inhibitor (TKI). These two drugs have been shown to have overlapping but also distinct anti-angiogenic effects. Consequently, we investigated the pharmacokinetics (PK) and pharmacodynamics (PD) of their combination in vivo.
Methods Murine melanoma B16/BL6 cells were grown orthotopically in BL6/C57 mice by injection into the derma of both ears to create a primary tumour which metastasized rapidly to the cervical lymph nodes. Mice were treated daily p.o. with PTK/ZK (100 mg/kg) or everolimus (1 mg/kg) or their combination, and anti-tumour efficacy (PD) assessed. In the same model, plasma PK of everolimus was measured following single doses of the monotherapy or combination schedules.
Results Two independent experiments showed that combination of everolimus and PTK/ZK caused at least additive increases in anti-tumour activity compared to either monotherapy, without increases in toxicity. Pooling the data to improve the statistical power demonstrated the interactions to be synergistic. PK modelling showed that although PTK/ZK increased everolimus plasma concentrations by about twofold, this PK drug–drug interaction could not account for the increased anti-tumour effect of the combination. Modelling of the PTK/ZK dose–response curve in this model suggested that any effect of everolimus on the PK of PTK/ZK was unlikely to affect efficacy. Measurement of changes in tumour and plasma VEGF levels at the endpoint of therapy confirmed earlier observations of differential effects of these two agents.
Conclusions The combination of everolimus and PTK/ZK hold promise for the treatment of human cancers.

Keywords Everolimus · PTK/ZK · PK-PD · VEGF-R TKI · mTOR · Anti-tumour activity · Angiogenesis

Abbreviations

bFGF	Basic fibroblast growth factor
LN	Lymph node
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor

Introduction

Everolimus (RAD001, Afinitor) is an orally active inhibitor of mTORC1, a multi-functional signal transducing protein implicated in cancer [1]. Everolimus possesses both anti-tumour cell and anti-angiogenic/anti-vascular activities [2–5]. Previously, it was shown that the anti-angiogenic/anti-vascular activities afforded by mTORC1 inhibition by everolimus are partly overlapping but also distinct from those following VEGF-R inhibition by the tyrosine kinase inhibitor (TKI) PTK/ZK [5, 6]. The purpose of the present study was to evaluate whether the combination of these two compounds would result in a greater anti-tumour effect. To evaluate this, the syngeneic orthotopic murine melanoma model B16/BL6 was selected. This model forms metastases to the regional lymph nodes, has highly vascularized tumours [5, 7–9] and is categorized as sensitive to everolimus in vitro and in vivo and to PTK/ZK in vivo only [5, 7,

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9, 10]. Anti-tumour efficacy, compound pharmacokinetics, histology and VEGF levels were used to characterize the anti-tumour effects of each compound, and their combination.

Materials and methods

Compounds, cells and animals

Everolimus was synthesized at Novartis Institutes for Biomedical Research (NIBR) and PTK/ZK at Schering AG, Berlin. For all in vivo experiments, a microemulsion of everolimus was freshly diluted in a vehicle of 5% glucose at an administration volume of 10 ml/kg. PTK/ZK was administered orally (p.o.) in a vehicle of 50% PEG-300 to 50% normal saline in a volume of 5 ml/kg. Formulated substances were prepared each day just prior to administration to mice and the administration volume individually adjusted based upon animal body weight.

The murine melanoma cell line B16/BL6 was obtained from Dr IJ Fidler (MD Anderson Cancer Center, Dallas, Texas), and the cells were cultured in vitro as previously described [7]. Healthy pathogen-free female C57BL/6 mice (20 g) were obtained from Iffa Crédo animal breeding facility (L'Arbresle, France). Isoflurane was purchased from Abbott AG, CH. All experimental approaches involving animals were approved by the local Veterinary Department, and all animals had free access to food and water at all times.

Murine B16/BL6 orthotopic melanoma model

This model was first described in 1984 [11] and subsequently has been used for investigations of anti-angiogenic agents both as monotherapy and in combination [5, 7–10]. Melanoma cells in the primary (ear) tumour metastasize to local lymph nodes, primarily in the neck (cervical region) and after 21 days 99% of these cervical LNs are black melanoma cells [11]. B16/BL6 cells (5×10^4 cells/μl) were injected intradermally (i.d.) into the dorsal pinna of both ears of syngeneic C57BL/6 mice under isoflurane anaesthesia. Tumour size was quantified by (a) measurements of primary tumour area (mm^2) using a microscope with computer-assisted image analysis software and (b) weighing the cervical LN at the endpoint. One week after cell inoculation, mice were treated daily for two further weeks with vehicle or everolimus (1 mg/kg p.o.) or PTK/ZK (100 mg/kg p.o.) or both drugs. Dose–response curves for everolimus (range 0.1–10 mg/kg) and PTK/ZK (range 20–100 mg/kg) were determined using once daily p.o. administration. Pharmacokinetic studies used a single dose of everolimus (1 mg/kg) alone or in combination with PTK/ZK (100 mg/kg), or PTK/ZK at 10, 50, 100 or 200 mg/kg. At the end of the experiments, the animals were killed by exposure to an atmosphere highly enriched with CO_2, a blood sample taken and the cervical LN removed and weighed.

Determination of everolimus drug levels in plasma

Everolimus levels in plasma were determined as previously described, see supplemental material of O'Reilly et al. [12].

Tumour histology

Endothelium of perfused vessels in the tumours was visualized using nuclear staining Hoechst-33342 dye and staining of tumours for CD31 and smooth muscle actin (SMA) were performed as previously described [5]. The primary antibodies were rat anti-mouse CD31 or mouse anti-mouse smooth muscle actin-FITC conjugated.

Measurement of VEGF

VEGF was measured by ELISA as described previously [5, 9]. Mouse VEGF ELISAs were obtained from R&D Systems (London, UK).

Statistical and other analyses

Results are presented as mean ± one standard error of the mean (SEM). The experiments were analysed separately (each $n = 6$) and as a pooled dataset ($n = 12$). A one-way ANOVA (OWA) was used with Dunnett or Tukey tests or with two-way ANOVA (TWA) employing Tukey tests for post hoc comparisons. Where the data was not normally distributed, the data was normalized by taking Log_{10} prior to statistical analyses. For all tests, the level of significance was set at $P < 0.05$ (two-tailed). Statistical calculations were performed using SigmaStat 2.0 (Jandel Scientific). Curve-fitting (linear and non-linear) was performed using GraphPad 3.0. Pharmacokinetic modelling used the WinNonLin program (Mountain View, CA, USA). Based upon constants estimated from non-compartmental analysis of pharmacokinetic data, a series of plasma pharmacokinetic models were assessed for fit to the data using AIC and correlation co-efficients as indices of fit.

Efficacy at the endpoint was summarized as the treatment/control ratio (T/C), calculated as the mean change in tumour index of the treated group divided by the mean change in tumour index of the control group, which was determined for the primary (ear) tumour using tumour area as the index and for cervical lymph nodes using weight as the index. Our unpublished data shows that LN weight strongly correlates with the number of viable tumour cells.

The interaction of the two drugs in combination was also assessed by the method described by Clarke [13] to provide

the Clark combination index (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 [14].

Results

Effects of everolimus/PTK/ZK combinations on tumour growth in B16/BL6 melanoma

In two independent experiments, the effects of 14-day treatment with a near optimal dose of PTK/ZK (100 mg/(kg day) or a sub-effective dose of everolimus alone and in combination was assessed in the orthotopic B16/BL6 melanoma model (Fig. 1; Table 1).

In the first experiment, treatment of tumour-bearing mice with PTK/ZK or everolimus reduced the growth of the primary tumour: $T/C = 0.77$ and 0.70, respectively (Fig. 1a, b), but in combination, the effects on tumour growth were more profound than could be achieved by either agent given alone: $T/C = 0.29$ ($P < 0.05$ versus vehicle controls and single agents). TWA demonstrated a significant effect of PTK/ZK ($P = 0.031$) and everolimus ($P = 0.001$), although no significant interaction (indicating an additive effect) between the two drugs ($P = 0.51$). The CCI (±95% CL) indicated an additive-synergistic response against the primary tumour (CCI: -0.25 ± 0.5). An equivalent interaction was observed with regard to cervical LN metastases (Fig. 1b). Here, PTK/ZK ($T/C = 0.42$) and everolimus ($T/C = 0.75$) significantly

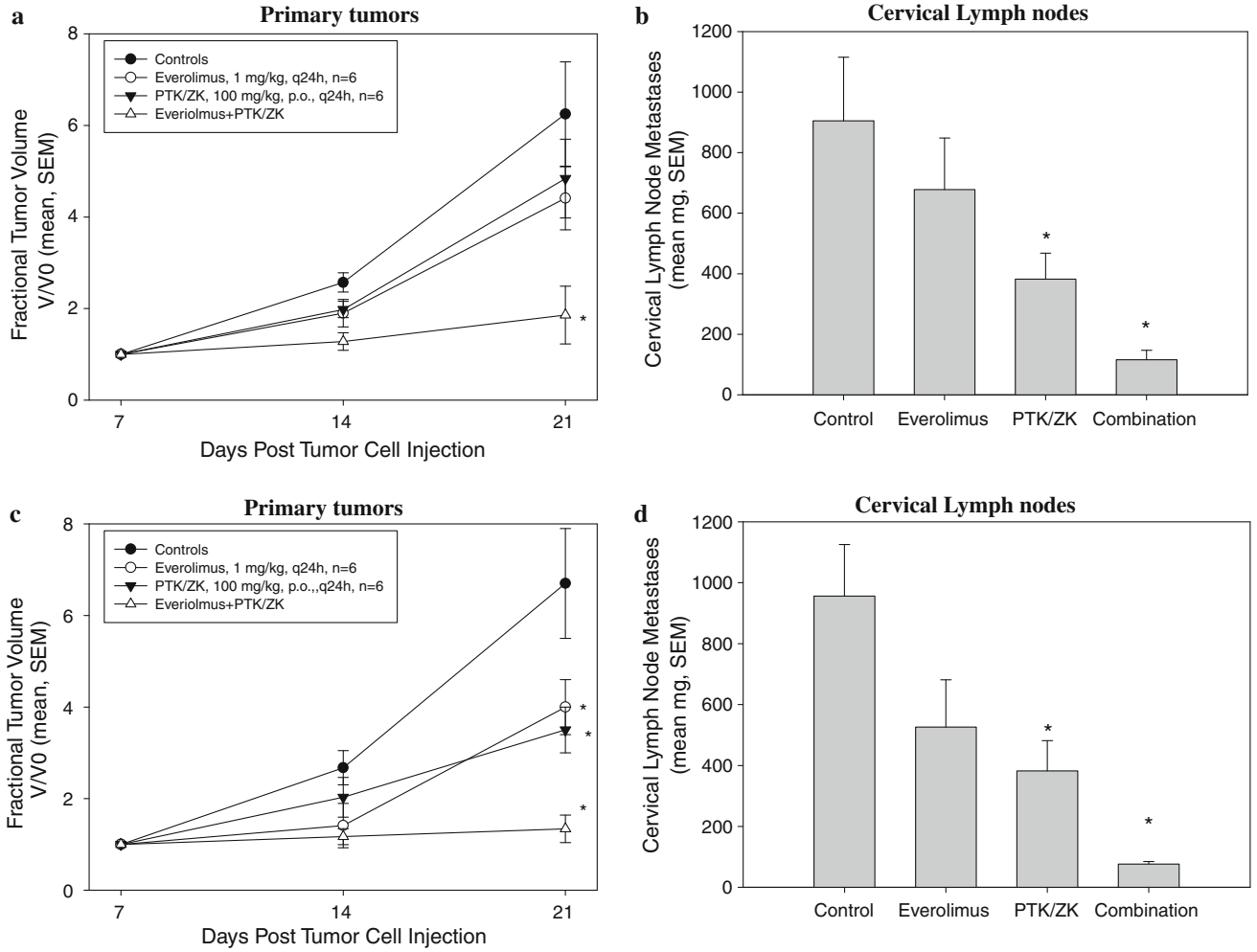


Fig. 1 Anti-tumour effects of everolimus and PTK/ZK, alone and in combination, against orthotopic murine B16/BL6 melanoma. B16/BL6 cells (5×10^4) were injected intradermally in the ears of C57BL/6 mice. Treatment with everolimus, PTK/ZK or a combination of the two was initiated 7 days after cell inoculation and continued until day 21. The animals were killed on day 21, and the cervical lymph nodes collected and weighed. Values are mean ± SEM; ($n = 6$ per experiment, 1–2 experiments). *$P < 0.05$, significant inhibition compared to control (OWA). **a** and **b** are from the first experiment, and **c** and **d** are from the second. In both experiments, in the vehicle-treated mice, the mean lymph node weight on day 21 was 4–5% of the mouse body weight; none of the drug treatments had a significant effect on body weight compared to vehicle

Table 1 Anti-tumour effects of everolimus, PTK/ZK and the combination in the B16/BL6 murine melanoma model

Compound	Primary tumour response			LN metastases response		
	Δ TV (V/V_0)	*T/C*	CCI	Δ TV (mg)	*T/C*	CCI
First experiment ($n = 6$)						
Vehicle controls	6.2 ± 1.1	1.00	*−0.25 (0.5)*	905 ± 210	1.00	*−0.19 (0.2)*
Everolimus	4.8 ± 0.9*	0.77		678 ± 170*	0.75	
PTK/ZK	4.4 ± 0.7*	0.70		382 ± 86*	0.42	
Everolimus plus PTK/ZK	1.8 ± 0.6*,#	0.29		116 ± 31*	0.13	
Second experiment ($n = 6$)						
Vehicle controls	6.7 ± 1.2	1.00	*−0.11 (0.3)*	956 ± 169	1.00	*−0.14 (0.3)*
Everolimus	4.0 ± 0.6*	0.59		526 ± 155*	0.54	
PTK/ZK	3.5 ± 0.5*	0.52		382 ± 99*	0.4	
Everolimus plus PTK/ZK	1.3 ± 0.3*,#	0.2		76 ± 9*	0.08	
Pooled experiments ($n = 12$)						
Vehicle controls	6.5 ± 0.8	1.00	*−0.17 (0.04)*	930 ± 128	1.00	*−0.17 (0.02)*
Everolimus	4.2 ± 0.5*	0.65		530 ± 104*	0.57	
PTK/ZK	4.2 ± 0.4*	0.65		454 ± 87*	0.49	
Everolimus plus PTK/ZK	1.6 ± 0.3*,†	0.25		96 ± 17*,‡	0.1	

C57/BL6 mice bearing B16/BL6 tumours were treated with everolimus (1 mg/kg, p.o.) or 100 mg/kg PTK/ZK. After 14 days of treatment, some of the animals were killed and the LN excised and weighed, results show mean ± SEM. The *CCI* was calculated as described in "Methods" and shows the 95% CL in parentheses

* $P < 0.05$ versus controls (OWA with Tukey), # $P > 0.05$ demonstrating no interaction (hence an additive effect) and † $P = 0.011$ ‡ $P = 0.015$ demonstrating a significant interaction (TWA)

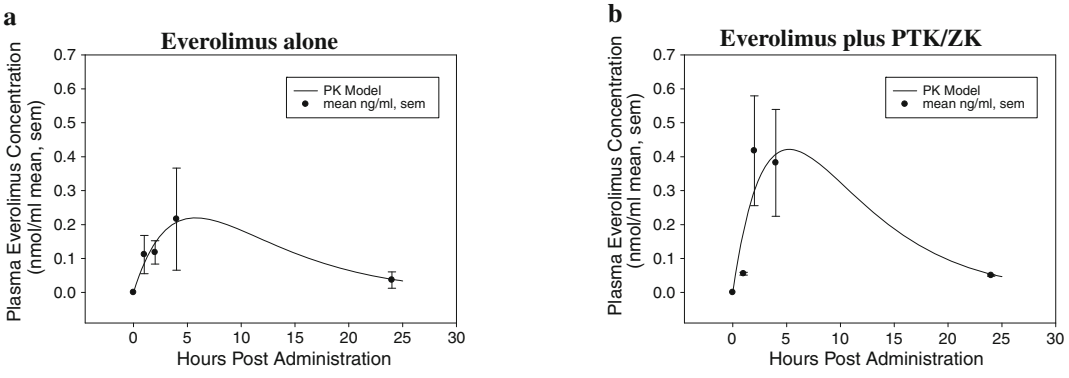
reduced LN weights and the combination further reduced LN weight ($T/C = 0.13$), although this did not reach statistically superiority to PTK/ZK alone ($P > 0.05$) (Table 1). TWA demonstrated a significant effect of PTK/ZK ($P = 0.007$) and everolimus ($P < 0.001$), and a strong trend towards a significant interaction between the two drugs ($P = 0.079$). The CCI indicated an additive-synergistic effect of the combination against LN metastases (CCI: −0.19 ± 0.2). The combination regimen was also effective in reducing the incidence of lung metastases, but not to a greater degree than either agent alone (Controls, 4/6; PTK787-treated, 1/6, everolimus, 2/6 and combination, 1/6 mice positive for lung metastases); however, likely owing to the small number of subjects, this did not reach statistical significance compared with controls (Chi-square test). The second experiment demonstrated similar results (Fig. 1c, d; Table 1), with the CCI again indicating an at least additive response against both primary (−0.11 ± 0.3) and LN metastases (−0.14 ± 0.3).

Pooling the data to provide greater statistical power ($n = 12$) demonstrated a statistically significant interaction by TWA of everolimus and PTK/ZK against both primary ear tumours ($P = 0.011$) and LN metastases ($P = 0.015$) which was confirmed by the CCI values which indicated synergy: primary ear tumours (−0.17 ± 0.04) LN metastases (−0.17 ± 0.02), see Table 1.

Importantly, all treatments, including the combination, were well tolerated and significantly promoted body weight gains (everolimus: 11 ± 2.7%; PTK/ZK: 8.6 ± 1.2%; combination: 5.4 ± 1.6%) when compared to controls (−0.5 ± 1.4%, $P > 0.05$), with no statistically significant differences between the active treatment groups (data from experiment 1).

Compound plasma pharmacokinetics and influence on efficacy

The pharmacokinetic profile of a single p.o. administration of 1 mg/kg everolimus in the absence (panel a) or the presence (panel b) of 100 mg/kg PTK/ZK is presented in Fig. 2, along with data from non-compartmental and 1st-order elimination pharmacokinetic models. There was a significant drug interaction characterized by PTK/ZK increasing the everolimus C_{max} (51%), lowering the T_{max} and increasing the C_{last} (50%) and AUC (76%), while decreasing the $t_{1/2}$ (13%). Thus, PTK/ZK effectively increased everolimus exposure given at a dose of 1 mg/kg to levels similar to that which would be obtained with a dose of approximately 1.8 mg/kg (based upon a 1st-order model). Further evaluation was made to determine whether this alteration alone was sufficient to account for the increased anti-tumour effects of the combination treatment. Based upon the dose–response curve of everolimus (Fig. 3) for either the primary (panel a) or lymph node metastases (panel b), increasing the dose of everolimus from 1 to 1.8 mg/kg would result in a small decrease in *T/C* values



Non-compartmental analysis

Tmax h	4	Tmax h	2-4
Cmax nmol/ml	0.22	Cmax nmol/ml	0.42
Clast nmol/h	0.04	Clast nmol/h	0.05
AUClast nmol*h/ml	3.0	AUClast nmol*h/ml	5.4
t1/2 h	7.8	t1/2 h	6.8

First Order Model

Volume/F	1.737	CL/F	0.3	Volume/F	0.864	CL/F	0.17
K01	0.172	Tmax	5.8	K01	0.1811	Tmax	5.2
K01	0.172	Cmax	0.22	K10	0.201	Cmax	0.42
		AUC	3.4			AUC	6.0
		Correlation	0.95			Correlation	0.87
		AIC	35			AIC	47

Fig. 2 Pharmacokinetics of everolimus alone (**a**) and in combination with PTK/ZK (**b**). Everolimus (1 mg/kg, p.o.) alone or with PTK/ZK (100 mg/kg) were administered p.o. once to C57BL/6 mice. At the indicated times, mice were killed, and the level of everolimus in blood determined by HPLC/MS. Pharmacokinetic modelling was performed using WinNonLin

from 0.67 to 0.52 for primary tumours and *T*/*C* of 0.55 to 0.47 for metastases. This represents an increase the anti-tumour effect of just 15–22%. However, the actual effect of the combination of everolimus and PTK/ZK markedly increased the anti-tumour effect by 62% and 70% for the primary tumours and LN metastases, respectively, in the first experiment and 82% and 85% for the second experiment. These analyses indicate that the increased exposure of everolimus by PTK/ZK is not sufficient to account for the increased activity of the combination.

Figure 4 shows the dose–response of PTK/ZK when compared with the activity of everolimus alone or in combination with 100 mg/kg PTK/ZK for primary (panel a) and LN metastases (panel b). The data was modelled, and this showed that the activity of PTK/ZK reached a maximum activity at doses above approximately 50 mg/kg against both primary tumours and LN metastases. Other experiments in the B16/BL6 model (not shown), and a rat mammary model [15] demonstrated that even daily doses of 200 mg/kg did not increase efficacy further. However, in both experiments shown here (Fig. 1; Table 1), the combination of everolimus and PTK/ZK produced an anti-tumour effect much greater than the maximal effect of PTK/ZK alone.

Effects of everolimus/PTK/ZK combinations on B16/BL6 melanoma vasculature

Plasma VEGF concentrations at the endpoint on day 21 (after 14-day treatment) declined in response to treatment, with everolimus being slightly more effective than PTK/ZK as monotherapy, although both single agents were significantly inferior to the combination (Fig. 5a). Similar effects were seen in the cervical metastatic tumour mass (Fig. 5b), and the plasma levels reflected the tumour tissue VEGF levels ($r = 0.56$, $P = 0.001$, $n = 31$; Spearman Rank correlation coefficient). However, when expressed as ng VEGF/mg tumour mass, a striking increase in the tumour concentration of VEGF occurred after treatment with PTK/ZK compared to the controls and everolimus monotherapy groups (Fig. 5c). IHC analysis of the cervical LN tumours indicated that tumour vascularization (CD31) was reduced by both therapies, but everolimus also strongly reduced levels of smooth muscle actin, a marker of fibroblasts, pericytes and smooth muscle cells. This effect on the SMA was maintained in the combination while changes in CD31 were less marked (see Figure S1 Supplemental Data).

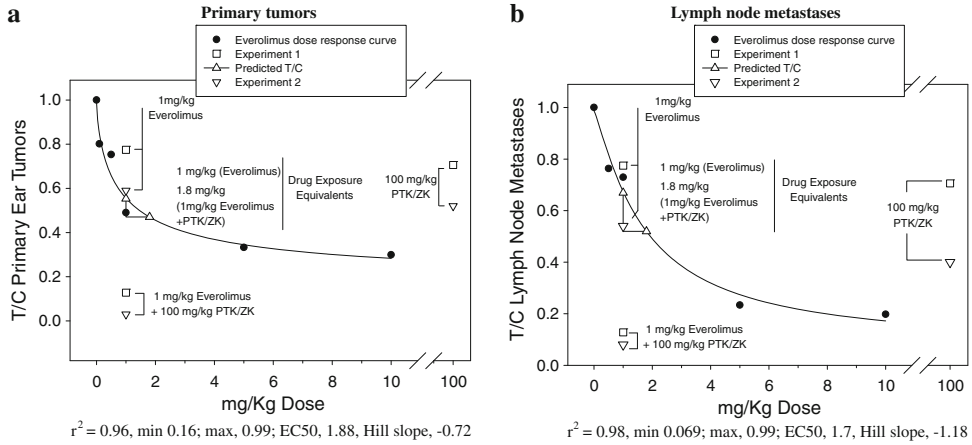


Fig. 3 Pharmacodynamic analysis of everolimus alone and in combination with PTK/ZK. The dose–response curves of everolimus (*filled circles*, compiled from two experiments) were modelled using the Hill plot function of SigmaPlot (*smooth line*). *Open squares* (experiment 1) and *inverted open triangles* (experiment 2) represent the anti-tumour effect obtained by treatment of tumour-bearing mice with everolimus (1 mg/kg) or PTK/ZK (100 mg/kg) and the combination. The *open triangles* represent the predicted change in *T/C* that would be obtained upon changing the dose from 1 mg/kg to 1.8 mg/kg, which is the dose predicted to be the equivalent dose of everolimus needed to be administered alone to provide a similar AUC, $t_{½}$, C_{max} and C_{last} to obtain the everolimus exposure when in the presence of PTK/ZK

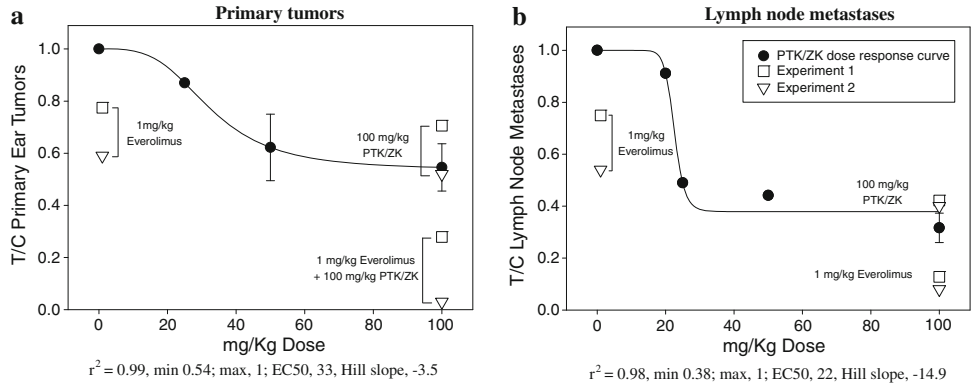


Fig. 4 Pharmacodynamic analysis of PTK/ZK alone and in combination with PTK/ZK. The dose–response curves of PTK/ZK (*filled circles*, compiled from three experiments; mean SEM where possible) were modelled using the Hill plot function of SigmaPlot (*smooth line*). *Open squares* (experiment 1) and *inverted open triangles* (experiment 2) represent the anti-tumour effect obtained by treatment of tumour-bearing mice with everolimus (1 mg/kg) or PTK/ZK (100 mg/kg) and their combination

Discussion

Everolimus and PTK/ZK have been shown to possess some overlapping as well as distinctive anti-angiogenic/anti-vascular effects in several different models [5]. In vitro, both compounds inhibited proliferation of VEGF-stimulated human endothelial cells, but only everolimus was effective against bFGF-stimulated cells. However, only PTK/ZK inhibited migration of human endothelial cells in vitro and VEGF-induced vascular leakage in vivo, although everolimus has been shown to inhibit migration of tumour cells in vitro [3]. In vivo, both compounds reduced CD31 staining in tumours, but only everolimus reduced the extent of SMA staining [3]. Given their different mechanism-of-action and differential effects on tumour vasculature, we investigated here their effects in combination on inhibiting tumour growth in vivo.

In the B16/BL6 melanoma model, both everolimus and PTK/ZK were effective as monotherapies but a dramatic increase in anti-tumour efficacy was obtained by the combination of the two agents. In the two independent experiments, the CCI and the statistical analysis demonstrated an at least additive effect against both primary tumours and LN metastases. Pooling the dataset increased the statistical power, resulting in the analyses showing a statistically significant synergy. Although the presence of PTK/ZK increased everolimus exposure, use of PK modelling indicated that this increase in exposure could not account for the markedly improved anti-tumour efficacy of the combination. However, one must also consider the potential effect

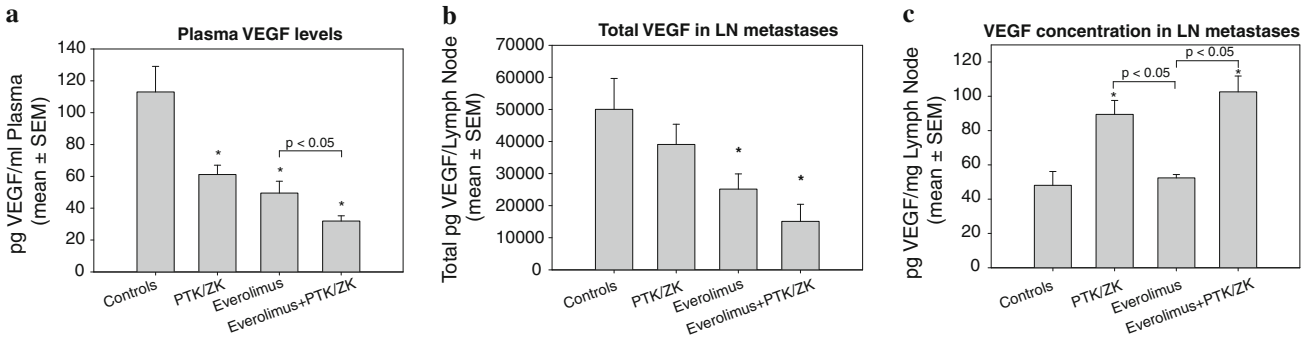


Fig. 5 Effects of everolimus or PTK7/ZK alone or in combination on VEGF levels in plasma and in tumours. Plasma VEGF levels (**a**) and cervical lymph node (LN) metastases levels expressed as total (**b**) and concentration of VEGF in the LN metastases (**c**). B16/BL6 cells (5×10^4) were injected intradermally in both ears of C57BL/6 mice. Treatment with everolimus, (1 mg/kg daily) or PTK/ZK (100 mg/kg daily) alone or in combination was initiated 7 days after cell inoculation and continued until day 21. The animals were killed on day 21, and plasma (heparin) prepared, and the cervical LN collected, weighed and extracted. Murine VEGF levels were determined by ELISA. Total amounts were estimated using the determined concentration multiplied by the total LN weight. Values are means ± SEM ($n = 6$–12). * $P < 0.05$, significant inhibition compared to control and pairwise comparisons are indicated by the *bars* and *P* values (ANOVA with Tukey)

of everolimus on PTK/ZK exposure. PTK/ZK (data on file, Novartis Pharma AG) and everolimus [16] are both primarily metabolized by CYP3A4 enzymes and thus competition would increase exposure of one or both compounds. Nevertheless, the high concentration of PTK/ZK relative to everolimus (approximately 100-fold higher concentration of PTK/ZK) suggests that the effect of everolimus on the pharmacokinetics of PTK/ZK would be minor when compared with the demonstrated effect of PTK/ZK on everolimus. An additional complication is that PTK/ZK demonstrates dose-dependent (Figure S2, supplemental data) and multiple-administration-dependent pharmacokinetics (Figure S3 supplemental data). Whereas plasma exposure is under-proportional to dose, multiple-administration of higher doses of PTK/ZK result in diminished exposure in primary tumours and LN metastases. An induction of metabolism that is both dose and time dependent could explain this and would likely also affect the exposure of everolimus in everolimus-PTK/ZK combinations upon multiple administrations. Despite this complication, dose–response curves indicated that the anti-tumour effect of PTK/ZK is maximal at doses of ≥50 mg/kg in both the B16/BL6 models used here and also an orthotopic rat mammary model [15]. Consequently, any modification of the complex metabolism of PTK/ZK by everolimus will likely not affect its efficacy.

Consistent with the increased efficacy of the combination, there was also a further decrease in plasma and tumour VEGF levels, although measurement of the *concentration* of VEGF in the LN, showed that everolimus had no effect, while both PTK/ZK monotherapy and the combination actually caused increased tumour VEGF. This may reflect that tumour cells increase VEGF release in an attempt to overcome the blockade in VEGF pathway signalling. In addition, treatment with VEGF pathway inhibitors can lead to an activation of other angiogenic parameters, such as an influx of macrophages and bone-marrow-derived cells which also secrete VEGF [17, 18]. These various factors may also explain why the combination was apparently less effective at reducing CD31 staining in tumours, although the strong effect of everolimus on SMA on mature vessels was maintained.

In human cancers, the character of the tumour vasculature appears to reflect the tumour histotype, both in terms of relative density of blood vessels and in terms of maturation state, e.g. size, extent of smooth muscle cell covering the endothelial vessels [19]. There is evidence that interruption of VEGF-R signalling for example by VEGF-R TKIs is most effective against immature vessels, but mature vessels (those bearing some degree of SMA/pericyte coverage) are more resistant to VEGF ablation [19, 20]. Thus, the evidence of a strong effect against the mature vessels by everolimus monotherapy and also in combination with the TKI is further support for the use of such combinations in the clinic. Recent reports show that combination of these two drugs is tolerable in patients and can provide meaningful clinical benefit [21, 22].

In summary, we have shown that everolimus and PTK/ZK show synergistic anti-tumour effects in an orthotopic well-vascularized syngeneic murine melanoma model. Although there was a drug–drug interaction, so that PTK/ZK increased everolimus exposure, PK modelling showed this was likely to have only a minor impact on overall efficacy. Furthermore, we confirmed distinct differences in the effects of mTORC1 or VEGF-R inhibition by a TKI on the tumour vasculature that may contribute to the more profound effects of these agents when used in combination.

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