

## Metformin and the mTOR Inhibitor Everolimus (RAD001) Sensitize Breast Cancer Cells to the Cytotoxic Effect of Chemotherapeutic Drugs *In Vitro*

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**Abstract.** Aim: Metformin appears to interfere directly with cell proliferation and apoptosis in cancer cells in a non-insulin-mediated manner. One of the key mechanisms of metformin's action is the activation of adenosine monophosphate activated protein kinase (AMPK). AMPK is linked with the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/protein kinase B (AKT) pathway and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) cascades – all known for being frequently dysregulated in breast cancer. Therefore, simultaneously targeting AMPK through metformin and the PI3K/AKT/mTOR pathway by an mTOR inhibitor could become a therapeutic approach. The aim of this study was to evaluate the anticancer effect of metformin alone and in combination with chemotherapeutic drugs and the mTOR inhibitor RAD001. Materials and Methods: The proliferation of breast cancer cells was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; and the cell apoptosis with enzyme-linked immunosorbent assay (ELISA). Gene expression at the protein level was determined by western blot. Results: We tested metformin alone and in combination with RAD001 and/or chemotherapeutic agents (carboplatin, paclitaxel and doxorubicin, respectively) on several human breast cancer cell lines with respect to cell proliferation, apoptosis and autophagy. Metformin alone inhibited cell proliferation and induced apoptosis in different

breast cancer cell lines (ERα-positive, HER2-positive, and triple-negative). The cytotoxic effect of metformin was more remarkable in triple-negative breast cancer cell lines than in other cell lines. The cell apoptosis induced by metformin is, at least partly, caspase-dependent and apoptosis inducing factor (AIF)-dependent. Interestingly, we demonstrated that metformin induced cell autophagy. Inhibiting autophagy with chloroquine, enhanced the treatment efficacy of metformin, indicating that autophagy induced by metformin may protect breast cancer cells from apoptosis. We further demonstrated that co-administration of metformin with chemotherapeutic agents and RAD001 intensified the inhibition of cell proliferation. The analysis of cell cycle-regulating proteins cyclin D, cyclin E and p27 by western blot indicated that the synergistic inhibition of G1 phase of the cell cycle by the combination treatment of metformin, chemotherapeutic drugs and/or RAD001 contributed to the synergistic inhibition of cell proliferation. Conclusion: Our investigation provides a rationale for the clinical application of metformin within treatment regimens for breast cancer.

The relationship between obesity, metabolic syndrome, diabetes and cancer has been recognized since many years. Multiple studies conducted in the last 20 years have identified several molecular mechanisms responsible for this phenomenon. The adenosine monophosphate-activated protein (AMP)-activated protein kinase (AMPK) pathway and the related mammalian target of rapamycin (mTOR) pathway have been determined as playing an important role in controlling the energy status of the cell in cancer biology (1). In this context, a biguanide that is most widely prescribed for the treatment of type 2 diabetes, namely metformin, has gained even more attention as an anticancer agent.

The initial interest in metformin came from clinical and epidemiological research. Several population-based studies have suggested that metformin reduces cancer incidence or mortality among type-2 diabetic patients treated with metformin (2-6). Some prospective clinical trials aimed at investigating the safety or the efficacy of metformin in cancer

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patients were initiated. Interim analyses of ongoing studies involving neoadjuvant treatment with metformin, of patients newly diagnosed with breast cancer, have demonstrated that metformin is a safe and well-tolerated drug with favourable effects on tumor cell proliferation and apoptosis (7).

Accumulated evidence from preclinical research suggests that metformin exerts its positive effect on the clinical course of neoplastic diseases and, in particular, on breast cancer, primarily through the stimulation of AMPK in association with the upstream liver kinase B1 (LKB1) (8, 9). AMPK is a key cellular energy sensor, activation of which by metformin leads to suppression of energy-consuming processes, such as gluconeogenesis, protein and fatty acid synthesis and, in type-2 diabetics, results in a (at least partial) metabolic normalization of hyperglycaemia and of insulin resistance (10, 11). In carcinoma cells, the stimulation of AMPK, mediated by metformin resulted in the inhibition of the mTOR/ribosomal S6 kinase pathway and thus in inhibition of pathological cell cycle progression, cell growth and angiogenesis (12, 13). Likewise, the stimulation of AMPK by metformin led to significant repression of cell proliferation in both estrogen receptor  $\alpha$  (ER $\alpha$ )-negative and -positive human breast cancer cell lines (14-16).

Dysregulation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) activities occurs frequently in breast cancer. Inhibition of the mTOR activity by rapamycin or its analogs results in the translational inhibition of the proteins required for cell cycle progression, survival and resistance to apoptosis, thereby inhibiting the growth and the progression of breast tumours, both *in vitro* and *in vivo* (17, 18). However, the activity of the mTOR inhibitors as single agents in solid tumours is limited. The limited clinical benefit of rapamycin and its analogs may be associated with the mTOR-dependent feedback loop that acts to inhibit PI3K/AKT activity. Rapamycin and its analogs, by inhibiting mTOR, not only inhibit protein translation, but also inhibit this feedback loop, leading to increased AKT activation. This in turn may limit some of the antiproliferative actions of this class of agents (19, 20). Recently published data indicated that mTOR inhibition, resulting from metformin exposure through AMPK activation, reduced AKT activation, an action opposite to that of rapamycin (21). These findings suggested that by integrating metformin into the treatment regime of the mTOR inhibitor in combination with chemotherapeutic agents, the antineoplastic efficacy might be further enhanced.

mTOR inhibitors are applied in order to enhance the efficacy of cytotoxic agents in various types of human cancers, preclinically and clinically (17, 22-27). We have previously demonstrated that the combinatorial use of RAD001 with carboplatin synergistically inhibited the cell cycle progression by inducing a G<sub>2</sub>-M phase arrest and thereby inhibited cell proliferation in different breast cancer cell lines (28). Similarly, clinical data revealed that diabetic

patients with breast cancer receiving metformin and neoadjuvant chemotherapy have a higher pathological complete response rate than diabetics not receiving metformin (29). Additional studies to evaluate the combinatorial effect of metformin with chemotherapeutic agents for breast cancer treatment have not been performed yet. In this study, we evaluated if the combination of metformin with different drugs could become a treatment concept for breast cancer.

## Materials and Methods

**Cell lines, reagents and culture conditions.** Human breast cancer cell lines BT-474, MCF-7, MDA-MB-231, T47D and human breast epithelial cell line MCF-10A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PAA, Cölbe, Germany), and incubated at 37°C, in a humidified atmosphere with 5% CO<sub>2</sub>.

Metformin and chloroquine (Sigma-Aldrich, Munich, Germany) were dissolved in cell culture media at a concentration of 1 M and 10<sup>-2</sup> M respectively. 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 dimethyl sulfoxide (DMSO) (Sigma-Aldrich), was stored at -20°C and diluted with fresh culture medium immediately before use. Carboplatin, doxorubicin and paclitaxel were provided by the pharmacy of the Charité-Universitätsmedizin Berlin (Berlin, Germany). These drugs were dissolved with 0.9% NaCl at a concentration of 10 mg/ml (carboplatin), 10<sup>-3</sup> M (doxorubicin and paclitaxel), and stored at 4°C. The pan-caspase inhibitor Z-VAD-FMK was purchased from R&D (Wiesbaden, Germany).

**Determination of cell viability by the 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.** Breast cancer cells were seeded into a 96-well plate at a density of 5×10<sup>3</sup> cells per well. After 24 h, cells were exposed to different concentrations of metformin, with or without the presence of different concentrations of different chemotherapeutics and RAD001 in culture medium. After incubation for the indicated times, the MTT reagent (Sigma-Aldrich) was added to each well and was 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 (30).

**Apoptosis analysis.** For the cell apoptosis analysis, cells were seeded into a 96-well plate at a density of 2×10<sup>3</sup> cells per well and were exposed to different concentrations of metformin, with or without carboplatin in the culture medium. After different culture durations, the 96-well plate was centrifuged, the cell culture medium was aspirated, and the cells were lysed with 200  $\mu$ l lysis buffer per well. Apoptotic cell death was determined with the cell death detection kit ELISA<sup>plus</sup> (Roche Diagnostics, Heidelberg, Germany) according to the manufacturer's recommendations with modifications described elsewhere (31).

**Western blot analysis.** Cells were lysed after culture and protein concentrations of whole cell lysates, as well as those of cytoplasmic and nuclear protein extracts (30), were measured using a BCA

protein assay kit (Pierce, Bonn, Germany). Western blot analysis was performed as described previously (30). Antibodies towards AIF, cleaved-caspase-3, phosphorylated eukaryotic translation initiation factor 4E (eIF4E), binding protein (p-4E-BP1), p-AKT (S473), microtubule-associated protein 1, light chain-3B (LC3B), poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signalling Technology (New England Biolabs, Frankfurt am Main, Germany); antibodies towards  $\beta$ -Actin, cyclin D, cyclin E and p27 were from Santa Cruz Biotechnology (Heidelberg, Germany).

**Statistical analysis.** All numerical data are reported as the mean $\pm$ SD unless otherwise specified. All data represent the results of at least three independent experiments. Groups of data were compared by means of Student's *t*-test. Synergistic and additive effects on cell proliferation, resulting from the combination of different chemotherapeutics and of metformin were assessed using the Chou-Talalay method (32) and the Calcsyn software (Biosoft, Ferguson, MO, USA). Briefly, the dose effect curve of proliferation for each drug alone was determined based on the experimental observations of the 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)<sub>1</sub> and (D)<sub>2</sub> were the doses of drug 1 and drug 2 that have x effect when used in combination, and (D<sub>x</sub>)<sub>1</sub> and (D<sub>x</sub>)<sub>2</sub> were the doses of drug 1 and drug 2 that have the same effect when used alone. A value of CI=1 indicates additive effects; CI<1 indicates more than the expected additive effect (*i.e.* synergism).

## Results

**Metformin inhibited cell proliferation on different breast cancer cell lines, but had no effect on a breast epithelial cell line.** To explore the effect of metformin on breast cancer cell proliferation, different breast cancer cell lines, including ER $\alpha$ -positive (MCF-7, T47D), ER $\alpha$ - and HER2-negative (MDA-MB-231) as well as HER2-overexpressing (BT-474) breast cancer cell lines were cultured in the presence of metformin at different concentrations and cell proliferation measured by the MTT assay. Proliferation of all cell lines tested in the presence of metformin for 4 days was inhibited in a concentration-dependent manner (Figure 1).

Among the breast cancer cell lines, the triple-negative cell line MDA-MB-231 was most sensitive to the metformin treatment, with proliferation being reduced to less than 20% of that of the control, at a concentration of 15 mM (Figure 1B).

As a next step, we tested the sensitivity of the non-malignant breast epithelial cell line MCF-10A to this drug. As shown in Figure 1B, this cell line was much more resistant to the metformin treatment than the other breast cancer cell lines, resulting in only about 30% inhibition of cell proliferation at the highest used concentration (25 mM). This result indicates a specific effect of metformin on breast cancer cells.

**Metformin induced a partly caspase-dependent cell death in the triple receptor-negative breast cancer cell line MDA-MB-231.** Metformin was shown to induce cell death in breast cancer cells by both a caspase-dependent and a PARP-dependent manner (16). Consistently, we demonstrated that the treatment of cells with metformin resulted in a concentration-dependent cell death after 24 h treatment. In the presence of pan-caspase inhibitor Z-VAD-FMK, the cell apoptosis induced by metformin treatment was significantly reduced, indicating that caspases were partially involved in the cell death mechanism (Figure 2A). However, the role of caspases in the metformin-induced effect on breast cancer cells was not pronounced under our experimental conditions, as was described by Zhang *et al.* (16); Cell proliferation in the presence of the pan-caspase inhibitor Z-VAD-FMK was not statistically significantly enhanced after 96 h treatment with metformin at different concentrations, as indicated by the *p*-values (*p*>0.05, Figure 2B). Cell apoptosis was further demonstrated with enhanced PARP cleavage and cleaved caspase-3 induced by metformin treatment, as examined with western blot (Figure 2C).

Due to the central role of AIF in the caspase-independent cell apoptosis (33, 34), we determined the nuclear and the cytoplasmatic AIF levels by western blot and found AIF to be enhanced in the nuclear protein fraction and reduced in the cytoplasmatic protein compartment after treatment with metformin in the MDA-MB-231 cell line. This indicates an increased translocation rate of AIF into the nucleus and subsequently an increased cell death rate (Figure 2D).

**Inhibition of autophagy enhanced the metformin-induced effect on breast cancer cells.** Under certain circumstances, autophagy constitutes a stress adaptation that prevents from cell death (and suppresses apoptosis), whereas in other cellular settings, it constitutes an alternative cell death pathway (35). This phenomenon was observed in melanoma where metformin exerts its anti-neoplastic effect by inducing cancer cell autophagy (36, 37).

We analyzed whether metformin might induce autophagy in breast cancer and the role of autophagy in metformin-mediated cell death by determining characteristic hallmarks of autophagy. During autophagy, LC3-I is converted to LC3-II through the autophagy related 4 homolog B (ATG4)-dependent insertion of a phosphoethanolamine moiety and it is recruited into the membrane of the phagophore formed, a double membrane required for the recycling of protein aggregates and organelles (38). Increased LC3-II expression, indicating autophagy, was detected for two doses of metformin (30 mM and 50 mM) within our experimental setting in MDA-MB-231 cells. By addition of the known autophagy-inhibitor chloroquine to metformin-treated cells, the conversion of LC3-I to LC3-II was inhibited, which indicated that cell autophagy was inhibited (Figure 3A). We further demonstrated that autophagy occurring under our experimental conditions

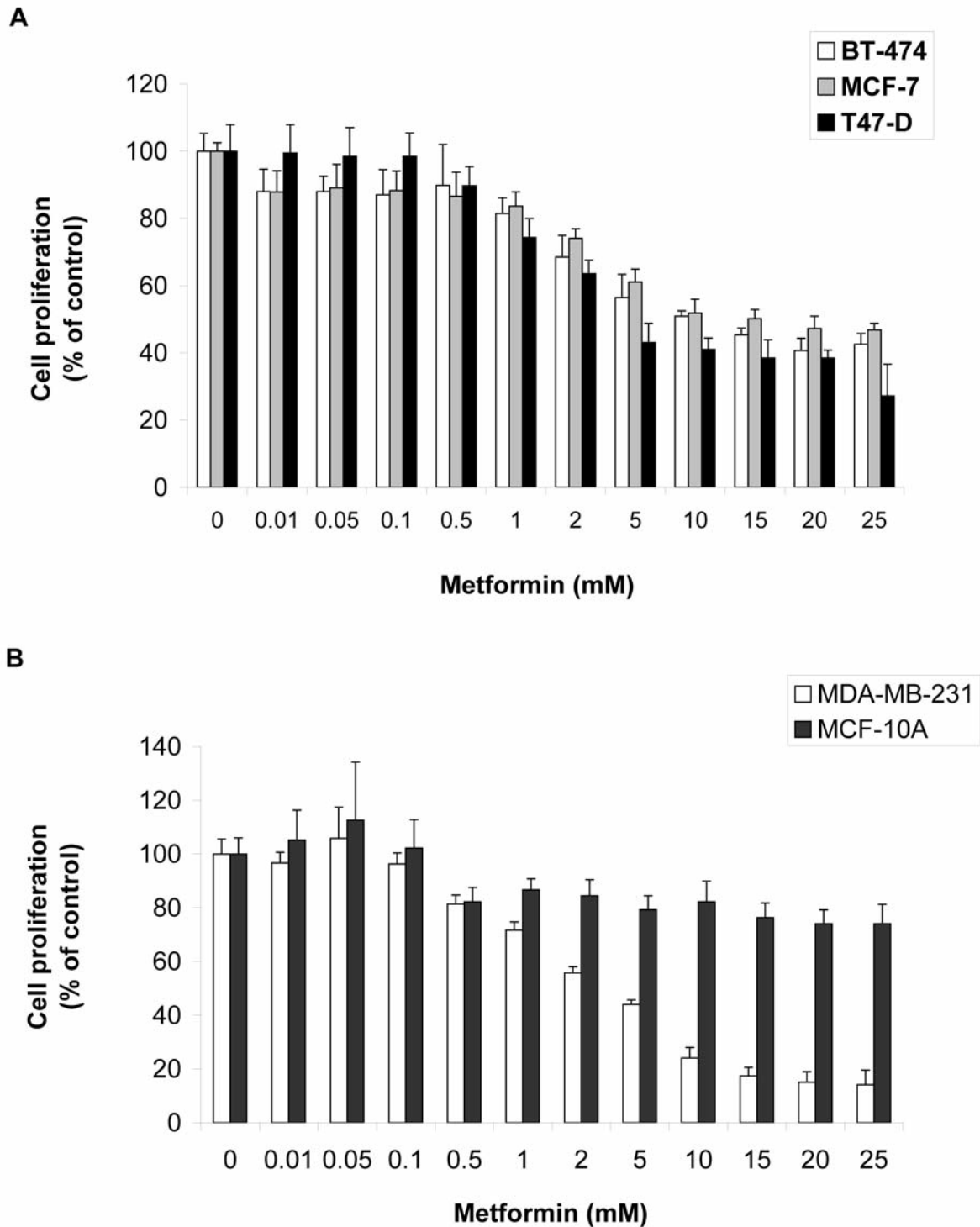


Figure 1. Metformin inhibited cell proliferation of breast cancer cell lines in a concentration-dependent manner but had no effect on a normal breast epithelial cell line. A: Cells of different breast cancer cell lines MCF-7, BT-474, and T47D ( $5 \times 10^4$ /ml) were cultured in the presence of escalating doses of metformin (0.01-25 mM) for 4 days. After incubation, cell proliferation was measured with the MTT assay. Results are expressed as a percentage of the corresponding controls (without treatment). Values are the mean $\pm$ SD of six parallel experiments. B: Breast cancer cells MDA-MB-231 and non-malignant human breast epithelial cells MCF-10A were cultured under the same conditions as described in Figure 1A. After incubation, cell proliferation was measured with the MTT assay. Results are expressed as a percentage of the corresponding controls (without treatment). Values are the mean $\pm$ SD of six parallel experiments.

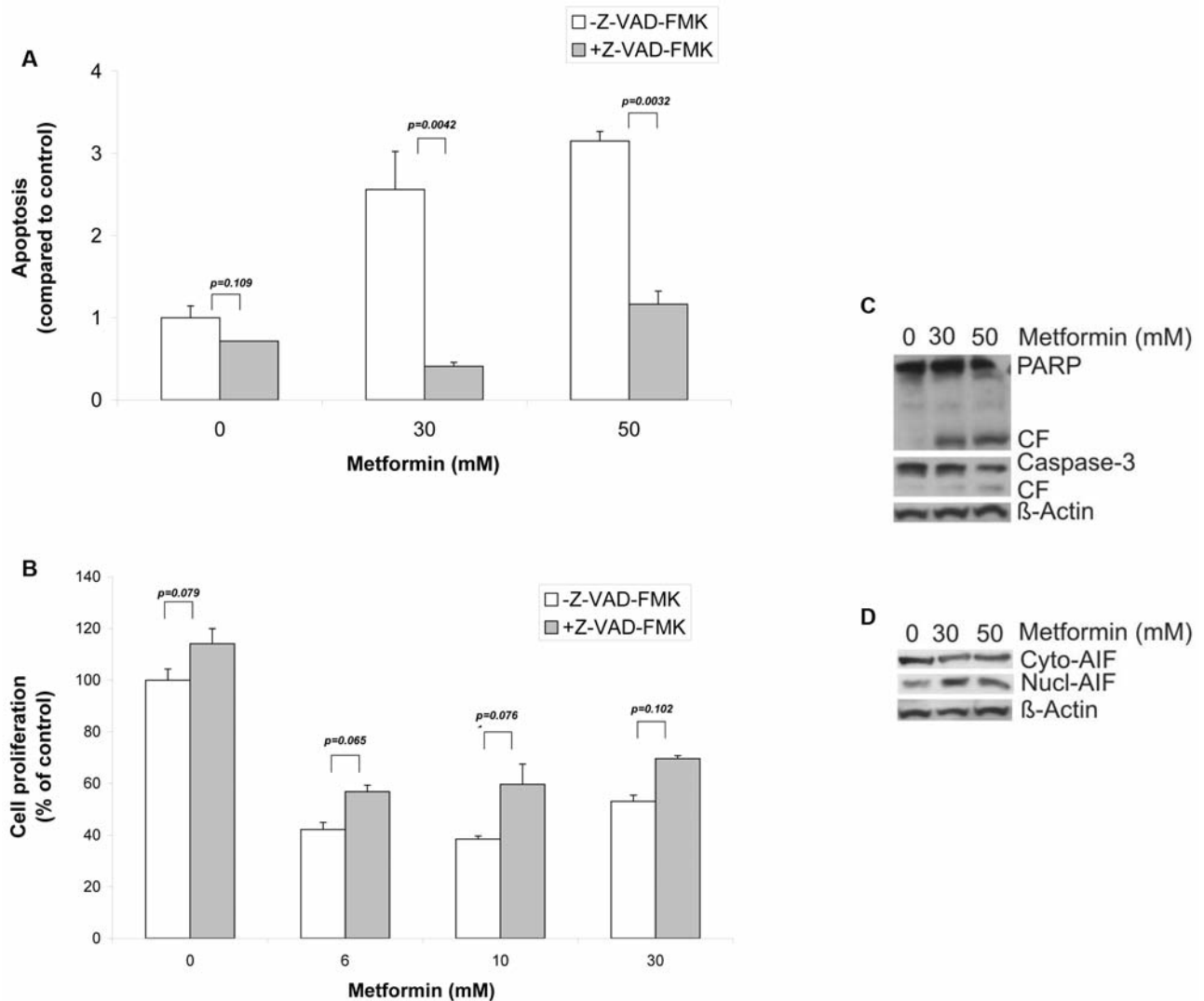


Figure 2. Metformin induced apoptotic cell death through caspase-independent and -dependent manners. A: MDA-MB-231 cells were treated with metformin in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (20  $\mu$ M) for 4 days. Internucleosomal DNA fragmentation was quantified by assaying for cytoplasmic mononucleosome- and oligonucleosome-associated histones 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 $\pm$ SD of triplicates. B: MDA-MB-231 cells were treated with metformin in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (20  $\mu$ M) for 4 days. After incubation, cell proliferation was measured with the MTT assay. Results are expressed as a percentage of the corresponding controls (without treatment). Values are the mean $\pm$ SD of six parallel experiments. Student's t-test was used to test for significance. C: Expression changes of poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3 after 4 days treatment with metformin were examined by western blot. The expression of  $\beta$ -actin served as loading control. CF: Cleaved fragments. D: Cytoplasmic (Cyto) and nuclear (Nucl) expression of apoptosis-inducing factor (AIF) in MDA-MB-231 cells after treatment with metformin at 30 mM and 50 mM for 24 h.

did not contribute to the anti-neoplastic effect of metformin in breast cancer, as reported in melanoma (39,40). On the contrary, autophagy occurring in the presence of metformin exerted a cytoprotective effect here, since chloroquine enhanced the metformin-induced cell apoptosis and consequently it intensified the metformin-induced inhibition of cell proliferation (41) (Figure 3B and C).

Addition of RAD001 or metformin to chemotherapy led to synergistic inhibition of proliferation in different breast cancer cell lines. The effect of carboplatin and metformin alone and in combination at different concentrations on MCF-7, T47-D, BT-474, and MDA-MB-231 cell proliferation was determined by the MTT assay after a 4-day treatment. Both carboplatin and metformin as monotherapy affected cell proliferation in a



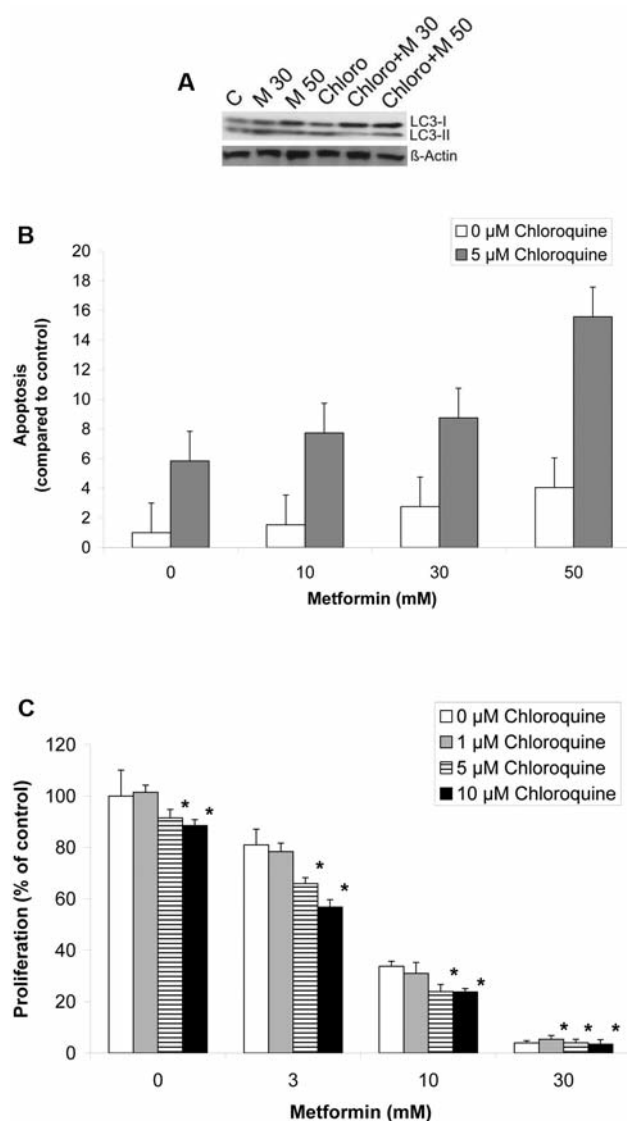


Figure 3. Metformin partially induced autophagic cell death on breast cancer cells. A: MDA-MB-231 cells were treated with metformin (30 mM or 50 mM) in the presence or absence of the autophagy inhibitor chloroquine (5  $\mu$ M) for 24 h. Changes of the LC-II expression after treatment were examined by western blot.  $\beta$ -Actin expression served as a loading control. C: Control; M 30: metformin, 30 mM; M 50: metformin, 50 mM; Chloro: chloroquine, 5  $\mu$ M. B: MDA-MB-231 cells were treated with metformin (10, 30, or 50 mM) for 4 days in the presence or absence of chloroquine (5  $\mu$ M) for 48 h. The internucleosomal DNA fragmentation was quantified by assaying for cytoplasmic mononucleosome- and oligonucleosome-associated histones 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 $\pm$ SD of triplicates. C: MDA-MB-231 cells were treated with metformin (3, 10, 30 mM) in the presence or absence of the chloroquine (1, 5, 10  $\mu$ M) for 4 days. After incubation, the cell proliferation was measured with the MTT assay. Results are expressed as a percentage of the corresponding controls (without treatment). Values are the mean $\pm$ SD of six parallel experiments. \* $p$ <0.05 in comparison with the group treated with the same concentration of metformin but in the absence of chloroquine.

dose-dependent manner. Combination of carboplatin with metformin significantly increased the growth inhibition in all cell lines as compared to monotherapy (Figure 4A-D).

The combination effect of metformin and carboplatin on cell proliferation was analyzed using a constant ratio combinatorial design with the CalcuSyn software. Synergistic inhibition of cell proliferation was observed in MCF-7, BT-474, T47-D and MDA-MB-231 cell lines as shown by the CI values <1 (Table I).

Similar results were obtained when the breast cancer cells were treated with doxorubicin or paclitaxel in combination with metformin (Table II). When RAD001 was further integrated into the treatment scheme, a synergistic effect on cell proliferation was achieved as shown by CI<1 (Table III).

*Prolonged combinatorial treatment with carboplatin and metformin resulted in synergistic G<sub>1</sub>-phase arrest of cell cycle progression.* In order to examine the mechanism responsible for the synergistic antiproliferative activity of carboplatin and metformin in breast cancer cells, the effect of the two drugs on the expression of cell-cycle-regulating factors was assessed by western blot. Importantly, p27 levels were found to be additionally enhanced in the MDA-MB-231 cell line, whereas cyclin D and cyclin E were attenuated through the combinatorial treatment in this cell line. This result indicated that the synergistically induced decrease in cell proliferation, through the combinatorial treatment with chemotherapeutics, could be due to the synergistic effect of the two drugs on the cell cycle transition. Interestingly, synergistic down-regulation of phosphorylated 4E-BP1 by the combined treatment with metformin and carboplatin was observed in the MDA-MB-231 cell line (Figure 5), suggesting a synergistic inhibition of the AKT/mTOR pathway when the two drugs were combined, contributing to the reduced cell cycle progression and cell proliferation. p27 levels were further increased and the down-regulation of phosphorylated 4E-BP1 was slightly intensified when RAD001 was integrated into the treatment regime (Figure 5).

## Discussion

Breast cancer, the leading neoplastic malignancy among females, affecting 1 out of 7-10 women, and diabetes mellitus type 2, affecting 7% of adults and 15% of those older than 60 years, are two major causes of morbidity and death worldwide (42). Generally, up to 20% of elderly breast cancer patients additionally suffer from diabetes (43). Diabetic patients have an increased risk for breast cancer and an adverse outcome (44). Metformin, a broadly prescribed biguanide class oral antidiabetic drug, has gained increasing attention as a potential anticancer agent. Long-term observations and clinical trials have indicated that it may reduce overall breast cancer risk and the mortality of breast cancer patients, especially in long-term users (45).

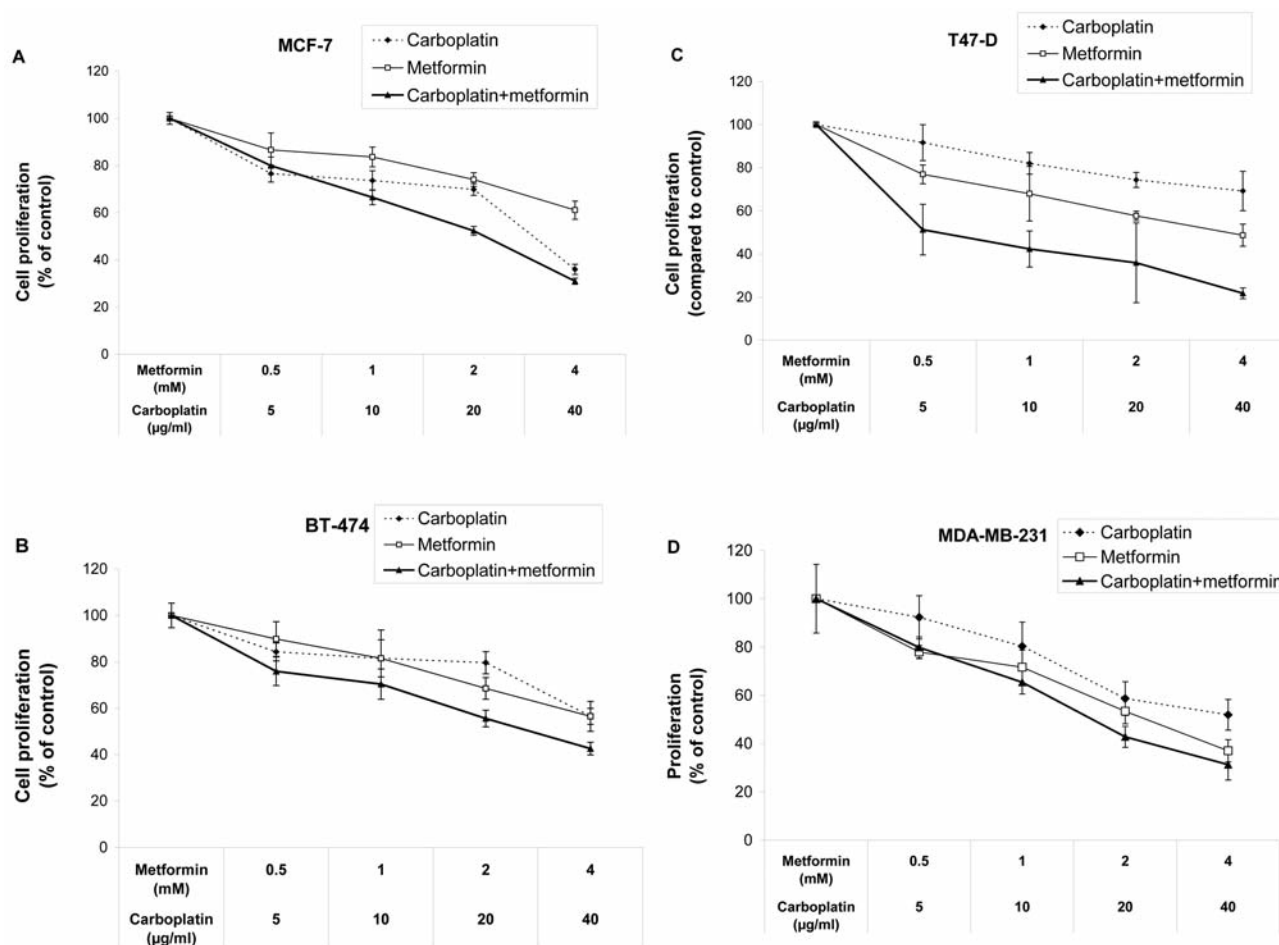


Figure 4. The combination of low concentrations of metformin and of chemotherapeutics, synergistically induced the inhibition of cell proliferation in different breast cancer cell lines. Breast cancer cells ( $2 \times 10^4$ /ml) were seeded in a 96-well-plate and were incubated with metformin (0.5, 1, 2, 4  $\mu$ M) either with or without carboplatin (5, 10, 20, 40  $\mu$ g/ml) for 4 days. The cell proliferation was measured by the MTT test. Results are expressed as a percentage compared to the corresponding control (without treatment). Values are the mean  $\pm$  SD of six parallel experiments. A: MCF-7; B: BT-474; C: T-47D; D: MDA-MB-231.

We have studied the *in vitro* anticancer effects of metformin on breast cancer cell lines and showed that metformin suppresses the proliferation of different breast cancer cell lines, including ER $\alpha$ -positive, HER2-overexpressing, and triple-negative breast cancer cell lines, in a concentration-dependent manner (Figure 1A and B). The non-malignant breast epithelial MCF-10A cells were much more resistant to the metformin treatment than were other malignant cell lines (Figure 1B). Among the breast cancer cell lines, the triple-negative cell line MDA-MB-231 was most sensitive to the metformin treatment, with its proliferation reduced to 20% compared to that of the control at a concentration of 15 mM (Figure 1B), which correlated well with the result of Liu *et al.* (14).

Our data indicate that metformin has unique biological and molecular effects on triple-negative breast cancer cells. It reduces cellular proliferation and induces apoptosis *via* the induction of specific and selective molecular effects. We presented here, for the first time, that caspases appear to be involved in cell death induced by metformin, not only by demonstrating the elevated cleavage of caspase-3, but also by showing that the pan-caspase-inhibitor Z-VAD-FMK significantly antagonized the cell death-inducing effect of metformin. However, the presence of Z-VAD-FMK in the culture media did not significantly inhibit cell proliferation as it did in the cell death assay, indicating the relevance of other mechanisms, also involved in the effects of metformin, on breast cancer cell death and proliferation (Figure 2A-C).

Table I. Summary of combination index (CI) values at the effective dose (ED) for 25%, 50%, 75% and 90% inhibition of proliferation of different breast cancer cell lines. Breast cancer cells ( $2 \times 10^4$ /ml) were seeded in a 96-well plate, incubated either with metformin alone, carboplatin alone, or with combination of the two drugs for 4 days. Cell proliferation was measured by the MTT test. CI values for each data point of the combination treatment were analyzed using the Calcsyn software. Values are the representative of three experiments.

Fraction affected (Fa)	Combination index (CI)			
	MCF-7	BT-474	T47-D	MDA-MB-231
0.25	1.041	0.735	0.116	1.033
0.5	0.814	0.760	0.517	0.670
0.75	0.638	0.791	0.333	0.472
0.90	0.503	0.825	0.694	0.378

Table II. Breast cancer cell lines were cultured in the presence of either paclitaxel, doxorubicin, metformin, or a combination of metformin plus one chemotherapeutic drug for 4 days. Cell proliferation was measured by the MTT test. Results are expressed as a percentage of cell proliferation compared to the corresponding control (without treatment). Values are the representative of three experiments.

	Control	Metformin (0.5 mM)	Paclitaxel (0.05 $\mu$ M)	Metformin+ paclitaxel
MCF-7	100	88.15	59.30	49.81
BT-474	100	86.61	47.26	42.63
MDA-MB-231	100	81.41	66.67	65.83

	Control	Metformin (5 mM)	Doxorubicin (0.5 $\mu$ M)	Metformin+ doxorubicin
MCF-7	100	65.03	79.30	57.03
BT-474	100	54.24	77.78	51.82
MDA-MB-231	100	67.47	69.10	58.54

The ability of metformin to induce apoptosis *via* PARP cleavage and the activation of both intrinsic and extrinsic caspase signaling cascades was demonstrated by the increase of the levels of cleaved PARP and cleaved caspase-3 (Figure 2C). We determined the nuclear and the cytoplasmatic AIF expression level by western blot and found that treatment with metformin resulted in an enhanced level of nuclear AIF and in a decreased level of the cytoplasmatic protein in MDA-MB-231 cells (Figure 2D). This result indicates that AIF-mediated cell death contributes to metformin-induced cell death in breast cancer cells. AIF is a mitochondrial protein which participates in more programmed cell death systems than initially thought. Research in recent years, has revealed that truncated AIF participates in caspase-independent apoptosis programs by binding to DNA and by

Table III. Breast cancer MCF-7 and BT-474 cells were cultured in the presence of a combination of carboplatin with metformin, of doxorubicin with metformin, or of paclitaxel with metformin in the presence or absence of RAD001 for 4 days. Cell proliferation was measured by the MTT test. Combination index (CI) values for each data point of the combination treatment were analyzed using the Calcsyn software. Values are representative of three experiments.

Carboplatin ( $\mu$ g/ml)	RAD001 (nM)	Metformin (mM)	CI	
			BT-474	MCF-7
10	10	5	0.74	0.677
10	10	10	0.806	0.72
10	10	25	0.82	0.777
10	10	50	0.852	0.834

Doxorubicin ( $\mu$ M)	RAD001 (nM)	Metformin (mM)	CI	
			BT-474	MCF-7
0.5	10	5	0.768	0.695
0.5	10	10	0.776	0.712
0.5	10	25	0.82	0.787
0.5	10	50	0.875	0.854

Paclitaxel ( $\mu$ M)	RAD001 (nM)	Metformin (mM)	CI	
			BT-474	MCF-7
0.5	10	5	0.82	0.772
0.5	10	10	0.828	0.772
0.5	10	25	0.847	0.782
0.5	10	50	0.88	0.884

mediating large-scale DNA fragmentation (33,34). This result indicates that the caspase-independent cell death mediated by AIF possibly occurs through metformin treatment and contributes to the observed effects of metformin on breast cancer cells.

Autophagy is an evolutionarily conserved catabolic process whereby intracellular proteins and complete organelles are sequestered in double-membrane vesicles (autophagosomes). Autophagy is a cellular process which degrades long-lived or malfunctioning proteins and obsolete or damaged organelles. It maintains cellular homeostasis and helps cells survive stressful conditions (46). The relationship between autophagy and tumor growth or suppression is complex. Cell viability under stress seems to be regulated by a complex interplay between autophagy and apoptosis. Whilst autophagy delays stress-induced apoptosis, it also limits necrotic death when apoptosis is impaired. Consistent with the cytoprotective role of autophagy, increasing evidence suggests that autophagy may contribute to resistance to cancer therapy. Thus, considerable attention has been drawn to targeting autophagy in the treatment of cancer.



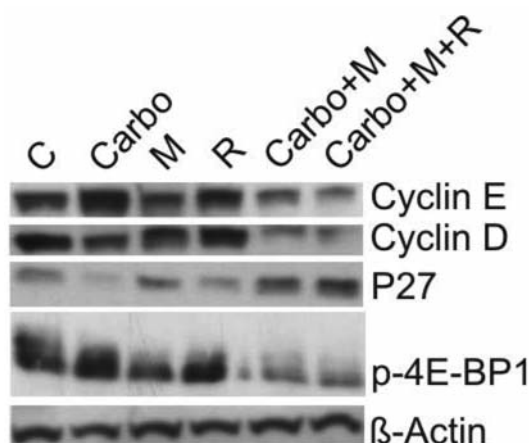


Figure 5. The combination of low concentrations of carboplatin and metformin resulted in the alteration of cell cycle-related proteins after long-term culture of the MDA-MB-231 cell line. Breast cancer MDA-MB-231 cells were cultured in the presence of either carboplatin (Carbo, 20  $\mu$ g/ml) alone, metformin (M, 20 mM) alone or in their combination, or in their combination and RAD001 (R, 10 nM) for 4 days. Cell lysates were analyzed by immunoblotting using specific antibodies towards different cell cycle-related proteins. The expression of  $\beta$ -actin served as a loading control. Representative blots of three independent experiments are shown.

A number of studies in various types of cancer have shown that inhibition of autophagy, either pharmacologically or by knocking-down autophagy genes, sensitizes cancer cells to a wide range of therapeutic modalities, including genotoxic chemo- and radiotherapy, hormonal therapy and receptor tyrosine kinase inhibition (47, 48).

mTOR has been identified to inhibit autophagy in response to growth factor and nutrient signals, but the downstream effectors of mTOR, that regulate autophagy, have not yet been discovered. AMPK is reported to promote autophagy through the inhibition of mTOR (49). Metformin-induced autophagy has been observed in different types of tumors, including colon cancer cell lines (50, 51). However, the role of metformin induced-autophagy in cancer cell death remains controversial. Metformin-mediated autophagy has been demonstrated to contribute to cell death in mouse and human melanoma cell lines (39, 40), while it protected glioma, rat C6 glioma and mouse L929 fibrosarcoma cell lines from cisplatin-induced cytotoxic effects (36). We observed that metformin induced not only apoptosis but also autophagy in the MDA-MB-231 cells since the elevated expression of an autophagy hall marker, LC3-II, was shown by western blot. Increased cell apoptosis and reduced cell proliferation were observed by adding the autophagy inhibitor chloroquine to the cell culture, indicating that metformin-induced autophagy attenuated its treatment efficacy for breast cancer. The overcoming of this negative effect of metformin could be achieved by integrating an autophagy inhibitor into the treatment regime (Figure 3).

We investigated for the first time the combinatorial effect of different chemotherapeutic drugs and metformin on tumor cell proliferation in a panel of breast cancer cell lines with different ER $\alpha$ - and Her2-status (Figure 4, Tables I and II). To our surprise, the cell proliferation inhibition was further intensified when the mTOR inhibitor RAD001 was added in the treatment regime of metformin in combination with the other chemotherapeutic drugs (Table III). Several mechanisms have been described which are responsible for the observed anticancer activity of metformin. Among them, a well-known mechanism is the metformin-mediated activation of AMPK, which leads to mTOR inhibition and subsequently to the decrease of S6 kinase expression (1). However, the consequence of mTOR inhibition by metformin is distinct and differs from that of rapamycin and its analogs: consistent with Zakikhani *et al.* (21), metformin strongly reduced the phospho-4E-BP1 levels, as shown in Figure 5, while RAD001 had only a minimal reduction effect on this protein. Exposure to metformin resulted in dose dependent cell proliferation inhibition, whereas RAD001 had only dose dependent cell proliferation inhibition at the concentrations up to 10 nM (data not shown). We believe that the observed synergistic effect on cell proliferation of metformin in combination with RAD001, demonstrated in Table III, was possibly due to the difference of the functional mechanism of the two drugs, so that more signalling pathways related to cell proliferation were inhibited by the combination of the two drugs than by each of them separately. Our results suggest that this combination can provide a new strategy for breast cancer treatment.

In conclusion, the present *in vitro* study with breast cancer cell lines demonstrates that metformin alone or with chemotherapeutic agents and/or the mTOR inhibitor RAD001 could be a promising approach for the treatment of breast cancer. Inhibition of cell proliferation and of cell apoptosis induced by the metformin treatment contributed to its effect on breast cancer cells. The integration of chemotherapeutic drugs enhanced the treatment efficacy of metformin. The mTOR inhibitor RAD001 further intensified the treatment effect. These results provide a rationale for future clinical trials with metformin in combination with chemotherapy and/or RAD001 for patients with breast cancer.

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