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PRECLINICAL STUDY

In vitro activity of the mTOR inhibitor everolimus, in a large panel of breast cancer cell lines and analysis for predictors of response

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Abstract Everolimus (RAD001, Afinitor®) is an oral, selective mTOR inhibitor recently approved by the US-FDA in combination with exemestane for treatment of hormone receptor positive advanced breast cancer. To date, no molecular predictors of response to everolimus in breast cancer have been identified. We hypothesized predictive markers could be identified using preclinical models. Using a molecularly characterized panel of human breast cancer and immortalized breast epithelial cell lines, we determined sensitivity to everolimus alone or in combination with ER− or HER2− targeted therapy. Gene expression microarrays and comparative genomic hybridization were performed on the cell lines to identify predictors of response to everolimus. Among 13 everolimus-sensitive cell lines, 10/13(77 %) were luminal, while in 26 resistant cell lines, 16/26(62 %) were non-luminal, and 10/26(38 %) were luminal. Only 3/24 non-luminal lines were sensitive, two of which were HER2+. Everolimus enhanced the anti-proliferative effect of both tamoxifen (TAM) and fulvestrant (FUL) in ER+ breast cancer cell lines, as well as trastuzumab in HER2+ cell lines. Everolimus + FUL but not everolimus + TAM reversed acquired resistance to TAM. Everolimus inhibited mTOR in tested cell lines by decreasing S6 phosphorylation, mediating its anti-proliferative effect by G0/G1 cell cycle arrest and induction of apoptosis. Chromosomal amplifications of *AURKA* (p value = 0.04) and *HER2* (p value = 0.03) were each associated with increased sensitivity to everolimus. Transcript expression microarrays identified *GSK3A, PIK3R3, KLF8*, and *MAPK10* among the genes overexpressed in sensitive luminal lines, while *PGP, RPL38, GPT*, and *GFAP* were

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among the genes overexpressed in resistant luminal cell lines. These preclinical in vitro data provide further support for continued clinical development of everolimus in luminal (ER+ or HER2+) breast cancer in combination with targeted therapies. We identified several potential molecular markers associated with response to everolimus that will require validation in clinical material.

Keywords Everolimus · mTOR · Breast cancer · Predictors of response

Abbreviations

ANOVA	Analysis of variance
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FITC	Fluorescein isothiocyanate
FUL	Fulvestrant
IC_{50}	Drug concentration that provides 50 % growth inhibition
mTOR	Mammalian target of rapamycin
HER2	Human epidermal growth factor receptor 2
Nim-DAPI	Nuclear isolation medium–4,6-diamidino-2-phenylindole dihydrochloride
OS	Overall survival
PI3K	Phosphoinositide 3-kinase
PFS	Progression-free survival
PTEN	Phosphatase and tensin homolog
RR	Relative risk
TAM	Tamoxifen
TNBC	Triple-negative breast cancer
TTP	Time to progression

Introduction

Molecular profiling of human breast cancers has revealed distinct subtypes that are characterized and often driven by altered signaling pathways [1, 2]. One such signaling pathway that has been shown to be altered in many human cancers is the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. This pathway has been shown to play a critical role in cell proliferation, migration, differentiation, and survival [3]. The mTOR protein is a 289-kD intracellular serine/threonine kinase with activity important in regulating the PI3K pathway through modulation and integration of signals from the intracellular and extracellular environments [4]. mTOR is a component of two multiprotein complexes: mTORC1 and mTORC2 [5]. Activation of mTORC1 leads to production of proteins via phosphorylation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase 1 (S6K1) [4]. The activation and downstream effects of mTORC2 are less well defined, but evidence suggests a role in cell survival, proliferation, metabolism, and cytoskeletal organization [4, 6–9].

While the PI3K/AKT/mTOR pathway appears to be critically important in numerous normal cell processes, its dysregulation has been demonstrated to be a significant factor in the pathogenesis of multiple cancers [3, 10, 11]. Breast cancers often bear molecular alterations that activate the PI3K/AKT/mTOR pathway such as overexpression of tyrosine kinases, downregulation of the tumor suppressor PTEN [12–15], activation of PI3K [16–18], or overexpression/hyperactivation of AKT [19]. Given the fundamental role of PI3K/AKT/mTOR signaling in cancer pathophysiology, therapeutic agents have been developed to target various components of the pathway. One such agent is everolimus (RAD001, Afinitor®, Novartis, Basel, Switzerland), an orally administered derivative of rapamycin that is a selective allosteric mTORC1 inhibitor [20–23]. Everolimus forms a complex with the intracellular receptor FKBP12 [24, 25] and may indirectly inhibit mTORC2 by sequestering free mTOR so that the assembly of mTORC2 complexes is precluded [8].

Everolimus has received approval by the US Food and Drug Administration (FDA) for treatment of hormone receptor-positive advanced breast cancer in combination with exemestane in post-menopausal patients with non-steroidal aromatase inhibitor-refractory disease, based on the results of a phase III randomized study [26]. Studies evaluating the use of everolimus in combination with HER2-targeted agents have also reported promising initial results [27–31].

To date, no consistent molecular determinants of responsiveness to everolimus have been defined, nor have studies clearly delineated subsets of breast cancer most likely to be sensitive to mTOR inhibition. Some data suggest that activation status of the PI3K/AKT/mTOR pathway may be an indicator of mTOR sensitivity [20, 32–34], while other data show no such association [35]. Large panels of molecularly characterized breast cancer cell lines are valuable tools for better defining molecular subtypes and screening biologically targeted therapies [36–38]. To assess the activity of everolimus in different molecular subtypes of breast cancer and to better understand molecular determinants of response to mTOR inhibition, we assessed the in vitro effects of everolimus as a single agent across a panel of 49 human breast cancer and 3 immortalized breast epithelial cell lines. In addition, we assessed the activity of everolimus in combination with tamoxifen, fulvestrant, and trastuzumab in selected estrogen receptor positive (ER+) and HER2 amplified cell lines, respectively.

Materials and methods

Cell lines, cell culture, and reagents

Our molecularly characterized cell line panel included 49 breast cancer cell lines and 3 immortalized breast epithelial cell lines representing the known molecular subgroups of breast cancer as previously described [38, 39]. Cell lines conditioned for acquired everolimus resistance were established in our laboratories as described previously [38, 39]. Everolimus (Afinitor®) was obtained from Novartis (Basel, Switzerland) and dissolved in DMSO (20 μM).

Point mutation data from the cancer cell line encyclopedia

The cancer cell line encyclopedia (CCLE) project has sequenced 4059 protein-coding genes via solution phase hybrid capture and massively parallel sequencing. These point mutation data were downloaded from the Broad Institute's publicly available database (http://www.broadinstitute.org/ccle/home) and incorporated into the UCLA Translational Oncology Research Laboratory (TORL)'s database for those cell lines where the two panels overlap. Data were available on 38 of the cell lines in UCLA-TORL's everolimus response panel. The CCLE has pre-restricted its whole-exome sequencing dataset for several inclusion criteria with the intention of enriching for oncogenic "driver" alterations [40].

Detailed information on cell lines, proliferation assays, comparative genomic hybridization, microarray analysis, flow cytometry analysis of cell cycle and apoptosis, western blot, and statistical analysis have been previously described [37, 38, 41] and can be found in online resource Supplementary Materials and Methods.

Results

Response to everolimus and molecular subtypes of breast cancer

A panel of 49 human breast cancer cell lines and 3 immortalized breast epithelial cell lines were used to test the anti-proliferative effects of everolimus. We observed relatively flat dose response curves over a wide range of concentrations in a majority of cell lines. For this reason, IC_{50} alone was deemed to be inadequate to classify response. Instead, a non-standard two-criterion cutoff was used to determine sensitivity such that those cell lines with an IC_{50} below 1 nM and those with more than 80 % growth inhibition at 100 nM were classified as sensitive. Those cell lines with an IC_{50} over 50 nM were classified as resistant. The rest were classified as intermediately sensitive. Based on these response criteria, 13 cell lines were classified as sensitive, 13 as intermediate, and 26 as resistant to everolimus (Table 1).

Four HER2-amplified lines that were conditioned for acquired resistance to trastuzumab or lapatinib were included in the panel (Table 1). While the BT-474 parental line was everolimus sensitive, the two resistant lines showed intermediate sensitivity. The SK-BR-3 parental line and SK-BR-3-LR were resistant to everolimus, and the SK-BR-3-TR line had intermediate sensitivity.

A total of 13 cell lines were determined to be sensitive to everolimus, and the majority (10/13; 77 %) represented the luminal subtype. Of these, 10/13 (77 %) were ER+ (5 HER2+, 5 HER2−), and 3 were basal/post-EMT (2 ER−/HER2+, 1 ER−/HER2−). While the sensitive lines were enriched for ER expression, not all of the ER+ responded to everolimus with 8/21 (38 %) ER+ classified as resistant (Table 1). The remainder of the lines in the panel was determined to have intermediate sensitivity. Similar response distributions were observed for the cell lines constituting the HER2+ subtype, i.e., 7/21 (33 %) sensitive versus 6/21 (29 %) resistant.

Of 26 resistant cell lines, 16 (62 %) were non-luminal (either basal or post-EMT) or immortalized (ER− and HER2−). In contrast only 3/24 (13 %) non-luminal (basal/post-EMT) lines were sensitive to everolimus, two of which were HER2+. All three immortalized cell lines were classified as resistant.

These findings suggest that, despite enrichment for ER and HER2 in sensitive cell lines and loss of these markers in resistant cell lines, these subtype classifications alone do not fully explain the differences in response to everolimus, and a search for other molecular predictors of response is warranted.

Everolimus enhances the anti-proliferative effect of tamoxifen and fulvestrant in ER-positive breast cancer cell lines

The combinations of everolimus with the estrogen receptor antagonists, TAM and FUL, were evaluated on three ER + luminal breast cancer cell lines with variable everolimus single-agent activity (sensitive; MDA-MB-415 and CAMA-1, and resistant; MCF-7). We observed an enhancement of the anti-proliferative effect of TAM and FUL with everolimus in all three tested cell lines (Supplementary Fig. S1).

Everolimus in combination with hormone pathway and HER2-targeting agents

Combinations of everolimus with TAM or FUL were evaluated in MCF-7 cells conditioned for acquired resistance to tamoxifen by a long-term estrogen deprivation

Table 1 The calculated response parameters for each cell line and its molecular classification

Cell line	Average inhibition at 100 nM (%)	Average IC_{50} (nM)	Classification	Breast cancer subtype	HER2 status	ER status
HCC-1500	>100	<0.05	Sensitive	Luminal	Normal	Positive
MDA-MB-175	>100	<0.4	Sensitive	Luminal	Normal	Positive
ZR-75-30	>100	< 0.05	Sensitive	Luminal	Amplified	Positive
HCC-202	>100	0.08	Sensitive	Luminal	Amplified	Positive
BT-474	>100	0.06	Sensitive	Luminal	Amplified	Positive
EFM-192A	>100	0.12	Sensitive	Luminal	Amplified	Positive
HCC-1419	>100	0.20	Sensitive	Luminal	Amplified	Positive
CAMA-1	>100	< 0.4	Sensitive	Luminal	Normal	Positive
HCC-1569	91.8	0.15	Sensitive	Post-EMT	Amplified	Negative
MDA-MB-415	91.7	< 0.4	Sensitive	Luminal	Normal	Positive
EFM-19	90.3	0.72	Sensitive	Luminal	Normal	Positive
HCC-1954	88.5	0.52	Sensitive	Basal	Amplified	Negative
HCC-38	87.6	0.28	Sensitive	Basal	Normal	Negative
BT-474-LR	>100	3.19	Intermediate	Luminal	Amplified	n/a
HCC-1187	91.2	2.32	Intermediate	Basal	Normal	Negative
UACC-893	86.9	7.21	Intermediate	Luminal	Amplified	Positive
BT-474-TR	67.9	< 0.05	Intermediate	Luminal	Amplified	n/a
HCC-1395	66.8	16.81	Intermediate	Post-EMT	Normal	Negative
ZR-75-1	66.3	13.72	Intermediate	Luminal	Normal	Positive
BT-549	61.3	3.07	Intermediate	Post-EMT	Normal	Negative
SK-BR-3-TR	60.7	37.96	Intermediate	Luminal	Amplified	n/a
SUM-190	59.6	12.30	Intermediate	Luminal	Amplified	Positive
SUM-225	59.4	29.74	Intermediate	Luminal	Amplified	Negative
BT-20	58.4	0.73	Intermediate	Basal	Normal	Negative
JIMT-1	53.2	<0.05	Intermediate	n/a	Amplified	n/a
MDA-MB-453	51.7	6.56	Intermediate	Luminal	Amplified	Negative
T-47D	50.2	67.37	Resistant	Luminal	Normal	Positive
MDA-MB-361	50.0	>100	Resistant	Luminal	Amplified	Positive
MDA-MB-468	48.9	>100	Resistant	Basal	Normal	Negative
SK-BR-3	48.5	>100	Resistant	Luminal	Amplified	Negative
DU-4475	47.5	>100	Resistant	Basal	Normal	Negative
HCC-1806	45.8	>100	Resistant	Basal	Normal	Negative
KPL-1	43.0	>100	Resistant	Luminal	Normal	Positive
HCC-1143	42.6	>100	Resistant	Basal	Normal	Negative
UACC-812	42.4	>100	Resistant	Luminal	Amplified	Positive
Hs578T	41.3	>100	Resistant	Post-EMT	Normal	Negative
MCF-7	40.4	>100	Resistant	Luminal	Normal	Positive
MDA-MB-134	38.6	>100	Resistant	Luminal	Normal	Positive
SK-BR-3-LR	38.1	>100	Resistant	Luminal	Amplified	n/a
MDA-MB-435	32.9	>100	Resistant	Post-EMT	Normal	Negative
HCC-1937	31.1	>100	Resistant	Post-EMT	Normal	Negative
CAL-51	30.4	>100	Resistant	Post-EMT	Normal	Negative
MDA-MB-231	29.3	>100	Resistant	Post-EMT	Normal	Negative
UACC-732	26.8	>100	Resistant	Luminal	Amplified	Positive
184B5	26.4	>100	Resistant	Immortalized	Normal	Negative
MDA-MB-157	25.9	>100	Resistant	Post-EMT	Normal	Negative
MDA-MB-436	22.8	>100	Resistant	Post-EMT	Normal	Negative

Table 1 continued

Cell line	Average inhibition at 100 nM (%)	Average IC_{50} (nM)	Classification	Breast cancer subtype	HER2 status	ER status
HCC-2218	17.0	>100	Resistant	Luminal	Amplified	Positive
COLO-824	14.6	>100	Resistant	Basal	Normal	Negative
MCF-10A	14.6	>100	Resistant	Immortalized	Normal	Negative
184A1	12.0	>100	Resistant	Immortalized	Normal	Negative
HCC-70	8.1	>100	Resistant	Basal	Normal	Negative

Sensitivity to everolimus was defined as the average inhibition at 100 nM > 80 % and IC_{50} < 1 nM. Resistance was defined as IC_{50} > 50 nM. Included are molecular subtype and HER2 and estrogen receptor (ER) status. n/a, not applicable; Post-EMT, cell lines classified as representing breast cancers that had undergone an epithelial-to-mesenchymal transition

(MCF7-TAMR). The anti-proliferative effect of the everolimus + tamoxifen combination in tamoxifen-resistant MCF7 cells was similar to the effect of single-agent tamoxifen (Supplementary Fig. S2). Conversely, everolimus potentiated the anti-proliferative effect of fulvestrant in this cell line suggesting that everolimus + fulvestrant may be a combination that reverses acquired resistance to tamoxifen.

The combination of everolimus with trastuzumab was evaluated in two HER2+ breast cancer cell lines: one everolimus sensitive (BT-474) and one everolimus resistant (SK-BR-3). In both cell lines, growth inhibition was enhanced with the combination of everolimus and trastuzumab compared to either agent used alone (Supplementary Fig. S3).

Biochemical effects of everolimus on signal transduction

To evaluate differential effects of everolimus on S6 and AKT activation, western blot analyses were performed in a subset of cell lines with variable sensitivities to everolimus. Cells were treated with 100 nM everolimus and harvested at 6 time points between 10 min and 48 h as described in Supplementary Materials and Methods.

We observed a significant inhibition of S6 phosphorylation in all tested cell lines after 30 min exposure to everolimus regardless of sensitivity to growth inhibitory effects (Fig. 1). This was accompanied by a less pronounced decrease in total S6 in sensitive cell lines. Feedback activation of AKT phosphorylation was observed in RAD001 sensitive (BT-474), intermediate (ZR-751), and resistant (KPL-1) cell lines. The effect of everolimus on total AKT was variable among the tested cell lines (Fig. 1).

In summary, we confirmed that everolimus inhibits mTOR in all tested cell lines by decreasing the phosphorylation of its downstream target S6. In addition, we observed a decrease in the total S6 protein in sensitive cell lines. AKT phosphorylation increased most of the cell lines tested irrespective of sensitivity.

Effects of everolimus on cell cycle and apoptosis

The effects of everolimus on the cell cycle were analyzed in a subset of sensitive, intermediate, and resistant cell lines. Cells were exposed to everolimus at 10 nM for 5 days, and then flow cytometry was performed using Nim-DAPI staining. G0/G1 cell cycle arrest was observed in the two sensitive and the one intermediate cell line tested but was not observed in resistant lines (Fig. 2).

Similarly, the effect of everolimus on apoptosis was determined in the same subset of cell lines. For this assay, cells were exposed to 100 nM of everolimus for 5 days and then analyzed with a dual stain flow cytometry protocol using Annexin V-FITC and propidium iodide. A significant increase in Annexin-V positive cells was seen in sensitive cell lines (Fig. 3). Together, these data suggest that the anti-proliferative effect of everolimus is mediated by both inhibition of the cell cycle and the induction of apoptosis.

Genotype-response association screening

After determining response to everolimus, we interrogated two large genomic datasets for candidate sensitivity or resistance biomarkers including point mutations from the CCLE database and copy number alterations (CNAs) from our own CGH arrays. Due to the large number of genetic alterations in these datasets relative to the number of cell lines in our study, false positives were a major concern. To minimize this problem, we used the publicly available gene ontology (GO) database to restrict our candidate biomarker set to those alterations most likely to be involved in PI3K/AKT/mTOR signaling (GO IDs: GO:0048015, GO:0014068, GO:0043552, GO:0014065). This gene list was cross referenced against the COSMIC gene census to further limit the list to genes where oncogenic driver mutations have been described. Our final candidate biomarker set consisted of 23 genes where either point mutations or CNAs were identified in our panel. Alterations in each of these genes were tested for an association with everolimus sensitivity or resistance (Table 2).

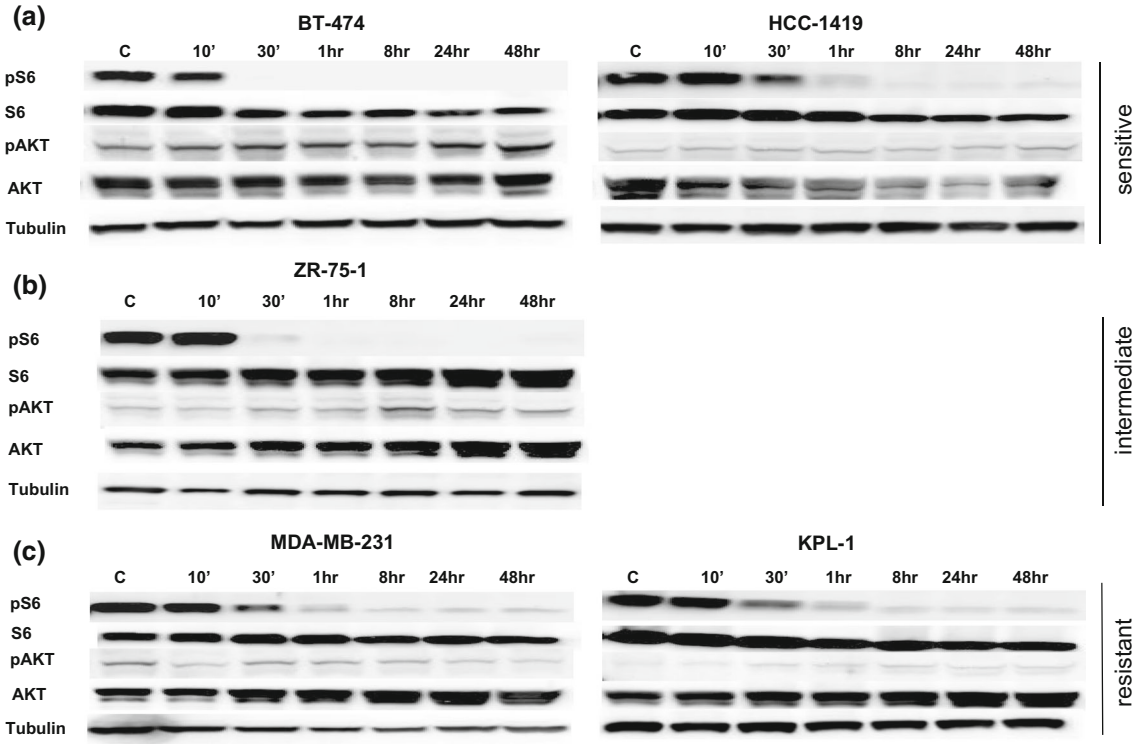

Fig. 1 The effects of everolimus on total and phosphorylated S6 and AKT. The effects of everolimus were measured in a subset of cell lines with a variable sensitivity to everolimus by western blot as described in Supplementary Materials and Methods. All cell lines were treated with 100 nM everolimus for 10 min–48 h. **a** Sensitive cell lines BT-474 and HCC-1419, **b** intermediate ZR-75-1, **c** resistant MDA-MB-231 and KPL-1. There was a significant inhibition of S6 phosphorylation in all tested cell lines and a less pronounced decrease in total S6 in sensitive cell lines. There was an increase in AKT phosphorylation in sensitive BT-474, intermediate ZR-75-1 (peak at 8 h), and resistant KPL-1. The effect of everolimus on total AKT was variable

Only two genotype-response associations were found to be statistically significant at $\alpha = 0.05$. Chromosomal amplifications of *AURKA* (p value $= 0.04$) and *HER2* (p value $= 0.03$) were each associated with increased in vitro sensitivity to everolimus. Of note, mutation of neither *PIK3CA* nor *PTEN* was significantly associated with response to everolimus.

Identification of differentially expressed genes and sensitivity to everolimus

Gene expression profiles were next used to identify genes associated with response to everolimus. First, we compared sensitive cell lines ($n = 13$) and resistant cell lines ($n = 25$) (acquired resistance cell lines were excluded). This approach identified 232 differentially expressed probes ($p \leq 0.01$), where 137 genes demonstrated increased expression in sensitive cell lines and 79 genes were increased in *de novo* resistant lines (Supplementary Table S1). Hierarchical clustering of the 38 cell lines across the 232 probes was performed. Two major branches were identified: one branch included 19 luminal lines and one HER2+ non-luminal cell line, and the other branch included all of the non-luminal lines ($n = 18$) (Supplementary Fig. S4). The 10 everolimus-sensitive luminal cell lines clustered together within the luminal branch.

We next compared only luminal breast cell lines classified as sensitive ($n = 10$) or resistant ($n = 9$) to everolimus. A total of 124 probes were differentially expressed ($p \leq 0.01$), where 72 genes demonstrated increased expression in sensitive lines, and 48 genes were increased in resistant lines (Supplementary Table S2). Two major branches were identified corresponding to everolimus sensitivity (Supplementary Fig. S5). Genes having a higher average expression level in luminal cell lines sensitive to everolimus included *GSK3A, PIK3R3, KLF8,* and *MAPK10*, whereas *PGP, RPL38*, *GPT*, and *GFAP* were found to be higher in resistant luminal cell lines.

Discussion

Dysregulation of the PI3K/AKT/mTOR pathway has been shown to play a role in breast cancer growth, progression, and treatment resistance, making it an attractive target for novel therapeutic agents. Using a large panel of well-characterized human breast cancer cell lines representing

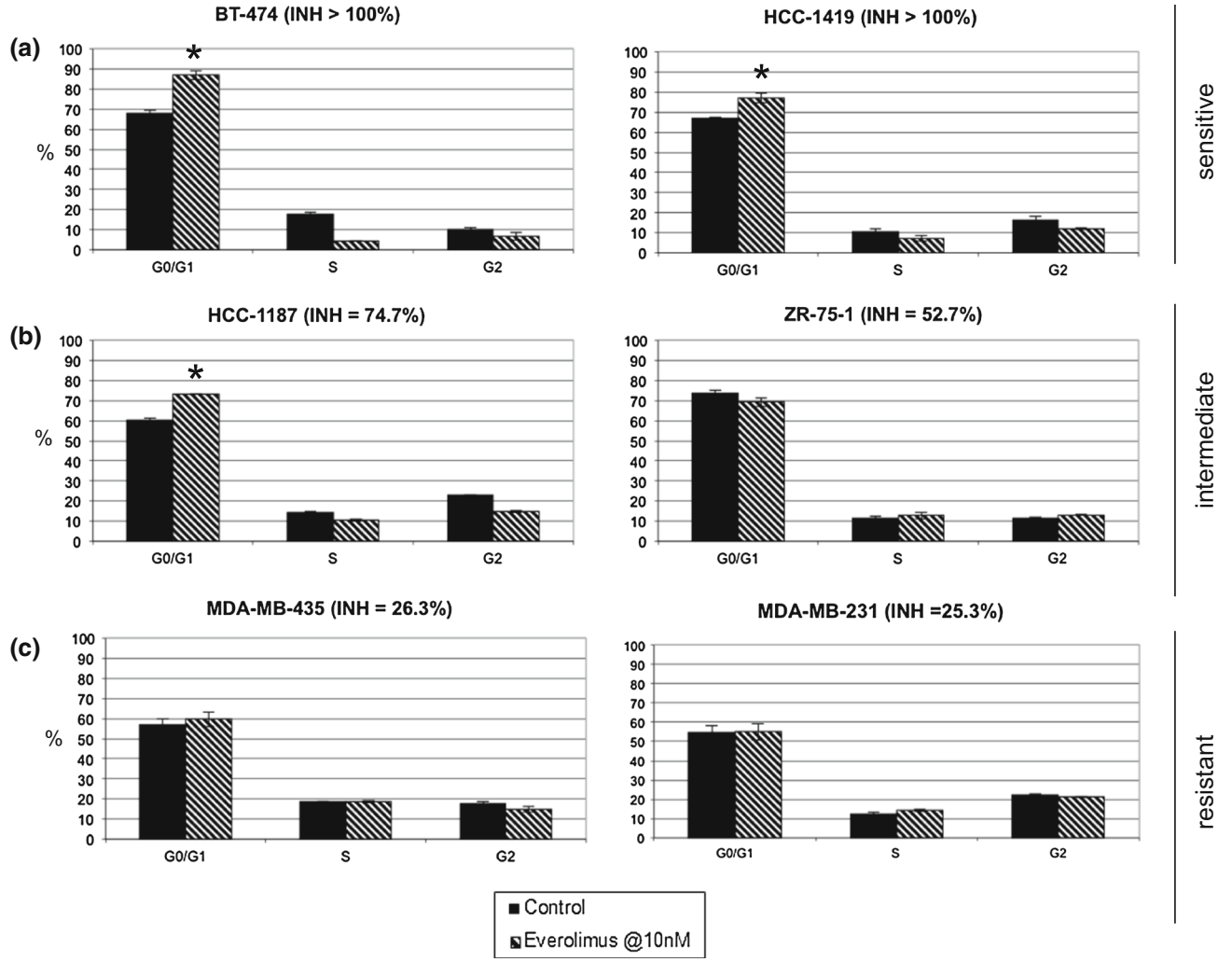

Fig. 2 Effects of everolimus on cell cycle. Cell lines with variable sensitivity to everolimus were treated with 10 nM everolimus for 5 days. **a** Sensitive cell lines show a G_0/G_1 arrest. **b** In intermediate cell lines, everolimus caused variable effects on the cell cycle. **c** No effect on the cell cycle was observed in resistant cell lines. The average growth inhibitory effect of 10 nM everolimus for each cell line is shown in parentheses. *Solid bars*- control samples; *striped bars*- treated samples. *Error bars* represent standard error for two separate experiments. *Indicates $p < 0.05$ compared to control

the known molecular subtypes of breast cancer, we identified significant enrichment of ER+ and HER2+ cell lines among those classified as sensitive to everolimus. Biochemical studies did not reveal any correlation with the ability of everolimus to block downstream mTOR signaling with growth inhibition given that everolimus blocked S6 activation in both sensitive and resistant cell lines. However, the drug's ability to induce G0/G1 arrest and apoptosis was only seen in the cell lines classified as sensitive. These observations are consistent with other studies [23]. Interestingly, we also demonstrated that everolimus induces feedback activation of AKT which was a phenomenon thought to be important in escaping the drug's effect; however, it was observed in both sensitive and resistant cell lines in the current study.

Additionally, combination therapy data indicate that everolimus enhances the anti-proliferative effect of both tamoxifen and fulvestrant in ER+ luminal cell lines. The combination of everolimus plus fulvestrant was active in MCF-7 tamoxifen-resistant cells that had been generated by long-term estrogen deprivation. These findings as well as those from other preclinical studies [42, 43] provide evidence that everolimus may reverse endocrine resistance. Two relevant randomized clinical trials have now been reported in which post-menopausal women with ER+, aromatase inhibitor-resistant advanced breast cancer were randomly assigned to receive an endocrine agent plus placebo or everolimus. In both studies, patient outcomes (clinical benefit rate, time to progression, and progression-free survival) were significantly better when everolimus was

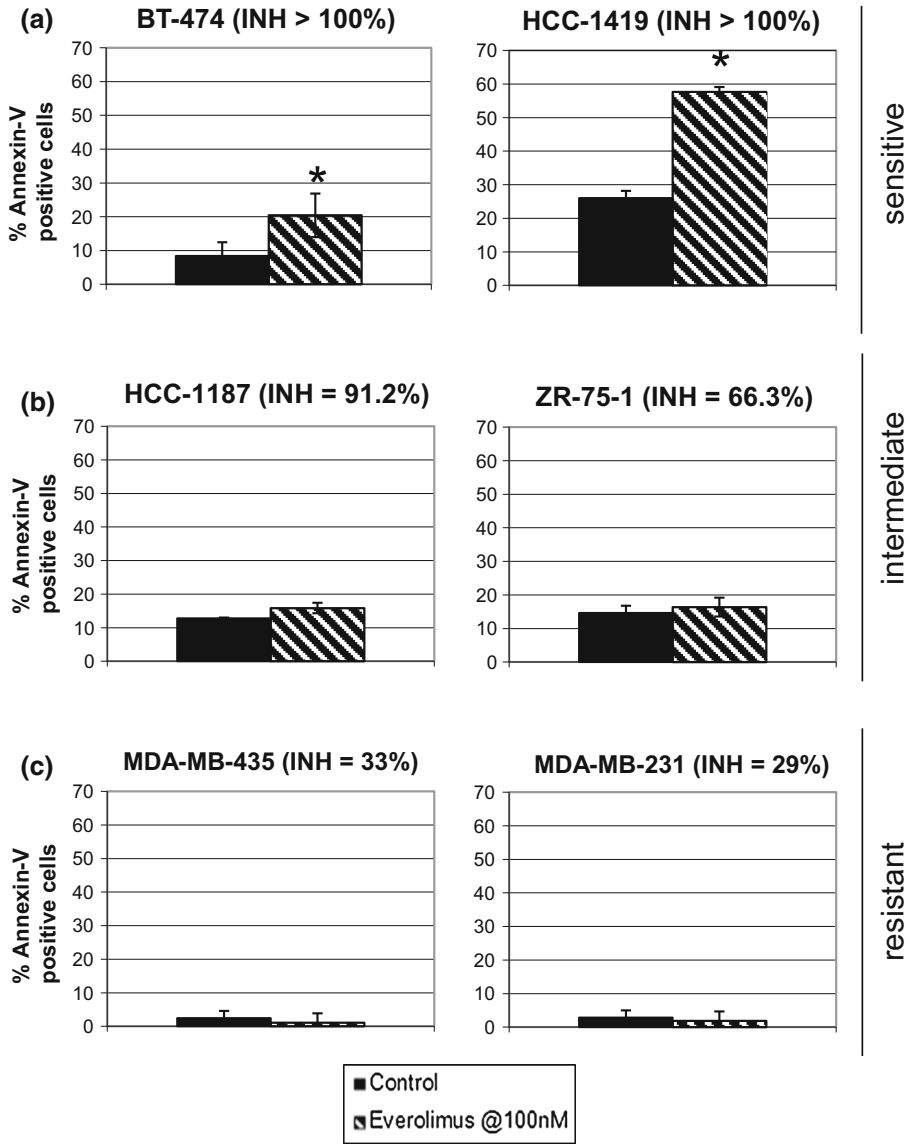

Fig. 3 Effects of everolimus on apoptosis. Cell lines with variable sensitivity to everolimus were treated with 100 nM everolimus for 5 days **a** Sensitive cell lines show an increase in Annexin-V positive cells as compared with no increase in **b** intermediate or **c** resistant cell lines. The average growth inhibitory effect (INH) of 100 nM everolimus for each cell line is shown in parentheses. *Solid bars*- control samples; *striped bars*- treated samples. *Error bars* represent standard error for two separate experiments. *Indicates $p < 0.05$ compared to control

added to either tamoxifen [44] or exemestane [26]. While our in vitro experiments did not combine everolimus with an aromatase inhibitor, the data generated add to the growing preclinical and clinical literature that shows inhibiting mTOR may restore sensitivity to hormonal manipulation in the setting of endocrine resistance.

Our in vitro analyses also indicate that HER2-amplified breast cancer cell lines are sensitive to everolimus. These data support the findings from our group [45] and others showing preclinical activity of everolimus and everolimus + trastuzumab in HER2+ breast cancer cells. The clinical activity of everolimus, trastuzumab, and taxane chemotherapy in treatment naïve HER2+ metastatic breast cancer is currently being evaluated in an ongoing randomized phase III study (BOLERO-1, NCT00876395). In addition, we have observed robust preclinical activity of everolimus in HER2+ breast cancer with *de novo* resistance to trastuzumab [45]. These preclinical data have been validated in several phase I and II clinical trials demonstrating that addition of everolimus to trastuzumab may reverse trastuzumab resistance in some patients [27–29]. Recently, a phase III randomized trial (BOLERO-3) evaluating trastuzumab and vinorelbine plus everolimus or placebo in trastuzumab-pretreated advanced HER2+ disease reported a statistically significant improvement in investigator assessed PFS for everolimus-treated patients [31].

Cell lines classified as non-luminal (basal or post-EMT), or immortalized (ER− and HER2−) were much more likely to be resistant to everolimus. Only one triple-negative cell line was classified as sensitive in our study. These findings are in contrast to a recently reported preclinical evaluation of everolimus in 9 triple-negative breast cancer

Table 2 Gene alterations tested for association with response to everolimus

Gene	Alteration	Mutations (n)	Sensitive (n)	Intermediate (n)	Resistant (n)	*p* value
ERBB2	CHR AMP	17	7	5	5	0.03*
AURKA	CHR AMP	14	7	2	5	0.04*
SPAG5	CHR AMP	5	3	0	2	0.08
FGF19	CHR AMP	12	5	3	4	0.15
RPS6KB1	CHR AMP	7	2	0	5	0.16
MDM2	CHR AMP	2	0	0	2	0.19
EGFR	CHR AMP	3	0	1	2	0.26
FGFR1	CHR AMP	5	1	2	2	0.27
FGF10	CHR AMP	1	0	0	1	0.31
TOP2A	CHR AMP	3	1	0	2	0.33
PTEN	CHR DEL	1	0	0	1	0.31
CBL	LOF PM	1	1	0	0	0.17
TSC2	LOF PM	1	1	0	0	0.17
GATA3	LOF PM	1	0	0	1	0.27
BUB1B	LOF PM	3	1	1	1	0.43
PTEN	LOF PM	8	3	2	3	0.43
NF1	LOF PM	6	2	1	3	0.47
PTPN11	ACT PM	2	2	0	0	0.06
KDR	ACT PM	1	0	1	0	0.08
PIK3CA	ACT PM	13	6	3	4	0.17
AKT1	ACT PM	1	0	0	1	0.27
ERBB2	ACT PM	1	0	0	1	0.27
KIT	ACT PM	1	0	0	1	0.27
PDGFRA	ACT PM	1	0	0	1	0.27
FGFR2	ACT PM	2	1	0	1	0.36

Gene alterations selected as described in Methods. Copy number variations were obtained from CGH analysis in 48 cell lines. Point mutations obtained from CCLE database for 38 cell lines in our panel

CHR AMP chromosomal amplification, *CHR DEL* chromosomal deletion, *LOF PM* loss-of-function point mutation, *ACT PM* activating point mutation

* $p < 0.05$

(TNBC) cell lines [46]. There are several important differences in both experimental design and analytical methodology that may explain this discrepancy. The proliferation assays performed differed in duration of treatment (5 days vs. 4 days), method of cell quantification (cell counting vs. CCK-8 colorimetric assay), and method of calculation of cell growth inhibition. In addition, everolimus is an allosteric inhibitor and has a flat dose–response curve. This observation makes the use of IC_{50} alone a difficult measure by which to stratify sensitivity/resistance outcomes. Given these concerns, we chose to use a multi-factorial cutoff for sensitivity in our analyses.

Through an unbiased analysis on a large panel of cell lines, we determined that only 38 % of ER+ and 33 % of HER2+ breast cancer cell lines were classified as sensitive to everolimus. These data recapitulate the clinical observations with everolimus [26–29] suggesting that the commonly used subtypes of breast cancer alone do not fully explain the differential response to everolimus and that additional other predictive biomarkers may exist.

In order to identify other genomic biomarkers that may predict differential sensitivity, we used several large genomics datasets with a particular focus on genes involved in PI3K/AKT/mTOR signaling and its regulation. In this analysis, chromosomal amplifications of *HER2* and *AURKA* were each associated with increased sensitivity to everolimus in vitro.

AURKA encodes for the serine/threonine kinase Aurora A and is amplified and overexpressed in multiple malignancies, including breast cancers. It regulates centrosome maturation and mitotic spindle assembly during mitotic progression [47, 48]. Overexpression of Aurora A results in the activation of AKT [49, 50] and cooperates with PI3K/AKT/mTOR pathway in oncogenic transformation [51]. Our findings indicating an association of Aurora A amplification with everolimus sensitivity suggests that activation of PI3K/AKT/mTOR pathway by Aurora A, possibly via activation of AKT, may sensitize breast cancer cells to everolimus. Whether *AURKA* amplification is a biomarker for everolimus sensitivity will need to be further explored in mechanistic studies and confirmed in clinical settings.

Microarray profiles were used to identify genes whose expression pattern is associated with response to everolimus. When we compared sensitive luminal breast cell lines to resistant luminal cell lines and performed

hierarchical clustering, two major branches were identified corresponding to everolimus sensitivity. Genes that had higher average expression in luminal cell lines sensitive to everolimus included *GSK3A, PIK3R3, KLF8,* and *MAPK10*, whereas *PGP, RPL38, GPT,* and *GFAP* were higher in resistant luminal cell lines. *GSK3A*, *PIK3R3*, and *KLF8* are known to interact with PI3K/AKT/mTOR signaling, and *PGP* is reported to be downregulated by PI3K/AKT inhibition in lymphoma cell lines [52–54]. We did not detect any significant difference in gene expression of *LKB11* (*STK11*) or *4EBP1* (*EIF4EBP1*) in sensitive versus resistant lines. These data require further exploration and validation and currently serve as hypothesis-generating observations.

To date, exploratory analyses from clinical trials evaluating everolimus have failed to consistently identify any predictive biomarkers of response including activation of the PI3K pathway (via mutations or changes in expression of protein components). Similar to other studies [33, 55], we observed no association between *PTEN* mutation and response to everolimus in vitro. In contrast, an exploratory subset analysis of BOLERO-3 suggests that low *PTEN* expression may predict greater benefit from everolimus in HER2+ metastatic breast cancers [56]. We also do not observe any association between *PIK3CA* mutations and response to everolimus in the current study. This is consistent with findings from a subset analysis of the BOLERO-2 trial in which gene alterations in the PI3K pathway did not correlate with response to everolimus [35]. Conversely, the BOLERO-2 analysis did show that patients with tumors that were either wild type or bearing only one alteration among the *PIK3CA*, *PTEN*, *CCND1*, and *FGRF1/2* genes had a better PFS compared to those with multiple alterations [35].

Data from the current broad analysis of everolimus response in a large breast cancer cell line panel continue to support further development of everolimus in luminal ER+ and HER2+ breast cancer. These analyses have identified several candidate biomarkers that may predict response to everolimus; however, these markers will require further validation in ongoing clinical trials of the drug in this disease.

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Conflict of interest Sara Hurvitz: travel to international conferences reimbursed by Novartis, Boehringer-Ingelheim, OBI Pharma and Roche, honorarium from Genentech, research funding by Novartis Pharmceuticals, Corp. Dennis J. Slamon: honoraria from Speakers Bureau- Genentech and Sanofi; ownership interest, Amgen; consultant/advisory board, Novartis Pharmaceuticals. Richard Finn: Novartis Pharmaceuticals, Corp research funding. David Chen and Ronald Linnartz: Novartis Pharmaceuticals, Corp. employment and stock ownership. This work was funded in part by Novartis. Other authors have no potential conflict of interest to disclose.

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