mTOR, p70S6K, AKT, and ERK1/2 levels predict sensitivity to mTOR and PI3K/mTOR inhibitors in human bronchial carcinoids

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Abstract

Bronchial carcinoids (BCs) are rare neuroendocrine tumors that are still orphans of medical treatment. Human BC primary cultures may display resistance to everolimus, an inhibitor of the mammalian target of rapamycin (mTOR), in terms of cell viability reduction. Our aim was to assess whether the novel dual phosphatidylinositol 3-kinase (PI3K)/mTOR inhibitor NVP-BEZ235 is effective in everolimus-resistant human BC tissues and cell lines. In addition, we searched for possible markers of the efficacy of mTOR inhibitors that may help in identifying the patients who may benefit from treatment with mTOR inhibitors, sparing them from ineffective therapy. We found that NVP-BEZ235 is twice as potent as everolimus in reducing cell viability and activating apoptosis in human BC tissues that display sensitivity to mTOR inhibitors, but is not effective in everolimus-resistant BC tissues and cell lines that bypass cyclin D1 downregulation and escape G0/G1 blockade. Rebound AKT activation was not observed in response to treatment with either mTOR inhibitor in the 'resistant' BC cells. In addition to total mTOR levels, putative markers of the sensitivity of BCs to mTOR inhibitors are represented by AKT, p70S6K (RPS6KB2), and ERK1/2 (MAPK3/1) protein levels. Finally, we validated these markers in an independent BC group. These data indicate that the dual PI3K/mTOR inhibitor NVP-BEZ235 is more potent than everolimus in reducing the proliferation of human BC cells. 'Resistant' cells display lower levels of mTOR, p70S6K, AKT, and ERK1/2, indicating that these proteins may be useful as predictive markers of resistance to mTOR and PI3K/mTOR inhibitors in human BCs.

Key Words

- bronchial carcinoids
- mTOR inhibitors
- mTOR resistance
- predictive markers

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Introduction

Bronchial carcinoids (BCs) are rare neuroendocrine tumors (NETs) that originate from endocrine cells dispersed in the respiratory epithelium and can be divided into typical BCs (TBCs) and atypical BCs (ABCs) (Cakir & Grossman 2011). TBCs are, in general, less aggressive, smaller, and much less likely to metastasize when compared with ABCs, which are aggressive and may metastasize to the brain, bone, and liver (Bertino *et al.* 2009). Currently, the main treatment for BCs is surgery, which can be curative in most of the cases (Carretta *et al.* 2000), but is not feasible for large, infiltrating, and metastatic disease (Gregory *et al.* 2004). In these settings, medical therapy is often tried (Fink *et al.* 2001) and is mainly represented by chemotherapy and radiation (Bertino *et al.* 2009) in the attempt to reduce tumor mass, while somatostatin analogs are employed for symptomatic control (Srirajaskanthan *et al.* 2009).

The mammalian target of rapamycin (mTOR) pathway plays a central role in the regulation of cell growth, metabolism, and apoptosis (Jiang & Liu 2008), representing a novel molecular target for anticancer drugs (Guertin & Sabatini 2005). mTOR associates with two sets of different proteins, forming either the mTORC1 complex or the mTORC2 complex, with the former being more sensitive to rapamycin than the latter (Loewith *et al.* 2002). It has been demonstrated that the mTOR pathway is constitutively activated in NETs (Capdevila *et al.* 2011), providing the basis for the development of specific mTOR inhibitors as new therapeutic tools for NETs (Dong *et al.* 2012), including BCs (Dong & Yao 2011). Despite their potential efficacy as anticancer agents, mTOR inhibitors have demonstrated erratic clinical activity (Wang & Sun 2009), indicating the need to identify possible efficacy markers. Recently, a differential mTOR activation status has been demonstrated in the spectrum of bronchopulmonary NETs, possibly suggesting that profiling of the mTOR pathway might predict patients' responsiveness to mTOR-targeted therapies (Righi *et al.* 2010). In keeping with this finding, we have recently demonstrated that ∼70% of human BC primary cultures respond to a mTOR inhibitor, everolimus, in terms of cell viability reduction. In these settings, the efficacy of everolimus correlated with mTOR expression, tumor size, mitotic index, angiogenic markers, and plasma chromogranin A levels (Zatelli *et al.* 2010*a*,*b*). In other settings, resistance to everolimus has been attributed to rebound AKT activation by the mTORC2 complex. It has indeed been demonstrated that AKT phosphorylation is increased in cancer biopsies of patients treated with everolimus when compared with the controls (O'Reilly *et al.* 2006). However, the mechanisms underlying the resistance of BCs to everolimus have not been clarified, so far.

The relative lack of sensitivity to currently employed mTOR inhibitors may be bypassed by using multi-target agents, such as NVP-BEZ235. The latter is an imidazo (4,5-c)quinoline derivative that inhibits both phosphatidylinositol 3-kinase (PI3K) and mTOR activities, inducing G1 arrest in cell-cycle progression (Maira *et al.* 2008). It has been demonstrated previously that NVP-BEZ235 dose dependently reduces cell viability and promotes apoptosis in a human TBC cell line, the NCI-H727 cells, as well as in other human NET cell lines, with a greater potency than everolimus (Zitzmann *et al.* 2010). However, there is no evidence, so far, that NVP-BEZ235 may be effective in BCs not responding to everolimus. In addition, the markers of efficacy of both drugs are yet to be identified.

Therefore, the aim of our study was to assess whether the novel dual PI3K/mTOR inhibitor is effective in everolimus-resistant human BC tissues and cell lines. In addition, we searched for possible markers of the efficacy of mTOR inhibitors that may help in identifying the patients who may benefit from treatment with mTOR inhibitors, sparing them from ineffective therapy.

Materials and methods

Human BC tissue collection and primary culture

Samples were derived from 21 patients diagnosed with BCs, whose characteristics are given in Table 1, who were operated on at the University of Ferrara (Section of Endocrinology, Institute of Surgery) and at the University of Padova (Department of Medical and Surgical Sciences). All patients (12 males and 9 females; age $=52\pm4.3$ years; median $=53$ years) underwent BC resection, and all but two had histological and immunohistochemical diagnosis of TBCs, according to the WHO classification (Travis & Brambilla 2004; Table 1).

Tissues were collected following the guidelines of the local committee on human research and immediately minced in RPMI-1640 medium under sterile conditions. Primary cultures were prepared as described previously (Zatelli *et al.* 2005, 2010*a*,*b*). Experiments were performed within 3 days in order to prevent the decrease in cell viability due to culture conditions and to avoid fibroblast

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Table 1 Clinical characteristics of BC patients.

Patient no.	Sex	Age	Side	Diameter (cm)	Histology	TNM
1	M	38	dx	1.7	Typical carcinoid	T1a N0 Mx
2	F	61	dx	2	Typical carcinoid	T1a N0 Mx
3	M	46	dx	2	Typical carcinoid	T1a N0 Mx
4	M	75	sx	1.5	Typical carcinoid	T1a N0 Mx
5	M	18	dx	2	Typical carcinoid	T1a N0 Mx
6	F	65	dx	2	Typical carcinoid	T1a N0 Mx
7	M	68	dx	1.7	Typical carcinoid	T1a N0 Mx
8	F	21	dx	1.4	Typical carcinoid	T1a N0 Mx
9	F	53	dx	3	Typical carcinoid	T1a N0 Mx
10	F	20	dx	2.8	Typical carcinoid	T1b N1 Mx
11	M	52	dx	1.8	Atypical carcinoid	T4 N3 M1b
12	M	32	sx	1.3	Typical carcinoid	T1a N0 Mx
13	M	70	sx	2	Typical carcinoid	T1a N2 Mx
14	F	76	sx	0.7	Typical carcinoid	T1a N0 Mx
15	F	42	sx	3.5	Typical carcinoid	T2a N0 Mx
16	M	69	dx	3.5	Typical carcinoid	T2a N1 Mx
17	M	70	dx	2.5	Typical carcinoid	T1b N2 Mx
18	F	47	dx	1.8	Typical carcinoid	T1a N0 Mx
19	F	35	dx	4.5	Typical carcinoid	T3 N0 Mx
20	M	58	sn	1	Typical carcinoid	T1a N0 Mx
21	M	76	dx	6	Atypical carcinoid	T2b N1 Mx

overgrowth, which is observed after 4 days of culture. Informed consent of the patients was obtained for disclosing clinical investigation and performing the *in vitro* study.

Cell line culture

NCI-H727, derived from a TBC, and NCI-H720, derived from an ABC, cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in RPMI-1640 medium (Euroclone, Milano, Italy), supplemented with 10% fetal bovine serum, at 37 °C in a humidified atmosphere with 5% CO_2.

Compounds

Everolimus and NVP-BEZ235 were provided by Novartis. All other reagents, if not otherwise specified, were purchased from Sigma.

Viable cell number assessment

Variations in viable cell number were assessed using the ATPlite kit (Perkin Elmer Life Sciences, Boston, MA, USA), seeding 2×10^4 cells/well in 96-well white plates, as described previously (Molè *et al*. 2011), and treated with the indicated compounds for 72 h. Control cells were treated with the vehicle alone (0.1% DMSO). After incubation, the revealing solution was added, and the luminescent output (relative luminescence units (RLU)) was recorded using the Envision Multilable Reader (Perkin Elmer, Monza, Italy). The results are expressed as mean value±s.e.m. percent RLU vs the vehicle-treated control cells from three independent experiments in six replicates.

Caspase activity

Caspase activity was measured using the Caspase-Glo 3/7 assay (Promega) as described previously (Zatelli *et al*. 2010*a*,*b*). Briefly, 2×10^4 cells/well were seeded in 96-well, white-walled plates and treated with the indicated compounds for 72 h. Then, the Caspase-Glo 3/7 reagent was added at room temperature directly to the cell culture plates, which were shaken at 12.7 ***g*** for 30 s, incubated for 1 h, and then measured for luminescent output (relative luminescence unit (RLU)) using the Envision Multilabel Reader (Perkin Elmer). The results are expressed as mean value±s.e.m. percent RLU vs the vehicle-treated control cells from three independent experiments in six replicates.

Flow cytometry

Cell-cycle analysis was performed using the CyFlow space cytometer (Partec, Münster, Germany). The NCI-H727 and NCI-H720 cells were treated with 100 nM everolimus or NVP-BEZ235 for 72 h. Vehicle-treated cells served as the controls. At the end of the incubation period, the cells were washed with PBS and incubated with 1 ml of staining solution (5 μg/ml propidium iodide, 10 μg/ml

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ribonuclease A, 0.1% sodium citrate, and 0.1% Triton X-100) overnight. A total of 2×10^4 events were acquired and analyzed using the FloMax Software (Partec), as described previously (Minoia *et al*. 2012).

RNA extraction and quantitative PCR

Total RNA was extracted from the cell lines using the TRIzol reagent following the manufacturer's instructions (Invitrogen). RNA integrity was evaluated using the Experion automated electrophoresis system (Bio-Rad Laboratories). Only RNA samples with a 28S:18S rRNA ratio >1.6 and an RNA quality indicator >9 were processed. All RNA samples were subjected to DNase I treatment. The RNA samples were subjected to RT with random hexamers, as described previously (Zatelli *et al*. 2002). Real-time quantitative PCR (Q-PCR) was performed to assess the expression of human mTOR (Hs.338207) using the TaqMan gene expression assay (Applied Biosystems). The samples were run in triplicate on an Applied Biosystems 7700 ABI Prism thermal cycler and analyzed with the SDS 1.9 Software (Applied Biosystems), by applying the method described by Pfaffl (2001).

To ensure the fidelity of mRNA extraction and RT, human target gene signals from all samples were normalized against five different housekeeping genes: 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*), human PO (ribosomal protein large), and human *GUS* (*GUSB*) (glucuronidase, β). All primers and probes were commercially available (Applied Biosystems). Calculations to estimate the expression stability and the pairwise variation were performed with the freely available GeNorm program. Data were normalized on human PO as described previously (Tagliati *et al*. 2010).

Western blot analysis

Frozen human BC tissues were disrupted using TissueRaptor (Qiagen) according to the manufacturer's instructions. For immunoblotting, human BC cell lines and disrupted tissues were dissolved in RIPA buffer (Pierce, Rockford, IL, USA), kept in ice for 30 min, and then centrifuged for 10 min. The supernatant, containing the proteins, was then transferred to a new tube and protein concentration was measured using the BCA Protein Assay Reagent Kit (Pierce), as described previously (Tagliati *et al*. 2006). For protein evaluation, lysates were fractionated on 10% SDS–PAGE, as described previously (Tagliati *et al*. 2006), and transferred by electrophoresis to nitrocellulose transfer membranes (PROTRAN, Dassel, Germany). The membranes were incubated with the following antibodies: polyclonal rabbit anti-human β-actin (Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-mTOR (Santa Cruz Biotechnology), polyclonal rabbit anti-phosho (Ser2448) mTOR (Abcam, Cambridge, UK), polyclonal rabbit anti-human AKT (Cell Signaling), polyclonal rabbit anti-human phosho (Ser473) AKT (Cell Signaling), polyclonal rabbit anti-human glycogen synthase kinase 3β (GSK3β) and anti-phospho (Ser9) GSK3β (Cell Signaling), polyclonal rabbit anti-human ERK1/2 and phospho (Thr202/Tyr204) ERK1/2 (Cell Signaling), and monoclonal mouse anti-human p70S6K and phospho (Thr389) p70S6K (Cell Signaling). All the antibodies were diluted at 1:1000. Anti-rabbit or anti-mouse HRP-conjugated IgG antibodies (Dako Italia, Milano, Italy) were used at a dilution of 1:5000, and binding was revealed using ECL (Pierce). The blots were then stripped and used for further blotting. Quantification of band intensity was done using a Gel Doc System with the Quantity One Software (Bio-Rad).

Kinase activity assay

Total and phosphorylated p70S6K (RPS6KB2) levels and total and phosphorylated ERK1/2 (MAPK3/1) levels were measured using the AlphaScreen SureFire total p70S6K assay, p-p70S6K (Thr389) assay, total ERK1/2 assay, and p-ERK1/2 (Thr202/Tyr204) assay (Perkin Elmer). Briefly, the cells were seeded at 2×10^4 cells/well in 96-well plates and, after overnight attachment, were incubated with or without everolimus or NVP-BEZ235 and evaluated as per the manufacturer's protocol. The plates were measured in Read plate on an Envison plate reader (Perkin Elmer), using standard AlphaScreen settings, and the output was recorded as counts per second (cps).

Statistical analysis

Concerning the results of cell viability and caspase 3/7 activation experiments, a preliminary analysis was carried out to determine whether the datasets conformed to a normal distribution, and a computation of homogeneity of variance was performed using Bartlett's test. The results were compared within each group and between the groups using ANOVA. If the *F* values were significant ($P<0.05$), Student's paired or unpaired *t*-test was used to evaluate individual differences between the means. *P* values <0.05 were considered significant. For all the other experiments, Student's paired or unpaired *t*-test was used to evaluate individual differences between the means, and *P* values <0.05 were considered significant.

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Results

Effects of NVP-BEZ235 and everolimus on BC primary cultures

We evaluated the ability of NVP-BEZ235 and everolimus to influence cell viability in 21 dispersed human BC primary cultures, which were divided into primary cultures displaying a significant reduction ($P<0.05$) in cell viability under everolimus treatment, referred to as 'sensitive', and those that did not, referred to as 'resistant'. Table 2 reports cell viability reduction observed under NVP-BEZ235 or everolimus treatment when compared with the control in each BC primary culture. According to this criterion, cultures from 13 BCs were considered as 'sensitive' and those from 8 BCs as 'resistant'. All BC primary cultures that were considered as 'sensitive' to everolimus also displayed a significant reduction ($P<0.05$) in cell viability under treatment with NVP-BEZ235. As shown in Fig. 1A, in the 'sensitive' group (black bars), both NVP-BEZ235 and everolimus significantly ($P<0.01$) reduced cell viability (−32.3 and −18.4% respectively). Furthermore, in the 'sensitive' group, NVP-BEZ235 was 1.8-fold more potent than everolimus in terms of cell viability reduction ($P<0.01$). In the 'resistant' BCs (white bars), both NVP-BEZ235 and everolimus did not significantly modify the viability of BC cells.

In order to verify whether the reduction in cell viability caused by NVP-BEZ235 and everolimus was due to the induction of apoptotic mechanisms, caspase 3/7 activation was evaluated. As shown in Fig. 1B, in the 'sensitive' group (black bars), both NVP-BEZ235 and everolimus significantly ($P<0.01$) induced caspase activation (+43.6 and +29.6% respectively); NVP-BEZ235 was 1.5-fold more potent than everolimus. In the 'resistant' BCs (white bars), both NVP-BEZ235 and everolimus did not significantly modify the caspase 3/7 activity of BCs.

Western blot analysis showed that both the total and phosphorylated forms of the mTOR protein were higher in the 'sensitive' group than in the 'resistant' group (Fig. 1C).

Regarding the patients' characteristics (Table 1), no difference was found between patients with 'sensitive' vs 'resistant' BCs concerning gender, tumor site, TNM, staging, and tumor size. However, patients whose BCs were 'sensitive' to mTOR inhibitors were significantly ($P<0.01$) younger than patients whose BCs were 'resistant (42.6±3.8 vs 67.3±2.5 years).

Table 2 Percent cell viability reduction under NVP-BEZ235 or everolimus treatment.

Patient no.	NVP-BEZ235	R/S	Everolimus	R/S
1	−33	S	−26	S
2	13	R	1	R
3	−20	S	−10	S
4	−2	R	10	R
5	−41	S	−27	S
6	−19	S	−12	S
7	−7	R	−5	R
8	−18	S	−12	S
9	−58	S	−50	S
10	−39	S	−20	S
11	−31	S	−12	S
12	−37	S	−11	S
13	1	R	1	R
14	−3	R	−3	R
15	3	R	1	R
16	−35	S	−16	S
17	−2	R	−1	R
18	−34	S	−15	S
19	−29	S	−13	S
20	−26	S	−15	S
21	−2	R	−1	R

Human BC primary cultures were each incubated with 100 nM NVP-BEZ235 or everolimus, and control cells were treated with a vehicle solution; cell viability was measured, and it is expressed as the mean percent cell viability reduction vs the untreated control cells. Human BC primary cultures were divided into cultures displaying a significant reduction ($P<0.05$) in cell viability under everolimus treatment, referred to as 'sensitive' (S), and those that did not, referred to as 'resistant' (R).

mTOR expression in human BC cell lines

Both the NCI-H727 and NCI-H720 cell lines were characterized for mTOR expression. As shown in Fig. 2A, mTOR expression levels in terms of mRNA were 1.5-fold higher ($P<0.05$) in the NCI-H720 cells than in the NCI-H727 cells. These data were confirmed by western blot analysis (Fig. 2B): the NCI-H720 cells exhibited higher levels of mTOR protein (both total and phosphorylated forms) than the NCI-H727 cells. In terms of mTOR expression, the NCI-H720 and NCI-H727 cells resembled the 'sensitive' BC group and the 'resistant' BC group respectively.

Influence of NVP-BEZ235 and everolimus on cell viability, apoptosis, and cell-cycle progression in human BC cell lines

As shown in Fig. 3A, the viability of the NCI-H720 cells was significantly reduced by treatment with 50 nM–1 μM NVP-BEZ235 by 55–69% ($P<0.01$ vs the control; $IC_{50}=0.32$ μM) and by treatment with 50 nM–1 μM everolimus by 31–54% ($P<0.01$ vs the control; $IC_{50}=0.7$ μM). Therefore, NVP-BEZ235 showed a twofold potency when compared with everolimus in reducing the viability of the NCI-H720 cells (Fig. 3A, upper panel). Caspase activation in the NCI-H720 cells was significantly induced by

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treatment with 50 nM–1 μM NVP-BEZ235 by 160–290% ($P<0.01$ vs the control; $IC_{50}=0.073$ μM) and by treatment with 250 nM–1 μM everolimus by 13–44% ($P<0.01$ vs the control; $IC_{50}=0.69$ μM). Thus, NVP-BEZ235 showed a tenfold potency when compared with everolimus in inducing caspase activation in the NCI-H720 cells (Fig. 3A, lower panel).

As shown in Fig. 3B, the viability of the NCI-H727 cells was significantly reduced by treatment with 50 nM–1 μM NVP-BEZ235 by 30–47% ($P<0.01$ vs the control; $IC_{50}=0.99$ μM) and by treatment with 50 nM–1 μM everolimus by 10–42% ($P<0.01$ vs the control; $IC_{50}=1.07$ μM). Therefore, NVP-BEZ235 showed a potency that was similar to that shown by everolimus in reducing the viability of the NCI-H727 cells (Fig. 3B, upper panel). Caspase activation in the NCI-H727 cells was significantly induced by treatment with 500 nM–1 μM NVP-BEZ235 by 20% ($P<0.01$ vs the control; $IC_{50}=1.65$ μM) and by treatment with 500 nM–1 μM everolimus by 10–15% ($P<0.01$ vs the control; $IC_{50}=3.14$ μM). Therefore, NVP-BEZ235 showed a twofold potency when compared with everolimus in inducing caspase activation in the NCI-H727 cells (Fig. 3B, lower panel).

These data show that the NCI-H720 cells are more sensitive than the NCI-H727 cells to the antiproliferative effects of both the employed drugs. In addition, the reduction in cell viability induced by treatment with either NVP-BEZ235 or everolimus was not completely mirrored by apoptosis activation. Therefore, the influence of NVP-BEZ235 and everolimus on cell-cycle progression was verified.

As shown in Fig. 4A, in the NCI-H720 cells, treatment with 100 nM NVP-BEZ235 caused a 11.5% increase ($P<0.05$ vs the control) in the number of cells in the G0/G1 phase of the cell cycle, with 7 and 4.5% reductions ($P<0.05$) in the number of cells in the S and G2/M phases respectively. Similarly, treatment with 100 nM everolimus caused an 8.7% increase ($P<0.05$) in the number of cells in the G0/G1 phase of the cell cycle, with 5.2 and 3.4% reductions ($P<0.05$) in the number of cells in the S and G2/M phases respectively.

As shown in Fig. 4B, in the NCI-H727 cell line, treatment with 100 nM NVP-BEZ235 caused a 4.7% increase in the number of cells in the G0/G1 phase of

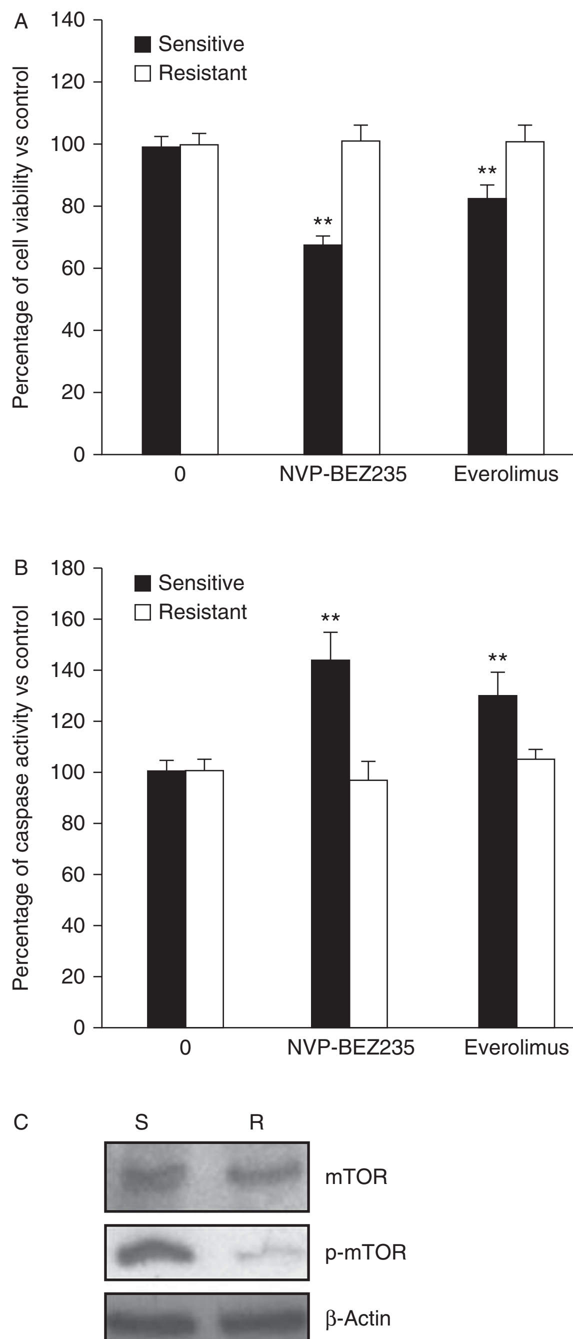

Figure 1
Effects of mTOR inhibitors and mTOR expression in human BC primary cultures. BC cells were incubated in 96-well plates for 72 h in a culture medium supplemented with 100 nM NVP-BEZ 235 or everolimus, and control cells were treated with a vehicle solution. (A) Cell viability of each primary culture was measured as a luminescent output. As described in the Results section, the samples were divided according to cell viability inhibition after treatment with everolimus into 'sensitive' (13 samples, black bars) and 'resistant' (eight samples, white bars) groups. Data from BC primary cultures were evaluated independently with six replicates each, and they are expressed as the mean ± S.E.M. percent cell viability inhibition vs the untreated control cells. $**P<0.01$ vs the untreated control cells. (B) Caspase activity was measured as a luminescent output in the 'sensitive' (black bars) and 'resistant' (white bars) primary cultures. Data from BC primary cultures were evaluated independently with six replicates each, and they are expressed as the mean ± S.E.M. percent caspase activity vs the untreated control cells. $**P<0.01$ vs the untreated control cells. (C) Western blot analysis for total mTOR and phosphorylated mTOR (p-mTOR) expression in a pool of 'sensitive' (S) human BC tissues and in a pool of 'resistant' (R) human BC tissues. β-Actin is shown as a loading control.

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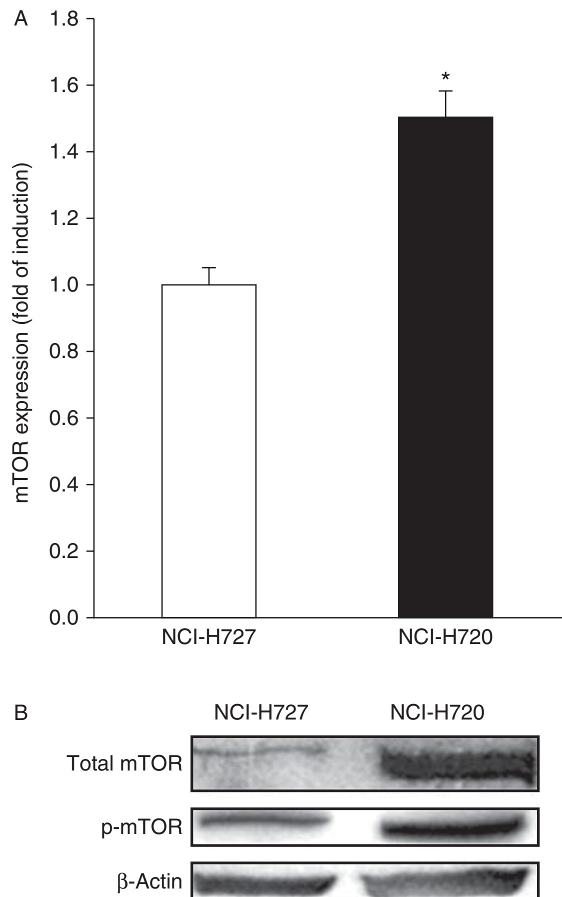

Figure 2
mTOR expression in human BC cell lines. (A) Total RNA was isolated from the NCI-H727 and NCI-H720 cells and relative Q-PCR for mTOR expression was performed. The experiment was repeated thrice, and the results are expressed as a fold of induction of mTOR expression levels compared with the NCI-H727 cells, considered as the reference. (B) Total proteins were isolated from the NCI-H727 and NCI-H720 cells and western blot analysis for total mTOR and phosphorylated mTOR (p-mTOR) protein expression was performed. β-Actin is shown as a loading control. *$P<0.05$ vs NCI-H727.

the cell cycle, with 6.9 and 2.1% reductions in the number of cells in the S and G2/M phases respectively. Similarly, treatment with 100 nM everolimus caused a 4.7% increase in the number of cells in the G0/G1 phase of the cell cycle, with 6.9 and 2.1% reductions in the number of cells in the S and G2/M phases respectively. All the variations in cell-cycle distribution recorded in the NCI-H727 cells did not reach statistical significance.

These data show that both NVP-BEZ235 and everolimus influence cell-cycle progression by inducing a delay in the G1 phase only in the NCI-H720 cells. Since the transition from the G1 to the S phase is also regulated by cyclin D1 expression levels, the latter were assessed in the NCI-H720 and NCI-H727 cells treated with 100 nM NVP-BEZ235 or everolimus (Fig. 4C). Western blot analysis showed that treatment with NVP-BEZ235 caused a sharp reduction in cyclin D1 protein levels in both the NCI-H720 and NCI-H727 cells, while everolimus reduced cyclin D1 protein levels in both the NCI-H720 and NCI-H727 cells to a smaller extent. It has been demonstrated previously that GSK3β activation promotes cyclin D1 degradation (Kunnimalaiyaan *et al*. 2007). Figure 4C shows that both NVP-BEZ235 and everolimus are capable of reducing the phosphorylation levels of GSK3β at Ser 9 in both the NCI-H720 and NCI-H727 cells, indicating an increase in kinase activity of the enzyme (Martin *et al*. 2005).

PI3K/AKT/mTOR pathway expression and activation in BC cell lines

In order to understand whether the sensitivity to mTOR inhibitors is related to the expression levels of mTOR pathway components, protein expression was evaluated by western blot analysis. As shown in Fig. 5A, in keeping with the results shown in Fig. 2, total and phosphorylated mTOR basal levels were higher in the NCI-H720 cells than in the NCI-H727 cells. In addition, phosphorylated mTOR levels were reduced by both 100 nM NVP-BEZ235 and everolimus, with the latter being more potent, in both the NCI-H720 and NCI-H727 cells. On the other hand, total mTOR protein levels were not significantly influenced by treatment with either mTOR inhibitor.

Similarly, both total and phosphorylated basal AKT protein levels were higher in the NCI-H720 cells than in the NCI-H727 cells; basal AKT levels were not influenced by treatment with mTOR inhibitors. On the other hand, phosphorylated AKT protein levels were slightly reduced by treatment with NVP-BEZ235 but not by treatment with everolimus in the NCI-H720 cells. In the NCI-H727 cells, phosphorylated AKT levels were not affected by treatment with either mTOR inhibitor (Fig. 5A). One of the main downstream mTOR targets is represented by p70S6K, a serine/threonine kinase, the phosphorylation of which induces protein synthesis at the ribosome (Loewith *et al*. 2002). Therefore, we investigated p70S6K phosphorylation and found this parameter to be significantly reduced by both NVP-BEZ235 and everolimus by 55–60% ($P<0.01$) in the NCI-H720 cells but not in the NCI-H727 cells (Fig. 5B). In addition, basal total p70 levels were twofold higher in the NCI-H720 cells than in the NCI-H727 cells (data not shown), as evident for the phosphorylated basal p70 levels (Fig. 5B).

The ERK1/2 pathway actively participates in the signals inducing cell proliferation also by promoting cyclin D1 transcription (Kumar *et al*. 2013). Moreover,

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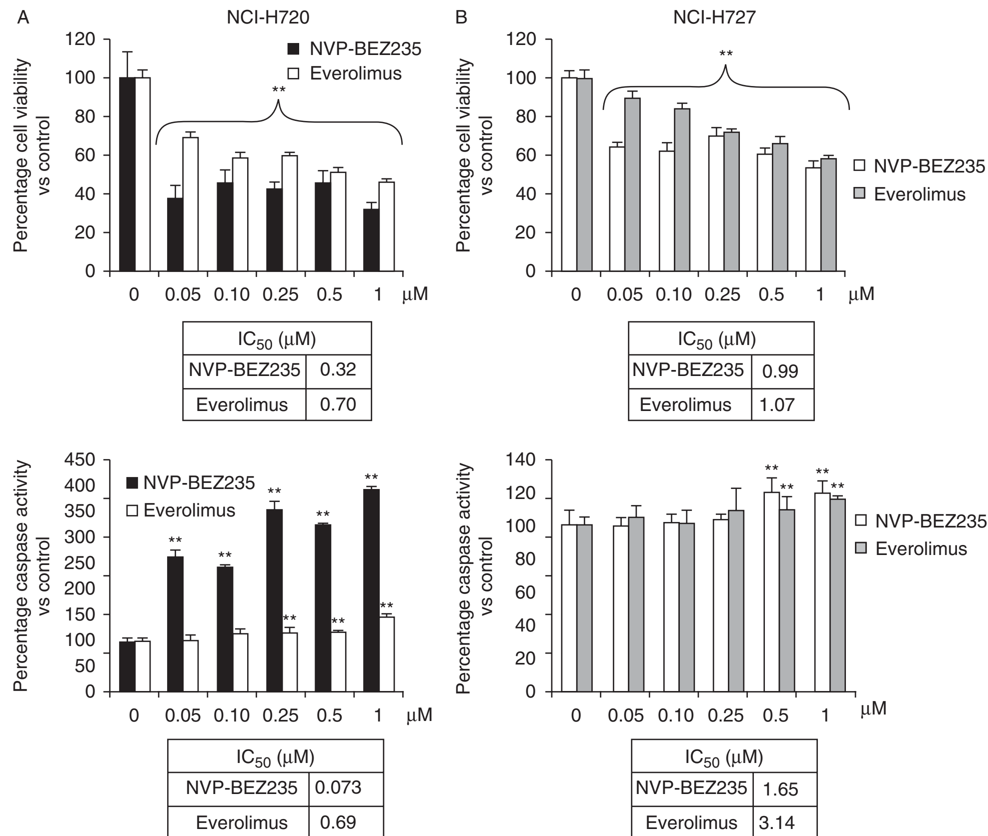

Figure 3

Effects of mTOR inhibitors on cell viability and caspase activation in human BC cell lines. (A) The NCI-H720 cells were incubated in 96-well plates for 72 h in a culture medium supplemented with 100 nM NVP-BEZ 235 (black bars) or everolimus (white bars), and control cells were treated with a vehicle solution. Upper panel: cell viability was measured as a luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± s.e.m. percent cell viability inhibition vs the untreated control cells. $^{**}P<0.01$ vs the untreated control cells. IC_{50} (µM) for cell viability reduction is reported in Table 2. Lower panel: caspase activity was measured as a luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± s.e.m. percent caspase activity vs the untreated control cells. $^{**}P<0.01$ vs the untreated control cells. IC_{50} (µM) for caspase 3/7 activity induction is reported in Table 2. (B) The NCI-H727 cells were incubated in 96-well plates for 72 h in a culture medium supplemented with 100 nM NVP-BEZ 235 (white bars) or everolimus (grey bars), and control cells were treated with a vehicle solution.

ERK1/2 is a well-known downstream PI3K pathway effector, independent of mTOR and AKT activation. Therefore, we investigated ERK1/2 phosphorylation and found this parameter to be significantly reduced by both NVP-BEZ235 and everolimus in the NCI-H720 cells by 20–60% ($P<0.01$), but not in the NCI-H727 cells (Fig. 5C). In addition, basal total ERK1/2 levels were 25-fold higher in the NCI-H720 cells than in the NCI-H727 cells (data not shown), as evident for phosphorylated ERK1/2 basal levels (Fig. 5C).

mTOR, p70S6K, AKT, and ERK1/2 levels in independent human BC tissues

In order to validate the putative markers identified in the two human BC cell lines, we evaluated a group of independent human BC tissues, already described in a previously published study as either 'sensitive' or 'resistant' to everolimus (Zatelli *et al*. 2010*a*,*b*). We randomly selected five 'sensitive' and five 'resistant' human BC tissues and analyzed the expression of total and phosphorylated mTOR, p70S6K, AKT, and ERK1/2 protein levels by western blot analysis. As shown in Fig. 5D, we found that both total and phosphorylated mTOR and AKT protein levels were ~20-fold higher in the 'sensitive' BCs than in the 'resistant' BCs. Similarly, both total and phosphorylated p70S6K levels were approximately sixfold higher in the 'sensitive' BCs than in the 'resistant' BCs. Total and phosphorylated ERK1/2 protein levels were five- and twofold higher in the 'sensitive' BCs than in the 'resistant' BCs respectively.

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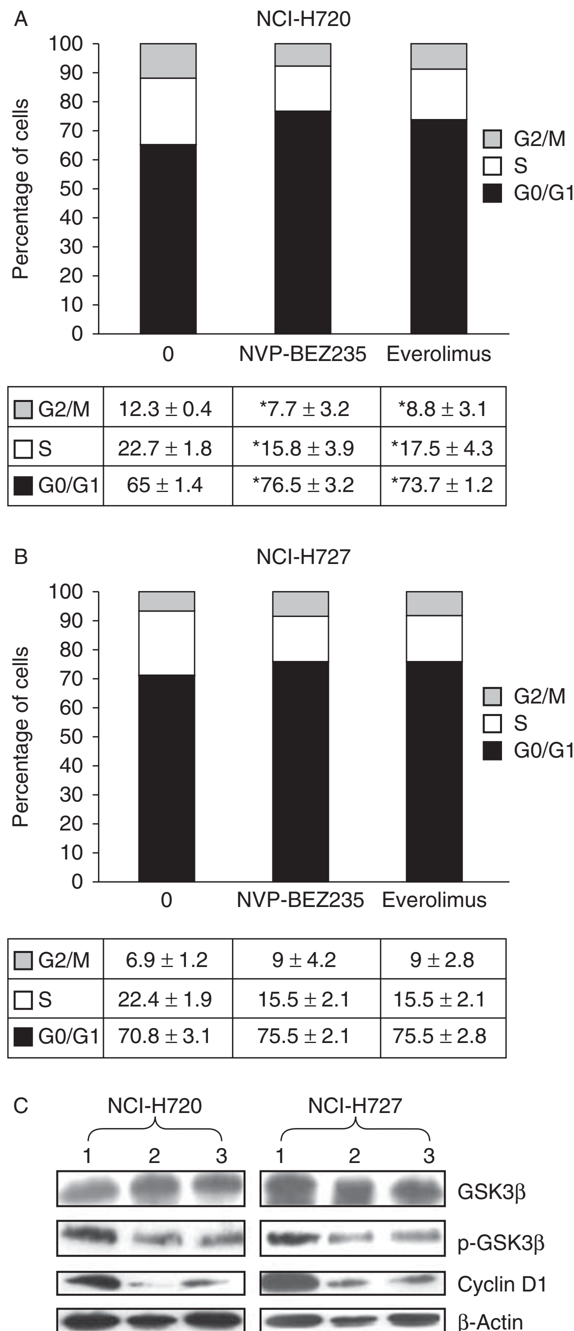

	0	NVP-BEZ235	Everolimus
G2/M	12.3 ± 0.4	*7.7 ± 3.2	*8.8 ± 3.1
S	22.7 ± 1.8	*15.8 ± 3.9	*17.5 ± 4.3
G0/G1	65 ± 1.4	*76.5 ± 3.2	*73.7 ± 1.2

	0	NVP-BEZ235	Everolimus
G2/M	6.9 ± 1.2	9 ± 4.2	9 ± 2.8
S	22.4 ± 1.9	15.5 ± 2.1	15.5 ± 2.1
G0/G1	70.8 ± 3.1	75.5 ± 2.1	75.5 ± 2.8

Figure 4
Effects of mTOR inhibitors on cell-cycle progression, GSK3β, and cyclin D1 expression in human BC cell lines. The NCI-H720 and NCI-H727 cells were incubated for 72 h in a culture medium supplemented with 100 nM NVP-BEZ 235 or everolimus, and control cells were treated with a vehicle solution. Cell cycle was analyzed in three independent experiments in the NCI-H720 (A) and NCI-H727 (B) cells. *$P<0.05$ vs the untreated control cells. (C) Total proteins were isolated from the NCI-H727 and NCI-H720 cells and western blot analysis for total GSK3β, phosphorylated GSK3β (p-GSK3β), and cyclin D1 (cyclin D1) protein expression was performed. β-Actin is shown as a loading control. Lane 1, mock-treated cells. Lane 2, cells treated with NVP-BEZ235. Lane 3, cells treated with everolimus.

Discussion

In this study, we confirmed that, in keeping with previously published evidence (Zatelli *et al.* 2010*a*,*b*), 62% of human BC primary cultures respond to treatment with everolimus with a significant reduction in cell viability paralleled by apoptosis activation. We also showed for the first time that the novel PI3K/mTOR inhibitor NVP-BEZ235 is twice as potent as everolimus in inhibiting the viability of BC primary culture cells, indicating that the PI3K pathway plays an important role in the regulation of the proliferation of BC cells. This hypothesis is further supported by the evidence that mTOR inhibitor-sensitive BC tissues display higher levels of p70S6K, AKT, and ERK1/2 than the resistant BC tissues. In addition, we found that human BC tissues that are resistant to everolimus are not responsive to NVP-BEZ235 either, suggesting that pathways different from the PI3K pathway should be inhibited to overcome the resistance to mTOR inhibitors in BCs.

In agreement with our previous results (Zatelli *et al.* 2010*a*,*b*), ‘sensitive’ BC tissues showed higher levels of total and phosphorylated mTOR than the ‘resistant’ BCs, supporting the hypothesis that mTOR expression levels may be useful to separate ‘sensitive’ tumors from ‘resistant’ tumors (Rossi *et al.* 2012). It has been hypothesized previously that resistance to everolimus may be overcome by the use of drugs inhibiting PI3K/AKT (Bousquet *et al.* 2012). Indeed, everolimus inhibits mTOR by interacting with the mTORC1 complex, which, in turn, inhibits p70S6K phosphorylation. The latter normally exerts a negative feedback on the PI3K/AKT/mTOR pathway activated by growth factors. Therefore, treatment with everolimus may cause rebound activation of the PI3K/AKT pathway by reducing p70S6K phosphorylation (Haruta *et al.* 2000, Shi *et al.* 2005, Sun *et al.* 2005). However, in our experimental settings, NVP-BEZ235, which inhibits both PI3K and mTOR, did not overcome the resistance of BCs to mTOR inhibitors. Therefore, it is crucial to identify possible markers that may predict tumor sensitivity to mTOR inhibitors in order to employ mTOR inhibitor therapy only in those patients who are likely to respond. We employed two human BC cell lines, the NCI-H720 and the NCI-H727 cells, displaying different levels of total and phosphorylated mTOR protein. Indeed, our data show that the NCI-H720 cells display higher total and phosphorylated mTOR protein levels than the NCI-H727 cells, similar to what has been observed in the ‘sensitive’ and ‘resistant’ BCs respectively. These human cell lines can be considered a reliable model, since the NCI-H720 cells, which have

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higher mTOR levels, are much more sensitive to everolimus in terms of cell viability reduction and apoptosis activation than the NCI-H727 cells, which exhibit lower mTOR levels. In addition, our data show that in the 'sensitive' group of human BC primary cultures, NVP-BEZ235 is two- to threefold more effective in terms of cell viability reduction and apoptosis activation than everolimus. Similarly, in 'sensitive' NCI-H720 cells, NVP-BEZ235 is twice as potent as everolimus. On the other hand, the efficacy of NVP-BEZ235 is very much reduced in 'resistant' NCI-H727 cells. Indeed, the IC_{50} for cell viability reduction is very similar for NVP-BEZ235 and everolimus in the NCI-H727 cells, as has been also reported previously by Zitzmann *et al*. (2010). Moreover, the reduction in the viability of the NCI-H727 cells observed under treatment with both mTOR inhibitors is not mirrored by a parallel apoptosis induction (if not at the higher concentrations), indicating that the antiproliferative effects of the employed compounds are mediated by other mechanisms in the 'resistant' cell line. Therefore, we examined the effects of these compounds on cell-cycle progression, since NVP-BEZ235 has been shown to induce G1 arrest (Maira *et al*. 2008). Our data show that both NVP-BEZ235 and everolimus induce an accumulation in the G0/G1 phase of the cell cycle only in the 'sensitive' NCI-H720 cells. This phenomenon is paralleled by a reduction in the levels of phosphorylated GSK3β, which regulates cyclin D1 protein levels, which are, in turn, downregulated. However, in the NCI-H727 cells, the reduction in both phosphorylated GSK3β and total cyclin D1 levels does not correspond to a significant cell-cycle phase modification, suggesting that further mechanisms allow 'resistant' BC cells to bypass cyclin D1 downregulation and that the antiproliferative effects of NVP-BEZ235 may be due to direct toxic effects (necrosis). Further studies will clarify whether mTOR resistance is due to altered cell-cycle progression.

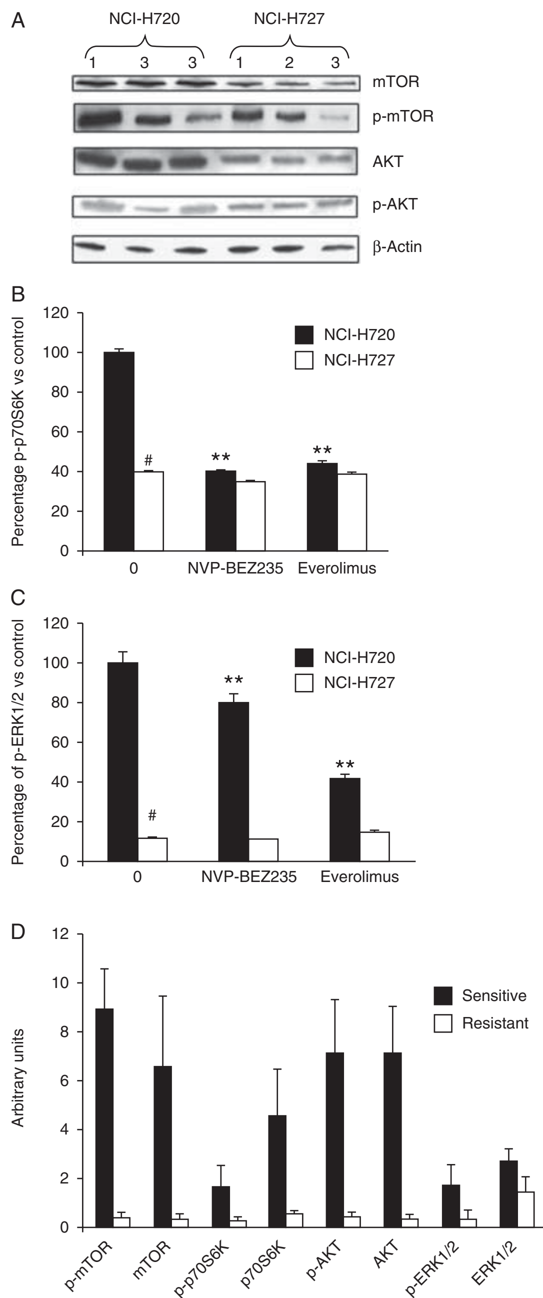

Figure 5
PI3K/AKT/mTOR pathway protein levels in BC cell lines and tissues. The NCI-H720 and NCI-H727 cells were incubated for 72 h in a culture medium supplemented with 100 nM NVP-BEZ 235 or everolimus, and control cells were treated with a vehicle solution. (A) Total proteins were isolated from the NCI-H727 and NCI-H720 cells and western blot analysis for total mTOR, phosphorylated mTOR (p-mTOR), total AKT, and phosphorylated AKT (p-AKT) expression was performed. β-Actin is shown as a loading control. Lane 1, mock-treated cells. Lane 2, cells treated with NVP-BEZ235. Lane 3, cells treated with everolimus. (B) The NCI-H720 (black bars) and NCI-H727 (white bars) cells were lysed and processed for total and phosphorylated p70S6K (p-p70S6K) SureFire assays in duplicate in three independent experiments. p-p70S6K levels were calculated as the ratio of p-p70S6K:total p70S6K cps output for each sample. Data are expressed as percentage of p-p70S6K vs the control, considering as the control sample the untreated NCI-H720 cells. $^{**}P<0.01$ vs the untreated control cells. $^{\#}P<0.01$ vs the control NCI-H720 cells. (C) The NCI-H720 (black bars) and NCI-H727 (white bars) cells were lysed and processed for total and phosphorylated ERK1/2 (p-ERK1/2) SureFire assays in duplicate in three independent experiments. p-ERK1/2 levels were calculated as the ratio of p-ERK1/2:total ERK1/2 cps output for each sample. Data are expressed as percentage of p-ERK1/2 vs the control, considering as the control sample the untreated NCI-H720 cells. $^{**}P<0.01$ vs the untreated control cells. $^{\#}P<0.01$ vs the control NCI-H720 cells. (D) Five 'sensitive' (black bars) and five 'resistant' (white bars) BC human tissues were disrupted, lysed, and processed for western blot analysis for p-mTOR, total mTOR, p-p70S6K, total p70S6K (p70S6K), p-AKT, total AKT, p-ERK1/2, ERK1/2, and β-actin expression for normalization purposes. Protein levels were measured as the ratio of the protein optical density (OD) to the β-actin OD of the same sample, and they are expressed as arbitrary units (mean±s.e.m.).

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Endocrine-Related Cancer

In summary, we found that the novel dual PI3K/mTOR inhibitor NVP-BEZ235 is twofold more effective than everolimus in 'sensitive' human BC tissues and cell lines but not in 'resistant' ones. Therefore, we set out to identify possible markers of sensitivity to mTOR inhibitors that may enable the selection of the correct drug for BCs.

In our settings, 'sensitive' BC primary cultures were derived from significantly younger patients when compared with those bearing 'resistant' BCs. Clinical studies evaluating the efficacy of everolimus in NETs have reported that the overall survival is greater in patients in the treatment arm than in those in the placebo arm independent of age (Pavel *et al*. 2011, Yao *et al*. 2011), even in the settings of BCs (Fazio *et al*. 2013). However, these studies did not examine the relationship between age and the efficacy of mTOR inhibitors in terms of tumor bulk reduction. Further analyses of the data collected during these interventional studies are necessary to clarify whether age may be considered a prognostic index of sensitivity to mTOR inhibitor therapy.

In addition, the expression level of mTOR pathway components was evaluated in the NCI-H720 and NCI-H727 cells. As has been reported already, the 'sensitive' BC cell line showed higher total and phosphorylated mTOR levels than the 'resistant' BC cells, in keeping with the higher sensitivity of these cells to mTOR inhibitors. In addition, our data show that in both 'sensitive' and 'resistant' BC cells, everolimus is more effective than NVP-BEZ235 in reducing mTOR phosphorylation, in keeping with the reported lower IC_{50} of the first compound (1.6–2.4 nM) when compared with the second compound (6.5–21 nM) (Sedrani *et al*. 1998, Liu *et al*. 2009). Therefore, the reduction in mTOR phosphorylation shall not be considered as a marker of sensitivity to the antiproliferative effects of mTOR inhibitors.

Similarly to total mTOR levels, basal AKT protein levels were also higher in the 'sensitive' BC cell line, where phosphorylated AKT levels were reduced only by NVP-BEZ235. This evidence is in agreement with the 'rebound' hypothesis, which might explain the resistance of mTOR inhibitors. However, in the 'resistant' BC cell line, phosphorylated AKT levels were not modified by either mTOR inhibitor, in keeping with previous results (Zitzmann *et al*. 2010), indicating that the 'rebound' hypothesis might not hold true in 'resistant' BC cells. In addition, p70S6K phosphorylation was strongly reduced by mTOR inhibitors in the 'sensitive' BC cells, while the 'resistant' BC cells displayed much lower basal p70S6K phosphorylation levels, which were not modified by the employed drugs, despite the reduction in phosphorylated mTOR levels. The latter evidence may indicate that in the 'resistant' BC cells mTOR inhibition is not mirrored by a downstream downregulation and that p70S6K may be the key for mTOR resistance in human BCs. Therefore, these data suggest that the 'resistant' phenotype in BCs is correlated with a decreased signaling efficiency of the AKT/mTOR/p70S6K pathway. In addition, basal AKT and p70S6K levels may differentiate between BCs that are likely to respond to mTOR inhibitors and those that are not likely to respond. Moreover, the evidence that ERK1/2 phosphorylation is not affected by either mTOR inhibitor in the 'resistant' BC cells supports the hypothesis that drugs inhibiting PI3K may not be useful to overcome the resistance to mTOR inhibitors in BCs. Our data indeed show that the ERK1/2 pathway, which is triggered by PI3K, is not affected by either mTOR inhibitor in 'resistant' BC cells. On the other hand, ERK1/2 protein levels are higher in the 'sensitive' BC cells than in the 'resistant' BC cells, possibly indicating that this pathway is important in regulating the growth of 'sensitive' BC cells and may represent a putative sensitivity marker.

The hypothesis that the identified proteins may be considered as putative markers of sensitivity to mTOR inhibitors is further strengthened by the evidence that the same proteins have been found to be overexpressed in an independent group of human BCs, whose sensitivity to mTOR inhibitors had been tested previously. Our data indeed show that higher basal mTOR, p70S6K, AKT, and ERK1/2 protein levels characterize BC tissues that respond to everolimus in terms of cell viability reduction *in vitro*.

In conclusion, our data indicate that, among the signaling molecules participating in the AKT/mTOR pathway, basal mTOR, p70S6K, AKT, and ERK1/2 proteins may be useful markers to identify human BCs that may benefit from medical therapy with mTOR inhibitors. The suitability of such candidate markers, however, needs to be confirmed in clinical trials. Our results provide the scientific grounds on which *in vivo* studies could be designed in the near future.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T Gagliano wrote the manuscript, designed the experiments, collected *in vitro* and clinical data, and performed the protein expression quantitative studies; M Bellio performed the primary culture studies; E Gentilin performed the statistical evaluation; D Molè performed the tissue protein isolation; F Tagliati supervised the Molecular Biology studies; M Schiavon provided surgical and biochemical information for patients from Padova; N G Cavallesco provided surgical and biochemical information for patients from Ferrara; L G Andriolo helped in collecting the surgical samples; M R Ambrosio provided clinical and biochemical information for patients from Ferrara; F Rea supervised and coordinated the surgical aspects; E degli Uberti supervised and coordinated the medical aspects; M C Zatelli contributed to manuscript writing and re-elaborated and matched *in vitro* and clinical data.

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