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The Impact of Insulin on Low-dose Metronomic Vinorelbine and Mafosfamide in Breast Cancer Cells

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Abstract. *Background/Aim: Breast cancer (BC) may be affected by diabetes and anti-diabetic medication, as well as its therapeutic agents. Low-dose metronomic chemotherapy (LDMC) is an available treatment option in BC. We investigated the impact of insulin on low-dose metronomic vinorelbine and mafosfamide in BC cell lines. Materials and Methods: Human BC cell lines T-47D, MCF-7, MDA-MB-231, BT-549 and non-tumorigenic breast cell line MCF-10A were exposed to 0.01 μg/ml and 10 μg/ml insulin in combination with low-dose metronomic vinorelbine or mafosfamide. The cell viability was determined after 24-72 hours using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results: Insulin, especially at a concentration of 10 μg/ml, seemed to increase viability of vinorelbine-treated hormone receptor-positive BC cells, whereas low-dose mafosfamide treatment tended to be potentiated by insulin in triple-negative cells. Conclusion: Our findings suggest that insulin may influence the cytotoxic activity of LDMC depending on insulin concentration, type of cytotoxic drug used and BC cell line.*

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer death among women, accounting for about 2.1 million new cases and 0.6 million deaths in 2018, worldwide. Incidence rates of BC have been slightly rising over the past decades (1). This trend likely reflects a combination of higher prevalence of known risk factors, including diabetes, obesity and physical inactivity, as well as nulliparity, late age at first birth, exogenous hormone intake and an increase in BC screening, awareness and rising life expectancy (1). Diabetes also has a high mortality rate, with 1.37 million deaths in 2017 (2). In Germany, 21% of all deaths can be attributed to diabetes (3). Larsson *et al*. showed in a meta-analysis women with diabetes to have a statistically significant 20% increased risk of BC (risk ratio=1.20, 95% confidence interval=1.12-1.28) (4). This may be associated with the fact that hyperglycemia and hyperinsulinemia, conditions associated with diabetes, may increase BC risk (5, 6). Moreover, diabetes seems to have a negative impact on the prognosis of BC (7-9). In a meta-analysis by Peairs *et al*., patients with BC and diabetes had a significantly higher all-cause mortality risk (pooled hazard ratio (HR)=1.49, 95% confidence interval=1.35-1.65) compared with their non-diabetic counterparts (10). On the one hand metabolic alterations related to diabetes and on the other anti-diabetic drugs may both contribute to higher incidence and mortality of BC (7, 11-13).

Low-dose metronomic chemotherapy (LDMC), defined as a continuous administration of low-dose cytotoxic drug with effects especially through immunomodulation, inhibition of angiogenesis and direct cytotoxicity (14, 15), is a feasible treatment option in BC, with less toxicity compared to conventional chemotherapy (16-18). In particular, the effectiveness of low-dose oral vinorelbine and cyclophosphamide has already been proven in several clinical studies and can be recommended for patients with hormone receptor-positive (HR^+), human epidermal growth factor receptor 2-negative ($HER2^-$) metastatic BC previously treated with anthracyclines and taxanes (19, 20).

Due to the fact that diabetes and anti-diabetic medication may affect BC, it is crucial to gain more experience about the interaction between anti-diabetics and therapeutic agents in BC. There is evidence that insulin may affect the cytotoxic effects of chemotherapy in BC (21, 22). The aim of this study was to investigate the effects of insulin on low-dose metronomic vinorelbine and mafosfamide in various BC cell lines.

Materials and Methods

Cell culture and treatment. Human BC cell lines T-47D, MCF-7 (both HR^+ and $HER2^-$), MDA-MB-231, BT-549 (both triple-negative) and non-tumorigenic breast cell line MCF-10A, were all obtained from

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American Type Culture Collection (Rockville, MD, USA). BC cells were cultured in RPMI 1640 culture medium (Thermo Fisher Scientific, Waltham, MA, USA), containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin; MCF-10A in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Thermo Fisher Scientific) supplemented with 5% FCS, 1% penicillin-streptomycin (both Thermo Fisher Scientific), epidermal growth factor (20 μg/ml), hydrocortisone (1.4×10^{-6} M), cholera toxin (100 ng/ml) and human insulin (10 μg/ml) (all Sigma Aldrich, St. Louis, MO, USA). Cells were incubated in a moistened atmosphere at 37˚C and 5% CO_2. For all functional assays, cells were detached by trypsin-ethylenediaminetetra-acetic acid (Sigma Aldrich) and seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) at 2,500 cells/well (passage 10/semiconfluence).

In order to demonstrate the influence of insulin on overall cell viability, culture medium was replaced by medium containing 5% FCS 24 hours before the experiment. Then the cells were treated with insulin (Sigma Aldrich) at concentrations of between 0.01 and 100 μg/ml for another 24 hours. In analyzing the influence of insulin on the effect of cytotoxic agents, cells were pretreated with insulin at 0.01 μg/ml (concentration in serum from healthy individuals) and 10 μg/ml for 24 hours as described elsewhere (23). Afterwards, vinorelbine (Navirel®; Medac, Wedel, Germany) or mafosfamide (Niomech-IIT GmbH, Bielefeld, Germany) were added at levels corresponding to the concentration in serum of metronomically treated patients (0.16-10 ng/ml vinorelbine; 0.15-9.6 μg/ml mafosfamide) *i.e.* at much lower concentrations compared to maximum tolerated doses of conventional chemotherapy, for a further 72 hours to simulate the metronomic dosing schedule (24, 25). To maintain a constant concentration of drugs, the drug and insulin-enriched medium was replaced every 24 hours.

Cell viability assay. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma Aldrich) as previously described (26). In brief, 20 μl/well MTT (0.5%) was added to cultures which were then incubated for 4 hours at 37˚C and 5% CO_2. After washing with Dulbecco's phosphate buffered saline, the tetrazolium precipitate was solubilized by isopropanol for 15 minutes and the absorbance was measured at 570 nm and 650 nm (reference wavelength) using a GloMax® multi detection system microplate reader (Anthos Labtec Instruments, Cambridge, UK).

Statistical analysis. All experiments were performed in quadruplicates and repeated three times. The absorbance in MTT assay for the untreated control group was regarded as 100% cell viability. The results of absorbance for the treated groups are indicated as the mean±standard deviation of three separate experiments and displayed as percentages relative to that of the control group. Student's *t*-test (Microsoft Excel 2013 v15.0; Microsoft Corporation, Redmond, WA, USA) was used to evaluate the statistical significance of the results. All *p*-values represented two-sided tests and statistical significance was assumed at a value of $p<0.05$.

Results

The impact of insulin on cell viability. Cell viability of HR^+ cell lines T-47D and MCF-7 decreased by 11.1% ($p=0.041$) and 13.3% ($p=0.035$) after 0.01 μg/ml insulin treatment, respectively, but was not significantly affected by insulin at concentrations of 0.1-10 μg/ml (Figure 1). Insulin at over 20 μg/ml reduced cell viability of T-47D cells (up to 25.7% at 50 μg/ml insulin, $p<0.001$) and MCF-7 cells (up to 37.1% at 100 μg/ml insulin, $p<0.001$). Cell viability of triple-negative cell lines MDA-MB-231 and BT-549 was not significantly altered by insulin in concentrations of up to 10 μg/ml except for 1 μg/ml insulin in MDA-MB-231 cells (increase by a maximum of 11.8%, $p=0.011$). Insulin at concentrations above 20 μg/ml reduced cell viability in MDA-MB-231 cells (up to 20.1% at 100 μg/ml insulin, $p<0.001$) and BT-549 cells (up to 18.0% at 100 μg/ml insulin, $p<0.001$). In contrast, for the non-tumorigenic cell line MCF-10A, viability significantly increased after treatment with up to 10 μg/ml insulin (by a maximum of 14.7% at 0.1 μg/ml insulin, $p=0.005$) and decreased after treatment with insulin at concentrations over 50 μg/ml (by a maximum of 31.2% at 100 μg/ml insulin, $p<0.001$). In all cell lines studied, the observed effects of insulin were marginal and concentration-independent up to a threshold of 10 μg/ml. Cell viability above 10 μg/ml insulin decreased linearly with insulin dose.

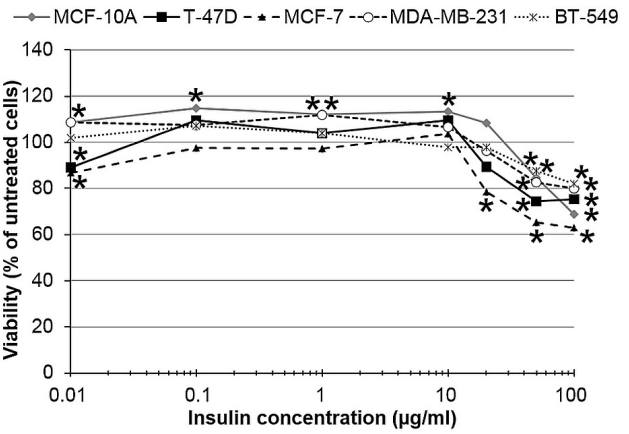

Figure 1. *The impact of insulin on cell viability. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure cell viability after 24-hour treatment with insulin at various concentrations. The results are shown as the mean values from three separate experiments. *Significantly different from the control at $p<0.05$.*

The impact of insulin on the cell response to vinorelbine. Vinorelbine at concentrations from 0.31 ng/ml for T-47D, 0.63 ng/ml for MCF-7, 0.31 ng/ml for MDA-MB-231 and 0.16 ng/ml for BT-549 and above significantly reduced cell viability (Figure 2A-D). The greatest reduction of viability was achieved in T-47D cells by 56.5% at 5 ng/ml vinorelbine and in MCF-7, MDA-MB-231 and BT-549 cells by 73.6%, 67.7 and 80.7% at 10 ng/ml vinorelbine, respectively. Cell viability of the non-tumorigenic MCF-10A cell line was reduced significantly only at vinorelbine concentrations of above 1.25 ng/ml by a maximum of 79.5% ($p<0.001$; Figure 2E).

Adding 0.01 μg/ml insulin led to significantly increased viability of T-47D cells treated with 0.31 ng/ml (p=0.024) and 1.25 ng/ml (p=0.002) vinorelbine (Figure 2A). Another tested HR^+ cell line (MCF-7) did not show any significant alterations regarding cell viability when treated with 0.01 μg/ml insulin and vinorelbine compared with the control without insulin (Figure 2B). In triple-negative cell lines treated with 0.01 μg/ml insulin, no significant impact on the cell response was detected except in MDA-MB-231 cells treated with 0.31 ng/ml vinorelbine (increased viability by 13.4%, p=0.004; Figure 2C and D). Insulin at 0.01 μg/ml significantly increased viability of non-tumorigenic MCF-10A cells treated with up to 0.63 ng/ml vinorelbine, by a maximum of 14.6% at 0.16 ng/ml vinorelbine (p=0.015; Figure 2E).

Insulin at 10 μg/ml combined with vinorelbine led to significantly higher cell viability than vinorelbine alone in both HR^+ cell lines. In particular, cell viability of T-47D cells increased after treating with up to 5 ng/ml vinorelbine, by a maximum of 27.4% at 0.63 ng/ml vinorelbine (p=0.005; Figure 2A). Viability of MCF-7 cells increased significantly at all vinorelbine concentrations tested, by a maximum of 31.3% at 0.16 ng/ml vinorelbine (p=0.002; Figure 2B). In triple-negative cell lines, 10 μg/ml insulin had no significant influence on the effect of vinorelbine except in MDA-MB-231 cells treated with 0.31 and 2.5 ng/ml vinorelbine (increasing cell viability by 11.6%, p=0.028; Figure 2C and D). Insulin treatment at 10 μg/ml of MCF-10A cells led to a significantly higher viability than in the control treated with vinorelbine alone, independent of the vinorelbine concentration (by 33.7% at 0.63 ng/ml vinorelbine, p<0.001; Figure 2E).

The impact of insulin on the cell response to mafosfamide. Mafosfamide, at a concentration starting from 0.3 μg/ml for T-47D and 0.6 μg/ml for MCF-7, MDA-MB-231 and BT-549 cells led to a significant reduction of cell viability (Figure 3A-D). The greatest reduction of viability was observed at 9.6 μg/ml mafosfamide in T-47D, MCF-7, MDA-MB-231 and BT-549 by 95.9%, 57.4%, 50.3% and 44.9%, respectively. Cell viability of the non-tumorigenic MCF-10A cell line decreased significantly only at mafosfamide concentrations of above 4.8 μg/ml, by a maximum of 45.1% (p<0.001; Figure 3E).

In T-47D, 0.01 μg/ml insulin combined with 1.2-4.8 μg/ml mafosfamide significantly reduced viability than mafosfamide alone, by a maximum of 14.7% (p=0.002; Figure 3A). MCF-7 cells treated with 0.01 μg/ml insulin and mafosfamide did not display any significant alterations in viability when compared with the control cells without insulin (Figure 3B). In triple-negative cell lines, 0.01 μg/ml insulin had no significant impact except in BT-549 cells treated with 0.15 μg/ml (increased cell viability by 8.5%, p=0.018) and 4.8 μg/ml mafosfamide (reduced cell viability by 7.2%, p=0.003) (Figure 3C and D). Viability of mafosfamide-treated non-tumorigenic MCF-10A cells was not significantly influenced by treatment with 0.01 μg/ml insulin (Figure 3E).

The cell response to mafosfamide in HR^+ cell lines was not significantly affected by treatment with 10 μg/ml insulin except in T-47D cells treated with 0.3 μg/ml mafosfamide in which cell viability was 12.3% higher (p<0.048; Figure 3A and B). In triple-negative MDA-MB-231 cells, 10 μg/ml insulin led to a lower cell viability at 2.4-9.6 μg/ml mafosfamide (maximum reduction of 21.0%, p=0.003) than with mafosfamide alone (Figure 3C). Cell viability of BT-549 cells after the treatment with 10 μg/ml insulin was higher at 0.3 μg/ml mafosfamide (by 6.7%, p=0.014) and lower at higher concentrations (by a maximum of 6.6%) without significance when compared to treatment with mafosfamide alone (Figure 3D). In MCF-10A cells, 10 μg/ml insulin did not affect the viability of mafosfamide-treated cells (Figure 3E).

Discussion

We showed in our *in vitro* experiments that insulin can influence drug cytotoxic activity depending on the insulin concentration, type of cytotoxic drug used and the BC cell line. While there is no compelling evidence that any insulin analog, nor human insulin is involved in BC initiation, insulin may contribute to tumor progression by up-regulating mitogenic signaling pathways (11). Insulin is a well-known growth factor which may initiate growth-promoting processes and metabolic effects through its interaction with the insulin receptor and the insulin-like growth factor 1 receptor, followed by activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways (27, 28). Various studies have already examined the influence of insulin on cellular parameters such as proliferation and viability *in vitro*. Twenty-four-hour treatment with 0.1-250 nM insulin, which corresponds to 0.00058-1.45 μg/ml insulin, promoted MCF-7 cell proliferation at different rates (29-31). Therefore, at the beginning of the present work, insulin was used at different concentrations for testing the viability of two HR^+, two triple-negative BC cell lines, as well as one non-tumorigenic breast cell line. The cell viability was mostly not affected by 0.01-10 μg/ml insulin, but concentrations above 20 μg/ml led to a significant reduction of cell viability in all BC cells. The aim of our study was to use insulin at physiological serum concentration (0.01 μg/ml), as well as at the highest possible concentration with no negative effect on cell viability (10 μg/ml) (23).

There is evidence that the mechanism of the reaction to some anticancer agents can be different depending on the scheduling of their administration (32, 33). Browder *et al*. demonstrated effective anticancer effects of metronomic chemotherapy, even in tumors that were initially resistant to conventional chemotherapy (34). The Victor-0-study examined the effect of metronomic versus standard schedule of vinorelbine and 5-fluorouracil administration in triple-negative BC cell lines. Cells treated with low-dose metronomic

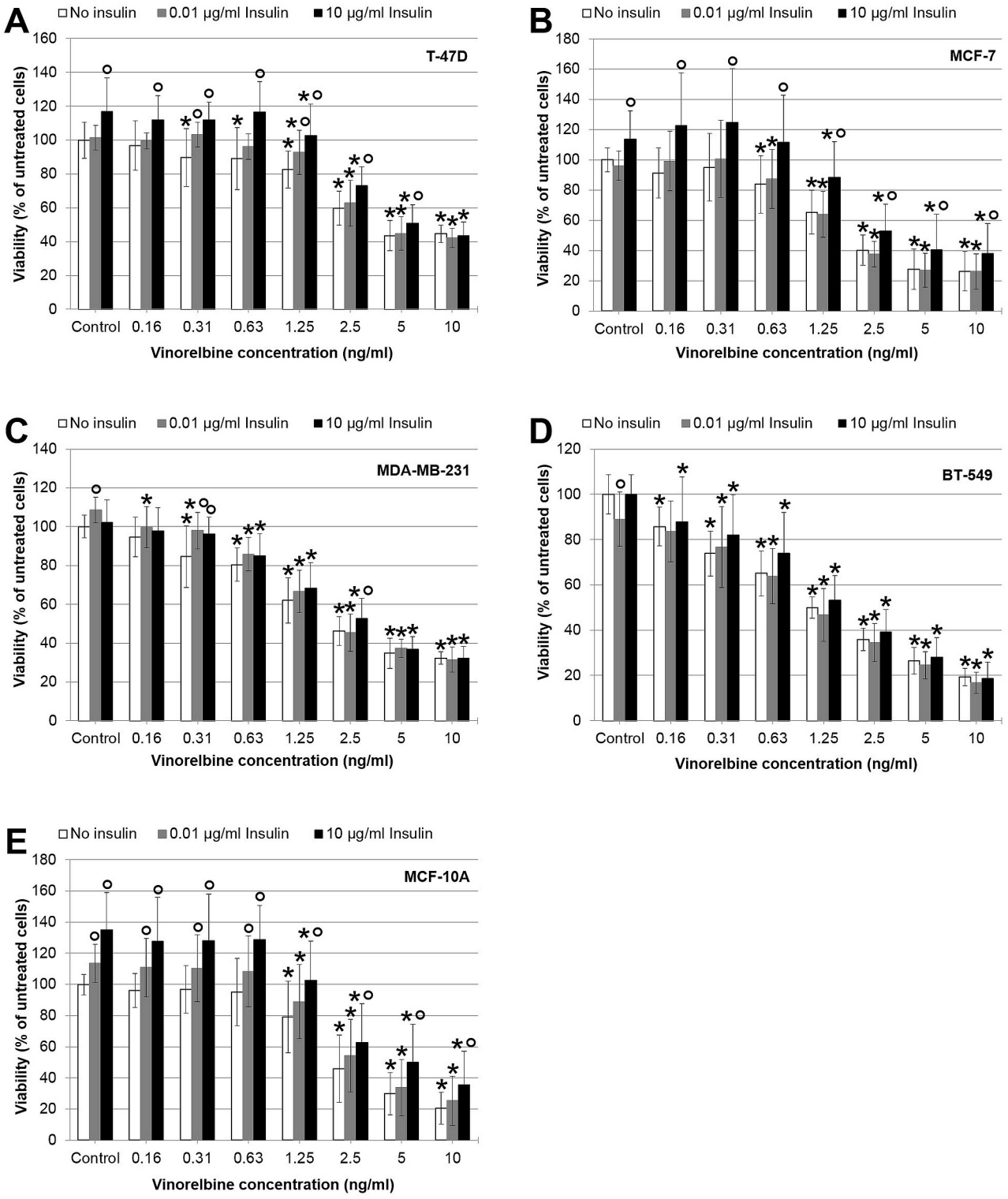

Figure 2. *The impact of insulin on the response of hormone receptor-positive breast cancer cell lines T-47D (A) and MCF-7 (B), triple-negative breast cancer cell lines MDA-MB-231 (C) and BT-549 (D), and non-tumorigenic breast cell line MCF-10A (E) to vinorelbine. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure cell viability after 24-hour treatment with insulin followed by 72-hour treatment with or without insulin and vinorelbine. The results are shown as the mean±standard deviation from three separate experiments. Significantly different at *p<0.05 compared with the control group, and °p<0.05 compared with the group without insulin.*

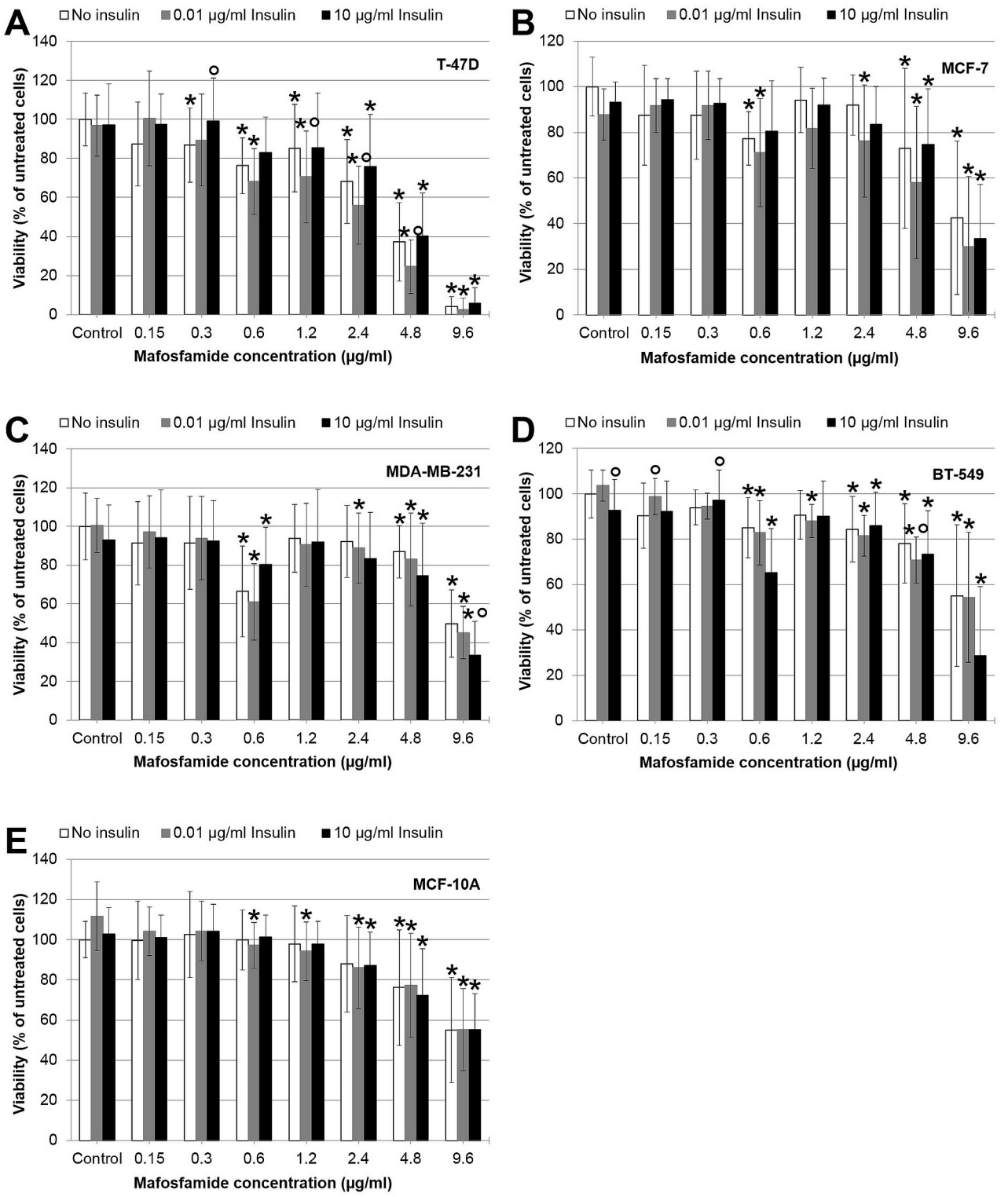

Figure 3. *The impact of insulin on the response of hormone receptor-positive breast cancer cell lines T-47D (A) and MCF-7 (B), triple-negative breast cancer cell lines MDA-MB-231 (C) and BT-549 (D), and non-tumorigenic breast cell line MCF-10A (E) to mafosfamide. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure cell viability after 24-hour treatment with insulin followed by 72-hour treatment with or without insulin and mafosfamide. The results are shown as the mean±standard deviation from three separate experiments. Significantly different at *p<0.05 compared with the control group, and °p<0.05 compared with the group without insulin.*

vinorelbine and 5-fluorouracil expressed high levels of autophagosome proteins, were senescence-associated β-galactosidase-positive and expressed a low level of cleaved caspase-3, suggesting that autophagy and cellular senescence contribute more than apoptosis to the growth-suppressive effect triggered by metronomic chemotherapy. On the contrary, the major contribution to the growth-suppressive effect of standard regimen seemed to be attributed to the induction of apoptosis (32). Here we demonstrated that low-dose metronomic vinorelbine at concentrations up to 10 ng/ml and mafosfamide at concentrations up to 9.6 μg/ml significantly reduced cell viability in all BC cell lines tested. In particular, even concentrations of 1.25 ng/ml vinorelbine and 0.6 μg/ml mafosfamide led to a significant reduction of cell viability regardless of insulin concentration. Furthermore, the reduction of cell viability was observed to be linearly related to the concentration of chemotherapeutic agents.

In the present study, compared to the cells without insulin, 0.01 μg/ml insulin, a concentration that can be detected in serum of healthy individuals, led to a significant increase in cell viability at vinorelbine concentrations of up to 1.25 ng/ml in T-47D cells. Insulin at 10 μg/ml significantly enhanced viability of both HR^+ cell lines at almost all vinorelbine concentrations. Mafosfamide-treated T-47D cells exposed to 10 μg/ml insulin, displayed a tendency for higher cell viability than did cells without insulin. It can be assumed that the cytotoxic effects of low-dose vinorelbine and mafosfamide might be suppressed by the proliferation-stimulating effects of insulin in HR^+ cells. These results may support the interplay between insulin and estrogen, as well as their receptors, in HR^+ BC (27, 35). Panno *et al*. showed that insulin can increase estrogen and progesterone receptor content and their binding capacity in MCF-7 cells (36). It may be assumed that insulin is able to affect the phosphorylation status of estrogen and progesterone receptors, inducing functional changes in both proteins. Moreover, there is evidence that insulin may enhance estrogen production followed by initiation of HR^+ BC development (27, 35). Contrary to this, in the triple-negative cell lines studied here, insulin affected the cytotoxic activity of vinorelbine little or not at all. The combination of mafosfamide and insulin led to similar results, but at a relatively high mafosfamide concentration of 9.6 μg/ml, we noted a tendency towards a potentiating effect of mafosfamide through insulin in both triple-negative cell lines. Comparing the two insulin doses, 10 μg/ml insulin showed greater effects than 0.01 μg/ml insulin on the response to cytostatic agents. In particular, the observed increase of viability in vinorelbine-treated HR^+ BC cells and the decrease of viability in mafosfamide-treated triple-negative BC cells were greater or only detectable after treatment with 10 μg/ml insulin. Agrawal *et al*. demonstrated that combination of 4000 μg/ml cyclophosphamide with 40 μg/ml insulin produced a significant inhibition in viability of MCF-7 cells with enhanced apoptosis when compared with chemotherapy alone and they proposed a model for insulin-induced sensitization process (21). It was suggested that insulin may act as a sensitizer of cancer cells to cytotoxic therapy through various mechanisms, including enhanced permeability of the cancer cell membrane to chemotherapeutic agents, known as insulin-mediated endocytosis, an increase in the S-phase fraction, and activation of metabolic and mitogenic pathways (21, 22). In studies using cytotoxic agents at standard doses, a potentiating effect of insulin was found (21, 22, 37). In contrast, our experiments were conducted with cytostatics at much lower doses to simulate LDMC treatment. The high cytostatic doses used in the study by Agrawal *et al*. (21) are intolerable for humans and were not applied in the present study. Furthermore, in our experiments we used mafosfamide instead of cyclophosphamide. Mafosfamide like cyclophosphamide belongs to the group of oxazaphosphorine derivatives with similar antineoplastic properties and mechanisms of action (38). Although closely related to cyclophosphamide, mafosfamide does not require hepatic activation to generate its active metabolite 4-hydroxy-cyclophosphamide and is a suitable alternative to cyclophosphamide in *in vitro* studies (39). Therefore, our results cannot be compared with those of Agrawal *et al*. (21), but nonetheless, it may be assumed that the scheduling of drug administration plays a key role in mechanisms of action of anticancer drugs, even in the presence of insulin. This rationale can also be supported by a study in colorectal cells by Volkova *et al*. Whereas they showed only a marginal impact of insulin and insulin-like growth factor 1 on the chemotherapy response at clinically relevant concentrations of cytostatics, the combination of insulin-like growth factor 1 and low-dose chemotherapy significantly increased cell viability in colorectal cells (40). Accordingly, in addition to the type of cell line and insulin concentration, the dose as well as the scheduling of cytostatic administration may be relevant for the drug interaction creating various effects in the anticancer treatment.

The present study provides the first insights into the effects of insulin on low-dose chemotherapeutic treatment corresponding to LDMC in BC cells. A potential limitation of our study is that we performed only *in vitro* experiments and the results cannot be simply transferred to humans *in vivo*. We investigated only the impact of insulin on cytotoxic activity of low-dose chemotherapy, whereas for the adequately assessment of the multimodal effects of LDMC, including immunomodulatory and anti-angiogenic properties, *in vivo* models are necessary.

In conclusion, our findings suggest that insulin may influence the cytotoxic activity of low-dose metronomic vinorelbine and mafosfamide. Insulin seemed to increase cell viability of vinorelbine-treated HR^+ BC cells, whereas low-dose mafosfamide treatment tended to be potentiated by insulin in triple-negative cells. Due to the rising incidence of hyperinsulinemia associated with *e.g*. diabetes and link

between hyperinsulinemia and pathogenesis of BC, it is crucial to conduct further investigations about the interaction between insulin and LDMC in BC.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Conceptualization: SK, MJB, ASH, RS and WB; investigation and formal analysis: SK, AL and WB; writing – original draft: SK and WB; writing – review and editing: MJB, AH, MS and RS.

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