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# Differential effects of integrin αv knockdown and cilengitide on sensitisation of triple-negative breast cancer and melanoma cells to microtubule poisons

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**Running title: Different effects of integrin αv knockdown and cilengitide**

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**ABBREVIATIONS:** Ad5, Adenovirus type 5; cDDP, cisplatin; cilengitide or SML1594, Cyclo(L-arginylglycyl-L-a-aspartyl-D-phenylalanyl-N-methyl-L-valyl), 2,2,2-trifluoroacetate; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FAK, focal adhesion kinase; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PF-573228 or PF-228, 6-[4-(3-Methanesulfonyl-benzylamino)-5-trifluoromethyl-pyrimidin-2-ylamino]-3,4-dihydro-1H-quinolin-2-one; PTX, paclitaxel; siRNA, small interfering RNA; TNBC, triple-negative breast cancer; VCR, vincristine.

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**ABSTRACT**

Low survival rates of patients with metastatic triple negative breast cancer (TNBC) and melanoma, in which current therapies are ineffective, emphasize the need for new therapeutic approaches. Integrin β1 appears to be a promising target when combined with chemotherapy, but recent data have shown that its inactivation increases metastatic potential due to the compensatory upregulation of other integrin subunits. Consequently, we decided to analyze the potential of integrin subunits αv, α3, or α4 as targets for improved therapy in seven TNBC and melanoma cell lines. Experiments performed in integrin αvβ1 negative melanoma cell line MDA-MB-435S showed that knockdown of integrin subunit αv increased sensitivity to microtubule poisons vincristine or paclitaxel, and decreased migration and invasion. In MDA-MB-435S cell line we also identified a phenomenon in which change in expression of one integrin subunit changes the expression of other integrin/s, leading to an unpredictable influence on sensitivity to anticancer drugs and cell migration referred to as the integrin switching effect. In a panel of six TNBC and melanoma cell lines the contribution of integrins αv *versus* integrins αvβ3/β5 was assessed by the combined action of αv-specific siRNA or αvβ3/β5 inhibitor cilengitide with paclitaxel. Our results suggest that, for TNBC, knockdown of integrin αv in combination with paclitaxel presents a better therapeutic option than combination of cilengitide with paclitaxel. However, in melanoma neither of these combinations is advisable because decreased sensitivity to paclitaxel was observed.

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**Introduction**

Integrins are a family of non-covalently associated heterodimeric adhesive receptors, composed of one α and one β subunit, that play roles in mediating cell-extracellular matrix (ECM) and cell-cell interactions. Ligation of integrins with ECM ligands induces a variety of intracellular signals and regulates several cellular responses including migration, differentiation, proliferation and survival (Guo and Giancotti, 2004). Integrins control a diverse array of cellular functions crucial to the initiation, progression and metastasis of solid tumors and are generally overexpressed in aggressive breast cancer and melanoma (Desgrosellier and Cheresh, 2010).

The integrin αv subunit is known to associate with five different β subunits (β1, β3, β5, β6 and β8) to form integrin heterodimers. Among these, integrins αvβ3 and αvβ5 have been extensively studied. In normal epithelial cells the expression of integrin αvβ3 is low while high levels have been shown in some tumors like breast carcinoma (Parvani et al., 2015) and melanoma (Danen et al., 1994; Nip et al., 1992). Expression of integrin αvβ3 is increased in brain metastases compared to primary tumors, in breast adenocarcinoma and malignant melanoma (Vogetseder et al., 2013). It has a pivotal role in human melanoma growth (Mitjans et al., 2000) and initiates the transition from the benign radial growth phase to the malignant vertical growth phase (Albelda et al., 1990). Bianchi-Smiraglia and colleagues (2013) demonstrated a critical role for integrin αvβ5 in the tumorigenic potential of triple negative breast cancer (TNBC) cells. The importance of integrin αvβ5 was also demonstrated in melanoma showing its involvement in the highly aggressive phenotype of melanoma cells expressing neuropilin 1 (Ruffini et al., 2015). Preclinical studies have revealed that α3β1 promotes pro-tumorigenic gene expression and function in breast cancer cells (Subbaram and Dipersio, 2011). Primary melanomas express low levels of α3β1, whereas metastatic melanomas express high levels of this integrin (Melchiori et al., 1995). It has been shown that downregulation of cell surface α4 integrin, and the consequent loss of α4β1 plasma fibronectin receptors in ERBB-2 overexpressing breast cancer cells is likely to be of functional significance to their ability to invade basement membranes and to metastasize (Ignatoski et al., 2000). In experimental models of melanoma metastasis, incubation of melanoma cells with antibodies directed against α4β1 can significantly reduce the frequency of lung metastases in mice that have been pretreated with proinflammatory cytokines (Kuphal et al., 2005).

Metastatic melanoma and TNBC of patients that have become refractory to immune-based or targeted therapies are still treated by chemotherapy. Several studies indicate that β1 integrins mediate drug resistance and stimulate metastasis in many different tumor types, including TNBC (Aoudjit and Vuori, 2001; Lahlou and Muller,

2011) and melanoma (Aoudjit and Vuori, 2001). It would be favorable for TNBC and melanoma treatment to find a way to enhance the efficacy of chemotherapy and concomitantly reduce metastasis. Many studies have used RNA interference to analyze whether integrins may be a target for combined cancer therapy strategies and most of them have targeted integrin subunit β1 (Aoudjit and Vuori, 2012). However, few reports analyzing integrin β1 knockdown identified integrin switching, a phenomenon in which change of expression of one integrin subunit can change the expression of other integrin/s, and observed increased metastasis (Parvani et al., 2013; Truong et al., 2014). These data raised concerns with respect to use of β1 integrins as drug targets to sensitize tumors to radio- or chemotherapy.

Results presented in this paper evaluate in TNBC and melanoma cell lines the use of small interfering RNA (siRNA) specific for integrin α subunits (αv, α3 and α4) in enhancing the efficacy of chemotherapy and reducing migration and invasion. Integrin αv knockdown in combination with vincristine (VCR) or paclitaxel (PTX) but not with cisplatin (cDDP), showed beneficial effect in TNBC but not in melanoma cell lines. The integrin αv knockdown also reduced cell migration and invasion. The therapeutic effect of combined action of αvβ3/β5 inhibitor cilengitide and PTX in TNBC and melanoma cell lines did not entirely reflect results of integrin αv knockdown. Since this combination showed either beneficial or detrimental effects in TNBC and melanoma, it cannot be recommended for therapy.



## Materials and Methods

*Cell culture*

The TNBC mesenchymal stem like (MDA-MB-231 and MDA-MB-436) and basal like MDA-MB-468 cell lines as well as human melanoma MDA-MB-435S (a spindle shaped variant of the parental MDA-MB-435), RPMI-7951, MeWo and A375 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), grown in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) at 37°C with 5% $CO_2$ in a humidified atmosphere.

*Drugs and chemicals*

cDDP (Sigma, USA) was dissolved in water and stored at -20°C while VCR (Sigma, USA) and PTX (Sigma, USA) were dissolved in phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO), respectively, and stored at

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-20°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Millipore, USA) was dissolved in PBS and stored at 4°C. Cilengitide (SML1594, Cyclo(L-arginylglycyl-L-a-aspartyl-D-phenylalanyl-N-methyl-L-valyl), 2,2,2-trifluoroacetate, Sigma-Aldrich, Germany), a cyclized Arg-Gly-Glu(RGD)-containing pentapeptide that selectively blocks activation of αvβ3 and αvβ5 integrins, was dissolved in water and stored at -20°C, while PF-228 (PF-573228, 6-[4-(3-Methanesulfonyl-benzylamino)-5-trifluoromethyl-pyrimidin-2-ylamino]-3,4-dihydro-1H-quinolin-2-one, Sigma Aldrich, Germany), a focal adhesion kinase (FAK) inhibitor, was dissolved in DMSO and stored at -20°C.

*Small interfering RNA (siRNA), transfection and determination of cell survival*

For silencing of integrin subunits β3, β5, αv, α3 and α4, the predesigned human integrin-specific siRNA sequences (Silencer Select Predesigned siRNA; Ambion, USA; siRNA ID# s7581 (si(β3)), s7591 (si(β5)), s7568 (si(αv)), s7569 (si(αv)), s7543 (si(α3)), s7544 (si(α4)) and control nonspecific siRNA (si(-)) (Silencer Select Predesigned siRNA Negative Control #1 siRNA; Ambion, USA) were used. The transfections of siRNAs were performed using Lipofectamine RNAiMAX Reagent (Invitrogen, USA) according to the manufacturer's instructions. For all experiments 50 nM of β3-, β5-, αv-, α3- and α4-specific siRNA was used, except for knocking down β3 in MDA-MB-435S and MDA-MB-231 where 120 nM β3-specific siRNA was applied. The success of silencing was assessed using flow cytometry 48 h after siRNA transfection, i.e. in the moment when cells were treated with anticancer drugs for assessment of sensitivity.

The sensitivity of cells to anticancer drugs was determined using MTT assay as described in (Stojanovic et al., 2016). Briefly, 24 h after siRNA transfection, cells were seeded in 96-well tissue culture plate (0.4-2x$10^4$ cells/well depending on the cell line). Twenty-four hours later, i.e. 48 h upon siRNA transfection, cells were treated with different concentrations of anticancer drugs. Seventy-two hours later, the absorbance of MTT-formazan product was measured with a microplate reader (Awareness Technology Inc., USA) at 600 nm. The only exception of this protocol were MDA-MB-231 cells which were seeded into 96-well plate, 48 h later transfected with siRNA and 48 h later exposed to anticancer drugs for assessment of cell survival. Absorbance data used for analysis were obtained by subtracting the signal in a blank well.

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*Determination of integrin αvβ3, αvβ5, αv, α3 and α4 levels by flow cytometry*

Flow cytometry was used to analyze expression of αvβ3, αvβ5, αv, α3β1 and α4 in cells. Briefly, adherent cells were grown in tissue culture dishes, detached by Versene (Invitrogen, USA) and washed twice with PBS. Membrane fluorescence staining for αvβ3, αvβ5, αv, α3β1 and α4 was performed (1 h, 4°C) with monoclonal antibodies directed against αvβ3, αvβ5, α3β1 and α4 (Chemicon, USA) and αv (Calbiochem, Germany). Binding of the unlabeled primary antibodies was revealed by incubation (30 min, 4°C) of PE-conjugated anti-mouse (DAKO, USA) or FITC-conjugated anti-mouse antibody (BD Pharmingen, USA). Isotype control samples were incubated with mouse IgG1 (Sigma, Germany) followed by PE- or FITC-conjugated anti-mouse antibody.

*Determination of cell migration and invasion*

For monitoring cell migration and invasion, cells transfected with control siRNA and integrin-specific siRNA (48 h after transfection) were serum starved (24 h), seeded (number depending on cell type) in migration or Matrigel-coated Invasion Transwell Cell Culture Inserts (pore size, 8 μm) (Corning, USA) and left to migrate/invade for 22 h toward 10% FBS in DMEM as a chemoattractant. The number of cells migrated/invaded to the underside of the filter was determined using ImageJ as previously described (Christmann et al., 2017).

*Confocal microscopy of actin, paxillin and phospho-paxillin*

For confocal microscopy analysis, MDA-MB-435S cells were seeded on cover slips, transfected with control siRNA or integrin αv- or α4-specific siRNA. Forty eight hours after transfection, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% TritonX-100 and incubated with primary rabbit polyclonal anti-Paxillin antibody or rabbit monoclonal anti-phospho Paxillin antibody [Y113] (Abcam, USA) for 1 h, washed and incubated with donkey Alexa-Fluor 555-conjugated-anti-rabbit secondary antibody (Molecular Probes, USA) for 1 h. F-actin fibers were stained with phalloidin-FITC (Sigma-Aldrich, USA), nuclei were counterstained with TO-PRO®-3 iodide (Invitrogen, USA) and slides mounted in Dako Fluorescent Mounting Media (DAKO, USA). Analysis was performed using Leica TSC SP2 (Lasertechnik GmbH, Germany) microscope and obtained images were processed with ImageJ.

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*Western blot analysis*

Cells grown in 6-well plate were lysed with 96°C heated 1× Laemmli buffer, scraped off the plate, boiled for 3 min at 96°C and sonicated. Proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane (Amersham, Germany), blocked in 5% non-fat dry milk, and membranes were incubated with rabbit monoclonal antibody against phosphorylated focal adhesion kinase Y397, pFAK(Y397) or total FAK (FAK) (Abcam, UK), followed by incubation with horseradish peroxidase-coupled secondary antibody (GE Healthcare, USA, Invitrogen, USA). Detection was done using a chemiluminescence reagent (GE Healthcare).

*Statistical analysis*

Each experiment was repeated at least three times and GraphPad Prism software v5.0 was used to analyze data. All data from MTT experiments were analyzed by related measure Two-way ANOVA with Bonferroni posttest. Data obtained from migration, invasion and immunofluorescence were analyzed by related measure One-way ANOVA with Dunnett's Multiple Comparison. * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$.

**Results**

*Integrin expression patterns in TNBC and melanoma cell lines*

To investigate the role of knockdown of αv, α3 and α4 integrins in TNBC and melanoma cells in sensitivity to different anticancer drugs, migration and invasion, the expression of integrins αv, αvβ3, αvβ5, α3β1 and α4 was analyzed in TNBC and melanoma cell lines. Cell lines used were three TNBC cell lines (MDA-MB-231, MDA-MB-468 and MDA-MB-436), three melanoma cell lines (RPMI-7951, MeWo and A375) and cell line MDA-MB-435S which has been used for years as TNBC cell line but recent data clearly showed that these cells originate from melanoma (Korch et al., 2018). The debate regarding the authenticity of this cell line started when this cell line was shown to be identical to M14 melanoma cell line (Rae et al., 2007). It has just been resolved, by authentication testing of M14 from 1975 (prior to the establishment of MDA-MB-435S) with comparison to donor serum and lymphoblastoid cell line ML14, that M14 is the authentic cell line and MDA-MB-435S is a misidentified derivative (Korch et al., 2018).

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Cell-surface expression of integrins in live cells of all seven cell lines was measured by flow cytometry (Fig. 1). Integrin αv was highly expressed in all cells except in MDA-MB-436 cells which express moderate amount of this subunit. The αv subunit forms a complete integrin complex through heterodimerisation with one of five β subunit binding partners: β1, β3, β5, β6 and β8. We were particularly interested in expression of integrins αvβ3 and αvβ5 and less interested in αvβ6 and αvβ8. Namely, integrin αvβ6 is weakly expressed in MDA-MB-435S, MDA-MB-231 and MDA-MB-468 but absent in majority of melanoma cells, similarly to integrin αvβ8 (Goodman et al., 2012). Flow cytometry analysis showed that integrin αvβ3 was highly expressed in MDA-MB-435S and A375 cells, moderately expressed in MDA-MB-231, MDA-MB-468 and RPMI-7951 cells, while the expression was absent in MDA-MB-436 and MeWo cells. On the other hand, integrin αvβ5 was highly expressed in all cell lines except for MDA-MB-468 and MDA-MB-436 which express low amount of this heterodimer. The most evenly expressed integrin in TNBC and melanoma cells was integrin α3β1, while the least represented integrin heterodimer in our cell panel was integrin α4β1 (α4 subunit forms heterodimer with β1 or β7 (Raab-Westphal et al., 2017)) with high expression in melanoma cell lines MDA-MB-435S, RPMI-7951 and A375 and very low expression in TNBC cell line MDA-MB-436. Expression of integrin subunits αv and α4 and integrin heterodimers αvβ3, αvβ5 and α3β1 in all cell lines is summarized in Supplemental Table ST1.

*Integrin subunit β3 or β5 knockdown alters expression of both αv integrin heterodimers αvβ3 and αvβ5: observation of the integrin switching effect in MDA-MB-435S cells*

We started our investigation with the melanoma cell line MDA-MB-435S because it has been previously shown that silencing of integrin αv in this cell line enhanced radiosensitivity (Cao et al., 2006). Another reason for using this cell line is that it expresses high level of integrins αvβ3, αvβ5, α3β1 and α4β1, but does not express integrin αvβ1 (Palmieri et al., 2002; Taherian et al., 2011; Wong et al., 1998). Therefore, by inclusion of this cell line, we aimed to differentiate between the contribution of integrins αvβ3 and αvβ5 in drug sensitivity, migration and invasion and to analyze the function of these integrins independent of integrin αvβ1 interference. Our goal was to determine which of the integrin heterodimers might be used as candidates for increasing sensitivity to anticancer drugs and inhibition of migration and invasion. Therefore, we decided to knockdown integrin subunits β3 or β5 in order to decrease the expression of integrin heterodimers αvβ3 or αvβ5, respectively, and knockdown integrin subunits αv, α3 or α4 with the aim to decrease the expression of all αv integrin heterodimers, integrin α3β1 or α4β1, respectively. The

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cell surface expression of integrin heterodimers αvβ3, αvβ5 and α3β1, the amount of integrin subunit αv and α4 was measured 48 h after siRNA transfection using flow cytometry and compared to the expression of corresponding molecules in cells transfected with control siRNA. The control siRNA had minimal effect on the expression of all integrin subunits and heterodimers as compared to non-transfected MDA-MB-435S cells (data not shown). Upon transfection with integrin subunit β3-specific siRNA (Fig. 2, first row) integrin αvβ3 expression decreased to 71% of the value of control siRNA. Concomitantly, we observed significant up-regulation of integrin αvβ5, i.e. 59% higher level as compared to control siRNA. Silencing of integrin subunit β3 decreased the total amount of integrin subunit αv very slightly. Similar balance effect was also observed when MDA-MB-435S cells were transfected with integrin subunit β5-specific siRNA (Fig. 2, second row), which resulted in significant decrease of integrin αvβ5 expression (44% of the value for control siRNA) and simultaneous increase of integrin αvβ3 expression by 27%. Silencing of integrin subunit β5 slightly decreased the total amount of integrin αv. In order to simultaneously reduce the expression of integrins αvβ3 and αvβ5, cells were transfected with integrin subunit αv-specific siRNA (Fig. 2, third row). We observed decreased expression of αv (26% of the value for control siRNA) and integrins αvβ3 and αvβ5, down to 72% and 35% of the value for control siRNA, respectively. MDA-MB-435S cells were also transfected with integrin subunit α3- (Fig. 2, fourth row) or α4-specific siRNA (Fig. 2A, fifth row) that led to significant down-regulation of integrin α3β1 (22% of the value for control siRNA) or α4β1 (8% of the value for control siRNA). We hypothesize that in MDA-MB-435S cell line, expressing principally two αv integrin heterodimers αvβ3 and αvβ5, knockdown of either integrin subunit β3 or β5 releases integrin subunit αv. This subunit very likely heterodimerises with free β5 or β3 subunits in the cell, increasing αvβ5 or αvβ3, respectively. In order to test this hypothesis, we measured the adenovirus type 5 (Ad5)-mediated transgene expression and the amount of adenoviral DNA upon transduction. Briefly, we used the property of integrins αvβ3 and αvβ5 to have analogous role in Ad5 attachment (binding) to the cell surface and internalization (entry) into the cell. When the ratio of αvβ3/αvβ5 is disturbed, the amount of attached/internalized Ad5 remains the same but integrin αvβ5 has a crucial role in Ad5 release from endosome i.e. the amount of integrin αvβ5 that modulates Ad5 release can be measured as transgene expression (Majhen et al., 2009). Therefore, the αvβ3/β5 balance effect in MDA-MB-435S cells was indeed confirmed in this way since the Ad5 transgene expression was increased upon integrin β3-specific siRNA transfection (2.36-fold due to the integrin αvβ5 up-regulation) or decreased upon integrin β5-specific siRNA transfection (0.42 fold due to the integrin αvβ5 down-

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regulation) (Supplemental Fig. S1A). Concurrently the amount of attached (Supplemental Fig. S1B) and internalized (Supplemental Fig. S1C) Ad5 DNA did not change significantly.

*The beneficial effect of integrin subunit β5, αv, or α4 knockdown in combination with VCR and PTX in melanoma cell line MDA-MB-435S*

To test whether silencing of integrins could be used to increase sensitivity of MDA-MB-435S cells to chemotherapy, the sensitivity was measured by MTT assay (Fig. 3). The control siRNA had minimal effect on the survival of MDA-MB-435S cells upon treatment with all selected anticancer drugs (data not shown). Silencing of integrin subunits decreased the survival of MDA-MB-435S cells and therefore in Fig. 3 we present absorbance data from MTT test for each combination of integrin subunit knockdown and anticancer drug. The various effects of combinations are summarized in Table 1. We determined whether integrin knockdown increases sensitivity (marked as S), decreases sensitivity (marked as R) or showed no interference (marked as NI), which can also be considered as beneficial effect since additive effect results in overall lower cell survival. Our results show that silencing of integrin subunits αv, β3, β5 or α3 decreased sensitivity of MDA-MB-435S cells to cDDP, while silencing of integrin subunit α4 showed no interaction with cDDP sensitivity (Fig. 3, left column; Table 1). On the other hand, silencing of integrin subunits αv, β5 or α4 increased sensitivity of MDA-MB-435S cells to VCR and PTX, while silencing of integrin subunits β3 or α3 decreased sensitivity to VCR and PTX (Fig. 3, middle and right column; Table 1). We conclude that knockdown of integrin subunits αv, β5 or α4 could be used for sensitization of MDA-MB-435S cells to VCR and PTX.

The activation of apoptosis is the main mode of cell death induced by chemotherapeutic agents. By binding to β tubulin paclitaxel and its derivatives stabilize cytoskeletal microtubules, which leads to cell cycle arrest via G2/M phase block. Prolonged arrest of cell division eventually activates a checkpoint and induces programmed cell death (Jordan and Wilson, 2004). In order to test whether knockdown of integrin subunit αv increases the fraction of apoptotic cells in MDA-MB-435S upon PTX treatment, we determined the apoptosis rate by Annexin V/PI staining. Cells transfected with integrin subunit αv siRNA demonstrated a significantly higher amount of cells in apoptosis 48 h upon PTX treatment in comparison to cells transfected with control siRNA (Supplemental Fig. S2A). We conclude that knockdown of integrin subunit αv increases sensitivity of MDA-MB-435S cells to PTX through the mechanism that increases apoptosis. Integrin αv knockdown had no effect on MDA-MB-435S cell cycle progression.

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Nevertheless, the percentage of sub-G1 population after PTX treatment was higher in cells transfected with integrin subunit αv siRNA, as compared to control siRNA (Supplemental Fig. S2B).

*Integrin subunit αv or α4 knockdown in MDA-MB-435S cells differently influence migration, organization of actin network and focal adhesions*

We next compared the ability of MDA-MB-435S cells transfected with control siRNA or integrin subunits αv, β3, β5 or α4 siRNA to migrate using FBS as the chemoattractant (Fig. 4A). Migration of the MDA-MB-435S cells transfected with control siRNA resembled the parental MDA-MB-435S cells (data not shown). Migration of MDA-MB-435S cells transfected with integrin subunit αv-specific siRNA was dramatically reduced. To specifically investigate the role of integrins αvβ3 or αvβ5 in migration of MDA-MB-435S cells, we determined cell migration upon integrin subunit β3 or β5 silencing. Silencing of neither of integrin subunits β3 or β5 significantly influenced MDA-MB-435S cell migration. In the light of previously shown integrin balance (switching) effect (Fig. 2), we conclude that both integrins αvβ3 and αvβ5 are equally important for cell migration and consequently their altered ratio does not significantly change the overall migration. Interestingly, silencing α4β1, an integrin that generally has a pro-migratory role (Wang et al., 2005), significantly increased cell migration (Fig. 4A). We hypothesize that this could be a consequence of released integrin subunit β1 upon integrin subunit α4-specific siRNA transfection, but we can only speculate which of β1 heterodimers could be formed. Our hypothesis is somewhat supported by the fact that transfection of MDA-MB-435S cells with integrin subunit α3-specific siRNA results in significantly increased migration as compared to cells transfected with control siRNA (Supplemental Fig. S3). Since integrin subunit α3 silencing does not have any effect on sensitivity to VCR and PTX, we postulate that increased sensitivity to microtubule poisons in cells transfected with integrin subunit α4-specific siRNA is more likely the consequence of integrin α4β1 downregulation than formation of some integrin β1 containing integrin heterodimer in the cell upon α4-specific siRNA transfection.

In order to evaluate *in vitro* invasiveness through Matrigel, we performed invasion assay upon integrin αv knockdown (Fig. 4B), which mimics the three-step hypothesis of invasion-adhesion, proteolytic dissolution of the extracellular matrix and migration (Albini, 1998), and observed significantly decreased invasion.

Next we monitored whether knockdown of integrin subunits αv and α4 influences actin remodeling and focal adhesions. MDA-MB-435S cells transfected with control siRNA resembled the parental MDA-MB-435S cells (data

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not shown), with cells well-spread, prominent actin stress fibers and cortical actin apparent. We observed punctuate paxillin staining in MDA-MB-435S cells (not shown), cells transfected with control, integrin subunit αv- or α4-specific siRNA (Fig. 4C, left panel). Tyrosine phosphorylation of paxillin is important for focal adhesion formation and for function of paxillin as a docking molecule in focal adhesions (Nakamura et al., 2000). The phospho-paxillin (Y113) staining in MDA-MB-435S cells transfected with control siRNA showed typical discrete short clusters of focal adhesion complex staining enriched at both the central region and the periphery of cells, which was coincident with the F-actin at focal adhesion sites (Fig. 4C, right panel, first row; enlarged focal adhesion sites are presented). Upon silencing integrin subunit αv, MDA-MB-435S cells were less well-spread and appeared smaller, paxillin staining therefore seemed to be more intense (Fig. 4C, left panel, second row). However, we verified that upon integrin αv knockdown the size of the cells was similar to the size of MDA-MB-435S cells transfected with control siRNA because their FSC/SSC ratio in flow cytometry analysis was similar (data not shown). Upon integrin αv knockdown MDA-MB-435S cells were showing disorganization of actin with loss of stress fibers and significant loss of phospho-paxillin (Y113) staining, indicating focal adhesion number reduction (Fig. 4C, right panel, second row; 4D). An increase in phospho-paxillin staining was observed in MDA-MB-435S cells following integrin subunit α4-specific siRNA transfection (Fig. 4C, right panel, third row; 4D), especially at the leading edge of migrating cells, which is in line with increased migration (Fig. 4A).

*Integrin subunit αv knockdown differently affects sensitivity of TNBC and melanoma cell lines to microtubule poisons*

Given results obtained in MDA-MB-435S cell line (Fig. 2; Fig. 3; Fig. 4; Supplemental Fig. S3), we decided not to proceed with integrin α3 or α4 silencing because we observed either decreased sensitivity to anticancer drugs or increased migration, which would not benefit cancer treatment. We decided to analyze the influence of integrin αv knockdown (Fig. 5A) on sensitivity to cytotoxic activity of cDDP, VCR and PTX (Fig. 5B) in three TNBC and three melanoma cell lines. We verified in MDA-MB-435S cell line the absence of integrin αv siRNA off target effects by comparison with another integrin αv-specific siRNA and obtained similar results by flow cytometry as well as MTT using PTX (data not shown). The control siRNA had minimal effect on the survival of cells upon treatment with all selected anticancer drugs (data not shown). The various effects of integrin subunit αv knockdown and anticancer drug combinations are summarized in Table 2, with increased sensitivity marked as S, decreased sensitivity marked as R and NI showing no interference. Our results show that knockdown of integrin αv achieved by αv-specific siRNA

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transfection in all cell lines was successful (Fig. 5A) and resulted in beneficial effect in all three TNBC cell lines in combination with VCR and PTX (Fig. 5B, Table 2). Conversely, in additional three melanoma cell lines we observed beneficial effect (similar as observed in MDA-MB-435S) in one (RPMI-7951) but in the other two (A375 and MeWo) cell lines we observed clear detrimental effect i.e. decreased sensitivity to all three drugs cDDP, VCR and PTX (Fig. 5B, Table 2). As regards cDDP, in two TNBC (MDA-MB-468 and MDA-MB-436) and two melanoma (A375 and MeWo) cell lines we observed decreased sensitivity which, if we take into account that similar result was obtained in MDA-MB-435S melanoma cell line (Fig. 3, Table 1), suggests against combination of integrin subunit αv knockdown and cDDP for improved therapy. In conclusion, knockdown of integrin subunit αv could be used for sensitization to VCR and PTX of TNBC but not melanoma.

In order to analyze the involvement of integrin heterodimers αvβ3 or αvβ5, in sensitization to VCR or PTX observed upon integrin αv knockdown, we performed silencing of integrin subunits β3 or β5 in MDA-MB-231 and RPMI-7951 cell lines and measured cell survival. Data are presented in Supplemental Fig. S4, while the various effects of combinations are summarized in Supplemental Table ST2. Surprisingly, in MDA-MB-231, integrin β5 knockdown did not show interaction with sensitivity to VCR or PTX while integrin β3 knockdown decreased cell sensitivity to both drugs. On the contrary, in melanoma cell line RPMI-7951 knockdown of either integrin β3 or β5 led to increased sensitivity to VCR or PTX (Supplemental Fig. S4A, Supplemental Table ST2). It should be noted that both cell lines express integrin αvβ1 and it is likely that this integrin also plays a role in sensitivity to VCR or PTX upon integrin αv knockdown. Due to the observed decreased sensitivity of MDA-MB-231 cells to VCR or PTX upon integrin β3 knockdown we also checked whether knockdown of integrin subunits β3 or β5 causes the balance effect we observed in MDA-MB-435S cells. We did not observe it (Supplemental Fig. S4B), however, this result does not exclude the possibility of other integrin switching effects.

*The knockdown of integrin αv significantly decreases migration of TNBC and melanoma cell lines; the key role of integrin αvβ5*

Integrins αv have a well-established role in migration and invasion of tumor cells (Desgrosellier and Cheresh, 2010). Thus we examined the *in vitro* migration of TNBC MDA-MB-231 and MDA-MB-468 and melanoma cell line RPMI-7951 upon integrin αv knockdown, achieved by αv-specific siRNA transfection, using Transwell inserts and FBS as a chemoattractant (Fig. 6A). Migration of MDA-MB-231 and MDA-MB-468 cells upon integrin αv

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knockdown was reduced to 40% or 35% of the migration capacity of parental cells transfected with control siRNA, respectively, while in melanoma cell line RPMI-7951, integrin αv knockdown dramatically reduced cell migration to the extent that it was difficult to find cells that migrated to the bottom side of the Transwell insert membrane. To specifically investigate which integrin heterodimer, αvβ3 or αvβ5, is more important for inhibition of migration, we measured cell migration upon integrin β3 or β5 knockdown that led to decreased expression of integrin αvβ3 or αvβ5. Cell migration was not altered upon knockdown of integrin β3 in either MDA-MB-231, MDA-MB-468 or RPMI-7951 cell lines. It was the decreased expression of integrin αvβ5, obtained by integrin β5 knockdown, that significantly inhibited cell migration in all three cell lines; although in MDA-MB-231 and RPMI-7951 it was not as successfully as knockdown of all integrin αv heterodimers as observed in MDA-MB-468 cells.

In order to evaluate *in vitro* invasiveness of representative TNBC and melanoma cell lines through Matrigel, we also performed Transwell insert invasion assays (Fig. 6B). In MDA-MB-231 cells we observed decreased invasion, in values similar to the migration inhibition, down to 40% of the control, while in RPMI-7951 cells the invasion was even more dramatically inhibited, down to 15% of the control. The effects of integrin subunit knockdown on migration and invasion are summarized in Table 3.

*Targeted inactivation of integrin heterodimers αvβ3 and αvβ5 using cilengitide combined with PTX does not mimic the combination of integrin αv knockdown and PTX*

Integrin/anticancer drug combination shown to be the most promising in our experiments was integrin αv knockdown/VCR or PTX in TNBC cell lines because of the beneficial effect of the combination which simultaneously decreases migration and invasion. To further analyze the importance of integrins αvβ3 and αvβ5 in response to PTX we decided to assess the combination therapy of cilengitide (a potent and selective inhibitor of integrins αvβ3 and αvβ5) and PTX. Cilengitide enabled us, as opposed to integrin αv knockdown, to analyze the role of inhibition of integrins αvβ3 and αvβ5 signaling without affecting the expression of other integrins αv.

In order to measure the combined effect of cilengitide and PTX, first we assessed the cytotoxic activity of different cilengitide concentrations in our panel of seven cell lines and found a dose dependent effect of cilengitide on cell survival (Fig. 7). The highest sensitivity was observed in melanoma cell line MeWo while TNBC cell line MDA-MB-468 was the least sensitive cell line. Interestingly, MDA-MB-468 cells were highly resistant to cilengitide treatment, which was not expected. Namely, they express comparable amount of integrin αvβ5 as MDA-MB-436 but

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do express integrin αvβ3, which is not the case in MDA-MB-436 (Fig. 1; Supplemental Table ST1). Nevertheless, MDA-MB-436 showed cytotoxic effect with 20-fold lower concentration of cilengitide. All other cell lines were similarly sensitive to cilengitide. In Fig. 7 we present data for each combination of cilengitide and PTX, while the various effects of combinations are summarized in Table 4. Cilengitide showed beneficial effect in all cell lines except TNBC cell line MDA-MB-468 and melanoma cell line MeWo, in which detrimental effect was observed. More specifically, cilengitide increased sensitivity of TNBC MDA-MB-231 and melanoma RPMI-7951 and A375 cell lines while demonstrating additive effect with PTX in TNBC MDA-MB-436 and melanoma cell line MDA-MB-435S. In conclusion, the comparison of the combination of integrin αv knockdown/PTX (Table 2) *versus* cilengitide/PTX (Table 4) shows discrepancy in TNBC cell line MDA-MB-468 and melanoma cell line A375.

*The inhibition of FAK phosphorylation at tyrosine 397 decreases sensitivity to PTX in six out of seven TNBC and melanoma cell lines*

Focal adhesion kinase is involved in integrin-induced signal transduction pathways. It plays a central role in tumor progression and metastasis and represents the link to growth factor receptors. In FAK, several sites of tyrosine phosphorylation have been identified. Tyrosine 397, the major autophosphorylation site in FAK, is essential for the majority of FAK functions (Zhao and Guan, 2011). In a variety of human cancers, the increased expression and/or activation of FAK has been found. Therefore, small molecular inhibitors of FAK kinase activity might be used in treatment of metastatic cancer (Sulzmaier et al., 2014). We hypothesized that integrin αv knockdown or cilengitide-mediated inhibition of pFAK(Y397) phosphorylation might be a crucial event in increased sensitivity to PTX. Therefore, we analyzed the phosphorylation of FAK(Y397) *versus* total amount of FAK using Western blot in all cell lines upon silencing of integrin αv or exposure to cilengitide. Decreased phosphorylation of FAK(Y397) upon knockdown of integrin αv (Fig. 8A) was found in all three TNBC cell lines and melanoma cell line MDA-MB-435S but not in the other three melanoma cell lines RPMI-7951, A375 and MeWo. There was no correlation to increased sensitivity to PTX because in cell line MDA-MB-436 there was no change in sensitivity after integrin αv knockdown (Fig. 5B, Table 2), even though integrin αv-specific siRNA transfection strongly reduced pFAK(Y397) level down to 20% of the control value. The level of pFAK(Y397) upon cilengitide exposure was also reduced in all three TNBC cell lines and in all melanoma cell lines except MeWo (Fig. 8B). There was no correlation to increased sensitivity to PTX since in cell line MDA-MB-468, demonstrating reduced pFAK(Y397) upon cilengitide exposure, the decreased

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sensitivity to PTX was observed (Fig. 7, Table 4). Interestingly, in melanoma cell lines RPMI-7951 and A375 we didn't observe reduction of pFAK(Y397) levels upon integrin αv knockdown but we observed reduction upon cilengitide exposure. We hypothesize that this discrepancy might be the consequence of integrin switching effects.

Finally, we investigated whether targeted inhibition of pFAK(Y397) could increase sensitivity of TNBC and melanoma cells to PTX. We first analyzed the reduction of pFAK(Y397) upon exposure to inhibitor PF-228 using Western blot (Supplemental Fig. S5). Then, the combined effect of pFAK(Y397) inhibitor PF-228 and PTX was analyzed in all seven cell lines. In Fig. 9 we present data for each combination of PF-228 and PTX while the various effects of combinations are summarized in Table 5. Surprisingly, PF-228 showed detrimental effect in all cell lines except melanoma cell line A375, in which no interaction between pFAK(Y397) inhibition and PTX was observed. We conclude that pFAK(Y397) is downstream from integrins αv or only integrins αvβ3 and αvβ5 in TNBC and melanoma cell lines (Fig. 8), but it is not implicated in integrin-mediated response to PTX. The discrepancy between change of sensitivity to PTX upon integrin αv knockdown or cilengitide exposure and pFAK(Y397) inhibitor might be explained by the fact that FAK is a cytoplasmic tyrosine kinase that plays critical roles in integrin, but also in other cell surface receptor signaling pathways (Kleinschmidt and Schlaepfer, 2017). Therefore, the use of pFAK(Y397) inhibitors in combination with PTX is not recommended for therapy of TNBC and melanoma.

**Discussion**

Integrins may represent candidate targets for combination treatments that can increase sensitivity to anticancer drugs and inhibit metastasis (Dickreuter and Cordes, 2017). However, change of expression of one integrin can lead to change in expression of other integrins in an event called integrin switching, which can have both mechanistic and therapeutic implications. Data showing that the specific inactivation of β1 integrin elicits metastatic progression due to compensatory upregulation of β3 integrin (Parvani et al., 2013; Truong et al., 2014) raised concerns with respect to the use of β1 integrins as drug targets to sensitize tumors to radio- or chemotherapy. Therefore, we decided to analyze the potential of different integrin α subunit knockdowns (αv, α3 or α4) to increase sensitivity of TNBC and melanoma cell lines to different anticancer drugs cDDP, VCR and PTX as well as its potential to inhibit migration and invasion.

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We started our investigation in melanoma cell line MDA-MB-435S (Korch et al., 2017) in which we observed integrin switching between integrin heterodimers αvβ3 and αvβ5. It has been shown in WM-266-4 melanoma cells that the number of αvβ3 and αvβ5 heterodimers is regulated at the level of the β3 and β5 genes, respectively, but that the activity of the αv gene dictates the number of αvβ1 heterodimers (Koistinen and Heino, 2002). Since MDA-MB-435S cells lack integrin αvβ1 (Wong et al., 1998), the observed balance is in line with proposed regulation model.

Knockdown of integrin subunits αv, β5 or α4 increased sensitivity of MDA-MB-435S cells to VCR and PTX, while knockdown of integrin subunit α3 or β3 produced the opposite effect. The knockdown of αvβ3 using β3-specific siRNA, which simultaneously increases the expression of integrin αvβ5, resulted in decreased sensitivity to VCR or PTX. This indicates that the signaling pathway triggered by integrin αvβ5 protects MDA-MB-435S cells, and can be augmented by increased expression of integrin αvβ5, which is somewhat different from our previously published results on the absence of dose response effect in conferring resistance to cDDP upon *de novo* expression of integrin αvβ3 in head and neck cell lines (Brozovic et al., 2008; Stojanović et al., 2016). Silencing of integrin subunits αv, β3, β5 or α3 decreased sensitivity of MDA-MB-435S cells to cDDP, while silencing of integrin subunit α4 did not have a significant effect. We do not know whether there is a common mechanism between increased sensitivity to VCR and PTX and decreased sensitivity to cDDP. However, according to our results in melanoma cell line MDA-MB-435S, it seems that these two events have separate signaling pathways. The concomitant sensitivity to cDDP in cells that develop resistance to PTX has been frequently described in literature (Stordal and Davey, 2009).

Integrins have an important role as migration/invasion-promoting receptors (Ganguly et al., 2013). The knockdown of integrin αv in MDA-MB-435S cell line resulted in dramatically decreased cell migration and invasion. Interestingly, knockdown of integrin subunits β3 or β5 did not influence migration at all, very likely because of the balance effect between integrin heterodimers αvβ3 and αvβ5. Our results are in line with animal studies showing that integrin αvβ3 is associated directly with the metastatic potential of MDA-MB-435S cells (Felding-Habermann et al., 2001; Harms et al., 2004). Integrin α4β1 generally has a pro-migratory role (Jordan and Wilson, 2004). However, integrin subunit α4 siRNA transfection in MDA-MB-435S cells unexpectedly increased cell migration. We hypothesize that this could be due to integrin switching effect i.e. release of integrin subunit β1 upon integrin subunit α4-specific siRNA transfection, but we can only speculate which of the β1 heterodimers could have formed.

A key antitumor action of the microtubule poisons is kinetic stabilization of spindle microtubule dynamics, leading to mitotic arrest and subsequent cell death, however, they may induce apoptotic cell death independent of cell

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cycle arrest (Jordan and Kamath, 2007). The first potential mechanism proposed to account for the increased sensitivity to VCR and PTX is decreased expression of drug transporters that efflux (pump out) drugs from cells (Jang et al., 2001). However, we found no difference in the ability of MDA-MB-435S cells to efflux Calcein AM, a process dependent on the activity of both multidrug resistance protein (MRP) and P-glycoprotein, upon integrin αv knockdown (data not shown). Since tubulin mutations, the second potential cause of modulation of sensitivity to microtubule drugs, are not expected upon integrin αv knockdown, we presume that a third potential mechanism i.e. modulation of apoptosis is very likely involved. Indeed, we showed in melanoma cell line MDA-MB-435S increased amount of apoptotic cells upon combination of integrin αv knockdown and PTX, which might be a consequence of modulation of apoptotic proteins or changes in expression of drug target i.e. microtubules. Deschesnes and colleagues (2007) showed that, regardless of the drug that depolymerises microtubules, a similar sequence of molecular and cellular alterations occur that culminate by cell death program reminiscent to anoikis. In addition, their study supports that remodeling of focal adhesion structures is closely linked to the changes in response to microtubule-depolymerizing agents. This is in line with our results showing that integrin αv knockdown significantly reduces the number of focal adhesions in MDA-MB-435S cells.

We show here that in TNBC cell lines integrin αv knockdown in combination with VCR or PTX resulted in beneficial effect, while in melanoma cell lines either beneficial (MDA-MB-435S and RPMI-7951) or detrimental (A375 and MeWo) effect was observed. In two TNBC and two melanoma cell lines, in which beneficial effect of integrin αv knockdown in combination with microtubule poisons was observed, we detected decreased migration and invasion. We have also shown the key role of integrin αvβ5 in migration, except in melanoma cell line MDA-MB-435S in which both integrins, αvβ3 and αvβ5, are implicated. Our results are in line with data obtained in cell line MDA-MB-231, in which integrin αv knockdown inhibited migration and invasion *in vitro* and metastasis *in vivo* (Li et al., 2015) and with the report showing that depletion of integrin β5 in MDA-MB-231 cells markedly reduced tumor growth and angiogenesis, whereas re-expression of integrin β5 rescued this phenotype (Bianchi-Smiraglia et al., 2013). This is also in accordance with published results showing that MDA-MB-231-derived β3 knockdown cells exhibited no changes in migration speed (Costa et al., 2013). However, it has been shown that integrin β3 knockdown in MDA-MB-231-derived tumor-bearing mice inhibits metastasis (Parvani et al., 2015). Integrin αvβ3 in TNBC contributes to the spontaneous metastasis of breast tumors to bone (Sloan et al., 2006) and is a pivotal integrin for the growth of

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human melanomas (Mitjans et al., 2000). In conclusion, αv siRNA-mediated knockdown of both integrins, αvβ3 and αvβ5, might be beneficial for therapy.

Cilengitide-driven inactivation of integrin heterodimers αvβ3 and αvβ5 showed the beneficial effect in combination with PTX in five out of seven TNBC and melanoma cell lines, although in a different set of cells as compared to integrin αv knockdown. The extreme example is melanoma cell line A375, in which decreased sensitivity to PTX was observed upon integrin αv knockdown, while cilengitide exposure led to increased sensitivity to PTX. These discrepancies might be explained by the fact that integrin αv knockdown affects all integrin αv heterodimers, while cilengitide inhibits signaling from integrins αvβ3 and αvβ5 only. The additive effect of cilengitide in integrin αvβ1 negative MDA-MB-435S cells *versus* the sensitizing effect observed upon integrin αv knockdown might be explained by 16-fold higher inhibitor concentration 50% (IC50) for αvβ5 (79 nM) (Nisato et al., 2003), shown to be responsible for sensitivity to PTX, than for αvβ3 (4.1 nM) (Reardon and Cheresh, 2011). Further, integrin switching might occur and change cell response to anticancer drugs. Although it seems less likely that integrin inhibition induces integrin switching, this possibility has been confirmed in TNBC cell line MDA-MB-231 in which two different monoclonal antibodies against integrin α3β1 increased attachment of MDA-MB-231 cells to collagen (Lichtner et al., 1998). Interestingly, a phase I study of cilengitide and PTX in patients with advanced solid tumors (one third of the patients had breast cancer) has been recently published showing antitumor activity and clinical benefit in 6 of the 12 evaluable patients (Haddad et al., 2017).

While decreased levels of pFAK(Y397) upon integrin αv knockdown or cilengitide were found in the majority of used cell lines, we didn't find correlation with modulation of sensitivity to PTX. Moreover, direct inhibition of pFAK(Y397) by PF-228 consistently decreased sensitivity to PTX in all cell lines except A375, where combination of PF-228 and PTX showed additive effect on cell viability, indicating that pFAK(Y397) is not a suitable target for therapy. Opposite results were obtained in ovarian cancer cells by Kang and colleagues (2013) who showed that pFAK(Y397) inhibition by VS-6063 sensitizes taxane-resistance ovarian cancer cells to PTX, and by Halder and colleagues (2005) who showed that FAK knockdown promotes *in vitro* efficacy of docetaxel in both taxane-sensitive and taxane-resistant cell lines.

In conclusion, our results suggest that for TNBC the knockdown of integrin αv in combination with PTX presents a better therapeutic option than cilengitide, and decreases cell migration and invasion at the same time.

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Contrarily, in melanoma neither of these combinations is advisable because decreased sensitivity to PTX was observed. Further study will be needed to reveal the mechanism behind this phenomenon.

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**Authorship contributions**

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Wrote or contributed to the writing of the manuscript: Stojanović, Dekanić, Paradžik, Majhen, Tomicic, Ambriović-Ristov

**References**

Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M and Buck CA (1990) Integrin distribution in malignant melanoma: association of the β3 subunit with tumor progression. *Cancer Res* **50**(20): 6757-6764.

Albini A (1998) Tumor and endothelial cell invasion of basement membranes. The matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. *Pathol Oncol Res* **4**(3): 230-241.

Aoudjit F and Vuori K (2001) Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene* **20**(36): 4995-5004.

Aoudjit F and Vuori K (2012) Integrin signaling in cancer cell survival and chemoresistance. *Chemother Res Pract* **2012**: 283181.

Bianchi-Smiraglia A, Paesante S and Bakin AV (2013) Integrin β5 contributes to the tumorigenic potential of breast cancer cells through the Src-FAK and MEK-ERK signaling pathways. *Oncogene* **32**(25): 3049-3058.

Brozovic A, Majhen D, Roje V, Mikac N, Jakopec S, Fritz G, Osmak M and Ambriovic-Ristov A (2008) αvβ3 integrin-mediated drug resistance in human laryngeal carcinoma cells is caused by glutathione-dependent elimination of drug-induced reactive oxidative species. *Mol Pharmacol* **74**(1): 298-306.

Cao Q, Cai W, Li T, Yang Y, Chen K, Xing L and Chen X (2006) Combination of integrin siRNA and irradiation for breast cancer therapy. *Biochem Biophys Res Commun* **351**(3): 726-732.

Christmann M, Diesler K, Majhen D, Steigerwald C, Berte N, Freund H, Stojanovic N, Kaina B, Osmak M, Ambriovic-Ristov A and Tomicic MT (2017) Integrin αVβ3 silencing sensitizes malignant glioma cells to temozolomide by suppression of homologous recombination repair. *Oncotarget* **8**(17): 27754-27771.

Costa P, Scales TM, Ivaska J and Parsons M (2013) Integrin-specific control of focal adhesion kinase and RhoA regulates membrane protrusion and invasion. *PloS one* **8**(9): e74659.

Danen EH, Ten Berge PJ, Van Muijen GN, Van 't Hof-Grootenboer AE, Brocker EB and Ruiter DJ (1994) Emergence of α5β1 fibronectin- and αvβ3 vitronectin-receptor expression in melanocytic tumour progression. *Histopathology* **24**(3): 249-256.

Deschesnes RG, Patenaude A, Rousseau JL, Fortin JS, Ricard C, Cote MF, Huot J, R CG and Petitclerc E (2007) Microtubule-destabilizing agents induce focal adhesion structure disorganization and anoikis in cancer cells. *J Pharmacol Exp Ther* **320**(2): 853-864.

Desgrosellier JS and Cheresh DA (2010) Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* **10**(1): 9-22.

Dickreuter E and Cordes N (2017) The cancer cell adhesion resistome: mechanisms, targeting and translational approaches. *Biol Chem* **398**(7): 721-735.

Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A and Mueller BM (2001) Integrin activation controls metastasis in human breast cancer. *Proc Natl Acad Sci U S A* **98**(4): 1853-1858.

Ganguly KK, Pal S, Moulik S and Chatterjee A (2013) Integrins and metastasis. *Cell Adh Migr* **7**(3): 251-261.

Goodman SL, Grote HJ and Wilm C (2012) Matched rabbit monoclonal antibodies against αv-series integrins reveal a novel alphavbeta3-LIBS epitope, and permit routine staining of archival paraffin samples of human tumors. *Biol Open* **1**(4): 329-340.

Guo W and Giancotti FG (2004) Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* **5**(10): 816-826.

Haddad T, Qin R, Lupu R, Satele D, Eadens M, Goetz MP, Erlichman C, Molina J (2017) A phase I study of cilengitide and paclitaxel in patients with advanced solid tumors. *Cancer Chemother Pharmacol*. **79**(6):1221-1227.

Halder J, Landen CN Jr, Lutgendorf SK, Li Y, Jennings NB, Fan D, Nelkin GM, Schmandt R, Schaller MD, Sood AK. (2005) Focal adhesion kinase silencing augments docetaxel-mediated apoptosis in ovarian cancer cells. *Clin Cancer Res.* **11**(24 Pt 1):8829-36.

Harms JF, Welch DR, Samant RS, Shevde LA, Miele ME, Babu GR, Goldberg SF, Gilman VR, Sosnowski DM, Campo DA, Gay CV, Budgeon LR, Mercer R, Jewell J, Mastro AM, Donahue HJ, Erin N, Debies MT, Meehan WJ, Jones AL, Mbalaviele G, Nickols A, Christensen ND, Melly R, Beck LN, Kent J, Rader RK, Kotyk JJ, Pagel MD, Westlin WF and Griggs DW (2004) A small molecule antagonist of the αvβ3 integrin suppresses MDA-MB-435 skeletal metastasis. *Clin Exp Metastasis* **21**(2): 119-128.

Ignatoski KM, Maehama T, Markwart SM, Dixon JE, Livant DL and Ethier SP (2000) ERBB-2 overexpression confers PI 3' kinase-dependent invasion capacity on human mammary epithelial cells. *Br J Cancer* **82**(3): 666-674.

Jang SH, Wientjes MG and Au JL (2001) Kinetics of P-glycoprotein-mediated efflux of paclitaxel. *J Pharmacol Exp Ther* **298**(3): 1236-1242.

Jordan MA and Kamath K (2007) How do microtubule-targeted drugs work? An overview. *Curr Cancer Drug Targets* **7**(8): 730-742.

Jordan MA and Wilson L (2004) Microtubules as a target for anticancer drugs. *Nat Rev Cancer* **4**(4): 253-265.

Kang Y, Hu W, Ivan C, Dalton HJ, Miyake T, Pecot CV, Zand B, Liu T, Huang J, Jennings NB, Rupaimoole R, Taylor M, Pradeep S, Wu SY, Lu C, Wen Y, Liu J and Sood AK (2013) Role of focal adhesion kinase in regulating YB-1-mediated paclitaxel resistance in ovarian cancer. *J Natl Cancer Inst* **105**(19): 1485-1495.

Kleinschmidt EG and Schlaepfer DD (2017) Focal adhesion kinase signaling in unexpected places. *Curr Opin Cell Biol* **45**: 24-30.

Koistinen P and Heino J (2002) The selective regulation of αVβ1 integrin expression is based on the hierarchical formation of αV-containing heterodimers. *J Biol Chem* **277**(27): 24835-24841.

Korch C, Hall EM, Dirks WG, Ewing M, Faries M, Varella-Garcia M, Robinson S, Storts D, Turner JA, Wang Y, Burnett EC, Healy L, Kniss D, Neve RM, Nims RW, Reid YA, Robinson WA and Capes-Davis A (2018) Authentication of M14 melanoma cell line proves misidentification of MDA-MB-435 breast cancer cell line. *Int J Cancer* **142**(3): 561-572.

Kuphal S, Bauer R and Bosserhoff AK (2005) Integrin signaling in malignant melanoma. *Cancer Metastasis Rev* **24**(2): 195-222.

Lahlou H and Muller WJ (2011) β1-integrins signaling and mammary tumor progression in transgenic mouse models: implications for human breast cancer. *Breast Cancer Res* **13**(6): 229.

Li Y, Drabsch Y, Pujuguet P, Ren J, van Laar T, Zhang L, van Dam H, Clement-Lacroix P and Ten Dijke P (2015) Genetic depletion and pharmacological targeting of αv integrin in breast cancer cells impairs metastasis in zebrafish and mouse xenograft models. *Breast Cancer Res* **17**: 28.

Lichtner RB, Howlett AR, Lerch M, Xuan JA, Brink J, Langton-Webster B and Schneider MR (1998) Negative cooperativity between α3β1 and α2β1 integrins in human mammary carcinoma MDA MB 231 cells. *Exp Cell Res* **240**(2): 368-376.

Majhen D, Nemet J, Richardson J, Gabrilovac J, Hajsig M, Osmak M, Eloit M and Ambriovic-Ristov A (2009) Differential role of αvβ3 and αvβ5 integrins in internalization and transduction efficacies of wild type and RGD4C fiber-modified adenoviruses. *Virus Res* **139**(1): 64-73.

Melchiori A, Mortarini R, Carlone S, Marchisio PC, Anichini A, Noonan DM and Albini A (1995) The α3β1 integrin is involved in melanoma cell migration and invasion. *Exp Cell Res* **219**(1): 233-242.

Mitjans F, Meyer T, Fittschen C, Goodman S, Jonczyk A, Marshall JF, Reyes G and Piulats J (2000) In vivo therapy of malignant melanoma by means of antagonists of αv integrins. *Int J Cancer* **87**(5): 716-723.

Nakamura K, Yano H, Uchida H, Hashimoto S, Schaefer E, Sabe H (2000) Tyrosine phosphorylation of paxillin alpha is involved in temporospatial regulation of paxillin-containing focal adhesion formation and F-actin organization in motile cells. *J Biol Chem.* **275**(35):27155-64.

Nip J, Shibata H, Loskutoff DJ, Cheresh DA and Brodt P (1992) Human melanoma cells derived from lymphatic metastases use integrin αvβ3 to adhere to lymph node vitronectin. *J Clin Invest* **90**(4): 1406-1413.

Nisato RE, Tille JC, Jonczyk A, Goodman SL, Pepper MS (2003) αvβ3 and αvβ5 integrin antagonists inhibit angiogenesis in vitro. *Angiogenesis* **6**(2):105-19.

Palmieri D, Lee JW, Juliano RL and Church FC (2002) Plasminogen activator inhibitor-1 and -3 increase cell adhesion and motility of MDA-MB-435 breast cancer cells. *J Biol Chem* **277**(43): 40950-40957.

Parvani JG, Galliher-Beckley AJ, Schiemann BJ and Schiemann WP (2013) Targeted inactivation of β1 integrin induces β3 integrin switching, which drives breast cancer metastasis by TGF-β. *Mol Biol Cell* **24**(21): 3449-3459.

Parvani JG, Gujrati MD, Mack MA, Schiemann WP and Lu ZR (2015) Silencing β3 Integrin by Targeted ECO/siRNA Nanoparticles Inhibits EMT and Metastasis of Triple-Negative Breast Cancer. *Cancer Res* **75**(11): 2316-2325.

Raab-Westphal S, Marshall JF and Goodman SL (2017) Integrins as Therapeutic Targets: Successes and Cancers. *Cancers (Basel)* **9**(9).

Rae JM, Creighton CJ, Meck JM, Haddad BR and Johnson MD (2007) MDA-MB-435 cells are derived from M14 melanoma cells--a loss for breast cancer, but a boon for melanoma research. *Breast Cancer Res Treatment* **104**(1): 13-19.

Reardon DA, Cheresh D (2011) Cilengitide: a prototypic integrin inhibitor for the treatment of glioblastoma and other malignancies. *Genes Cancer*. **2**(12):1159-65.

Ruffini F, Graziani G, Levati L, Tentori L, D'Atri S and Lacal PM (2015) Cilengitide downmodulates invasiveness and vasculogenic mimicry of neuropilin 1 expressing melanoma cells through the inhibition of αvβ5 integrin. *Int J Cancer* **136**(6): E545-558.

Sloan EK, Pouliot N, Stanley KL, Chia J, Moseley JM, Hards DK and Anderson RL (2006) Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Cancer Res* **8**(2): R20.

Stojanovic N, Brozovic A, Majhen D, Bosnar MH, Fritz G, Osmak M and Ambriovic-Ristov A (2016) Integrin αvβ3 expression in tongue squamous carcinoma cells Cal27 confers anticancer drug resistance through loss of pSrc(Y418). *Biochim Biophys Acta* **1863**(8): 1969-1978.

Stordal B and Davey R (2009) A systematic review of genes involved in the inverse resistance relationship between cisplatin and paclitaxel chemotherapy: role of BRCA1. *Curr Cancer Drug Targets* **9**(3): 354-365.

Subbaram S and Dipersio CM (2011) Integrin α3β1 as a breast cancer target. *Expert Opin Ther Targets* **15**(10): 1197-1210.

Sulzmaier FJ, Jean C and Schlaepfer DD (2014) FAK in cancer: mechanistic findings and clinical applications. *Nat Rev Cancer* **14**(9): 598-610.

Taherian A, Li X, Liu Y and Haas TA (2011) Differences in integrin expression and signaling within human breast cancer cells. *BMC Cancer* **11**: 293.

Truong HH, Xiong J, Ghotra VP, Nirmala E, Haazen L, Le Devedec SE, Balcioglu HE, He S, Snaar-Jagalska BE, Vreugdenhil E, Meerman JH, van de Water B and Danen EH (2014) β1 integrin inhibition elicits a prometastatic switch through the TGFβ-miR-200-ZEB network in E-cadherin-positive triple-negative breast cancer. *Science Signaling* **7**(312): ra15.

Vogetseder A, Thies S, Ingold B, Roth P, Weller M, Schraml P, Goodman SL and Moch H (2013) αv-integrin isoform expression in primary human tumors and brain metastases. *Int J Cancer* **133**(10): 2362-2371.

Wang HS, Hung Y, Su CH, Peng ST, Guo YJ, Lai MC, Liu CY and Hsu JW (2005) CD44 cross-linking induces integrin-mediated adhesion and transendothelial migration in breast cancer cell line by up-regulation of LFA-1 (αLβ2) and VLA-4 (α4β1). *Exp Cell Res* **304**(1): 116-126.

Wong NC, Mueller BM, Barbas CF, Ruminski P, Quaranta V, Lin EC and Smith JW (1998) αv integrins mediate adhesion and migration of breast carcinoma cell lines. *Clin Exp Metastasis* **16**(1): 50-61.

Zhao X and Guan JL (2011) Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev* **63**(8): 610-615.

**Footnotes**

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**Figure legends**

**Fig. 1 Surface expression of integrin subunit αv, integrins αvβ3, αvβ5, α3β1 and integrin subunit α4 in melanoma cell lines MDA-MB-435S, RPMI-7951, A375, MeWo and TNBC cell lines MDA-MB-231, MDA-MB-468 and MDA-MB-436.** Cells were detached by Versene and analyzed by flow cytometry using antibodies against integrin subunit αv, α4 or integrins αvβ3, αvβ5 (black histogram) and isotype-matched antibody as a negative control (grey histogram), followed by rabbit FITC-conjugated-antimouse antibody. The representative data of three independent experiments yielding similar results are shown.

**Fig. 2 Integrin subunit β3, β5, αv, α3 or α4 knockdown in MDA-MB-435S cells reduces expression of integrins αvβ3, αvβ5, integrin subunit αv, integrin α3β1 or integrin subunit α4, respectively.** Surface expression of integrins αvβ3 and αvβ5, integrin subunit αv, integrin α3β1 or integrin subunit α4 in MDA-MB-435S cells was analyzed by indirect flow cytometry 48 h following transfection with integrin subunit β3-, β5-, αv-, α3- or α4-specific siRNA (black histogram) and compared to cells transfected with control siRNA (grey histogram). Representative data of three independent experiments yielding similar results are shown. Mean fluorescence intensities (MFI) relative to cells transfected with control siRNA for corresponding histograms are shown in upper right corners.

**Fig. 3 Modulation of MDA-MB-435S cell sensitivity to cDDP, VCR and PTX upon integrin subunit β3, β5, αv, α3 or α4 knockdown.** MDA-MB-435S cells were transfected with control or integrin subunit β3-, β5-, αv-, α3- or α4-specific siRNA. Twenty-four hours upon transfection cells were seeded in 96-well plates, treated next day with cDDP, VCR or PTX, and cytotoxicity was measured by MTT 72 h later. Average absorbance data ± s.d. shown are representative of at least three independent experiments yielding similar results. Data were analyzed by Two-way ANOVA with Bonferroni posttest. Asterisks denote: * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

**Fig. 4 Alteration of migration, invasion, actin network organization and focal adhesions upon integrin subunit αv or α4 knockdown in MDA-MB-435S cells. (A) In MDA-MB-435S cells integrin αv knockdown decreases while integrin α4 knockdown increases migration as compared to cells transfected with control siRNA, while no change was observed upon integrin subunit β3 or β5 knockdown.** Forty-eight hours after transfection with control, integrin subunit αv-, β3-, β5- or α4-specific siRNA, cells were serum starved for 24 h and then seeded in

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Transwell Cell Culture Inserts and left to migrate for 22 h toward serum. Cells on the underside of the inserts were stained with crystal violet, photographed and counted. Averages of five microscope fields of three independently performed experiments ± s.d. are shown as compared to control cells transfected with control siRNA set as 1. Data were analyzed by One-way ANOVA with Dunnett's Multiple Comparison. Asterisks denote: * $p<0.05$, ** $p<0.01$, *** $p< 0.001$. **(B) Decreased invasion of integrin subunit αv-specific siRNA transfected MDA-MB-435S cells as compared to cells transfected with control siRNA.** Forty-eight hours after transfection with control or integrin subunit αv-specific siRNA, cells were serum starved for 24 h and then seeded in Matrigel-coated Invasion Transwell Cell Culture Inserts and left to invade for 22 h toward serum. Cells on the underside of the inserts were fixed, stained with crystal violet, photographed and counted. Averages of five microscope fields of three independently performed experiments ± s.d. are shown as compared to control cells transfected with control siRNA set as 1. Data were analyzed by One-way ANOVA with Dunnett's Multiple Comparison. Asterisks denote: ** $p<0.01$. **(C) Integrin subunit αv or α4 knockdown influences actin network organization and focal adhesions in MDA-MB-435S cells.** Cells were seeded onto cover-slips one day before transfection with control (first row), integrin subunit αv- (second row) or α4-specific (third row) siRNA. Forty-eight hours after transfection cells were fixed, permeabilized and stained for anti-paxillin or anti-phospho-paxillin [Y117] antibody, followed by Alexa-Fluor 555-conjugated antibody (red color). F-actin staining (green color) was performed in all samples and the nuclei were stained with TO-PRO®-3 iodide (blue color). Analysis was performed using TCS SP Leica. **(D) MDA-MB-435S cells transfected with integrin subunit αv-specific siRNA are smaller, have less focal adhesions per cell and have lower amount of stress fibers as compared to cells transfected with control siRNA.** Data represent measurements of >20 cells and are plotted as mean ± s.d. (n = 3). Data were analyzed by One-way ANOVA with Dunnett's Multiple Comparison. Asterisks denote: * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

**Fig. 5 Integrin subunit αv knockdown in TNBC (MDA-MB-231, MDA-MB-468 and MDA-MB-436) and melanoma (RPMI-7951, A375 and MeWo) cells reduces expression of αv integrins and modulates sensitivity to cDDP, VCR and PTX. (A) Surface expression of integrin subunit αv in TNBC and melanoma cells following transfection with integrin subunit αv-specific siRNA compared to cells transfected with control siRNA.** Forty-eight hours after transfection cells were analyzed by flow cytometry using integrin subunit αv-specific antibody followed by FITC-conjugated antibody. Grey histogram represents control siRNA and black histogram represent

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integrin subunit αv-specific siRNA. Mean fluorescence intensities (MFI) relative to cells transfected with control siRNA for each histogram are shown in upper right corners. Representative data of three independent experiments yielding similar results are shown. **(B) Integrin subunit αv knockdown modulates sensitivity of TNBC and melanoma cells to cDDP, VCR and PTX.** Cells were transfected with control or integrin αv-specific siRNA. Twenty-four hours after transfection cells were seeded in 96-well plates, treated the next day with cDDP, VCR or PTX, and cytotoxicity was measured by MTT 72 h later. Average absorbance data ± s.d. shown are representative of at least three independent experiments yielding similar results. Data were analyzed by Two-way ANOVA with Bonferroni posttest. Asterisks denote: * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

**Fig. 6 Decreased migration and invasion of TNBC and melanoma cells upon integrin subunit αv knockdown. (A) Migration of MDA-MB-231, MDA-MB-468 and RPMI-7951 cells transfected with integrin αv, β3 or β5-specific siRNA compared to cells transfected with control siRNA.** Cell migration was determined in cells upon transfection with control, integrin subunit αv-, β3- or β5-specific siRNA. Forty-eight hours after transfection, cells were serum starved for 24 h, then seeded on Transwell Cell Culture Inserts and left to migrate toward serum. After 22 h, cells on the underside of the inserts were fixed, stained with crystal violet, photographed and counted. Representative photographs are shown. Averages of five microscope fields from at least two independent experiments ± s.d. is shown as relative to migration of cells transfected with control siRNA set as 1. Data were analyzed by One-way ANOVA with Dunnett's Multiple Comparison. Asterisks denote: * $p<0.05$, *** $p< 0.001$. **(B) Invasion of TNBC cells MDA-MB-231 and melanoma cells RPMI-7951 upon integrin subunit αv knockdown.** Cell invasion was determined in cells upon transfection with control or integrin subunit αv-specific siRNA. Forty-eight hours after transfection, cells were serum starved for 24 hours and cell invasion was measured in Transwell Cell Culture Inserts coated with Matrigel. After 22 h, cells on the underside of the filters were fixed, stained with crystal violet, photographed and counted. Representative photographs are shown and average number of five microscopic fields of invaded cells ± s.d. from at least two independent experiments is shown as relative to invasion of cells transfected with control siRNA set as 1. Data were analyzed by One-way ANOVA with Dunnett's Multiple Comparison. Asterisks denote: *** $p<0.001$.

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**Fig. 7 Sensitivity of TNBC (MDA-MB-231, MDA-MB-468 and MDA-MB-436) and melanoma (MDA-MB-435S, RPMI-7951, A375 and MeWo) cells to PTX upon cilengitide exposure.** Cells were seeded in 96-well plate and 24 h later treated with cilengitide and PTX. Survival was measured by MTT assay 72 h later. Absorbance data presented are representative of at least three independent experiments with similar results ± s.d. Data were analyzed by Two-way ANOVA with Bonferroni posttest. Asterisks denote: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

**Fig. 8 Levels of pFAK(Y397) in TNBC (MDA-MB-231, MDA-MB-468 and MDA-MB-436) and melanoma (MDA-MB-435S, RPMI-7951, A375 and MeWo) cells upon integrin subunit αv knockdown (A) or cilengitide exposure (B).** Cells were seeded in 6-well plates, transfected with αv-specific siRNA (A) or exposed to cilengitide (IC70) (B). Forty-eight hours upon transfection (A) or 1 h upon cilengitide exposure (B), whole cell extracts were prepared and analyzed by Western blot. The level of pFAK(Y397) was normalized against total FAK and presented as relative to the expression in cells transfected with control siRNA or untreated cells. The results presented are average of at least three independent experiments ± s.d.

**Fig. 9 Sensitivity of TNBC (MDA-MB-231, MDA-MB-468 and MDA-MB-436) and melanoma (MDA-MB-435S, RPMI-7951, A375 and MeWo) cells to PTX upon inhibition of pFAK(Y397) using PF-228.** Cells were seeded in 96-well plate and 24 h later treated with PF-228 and PTX. The survival was measured by MTT assay 72 h later. Absorbance data presented are representative of three independent experiments with similar results ± s.d. Data were analyzed by Two-way ANOVA with Bonferroni posttest. Asterisks denote: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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**Table 1: The effect of different integrin subunits knockdown on sensitivity of MDA-MB-435S cells to cDDP, VCR and PTX.**

| | Antitumour drug | | |
|---|---|---|---|
| Integrin subunit | cDDP | VCR | PTX |
| **β3** | R | R | R |
| **β5** | R | **S** | **NI** |
| **αv** | R | **S** | **S** |
| **α3** | R | R | R |
| **α4** | **NI** | **S** | **S** |

*S = sensitization (increased sensitivity to combination)*

*NI = no interaction between treatments in combination*

*R = resistance (decreased sensitivity to combination)*

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**Table 2: The effect of integrin subunit αv knockdown on sensitivity of TNBC and melanoma cell lines to cDDP, VCR and PTX.**

| | Anticancer drug | | |
|---|---|---|---|
| Cell line | cDDP | VCR | PTX |
| MDA-MB-231 | **S** | **S** | **S** |
| MDA-MB-468 | R | **NI** | **NI** |
| MDA-MB-436 | R | **NI** | **NI** |
| RPMI-7951 | **NI** | **NI** | **S** |
| A375 | R | R | R |
| MeWo | R | R | R |

*S = sensitization (increased sensitivity to combination)*

*NI = no interaction between treatments in combination*

*R = resistance (decreased sensitivity to combination)*

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**Table 3: The effect of integrin subunit αv, β3 or β5 knockdown on migration and invasion of TNBC and melanoma cell lines.**

| | Cell line | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Integrin subunit specific siRNA | MDA-MB-435S | | MDA-MB-231 | | MDA-MB-468 | | RPMI-7951 | |
| | migration | invasion | migration | invasion | migration | invasion | migration | invasion |
| **si(αv)** | ↓ | ↓ | ↓ | ↓ | ↓ | na | ↓ | ↓ |
| **si(β3)** | = | na | = | na | = | na | = | na |
| **si(β5)** | = | na | ↓ | na | ↓ | na | ↓ | na |

*↓ = decreased migration/invasion; =no change in migration/invasion; na = not analysed*

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**Table 4: The effect of αvβ3/β5 inhibitor cilengitide on sensitivity of TNBC and melanoma cell lines to PTX.**

| | Inhibitor |
|---|---|
| Cell line | cilengitide |
| MDA-MB-435S | **NI** |
| MDA-MB-231 | **S** |
| MDA-MB-468 | R |
| MDA-MB-436 | **NI** |
| RPMI-7951 | **S** |
| A375 | **S** |
| MeWo | R |

*S = sensitization (increased sensitivity to combination)*

*NI = no interaction between treatments in combination*

*R = resistance (decreased sensitivity to combination)*

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Table 5: The effect of pFAK(Y397) inhibitor on sensitivity of TNBC and melanoma cell lines to PTX.

| | Inhibitor |
|---|---|
| Cell line | PF-228 |
| MDA-MB-435S | R |
| MDA-MB-231 | R |
| MDA-MB-468 | R |
| MDA-MB-436 | R |
| RPMI-7951 | R |
| A375 | **NI** |
| MeWo | R |

*S = sensitization (increased sensitivity to combination)*

*NI = no interaction between treatments in combination*

*R = resistance (decreased sensitivity to combination)*

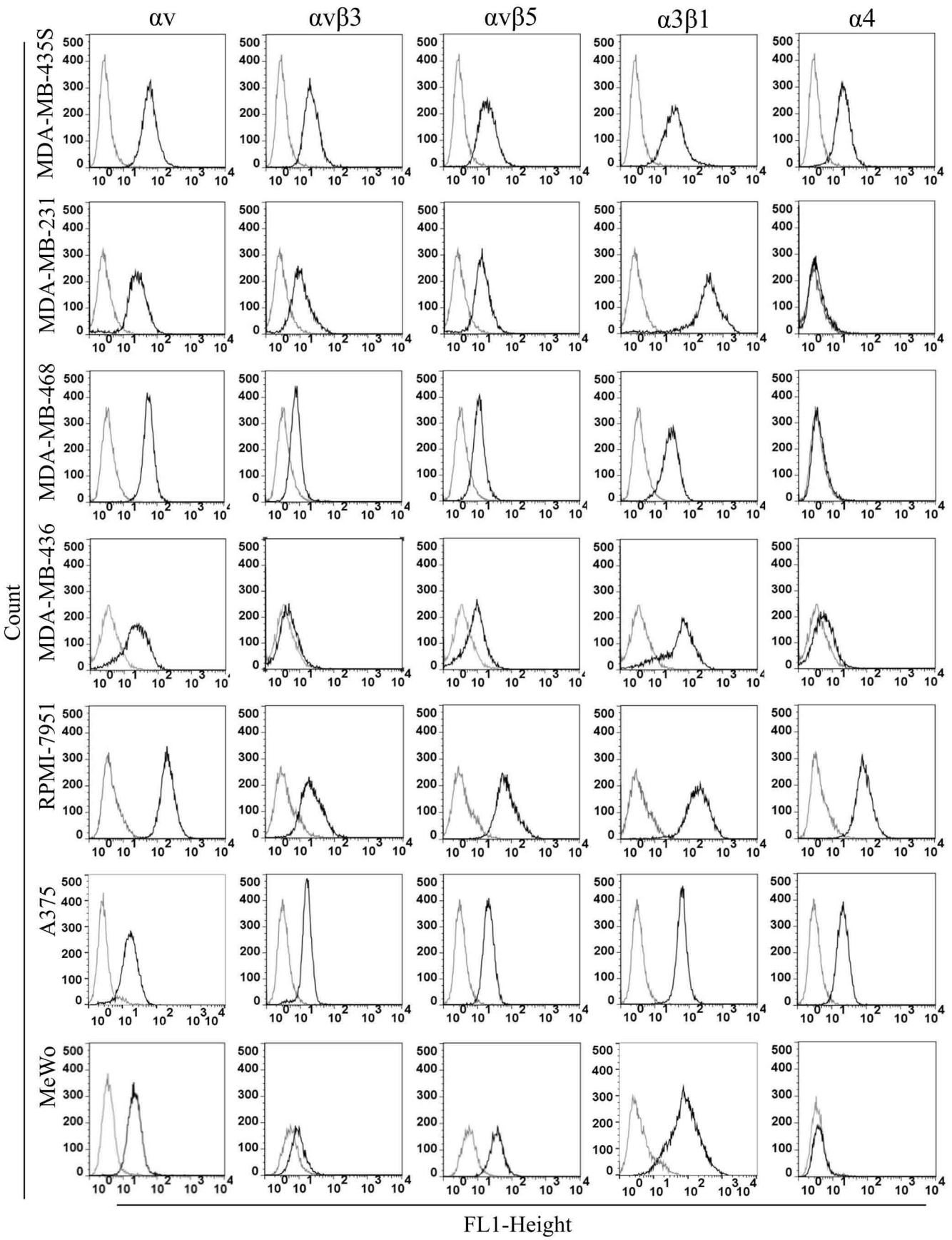
αv
αvβ3
αvβ5
α3β1
α4
MDA-MB-435S
MDA-MB-231
MDA-MB-468
MDA-MB-436
RPMI-7951
A375
MeWo
Count
FL1-Height

Figure 1.

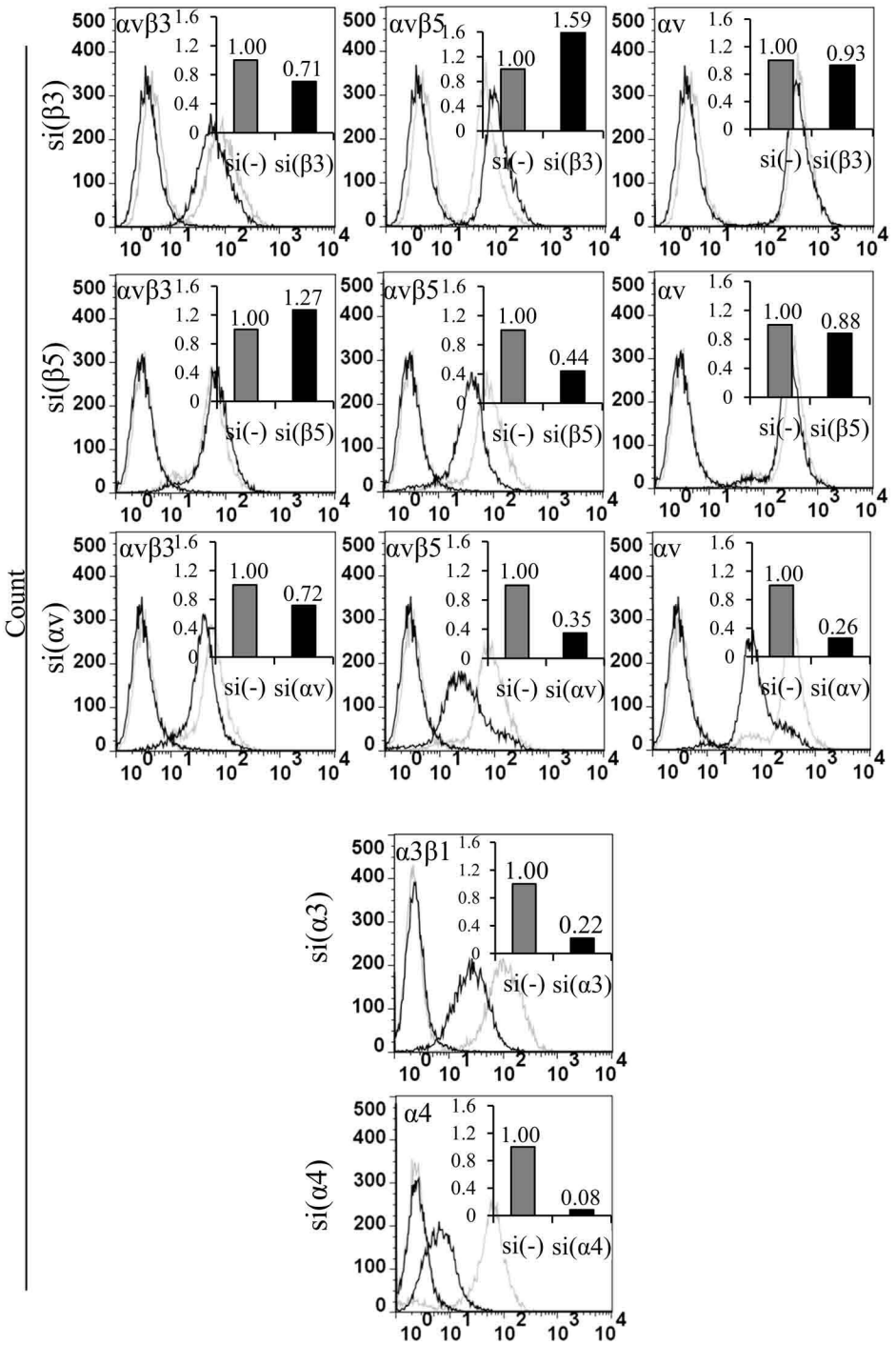
Count
si(β3)
si(β5)
si(αv)
si(α3)
si(α4)
αvβ3
αvβ5
αv
α3β1
α4
1.00
0.71
1.59
0.93
1.27
0.44
0.88
0.72
0.35
0.26
0.22
0.08
si(-) si(β3)
si(-) si(β5)
si(-) si(αv)
si(-) si(α3)
si(-) si(α4)
FL2-Height

Figure 2.

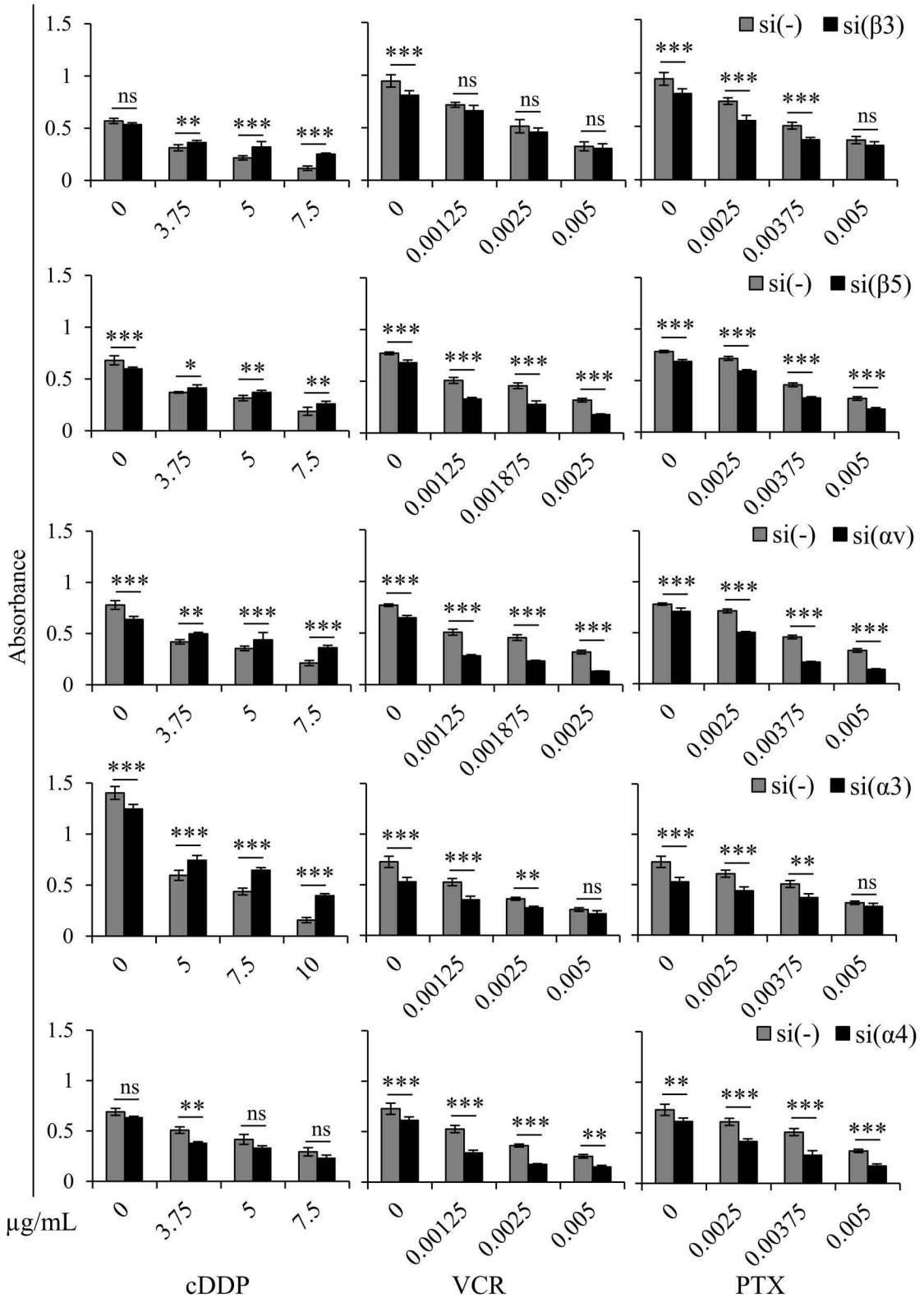
si(-)
si(β3)
si(β5)
si(αv)
si(α3)
si(α4)
Absorbance
μg/mL
cDDP
VCR
PTX
0
3.75
5
7.5
10
0.00125
0.001875
0.0025
0.00375
0.005
ns

Figure 3.

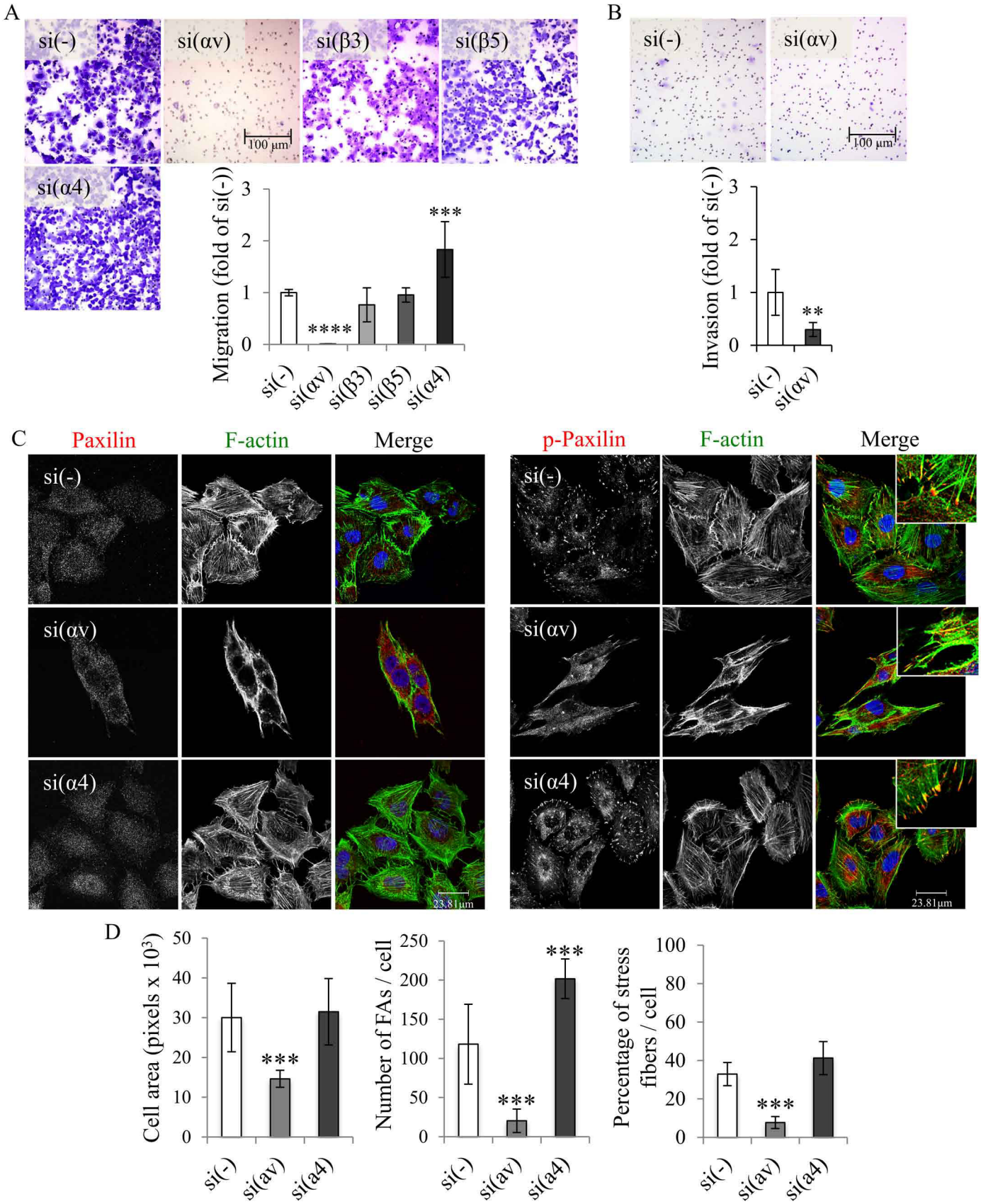
A
si(-)
si(αv)
si(β3)
si(β5)
si(α4)
100 μm
Migration (fold of si(-))
****
***
si(-)
si(αv)
si(β3)
si(β5)
si(α4)
B
si(-)
si(αv)
100 μm
Invasion (fold of si(-))
**
C
Paxilin
F-actin
Merge
p-Paxilin
F-actin
Merge
23.81μm
D
Cell area (pixels x 10³)
Number of FAs / cell
Percentage of stress fibers / cell
si(-)
si(αv)
si(α4)

Figure 4.

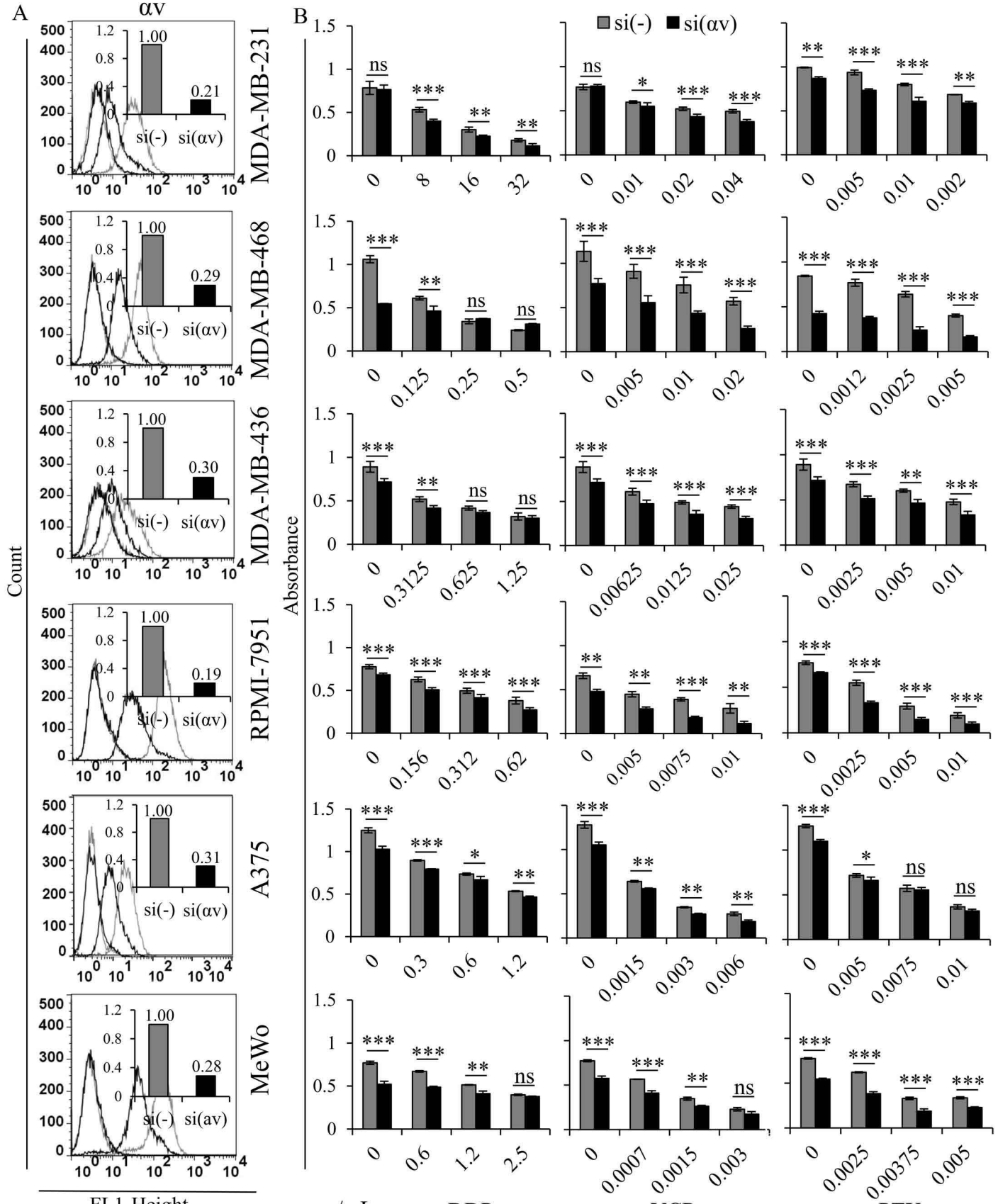
A
αv
MDA-MB-231
MDA-MB-468
MDA-MB-436
RPMI-7951
A375
MeWo
1.00
0.21
0.29
0.30
0.19
0.31
0.28
si(-)
si(αv)
si(av)
Count
FL1-Height
B
si(-)
si(αv)
Absorbance
μg/mL
cDDP
VCR
PTX

Figure 5.

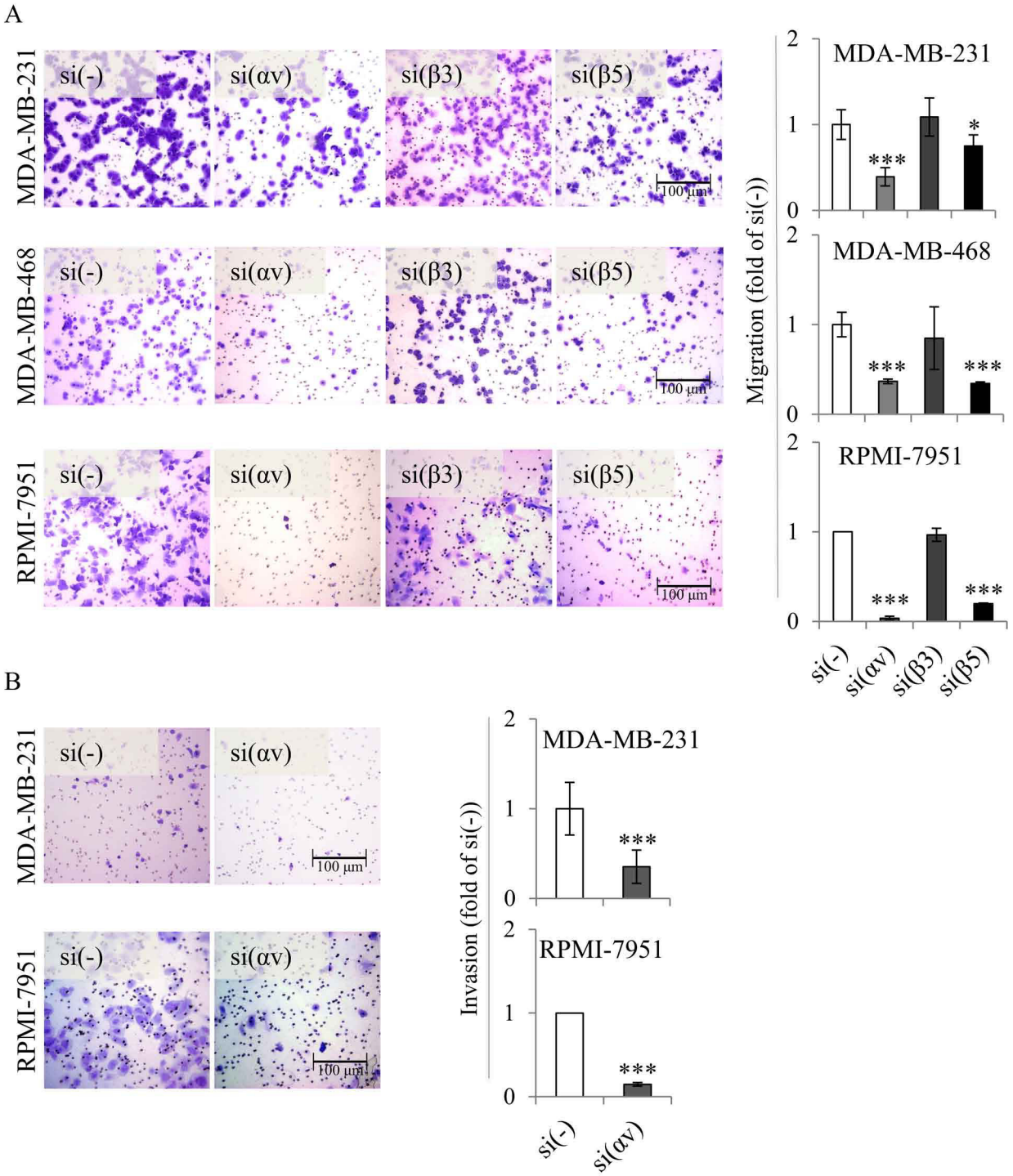
A
MDA-MB-231
MDA-MB-468
RPMI-7951
si(-)
si(αv)
si(β3)
si(β5)
100 μm
Migration (fold of si(-))
MDA-MB-231
MDA-MB-468
RPMI-7951
***
*
si(-)
si(αv)
si(β3)
si(β5)
B
MDA-MB-231
RPMI-7951
si(-)
si(αv)
100 μm
Invasion (fold of si(-))
MDA-MB-231
RPMI-7951
***
si(-)
si(αv)

Figure 6.

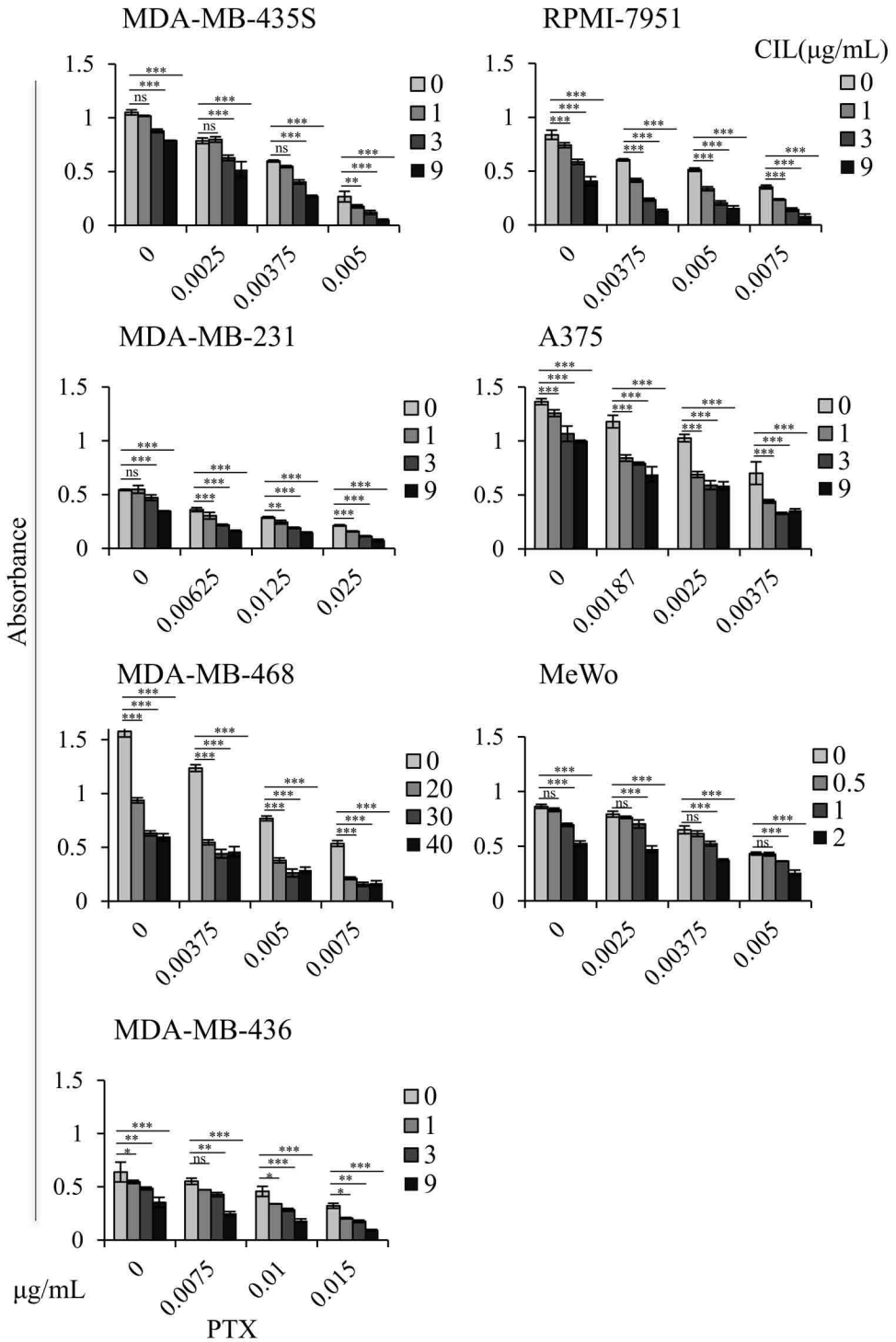
MDA-MB-435S
RPMI-7951
CIL(μg/mL)
MDA-MB-231
A375
MDA-MB-468
MeWo
MDA-MB-436
Absorbance
μg/mL
PTX
0
1
3
9
20
30
40
0.5
2
ns
0
0.0025
0.00375
0.005
0.0075
0.00625
0.0125
0.025
0.00187
0.01
0.015

Figure 7.

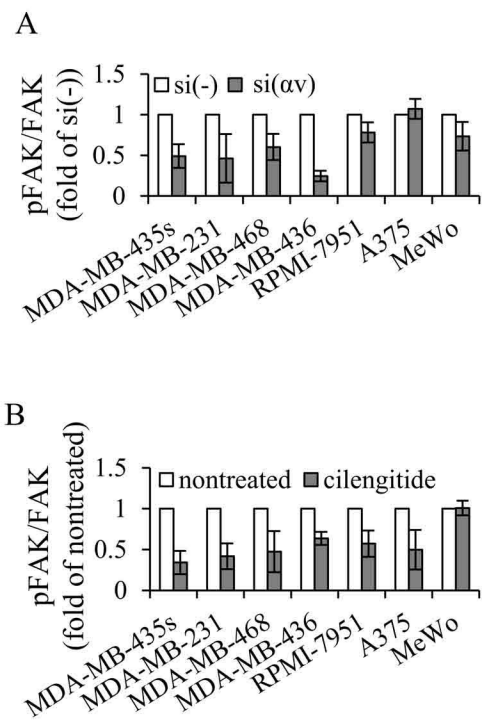
A
pFAK/FAK
(fold of si(-))
1.5
1
0.5
0
si(-)
si(αv)
MDA-MB-435s
MDA-MB-231
MDA-MB-468
MDA-MB-436
RPMI-7951
A375
MeWo
B
pFAK/FAK
(fold of nontreated)
1.5
1
0.5
0
nontreated
cilengitide
MDA-MB-435s
MDA-MB-231
MDA-MB-468
MDA-MB-436
RPMI-7951
A375
MeWo

Figure 8.

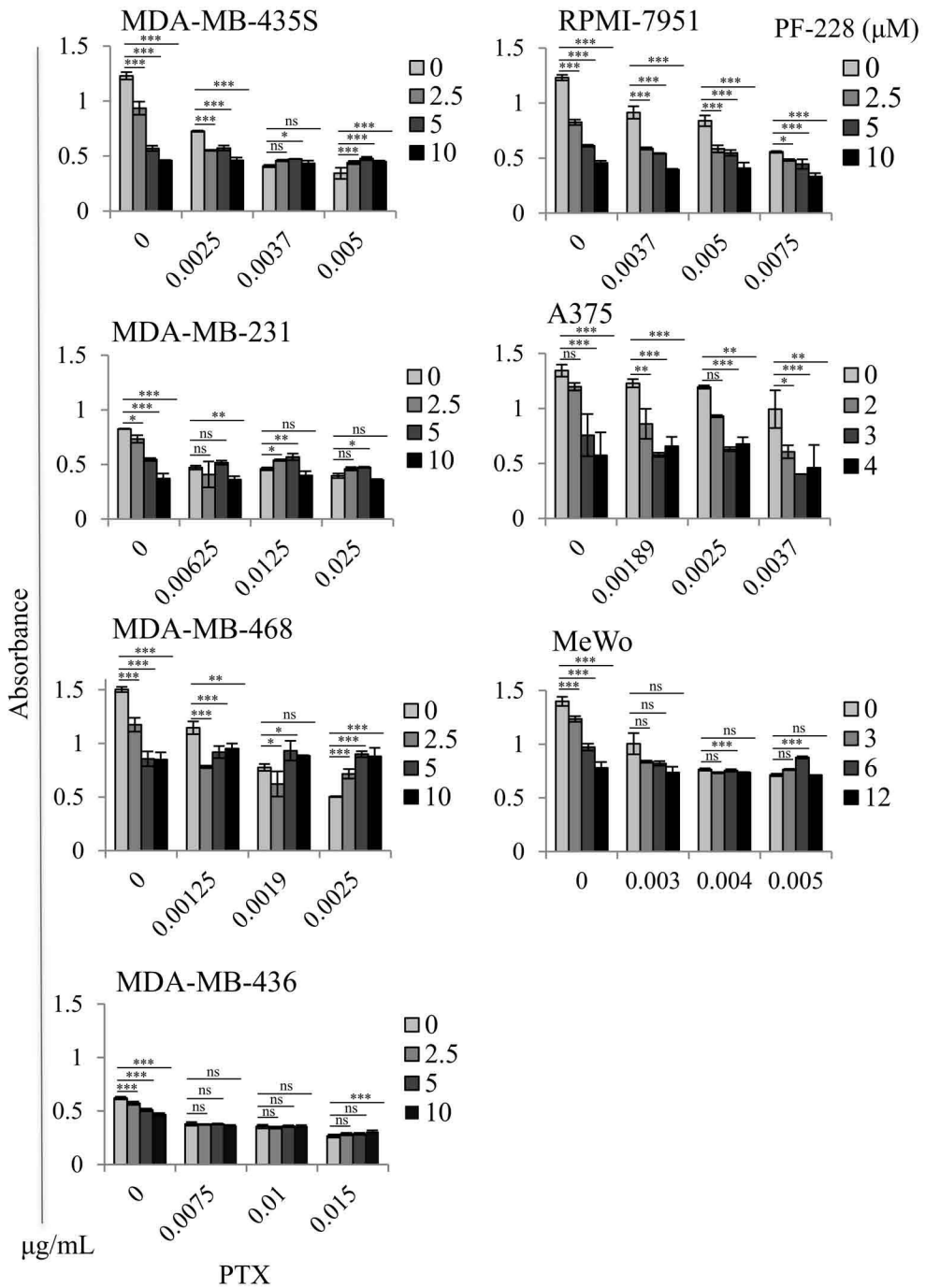
MDA-MB-435S
RPMI-7951
PF-228 (μM)
MDA-MB-231
A375
MDA-MB-468
MeWo
MDA-MB-436
Absorbance
μg/mL
PTX
0
2.5
5
10
2
3
4
6
12
0
0.0025
0.0037
0.005
0.0075
0.00625
0.0125
0.025
0.00189
0.00125
0.0019
0.003
0.004
0.01
0.015

Figure 9.