The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to the Cytotoxic Effect of Carboplatin in Breast Cancer *In Vitro*

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Abstract. *Aim: The phosphatidylinositol 3-kinase (PI3K)/protein kinase B(AKT)/mammalian target of rapamycin (mTOR) signaling pathway is aberrantly activated in many types of cancer, including breast cancer. It is recognized that breast cancer cells develop resistance to a variety of standard therapies through the activation of this pathway. We hypothesized that targeting this signaling by the mTOR inhibitor RAD001 may potentiate the cytotoxicity of a conventional chemotherapeutic drug, carboplatin, and enhance the treatment efficacy for breast cancer. Materials and Methods: Cell proliferation was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; cell apoptosis with enzyme-linked immunosorbent assay (ELISA). Flow cytometry was used for the analysis of cell cycle distribution and mitochondrial membrane function. Gene expression at the protein level was determined by Western blot. Results: MTOR inhibitor RAD001 enhanced the sensitivity of breast cancer cells to carboplatin. RAD001 in combination with carboplatin resulted in synergistic inhibition of cell proliferation and caspase-independent apoptosis in these cells. Moreover, in MCF-7 and BT-474 cells, synergistic effects of this combination on G_2/M cell cycle arrest and regulation of different molecules responsible for cell cycle transition and apoptosis were observed. The p53 pathway was involved in the synergism of RAD001 and carboplatin on breast cancer cell proliferation and apoptosis, since the synergistic effect was demonstrated in all tested breast cancer cell lines with wild-type p53 and the use of p53 inhibitor partially antagonized the effect of RAD001 and carboplatin on p53 and p21 expression, as well as their inhibitory effect on cell proliferation. However, a synergistic effect of the combination of the two drugs on cell proliferation was observed in two p53-mutated cell lines with high AKT expression, suggesting that an alternative mechanism underlying the observed synergism exists. Conclusion: Our results suggest that the combination of RAD001 and carboplatin is a promising treatment approach for breast cancer. On the basis of these results, we have initiated a phase I/II clinical trial with the combination of carboplatin and RAD001 in patients with metastatic breast cancer.*

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The chemotherapeutic platinum drugs cisplatin and carboplatin are classified as DNA alkylating agents. There is overwhelming evidence to support the view that the major mechanism of action of this type of drugs is that they covalently bind to purine DNA bases and form DNA adducts. Subsequently, this DNA damage activates various signal-transduction pathways, for example, those involved in DNA-damage recognition and repair, cell-cycle arrest, and apoptosis (1). Carboplatin was introduced in the late 1980s and has since then gained popularity in clinical treatment due to its reduced side-effects as compared to its parent compound cisplatin. Interest in platinum compounds for the treatment of breast and ovarian cancer has increased during recent years, partially because of encouraging results from clinical trials on taxane/platinum doublets (2, 3). Nevertheless, sensitivity to platinum-based therapy is a major obstacle for its successful clinical application. Several mechanisms, *e.g.* the alterations in the mitogen-activated protein kinase (MAPK) cascade pathway, deregulation of phosphatidyl-inositol 3-kinase (PI3K)/protein kinase B(AKT)/mammalian target of rapamycin (mTOR) signaling pathways, as well as dysfunction of tumor suppression genes (such as *p53*), have so far been identified to contribute to the sensitivity of breast cancer cells to carboplatin (4).

Dysregulation of PI3K/AKT/mTOR activities frequently occurs in breast cancer. The mTOR kinase is a key downstream molecule in the PI3K/AKT pathways. Inhibition of mTOR activity by rapamycin or its analogs results in a

translational inhibition of proteins required for cell cycle progression, survival and resistance to apoptosis, thereby inhibiting the growth and progression of several solid tumor types both *in vitro* and *in vivo* (5-7). The activity of mTOR inhibitor as a single agent in solid tumors is limited. However, inhibition of mTOR has been shown to enhance the efficacy of chemo-cytotoxic agents in various types of human cancer (8-14).

The mechanism underlying mTOR inhibitor-mediated enhancement of chemosensitivity remains largely unclear. It has been postulated that RAD001 sensitizes breast cancer cells to cisplatin treatment by inhibiting p53-induced p21 expression through inhibition of its protein translation. This effect of RAD001 was not observed in tumor cells with mutated *p53* (8). However, it has been demonstrated in hepatocellular carcinoma that RAD001 enhances the sensitivity of tumor cells to cisplatin in both p53-dependent and p53-independent fashions (11). Other researchers have reported that the sensitivity of cancer cells to mTOR inhibitors is independent of *p53* status in human ovarian carcinoma cells (9), but rather dependent on the AKT activity.

Since carboplatin is a derivate of cisplatin, we speculated that mTOR inhibitor RAD001 may also potentiate the cytotoxicity of carboplatin in breast cancer cells. In this study, we investigated whether RAD001 synergistically enhanced the sensitivity of breast cancer cells with wild-type *p53* and with mutated *p53* to carboplatin. The role of p53 pathway in the synergism of the two drugs was also investigated.

Materials and Methods

Cell lines, reagents and culture conditions. Human breast cancer cell lines BT-20, BT-474, MCF-7, MDA-MB-231, SKBR-3, T47D, and ZR-75 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PAA, Cölbe, Germany), at 37˚C in a humidified atmosphere with 5% CO_2.

RAD001 (everolimus) was kindly provided by Novartis Institutes for Biomedical Research (Basel, Switzerland). A 10 mM RAD001 stock solution for cell culture was prepared in dimethylsulfoxid (DMSO) (Sigma–Aldrich, Munich, Germany), stored at −20˚C and diluted with fresh culture medium immediately before use. Carboplatin was provided by the pharmacy of the University Hospital Charité of Humboldt University. This drug was dissolved with physiological saline at a concentration of 10 mg/ml and stored at 4˚C. The pan-caspase inhibitor Z-VAD-FMK was purchased from R&D (Wiesbaden-Nordenstadt, Germany) and the p53 inhibitor pifitrin from Sigma–Aldrich (Taufkirchen, Germany).

Determination of cell viability by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Breast cancer cells were seeded into a 96-well plate at a density of 5×10^3 cells per well. After 24 h, cells were exposed to different concentrations of RAD001 (0.5, 5, and 50 nM), with or without carboplatin in culture medium. After incubation for the indicated time points, MTT reagent (Sigma–Aldrich, Munich, Germany) was added to each well and incubated for another 4 h. The reaction was stopped with 0.01 N hydrochloric acid in 10% sodium dodecyl sulfate (SDS) solution overnight and the absorbance was measured at 550 nm (15).

Cell cycle analysis. For cell cycle analysis, treated cells were harvested and fixed in 70% ethanol at 4˚C for 30 min. Cells were washed twice with phosphate-buffered saline (PBS) then stained with propidium iodide using the CycleTest™ Plus DNA Reagent Kit (BD Biosciences, Heidelberg, Germany). Cell cycle analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

Apoptosis analysis. For the cell apoptosis analysis, cells were seeded into a 96-well plate at a density of 2×10^3 cells per well and were exposed to carboplatin, RAD001, or their combination, with and without the pan-caspase inhibitor Z-VAD-FMK in culture medium. After culture, the 96-well plate was centrifuged, cell culture medium was aspirated, and cells were lysed with 200 μl lysis buffer per well. Apoptotic cell death was determined with the cell death detection ELISA^{plus} (Roche Diagnostics, Heidelberg, Germany) according to the manufacturer's recommendations with modifications described elsewhere (16).

Evaluation of mitochondrial membrane potential. The mitochondrial membrane potential of breast cancer cells after treatment with the drugs was determined with MitoCapture™ Mitochondrial Apoptosis Detection Kit (BioVision, Heidelberg, Germany). The assay utilizes a novel cationic dye (called MitoCapture) that fluoresces differently in healthy *versus* apoptotic cells. In healthy cells MitoCapture accumulates and aggregates in the mitochondria, fluorescing bright red. In apoptotic cells, a shift in mitochondrial transmembrane potential prevents the dye from accumulating in the mitochondria. Instead, it remains in the cytoplasm in its monomer form, fluorescing green.

Briefly, MCF-7 cells were cultured in the presence of 50 μg/ml carboplatin, 20 nM RAD001, or both for 24 h. Cells were trypsinized, counted, and re-suspended in 1 ml of diluted MitoCapture solution. After incubation at 37˚C in a 5% CO_2 incubator for 15-20 min, cells were centrifuged at 500 × *g*, and the supernatant was discarded. Cells were re-suspended in 1 ml of the pre-warmed incubation buffer and the fluorescent signals were quantified by flow cytometry.

Western blot analysis. Protein concentrations of whole-cell lysates, as well as either cytoplasmic or nuclear protein extracts, were measured using a BCA protein assay kit (Pierce, Bonn, Germany). Western blot analysis was performed as described previously (15). Antibodies against apoptosis-inducing factor (AIF), caspase-3, cell division cycle 25 homolog C (CDC-25C), cyclin-dependent kinase (CDK) 1, phosphorylated eukaryotic translation initiation factor 4E binding protein 1 (p-4E-BP1) (Th37/46), p-p70S6 kinase (Thr389) were from Cell Signalling Technology (New England Biolabs, Frankfurt am Main, Germany); antibody against BCL-2 was from Dako Corp. (Hamburg, Germany); antibodies against β-actin, BAX, BAK, cyclin B1, cyclin D1, CDK-4, p21, p27, and p53 were from Santa Cruz Biotechnology (Heidelberg, Germany).

Statistical analysis. All numerical data are reported as the mean±SD unless otherwise specified. All data represent the results of at least three independent experiments. Groups of data were compared by

Table I. *Summary of combination index (CI) at effective dose (ED) for 25%, 50%, 75% and 90% proliferation inhibition of different breast cancer cell lines. Representative values of at least three experiments are shown.*

Cell line	*p53* status	CI values at			
		ED 25	ED 50	ED 75	ED 90
MCF-7	Wild-type	0.871	0.228	0.171	0.143
MCF7-tam (tamoxifen-resistant MCF-7)	Wild-type	0.605	0.611	0.615	0.619
ZR-75	Wild-type	0.937	0.599	0.416	0.329
BT-474	Mutation	0.592	0.423	0.318	0.251
SKBR-3	Mutation	0.642	0.623	0.662	0.708
T47D	Mutation	>1	>1	>1	>1
MDA-MB-231	Mutation	>1	>1	>1	>1
BT-20	Mutation	>1	>1	>1	>1

Table II. *The combination of low concentrations of carboplatin and RAD001 induced a significant G_2/M phase arrest of the cell cycle after long-term culture in BT-474 cell lines. Breast cancer BT-474 cells were cultured in the presence of either carboplatin (5 μg/ml) alone, RAD001 (10 nM) alone or the combination of both for different durations. Cell cycle analysis was carried out using flow cytometry after propidium iodide staining as described in the Materials and Methods. Data shown are representative of three independent experiments.*

		Control	RAD001 (10 nM)	Carboplatin (5 μg/ml)	RAD001+ Carboplatin
7 Days	$G_{0/1}$	73.72	69.92	52.6	41.23
	S	8.43	7.22	6.55	8.91
	G_2/M	14.34	16.98	34.67	45.8
8 Days	$G_{0/1}$	63.74	74.7	36.59	35.42
	S	12.94	5.91	4.98	6.75
	G_2/M	15.12	14.3	50.22	54.5
9 Days	$G_{0/1}$	77.62	77.94	48.09	27.13
	S	5.56	1.39	5.43	4.33
	G_2/M	12.37	15.83	39.23	60.81

means of Student's *t*-test. Synergistic and additive effects on cell proliferation by the combination of carboplatin and RAD001 were assessed using the Chou-Talalay method (17) and Calcusyn software (Biosoft, Ferguson, MO, USA). Briefly, the dose–effect curve on proliferation for each drug alone was determined based on the experimental observations of MTT assay using the median-effect principle; the combination index (CI) for each experimental combination was then calculated according to the following equation:

$$CI=\frac{(D)_1}{(D_x)_1}+\frac{(D)_2}{(D_x)_2}+\frac{(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

where $(D)_1$ and $(D)_2$ were the doses of drug 1 and drug 2 that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ were the doses of drug 1 and drug 2 that have the same x effect when used alone. CI=1 indicates additive effects; CI <1 indicates more than the expected additive effect (synergism).

Results

Synergistic inhibition of cell proliferation by the combination of carboplatin and mTOR inhibitor RAD001 in wild-type p53 and p53-mutated breast cancer cell lines. The effect of carboplatin and RAD001 alone, and in combination, at different concentrations on MCF-7 and BT-474 cell proliferation was determined by MTT assay after a 4-day treatment. Both carboplatin and RAD001 as monotherapy affected cell proliferation in a dose-dependent manner. Combination of carboplatin with RAD001 significantly increased the growth inhibition in both cell lines as compared to monotherapy (Figure 1A). The increase of growth inhibition was most pronounced if carboplatin and RAD001 were applied at relatively high concentrations.

Time-dependent cell proliferation of MCF-7 and BT-474 cells after exposure to carboplatin (10 μg/ml), RAD001 (20 nM), or their combination for 1, 2, 3, 7 days, respectively, is shown in Figure 1B. A significant combination effect on cell proliferation was observed after treatment of the cells for more than 4 days.

The combination effect of RAD001 and carboplatin on cell proliferation was analyzed now in more breast cancer cell lines using a constant ratio combination design with CalcuSyn software. Synergistic inhibition of cell proliferation was observed in MCF-7, tamoxifen-resistant MCF-7, and ZR-75 (all wild-type *p53*), as well as in SKBR-3 and BT-474 cell lines (all *p53*-mutated), as shown by the CI values <1 at 25%, 50% and 90% proliferation inhibition, respectively. In other *p53*-mutated breast cancer cell lines tested, the combination of the two drugs did not reveal an agonistic effect on inhibition of cell proliferation, as indicated by CI values >1 (Table I).

Prolonged combination treatment with carboplatin and RAD001 resulted in synergistic G_2 phase arrest of cell cycle progression. To examine the mechanism responsible for the synergistic antiproliferative activity of carboplatin and RAD001 in MCF-7 and BT-474 cells, the cell cycle distribution was first evaluated using flow cytometric analysis. As shown in Table II, carboplatin used alone resulted in G_2 phase arrest in the BT-474 cell line. Synergistic G_2 phase arrest was observed with the combination treatment of the two drugs in the late phase of treatment (from 7 to 9 days). The similar time-dependent effect of the combination of the two drugs on G_2 phase cells was also observed for the MCF-7 cell line (data not shown). Next, we assessed the effect of the two drugs on the expression of cell-cycle regulating factors by Western blot.

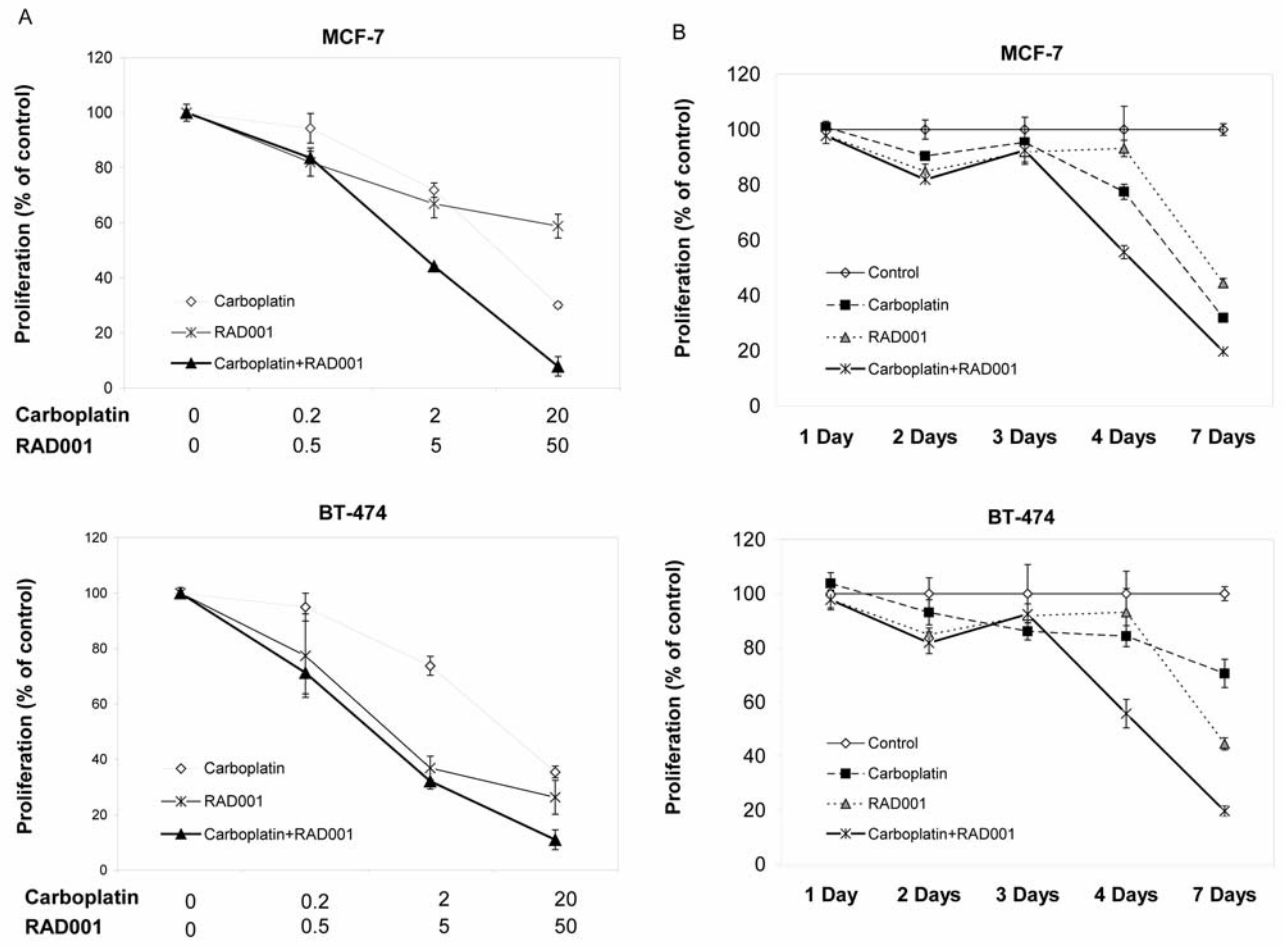

Figure 1. *The mTOR inhibitor RAD001 sensitized different breast cancer cell lines to the cytotoxic effect of carboplatin. A: Different breast cancer cells (5×10^4/ml) were cultured in the presence of escalating doses of carboplatin (μg/ml), RAD001 (nM), and the combination of the two drugs. After 4 days, cell proliferation was measured with MTT assay. Results are expressed as a percentage of the controls (without treatment). Values are the mean±SD of six parallel experiments. B: Breast cancer MCF-7 and BT-474 cells were cultured in the presence of either carboplatin (10 μg/ml), RAD001 (20 nM), or both. Cell proliferation was measured with MTT assay after different incubation times. Results are expressed as a percentage of the controls (without treatment). Values are the mean±SD of six parallel experiments.*

Protein expression of cyclin B1, cyclin D1, CDK-1, CDK-4, and CDC-25C were lower in cells treated with either carboplatin, or RAD001, as well as in cells treated with the combination for 7 days. Importantly, p21 and p27 levels were found to be additionally enhanced through the combination treatment in the MCF-7 cell line, which correlated with the synergistic effect of the two drugs on the G_2 cell-cycle phase arrest, whereas in the BT-474 cell line (*p53*-mutated), only additional enhancement of p27 occurred with the combination treatment (Figure 2).

Prolonged combination treatment with carboplatin and RAD001 resulted in synergistic caspase-independent cell death. RAD001 was reported to inhibit cell proliferation, but had little effect on cell viability and apoptosis (8, 11, 18). However, it rather enhanced the apoptotic induction effect of chemotherapeutics (8, 18). Consistent with these findings, we demonstrated here that co-treatment of cells with RAD001 enhanced carboplatin-induced cell death after a prolonged treatment time (Figure 3A). The cell death observed here was not typical caspase-dependent apoptosis, since it was not inhibited by the presence of the pan-caspase inhibitor Z-VAD-FMK (Figure 3A).

Mitochondrial membrane potential regulates the opening of the mitochondrial permeability transition pore, which plays an important role in triggering various apoptotic pathways. Using a cationic dye that fluoresces differently in cytoplasm and mitochondria, the membrane potential of mitochondria of MCF-7 cells was determined by flow cytometry. Significant loss of mitochondrial membrane function was detected in

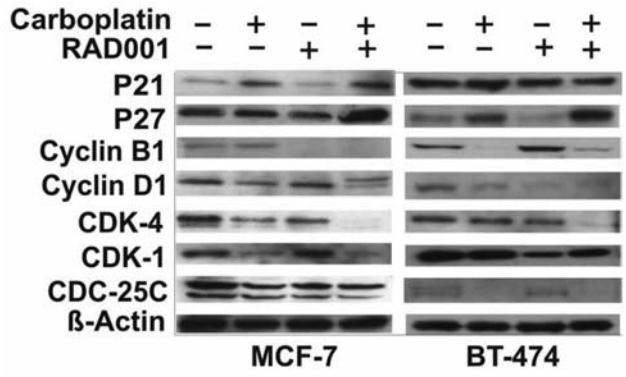

Figure 2. *The combination of low concentrations of carboplatin and RAD001 resulted in the alteration of cell-cycle related proteins after long-term culture in MCF-7 and BT-474 cell lines. Breast cancer cells (MCF-7 and BT-474) were cultured in the presence of either carboplatin (5 μg/ml) alone, RAD001 (10 nM) alone, or the combination of both at these concentrations for 7 days. Cell lysates were analyzed by immunoblotting using specific antibodies against different cell cycle-related proteins. β-Actin expression served as a loading control. Representative blots of three independent experiments are shown.*

MCF-7 cells treated with the combination of carboplatin and RAD001 in comparison with cells treated with carboplatin alone (35.62% *versus* 20.75%, Figure 3B).

Western blot analysis showed the expression of pro-apoptotic BCL-2 family proteins, namely BAX and BAK, was increased by the combination treatment of cells with the two drugs, whereas expression of the pro-survival protein BCL-2 was reduced. The changes of protein levels of BCL-2 family members were more significant in the MCF-7 cell line than in the BT-474 cell line. Furthermore, we found an elevation of the p53 level by carboplatin and/or RAD001 treatment in MCF-7 cells, but not in BT-474 cells, which harbour mutated *p53* (Figure 3C).

Due to the central role of AIF in caspase-independent cell apoptosis (19), we determined nuclear and cytoplasmatic AIF levels by Western blot and found an enhanced AIF level in nuclear and cytoplasmatic protein lysates of BT-474 cells treated with carboplatin and/or RAD001. In MCF-7 cells, AIF was expressed at a slightly higher level in nuclear protein, indicating an increased translocation rate of AIF in the nucleus and subsequently increased cell death rate (Figure 3D).

RAD001 sensitized MCF-7 breast cancer cells to carboplatin-induced cell cytotoxicity partially through a p53-dependent pathway. Previous works showed that RAD001 enhanced cisplatin-induced apoptosis in cells with wild-type *p53*, but not in *p53*-mutated tumor cells (8); transfection of wild-type *p53* into *p53*-mutant cells increased the sensitivity of cells to cisplatin in hepatocellular carcinoma (11). In order to analyze the role of p53 in our system, we blocked p53 activity using p53 blocker pifitrin in MCF-7 cells. As shown by Western blot, the enhanced expression of p53 and the p53 target gene *p21* induced by carboplatin and/or RAD001 was blocked after exposure to pifitrin, indicating that pifitrin suppressed the activity of p53 in our system (Figure 4A). Interestingly, we demonstrated here that attenuation of p53 activity by pifitrin partially antagonized the carboplatin/RAD001-induced cell proliferation inhibition in MCF-7 cell line with wild-type *p53* (Figure 4B). These results strongly suggest that p53 is partially responsible for the synergistic effect caused by the two drugs on cell death and proliferation inhibition in the MCF-7 cell line. However, other mechanisms may also be involved in this process.

Synergistic inhibition of AKT/mTOR downstream proteins by the combination of carboplatin and RAD001 in breast cancer cell lines. Western blot analysis of the downstream signaling proteins of mTOR, namely p70S6 kinase and 4E-BP1, were carried out with MCF-7 (wild-type *p53*) cells and BT-474 (*p53*-mutated) cells after exposure to 10 nM RAD001, 5 μg/ml carboplatin alone, or the combination of the two drugs for 7 days. Synergistic down-regulation of phosphorylated p70S6 kinase and phosphorylated 4E-BP1 by the combined treatment was observed in both cell lines (Figure 5A), suggesting a synergistic inhibition of the Akt/mTOR pathway when the two drugs were combined.

AKT is a crucial upstream mediator of mTOR signaling. We detected the expression of AKT in different cell lines tested by Western blot. Results revealed high expression of total AKT in BT-474, SKBR-3, MCF-7 and ZR-75 cell lines, in which synergistic proliferation-inhibitory effects of the combination of carboplatin and RAD001 were also observed (Figure 5B). In contrast, in cells such as T47D, BT-20 and MDA-MB-231, where no combination effects of the two drugs were noticed (Table I), very low levels of AKT were expressed. Interestingly, such a correlation was only associated with total AKT but not with the level of phosphorylated AKT.

Discussion

The present study demonstrates that mTOR inhibition through RAD001 enhances the sensitivity of breast cancer cells *in vitro* to carboplatin by amplifying the carboplatin-induced cytotoxic effect in different breast cancer cell lines. RAD001 alone suppressed cell proliferation but did not induce apoptosis in breast cancer cells. RAD001 in combination with carboplatin resulted in synergistic cell proliferation inhibition and caspase-independent apoptosis. In MCF-7 and BT-474 cell lines, synergistic effects of the combination treatment on cell cycle arrest in the G_2/M phase, on the regulation of different molecules responsible for the cell cycle transition, and on pro-apoptotic/anti-apoptotic BCL-2 family members were observed. The

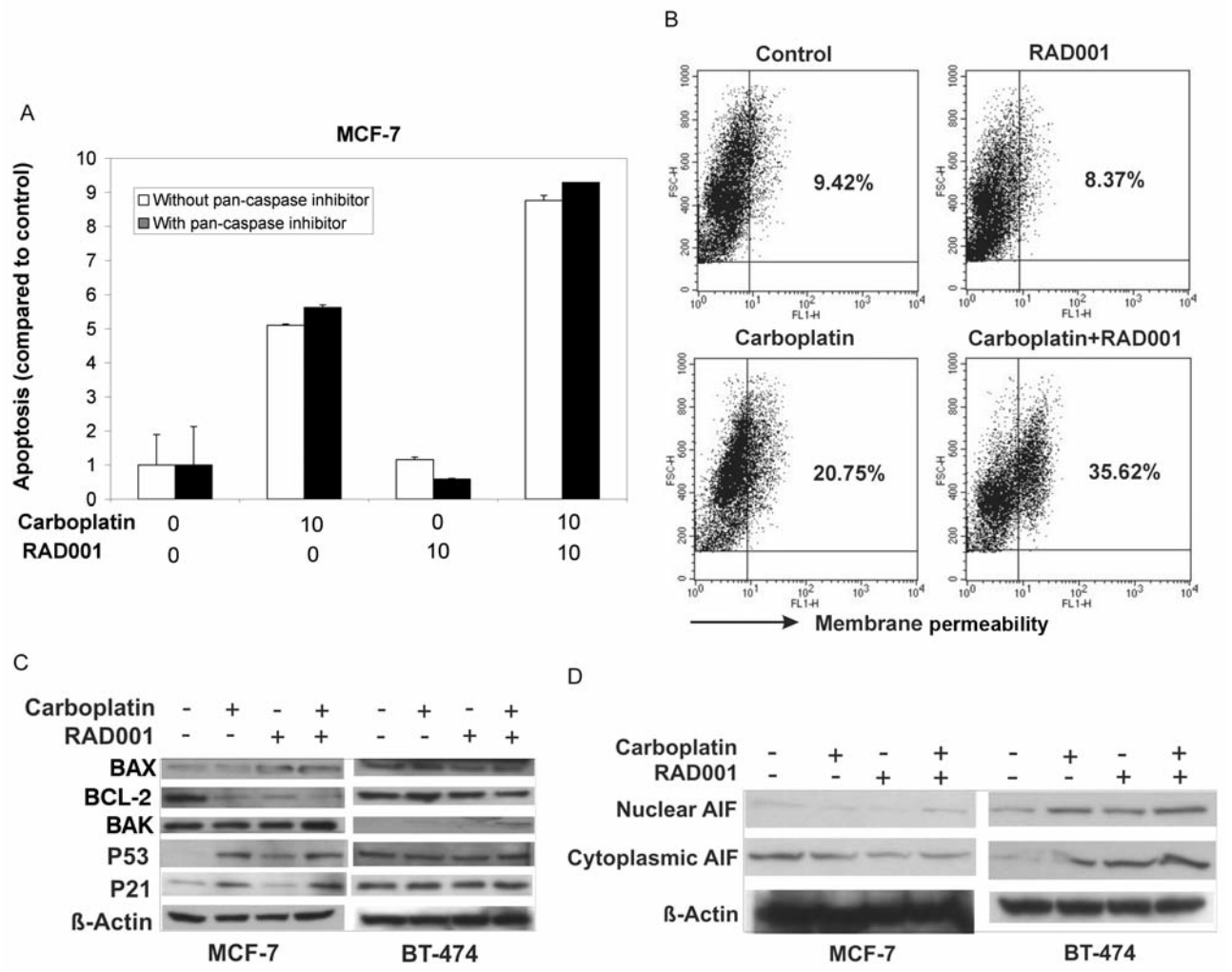

Figure 3. *RAD001 sensitized breast cancer cells to carboplatin-induced apoptotic cell death through a caspase-independent and apoptosis-inducing factor (AIF)-dependent pathway. A: MCF-7 cells were treated with carboplatin (μg/ml) alone, RAD001 (nM) alone, or the combination of both, either in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (20 μM) for 7 days. Internucleosomal DNA fragmentation was quantified by assaying for cytoplasmic mononucleosome- and oligonucleosome-associated histone using a cell death detection ELISA kit. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm and is expressed as the mean±SD of triplicates. B: MCF-7 cells were treated with carboplatin (10 μg/ml) alone, RAD001 (10 nM) alone, or combination of both for 7 days, and the mitochondrial membrane potential was determined by flow cytometry. Increased permeabilization of the mitochondrial membrane is shown by an increased intensity of green fluorescence (FL1). C: Expression changes of BCL-2, BAX, BAK, p53, and p21 after 7-day treatment with carboplatin (10 μg/ml), RAD001 (10 nM), or both drugs were examined by Western blot. β-Actin expression served as loading control. D: Cytoplasmic and nuclear expression of AIF in MCF-7 and BT-474 cells after treatment with carboplatin (10 μg/ml) alone, RAD001 (10 nM) alone, or their combination.*

synergism of RAD001 and carboplatin on breast cancer cell proliferation and apoptosis might be only partially dependent on the p53 pathway, since the synergistic effect was demonstrated not only in all tested breast cancer cell lines with wild-type *p53* but also in some breast cancer cell lines with mutated-*p53*. Moreover, the use of a p53 inhibitor only partially antagonized the effect of the two drugs on cell proliferation. Furthermore, the synergism of the combination of the two drugs was also observed in cell lines with high AKT expression, suggesting the importance of AKT signaling activity in the synergistic effect.

We investigated for the first time the combination effect of carboplatin and the mTOR inhibitor RAD001 on tumor cell proliferation in a panel of breast cancer cell lines with different estrogen receptor, Her-2, and *p53* status. The synergistic proliferation-inhibitory effect of the combination of the two drugs was observed in all tested cell lines with wild- type *p53*, including tamoxifen-resistant MCF-7 cells

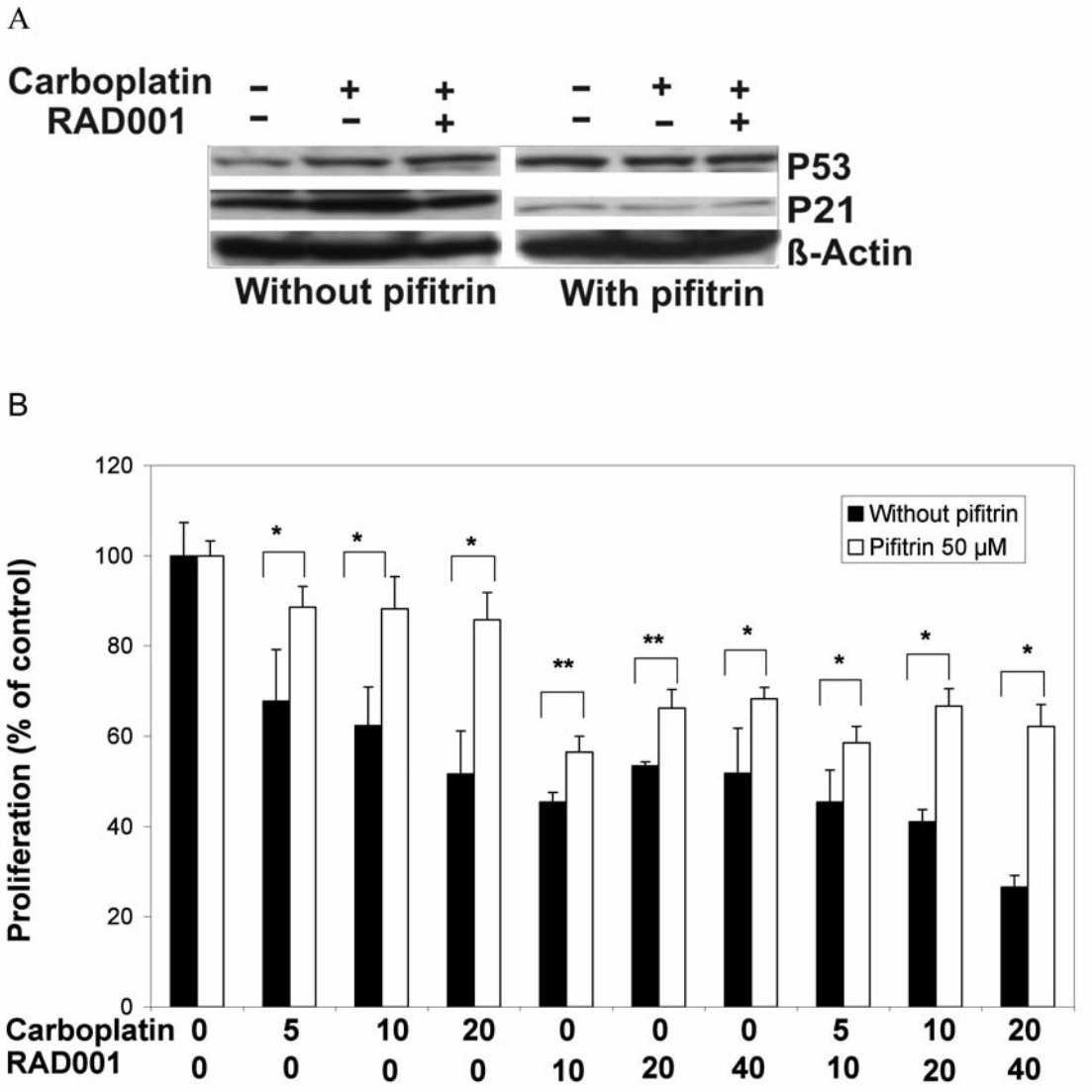

Figure 4. *RAD001 sensitized MCF-7 breast cancer cells to carboplatin-induced cell cytotoxicity partially through a p53-dependent pathway. A: MCF-7 (wild-type p53) cells were cultured in the presence of either carboplatin (50 µg/ml), RAD001 (20 nM), or the combination of the two drugs for 24 h, with and without 50 µM of p53 inhibitor pifitrin and then harvested for the Western blot analysis. B: MCF-7 cells (wild-type p53) were treated with either carboplatin (µg/ml), RAD001 (nM) or combination of the two drugs as indicated, in the presence or absence of 50 µM of p53 inhibitor pifitrin. Cell proliferation was measured with MTT assay after different incubation times. Results are expressed as a percentage of that controls (without treatment). Values are the mean±SD of six parallel experiments. Student's t-test was used for the significance test. *p<0.05; **p<0.01.*

and MCF-7 cells transfected with activated *AKT1* (data not shown), and in some cell lines with mutated *p53* but higher basal expression of AKT (BT-474 and SKBR-3).

RAD001 treatment alone resulted in G_1 phase arrest of cells in the cell cycle, while carboplatin alone caused G_2 phase arrest (20, 21). Combination effect of the two drugs on cell cycle progression was less pronounced in the early phase of treatment (data not shown); a significantly increased G_2 phase arrest of cell cycle progression by the combination treatment was observed when the treatment time was prolonged to more than 5 days. Similar results were obtained both in MCF-7 cells and BT-474 cells, indicating that p53 was not involved in the mechanism responsible for this phenomenon. The G_2/M cell cycle arrest was confirmed by assessing the effect of the two drugs on cell cycle-related regulating factors with immunoblotting. Protein expressions of cyclin B1, CDK-1 and CDC-25C, which regulate the G_2/M phase transition and onset of mitosis (22, 23), were lower in cells treated with carboplatin or RAD001 alone, as well as in cells treated with the combination. Interestingly, the combination of the two drugs

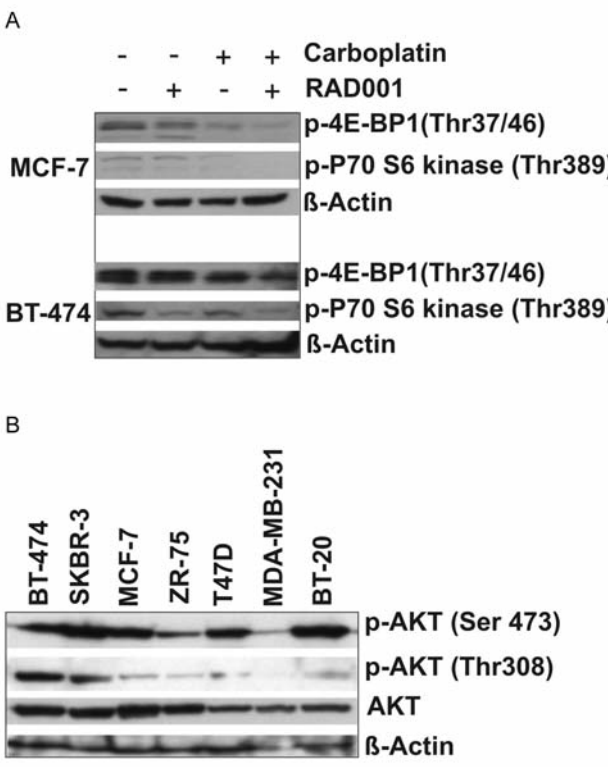

Figure 5. *Carboplatin and RAD001 synergistically inhibited mTOR downstream signaling in breast cancer cells. A: Breast cancer MCF-7 and BT-474 cells were cultured in the presence of either carboplatin (10 μg/ml), RAD001 (10 nM), or the combination of the two drugs for 7 days, after which the expression of phosphorylated 4E-BP-1 and p70S6K was determined by Western blot analysis. β-Actin was used as a loading control. B: The total protein was isolated from breast cancer BT-474, SKBR-3, MCF-7, ZR-75, T47D, MDA-MB-231, and BT-20 cells. AKT, p-AKT (Ser 473), and p-AKT (Thr 308) expressions were determined by Western blot analysis. β-Actin was used as a loading control.*

synergistically increased p27 and reduced cyclin D1/CDK4 levels in MCF-7 and BT-474 cells. Besides their well-known roles in the regulation of the G_1 to S phase transition, these factors are also involved in the control of cell apoptosis (24). We assumed that this regulation of p27 and cyclin D1/CDK4 induced by the combination treatment with RAD001 and carboplatin might be more involved in apoptosis than in cell cycle regulation.

In accordance with other work (8, 18), although RAD001 alone did not induce apoptosis in breast cancer cells, RAD001 combined with carboplatin induced a significant increase of apoptosis compared to carboplatin alone. We show here for the first time that caspases appear not to be involved in the cell death induced by the two drugs. The pan-caspase inhibitor Z-VAD-FMK did not antagonize the cell death-inducing and proliferation-inhibitory effects (Figure 3) of the two drugs. Moreover, no increase of caspase-3, -8, and -9 activity was detected in MCF-7 and BT-474 cell lines after the combined treatment (data not shown).

The significant loss of mitochondrial membrane function with the combination of carboplatin and RAD001 in comparison to cells treated with carboplatin alone in the MCF-7 cell line (Figure 3B) could be an indication of mitochondria-involved intrinsic apoptosis. Immunoblotting data revealed significant up-regulation of the pro-apoptotic BCL-2 family members BAX and BAK and down-regulation of anti-apoptotic BCL-2 in the MCF-7 cell line. In conjugation with the increased level of p53 and p21 in MCF-7 cells treated with the combination of the two drugs, these results suggest that p53 is involved, at least in part, in the mechanism leading to apoptosis in p53-functional breast cancer (25). Significant alterations of these molecules were not observed in BT-474 cells with mutated *p53* (Figure 3C). Further investigation will be required to elucidate the mechanism responsible for the occurrence of apoptosis in this breast cancer cell lines.

Recent evidence indicates that programmed cell death (PCD) can occur in the complete absence of caspase activation. Indeed, a large number of caspase-independent models are now defined and a key protein implicated in this type of PCD has been identified, namely AIF. AIF is a mitochondrial protein which participates in more PCD systems than initially thought. Importantly, intense DNA damage leads *via* PARP-1 to calpain activation. Calpain in turn activates BAX, resulting in its translocation from the cytosol to mitochondria, where it facilitates the release of truncated AIF. Activated calpain also regulates AIF release by cleaving the membrane-anchored AIF to the soluble form of AIF. Once liberated in the cytosol, truncated AIF translocates to the nucleus, binds to DNA and mediates large-scale DNA fragmentation and cell death (19, 26). We determined nuclear and cytoplasmatic AIF expression level by Western blot and found an enhanced AIF level in nuclear and cytoplasmatic protein lysates with the combination treatment in BT-474 cells. In MCF-7 cells, AIF cytoplasmatic protein was reduced through co-treatment with the two drugs, but there was an increase in the nuclear protein level, indicating an increased translocation of AIF into the nucleus. This result indicates that the intensified cell death observed on co-treatment of breast cancer cells with carboplatin and RAD001 might be mediated by AIF.

Our results show that RAD001 enhanced carboplatin-mediated cytotoxicity in different breast cancer cell lines irrespective of *p53* status, as the synergistic effect of combination treatment was observed both in the MCF-7 cell line with the wild-type *p53* and in the BT-474 cell line with the mutated *p53*. These findings suggest that RAD001 enhances carboplatin-induced cytotoxicity seemly in a p53-independent manner. However, in the presence of the p53 inhibitor pifitrin, the cytotoxic effect of RAD001 and

carboplatin on MCF-7 cells was significantly weaker in comparison to that in the absence of this inhibitor. In the presence of pifitrin, the combination of the two drugs did not induce a synergistic cytotoxic effect; no increase of the protein level of p53 or p21 by RAD001 or carboplatin treatment was detected by immunoblotting. Taking these findings together, we would suggest that p53 might be partially responsible for the synergism of the combination treatment in cells with wild-type *p53*.

Mabuchi *et al*. (9) and Wangpaichitr *et al*. (12) reported that mTOR inhibitors enhanced cisplatin-induced apoptosis in cells with high AKT/mTOR activity, with minimal effects on cells with low AKT/mTOR activity. We observed that p53 is not the only determinant for the synergism of the combined treatment with RAD001 and carboplatin, as RAD001 also sensitized the carboplatin-induced cytotoxic effect in two of the tested cell lines which harbour the mutated *p53* (BT-474 and SKBR-3). We hypothesize therefore that AKT/mTOR activity might play a role in the synergism of the combination treatment for this group of cell lines. Indeed, treatment with mTOR inhibitor attenuated phosphorylation of both 4E-BP1 and p70S6K in accordance with the results from other colleagues (9, 27). With the co-treatment of carboplatin, phosphorylation of these proteins was more remarkably attenuated. Detection of phosphorylated AKT and AKT expression in all of the tested breast cancer cell lines showed that the synergism of combination treatment was demonstrated only in cell lines with high expression of AKT (BT-474, SKBR-3, MCF-7 and ZR-75). In cell lines with low AKT expression and mutated *p53* (T47D, MDA-MB-231 and BT-20), no synergism occurred. Therefore, our results suggest a role of AKT in the efficacy of combination treatment.

In conclusion, the present study has demonstrated that use of the mTOR inhibitor RAD001, in combination with carboplatin, is a promising approach for the treatment of breast cancer, including some tumors with mutated *p53* and hyperactivation of the AKT/mTOR pathway. These results provide a rationale for future clinical trials with RAD001 in combination with carboplatin in breast cancer patients.

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