VOLUME 26 · NUMBER 10 · APRIL 1 2008

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Dose- and Schedule-Dependent Inhibition of the Mammalian Target of Rapamycin Pathway With Everolimus: A Phase I Tumor Pharmacodynamic Study in Patients With Advanced Solid Tumors

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Submitted September 20, 2007; accepted November 16, 2007; published online ahead of print at www.jco.org on March 10, 2008.

Presented in part at the 41st Annual Meeting of the American Society of Clinical Oncology, Orlando, FL, May 13-17, 2005; and at the 13th European Cancer Conference, Paris, France, October 30 to November 3, 2005.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/08/2610-1603/$20.00

DOI: 10.1200/JCO.2007.14.5482

ABSTRACT

Purpose
Everolimus is a selective mammalian target of rapamycin (mTOR) inhibitor with promising anticancer activity. In order to identify a rationally based dose and schedule for cancer treatment, we have conducted a tumor pharmacodynamic phase I study in patients with advanced solid tumors.

Patients and Methods
Fifty-five patients were treated with everolimus in cohorts of 20, 50, and 70 mg weekly or 5 and 10 mg daily. Dose escalation depended on dose limiting toxicity (DLT) rate during the first 4-week period. Pre- and on-treatment steady-state tumor and skin biopsies were evaluated for total and phosphorylated (p) protein S6 kinase 1, eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4E-BP1), eukaryotic initiation factor 4G (eIF-4G), AKT, and Ki-67 expression. Plasma trough levels of everolimus were determined on a weekly basis before dosing during the first 4 weeks.

Results
We observed a dose- and schedule-dependent inhibition of the mTOR pathway with a near complete inhibition of pS6 and peIF-4G at 10 mg/d and ≥ 50 mg/wk. In addition, pAKT was upregulated in 50% of the treated tumors. In the daily schedule, there was a correlation between everolimus plasma trough concentrations and inhibition of peIF4G and p4E-BP1. There was good concordance of mTOR pathway inhibition between skin and tumor. Clinical benefit was observed in four patients including one patient with advanced colorectal cancer achieving a partial response. DLTs occurred in five patients: one patient at 10 mg/d (grade 3 stomatitis) and four patients at 70 mg/wk (two with grade 3 stomatitis, one with grade 3 neutropenia, and one with grade 3 hyperglycemia).

Conclusion
Everolimus achieved mTOR signaling inhibition at doses below the DLT. A dosage of 10 mg/d or 50 mg/wk is recommended for further development.

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INTRODUCTION

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase, downstream of the phosphatidyl inositol 3′-kinase (PI3K)-AKT signaling pathway.[1,2] mTOR is activated in response to different stimuli such as nutrients and growth factor receptors.[3] With the involvement of the PI3K-AKT pathway, mTOR relays a signal to translational regulators, specifically enhancing the translation of mRNAs encoding proteins essential for cell growth and cell cycle progression through G1 to S phase.[2,4,5] As a result of its central position within this signal transduction pathway, mTOR has been considered an important target for new anticancer drug development.[2,6,7] In support of its role in cancer, the mTOR pathway is aberrantly activated in around half of human tumors[1,4,8] and plays a critical role in angiogenesis.[9-14]

mTOR signals to at least two downstream effectors, the translational repressor protein eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase 1 (S6K1).[15-17] Binding of 4E-BP1 to the translational activator eIF-4E is modulated by mTOR-dependent phosphorylation of multiple specific serine and threonine residues.[18,19] After a final phosphorylation at Ser65, 4E-BP1 dissociates from eIF4E,

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thereby allowing for the reconstitution of a translationally competent initiation factor complex with the involvement of eIF-4F[20] and eIF-4G.[21-23] eIF-4F activation results in the translation of a subset of capped mRNAs containing highly structured 5′-untranslated regions and encoding proteins involved in the G1 to S transition.[24,25]

Everolimus (RAD001), an oral rapamycin derivative, has demonstrated potent antiproliferative effects against a variety of mammalian cell lines. Everolimus inhibits cytokine-driven lymphocyte proliferation,[26] as well as the proliferation of human tumor-derived cells, both in vitro in cell culture and in vivo in animal xenograft models.[27-31] As a result of these properties, everolimus has been developed as an immunosuppressant[32-34] and is now being developed as an anticancer agent. In the syngenic CA20948 rat pancreatic tumor model, everolimus has been shown to inhibit 4E-BP1 phosphorylation and S6K1 signaling in tumor and normal tissue.[29,35,36] Treatment with everolimus demonstrated equivalent activity with daily and intermittent schedules, this activity being dose-dependent for the two administration schedules.[29]

Based on these considerations, the objectives of this trial were to assess the optimal dose and schedule of orally everolimus, administered weekly or daily, based on the safety profile and pharmacodynamic (PD) effects on mTOR dependent pathways in sequential tumor and skin biopsies. In addition, the effects of everolimus on tumor and skin specimens were correlated to plasma trough concentrations of the drug.

PATIENTS AND METHODS

Patient Population

Main inclusion criteria were histologically confirmed advanced tumors, unresponsive to standard therapy; presence of disease accessible to repetitive biopsies; age ≥ 18 years; life expectancy ≥ 12 weeks; WHO performance status of 0 to 2; and adequate bone marrow, hepatic, and renal function. All patients gave informed consent, and approval was obtained from the ethics committees at the participating institutions and regulatory authorities. The study followed the Declaration of Helsinki and good clinical practice guidelines.

Treatment and Dose Escalation Criteria

Everolimus was administered either as a single weekly oral dose (20, 50, and 70 mg) or as a continuous daily oral dose (5 and 10 mg) until progression. Initially, six to eight patients were to be enrolled to each dose level to have a minimum of four fully assessable patients for the PD end points of the study. Dose escalation proceeded in the absence of more than one of six patients with dose-limiting toxicity (DLT) in the first 28 days of treatment. If two or more patients presented DLT at a dose level, enrollment of patients to that dose level was discontinued and the immediately preceding dose level was considered the maximum tolerated dose for a given schedule. DLT was defined as any one of the following drug-suspected toxicities: grade 3 or higher National Cancer Institute Common Toxicity Criteria version 3.0 hematologic toxicity and grade 3 or higher nonhematologic toxicity despite the use of adequate/maximal medical intervention and/or prophylaxis.

Safety and Response Assessments

Routine clinical and laboratory assessments were conducted on a weekly basis during the first 4 weeks of treatment and thereafter every 2 weeks; after 6 months, assessments were conducted once per month. ECG monitoring was performed at baseline and at the fourth week of treatment after dosing. Adverse events were recorded, graded using the National Cancer Institute Common Toxicity Criteria version 3.0, and assessed by the investigator for any relationship with everolimus treatment. Objective measurement of tumor mass was assessed in accordance with the Response Evaluation Criteria in Solid Tumors criteria[37] after 8 weeks on treatment, and thereafter every 8 weeks.

Pharmacokinetic Analysis

A significant linear correlation between steady-state trough concentrations and overall exposure (area under the curve) to everolimus had been previously found when the drug was administered daily.[38] In this context, steady-state trough blood levels were chosen as a convenient monitoring pharmacokinetic (PK) parameter for this trial. Plasma levels of everolimus were determined on a weekly basis before dosing during the first 4 weeks of treatment.

The concentration of everolimus in whole blood was determined by liquid chromatography–mass spectrometry after liquid-liquid extraction. This method for blood sample has a lower limit of quantification of 0.3 ng/mL. Trough concentrations were reported as mean and standard deviation.

Pharmacodynamic Assessments

The PD effects by everolimus in tumor and skin were determined in all the patients included in the study. The timing of tumor and skin tissue biopsies was different in the two schedules: in the daily schedule at baseline and before dosing at day 2 in week 4; and in the weekly schedule at baseline, 24 hours and at day 6 postdose administration in week 4. The aim of the third biopsy in the weekly schedule was to assess whether everolimus-related inhibition was sustained between dosing. Processing of the samples, immunohistochemistry, and statistical analysis were performed as previously described (online-only Appendix A1).[39,40]

Briefly, immunohistochemical analysis of total AKT, phosphorylated AKT at Ser473 (pAkt), total 4E-BP1, phosphorylated 4E-BP1 at Thr70 (p4E-BP1), phosphorylated eIF-4G at Ser1108 (peIF-4G), total S6, phosphorylated S6 at Ser235/236 and at Ser240/244 (pS6), and proliferation marker Ki-67 were performed in formalin-fixed paraffin-embedded sections from tumor and skin samples. Qualitative changes in the expression of markers were assessed in a blinded fashion. For quantitative analysis, the histochemical score (Hscore) was calculated to evaluate complete tumor sections and epidermis on skin samples at high magnification using a light microscopy, as previously described. Paired pretherapy and on-therapy samples were analyzed using the Wilcoxon rank test by SPSS Data Analysis Program version 10.0 (SPSS Inc, Chicago, IL). Statistical tests were conducted at the two-sided .05 level of significance. Pearson linear correlation was employed to examine the potential relationships between trough concentration values of everolimus and PD effects in tumor and skin samples.

RESULTS

Characteristics of the 55 patients included in the weekly schedule (n = 31) and the daily schedule (n = 24) are listed in Table 1. The distribution of patients across dose levels is present in Table 2.

Clinical Toxicities

The numbers of patients reported with suspected toxicities were similar in the weekly and daily schedule (Table 2). Hematologic abnormalities were uncommon with only 12 patients (22%) presenting grade 1 to 3 neutropenia (only one patient presented grade 3) and 17 (31%) patients presenting grade 1 to 2 thrombocytopenia. Of these, only the grade 3 neutropenia was reported as drug related toxicity. The most frequent nonhematologic toxicities were skin rash/erythema (42%), stomatitis/oral mucositis (38%), headache (36%), and fatigue (29%). There were no grade 4 toxicities while grade 3 toxicities were reported in nine patients (16%), including stomatitis/oral mucositis (9%), hyperglycemia (4%), and fatigue (2%). DLT occurred in five patients: one patient at 10 mg daily (grade 3 stomatitis) and four patients at 70 mg weekly (two with grade 3 stomatitis, one with grade 3 neutropenia, and one with grade 3 hyperglycemia). Hence, at the dose of 70 mg weekly four of seven patients presented DLTs and this dose was considered too toxic for further study. The cumulative tolerance of everolimus was acceptable with only four additional patients presenting grade 3 toxicities after the first 28-day period: stomatitis/oral mucositis,[2] hyperglycemia,[1] and fatigue.[1]

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Table 1. Patient Characteristics

Characteristic	No.	%
Total No. of patients	55	
Sex		
Male	21	38
Female	34	62
Median age, years	59	
Range	27-85	
WHO PS		
0	34	62
1	20	36
2	1	2
Primary diagnosis		
Breast	19	35
Colorectal	16	29
Pancreas	4	7
NET	2	4
Renal	2	4
NSCLC	2	4
Gallbladder	2	4
Melanoma	2	4
Other	6	11

Abbreviations: PS, performance status; NET, neuroendocrine tumors; NSCLC, non–small-cell lung cancer.

Antitumor Activity

There was one partial response in a patient with a heavily pretreated metastatic colorectal cancer treated at 20 mg weekly that lasted 5.3 months (disease control during 9 months). Three patients presented stabilization of their disease for more than 5 months: one patient with an advanced renal cell cancer treated at 50 mg weekly lasting 14.6+ months and two patients with advanced breast cancer treated at 70 and 20 mg weekly lasting 10.7 and 5.6+ months, respectively.

PK

Table 3 depicts the trough concentrations of everolimus according to the different schedules and doses. Unweighted linear regression analysis of the relationship between dose and pharmacologic exposure was performed. Everolimus exhibited a linear dose-trough concentration relationship in the daily schedule (regression slope 0.96; 90% CI, 0.25 to 1.66), whereas this relationship could not be found in the weekly schedule due to insufficient serum sampling.

Tumor and Skin mTOR Pathway Signaling PD Studies

Inhibition of mTOR signaling was observed at all dose levels and schedules. Figure 1 shows the box-plots of the collated PD effect in tumor and skin in all the patients with paired pre- and on-therapy biopsies (30 patients with tumor and 43 with skin paired samples). As a whole, treatment with everolimus resulted in an almost complete inhibition of pS6 ($P < .001$) and peIF-4G ($P < .001$). p4E-BP1 (Thr70) was profoundly reduced in skin ($P < .001$), this reduction being of less magnitude in the tumor ($P = .058$). There was also an overall increase in AKT phosphorylation (Ser473) both in tumors ($P = .006$) and skin ($P < .001$). The observed effects on protein phosphorylation were not due to changes in protein expression, as total AKT, 4E-BP1, and S6 protein levels were unmodified as a result

Table 2. Frequency Distribution of Drug-Related AEs in the Daily and in the Weekly Schedule (reported in three or more patients)

Parameter	Schedule					
	Daily		Weekly			Total
Dose, mg	5	10	20	50	70	—
No. of patients						
Treated	12	12	12	12	7	55
With drug-related AEs	11	12	11	11	7	52
AE						
Skin rash and erythema	7	7	1	5	3	23
Stomatitis/oral mucositis	4 (1)	6 (1)	2	5 (1)	4 (2)	21 (5)
Headache	6	4	4	3	3	20
Fatigue	1	5 (1)	3	4	3	16 (1)
Anorexia	2	4	1	4	1	12
Vomiting	0	5	1	4	0	10
Hypercholesterolemia	5	1	1	2	0	9
Nausea	1	4	1	2	0	8
Anemia	1	1	2	0	3	7
Upper abdominal pain/dyspepsia	2	3	1	1	0	7
Diarrhea	0	2	0	3	1	6
Dry mouth	1	2	2	0	1	6
Abdominal distension	1	1	0	2	1	5
Pruritus	2	0	1	1	1	5
Dysguesia	3	1	1	0	0	5
Hyperglycemia	0	1	2 (1)	0	1 (1)	4 (2)
Constipation	1	2	0	1	0	4
Nail disorders	0	0	1	1	1	3
Epistaxis	0	0	1	1	1	3

NOTE. All grades (grade 3 in parentheses).
Abbreviation: AEs, adverse events.

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Table 3. Trough Concentrations According to the Different Schedules and Doses

Schedule and Dose (mg)	Trough Concentrations (ng/mL) No.	Mean	SD
Daily*			
5	12	8.5	5.5
10	11	17.0	12.4
Weekly			
20	10	0.7	0.5
50	12	1.0	1.5
70	7	4.2	4.4

Abbreviation: SD, standard deviation.
*Dose-trough concentration relationship: regression slope = 0.96 (90% CI, 0.25 to 1.66).

of everolimus administration (data not shown). Cellular proliferation was reduced both in tumor ($P = .014$) and skin ($P = .008$).

In order to dissect the effects of the studied doses and schedules of everolimus, we analyzed mTOR-dependent PD changes in individual patients (Fig 2). In the daily schedule, inhibition of pS6 was near complete at both dose levels, while inhibition of peIF-4G was only partial at 5 mg, and complete at 10 mg. Reductions in p4E-BP1 were also more profound at 10 mg, albeit with a greater interpatient variability. At both dose levels, the majority of patients presented a reduction in the proliferation index whereas pAKT increased in around half of the patients. In the weekly schedule, inhibition of tumor mTOR–dependent signaling was evaluated 24 hours after drug administration (early effect) and at 24 hours before the next weekly administration, in order to assess whether any effects persisted until the next dose (sustained or trough effect). Inhibition of pS6, both early and sustained, was almost complete at all doses. Early inhibition of peIF-4G was complete at all dose levels, but sustained inhibition was only observed at doses ≥ 50 mg. As with the daily schedule, p4E-BP1 inhibition was not observed in all patients, but in those patients achieving inhibition it was sustained. Increased tumor pAKT was greater at doses ≥ 50 mg than at 20 mg but it was not sustained. Proliferation was reduced in most of the patients 24 hours after the weekly dosing but, in most cases, it was not sustained. Because the number of patients that achieved clinical benefit is small, it is not possible to analyze the predictiveness of the PD markers. Representative biomarker expression changes in tumor and skin from two selected patients with advanced breast carcinoma treated at 10 mg/d and 50 mg/wk are shown in Figure 3.

We explored the potential relationships between plasma trough concentrations of everolimus and tumor and skin PD effects, only in patients on daily therapy as, for this schedule, a correlation is proven between trough concentrations and overall exposure.[33,34,38] The almost complete inhibition shown for pS6 in tumors from patients treated at the two daily dose levels did not permit any correlation analysis with respect to this biomarker. However, a trend could be observed in the relationship between everolimus trough values and tumor peIF-4G inhibition ($r = -0.49$; $P = .17$). Trough concentrations also correlated significantly with inhibition of tumor p4E-BP1 ($r = -0.6$; $P = .049$). Nevertheless, no trends in PD correlations between trough values and treatment-related upregulation of tumor pAkt were evident.

DISCUSSION

This phase I study was aimed at identifying a recommended dose and schedule of everolimus in patients with cancer defined by the achievement of a complete and sustained inhibition of mTOR

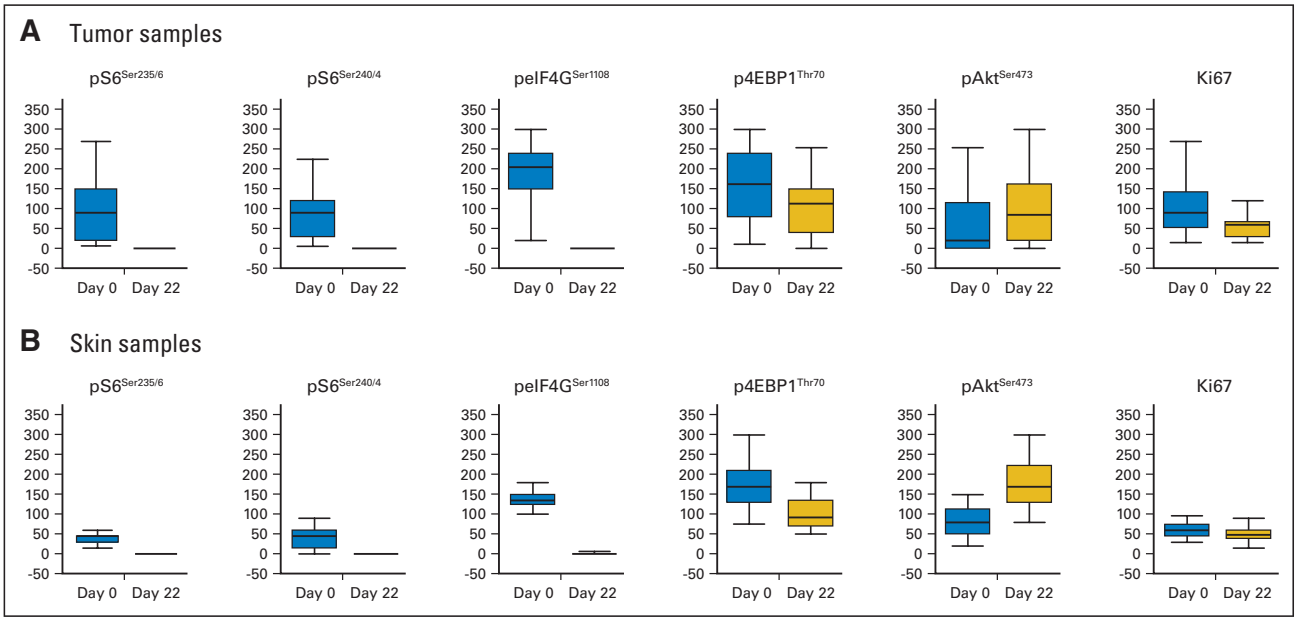

Fig 1. Pharmacodynamic effects after everolimus treatment in all the patients included in the study with (A) assessable paired tumor (n = 30) and (B) skin samples (n = 43). Box-plots showing the expression at baseline and on-treatment for the following markers: $pS6^{Ser235/6}$, $pS6^{Ser240/4}$, $peIF4G^{Ser1108}$, $p4E\text{-}BP1^{Thr70}$, $pAkt^{Ser473}$ and Ki-67. Boxes indicate 90% of the values. Bold lines indicate the mean of the values. External lines indicate the complete range when beyond 90% of the values. Day 0: baseline sample; day 22: sample obtained at fourth week 24 hours after dosing.

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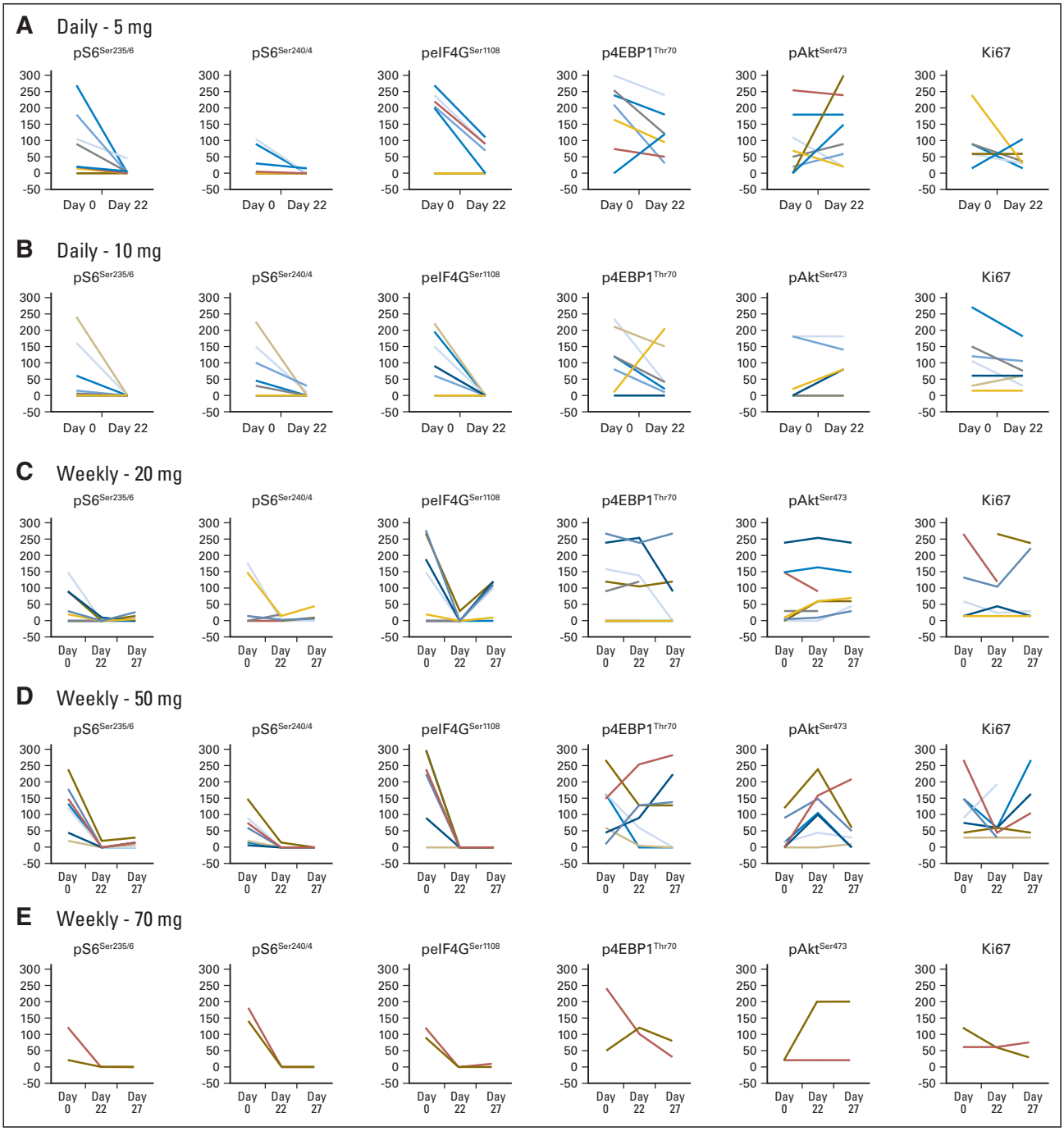

Fig 2. (A-E) Tumor pharmacodynamic effects in patients treated in the daily (treated at 5 and 10 mg) and in the weekly schedules (treated at 20, 50, and 70 mg). The lines show the individual evolution of the expression of the biomarkers (pS6$^{Ser235/6}$, pS6$^{Ser240/4}$, peIF4G^{Ser1108}, p4E-BP1^{Thr70}, pAkt^{Ser473}, and Ki-67) at baseline and on-treatment. In the daily schedule samples were obtained on day 0 (baseline) and on day 22 (on-treatment). In the weekly schedules samples were obtained on day 0 (baseline), on day 22 (on-treatment, 24 hours after dosing) and on day 27 (on-treatment, 144 hours after dosing). The aim of this third biopsy was to assess the persistent effect of everolimus administered on a weekly basis.

dependent–signaling pathways on tumor and skin. Our approach was based on the existing correlation in preclinical models between antitumor activity and mTOR-signaling inhibition. In a CA20948 syngenic rat pancreatic tumor model, doses of everolimus that inhibited tumor growth also dramatically inhibited mTOR signaling in tumor, skin, and peripheral blood mononuclear cells (PBMCs).[29] In this model, a decrease in p4E-BP1 (Thr70) and an increase in eIF-4E and 4E-BP1 association were consistently

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