Combination of rapamycin and 17-allylamino-17-demethoxygeldanamycin abrogates Akt activation and potentiates mTOR blockade in breast cancer cells

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Increased Akt phosphorylation was reported in cancer cell lines and tumor tissues of patients exposed to rapamycin, a response likely contributing to the attenuated antitumor activity of rapamycin. It is, therefore, necessary to develop and validate combination strategies to reverse rapamycin-induced Akt signaling. We now report that Akt activation in response to rapamycin is abrogated by 17-allylamino-17-demethoxygeldanamycin (17-AAG), a heat shock protein 90 (HSP90) inhibitor. Rapamycin/17-AAG combination results in an enhanced antiproliferative activity in both MCF-7 and MDA-MB-231 breast cancer cells. In combination 17-AAG confers potent suppression of Raf–MEK–extracellular signal-regulated kinase signaling, a pathway that is otherwise not inhibited by rapamycin individually. Importantly, 17-AAG cooperates with rapamycin to block the phosphorylation of the mammalian target of rapamycin at Ser2448, as well as its downstream effectors ribosomal p70 S6 kinase and eukaryotic initiation factor 4E binding protein 1, which is accompanied by a substantial reduction in cyclins D1 and E. The potency of rapamycin/17-AAG combination is not affected by the activation of insulin-like growth factor 1 receptor signaling, which has been previously shown to diminish the antiproliferative activity of rapamycin. Rapamycin/17-AAG combination alleviates the induction of HSP90 protein, a heat shock response frequently associated with 17-AAG monotherapy. Our findings establish a mechanistic rationale for a combination approach using rapamycin and 17-AAG in the treatment of breast cancer. *Anti-Cancer Drugs* 19:681–688 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Constitutive activation of phosphatidylinositol-3-OH kinase (PI3K) and Ras signaling pathways, owing to aberrant activation of growth factor receptors and mutations in key signaling components, has been attributed to the tumorigenesis of most human cancers including breast cancer. Remarkably, a converged downstream effector of otherwise distinct PI3K–Akt and Ras–RAF–MEK–extracellular signal-regulated kinase (ERK) signaling cascades, the mammalian target of rapamycin (mTOR) controls cell growth and proliferation via the regulation of protein translation [1]. Activation of mTOR is mediated by Akt [2] as well as ERK1/2 [3], indicating both synergy and redundancy between PI3K and Ras-mediated signaling on mTOR.

Rapamycin specifically disrupts the formation of mTORC1, a heterotrimeric protein complex consisting of mTOR, raptor, and mLST8, inhibiting the kinase activity of mTORC1 [4]. Preclinical studies indicate that breast cancer cells are particularly sensitive to mTOR inhibition [5]. Rapamycin and its analogs are currently undergoing clinical trials for the treatment of metastatic breast cancer and other malignancies. However, patient responsiveness to mTOR inhibition as monotherapy in breast cancer has been disappointing [6]. Increased Akt phosphorylation has been reported in several cancer cell lines and tumor tissues of patients receiving rapamycin treatment [7,8], implying a potential mechanism for tumors to circumvent the growth inhibition by rapamycin.

Heat shock protein 90 (HSP90) is a molecular chaperone that has an important role in the conformational maturation and stability of oncogenic signaling proteins, including HER-2, Akt, insulin-like growth factor 1 receptor (IGF-1R), and B-Raf among numerous others [9]. High expression of HSP90 in tumors is positively correlated with the decreased survival in breast cancer patients [10]. HSP90 inhibitor geldanamycin competes with ATP binding to HSP90 and inhibits its chaperone function, leading to ubiquitination and degradation of its client proteins by proteasome. Clinical trials are ongoing to evaluate geldanamycin analog 17-allylamino-17-demethoxygeldanamycin (17-AAG) in patients with metastatic breast cancer and other advanced solid tumors [11].

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We hypothesized that the combination of rapamycin and 17-AAG may abolish Akt phosphorylation and achieve an enhanced mTORC1 blockade. The premise is based on the observations that 17-AAG promotes the degradation of Akt and Raf oncoproteins, both of which are crucial in the signaling cascades, leading to mTORC1 activation (Fig. 1a). We report herein an emergent antiproliferative activity associated with the combination of rapamycin and 17-AAG. The elevated Akt activation owing to rapamycin treatment is ablated by 17-AAG. Furthermore, 17-AAG cooperates with rapamycin to augment mTORC1 inhibition in breast cancer cells.

Methods

Cell lines and chemicals

Human breast MCF-7 and MDA-MB-231 cells were kindly provided by Dr Jon Holy at University of Minnesota Medicine School, Duluth. Rapamycin, 17-AAG, LY294002, and UO126 were purchased from LC Laboratories (Woburn, Massachusetts, USA). IGF-1 was purchased from GroPep (Adelaide, Australia).

Cell proliferation assay

MCF-7 and MDA-MB-231 cells were plated in 96-well plates and treated for 72 h. Cells were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet (Sigma, St Louis, Missouri, USA), and dissolved in 0.5% Triton X-100 (Sigma). The plates were read at 560 nm on a spectrophotometer [12].

Cell cycle analysis

MCF-7 and MDA-MB-231 cells were incubated with 10 nmol/l rapamycin and/or 17-AAG (0.25 μmol/l for MCF-7 and 2.5 μmol/l for MDA-MB-231) for 72 h. The cells were then trypsinized, washed with PBS, and fixed in -20°C ethanol. Fixed cells were stained for 30 min with a staining buffer comprising propidium iodide (100 μg/ml) and RNase (20 μg/ml) in PBS buffer. Cells were counted on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, Mountain View, California, USA), and the percentage of cells in the sub-G_1, G_1, S, and G_2/M phases of the cell cycle were determined.

Western blot analysis

Whole cell lysates were resolved on 7.5 or 12% SDS-PAGE gels and transferred onto nitrocellulose membrane. Antibodies specific for cyclin E and B-Raf were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA), anticyclin D1 antibody from Calbiochem (San Diego, California, USA), and all other antibodies from Cell Signaling Technology (Beverly, Massachusetts, USA). Immunodetection was performed using corresponding horseradish peroxide-conjugated secondary antibodies. Horseradish peroxide activity was detected using enhanced chemiluminescence (Pierce, Rockford, Illinois, USA).

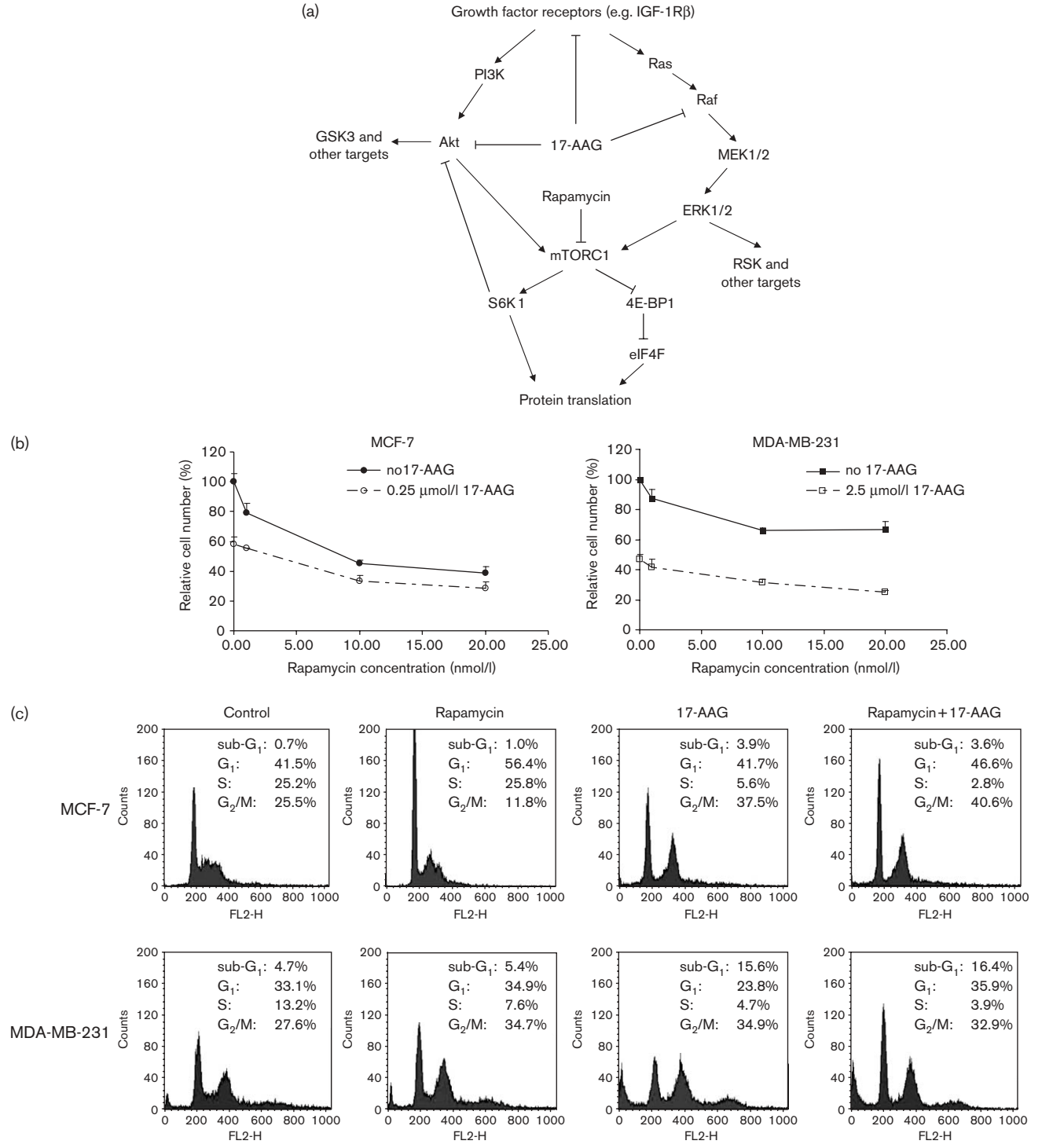
Results and discussion

Combination of rapamycin and 17-AAG exerts an enhanced antiproliferative effect

We performed cell proliferation assay to study the effect of rapamycin (1–20 nmol/l) and 17-AAG (0.05–10 μmol/l) either alone or in combination on growth inhibition of MCF-7 and MDA-MB-231 cells. As shown in Fig. 1b, rapamycin at a concentration of 10 nmol/l achieved approximately 55% growth inhibition in MCF-7 cells. The effect of 17-AAG was dose-dependent (data not shown) with 0.25 μmol/l causing about 40% growth inhibition (Fig. 1b). Compared with MCF-7, MDA-MB-231 cells were less sensitive to rapamycin. A plateau of less than 35% of growth inhibition by rapamycin was observed at concentrations ≥ 10 nmol/l. The effect of 17-AAG was also dose-dependent, but less potent (data not shown) with 2.5 μmol/l causing about 50% growth inhibition (Fig. 1b). The combination of rapamycin and 17-AAG significantly inhibited the growth of both MCF-7 and MDA-MB-231 to a greater extent compared with the same doses of each agent individually ($P < 0.05$). The combined effect was less than additive, which is likely owing to the partial redundancy of the inhibitory effects exerted by these two agents on the signaling leading to cell proliferation. Combinations of 10 nmol/l rapamycin and 0.25 μmol/l 17-AAG in MCF-7 cells and 10 nmol/l rapamycin and 2.5 μmol/l 17-AAG in MDA-MB-231 cells were chosen to further study the molecular mechanism underlying their combinatory effect, as these regimens resulted in an enhanced antiproliferative effect, and because they are within the ranges of average plasma concentrations reported in clinical trials [6,11].

To further investigate the effect of rapamycin and 17-AAG on cell viability, a cell cycle analysis was performed (Fig. 1c). In MCF-7 cells, the treatment of rapamycin led to a pronounced shift of cell population from G_2/M phase to G_1 phase, whereas 17-AAG caused a marked accumulation of cells in G_2/M phase while suppressing the cell population in S phase. The combination of rapamycin and 17-AAG resulted in cell cycle arrest in both G_1 and G_2/M phases. The number of cells undergoing apoptosis, as indicated by the sub-G_1 population, was slightly elevated after either 17-AAG or rapamycin/17-AAG treatment. These data indicate that the inhibitory effect of rapamycin and 17-AAG on MCF-7 cell proliferation is largely because of cell cycle arrest, and the enhanced antiproliferative effect observed with rapamycin/17-AAG coadministration is achieved via a more complete blockade of cell cycle progression. In MDA-MB-231 cells, individually both rapamycin and 17-AAG increased the cell population in G_2/M phase while reducing cell numbers in S phase. 17-AAG alone or in combination caused a marked increase in apoptotic cells, as indicated by a significant elevation in sub-G_1 population. The presence of apoptotic cells was confirmed by evaluating the cleavage of poly (ADP-ribose) polymerase using

Fig. 1



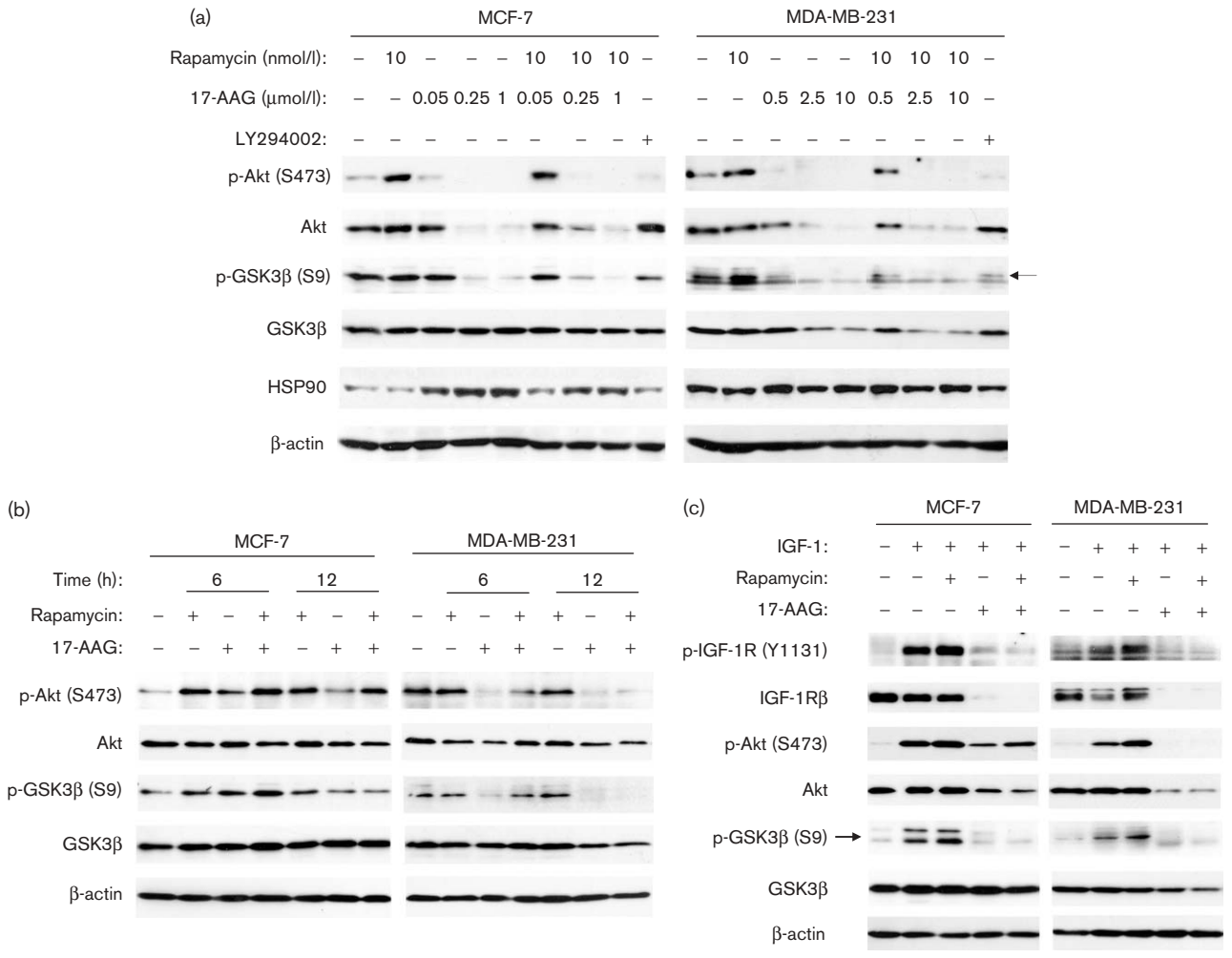
(a) Simplified scheme of phosphatidylinositol-3-OH kinase (PI3K)–Akt–mTORC1 and Ras–Raf–MEK–extracellular signal-regulated kinase (ERK)–mTORC1 signaling pathways and the molecular targets of rapamycin and 17-allylamino-17-demethoxygeldanamycin (17-AAG). (b) The combination of rapamycin and 17-AAG exerts an enhanced antiproliferative effect. MCF-7 and MDA-MB-231 cells (7500 cells/well) were incubated in 96-well plates in the presence of increasing concentrations of rapamycin and/or 17-AAG for 72 h. Results show representative data obtained from four independent experiments and are reported as the means (n=3); bars ± SD. (c) The combination of rapamycin and 17-AAG leads to cell cycle arrest and apoptosis. MCF-7 and MDA-MB-231 cells were incubated in the presence of 10 nmol/l rapamycin and/or 17-AAG (0.25 μmol/l for MCF-7 cells and 2.5 μmol/l for MDA-MB-231 cells) for 72 h. The cells were stained with propidium iodide and subjected to cell cycle analysis. Results show representative data obtained from three independent experiments. 4E-BP1, eukaryotic initiation factor 4E binding protein 1; eIF4F, eukaryotic initiation factor 4F; IGF-1Rβ, insulin-like growth factor 1 receptor β; GSK3, glycogen synthase kinase 3; mTOR, the mammalian target of rapamycin; mTORC1, mTOR complex 1; RSK, ribosomal p90 S6 kinase; S6K1, ribosomal p70 S6 kinase.

western blot analysis. When MDA-MB-231 cells were treated with 17-AAG alone or in combination, cleavage of poly (ADP-ribose) polymerase from a 116 kDa protein to a characteristic 85 kDa fragment was observed (data not shown), indicating these cells undergo apoptosis. These data demonstrate that the enhanced antiproliferative effect observed with rapamycin/17-AAG coadministration in MDA-MB-231 cells is achieved via the induction of apoptosis as well as a more complete blockade of cell cycle progression.

17-AAG abrogates rapamycin-induced Akt signaling

Two recent studies demonstrate that in lung, breast, and prostate cancer cell lines, as well as tumor biopsy samples of colorectal cancer patients, rapamycin exposure leads to an increase in Akt phosphorylation [7,8]. We asked whether 17-AAG was able to block rapamycin-induced Akt activation. The elevated Akt signaling in response to mTOR inhibition by rapamycin has been attributed to an increase in insulin receptor substrate 1, an adapter protein that mediates PI3K-Akt activation by IGF-1R, which is negatively regulated by ribosomal p70 S6 kinase (S6K1), an immediate downstream target of mTORC1 [13]. In both MCF-7 and MDA-MB-231 cells, 10 nmol/l rapamycin notably induced p-Akt (Fig. 2a). At concentrations 0.25 μmol/l or higher in MCF-7 cells, or 2.5 μmol/l or higher in MDA-MB-231 cells, 17-AAG individually or in cotreatment with rapamycin diminished p-Akt, paralleled by a marked reduction in phosphorylated glycogen synthase 3beta (p-GSK3β), an immediate Akt substrate (Fig. 2a). In addition to a substantial decline in total Akt protein resulting from 17-AAG treatment in both cell

Fig. 2



17-allylamino-17-demethoxygeldanamycin (17-AAG) abrogates rapamycin-induced Akt signaling. Cells were treated with rapamycin and 17-AAG individually or in combination at concentrations as indicated for 24 h (a), for shorter time intervals (b), or in the presence of 20 ng/ml insulin-like growth factor 1 receptor (IGF-1) with serum starvation for 24 h (c). Phosphatidylinositol-3-OH kinase (PI3K) inhibitor LY294002 was used as a positive control at 5 μmol/l. Whole cell lysates were analyzed by western blotting and probed with antibodies as indicated. β-actin level was used as a loading control. GSK3β, glycogen synthase kinase 3b; HSP90, heat shock protein 90; IGF-1R, insulin-like growth factor 1 receptor.

lines, there was also moderate loss of total GSK3β in MDA-MB-231 cells. In MCF-7 cells, 17-AAG induced HSP90 protein levels in a dose-dependent manner, which were partially reversed when coadministered with rapamycin (Fig. 2a). This effect was not discernible in MDA-MB-231 cells. Activation of heat shock response and the consequent induction of antiapoptotic HSPs is a general feature of HSP90 inhibitors and represents protective mechanisms against effective HSP90 inhibition [14]. Our result suggests that the rapamycin/17-AAG combination may provide a novel strategy to attenuate 17-AAG-induced heat shock response. As a positive control, the PI3K inhibitor LY294002 decreased p-Akt and p-GSK3β without affecting their total protein levels in both cell lines. A time-course study revealed that inhibition of p-Akt and p-GSK3β by 17-AAG in MCF-7 cells was not apparent at earlier time intervals, whereas in MDA-MB-231 cells, rapamycin/17-AAG cotreatment effectively blocked phosphorylation of Akt and GSK3β by 12 h (Fig. 2b).

Elevated IGF-1/IGF-1R signaling has been implicated in the development and progression of breast cancer and other solid tumors [15]. We tested whether 17-AAG could suppress Akt signaling induced by rapamycin in the presence of IGF-1. In serum-starved MCF-7 and MDA-MB-231 cells, IGF-1 activated IGF-1Rβ, which led to elevated p-Akt and p-GSK3β (Fig. 2c). Compared with IGF-1-treated control cells, rapamycin treatment further increased p-Akt and p-GSK3β, which were ablated when cotreated with 17-AAG. The treatment of 17-AAG alone or in combination resulted in a marked decline in total IGF-1R and Akt levels, leading to concomitant loss of p-IGF-1R, p-Akt, and p-GSK3β. Collectively, these results demonstrate that rapamycin/17-AAG combination abrogates Akt activation induced by rapamycin.

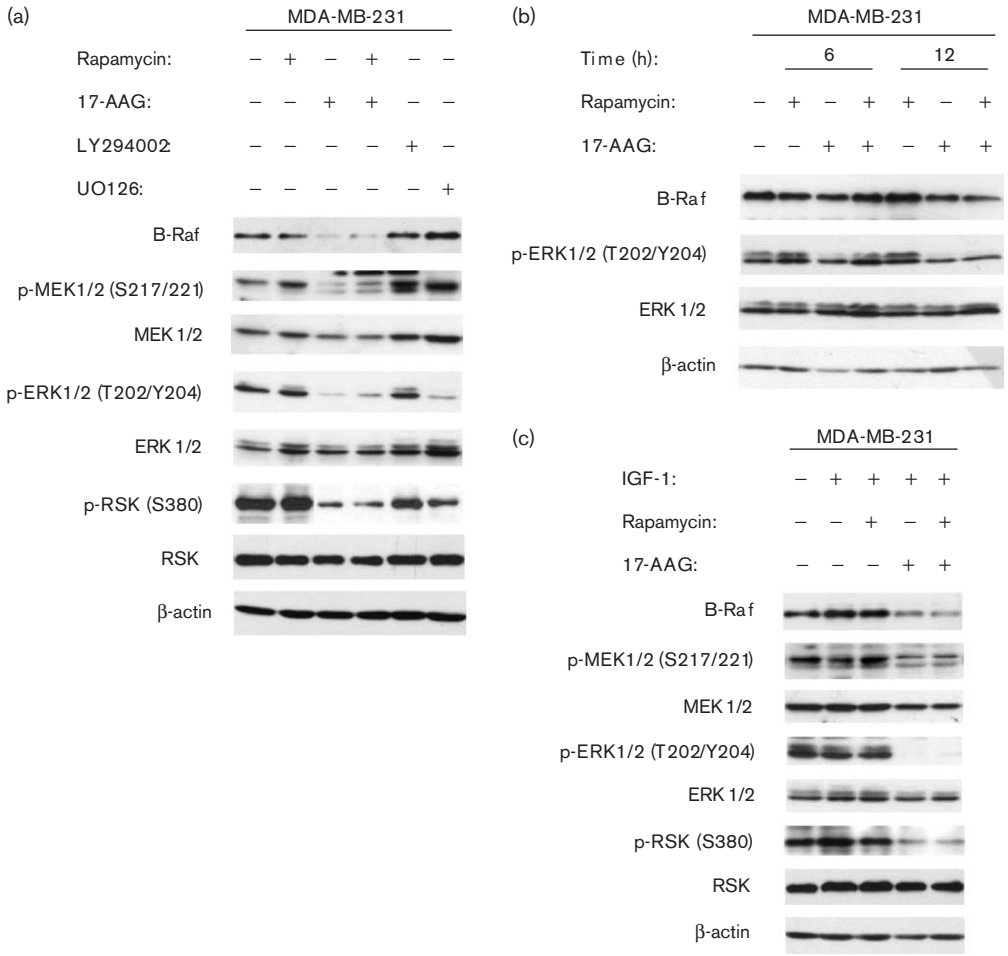
Combination of rapamycin with 17-AAG confers inhibition of Raf–MEK–ERK signaling

Hyperactivation of the Ras–Raf–MEK–ERK signaling pathway is frequently seen in breast cancer [16]. Mutated in K-Ras and B-Raf, which leads to the constitutive activation of ERK1/2 and their downstream targets, MDA-MB-231 cells were less sensitive than MCF-7 cells to the cytostatic effect of rapamycin. Phosphorylation of ERK1/2 and its direct target p90 ribosomal S6 kinase (RSK) was undetectable in MCF-7 cells (data not shown). To gain further insights into the perturbation in signaling cascades that may contribute to the emergent antiproliferative activity observed with rapamycin/17-AAG combination, we examined protein levels of B-Raf, MEK1/2, ERK1/2, and RSK in MDA-MB-231 cells. Rapamycin failed to alter the levels of any of these proteins, indicating its ineffectiveness in inhibiting Raf–MEK–ERK signaling cascade (Fig. 3a). In contrast, the treatment with 17-AAG, either alone or combined with rapamycin, resulted in a pronounced reduction in B-Raf and a concurrent decline in phosphorylation cascades of MEK1/2, ERK1/2, and RSK without affecting their total protein levels. As a positive control, MEK1/2 inhibitor UO126 specifically inhibited MEK–ERK signaling. In a time-course study, the effect of rapamycin/17-AAG combination was examined at earlier intervals in relation to B-Raf expression and phosphorylation of ERK1/2. Although a minor decline was noted only in cells treated with 17-AAG alone after 6-h exposure, a clear reduction in B-Raf and p-ERK1/2 was observed with rapamycin/17-AAG cotreatment by 12 h (Fig. 3b). The presence of IGF-1 had no effect on Raf–MEK–ERK signaling or 17-AAG action (Fig. 3c). These results indicate that 17-AAG confers potent inhibition of Raf–MEK–ERK signaling to rapamycin/17-AAG combination.

Rapamycin/17-AAG combination cooperatively inhibits mTORC1 signaling

mTORC1 regulates protein translation primarily by directly phosphorylating the key translation regulators S6K1 and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1, a negative regulator of translation initiation, by mTORC1 releases its binding to eIF4E, enabling the formation of eIF4F complex [17]. In breast cancer, hyperphosphorylation of 4E-BP1 is strongly correlated with lymph node metastasis and a poor prognosis [18]. We asked whether the combination of rapamycin and 17-AAG had an enhanced inhibitory effect on mTORC1 signaling. As expected, rapamycin blocked mTOC1 activity in MCF-7 cells, as indicated by marked inhibition of p-4E-BP1 and p-S6K1 (Fig. 4a). Most of the 4E-BP1 proteins were present in the hypophosphorylated fast migrating state, suggesting that 4E-BP1 is largely active as a translation repressor in rapamycin-treated MCF-7 cells. Interestingly, 17-AAG also potently reduced p-4E-BP1 and p-S6K1, which is consistent with the findings of a recent report [19]. Rapamycin/17-AAG cotreatment led to a further decline in p-4E-BP1 than either agent alone. Moreover, rapamycin/17-AAG combination synergistically blocked p-mTOR at Ser2448. mTOR is phosphorylated at this site by Akt [20] as well as S6K1 [21]. The decreased p-mTOR possibly reflects an augmented inhibition of S6K1 activity by rapamycin/17-AAG cotreatment, although p-S6K1 was undetectable when cells were treated with rapamycin or 17-AAG alone. In MDA-MB-231 cells, p-S6K1 level was below the detection limit (data not shown). A pronounced inhibition of p-4E-BP1 was observed with rapamycin/17-AAG regimen, compared with the modest decrease resulting from either agent individually. In these cells, 17-AAG individually or in combination noticeably inhibited p-mTOR (Fig. 4a). PI3K inhibitor LY294002, but not MEK1/2 inhibitor UO126 caused dephosphorylation of mTORC1 effectors in MCF-7 cells, suggesting the predominant PI3K-Akt regulation on mTORC1 activity. The partial reduction in p-4E-BP1 in MDA-MB-231 cells by both LY294002 and

Fig. 3



The combination of rapamycin with 17-allylamino-17-demethoxygeldanamycin (17-AAG) confers inhibition of Raf–MEK–extracellular signal-regulated kinase (ERK) signaling. MDA-MB-231 cells were treated with 10 nmol/l rapamycin and 2.5 μmol/l 17-AAG individually or in combination for 24 h (a), for shorter time intervals (b), or in the presence of 20 ng/ml insulin-like growth factor 1 receptor (IGF-1) with serum starvation for 24 h (c). LY294002 and UO126 were used at 5 μmol/l and 2.5 μmol/l, respectively. Whole cell lysates were analyzed by western blotting and probed with antibodies as indicated. β-actin level was used as a loading control. RSK, ribosomal p90 S6 kinase.

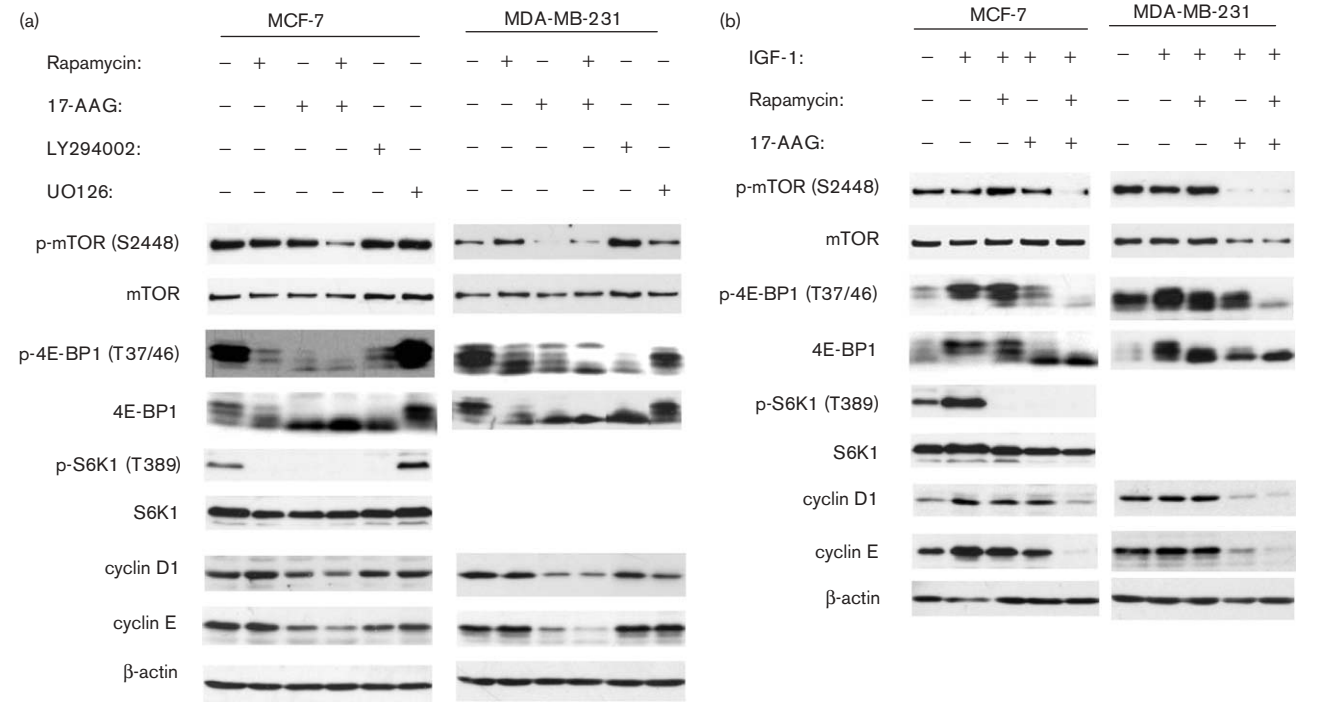
UO126 was in line with the notion that mTORC1 signaling in these cells is regulated by PI3K-Akt as well as Raf–MEK–ERK pathways.

Next, we asked whether rapamycin/17-AAG combination could inhibit mTORC1 signaling in IGF-1-treated cells. Compared with the serum-starved control cells, IGF-1 increased levels of p-S6K1 and p-4E-BP1, indicating that IGF-1R activation leads to an elevated mTORC1 activity (Fig. 4b). In MCF-7 cells, rapamycin abolished p-S6K1 induced by IGF-1, but was ineffective in decreasing p-4E-BP1, indicating that mTORC1 inhibition by rapamycin is compromised by the activation of IGF-1/IGF-1R–PI3K–Akt signaling (Fig. 4b). This result may explain the diminished antiproliferative effect of rapamycin in IGF-1-treated MCF-7 cells reported recently [8]. In contrast, 17-AAG as a single agent remained effective in down-regulating both p-S6K1 and p-4E-BP1. Rapamycin/17-AAG cotreatment synergistically ablated p-4E-BP1 as well as p-mTOR. In IGF-1-treated MDA-MB-231 cells, a substantial decline in p-4E-BP1 was also observed with rapamycin/17-AAG cotreatment, compared with the modest decrease resulting from either agent individually. In these cells, 17-AAG alone or in combination abolished p-mTOR (Fig. 4b). Collectively, our results demonstrate that the combination of rapamycin and 17-AAG enhances mTORC1 blockade.

Rapamycin/17-AAG combination reduces cyclins D1 and E levels

Cyclin D1, whose cap-dependent translation is regulated by mTORC1, is overexpressed in up to 50% of human

Fig. 4



Rapmycin/17-allylamino-17-demethoxygeldanamycin (17-AAG) combination enhances mTORC1 blockade and reduces cyclins D1 and E levels. Cells were treated with 10 nmol/l rapamycin and 0.25 μmol/l (in MCF-7 cells) or 2.5 μmol/l (in MDA-MB-231 cells) 17-AAG individually or in combination for 24 h (a) or in the presence of 20 ng/ml insulin-like growth factor 1 receptor (IGF-1R) with serum starvation (b). LY294002 and UO126 were used as at 5 μmol/l and 2.5 μmol/l, respectively. Whole cell lysates were analyzed by western blotting and probed with antibodies as indicated. β-actin level was used as a loading control. mTOR, the mammalian target of rapamycin; mTORC1, mTOR complex 1; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; S6K1, ribosomal p70 S6 kinase.

breast cancers of all histological types [22]. Importantly, overexpression of cyclin D1 has a causative role in the pathogenesis of breast cancer, as transgenic mice engineered to overexpress cyclin D1 in mammary glands succumb to breast cancer [23]. Cyclin D1 controls the activation of cyclin E, whose expression level has been positively correlated with the decreased survival in breast cancer patients [24]. We evaluated whether the treatment of rapamycin and 17-AAG-reduced cyclins D1 and E. Rapamycin had little effect on cyclins D1 and E in MCF-7 and MDA-MB-231 cells, whereas 17-AAG individually or in combination caused a marked decline in both cyclins (Fig. 4a). However, in MCF-7 cells in the presence of IGF-1 stimulation, a significant reduction in cyclins D1 and E was only achieved by rapamycin/17-AAG regimen, but not by either individual agent (Fig. 4b). In IGF-1-treated MDA-MB-231 cells, rapamycin/17-AAG cotreatment led to a more complete suppression of cyclins D1 and E than either individual agent. These results demonstrate that the combination of rapamycin/17-AAG potently decreases cyclins D1 and E in breast cancer cells.

As a master regulator of protein synthesis, mTORC1 is an important molecular target for cancer therapy. As both PI3K and Ras signaling pathways mediate mTORC1 activation, it is plausible that a more complete mTORC1 inhibition may be achieved by combining mTORC1 inhibitors with agents that block signaling transduction upstream of mTORC1. We demonstrate that the combination of rapamycin and 17-AAG inhibits rapamycin-induced Akt activation and confers inhibition of Raf–MEK–ERK signaling. Moreover, 17-AAG cooperates with rapamycin to achieve an augmented suppression of mTORC1 signaling. Rapamycin/17-AAG combination results in an enhanced antiproliferative effect in human breast cancer cells. These findings provide a mechanistic rationale for the combined administration of these two promising anticancer agents in patients with breast cancer.

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