COMBINATION OF PI3K/mTOR INHIBITORS: ANTITUMOR ACTIVITY AND MOLECULAR CORRELATES.

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Running title: In vitro and in vivo inhibition of PI3K/mTOR pathway

Key words: signal transduction, mTOR inhibitors, proteomics, xenograft

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ABSTRACT

The 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 harbour 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 analogues and caused more sustained inhibition of Akt phosphorylation. The combination also 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 may have therapeutic implications for inhibiting this pathway clinically.

INTRODUCTION

The phosphatidylinositol-3-kinase (PI3K) pathway is a key signal transduction pathway that regulates many physiological 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 PIP3. The lipid phosphatase PTEN counterbalances the process dephosphorylating PIP3 to PIP2, thus terminating PI3K-dependent signaling (6, 7).

PIP3 allows the recruitment of the main PI3K downstream effector Akt/PKB at membrane level and its subsequent activation through the phosphorylation of Thr 308 by PDK1 and 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, 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 those mRNAs with a long, highly structured 5′-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 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 anticancer 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. An interesting approach could also 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 in vitro and in vivo seem correlated with the molecular changes induced by the drugs alone and combined.

Our results provide evidence that targeting mTOR with two 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, human breast MCF-7 and MDA-MB-231 cells were grown in RPMI1640 medium. Human lung carcinoma A549 cells were cultured in DMEM.

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

Western blotting analysis

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

Real-time PCR

RNA was extracted using SV Total RNA Isolation (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 KARS. Primer sequences are reported in Supplemental Table S1. Real-time PCR was done using the 7900HT Sequence Detection System (Applied Biosystems).

Determination of eIF4E-4EBP1 complexes
One hundred μg of total protein extracts 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 IP buffer (50 mM Tris-hCl pH7.5, 150 mM NaCl, 10 mM NaF, 5 mM Na3VO4, 1% Triton X-100, proteases inhibitor); bound complexes were solubilized in 30 μl of loading buffer and proteins resolved by 15% SDS-PAGE. Immunoblots were done as previously described.

**Luciferase reporter assays**

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

**Two-dimensional gel electrophoresis (2-DE) image analysis and mass spectrometry**

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

**Protein network analysis**

MetaCore (GeneGo, St Joseph, MI) was used to map the differently expressed proteins into biological networks and for functional interpretation of the protein data as detailed in Supplemental 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 X 10^6 cells/mouse) were injected subcutaneously. Details of the in vivo experiments are available in Supplemental Methods.
RESULTS

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

To examine the *in vitro* cytotoxic effects of PI-103, the ovarian carcinoma cell line Skov-3 was 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%. PI-103 was also able to counteract the activation of Akt induced by insulin stimulation (Fig. 1C).

As shown in Fig. 1D, PI-103 inhibitd the phosphorylation of p-Akt(Ser473) and p-p70S6K(Thr389), while Erk phosphorylation remained unchanged. As for the mechanism of the G1 arrest, PI-103 treatment raised p27 levels, while p-cdc2 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 m-TOR 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 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 five cell lines with an altered PI3K/Akt/mTOR pathway were sensitive to various extent to both drugs and showed greater sensitivity when the two 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 (see Supplemental Figure 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 24h of treatment both PI-103-treated samples and the combination had more than 80% of cells arrested in G1-phase, while the effect on cell cycle was milder for rapamycin-treated sample (Fig. 2D and Supplemental Table S4). In the next 24h, 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 fewer G2 cells (7.4%) were present.

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

At 24h of recovery, cells treated with the rapamycin→PI-103 schedule and the concurrent one showed delayed cell cycle progression, indicating these schedules had a more lasting effect. The other sequence gave results similar to 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/m-TOR 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 compared to controls (Fig 3A) and the combination was more active than either drugs singly without any significant toxicity. Fig. 3B reports the densitometric analysis of the p-Akt/Akt ratio in tumors. Like in vitro, the combination lowered phosphorylation more than either PI-103 or RAD-001 alone.
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 to that of controls) using half the RAD-001 dose but the same dose of PI-103. This second cycle still inhibited the tumor growth but 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 seemed mainly reduced after the combined treatment, while p-S6 was almost completely lowered by RAD-001 alone (Fig. 3D).

"Co-targeting" mTOR enhances inhibition of mTORC1 and downstream pathways preventing Akt increase.

In Skov-3 cells (Fig. 4A left panel) rapamycin increased the amount of phospho-Akt (Ser473), while PI-103 had a partial inhibitory effect. The combination reduced phospho-Akt (Ser473) levels much more than the single treatments. Effects were similar in two other cell lines, Ovcar-3 and PC3 (Fig. 4A right panel).

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

As for downstream effectors of mTORC1, rapamycin strongly inhibited p70S6K phosphorylation. The addition of PI-103 did not boost this effect, though there was a partial decrease in S6 phosphorylation state, as expected, in the PI-103 treated sample (Fig. 4B). The other downstream effector of mTORC1 showed a different situation (Figure 4C and 4D). 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 while cells treated with the combination showed
only hypophosphorylated forms of 4EBP1. This was visible both in short experiments where 1h of treatment was followed by induction by insulin (Fig. 4C) and in longer experiments where cells were treated for 4 and 24 hours (Fig. 4D left panel). Once again we repeated the experiments 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 panel).

To better characterize the combination’s effects on translation-initiation events, total cellular protein extracts were incubated with the cap-analogue m7-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 to eIF4E. The different phosphorylation status of 4EBP1 can be seen as previously shown. Results were similar in Ovcar-3 and PC3 cells (Supplemental Figure S2).

A dual-luciferase reporter, that distinguishes cap-dependent from cap-independent translation was used in transfection experiments followed by 24h 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 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 5’-UTR, including c-myc, cyclin D1 and Bcl-XL. Fig. 5C right panel shows that c-myc protein levels were reduced by treatment, while c-myc mRNA levels (left panel), did not change.

We further examined the effect of treatment on c-myc translation by $[^{35}\text{S}]$ metabolic labeling. (for details see Supplemental Methods). As indicated in Fig 5D the combination greatly reduced the amount of newly synthesized c-myc, while 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 proteome profile of Skov-3 cells exposed to either the single drugs or the combination with their untreated counterpart.

Overall, 33 protein species out of 973 visualized showed a statistically significant change in abundance as a result of drug treatments (Supplemental Figure S3). All these species were positively identified by mass spectrometry and are reported in Supplemental Table S5.

Drug treatment induced a general down-regulation 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 analysed 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 down-regulated 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 (Supplemental Figure S4). Again, the most prominent regulatory element in this network was c-myc, which interacts directly with twelve 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, that generates sub-networks centered on transcription factors (Supplemental Figure S5). Again, c-myc was the most significant transcription factor regulating the cell proteomic profile affected by either the combination or rapamycin. The three top-ranked transcription factor sub-networks for rapamycin and the combination were merged and visualized in Figure 7, panels A and B. C-myc was regulating the expression of AHCY,
eIF4A1, VCP, MSN, EEF14G, TCP1 and LAMNA whose reduced abundance was common with both rapamycin and the combination, while the down-regulation of RUVBL1, KARS, GNAB1 was specific to the combination. The c-myc-related reduced expression of 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 ChIP (for details see Supplemental Methods) to measure c-myc binding to KARS promoter and Real-Time PCR for its mRNA levels. c-myc ChIP showed less precipitation of the target DNA after treatment with the two single drugs (Fig. 7C). The combined treatment further enhanced the effect. In agreement with this, KARS expression was down-regulated by the combination more than the two single treatments (Fig. 7D). Cells overexpressing an exogenous c-myc without UTRs (not sensitive to translational regulation, pCGN-cmyc kindly provided by Dr WP. Tansey) showed no drop in KARS (by Real Time PCR) and c-myc (by Western blotting) after both single drugs and the combination (Figure 7E).
DISCUSSION

Several mTOR inhibitors have been synthesized and their activity at 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 directly phosphorylates and activates Akt (10, 30), but also promotes eIF4E Ser209 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 two 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 pAkt was greater with the combination than with the single drugs. At cell cycle level too we found a positive correlation between the effects in terms of growth inhibition and the mechanism of action. The G1 arrest obtained when cells were treated with the two drugs was in fact more sustained and cells re-entered cell cycle more slowly. The schedule-dependence observed here agrees with the proposed effect on cell cycle and with other published observations. In vivo experiments confirmed the additive effect observed in vitro.

As for 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 led 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 while 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 the combination of rapamycin and PI-103 caused progressive de-phosphorylation of 4EBP1 which 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 towards 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 transcriptional level further corroborates the evidence that c-myc down-regulation 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 reduced as confirmed by Western Blot analysis and $^{35}$S pulse labeling.

Interestingly, only the drug combination almost halved the expression of KARS and reduced its transcript. 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 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 aminoacids in the cell. Since recent evidence suggests that mTOR might respond to tRNA and/or to aminoacids (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 the single treatments and the combination indicates not only that treatment mainly inhibits the mTOR pathway through 4EBP1 and not other members of the pathway, but also that the combination causes no generalized translation inhibition.
Differently from Choo et al (45, 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 after 24h of treatment. This effect might be related to the cell specificity or other unknown alterations affecting the pathway in the particular context.

In conclusion, we report here that the combination of a catalytic and an allosteric inhibitor of mTOR can be more active than the single drugs 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.
REFERENCES


LEGENDS TO THE FIGURES

Figure 1. PI-103 reduces the growth of Skov-3 modulating cell cycle and PI3K pathway

A) Cytotoxicity induced by 24h PI-103 treatment in Skov-3 cells. Results are reported as the percentage of inhibition relative to controls and are the mean +/- SD of at least six replicates. B) Cell cycle effects determined by FACS analysis in Skov-3 cells after 24h PI-103 treatment. C) Concentration-dependent inhibition of Akt phosphorylation (Ser473) after 1h PI-103 treatment with the concentrations indicated followed by 5 min of 100 nM insulin stimulation. Before treatment cells were starved for 12 h. D) Representative Western blot reporting the effects of PI-103 on components of the PI3K pathway and cell-cycle related proteins.

Figure 2. PI-103 exerts positive association in combination with rapamycin.

A) Isobologram analysis showing the synergistic effects of the combination considering both the IC30 and IC50.

B) Effects of PI-103 400nM and rapamycin 1nM singly or combined in Skov-3 (○), Ovcar-3 (■), mcf-7 (Δ), PC3 (□), A549 (○) and MDA-231 (●) cells. Values are the mean of three independent experiments each consisting of six replicates and are expressed as the percentage of inhibition relative to controls.

C) Sequence-dependence of the combination. Absolute cell counts were done every 24h. Values are the mean +/- SD of three independent experiments each run in triplicate. Untreated cells (♦), PI-103 (■), rapamycin (□), PI-103 plus rapamycin (Δ).

D) Effect of drugs, singly or combined on Skov-3 cell cycle phase distribution determined by FACS analysis. Cells were treated for 24h with the concentrations indicated of drugs then cultured for additional 72h. They were analysed every 24h. Images are representative of one experiment. Experiments were repeated twice with closely comparable results. “T” indicates treatment, and “R” stands for recovery (incubation in drug-free medium) after treatment.
Figure 3. The drug combination inhibits the growth of ovarian Skov-3 and prostate PC3 xenografts.

A) Antitumor activity on Skov-3. Mice were given a daily i.p. dose of PI-103 10 mg/kg (■) or RAD001 5 mg/kg orally (∆) or both (○) for 10 days (from day 15 to 24). (●) Untreated mice. * p<0.05 and ** p<0.01 compared to untreated animals.

B) Densitometric analysis showing the level of p-Akt S(473) 24h after a single dose. Results are the mean density of three replicates +/- SD and are the ratio of p-Akt Ser(473) to total Akt.

C) Antitumor activity on PC3. Mice were given a daily i.p. dose of PI-103 10 mg/kg (■) or RAD001 5 mg/kg orally (∆) or both (○) for 10 days (from day 20 to 29). In the second treatment cycle the RAD001 dose (from day 60 to 66) was reduced to 2.5 mg/kg while the PI-103 dose was not changed. (●) Untreated mice. * p<0.05 compared to RAD-001 treated animals.

D) Representative Western blot after a single treatment on PC3 xenografted mice.

Figure 4. Effects of treatment on PI3K pathway molecular effectors.

A) p-Akt (Ser473) levels after 4h or 24h treatment on Skov-3 (left panel) and 24h on Ovcar-3 and PC3 (right panel).

B) phospho-state of p70S6K(Thr389) and S6(Ser235/6) ribosomal protein after 24h of treatment on Skov-3.

C) p-4EBP1 (Thr 37/46) levels in Skov-3 cells starved O/N then treated with the indicated drugs concentrations for 1h. Cells were stimulated for 5 or 10 minutes with insulin 100 nM before protein extraction.

D) 4EBP1 levels in Skov-3 cells after 4h or 24h of treatment (left panel) and 24h (right panel) with the indicated drug concentrations.
**Figure 5.** The drug combination affects the phospho-state of 4EBP1, reducing the cap-dependent translation of c-Myc.

A) eIF4E, eIF4G and 4EBP1 co-precipitated with m7-GTP Sepharose beads. Actin was used as internal loading control and to check 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 three replicates +/- SD and show the difference in expression from untreated cells arbitrarily set at 1.

D) $^{35}$S pulse labeling experiment in Skov-3 cells after single and combined treatment for 24 hours. IP c-myc (upper panel) and total input (lower panel) are reported for each treatment.

D) PDCD4 protein levels after 24h of drug treatment.

**Figure 6.** Protein networks

The protein-protein interaction network generated by *the shortest path algorithm* using the list of differently expressed proteins in Skov-3 cells in response to the drug combination. Thick lines highlight direct interactions between c-Myc and a subset of proteins identified in the study. Nodes represent proteins and the different shapes of the nodes indicate their functional class. Circles represent proteins identified in the proteomic analysis. Lines connecting the nodes indicate interactions of activation or induction, modification or direct binding; the arrowheads indicate the direction of the interaction. The protein nodes SAHH, Lamin A/C, Glucosidase II, AL1A1, eEF1G refer to proteomic abbreviations AHCY, LMNA, GNAB1,ALDH1A1, EEF14G respectively.

**Figure 7.** Transcription factor networks (c-Myc, SP1, GCR-alpha) generated by the *transcription regulation algorithm* using the list of differently expressed proteins in Skov-3 cells in response to
rapamycin (panel A) and rapamycin + PI-103 (panel B). The protein nodes SAHH, Lamin A/C, Glucosidase II, AL1A1, eEF1G, GRP78, HSP60 refer to proteomics abbreviations AHCY, LMNA, GNAB1, ALDH1A1, EEF14G, HSPA5, HSPD1 respectively.

C) Chromatin Immunoprecipitation (ChIP) was done using anti c-myc antibody on KARS promoter after 24h treatment. D) KARS mRNA levels in untreated and treated Skov-3 cells. E) Upper panel: Real Time PCR showing the levels of KARS mRNA in Skov-3 cells previously transfected with a c-myc construct lacking 5’UTR and 12 hours later treated for another 24h with the drugs indicated. Lower panel shows exogenous c-myc expression levels to confirm transfection efficiency and the absence of treatments-mediated modulatory effects. 7D and 7E results were the mean of three replicates +/- SD and indicate the change in expression relative to untreated cells arbitrarily set at 1. In panels 7C, D and E, treatment conditions (PI-103 400nM, rapamycin 1nM and the combination) were identical.
**A**

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1 1.6 2.5 3.2

**B**

RIF Ratio

![Graph showing RIF Ratio](image)

- untreated
- Rapamycin
- PI-103
- Comb

**C**

Fold Change

![Graph showing Fold Change](image)

- Ctrl
- PI-103
- Rapamycin
- Comb

**D**

- c-myc IP
- input

![Graph showing c-myc](image)

**E**

- PDCD4
- Actin

![Graph showing PDCD4 and Actin](image)
COMBINATION OF PI3K/mTOR INHIBITORS: ANTITUMOR ACTIVITY AND MOLECULAR CORRELATES

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