Inhibition of mTOR pathway by everolimus cooperates with EGFR inhibitors in human tumours sensitive and resistant to anti-EGFR drugs

R Bianco¹, S Garofalo¹, R Rosa¹, V Damiano¹, T Gelardi¹, G Daniele¹, R Marciano¹, F Ciardiello² and G Tortora*¹
¹Cattedra di Oncologia Medica, Dipartimento Endocrinologia e Oncologia Molecolare e Clinica, Università di Napoli Federico II, via S. Pansini 5, Napoli 80131, Italy; ²Dipartimento Medico-Chirurgico di Internistica Clinica e Sperimentale, Seconda Università di Napoli, via S. Pansini 5, Napoli 80131, Italy

Inhibition of a single transduction pathway is often inefficient due to activation of alternative signalling. The mammalian target of rapamycin (mTOR) is a key intracellular kinase integrating proliferation, survival and angiogenic pathways and has been implicated in the resistance to EGFR inhibitors. Thus, mTOR blockade is pursued to interfere at multiple levels with tumour growth. We used everolimus (RAD001) to inhibit mTOR, alone or in combination with anti-EGFR drugs gefitinib or cetuximab, on human cancer cell lines sensitive and resistant to EGFR inhibitors, both in vitro and in vivo. We demonstrated that everolimus is active against EGFR-resistant cancer cell lines and partially restores the ability of EGFR inhibitors to inhibit growth and survival. Everolimus reduces the expression of EGFR-related signalling effectors and VEGF production, inhibiting proliferation and capillary tube formation of endothelial cells, both alone and in combination with gefitinib. Finally, combination of everolimus and gefitinib inhibits growth of GEO and GEO-GR (gefitinib resistant) colon cancer xenografts, activation of signalling proteins and VEGF secretion. Targeting mTOR pathway with everolimus overcomes resistance to EGFR inhibitors and produces a cooperative effect with EGFR inhibitors, providing a valid therapeutic strategy to be tested in a clinical setting.

Keywords: angiogenesis; drug resistance; EGFR; everolimus; mTOR

The mammalian target of rapamycin (mTOR), also known as FKBP12 rapamycin-associated protein, rapamycin target or sirolimus effector protein, is a serine/threonine-specific kinase responsible for mitogen-induced cell proliferation/survival signalling (Bjornsti and Houghton, 2004; Abraham and Gibbons, 2007). In mammalian cells, mTOR signalling depends on signal transmission through the phosphoinositide 3-kinase (PI3K)/Akt pathway. Activated Akt phosphorylates TSC2, a component of tuberous sclerosis complexes 1 and 2, sensitive to nutrient status, and favours mTOR activity (Manning et al, 2002; Zhang et al, 2003). An important function of mTOR is the translation control via activation of ribosomal p70S6 kinase (S6K1) and suppression of 4E-BPs, resulting in enhanced translation of mRNAs encoding cell-cycle regulators and promotion of G1–S cell-cycle transition (Schmelzle and Hall, 2000; Bjornsti and Houghton, 2004). The mammalian target of rapamycin signalling is dependent on insulin, growth factors, and nutrition status, and nutrient deficiency leads to mTOR inhibition.

Mammalian target of rapamycin is an essential part of tumour growth being capable of integrating proliferative, antiapoptotic and angiogenic signalling by connecting VEGF, hypoxia-inducible factor 1 (HIF-1) and HER family receptors. VEGF is a potent promoter of angiogenesis, and its overexpression is associated with tumour progression and poor clinical outcome. Hypoxia-inducible factor 1, a heterodimeric protein consisting of HIF-1α and HIF-1β, controls the transcription of genes involved in cell proliferation/survival, invasion/metastasis and angiogenesis, including VEGF. Hypoxia-inducible factor 1 is degraded through ubiquitylation involving the von Hippel–Lindau tumour-suppressor protein, whose loss of function, detected in renal cancer and neuroendocrine tumours, impairs HIF-1α degradation and favours the stimulation of mTOR signalling (Mellilo, 2007). Finally, it has been reported that HER2 activation in normoxic cells increases HIF-1α via PI3K, Akt and mTOR (Laughner et al, 2001), and that EGF induces HIF-1α, which can be prevented by using mTOR inhibitors under both normoxic and hypoxic conditions (Phillips et al, 2005). Taken together, these studies demonstrate that mTOR is a positive regulator of HIF-1α translation and an inducer of HIF-1α/VEGF-dependent angiogenesis. In addition, signalling through mTOR is stimulated by defects in the pathway components upstream of mTOR, such as growth factor receptors, PI3K, Akt, PTEN, TSC1/TSC2, or by stimulation of PI3K by effectors of the mutant Ras/Raf/MAPK pathway (Bjornsti and Houghton, 2004).

In view of the above-mentioned facts, several mTOR inhibitors rapamycin-analogues have been developed, including temsirolimus.
**mTOR inhibition in EGFR-drugs-resistant tumours**

R Bianco et al

(CCI-779), everolimus (RAD001) and AP23573 (Hidalgo and Rowinsky, 2000; Rowinsky, 2004). Clinical studies have been very encouraging in renal cell cancer. In a multicenter phase 3 trial, temsirolimus improved overall survival in metastatic renal-cell carcinoma patients with poor prognostic score, as compared with IFN-α; however, the addition of temsirolimus to IFN did not improve survival (Hudes et al, 2007). The potential efficacy of these new agents is now under evaluation in patients with other solid tumours (Janus et al, 2005; Smolewski, 2006; Fouladi et al, 2007).

Everolimus (40-O-(2-hydroxylpropyl)-rapamycin) is an orally bioavailable derivative of rapamycin under clinical evaluation in different types of cancer (Boulay et al, 2004), including renal cell cancer and endometrial cancer (Sansal and Sellers, 2004). Moreover, everolimus in combination with imatinib mesylate is under study in patients with gastrointestinal stromal tumours (Van Oosterom et al, 2004). Phase I/II trials of everolimus in combination with erlotinib, in patients with metastatic breast cancer and advanced NSCLC are currently undergoing.

EGFR is a major transducer of mitogenic signals involved in cancer pathogenesis and progression (Citri and Yarden, 2006) upstream of mTOR and an important target for anticancer therapy (Baselga and Arteaga, 2005; Hynes and Lane, 2005; Mendelsohn and Baselga, 2006). Several anti-EGFR drugs, such as monoclonal antibodies directed against the extracellular domain and small molecule inhibitors of the receptor-associated tyrosine kinase, have been approved for clinical use (Cunningham et al, 2004; Bezjak et al, 2006). However, a relevant percentage of patients treated with these drugs has no clinical benefit or incurs in the development of acquired resistance. Constitutive and acquired resistance to EGFR inhibitors in a large number of patients is a relevant clinical problem. Frequently, activation of signalling pathways downstream or alternative to EGFR may be responsible for the development of resistance to these inhibitors (Sansal and Sellers, 2004). We, and others, have previously shown that colon tumours that acquire resistance to anti-EGFR drugs cetuximab and gefitinib overexpress Akt and VEGF, which act as the escape pathways to overcome the EGFR blockade (Viloria-Petit et al, 2001; Ciardiello et al, 2004; Bianco et al, 2005). As mentioned above, the PI3K-AKT pathway connects EGFR and mTOR.

The link between HIF-1 and EGF and the reported ability of mTOR inhibitors rapamycin and everolimus to reduce VEGF secretion in vitro and in vivo, renders mTOR inhibitors potentially effective against cancer cells resistant to EGFR inhibitors (Viloria-Petit et al, 2001; Ciardiello et al, 2004; Bianco et al, 2005). In addition, targeting together mTOR and EGFR might cause a more profound effect on tumour growth control compared with a single agent (Hynes and Lane, 2005).

In this study, we have evaluated (1) the antitumour effect of everolimus on different human cancer cell lines, sensitive or resistant to EGFR inhibitors, in vitro and in nude mice; (2) the possibility to restore sensitivity to EGFR inhibitors, using everolimus in combination with gefitinib or cetuximab; (3) the effect of treatment on signalling pathways and VEGF.

**MATERIALS AND METHODS**

**Drugs**

Everolimus, gefitinib and cetuximab were provided by Novartis International AG (Basel, Switzerland), Dr Anderson Ryan (AstraZeneca Pharmaceuticals, Macclesfield, UK) and ImClone Systems (New York, NY, USA).

**Cell lines**

Human GEO colon, PC3 prostate and MDA-MB-468 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). GEO-GR (gefitinib resistant), GEO-GF and PC3-GR (gefitinib resistant) cells were established as described previously (Ciardiello et al, 2004).

**Growth in soft agar**

Cells (104 cells per well) were suspended in 0.3% Difco Noble agar (Difco, Detroit, MI, USA) in culture medium, layered over 0.8% agar medium base layer in 24-well plates (Becton Dickinson, Lincoln Park, NJ, USA) and treated with gefitinib, cetuximab or everolimus. After 10 – 14 days, cells were stained and colonies up to 0.05 mm were counted (Ciardiello et al, 2004).

**Cell-survival assay**

Cells were grown in 24-well plates in the presence of everolimus (1 nM to 1 µM), gefitinib (1 or 5 µM) or their combination. After removing supernatant, 1 mg ml–1 of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Milan, Italy) solution in medium was added to each well. After adding isopropanol, absorbance was measured at 570 nm.

**Western blot analysis**

Total cell lysates were obtained from homogenised tumour specimens or from cells. Protein extracts resolved by a 4 – 20% SDS-PAGE were probed with different antibodies: anti-Akt, anti-phospho (Ser473)-Akt (Cell Signaling, Beverly, MA, USA); anti-ERK 1/2, anti-phospho-ERK 1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-S6K1, anti-phospho S6K1, anti-VEGF (Santa Cruz Biotechnology); anti-actin (Sigma-Aldrich, Milan, Italy). Immunoreactive proteins were visualised by enhanced chemiluminescence (Amersham International, London, UK) (Ciardiello et al, 2004).

**ELISA assay**

VEGF concentrations in conditioned media, total cell lysates from homogenised tumour specimens and serum of sacrificed mice were determined by ELISA (Guba et al, 2002). The absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Hercules, CA, USA) and VEGF concentrations were determined using linear regression analysis.

**Xenografts in nude mice**

Balb/CAnNcrlBR athymic (nu/+nu+/) mice (Charles River Laboratories, Milan, Italy) were maintained in accordance with institutional guidelines of the University of Naples Animal Care Committee and Declaration of Helsinki. GEO and GEO-GR cells (106 cells per mice) resuspended in Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) were injected subcutaneously. After 7 days, groups of 10 mice were randomised to receive treatments: intraperitoneal gefitinib, 100 mg kg–1, three times a week for 3 weeks; everolimus (by gavage), 5 mg kg–1, three times a week for 3 weeks; or their combination, on days 7–11, 14–18 and 21–25. Tumour volume was measured using the formula π/6 × larger diameter × (smaller diameter)2 (Ciardiello et al, 2004). Two mice were sacrificed on day 25 for biochemical analysis.

**Vascular endothelial cell capillary tube and network formation**

Matrigel was diluted in DMEM, added into a 30-mm culture dish and incubated at 37°C for 30 min; then HUVECs (4 × 105) in RPMI medium were added in each dish, in presence of everolimus 0.1 µM, gefitinib 5 µM or the combination. The positive control was
matrigel with VEGF 100 ng ml\(^{-1}\) (R&D Systems, Minneapolis, MN, USA). Pictures were taken at 0 and 24 h.

**Statistical analysis**

The Student’s t-test and the Mantel–Cox log-rank test were used to evaluate the statistical significance of the results. All P-values were two-sided. Analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA, USA).

**RESULTS**

**Everolimus is active in cancer cell lines resistant to gefitinib and cetuximab and partially restores sensitivity to these EGFR inhibitors**

To evaluate the effect of everolimus on cancer cell growth, we have performed a soft agar assay on different cancer cells. Wild-type human colon cancer GEO cells are sensitive to both cetuximab and gefitinib, with an IC\(_{50}\) of less than 0.5 μg ml\(^{-1}\) and 0.5 μM, respectively. Wild-type human prostate cancer PC3 cells demonstrate the same pattern of sensitivity to gefitinib, but are resistant to cetuximab at doses up to 20 μg ml\(^{-1}\). GEO-CR, GEO-GR and PC3-GR human cancer cell lines were established *in vitro* from GEO or PC3 tumours treated continuously for 16 weeks with either gefitinib or cetuximab. These derivative cell lines are resistant to gefitinib or cetuximab. These derivative cell lines are resistant to gefitinib or cetuximab. These derivative cell lines are resistant to gefitinib or cetuximab.

Regardless of the degree of sensitivity to EGFR inhibitors and PTEN status, everolimus caused an efficient dose-dependent inhibition of soft agar growth in all cell lines, with an IC\(_{50}\) ranging between 1 and 5 μM, (Figure 1A). To evaluate the everolimus potential to restore sensitivity to gefitinib and cetuximab in cancer cell lines resistant to these anti-EGFR drugs, we performed a soft agar assay on cells treated with doses of cetuximab or gefitinib ranging from 0.1 to 1 μg ml\(^{-1}\) and from 0.1 to 1 μM, respectively, in presence of everolimus 0.1 μM. Wild-type GEO cells showed a reduction of cetuximab IC\(_{50}\) from 0.5 to 0.2 μg ml\(^{-1}\) and gefitinib IC\(_{50}\) from 0.5 to 0.2 μM. Wild-type PC3 cells showed a reduction of gefitinib IC\(_{50}\) from 0.5 to 0.2 μM, and they became sensitive to cetuximab (IC\(_{50}\) 0.8 μg ml\(^{-1}\)). In all the resistant cell lines, everolimus in combination with cetuximab or with gefitinib restored dose-dependent growth inhibition induced by anti-EGFR drugs (Figure 1B and C).

**Figure 1** (A) Cells were treated with everolimus (0.01–20 μM), (B and C) Cells were treated with cetuximab (0.1–1 μg ml\(^{-1}\)), and gefitinib (0.1–1 μM), in presence of everolimus, 0.1 μM. (D) Western blotting on total cell lysates from cells treated with 1 μM gefitinib, 1 μg ml\(^{-1}\) cetuximab or 0.1 μM everolimus. Lane 1: untreated control; lane 2: cetuximab; lane 3: gefitinib; lane 4: everolimus.
Everolimus inhibits mTOR pathway in resistant cancer cells

As mTOR signalling is dependent on growth factors, including EGF, we compared everolimus with gefitinib or cetuximab in both wild type and EGFR inhibitor-resistant cancer cell lines, focusing on the mTOR effector p70S6 Kinase. Western Blot analysis revealed that in wild-type GEO and PC3 cells, everolimus (0.1 μM) reduces p70S6K phosphorylation similarly to gefitinib or cetuximab, confirming the role of EGFR in mTOR signalling regulation. In resistant GEO-GR, GEO-GR, PC3-GR and MDA-468 cells, EGFR inhibitors were ineffective on p70S6K activity, whereas everolimus caused a complete suppression of p70S6K phosphorylation (Figure 1D).

Everolimus restores the survival-inhibitory effects of anti-EGFR drugs in resistant cancer cells

To verify the effects of everolimus alone or in combination with gefitinib on wild-type GEO and GEO-GR cell lines, we performed a cell-survival assay. As expected, gefitinib (5 μM) induced a 50% cell-survival inhibition in GEO cells, whereas it was totally ineffective on GEO-GR cells. Everolimus (0.1 μM) was effective on GEO cells, with a cell-survival inhibition of about 25% and it showed a slight activity on GEO-GR cells. Combination of everolimus and gefitinib improved the cell-survival inhibition caused by gefitinib alone in sensitive GEO cells, and it partially reverted the resistance to gefitinib in GEO-GR cells with a cell-survival inhibition of about 30% (Figure 2A). These results refer to the simultaneous administration of the two agents. This modality was chosen because previously we performed a series of experiments to test different sequences of administration of the two drugs, comparing either the use of everolimus 1, 6 or 24 h before gefitinib, or gefitinib before everolimus or their simultaneous treatment. We observed no significant differences among the different modalities used (data not shown).

As Akt is involved in pathways regulating cell survival, we investigated the effect of the combined treatment with everolimus and gefitinib on Akt phosphorylation/activation. As expected, gefitinib is able to inhibit Akt activation in GEO, but not in GEO-GR cells, whereas everolimus has no effect on Akt phosphorylation in both cell lines (Figure 2B). Conversely, the combination of everolimus and gefitinib strongly reduces pAkt levels, to a greater extent in GEO-GR than in GEO cells (Figure 2B).

Everolimus reduces the secretion of VEGF in wild type and resistant cancer cells

Based on the reported ability of mTOR to affect VEGF levels (Guba et al, 2002), we evaluated the effect of everolimus on VEGF production in our cancer cell lines. As shown in Figure 3A, everolimus reduces human VEGF (hVEGF) secretion of 20–45% both in wild type and in resistant cell lines, compared with untreated controls. We then measured hVEGF by ELISA assay on conditioned media derived from GEO and GEO-GR cancer cell culture treated for 24 h with everolimus (0.1 μM) and gefitinib (5 μM) alone or in combination. Gefitinib significantly reduces hVEGF levels only in wild-type GEO cells, whereas everolimus is active in both cell lines, especially in resistant GEO-GR cells. Combined treatment with gefitinib and everolimus causes a strong reduction of hVEGF levels both in GEO and in GEO-GR cells (Figure 3B and C).

Everolimus inhibits vascular endothelial cells proliferation and VEGF-mediated angiogenesis

Based on evidence that the mTOR-p70S6K signalling pathway is required for VEGF stimulation of endothelial cells (Yu and Sato, 1999), we verified whether everolimus could inhibit angiogenesis at a second level, where receptor-mediated stimulation of vascular endothelial cells occurs. To investigate this hypothesis, we first evaluated the capability of everolimus to inhibit HUVECs (human umbilical vein endothelial cells) survival: HUVECs are sensitive to everolimus, with a 35% survival inhibition at 0.1 μM (Figure 3D). As shown in Figure 3E, combined treatment with everolimus (0.01 μM) and gefitinib (1 μM) caused a 50% inhibition of HUVEC survival, with a cooperative effect compared with the single agents. We then examined the effects of everolimus on VEGF-stimulated capillary tube and network formation, and we found that this process is inhibited by everolimus 0.1 μM, by gefitinib 5 μM and almost suppressed by the combination of the two drugs (Figure 3F). Thus, VEGF-dependent HUVEC proliferation and tube formation seem to be very sensitive to everolimus and gefitinib inhibition.

Combination of everolimus with gefitinib cooperatively inhibits GEO and GEO-GR colon cancer xenografts growth

To further investigate the role of everolimus on cancer cells resistant to EGFR inhibitors, we established GEO and GEO-GR xenografts by using BalbC nude mice. As expected, treatment with gefitinib alone was effective on GEO xenografts, but ineffective on GEO-GR xenografts, because, on day 56, all mice in this group reached the maximum allowed tumour size of about 2 cm³. Conversely, treatment with everolimus produced a 50% tumour growth inhibition, with an average tumour size of about 1.0 cm³ at the same time point in both xenograft models. The combination of everolimus and gefitinib caused a cooperative antitumour activity, resulting in over 90% tumour growth inhibition on day 56 (tumour...
Treatment-related side effects were observed in the treated mice. Differences among the groups were calculated by log-rank test. No experiment, 80% of mice were still alive (Figure 4C and D).

In GEO-GR-xenografted mice, in fact at the end of the injection, with a median gain in survival compared with untreated animals. Mice treated with everolimus alone reached the tumour size of about 2 cm$^3$ 13–14 weeks after tumour injection, with a median gain in survival compared with untreated animals of 5 weeks in GEO-xenografted mice and 7 weeks in GEO-GR mice. Combined treatment with everolimus and gefitinib produced a dramatic survival prolongation not only in GEO but also in GEO-GR-xenografted mice (Figure 4B). At the end of the experiment, 10% of GEO-GR tumour growth inhibition was still evident and tumour size was about 1 cm$^3$ (Figure 4B). Comparison of tumour sizes among different treatment groups, evaluated by the Student’s t-test, was statistically significant.

The treatment with gefitinib alone prolonged strongly the survival of GEO-xenografted mice (about 20% of mice were still alive at the end of the experiment), but no significant gain of survival was observed for GEO-GR-xenografted mice, compared with untreated animals. Mice treated with everolimus alone reached the tumour size of about 2 cm$^3$ 13–14 weeks after tumour injection, with a median gain in survival compared with untreated animals of 5 weeks in GEO-xenografted mice and 7 weeks in GEO-GR mice. Combined treatment with everolimus and gefitinib produced a dramatic survival prolongation not only in GEO but also in GEO-GR-xenografted mice, in fact at the end of the experiment, 80% of mice were still alive (Figure 4C and D). Differences among the groups were calculated by log-rank test. No treatment-related side effects were observed in the treated mice.

Combination of everolimus and gefitinib inhibits the expression of signalling proteins in GEO-GR xenografts

We analysed the effect of treatment on the expression of a variety of proteins having a critical role in cancer cell proliferation and angiogenesis. Western blotting analysis was performed on cell lysates from tumours removed at the end of the third week of treatment, on day 25. Everolimus markedly reduced mTOR effector phospho-p70S6K in GEO-GR xenografts, whereas gefitinib, as expected, was ineffective. The combination of everolimus with gefitinib caused a complete suppression of p70S6K phosphorylation/activation. When used alone, gefitinib and everolimus produced only a slight reduction of activity of EGFR effectors Akt and MAPK; in addition, the combined treatment efficiently reduced phospho-Akt and phospho-MAPK expression without affecting the total amount of p70S6K, Akt and MAPK. Finally, gefitinib did not modify VEGF expression, whereas everolimus alone caused a moderate VEGF reduction further improved by the addition of gefitinib (Figure 5A).

Combination of everolimus and gefitinib reduces the levels of hVEGF, but not of murine VEGF, in GEO-GR tumour specimens and in mice serum

To further investigate the effect of treatment on VEGF levels, we performed ELISA assays on protein extracts from tumour specimens and on serum derived from GEO-GR xenografts. Treatment with gefitinib caused only a slight reduction of both intratumour and circulating hVEGF levels, whereas everolimus treatment reduces hVEGF of about 25% both in tumour specimens and in serum. Combined treatment with everolimus and gefitinib induces a more potent inhibition of hVEGF levels as compared with treatment with single agents (Figure 5B and C). Conversely, neither single agent nor their combination affects murine VEGF (mVEGF) as compared with untreated mice (data not shown).
In the past few years, we have learned that rational combination of targeted therapeutics may achieve a more potent antitumour effect and help to overcome the development of resistance, an emerging clinical issue often responsible for the failure of most modern antitumour approaches. In the case of EGFR and mTOR signalling pathways, many experimental data suggest that these pathways share overlapping signalling outputs (Adjei, 2006). Moreover, continued activation of PI3K/Akt signalling, which triggers mTOR, seems to contribute to the development and maintenance of an EGFR-resistant phenotype (Chakravarti et al., 2002; Vivanco and Sawyers, 2002; Bianco et al., 2003; Janmaat et al., 2003), and it has been found in tumour samples from cancers patients failed in EGFR-targeted therapy (Rojo et al., 2006). These evidences support the hypothesis that a more efficient antitumour effect may result from the combined blockade of EGFR and mTOR signalling pathways. Preclinical evidences demonstrate that this approach may be particularly effective, at least in some experimental models in which the combination of EGFR antagonists, such as gefitinib or erlotinib, and mTOR inhibitors, such as rapamycin and everolimus, resulted in an enhanced tumour cell growth control (Gemmill et al., 2005; Goudar et al., 2005; Rao et al., 2005; Wang et al., 2006; Jimeno et al., 2007). Recently, a pilot study including 28 heavily pretreated patients with recurrent malignant gliomas treated with the combination of gefitinib or erlotinib and rapamycin reported interesting results in terms of toxicity and tumour response (Doherty et al., 2006).

In the present study, we demonstrated that blocking mTOR activity might induce inhibition of cancer cell proliferation and survival. Interestingly, the antiproliferative effect exerted by the specific mTOR inhibitor everolimus does not strictly rely on the integrity of an EGFR-driven signal transduction pathway. In fact, everolimus is not only effective in tumour cells with acquired resistance to anti-EGFR drugs, but it is also able to induce a re-sensitisation against this class of inhibitors. In our experimental model, constitutive activation of the mTOR target p70S6K, which may be phosphorylated by Akt-dependent and Akt-independent mechanisms, invariably correlates with lack of response to EGFR antagonists. In addition, mTOR targeting by the specific inhibitor everolimus not only results in significant antiproliferative activity but also restores sensitivity to anti-EGFR drugs in resistant cancer cells, producing a strong reduction of Akt activation, when used in combination with anti-EGFR drugs. Although sequence has been reported to be important in some papers, in this study, we have chosen the simultaneous administration of everolimus and anti-EGFR drugs as treatment modality, because a series of experiments showed no significant differences compared with sequential administration.

It is known that inhibition of mTOR pathway correlates with decreasing of angiogenesis: in fact, rapamycin is able to reduce tumour vascularisation by promoting endothelial cell death and inducing increased susceptibility of tumour-specific vessels to thrombosis (Guba et al., 2005). Moreover, mTOR inhibition improves the antiangiogenic activity of anti-VEGF antibody (Stephan et al., 2004). It has also been clearly demonstrated that rapamycin impaires VEGF production and secretion in human cancer cells in vitro and endogenous VEGF in serum derived from mice bearing human tumours (Guba et al., 2002). These preclinical data have been confirmed by recently reported clinical data with temsirolimus in combination with antiangiogenic agents. In a phase I trial in patients with measurable stage IV clear cell renal cell carcinoma, combination therapy with temsirolimus and bevacizumab was safe and showed promising clinical antitumour activity.

DISCUSSION

In the present study, we demonstrated that blocking mTOR activity might induce inhibition of cancer cell proliferation and survival. Interestingly, the antiproliferative effect exerted by the specific mTOR inhibitor everolimus does not strictly rely on the
(Merchan, 2007). A phase I study of temsirolimus in combination with sorafenib in patients with advanced solid malignancies also produced good results, without the evidence of drug–drug interactions (Patnaik, 2007). Interestingly, although EGFR inhibition induces a VEGF reduction in both protein extracts and conditioned media of wild-type tumour cells, only everolimus efficiently inhibits VEGF levels in EGFR inhibitor-resistant cells. Moreover, we have shown that the antiangiogenic effect of everolimus correlates not only with the reduction of VEGF by cancer cells but also with a direct inhibitory effect on endothelial cells, as proven by its ability to inhibit HUVEC proliferation and tubular formation alone and in combination with gefitinib.

The combined treatment with gefitinib and everolimus potentiates antitumour and antiangiogenic effects also in mice bearing GEO and GEO-GR xenografts, in which we observed a cooperative antitumour activity resulting in over 90% tumour growth inhibition on day 56, a dramatic survival prolongation; these effects correlate with a potent inhibition of Akt activation and with a serum reduction of hVEGF but not of mVEGF. The antiangiogenic activity of an EGFR and mTOR combined inhibition has been reported by others. Jimeno and co-workers (Jimeno et al, 2007) demonstrated that the combination of temsirolimus and erlotinib results in a synergistic antitumour effect against squamous cell carcinoma cell lines, sensitive or resistant to EGFR inhibitors.

In conclusion, mTOR inhibition causes antitumour activity in EGFR-resistant cancer cell lines and xenografts, and this effect seems to be mediated by inhibition of survival signalling pathways and angiogenesis. The combination of everolimus with an EGFR inhibitor potentiates this effect, and it may resensitise resistant cancer cells to EGFR antagonists. In light of the recent approval of mTOR inhibitors, these results support the clinical development of anti-EGFR and mTOR drugs in combination.

ACKNOWLEDGEMENTS

This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), the Ministry of Health Strategic Project and the Regione Campania. We acknowledge the excellent technical assistance of Gaetano Borriello.

REFERENCES

Abraham RT, Gibbons JJ (2007) The mammalian target of rapamycin signaling pathway: twists and turns in the road to cancer therapy. *Clin Cancer Res* 13: 3109 – 3114

Adjei AA (2006) Novel combinations based on epidermal growth factor receptor inhibition. *Clin Cancer Res* 12: 4446s – 4450s

Baselga J, Arteaga CL (2005) Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* 23: 2445 – 2459

Bezjak A, Tu D, Seymour L, Clark G, Trajkovic A, Zukin M, Ayoub J, Lago S, de Albuquerque Ribeiro R, Gerogiani A, Cyjon A, Noble J, Laberge F, Chan RT, Fenton D, von Pawel J, Reck M, Shepherd FA (2006) Symptom improvement in lung cancer patients treated with erlotinib: quality of life analysis of the National Cancer Institute of Canada Clinical Trials Group Study BR.21. *J Clin Oncol* 24: 3831 – 3837

Bianco R, Shin I, Ritter CA, Yakes FM, Basso A, Rosen N, Tsurutani N, Dennis PA, Mills GB, Arteaga CL (2003) Loss of PTEN/MMAC1/TEP in
EGF receptor-expressing tumor cells counteracts the antitumor action of EGF tyrosine kinase inhibitors. Oncogene 22: 2812 – 2822
Bianco R, Troiani T, Tortora G, Ciardiello F (2005) Intrinsic and acquired resistance to EGFR inhibitors in human cancer therapy. Endocr Relat Cancer 12: 159 – 171
Bjornsti MA, Houghton PJ (2004) The TOR pathway: a target for cancer therapy. Nat Rev Cancer 4: 335 – 348
Boulay A, Zumstein-Mecke S, Stephan C, Beuvink I, Zilbermann F, Haller R, Tobbler S, Heusser C, O’Reilly T, Stolz B, Marti A, Thomas G, Lane HA (2004) Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. Cancer Res 64: 252 – 261
Chapman PB, Lenz H, Loo L, Dyson NJ (2002) Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res 62: 200 – 207
Ciardiello F, Bianco R, Caputo R, Caputo R, Damiano V, Troiani T, Melisi D, De Vita F, De Placido S, Bianco AR, Tortora G (2004) Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to anti-epidermal growth factor receptor therapy. Clin Cancer Res 10: 784 – 793
Citrine A, Yarden Y (2006) EGF-ERBB signalling: towards the systems level. British Journal of Cancer (2008) 98: 105 – 109
De Vita F, De Placido S, Tortora G, Ciardiello F (2004) Antitumor activity of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway in recurrent malignant gliomas. Neurology 67: 156 – 158
Doherty L, Gigas DC, Kesari S, Drappatz J, Kim R, Zimmerman J, Reese ED, Conrad CA, Traxler P, Lane HA, Reardon DA, Cavenee WK, Ostrowsky L, Wen PY (2006) Investigation of mammalian target of rapamycin (mTOR) inhibitors in pediatric patients with refractory solid tumors. J Clin Oncol 24: 4309 – 4316
Eftekhar H, Mclarnon J, Contractor S, Draper C, Thomas M, Mayo N, Zhang W, Lopes H, Murchison EJ, Yeo TC (2004) The phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin (mTOR) pathway in human cancer. J Clin Oncol 22: 2266 – 2277
Goudar RK, Shi Q, Hjelmeland MD, Keir ST, McLendon RE, Wikstrand CJ, Gemmill RM, Zhou M, Costa L, Korch C, Bukowski RM, Drabkin HA (2005) Dual EGFR and mTOR kinase pathways. J Clin Oncol 23: 2266 – 2277
Gudas LJ, Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, James CD, Zhang X, Kincaid E, Chen R, Clark DP, Forastiere A, Hjelmeland MD, Keir ST, McLendon RE, Wikstrand CJ, Gemmill RM, Zhou M, Costa L, Korch C, Bukowski RM, Drabkin HA (2005) Dual EGFR and mTOR kinase pathways. J Clin Oncol 23: 2266 – 2277
Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Hidalgo M, Rowinsky EK (2000) The rapamycin-sensitive signal transduction pathway in advanced prostate cancer: inhibition of tumor growth by the mTOR inhibitor RAD001 in combination with the antiandrogen flutamide. Clin Cancer Res 6: 2316 – 2326
Hynes NE, Lane HA (2005) mTOR and mTOR signaling in squamous cell carcinoma models, and development of early markers of efficacy. J Cancer Res 96: 952 – 959
Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 21: 3995 – 4004
Manning BD, Ree AE, Logsdon MN, Blenis J, Cantley LC (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/aKT pathway. Mol Cell 10: 151 – 162
Melillo G (2007) Targeting hypoxia cell signaling for cancer therapy. Cancer Metastasis Rev 26: 341 – 352
Mendelsohn J, Baselga J (2006) Epidermal growth factor receptor targeting in cancer. Semin Oncol 33: 369 – 385
Merchant JR (2007) Phase I/II trial of CCI-779 and bevacizumab in stage IV renal cell carcinoma: phase 1 safety and activity results. 2007 ASCO Annual Meeting Abstract - No. 5034
Patnaik A (2007) A phase I, pharmacokinetic and pharmacodynamic study of sorafenib (S), a multi-targeted kinase inhibitor in combination with temsirolimus (T), an mTOR inhibitor in patients with advanced malignancies. 2007 ASCO Annual Meeting Abstract - No. 3512
Phillips RJ, Restrepo E, Garhat-Kermar, Burdick MD, Sica A, Belperio JA, Keane MP, Strier RM (2005) Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin (mTOR) pathway and activation of hypoxia inducible factor-1α. J Biol Chem 280: 22473 – 22481
Rao RD, Malek AC, Lamont JD, Goble JM, Erlichman C, James CD, Sarkaria JN (2005) Disruption of parallel and converging signaling pathways contributes to the synergistic antitumor effects of simultaneous mTOR and EGFR inhibition in GBM cells. Neoplasia 7: 921 – 929
Rojo F, Tabernero J, Albarran J, Van Cutsem E, Ohtsu A, Doi T, Koizumi W, Shirao K, Takuchi H, Ramon y Cajal S, Baselga J (2006) Pharmacodynamic studies of gefitinib in tumor biopsy specimens from patients with advanced gastric carcinoma. J Clin Oncol 24: 4309 – 4316
Rowinsky EK (2004) Targeting the molecular target of rapamycin (mTOR). Curr Opin Oncol 16: 564 – 575
Salsal A, Sellers WR (2004) The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 22: 2954 – 2963
Schmelze PC, Hall MN (2000) TOR, a central controller of cell growth. Cell 103: 253 – 262
Smolewski P (2006) Investigating mammalian target of rapamycin inhibitors for their anticancer properties. Expert Opin Investig Drugs 1: 1201 – 1227
Stephan S, Datta K, Wang E, Li J, Breken RA, Parangi S, Thorpe PE, Mihopaladyay D (2004) Effect of rapamycin alone and in combination with antiangiogenesis therapy in an orthotopic model of human pancreatic cancer. Clin Cancer Res 10: 6993 – 7000
van Oosterom AT, Reichardt P, Blay J, Dumaz H, Fletcher J, Decuypere-Rychter M, Cachia C, Driessen NM, Dranovski S, Yapa A, Demetri G (2004) Combination signal transduction inhibition: a phase I/II trial of the oral mTOR-inhibitor everolimus (E, RAD001) and imatinib mesylate (IM) in patients (pts) with gastrointestinal stromal tumor (GIST) refractory to IMJ. Clin Oncol ASCO Annual Meeting Proceedings (Post-Meeting Edition). Abstract - No. 3002
Viloria-Petit A, Crombet T, Jothis S, Hoffin D, Bohlen P, Schlaeppi JM, Rak J, Kerbel RS (2001) Acquired resistance to the antitumor activity of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res 61: 5090 – 5101
Vivanco I, Sawyer’s DL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489 – 501
Wang MY, Lu KV, Zhu S, Dia EQ, Vivanco I, Cavenee WK, Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT/mammalian target of rapamycin (mTOR) pathway in human cancer: markers of efficacy. Clin Cancer Res 8: 4798 – 5011
Wang MY, Lu KV, Zhu S, Dia EQ, Vivanco I, Shackelford GM, Cavenee WK, Mellinghoff IK, Cloughesy TF, Sawyer’s DL, Mitchel PS (2006) Mammalian target of rapamycin inhibition promotes response to epidermal growth factor receptor kinase inhibitors in PTEN-deficient and PTEN-intact glioblastoma cells. Cancer Res 66: 7864 – 7869
Yu Y, Sato JD (1999) MAP kinases, phosphatidylinositol 3-kinase, and Akt signal mitogenic responses of human endothelial cells to vascular endothelial growth factor. J Cell Physiol 178: 235 – 246
Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol 5: 578 – 581