Lung cancer is a major cause of death in the developed world. Small cell lung cancer (SCLC) represents ~20% of all cases of lung cancer and is strongly correlated with cigarette smoking. Etoposide-based chemotherapy and radiotherapy treatment are commonly used. However, an initial therapeutic responsiveness is followed by disease recurrence and the overall 5-year survival rate is <5%. Consequently, novel therapeutic strategies are urgently required. In recent years, a rising number of biological dysfunctions involved in SCLC pathogenesis were reported, including ectopic expression of neuroendocrine regulatory peptides, overexpression of Myc family oncogenes and extracellular matrix proteins, as well genetic abnormalities in the tumor suppressor genes p53 and pRB (1, 2). Moreover, it has been shown that polypeptide growth factors such as stem cell factor (SCF) and fibroblast growth factor-2 (FGF-2) induce a variety of responses in human SCLC cells, including growth and proliferation, chemoresistance, and motility (3–6). Activation of two major intracellular signaling cascades, the phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and the mitogen-activated Erk kinase (MEK)/extracellular signal-regulated kinase (Erk) pathway, have been found to be involved in the survival and proliferation of SCLC (3, 5–8). Furthermore, it has been found that the ribosomal protein S6 kinases-1 and -2 (S6K1, 2) are highly overexpressed in SCLC cells compared with normal human type II pneumocytes, and that mTOR transduces mitogen-induced proliferation in SCLC (6). Therefore, targeting these pathways with novel anticancer agents may pave the way for more effective therapy of SCLC.

It has been shown that mTOR is a highly conserved, ubiquitously expressed signaling molecule, which is activated

Abstract Purpose: The Akt/mammalian target of rapamycin (mTOR) pathway is frequently activated in human cancers and plays an important role in small cell lung cancer (SCLC) biology. We investigated the potential of targeting mTOR signaling as a novel antitumor approach in SCLC.

Experimental Design: The expression of mTOR in patient specimens and in a panel of SCLC cell lines was analyzed. The effects on SCLC cell survival and downstream signaling were determined following mTOR inhibition by the rapamycin derivative RAD001 (Everolimus) or down-regulation by small interfering RNA.

Results: We found elevated expression of mTOR in patient specimens and SCLC cell lines, compared with normal lung tissue and normal lung epithelial cells. RAD001 treatment impaired basal and growth factor – stimulated cell growth in a panel of SCLC cell lines. Cells with increased Akt pathway activation were more sensitive to RAD001. Accordingly, a constitutive activation of the Akt/mTOR pathway was sufficient to sensitize resistant SCLC cells to the cytotoxic effect of RAD001. In the sensitive cells, RAD001 showed a strong additive effect to the proapoptotic action of the chemotherapeutic agent etoposide. Intriguingly, we observed low Bcl-2 family proteins levels in the SCLC cells with a constitutive Akt pathway activation, whereas an increased expression was detected in the RAD001-resistant SCLC cells. An antisense construct targeting Bcl-2 or a Bcl-2 – specific inhibitor was able to sensitize resistant SCLC cells to RAD001. Moreover, SCLC tumor growth in vivo was significantly inhibited by RAD001.

Conclusion: Together, our data show that inhibiting mTOR signaling with RAD001 potently disrupts growth and survival signaling in human SCLC cells.
Translational Relevance
Small cell lung cancer (SCLC) is a devastating disease. The present report highlights the importance of mammalian target of rapamycin (mTOR) and Bcl-2 family proteins for the chemoresistance of SCLC. Based on our data, targeting mTOR by the commercially available and clinically approved inhibitor RAD001 (Everolimus) significantly reduces SCLC cell growth and proliferation. Moreover, RAD001 further sensitizes SCLC cells to the antitumor effects of commonly used chemotherapeutic drugs. Importantly, the differences in SCLC cells’ responsiveness to RAD001 are based on the activation status of the Akt/mTOR pathway and the expression levels of Bcl-2 family proteins. Based on our findings, for a clinical relevant application it may be of advantage to screen SCLC patients for Akt/mTOR pathway activation status and Bcl-2 family expression prior to considering the use of RAD001 in combination with chemotherapeutic agents such as etoposide.

downstream of multiple distinct growth factor receptors and is of crucial importance for mediating cell proliferation and survival. These mTOR-dependent cell processes can be blocked by rapamycin and its derivatives RAD001 and CCI-779, and thus a new cancer-related therapy was proposed (9). Rapamycin was discovered as a fungicide produced by Streptomyces hygroscopicus and its antitumor activity was documented in many studies, but stability and solubility problems required the synthesis of derivatives such as RAD001 and CCI-779 with improved properties (9, 10).

mTOR is a 289-kDa serine/threonine kinase that belongs to the PI3K-related protein kinase family. Activation of mTOR leads to activation of S6K1 and phosphorylation of the ribosomal S6 protein and of the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), resulting in both ribosome biogenesis and cap-dependent mRNA translation. Thus, activation of mTOR is required for G1 cell-cycle progression and S-phase initiation. Inhibition of mTOR with rapamycin induces a G1 cell-cycle arrest. Furthermore, it was recently reported that rapamycin and its derivative RAD001 disrupt the cyclin D1/Cdk2/p21\textsuperscript{WAF1} complex in tumor cells, which leads to cell cycle arrest (11). RAD001 also dramatically enhances cisplatin-induced apoptosis in tumor cells expressing wild-type p53, by inhibiting p53-induced p21\textsuperscript{WAF1} expression (12). Findings by another group have suggested that rapamycin induces apoptosis in tumor cells through-down-regulation of Bcl-X\textsubscript{L} and up-regulation of Bax, whereas there was no significant effect on the cell cycle (13).

It was recently shown, however, that rapamycin derivatives inhibit a mTOR-dependent serine phosphorylation of the insulin receptor substrate-1, and thus enhance insulin-like growth factor-I signaling and downstream PI3K/Akt activation (14). This response was abrogated by pretreatment of the cells with a specific insulin-like growth factor-I receptor inhibitor (15). Thus, induction of Akt activity by mTOR inhibition leads to resistance to pharmacologic mTOR inhibitors, but blocking the insulin-like growth factor-I receptor reverses the Akt activation and the cell’s resistance.

Here we report that RAD001 blocks SCLC cell growth in vitro and in vivo and present evidence that the Akt/mTOR pathway expression modulate the cell’s sensitivity to the drug.

Materials and Methods

Reagents and antibodies. Antibodies against Akt1, caspase-3, Erk1/2, inhibitor of caspase-activated DNase (ICAD), mouse double minute 2 (MDM2), H-Ras, p21\textsuperscript{WAF1}, and poly (ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology. Antibodies against activated Akt (Ser473), Bcl-2, Bcl-X\textsubscript{L}, Bax, Thr37/46-phosphorylated-4E-BP, Ser235/236- or Ser240/244-phosphorylated-protein S6, and total S6 protein were from Cell Signalling Technology. Antibodies against β-actin, β-tubulin, and activated Erk1/2 (Thr202/Tyr204) were from Sigma Aldrich. Etoposide, z-VAD-FMK, SCF, GF-2, bombesin and vasopressin were from Calbiochem. 3-methyladenine was from Sigma-Aldrich. RAD001 was supplied by the Novartis Institutes for BioMedical Research Basel, Oncology, Switzerland. The Bcl-2 Inhibitor III, EM20-25, was obtained from Calbiochem.

Tissue samples. Normal lung tissue and SCLC tissue microarray were obtained from US Biomax, Inc., code BN04011\textsuperscript{7} and BS04111\textsuperscript{8} respectively.

Cell lines, cell culture, cell proliferation, and apoptosis. Human SCLC lines were cultured as described (5). For experimental purposes the SCLC cells were diluted into serum-free medium and grown for 3 to 5 d. Human type II pneumocytes were isolated and cultivated as described (6).

SCLC cells (2 x 10\textsuperscript{5}/mL) were grown for 3 d in serum-containing medium in the presence or absence of RAD001. Cell proliferation was analyzed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Alternatively, cell counting experiments based on Trypan Blue exclusion were done. SCLC cells grown in RPMI containing 10% FCS, were washed four times in RPMI, and resuspended in serum-free medium. Cells were then aliquoted in 24-well Falcon plates at a density of 1.5 x 10\textsuperscript{4} cells/mL in the presence or absence of inhibitors and/or growth factors. After the relevant incubation period, cell clumps within the cell suspension were disaggregated by passing the well content five times through a 19-gauge needle and the number of viable cells determined by Trypan Blue exclusion, using a hemocytometer. Each condition was done in quadruplicate and results obtained from averaging the values of three counts per replicate.

For detection of apoptosis, SCLC cells (2 x 10\textsuperscript{5}/mL) were incubated for 24 h in the presence or absence of RAD001 and/or etoposide. The cells were then lysed and samples analyzed by SDS-PAGE and Western blot with anti-PARP, anti-caspase-3 and anti-ICAD antibodies. Additionally, apoptosis was analyzed by caspase 3/7 activation using the Caspase-Glo 3/7 Assay (Promega), according to the manufacturer’s instruction.

In vivo experiments. The effects of RAD001 on growth of established H-69 tumors in vivo were assessed in adult ICR nude mice (Cancer Research-UK RSL, Clare Hall). The xenograft was established by injecting 10\textsuperscript{5} H-69 SCLC cells in 0.2 mL PBS s.c. into one flank and subsequently passaged as previously described (16). RAD001 (5 mg/kg) or placebo was administered daily by oral gavage (5 animals per group). Tumor growth was monitored over a 4-wk period.

SDS-PAGE and Western blotting. These assays were done exactly as described (3).

\textsuperscript{7} http://www.biomax.us/tissue-arrays/Lung/BN04011

\textsuperscript{8} http://www.biomax.us/tissue-arrays/Lung/BS04111

Cancer Therapy: Preclinical


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Reverse transcription-PCR and TaqMan analysis. Total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instruction. For each reverse transcription-PCR, 1 μg of total RNA was used with the QIAGEN One-Step RT-PCR Kit. The following primers were used: mTOR, sense 5'-CTG GGA CTC AAA TGT GTGCAGTTC-3', and antisense, 5'-GAACAATAGGGTGAATGATCCGGG-3'. The reaction condition were as follows: reverse transcription at 50°C for 30 min, initial PCR activation at 95°C for 15 min followed by 30 cycles at 95°C for 30 s, 54°C for 60 s, and 72°C for 45 s. The PCR products were analyzed in 1% agarose gel. TaqMan Gene Expression Assay for Bcl-2 was done according to the manufacturer's protocol.

Immunohistochemistry. mTOR immunoreactivity was evaluated on tissue microarray sections using a mTOR monoclonal antibody [Cell Signaling, mTOR (7C10) #2983] in a modification of the antigen retrieval technique of Shi and colleagues (17). Briefly, the sections were rehydrated in graded alcohols, heated in a microwave oven at 900W for 20 min in citrate buffer at pH 6. They were cooled at room temperature before the immunostains. mTOR monoclonal antibody was used at 1:20 dilution for 1 h at room temperature and then processed with Polymer-HRP Kit (BioGenex) with diaminobenzenzidine development and Mayer hematoxylin counterstaining. A mTOR-positive cell line cytoblock was used as positive external control. Negative controls were obtained by omitting the primary antibody.

Immunohistochemistry scoring. A semiquantitative immunohistochemical score was used including assessment of both the intensity of staining and the percentage of positive cells. In addition for the intensity, a score of 0 to 3, corresponding to negative, weak, moderate and strong positivity, was recorded. The range of possible scores was thus 0 to 300. Immunohistochemical score and similar semiquantitative scoring systems have been successfully used for tissue microarray evaluation (18–20). Each core was scored individually. If more than one core was evaluated from each tumor the mean score was calculated. If one core was uninformative the score applied was the mean of the remaining cores. One observer
Fig. 2. RAD001 blocks basal and growth factor–stimulated SCLC cell proliferation in vitro and inhibits H-69 tumor growth in vivo. A, type II pneumocytes, HC-33, H-69, H-209, H-510, H-524, H-1622, and H-2171 were incubated with increasing concentrations of RAD001 in serum-containing medium. Cell proliferation was assessed using the MTS assay after 5 d. The data are mean with SD from eight replicates and representative of at least three independent experiments. *, P < 0.05. B and C, H-69 (B) or H-510 (C) cells were incubated with vehicle (open bars), rapamycin (10 nmol/L; black bars), or RAD001 (1 nmol/L; grey bars) in serum-free medium in the presence or absence of FGF-2 (1.0 ng/mL) or SCF (10 ng/mL). Cell counting was done on different days, where indicated. The data are mean with SD from three experiments. D, xenografts of H-69 SCLC cells were grown s.c. in adult nude mice. RAD001 (5 mg/kg; closed bars) or placebo (open bars) was administered by oral gavage daily and tumor growth monitored over a 4-wk period. Tumor volume was determined at the times indicated. Data are mean from five animals in each group. *, P < 0.05.
scored all cases, which were rechecked randomly by the same investigator after a period of time. A good correlation was found between the two estimations.

**Transient transfection of SCLC cells.** H-69 or H-510 cells were transfected with either non-targeting small interfering RNA (siRNA) or SMARTpool siRNA duplexes targeting mTOR (Dharmacon), using the Amaxa Nucleofector system (Amaxa biosystems), according to the manufacturer’s protocol. The same protocol was used to transfect H-510 cells with the antisense oligonucleotide 4625 targeting Bcl-2 or a corresponding scrambled sequence control (21). Briefly, $1.5 \times 10^6$ SCLC cells grown in RPMI/10% FCS were transfected with 6 µL of 20 µmol/L siRNA in 100 µL of Nucleofector Solution V using the program T-16 on the Amaxa Nucleofector. Following transfection, cells were transferred into RPMI/10% FCS overnight before they were used for experiments.

**Statistical analysis.** ANOVA and Bonferroni multiple comparison test or Kruskal-Wallis nonparametric ANOVA test was used to assess statistical significance of differences between groups. The nonparametric Mann-Whitney test was used to compare the medians of two unpaired groups. $P < 0.05$ was considered significant.

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**Fig. 3.** RAD001 sensitizes SCLC cells to the cytotoxic effect of etoposide. A, H-69, H-510, and type II pneumocytes grown in serum-containing medium were incubated with increasing concentrations of RAD001 alone (1 µmol/L) or in combination with etoposide at concentrations indicated. Cell proliferation was assessed using the MTS assay after 5 d. The data are mean with SD from three experiments. B, H-69 cells grown in serum-containing medium were incubated with increasing concentrations of RAD001 in the presence of etoposide at the concentrations indicated. After 24 h, the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blot for the proteins indicated. C, H-69 cells grown in serum-containing medium were pretreated with 20 µmol/L z-VAD-FMK 1 h prior to incubation with RAD001 (100 nmol/L) alone, etoposide (10 µmol/L) alone, or combination treatment with both components. After 24 h, the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blot for the proteins indicated. D, H-69 cells grown in serum-containing medium were incubated with RAD001 (100 nmol/L) alone, or in combination with etoposide (10 µmol/L) in the presence or absence of zVAD (50 µmol/L) or 3-methyladenine (1 mmol/L). Cell proliferation was assessed using the MTS assay after 3 d. The data are mean with SD from three independent experiments performed in quadruplicate. ***, $P < 0.05$.**
Results

**mTOR and downstream targets are up-regulated in human SCLC cell lines and patient specimens.** The protein expression levels and the activation status of signaling molecules involved in the mTOR pathway were compared between purified type II lung epithelial cells and a panel of seven human SCLC cell lines. Our analysis revealed that mTOR and S6K1 were consistently expressed in the lung tumor cell lines (Fig. 1A). Moreover, the protein levels of mTOR, S6K1, and the phosphorylation of 4E-BP1 were elevated in SCLC cell lines, as compared with type II epithelial cells (Fig. 1A). Elevated levels of phosphorylated Akt were also found in some of the SCLC cell lines. In contrast, there was no increase in the levels of activated or total Erk1/2, or in the phosphorylation of the ribosomal S6 protein on Ser235/236 or Ser240/244 (Fig. 1A).

We further investigated the mTOR expression by immunohistochemistry in a total of 30 normal lung tissue samples and 30 SCLC patients samples (Fig. 1B). We found that 50% of the SCLC specimens were positive for mTOR, whereas mTOR expression was not detectable in normal lung tissue (Fig. 1C). Together, these data indicate that up-regulation of signaling enzymes involved in the mTOR pathway may contribute to the abnormal growth and survival properties of human SCLC cells.

**RAD001 inhibits SCLC cell growth in vitro.** To investigate the contribution of mTOR signaling to SCLC cell growth, the impact of the rapamycin derivative RAD001 (Everolimus) on the growth of HC-33, H-69, H-209, H-510, H524, H1622, and H2171 SCLC cell lines, and immortalized type II pneumocytes was studied. On the basis of RAD001 sensitivity we observed two groups of SCLC cell lines. H-209, H-510, and H-2171 cells showed high resistance to RAD001 (Fig. 2A, left), whereas the growth of HC-33, H-69, H-524, and H-1622 cells was inhibited at submicromolar concentrations of RAD001 in a serum-containing medium (Fig. 2A, right). Intriguingly, the relative sensitivity of H-69, H-524, and H-1622 cells to RAD001 treatment correlated with the enhanced phosphorylation of Akt and/or 4E-BP1 in these cell lines, as compared with H-209, H-510, and H-2171 cells (Fig. 1A). Type II pneumocytes were also relatively resistant to RAD001 (Fig. 2A), with 40% inhibition of cell growth observed only at 10 μmol/L (data not shown). In addition, RAD001 inhibited basal H-69 cell growth in serum-free medium (data not shown). The inhibitory effect of RAD001 was also observed when SCLC cell growth in liquid culture was investigated in response to bombesin, FGF-2, SCF, or vasopressin (Fig. 2B and C and Supplemental Fig. S1A and B). In H-69 and H-510 cells, low doses of rapamycin or RAD001 completely blocked growth induced by polypeptide (FGF-2, SCF) or neuropeptide (bombesin, vasopressin) growth factors in liquid culture (Fig. 2B and C and Supplemental Fig. S1A and B). Together these data show that the mTOR pathway is critical for basal and growth factor–stimulated proliferation in SCLC cells.

**RAD001 impairs basal and SCF-stimulated activation of the mTOR pathway.** To further elucidate the molecular mechanism of SCLC cells proliferation inhibition by RAD001, the impact of the drug on basal mTOR activation and early signaling events stimulated by SCF was investigated. The basal phosphorylation of the S6 protein in type II pneumocytes, H-69, H-1622, and H-510 cells was completely blocked after treatment with 1 nmol/L RAD001 after 20 hours in serum-containing medium (Supplemental Fig. S1C). Pretreatment of H-69 and H-510 cells with increasing concentrations of RAD001 completely abrogated SCF-stimulated phosphorylation of the S6 protein (Supplemental Fig. S1D and E). In contrast, the drug did not markedly impair activation of Erk1/2 by SCF (Supplemental Fig. S1E). A partial inhibition of SCF-stimulated Akt phosphorylation on Ser473 was observed upon RAD001 treatment (Supplemental Fig. S1D), whereas a weak induction of Akt activation was observed in resting H-69 cells pretreated with RAD001 at high doses (Supplemental Fig. S1D). Similar results were obtained when S6K activation by other polypeptide growth factors was studied in SCLC cells. RAD001 inhibited phosphorylation of the ribosomal S6 protein by hepatocyte growth factor and FGF-2 in the two cell lines under study (data not shown). The effect of RAD001 on basal and SCF-stimulated phosphorylation of the S6 protein in H-69 and H-510 cells was dose-dependent, with a maximal effect at 1 nmol/L and was apparent after 1 hour of pretreatment (Supplemental Fig. S1D and E). Together these results show that RAD001 selectively blocks the mTOR signaling pathway, which was previously implicated in SCLC cell growth, but not other pathways such as MEK/Erk (6).

**RAD001 impairs SCLC tumor growth in vivo.** To investigate the potential ability of RAD001 to inhibit SCLC tumor growth in vivo, a xenograft of H-69 SCLC cells was established by s.c. injection in nu/nu mice. The mice were then given placebo or RAD001 (5 mg/kg) daily by oral gavage over a 4-week period. The xenografts of placebo-treated animals displayed a 4-fold increase in tumor volume after 4 weeks, whereas no increase was observed in mice treated with RAD001 (Fig. 2D). Moreover, RAD001 induced a sustained decrease in tumor volume between 3 and 4 weeks of treatment (Fig. 2D).

**RAD001 sensitizes human SCLC cells to etoposide.** In view of the ability of RAD001 to block SCLC cell proliferation, we next investigated whether the drug could enhance the effect of the chemotherapeutic agent etoposide in these cells. H-69, H-510, and type II pneumocytes were treated with three different concentrations of etoposide (0.3-3.0 μmol/L) alone or in the presence of 1 nmol/L RAD001. In the H-69 cell line, RAD001 treatment markedly enhanced the cytotoxic effects of etoposide (Fig. 3A). A comparable effect, however, was not observed in H-510 cells and type II pneumocytes, which are less sensitive to RAD001 (Fig. 3A).

In view of the ability of RAD001 to sensitize SCLC cells to etoposide, we then investigated whether the drug contributes to an increase in apoptosis in H-69 cells. The induction of apoptosis was assessed by Western blot analysis to detect the cleaved fragments of caspase-3, PARP, and ICAD. RAD001 as a single agent induced apoptosis at high concentration (100 nmol) in H-69 cells, as evidenced by caspase-3, PARP, and ICAD cleavage (Fig. 3B). H-69 cells treated with different etoposide doses displayed a marked induction of caspase-3, PARP, and ICAD cleavage with increasing concentrations of RAD001, showing enhanced induction of apoptosis upon combination treatment (Fig. 3B). A comparable effect was not observed in H-510 cells and type II pneumocytes (Supplemental Fig. S2A). Interestingly, untreated H-69 cells displayed high levels of p21WAF1 and pMDM2, and RAD001 treatment decreased markedly the levels of both proteins (Fig. 3B). In contrast, RAD001 alone or in combination with etoposide did not alter the levels of Bcl-2, Bcl-XL, or Bax (Fig. 3B).
RAD001 at high concentration (100 nmol/L) and etoposide alone induced caspase-3 cleavage into the active 17-kDa and 12-kDa subunits, and the combination treatment further strongly enhanced the appearance of the two bands (Fig. 3C). Pretreatment of the cells with 20 \(\mu\)mol/L z-VAD-FMK led to the disappearance of the 12-kDa band and to the accumulation of the premature 20-kDa fragment (Fig. 3C). Furthermore, pretreating H-69 cells with 50 \(\mu\)mol/L z-VAD-FMK was sufficient to partially inhibit the effects of RAD001 alone, etoposide alone, or of the combination treatment on cell viability (Fig. 3D). Because an active mTOR pathway protects cells from autophagy and rapamycin treatment induces autophagy (22, 23), we tested whether the cytotoxic effect of RAD001 in SCLC cells is based on activated autophagy. H-69 cells were treated with 1 mmol/L 3-methyladenine, an inhibitor of autophagy, in the presence of RAD001 alone, etoposide alone, or of the combination treatment on cell viability (Fig. 3D). Because an active mTOR pathway protects cells from autophagy and rapamycin treatment induces autophagy (22, 23), we tested whether the cytotoxic effect of RAD001 in SCLC cells is based on activated autophagy. H-69 cells were treated with 1 mmol/L 3-methyladenine, an inhibitor of autophagy, in the presence of RAD001 alone, etoposide alone, or of the combination treatment on cell viability (Fig. 3D). 3-methyladenine was able to rescue almost completely the cytotoxic effect of RAD001 alone and showed significant effect in the combination treatment with etoposide alone (Fig. 3D), suggesting that induction of autophagy is a major contributor to the reduction in cell viability observed in response to RAD001 treatment in SCLC cells.

Together, these data show that blocking mTOR signaling with RAD001 induces autophagy and increases apoptosis induced by low doses of chemotherapeutic agents in human SCLC cells.

SCLC cells with enhanced activation of the Akt/mTOR pathway are more sensitive to the effects of RAD001. The results above indicated that the activation of the Akt/mTOR signaling in some SCLC cell lines may contribute to their enhanced sensitivity to RAD001. To further substantiate this hypothesis, we utilized H-510 cells transfected with a short hairpin RNA (shRNA) targeting H-Ras (H-510/H-Ras), which have a higher basal Akt activation compared with vector-transfected H-510 cells (Fig. 4A and Supplemental Fig. S2B), as previously reported (24). Moreover, phosphorylation of both 4E-BP1 and the S6 protein was also enhanced in the H510/H-Ras cells (Fig. 4A and Supplemental Fig. S2B). Intriguingly, the H-510/H-Ras cells displayed markedly decreased levels of Bcl-2 protein, whereas Bcl-XL levels were only partially reduced (Fig. 4A and Supplemental Fig. S2B). We next investigated the mechanism of Bcl-2 down-regulation in H-510/H-Ras cells. A quantitative gene expression analysis revealed a strong reduction in Bcl-2 mRNA levels in H-510/H-Ras cells, as compared with cells transfected with pRS-shGFp control vector (Fig. 4B).

RAD001 only moderately inhibited the proliferation of vector-transfected H-510 cells, although it did promote inhibition of S6 protein phosphorylation in these cells (Fig. 4C and data not shown). In contrast to vector-transfected H-510 cells, the growth of H-510/H-Ras cells in liquid culture was strongly inhibited by RAD001 at submicromolar concentrations (Fig. 4C and Supplemental Fig. S2C), showing that
Fig. 5. Bcl-2 protein expression modulates the sensitivity of SCLC cells to RAD001. A and B, equal amounts of lysates from purified human type II lung epithelial cells (N), or human SCLC cell lines (as indicated) were analyzed by SDS-PAGE and Western blotting with antibodies specific for the proteins indicated. The Western blots were quantified by densitometry (B). C, H-510 cells were transiently transfected with Bcl-2 antisense (AS) or scrambled control (Sc). Cell lysates were analyzed by SDS-PAGE and Western blotting with antibodies specific for Bcl-2. D, cell proliferation of H-510 cells transiently transfected with Bcl-2 antisense (open squares) or scrambled control (closed squares) was assessed using the MTS assay after 3 d. The data are mean from three independent experiments done in quadruplicate. *, P < 0.05. E, H-510 cells were incubated with increasing concentrations of RAD001 in the presence or absence of 40 μmol/L EM20-25. Cell proliferation was assessed using the MTS assay after 3 d. The data are mean from three independent experiments done in quadruplicate. *, P < 0.05. F, H-510 cells were incubated with increasing concentrations of RAD001 in the presence or absence of 40 μmol/L EM20-25. Caspase 3/7 activation was assessed using the Caspase-Glo 3/7 Assay after 3 d. The data are mean from three independent experiments done in quadruplicate. *, P < 0.05.
activation of the Akt/mTOR pathway is sufficient to sensitize H-510 cells to RAD001.

Expression of antiapoptotic Bcl-2 family members contributes to the resistance of SCLC cells to RAD001. Rapamycin was previously reported to affect the levels of proteins of the Bcl-2 family in different tumor cells, which contributes to its growth-suppressive effects (13). In view of these findings, we sought to investigate the expression pattern of Bcl-2 proteins in SCLC cell lines and its possible contribution to the sensitivity of the cells to RAD001. A comparative Western blot analysis revealed that the expression levels of the antiapoptotic proteins Bcl-2 and Bcl-XL were elevated in SCLC cell lines, as compared with type II pneumocytes (Fig. 5A and B). Intriguingly, the H-209 and H-510 cell lines displayed higher levels of both Bcl-2 and Bcl-XL than the H-69 cell line, indicating a possible contribution to RAD001 resistance (Figs. 2A and 5A and B). To test the latter hypothesis, a specific antisense construct targeting Bcl-2 was tested in H-510 SCLC cells. Effective down-regulation of the target protein Bcl-2 was confirmed by Western blot analysis (Fig. 5C). H-510 cells transfected with the specific Bcl-2 antisense displayed enhanced sensitivity to RAD001 (Fig. 5D).

RNA interference targeting mTOR selectively inhibits SCLC growth. The results above revealed differences in sensitivities to RAD001 in a panel of SCLC cells lines. In order to confirm these results by an alternative approach, H-69 and H-510 SCLC cells were transiently transfected with siRNA targeting mTOR. Western blot and reverse transcription-PCR analysis revealed down-regulation of mTOR in both cell lines (Fig. 6A-B). Transfection of H-69 and H-510 cells induced reduction in the phosphorylation of the mTOR downstream target 4E-BP1 (Fig. 6A). Growth of the transfected H-69 cells was significantly affected by the mTOR siRNA, whereas no effect was observed in the H-510 cell line (Fig. 6C). As shown in Fig. 6A, we did not observe a total mTOR pathway inhibition, as evidenced by residual phosphorylation of 4E-BP1, which may explain the partial inhibitory effect on H-69 cell growth. Together these data show that decreased levels of mTOR significantly affect proliferation in the H-69, but not in the H-510 cell line.

Discussion

In the present report, we have evaluated the potential of the rapamycin derivative RAD001 as an antitumor agent in human SCLC cells. An immunohistochemistry analysis revealed that mTOR is overexpressed in 50% of all tested SCLC patient tumor samples and we further found mTOR and S6K1 overexpression in a panel of SCLC cell lines, as compared with type II pneumocytes. These findings correlated with enhanced phosphorylation of components of the Akt/mTOR pathway observed in the SCLC cell lines. Surprisingly, despite overexpression of mTOR and S6K1, enhanced phosphorylation of the ribosomal S6 protein was not observed in the SCLC cell lines, as compared with type II pneumocytes. A possible explanation for these findings is that the constitutive activation of Erk1/2 observed in type II pneumocytes is in part responsible for the phosphorylation of the S6 protein, which was also observed in mice with a targeted deletion of the S6K1 and S6K2 genes (27). Moreover, there was a lack of correlation between
the phosphorylation levels of Akt and 4E-BP1 in the SCLC cell lines.

In selected SCLC cell lines displaying enhanced phosphorylation/activation of components of the Akt/mTOR pathway (HC-33, H-69, H-524, and H-1622), RAD001 inhibited basal growth in vitro at submicromolar concentrations corresponding to those inducing suppression of S6 protein phosphorylation. Moreover, growth of the H-69 cell line as xenografts in nude mice was completely inhibited by oral administration of RAD001. Interestingly, RAD001 treatment also induced tumor regression in H-69 xenografts. In other SCLC cell lines (H-209, H-510, and H-2171), and immortalized type II pneumocytes, cell proliferation was inhibited only at higher concentrations. This was supported by the findings that siRNA-mediated reduction of mTOR expression resulted in a significant effect on proliferation in the H-69, but not in the H-510 cell line. These observations may be attributed to the fact that these SCLC cell lines require different thresholds of mTOR expression for proliferation. The differences in sensitivities between the SCLC cell lines were not caused by a defect in the mTOR pathway, because basal and growth factor–stimulated phosphorylation of the S6 protein were inhibited by RAD001 in H-69 and H-510 cells with similar dose-dependencies. Moreover, in both cell lines, RAD001 inhibited cell growth induced by polypeptide or neuropeptide growth factors. Although, as previously observed in other cell systems (14), RAD001 treatment of H-69 cells induced a weak activation of Akt, as assessed by the induction of Ser473 phosphorylation, other additional factors seemed to modulate the resistance of SCLC cells to RAD001. In addition, we observed a partial inhibition of SCF-induced activation of Akt upon RAD001 treatment of H-69 cells, which could be due to inhibition of the mTORC2 complex implicated in the regulation of Akt Ser473 phosphorylation (28).

Previous reports have documented increased sensitivity of phosphatase and tensin homolog–deficient cancer cells to rapamycin derivatives, implying that constitutive activation of the PI3K/Akt/mTOR pathway renders tumor cells more sensitive to these drugs (29, 30). A similar observation was made here in SCLC cell lines, because cells with enhanced phosphorylation of components of the Akt/mTOR signaling pathway, were most sensitive to RAD001. Moreover, SCLC cells in which Akt/mTOR signaling was constitutively activated by transfection displayed enhanced sensitivity to RAD001. Surprisingly, Akt/mTOR pathway activation resulted in decreased levels of the proapoptotic protein Bcl-2 and, to a lesser extent, Bcl-XL. Accordingly, expression of Bcl-2 was high in RAD001-resistant SCLC cell lines (H-209, H-510, and H-2171), as compared with the sensitive cell lines (HC-33, H-69, and H524). A specific Bcl-2 antisense construct was able to restore RAD001 sensitivity in resistant SCLC cells (H-510). Similar findings were recently reported in ovarian cancer cells, in which the same antisense construct increased RAD001-mediated apoptosis (31). We could further confirm that the function of overexpressed Bcl-2 is sufficient to render SCLC cells resistant to RAD001. By cotreatment with RAD001 in the presence of a specific Bcl-2 inhibitor (EM20-25) at concentrations previously reported to sensitize cancer cells to the proapoptotic effects of chemotherapeutics (25), we observed strong reduction of cell viability in RAD001-resistant SCLC cell lines due to an increase in apoptosis. The simplest model accounting for these observations would be that RAD001-mediated apoptosis accounts in part for its growth-suppressing effects in SCLC cells. As a consequence, overexpression of antiapoptotic Bcl-2 family proteins would protect SCLC cells from RAD001-mediated cell death. The mechanism of Bcl-2 down-regulation in SCLC cell lines with enhanced Akt/mTOR signaling is unclear. Akt has previously been reported to phosphorylate and inactivate the proapoptotic Bcl-2 family member Bad (32). It is conceivable that, in SCLC cells displaying enhanced Akt activity, high Bcl-2 expression levels are not required, because Bad is inactivated by Akt-mediated phosphorylation.

Using a specific autophagy inhibitor we were able to rescue H-69 SCLC cells from the cytotoxic effect induced by RAD001 alone or in combination with etoposide, thus raising the possibility that RAD001 induces autophagy in the SCLC cells in addition to its effects on cell growth and apoptosis. Intriguingly, Bcl-2 has been reported to inhibit the autophagic response (33) and rapamycin also induced autophagy in malignant glioma cells (34).

A recent report has described the mechanism of RAD001-mediated chemosensitization in non-SCLC cells (A-549) through inhibition of p53-induced accumulation of p21WAF1 (12). The rapamycin derivative CCI-779 was also recently shown to sensitize SCLC cells to cisplatin-induced apoptosis (35). Here we show that RAD001 can increase etoposide-induced apoptosis, independently of p53, as the H-69 cell line does not express p53 (36, 37). Interestingly, we observed high levels of p21WAF1 and MDM2 in untreated H-69 SCLC cells, and RAD001 treatment resulted in decreased levels of both proteins. Because p21WAF1 and MDM2 have been described as proteins with a short half-life (38, 39) and a recent study reported that reduced p21WAF1 protein expression in non-SCLC cells (A-549) after RAD001 treatment was due to inhibition of global translation (12), we suppose that this could be a general mechanism facilitating the proapoptotic effect of RAD001. Recent studies further confirmed the antisurvival effect of rapamycin and its derivatives by decreasing p21WAF1 levels in different cancer types (40–42). Intriguingly, beside its established role as an apoptosis inhibitor (43), p21WAF1 has been recently reported to also block autophagy induction (44). Thus, inhibition of mTOR-dependent protein translation could affect the chemosensitivity of SCLC cells by decreasing the levels of p21WAF1 and MDM2. In contrast, Bcl-2, Bcl-XL, or Bax levels were unaffected by RAD001 in SCLC cells. The rapamycin derivative CCI-779 was recently evaluated in a phase II clinical trial in SCLC patients after induction chemotherapy (45). Although some beneficial effects were observed in the high-dose group, some have argued that rapamycin derivatives may not be further considered for clinical trials in SCLC (45). The present report highlights significant differences in SCLC cells responsiveness to the rapamycin derivative RAD001. Based on our findings, it may be of advantage to screen SCLC patients for Akt/mTOR pathway activation status and/or Bcl-2 family expression prior to considering the use of RAD001 in combination with chemotherapeutic agents such as etoposide.

**Disclosure of Potential Conflicts of Interest**

H. Lane, shares and previous employment in Novartis Pharma AG.
RAD001 Inhibits SCLC Cell Growth

References


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