Combination of PI3K/mTOR Inhibitors: Antitumor Activity and Molecular Correlates

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Abstract

The phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway is a major target for cancer therapy. As a strategy to induce the maximal inhibition of this pathway in cancer cells, we combined allosteric mTOR inhibitors (rapamycin and RAD001) with a dual PI3K/mTOR kinase inhibitor (PI-103). Both in vitro and in vivo, the combination exhibited more activity than single agents in human ovarian and prostate cancer cells that harbor alterations in the pathway. At the molecular level, combined inhibition of mTOR prevented the rebound activation of Akt that is seen after treatment with rapamycin and its analogues and caused more sustained inhibition of Akt phosphorylation. Furthermore, the combination strongly inhibited the expression of PI3K/Akt/mTOR downstream proteins. In particular, it showed greater activity than the single agents in inhibiting the phosphorylation of 4EBP1, both in vitro and in vivo, resulting in selective inhibition of CAP-dependent translation. A proteomic approach was used to confirm the identification of c-Myc as the key regulator for the reduction in downstream proteins affected by the combined inhibition of mTOR. In conclusion, the combination of a catalytic and an allosteric inhibitor of mTOR shows greater activity, without a concomitant increase in toxicity, than either drug alone, and this may have therapeutic implications for inhibiting this pathway in the clinical setting. Cancer Res; 71(13); 4573–84. ©2011 AACR.

Introduction

The phosphatidylinositol-3-kinase (PI3K) pathway is a key signal-transduction pathway that regulates many physiologic functions in cells, and its deregulation has been associated with malignant transformation and apoptotic resistance (1–5).

PI3Ks are activated in response to many extracellular stimuli such as growth factors and hormones. Activated PI3Ks localize in proximity of the plasma membrane lipid substrate phosphatidylinositol-4,5-diphosphate (PIP2), allowing its conversion to phosphatidylinositol-4,5-triphosphate (PIP3) (1). The lipid phosphatase PTEN counterbalances the process dephosphorylation of PIP3 (2) and signals the plasma membrane lipid substrate phosphatidylinositol-4,5,6-triphosphate (PIP3). The lipid phosphatase PTEN counterbalances the process dephosphorylation of PIP3 to PIP2, thus terminating PI3K-dependent signaling (6, 7).

PIP3 allows the recruitment of the main PI3K downstream effector Akt/PKB at the membrane level and its subsequent activation through the phosphorylation of threonine (Thr)-308, by PDK1, and serine (Ser)-473, by the mTORC2 complex (8–10). Akt targets several substrates and promotes protein synthesis and cell growth through phosphorylation of the negative regulator of mTOR, the TSC1/TSC2 complex (11). mTORC1 activation positively regulates mRNA translation initiation through its downstream effectors p70S6K and 4EBP1 (12). Activated p70 can phosphorylate the 40S ribosomal protein S6, increasing translation of mRNAs with a 5′-terminal oligopyrimidine (TOP) tract such as ribosomal proteins and elongation factors (13). Phosphorylation of 4EBP1 leads to its detachment from eIF4E, allowing eIF4E to complex with eIF4G to form the eIF4F initiation complex, driving increased cap-dependent translation of these mRNAs with a long, highly structured 5′-untranslated region (UTR) such as cyclin D1, c-Myc, and VEGF (12, 14).

PI3K and mTOR inhibitors have been extensively studied in various types of cancer with, at least at the preclinical level, promising effects. At a clinical level, the results have been less encouraging although clinical evidence of benefit has been reported (15, 16). Drug combinations are being given increasing importance in clinical settings as the most appropriate approach to target tumors and avoid acquired resistance. Activity of the PI3K/Akt/mTOR pathway inhibitors in combination with classical antitumor agents has proved highly effective in several experimental systems (17–19). Combining drugs targeting different pathways inside the cell is likely to be effective in cells that adopt alternative or compensatory pathway activation. Furthermore, an interesting approach could be to aim for the same pathway at different levels or with
molecules targeting different sites of the protein, thus enhancing the chances of completely shutting down the signaling cascade.

We analyzed the activity of the dual PI3K–mTOR inhibitor PI-103 either as single agent or in a combination. It exerts at least additive effects with rapamycin, an allosteric inhibitor also affecting the mTOR pathway. The results, both in vitro and in vivo, appear to correlate with the molecular changes induced by the drugs individually and in combination.

Our results provide evidence that targeting mTOR with 2 different inhibitors could be a new strategy worth exploring to achieve better antitumor activity.

Materials and Methods

Cell cultures and drug treatments

Human ovarian carcinoma Skov-3 and Ovcar-3, human prostate carcinoma PC3, and human breast MCF-7 and MDA-MB-231 cells were grown in RPMI 1640 medium. Human lung carcinoma A549 cells were cultured in Dulbecco’s modified Eagle medium (DMEM).

Cells were treated with different concentrations of rapamycin, PI-103 or both. The sulforhodamine B (SRB) assay was used to measure cell proliferation as reported previously (20). Bromodeoxyuridine (BrdU) incorporation (Cell proliferation ELISA; Roche) was used as an additional test. Combination effects of PI-103 and rapamycin were quantified as described (ref. 21; Supplementary Methods). Experiments were run at least in triplicate. The 3 replicates for each time point were pooled and used for fluorescence-activated cell-sorting (FACS) analysis of DNA content (see Supplementary Methods).

Western blotting analysis

Proteins were extracted and visualized using standard techniques, as reported (22) and detailed in Supplementary Materials.

Real-time PCR

RNA was extracted using SV Total RNA Isolation System (Promega) and retrotranscribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). GoTaq qPCR Master Mix (Promega) was used to quantify c-Myc and lysyl tRNA synthetase (KARS). Primer sequences are reported in Supplementary Table S1. Real-time PCR (RT-PCR) was carried out using the 7900HT Sequence Detection System (Applied Biosystems).

Determination of eIF4E-4EBP1 complexes

Total protein extracts (100 μg) were incubated with 7-methyl-GTP-Sepharose (Amersham) overnight at 4°C to capture eIF4E and its binding partners. The beads were washed twice with immunoprecipitation (IP) buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10 mmol/L NaF, 5 mmol/L Na3VO4, 1% Triton X-100, protease inhibitor); bound complexes were solubilized in 30 μL of loading buffer, and proteins were resolved by 15% SDS-PAGE. Immunoblots were done as previously described.

Luciferase reporter assays

Cells were seeded in 6-well plates 48 hours before transfection with 2 μg of bicistronic reporter plasmids (kindly provided by Dr. Ruggero; ref. 23) using Lipofectamine 2000 (Invitrogen). Eight hours after transfection, cells were treated for 24 hours with the drugs, individually or in combination. Renilla and firefly luciferase were measured using the Dual-Luciferase Reporter Assay System (Promega). Data are expressed as the mean ± SD of 2 experiments, each run in triplicate.

Two-dimensional gel electrophoresis image analysis and mass spectrometry

Differential protein expression in the total Skov-3 lysate from untreated and treated cells was analyzed using 2-dimensional gel electrophoresis (2-DE), computerized gel image, and univariate statistical analysis for comparative proteomics (Supplementary Methods). In-gel trypsin digestion and tandem mass spectrometry for protein identification were conducted as previously reported (24, 25). All information concerning mass spectrometry submission parameters and peptide/protein identification are available in Supplementary Tables S2 and S3.

Protein-network analysis

MetaCore (GeneGo) was used to map the differently expressed proteins into biological networks and for functional interpretation of the protein data as detailed in Supplementary Methods.

Xenograft models

Six-week-old NCr-nu/nu mice were obtained from Harlan, Italy. Mice were maintained under specific pathogen-free conditions. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies.

Exponentially growing Skov-3 and PC3 cells (7 × 106 cells per mouse) were injected subcutaneously. Details of the in vivo experiments are available in Supplementary Methods.

Results

PI-103 exerts a dose-dependent effect on cell proliferation

To examine the in vitro cytotoxic effects of PI-103, cells from the ovarian carcinoma cell line Skov-3 were treated with different PI-103 concentrations (Fig. 1A). Concentration-dependent growth inhibition was associated with a specific G1 phase arrest (Fig. 1B); in these conditions, the percentage of dead cells was similar in control and PI-103-treated cells and was less than 4%. Furthermore, PI-103 was able to counteract the activation of Akt induced by insulin stimulation (Fig. 1C).

As shown in Figure 1D, PI-103 inhibited the phosphorylation of phospho-Akt (p-Akt; Ser-473) and p-p70S6K (Thr-389), whereas Erk phosphorylation remained unchanged. As for the mechanism of the G1 arrest, PI-103 treatment raised p27 levels, whereas p-cdc2 levels decreased.
PI-103 with rapamycin exert at least additive effects

PI-103 was tested in combination with a panel of drugs with different mechanisms of action. The allosteric mTOR inhibitor rapamycin gave the best indications for further analysis in combination with PI-103. We used isobologram analysis for qualitative assessment of synergy, additivity, or antagonism. All of the plotted values lie below the diagonal, indicating a synergistic effect (Fig. 2A).

To extend the results to other cell lines, we tested the activity of PI-103 and rapamycin, singly or in combination, on different cancer cell lines (Fig. 2B). All 5 cell lines with an altered PI3K/Akt/mTOR pathway were sensitive, to various extents, to both drugs and showed greater sensitivity when the 2 compounds were combined. MDA-MB-231 cells, whose pathway is unaltered, did not respond significantly to either drug singly or to the combination. Similar results were obtained when BrdU incorporation was determined in the different cell lines at the end of treatment (Supplementary Fig. S1).

Combination treatment is influenced by the sequence of treatment

We investigated whether the effects observed on combining PI-103 and rapamycin were dependent on the treatment schedule (Fig. 2C). Results were comparable when rapamycin and PI-103 were given simultaneously (indicated as...
"concurrent") or when rapamycin was followed 24 hours later by PI-103 (indicated as "rapamycin→PI-103"). PI-103 followed by rapamycin (indicated as "PI-103→rapamycin") resulted in antagonistic effects.

Cell-cycle perturbation analysis helped clarify these effects. After 24 hours of treatment, both PI-103–treated samples and the combination had more than 80% of cells arrested in G1 phase, whereas the effect on cell cycle was milder for the rapamycin-treated sample (Fig. 2D; Supplementary Table S4). In the next 24 hours, single-drug–treated samples started re-entering the cell cycle, although with different strengths. Cells treated with the concurrent
combination progressed slower than PI-103-treated ones, and only S-phase (57.5%) and a few G2 cells (7.4%) were present.

When rapamycin was given as the second drug, it did not completely maintain the cells arrested in G1 by PI-103. When PI-103 was the second drug, a very high percentage of cells were in G1 phase, indicating that those still proliferating after rapamycin treatment had been arrested in G1 by addition of PI-103.

At 24 hours of recovery, cells treated with the rapamycin−PI-103 schedule and the concurrent one showed delayed cell-cycle progression, indicating that these schedules had a more lasting effect. The other sequence gave results similar to those of controls.

The in vitro combination can be translated in vivo

The combination used in vitro was tested in vivo in cells with an altered PI3K/Akt/mTOR pathway, using RAD001 instead of rapamycin because of its longer half-life and stability. In the Skov-3 xenograft model, daily treatment with PI-103 (10 mg/kg) and RAD001 (5 mg/kg) reduced the tumor mass as compared with controls (Fig. 3A), and the combination was more active than either drugs given singly and without any significant toxicity. Figure 3B reports the densitometric analysis of the p-Akt/Akt ratio in tumors. Similar to the in vitro experiments, the combination lowered phosphorylation more than either PI-103 or RAD001 individually.

The same experiment was done in PC3 xenografts (Fig. 3C). In this model, RAD001 was much more effective
than PI-103 and was so active that it was difficult to see any effect of the combination. We, therefore, gave a second treatment cycle (when the tumors started to re-grow at a rate comparable with that of controls) using half the RAD001 dose but the same dose of PI-103. This second cycle still inhibited the tumor growth, although we did note a stronger effect with the combination. Again, there was no evidence of toxicity.

In tumors explanted and analyzed for the expression of some PI3K downstream effectors, Akt and 4EBP-1 phosphorylation appeared to be mainly reduced after the combined treatment, whereas p-S6 was almost completely inhibited by RAD001 alone (Fig. 3D).

"Cotargeting" mTOR enhances inhibition of mTORC1 and downstream pathways preventing Akt increase

In Skov-3 cells (Fig. 4A, left), rapamycin increased the amount of p-Akt (Ser-473), whereas PI-103 had a partial inhibitory effect. The combination reduced p-Akt (Ser-473) levels much more than the single-drug treatments. Effects were similar in 2 other cell lines, Ovcar-3 and PC3 (Fig. 4A, right).

To exclude that the effect of the combination on p-Akt levels was simply due to the sum of the effects of the 2 drugs, we doubled the concentration of both drugs; the combination maintained its effect, which was higher than the effect of individual drugs (Fig. 4A, right).

As for downstream effectors of mTORC1, rapamycin strongly inhibited p70S6K phosphorylation. The addition of PI-103 did not boost this effect, although there was a partial decrease in the S6 phosphorylation state, as expected, in the PI-103 treated sample (Fig. 4B). The other downstream effector of mTORC1 showed a different situation (Fig. 4C and D). Cells treated with the combination had a lower level of phospho-4EBP1 (Thr-37/46) than the untreated or single-drug–treated samples. In addition, slower migrating forms (hyperphosphorylated forms) were visible in the untreated or single-drug–treated samples, whereas cells treated with the combination showed only hypophosphorylated forms of 4EBP1. This was visible both in short experiments where 1 hour of treatment was followed by induction with insulin (Fig. 4C) and in longer experiments where cells were treated for 4 and 24 hours (Fig. 4D, left). Once again, we repeated the experiments by doubling the concentrations of both drugs to...
show that the specificity of the combination was not simply due to the higher drug concentration (Fig. 4D, right).

To better characterize the combination’s effects on translation-initiation events, total cellular protein extracts were incubated with the cap-analogue 7-methyl-GTP-Sepharose to capture eIF4E and its cellular binding partners (Fig. 5A). mRNA cap-binding protein eIF4E co-precipitates with its most abundant repressor 4EBP1. PI-103 and rapamycin induced a slight association of eIF4E with 4EBP1. The combination strongly induced the amount of hypophosphorylated 4EBP1 that could be pulled down. As expected, there was a concomitant decrease in eIF4G-associated with eIF4E. The different phosphorylation status of 4EBP1 can be seen as previously shown. Results were similar in Ovcar-3 and PC3 cells (Supplementary Fig. S2).

A dual-luciferase reporter that distinguishes cap-dependent from cap-independent translation was used in transfection experiments, followed by 24 hours of treatment (Fig. 5B). We used Renilla-luciferase as the gene reporter under cap-dependent translation control and firefly luciferase, under the control of an internal ribosome-entry site (IRES), to normalize the signal from Renilla. Renilla activity was partially reduced after administration of the single drugs but the effect was much stronger in the combination-treated sample.

Cap-dependent translation enhances the translation of a subset of mRNAs characterized by a complex and structured

Figure 5. The drug combination affects the phospho-state of 4EBP1, reducing the cap-dependent translation of c-Myc. A, eIF4E, eIF4G, and 4EBP1 coprecipitated with 7-methyl-GTP Sepharose beads. Actin was used as internal loading control and to ensure that there was no contamination in the beads. B, cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (firefly luciferase activity) were quantified by measuring the luminescence in untreated cells or in cells treated with rapamycin, PI-103 or the combination. *, P < 0.05; **, P < 0.01; and ***, P < 0.001. C, c-Myc mRNA and protein expression after single and combined treatment for 24 hours. mRNA levels are the mean of 3 replicates ± SD and show the difference in expression from untreated cells arbitrarily set at 1. D, 35S pulse-labeling experiment in Skov-3 cells after single and combined treatment for 24 hours. IP c-Myc (top) and total input (bottom) are reported for each treatment. D, PDCD4 protein levels after 24 hours of drug treatment.
5’-UTR, including c-Myc, cyclin D1, and Bcl-XL. Figure 5C (right) shows that c-Myc protein levels were reduced by treatment, whereas c-Myc mRNA levels (Fig. 5C, left), did not change.

We further examined the effect of treatment on c-Myc translation by [35S] metabolic labeling (for details, see Supplementary Methods). As indicated in Figure 5D, the combination greatly reduced the amount of newly synthesized c-Myc, whereas the single drugs had only minor effects. In contrast, the expression of PDCD4, a direct Akt and p70S6K downstream target (26, 27; Fig. 5E), was induced by both drugs singly, and the combination did not further enhance its expression.

We applied an exploratory proteomic approach based on 2-DE coupled with tandem mass spectrometry to compare the proteomic profile of Skov-3 cells exposed to either the single drugs or the combination with their untreated counterpart. Overall, 33 of 973 protein species visualized showed a statistically significant change in abundance as a result of drug treatments (Supplementary Fig. S3). All these species were positively identified by mass spectrometry and are reported in Supplementary Table S5.

Drug treatment induced a general downregulation of the identified proteins. There were 11 proteins whose abundance was altered in the same direction by rapamycin and the combination, suggesting that rapamycin was driving the change observed with the combination.

The protein–protein interaction network was analyzed using the ”shortest path” algorithm in the MetaCore suite to map the shortest paths of interactions among these differentially expressed proteins. After the combination treatment, the majority of these proteins were brought together in the network with the interactions shown in Figure 6. Eleven downregulated proteins identified in the cells treated with the combination were directly connected to c-Myc able to regulate their transcription. Similarly, the shortest path algorithm connected the majority of proteins (74%) differently expressed by rapamycin (Supplementary Fig. S4). Again, the most prominent regulatory element in this network was c-Myc, which interacts directly with 12 proteins whose abundance was significantly reduced by rapamycin.

The interpretation of proteomic data provided by the protein-interaction-network analysis was further supported
by transcription-regulation analysis, which generates sub-networks centered on transcription factors (Supplementary Fig. S5). Again, c-Myc was the most significant transcription factor regulating the cell proteomic profile affected by either the combination or rapamycin. The 3 top-ranked transcription-factor sub-networks for rapamycin and the combination were merged and visualized in Figure 7A and B. c-Myc regulated the expression of AHCY, eIF4A1, VCP, MSN, EEF14G, TCP1, and LAMNA whose reduced abundance was common with both rapamycin and the combination, whereas the downregulation of RUVBL1, KARS, and GNAB1 was specific to the combination. The c-Myc–related reduced expression of

**Figure 7.** Transcription factor networks (c-Myc, SP1, and GCR-alpha) generated by the “transcription regulation algorithm” using the list of differently expressed proteins in Skov-3 cells in response to rapamycin (A) and rapamycin + PI-103 (B). The protein nodes SAHH, lamin A/C, glucosidase II, AL1A1, eEF1G, GRP78, and HSP60 refer to proteomics abbreviations AHCY, LMNA, GNAB1, ALDH1A1, EEF14G, HSPA5, and HSPD1, respectively. C, ChIP analysis was performed with anti-flag and anti-c-myc antibodies after on KRAS promoter 24-hour treatment. D, KARS mRNA levels in untreated and treated Skov-3 cells. E, top: RT-PCR showing the levels of KARS mRNA in Skov-3 cells previously transfected with a c-Myc construct lacking 5'-UTR and treated 12 hours later for another 24 hours with the drugs indicated. Bottom, shows exogenous c-Myc expression levels to confirm transfection efficiency and the absence of treatment-mediated modulatory effects. D and E, results were the mean of 3 replicates ± SD and indicate the change in expression relative to untreated cells arbitrarily set at 1. C–E, treatment conditions (PI-103 400 nmol/L, rapamycin 1 nmol/L, and the combination) were identical.
TPT1, HSPD1, and CLIC4 was only observed after rapamycin treatment.

To validate some of the findings suggested by our network analysis, where c-Myc is a key factor for the downregulated proteins with the drug combination, we used chromatin immunoprecipitation (ChIP; for details, see Supplementary Methods) to measure c-Myc binding to KARS promoter and RT-PCR for assessing its mRNA levels. c-Myc ChIP showed less precipitation of the target DNA after treatment with the 2 single drugs (Fig. 7C). The combined treatment further enhanced the effect. In agreement with this, KARS expression was downregulated by the combination more than it did with the 2 single treatments (Fig. 7D). Cells overexpressing an exogenous c-Myc without UTRs (not sensitive to translational regulation; pCGN—c-Myc kindly provided by Dr W.P. Tansey) showed no drop in KARS (by RT-PCR) and c-Myc (by Western blotting analysis) after both single drugs and the combination (Fig. 7E).

**Discussion**

Several mTOR inhibitors have been synthesized, and their activity at the preclinical level is recognized. Unfortunately, the promises of the preclinical results have not translated to the expected clinical activity, although RAD001 has proven activity and has been approved for the treatment of renal cell carcinoma refractory to VEGFR inhibitors (28, 29). Single treatment with rapamycin and its analogues (rapalogues) activates negative feedback mechanisms leading to increased formation of mTORC2 complex, which not only directly phosphorylates and activates Akt (10, 30) but also promotes eIF4E Ser-209 phosphorylation, favoring its role in the initiation complex (31).

Our strategy exploited the specificity of PI-103 for the catalytic site of PI3K and (although to a lesser extent) of mTOR with disassembling of mTORC1 by the allosteric inhibition of rapamycin. This should simultaneously inhibit mTOR at both the kinase domain and the structural level.

There are few examples showing that the combined use of 2 different molecules targeting the same cellular effector through independent inhibitory mechanisms can improve the effects of the single drugs (32–34). We showed here that simultaneous treatment with rapamycin/RAD001 and PI-103 blocked the rapamycin/RAD001-induced stimulation of Akt, as expected, and that the inhibition of p-Akt was greater with the combination than with the single drugs. At the cell-cycle level too, we found a positive correlation between the effects in terms of growth inhibition and the mechanism of action. The G1 phase arrest obtained when cells were treated with the 2 drugs was, in fact, more sustained and cells re-entered cell cycle more slowly. The schedule-dependence observed in this study agrees with the proposed effect on cell cycle and with other published observations. *In vivo* experiments confirmed the additive effect observed *in vitro*.

With regard to the mechanism of action, the inhibition of mTOR exerted by the combination not only prevented the rebound activation of Akt observed in many tumors after rapalogues treatment but also to more sustained inhibition of Akt phosphorylation. In addition, the combination selectively inhibited downstream targets of mTOR. It has been recently proposed that p70S6K mostly regulates mTOR control of cell size whereas its effects on proliferation are mainly attributable to 4EBPs (35). Our data suggest that the combination may augment the effect on the specific downstream signaling pathway involved in cell proliferation. Only combination therapy with rapamycin and PI-103 caused progressive de-phosphorylation of 4EBP1 that resulted in selective inhibition of translation for CAP-site-containing structures.

Components of the translational machinery are abundant and active in many cancers and their roles have been established (36–38). The selectivity toward the regulation of cap-dependent translation effectors was confirmed by the expression of some genes translated through these mechanisms (i.e., c-Myc). The fact that we did not find any change at the transcriptional level further corroborates the evidence that c-Myc downregulation is mediated by mTOR inhibition of translation machinery, and the proteomic analysis further supports this. Network generation helped with the interpretation of our proteomic data, pointing to c-Myc as the key regulator for the majority of downstream proteins (39–41) whose levels were reduced by the combination treatment and by rapamycin alone. Our proteomic and network analysis thus suggested that the c-Myc level might decrease, as confirmed by Western blot analysis and 35S pulse labeling.

Interestingly, only the combination treatment almost halved the expression of KARS and reduced its transcription. KARS has been reported to be regulated by c-Myc, and the fact that the combination can inhibit occupancy of the KARS promoter by c-Myc (more than the inhibition by the single drugs) strongly suggests that the effects on KARS are c-Myc dependent. KARS is a lysyl-tRNA synthetase that catalyzes the specific attachment of lysine to its cognate tRNA, with a plausible consequence of an altered pool of uncharged tRNA and/or amino acids in the cell. Because recent evidence suggests that mTOR might respond to tRNA and/or to amino acids (42–44), the lower expression of KARS after the combination treatment might be viewed as a cell response to reinforce the negative regulation of the mTOR pathway. That PDCD4 expression increased comparably after both single and combination treatments indicates not only that treatment mainly inhibits the mTOR pathway through 4EBP1, and not by other members of the pathway, but also that the combination causes no generalized translation inhibition.

Differing from the report by Choo and Blenis (45) and Choo and colleagues (46) who showed that with rapamycin, after an initial inhibitory effect on 4EBP1 phosphorylation, phosphorylation re-appeared, we saw no reactivation of the pathway at least until after 24 hours of treatment. This effect might be related to the cell specificity or other unknown alterations affecting the pathway in this particular context.

In conclusion, in this article, we report that the combination of a catalytic and an allosteric inhibitor of mTOR can be more active than the single drugs acting alone. The efficacy of this combination has been recently reported in melanoma cells both *in vitro* and *in vivo* (47), thus supporting our present observations. The simultaneous inhibition of the target by
action on distinct portions of the protein might be extended to other targets and, if proven, could offer an alternative approach to shut down a cascade of the signaling pathway. The evidence that the combination of inhibitors acting at different levels increased the activity but not the toxicity upholds this possibility, which needs to be tested in well-characterized systems and, probably, with the new and more selective inhibitors that are emerging, which might have clinical implications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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