

# Inhibition of focal adhesion kinase by antisense oligonucleotides enhances the sensitivity of breast cancer cells to camptothecins

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**ABSTRACT:** This study shows a strong association between cell attachment to substratum and activation of  $\beta$ 1-integrin-signaling with resistance to the camptothecin derivative topotecan (TPT) in breast cancer cells. We propose a mechanistic-driven approach to sensitize the cells to camptothecins. ZR-75-1 anchorage-dependent breast cancer cell line, its derivative 9D3S suspension cells (9D3S-S), and 9D3S cells attached to fibronectin-coated plates (9D3S-A) were treated with TPT (1  $\mu$ M) or CPT-11 (40  $\mu$ M) for 48 h. Programmed cell death (PCD), as shown by poly(ADP-ribose) polymerase (PARP), pro-caspase-3 and pro-caspase-9 cleavage, was observed in 9D3S-S cells but not in ZR-75-1 or 9D3S-A cells. Because p125 focal adhesion kinase (FAK) is a transducer in the  $\beta$ 1-integrin signaling pathway, it is essential to cell adhesion and it is overexpressed in metastatic breast cancer, we hypothesized that attenuation of FAK might enhance the sensitivity of breast cancer cells to camptothecins. Moreover, inhibition of *FAK* gene expression by a phosphorothioated antisense oligodeoxynucleotide targeting the portion of the gene encoding amino acids 262-268, increased the sensitivity of ZR-75-1, MDA-MB-231 and MCF7 breast cancer cells to treatment with TPT or CPT-11.

## Introduction

Camptothecin, a pentacyclic alkaloid isolated from the Chinese tree *Camptotheca acuminata* (Hertzberg *et al.*, 1989; Zhang *et al.*, 1990), and its derivative topotecan (TPT) and the MDR 1-independent irinotecan (CPT-11) are important chemotherapeutic agents (Kingsbury *et al.*, 1991; Rivory and Robert, 1995).

Camptothecins bind to the topoisomerase (Topo) I-DNA complex during DNA replication, resulting in DNA damage (Rivory and Robert, 1995; Zhang *et al.*, 1990) followed by programmed cell death (PCD), an important therapeutic indicator of their efficacy (Whitacre and Berger, 1997; Whitacre *et al.*, 1999). A marker of PCD commonly used is the cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) between Asp 216 and Gly 217. This proteolytic cleavage triggered by a cascade of caspases, splits the 116 kD enzyme into two fragments of about 90 kD and 26 kD (Whitacre and Berger, 1997; Whitacre *et al.*, 1999). It has been observed that camptothecins appear to be more effective

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for the treatment of leukemia, with complete responses reported in patients and mouse model systems (Potmesil, 1994; Uckun *et al.*, 1995), than for solid tumors. In concordance with these observations, the major toxicity described during treatment of solid tumors is hematologic (Kantarjian *et al.*, 1993; Potmesil, 1994). Furthermore, leukemia cells are known to have a distinctive propensity to undergo PCD following treatment with camptothecins compared to the relatively lower response of colon cancer cell lines (Darzynkiewicz *et al.*, 1996) and growth arrest rather than tumor regression in colon tumors (Rothenberg, 1996; Whitacre *et al.*, 1999). Moreover, cell adhesion processes induce resistance to genotoxic agents (Fridman *et al.*, 1990; Olive and Durand, 1994; St Croix and Kerbel, 1997; Uhm *et al.*, 1999; Whitacre and Berger, 1997) and to radiation (Olive and Durand, 1994).

We have previously reported a strong association between cell adhesion to substratum and resistance to TPT in isogenic cervical cancer cell lines that differ in their attachment properties (Whitacre and Berger, 1997) as well as in a broad range of human cancer cell lines of various origins (Uhm *et al.*, 1999; Whitacre and Berger, 1997). Focal adhesion kinase (FAK), a transducer in the  $\beta 1$ -integrin signaling pathway reported to be essential to intercellular adhesion (Maung *et al.*, 1999), has been suggested to also play an important role in cell survival (Frisch *et al.*, 1996). Activation of integrin-signaling by binding to substratum or fibronectin results in phosphorylation of specific kinases including FAK, and activation of second messenger (Frisch *et al.*, 1996; Hanks *et al.*, 1992; Schaller *et al.*, 1992). This activation of FAK subsequently suppresses induction of PCD (Frisch *et al.*, 1996; Ruoslahti and Reed, 1994) in normal epithelial and endothelial cells. FAK is overexpressed in 88% of invasive and metastatic human breast tumors compared to normal tissue from the same individuals (Owens *et al.*, 1995) and in several other types of cancer, including thyroid and lung, where it correlates with poor prognosis and aggressive phenotypes (Schaller *et al.*, 1992). FAK and integrin-associated proteins also play an important role in proliferation and survival of prostate tumors and prostate cancer cell lines (Tremblay *et al.*, 1996), and is therefore considered a marker of malignancy (Owens *et al.*, 1995). Because of the high levels of FAK in solid tumors, and its function in cell adhesion and survival, we hypothesized that attenuation of FAK may sensitize cells to camptothecins. Moreover, it has been shown (Xu *et al.*, 1998) that inhibition of FAK by targeting the portion of the gene encoding amino acids 262-268 by phosphorothioated antisense oligo-

nucleotides induces cell detachment. This experimental approach has been used in this study to evaluate FAK as a potential target for anticancer therapy.

## Materials and Methods

### Cell lines

The ZR-75-1, MDA-MB-231 and MCF-7 breast cancer cell lines were originally obtained from the American Type Culture Collection (ATCC, Maryland, VA). The 9D3S cell line was derived by limiting dilutions from the ZR-75-1 cell line by Dr. Okot Nyormoi when he was in Dr. Michael A. Tainsky's laboratory at the University of Texas M.D. Anderson Cancer Center (Nyormoi *et al.*, 2001). The ZR-75-1 and 9D3S cell lines were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). MDA-MB-231 breast ductal carcinoma cells were kept in IMEM and MCF7 breast adenocarcinoma cells were cultured in MEM containing 10% FBS. Cells were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Cells were observed under a conventional optical microscope (Hund Wetzlar Seiler W1352) and used during early logarithmic growth when both ZR-75-1 and its derivative 9D3S divided at a similar rate.

### Western blot

Cells were lysed and sonicated in a solution comprised of 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate (SDS), 1% Triton X-100, 5 mM EDTA, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin and 1 mM phenyl-methylsulfonyl fluoride (all reagents were from Sigma Chemical Co., St. Louis, MO). Samples containing 70  $\mu$ g protein measured by Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA) were separated by SDS-PAGE consisting of a 5% (w/v) acrylamide stacking gel and a 12.5% (w/v) separating gel containing 0.1% SDS (5). The running buffer was comprised of 0.1% SDS, 25 mM Tris and 250 mM glycine (pH 8.3). Electrophoretic fractionation was carried out at a constant current of 15 mA. Proteins were then electrotransferred onto an Immobilon p15 membrane (Milipore Corp., Bedford MA). The filters were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-Tween) for 5

min and then incubated overnight at 4°C with primary antibody at a concentration of 1 µg/ml in blocking solution. After washing in PBS-Tween, the filters were incubated overnight at 4°C in horseradish peroxidase-conjugated anti-immunoglobulin (1:1000). Following three washes in PBS-Tween, bands were visualized with enhanced chemiluminescence reagent and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham Life Science Inc, Arlington Heights, IL). Intensity of the bands was quantified by densitometric scanning (SCIscan 5000 USB densitometer, United States Biochemical, Cleveland, OH) and normalized with respect to actin.

#### Source of antibodies

The monoclonal antibody to purified human PARP (Ranjit *et al.*, 1995) is an IgG1κ that shows specificity for the NAD-binding domain of PARP (PharMingen, San Diego, CA). Monoclonal anti-human pro-caspase-3 antibody was from Transduction Laboratories (Lexington, KY), and monoclonal antibody to pro-caspase-

9 was from PharMingen. MAB2156 to FAK was from Chemicon International, Inc. (Tencula, CA). Monoclonal antibody to actin and the horseradish peroxidase-linked anti-mouse immunoglobulins were from Amersham Life Science, Inc.

#### Acridine orange staining

An aliquot of 10 µl of a 100 µg/ml acridine orange (AO) stock solution prepared in PBS was added to 100 µl of single cell suspension. Immediately thereafter a minimum of 100 cells per point were counted under a fluorescence microscope. Cells with condensed chromatin in apoptotic bodies and cell membrane blebbing were counted as acridine orange positive whereas cells with homogenous chromatin were counted as acridine orange negative (Whitacre and Berger, 1997).

#### Antisense oligonucleotides

We have utilized phosphorothioated oligodeoxynucleotides to prevent their rapid degradation by ubiquitous nucleases. Since it has been previously shown that a 20-mer oligonucleotide targeted to the portion of the *FAK* gene encoding amino acids 262-268 (Xu *et al.*, 1998) induces cell detachment from substrate we have selected this sequence for the present study. The oligonucleotide was named AS2 (5'-ATAATCCAGCTTGAACCAAG-3'). A 2-base mismatched oligonucleotide named MS2-2 (5'-ATAATCGAGCTTCAACCAAG-3'), 5-base mismatched oligonucleotide named MS2-5 (5'-ATAATCGACGTTCAAGCAAG-3') and 6-base mismatched oligonucleotide named MS2-6 (5'-ATAAGCGACGTTCAAGCAAG-3'), were used as negative controls. N-terminal fluorescein isothiocyanate (FITC)-labeled oligodeoxynucleotides were used to assess cellular uptake. The oligonucleotides were from (MWG-Biotech Inc, Highpoint, NC).

#### Antisense treatment

ZR-75-1, MDA-MB-231 and MCF7 cells were plated in 3 ml of their respective culture medium at a density of 10<sup>5</sup> cells per well in 6-well plates and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. Following 24 h incubation the culture medium was replaced with serum-free Opti-MEM (Life Technology Inc., Carlsbad, CA). Cells were then transfected with 0.1-10 µM AS2 or one of the MS2 oligonucleotides with 10 µg/ml lipofectamine (LPA) reagent (Life Technology Inc.) fol-

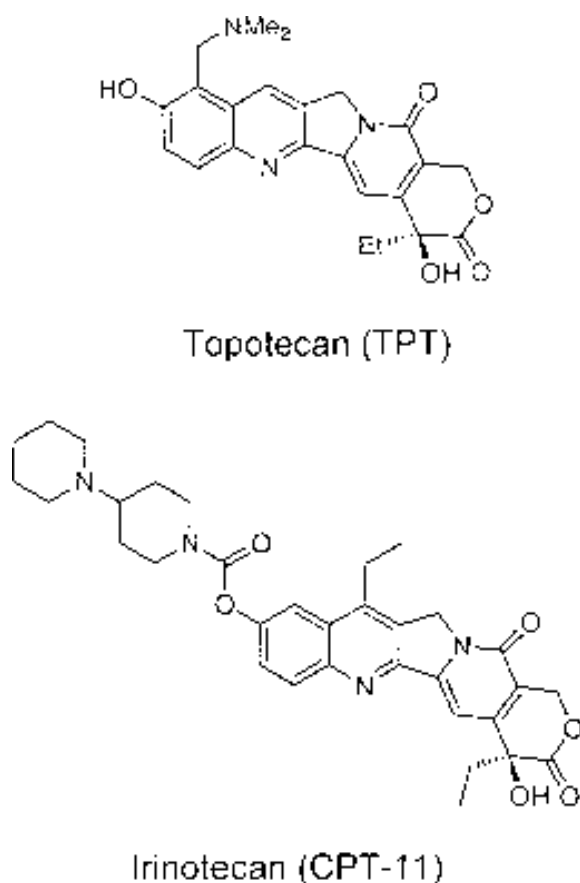


FIGURE 1. Chemical structure of TPT and CPT-11.

lowing the manufacturer's instructions. Time-course and dose-response for inhibition of FAK expression were assessed by Western blot.

#### *Treatment with camptothecins*

After pretreatment of cells with LPA alone, AS2 or MS2 in serum-free Opti-MEM, as described above, heat inactivated FBS was added to a final concentration of 10%. Stock solutions of TPT and CPT-11 were prepared in dimethyl sulfoxide (DMSO) and diluted 1:1000 in the culture media to final concentrations of 0.1-5  $\mu$ M TPT or 10-40  $\mu$ M CPT-11. Cells were then incubated for an additional 24-48 h at 37°C. Lysates were prepared from treated cells containing both adherent and non-adherent fractions, to assess PARP cleavage by Western blot.

## Results

#### *Cell adhesion and resistance to camptothecins*

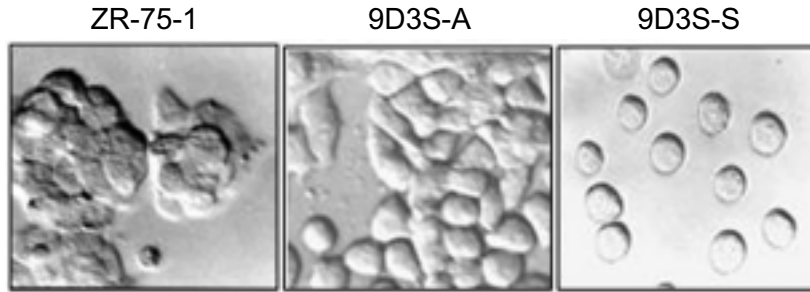
To demonstrate the relationship between cell adhesion and resistance to the pentacyclic alkaloids camptothecins (Fig. 1), we examined the ZR-75-1 and its derivative 9D3S breast cancer cell lines. Intercellular adhesion of ZR-75-1 cells is strong, and the cells anchor to polystyrene flasks, whereas 9D3S cells grow as a single cell suspension (9D3S-S) (Fig. 2A). 9D3S cells were induced to attach (9D3S-A) to 6-well plates coated with 40  $\mu$ g fibronectin per well (Fig. 2) to evaluate the effect of activation of  $\beta$ 1-integrin signaling pathway on the sensitivity of the cells to TPT or CPT-11. ZR-75-1, 9D3S-A and 9D3S-S cells, all in logarithmic growth and similar doubling time, were treated with increasing concentrations of TPT (0.1-1  $\mu$ M) or CPT-11 (10-40  $\mu$ M) for various times (5 min-72 h). The percentage of cells in PCD as assessed by AO staining was much greater for 9D3S-S cells than for 9D3S-A or ZR-75-1 cultures. Figure 2B shows the results obtained after 48 h treatment with 0.1  $\mu$ M TPT or 40  $\mu$ M CPT-11, conditions that enabled to visualize the most clear differential pattern of sensitivity between adherent and suspension cells. About 21% of 9D3S-S cells showed chromatin condensation following treatment with 1  $\mu$ M TPT for 48 h, whereas only ~2-5% of the ZR-75-1 or 9D3S-A cells were AO positive under the same experimental conditions (Fig. 2B). Likewise, treatment with 40  $\mu$ M CPT-11 for 48 h resulted in ~11% AO positive of 9D3S-S cells, whereas only about 3-4% of 9D3S-A

or ZR-75-1 cells were AO positive (Fig. 2B). Furthermore, the low percentage of TB positive cells with a parallel pattern to the AO response suggested that necrosis probably occurred secondary to PCD (Fig. 2B). PARP cleavage (Fig. 2CD) and degradation of pro-caspase-3 and pro-caspase-9 (Fig. 2C) were assessed as indicators of PCD. In agreement with the results shown with acridine orange staining, 9D3S-S cells showed markedly higher levels of PARP cleavage and degradation of pro-caspase-3 and pro-caspase-9 compared with the ZR-75-1 (Fig. 2C) or the 9D3S-A cells (Fig. 2D) after treatment with 1  $\mu$ M TPT or 40  $\mu$ M CPT-11 for 48 h. Actin was used as internal standard for protein loading. Our results suggest a strong association between breast cancer cell adhesion and resistance to TPT or CPT-11.

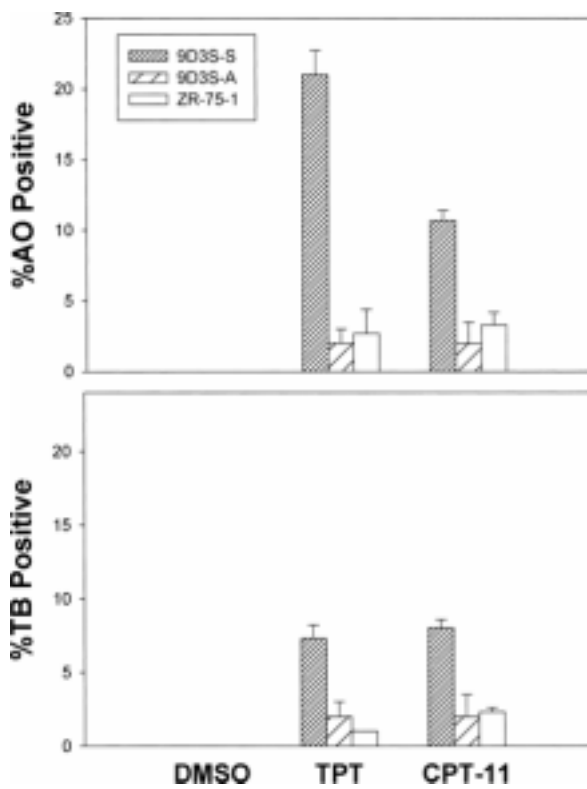
#### *Attenuation of FAK by antisense oligonucleotides*

The antisense oligonucleotides used in this study specifically inhibit the translation of encoded proteins by hybridizing to the target mRNA through Watson-Crick complementary base recognition. To evaluate the effect of attenuation of FAK on the sensitivity of cells to camptothecins we have selected three breast cancer cell lines expressing FAK (Fig. 3B): ZR-75-1, MDA-MB-231 and MCF7 cells. In order to assess uptake of the oligonucleotides, cells were transfected with FITC-labeled antisense as described in Materials and Methods. Fluorescence was observed within the cells under an inverted fluorescence microscope about 4 h after transfection and remained visible approximately for 24 h. Background fluorescence was absent from untreated cells (data not shown). We have evaluated various doses (0.1-10  $\mu$ M) and times (24-72 h) of treatment with the antisense oligonucleotides and found optimal inhibition of FAK at 3  $\mu$ M AS2 for 24 h. Figure 3A shows positive uptake of N-terminal FITC-labeled AS2 or MS2 (3  $\mu$ M) by ZR-75-1, MDA-MB-231 and MCF7 cells following 24 h incubation. Western blotting against FAK showed decrease of protein levels in the AS2 treated group but not in cells treated with LPA alone or the MS2 mismatched oligonucleotides (Fig. 3B). Since all three MS2 oligonucleotides analyzed (MS2-2, MS2-5 and MS2-6) resulted in identical responses, only the results obtained with MS2-2 are shown. The degree of attenuation of FAK varied from one experiment to another with apparently greater efficacy in the treatment of the MDA-MB-231 cells compared with the ZR-75-1 cells, despite the fact that both cell lines express similar high endogenous levels of the target protein. We suggest that this effect may be related to differences in the efficiency of

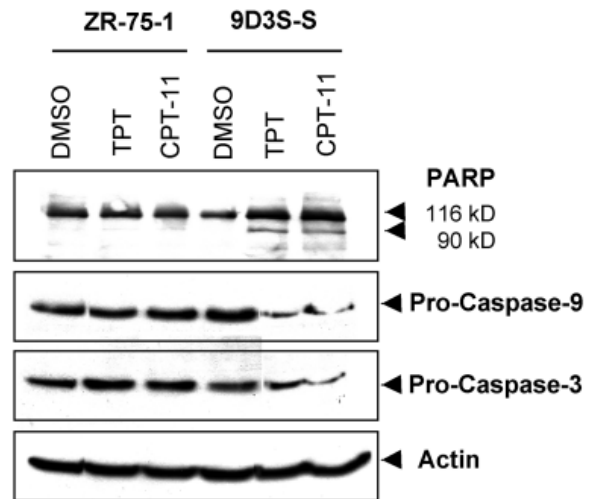
**Fig. 2A**



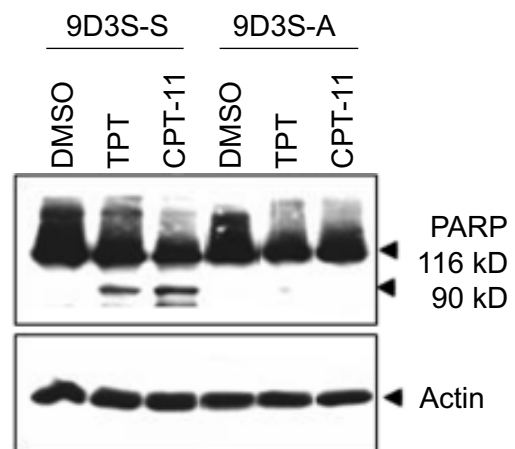
**Fig. 2B**



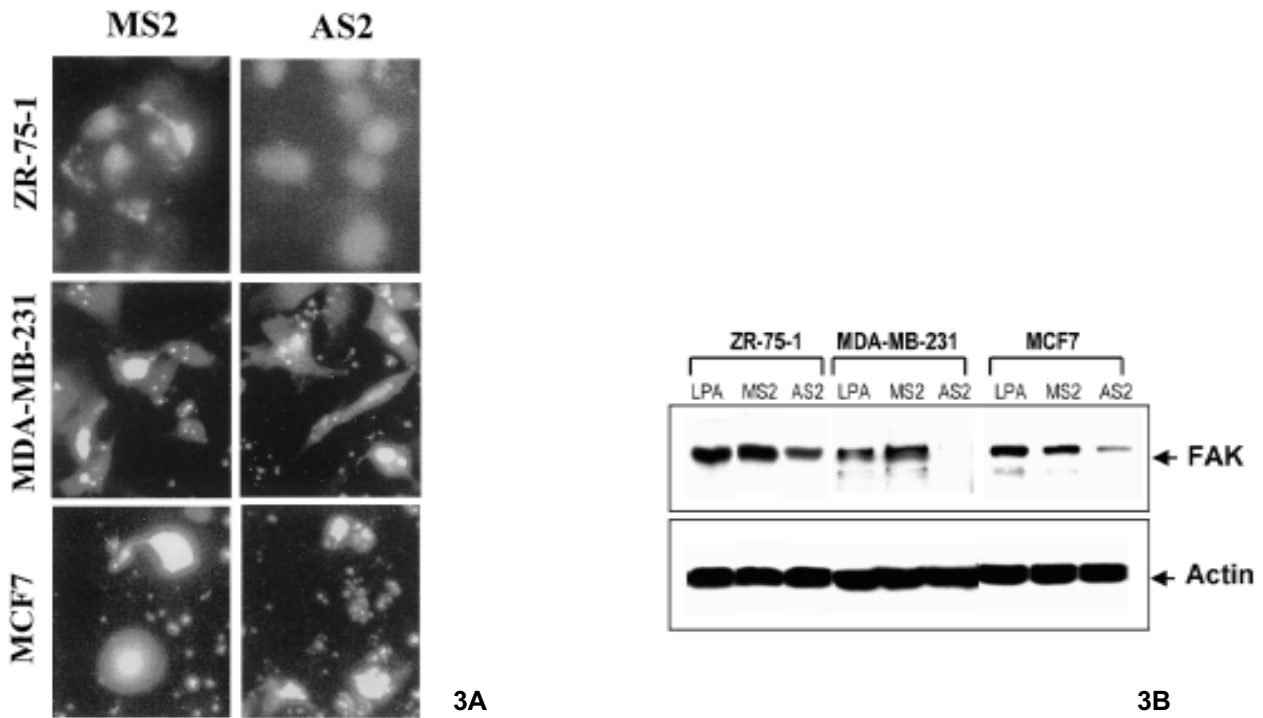
**Fig. 2C**



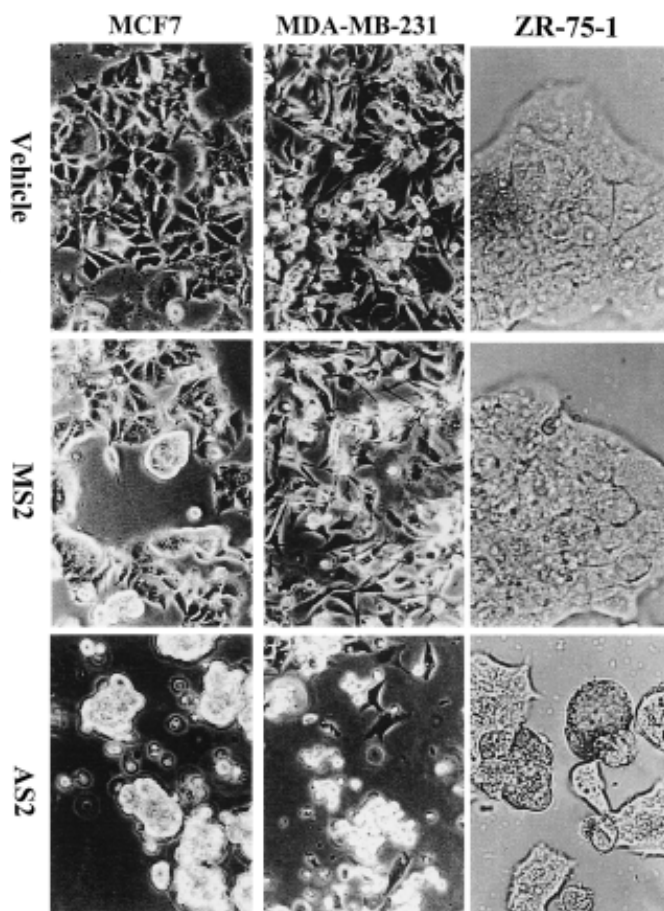
**Fig. 2D**



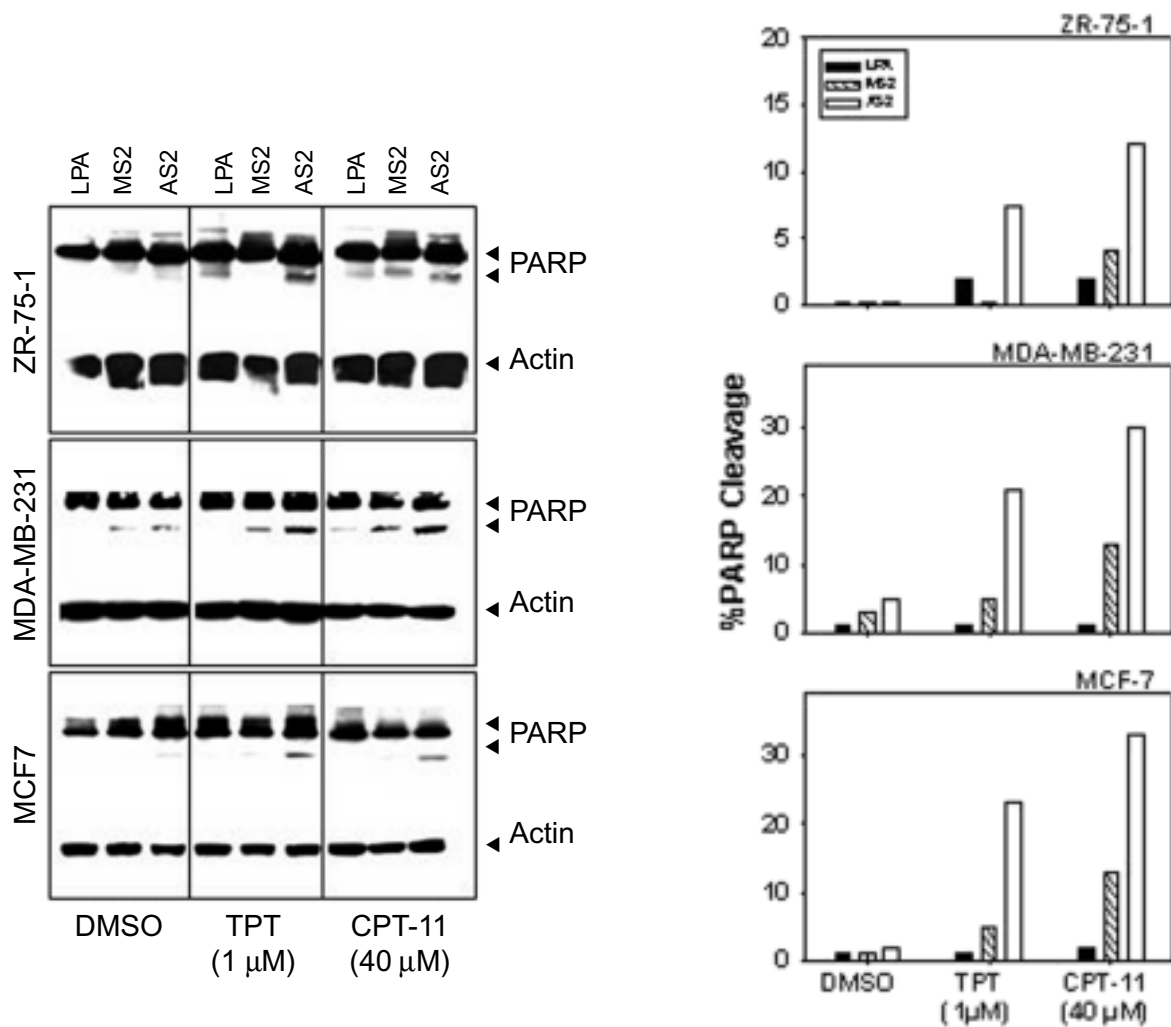
**FIGURE 2.** Association between cell adhesion status and resistance to camptothecins. (A). ZR-75-1 breast adenocarcinoma cells anchored to polystyrene flasks, 9D3S-A cells attached to fibronectin-coated plates and 9D3S-S suspension cell. (B). Treatment with 1  $\mu$ M TPT or 40  $\mu$ M CPT-11 for 48 h induced chromatin condensation and apoptotic acridine orange staining in 9D3S-S cells. Necrosis was assessed by trypan blue uptake. The results are the mean value of triplicates  $\pm$  standard deviation. (C). Western blot of ZR-75-1 and 9D3S-S cells treated with 1  $\mu$ M TPT or 40  $\mu$ M CPT-11 for 48 h. TPT and CPT-11 induced PARP cleavage and degradation of pro-caspase-9 and pro-caspase-3 in 9D3S-S cells at 48 h, whereas this response was markedly delayed in ZR-75-1 and (D) PARP cleavage was delayed in 9D3S-A cells compared with 9D3S-S cells. Actin was used as internal standard for protein loading.



**FIGURE 3.** Effect of AS2 on FAK expression. (A). ZR-75-1, MDA-MB-231 and MCF7 cells in logarithmic growth were treated with 3  $\mu$ M FITC-labeled AS2 or MS2 for 24 h. MDA-MB-231 and MCF7 cells show higher uptake of oligonucleotides than ZR-75-1 cells. (B). AS2 markedly reduced endogenous levels of FAK as measured by Western blotting. Actin was used as internal standard for protein loading. LPA and a two-base mismatched oligonucleotide (MS2) were used as negative controls.



**FIGURE 4.** Effect of AS2 on cell adhesion. ZR-75-1, MDA-MB-231 and MCF7 cells in logarithmic growth were treated with 3  $\mu$ M AS2 or MS2 for 24 h. AS2 transfected cells detached after rotation of the plates more readily than the MS2 or LPA-transfected control cells.



**FIGURE 5.** Attenuation of FAK sensitizes breast cancer cells to camptothecins. (A) Cells in logarithmic growth were treated with 3 μM AS2 for 24 h as described in Materials and Methods. Cells treated with LPA or MS2 were used as negative controls. Subsequently, 1 μM TPT or 40 μM CPT-11 was added onto the cells. Combination treatment with AS2 and TPT or CPT-11 showed greater toxicity, as assessed by PARP cleavage. The 116 and 90 kD bands correspond to reactivity with PARP antibody, whereas the 46 kD band corresponds to actin used as internal standard for protein loading. (B) Densitometric profile of the results shown in A.

transfection shown in Figure 3A with FITC-labeled oligonucleotides. AS2 did not affect the ability of the cells to produce colonies (data not shown) and showed little (<5% PARP cleavage) cytotoxicity (Fig. 5AB). Some cells treated with AS2 detached from the polystyrene flasks after gentle rotation whereas MS2 and LPA treated cells remained attached (Fig. 4).

*AS2 enhances the sensitivity of breast cancer cells to camptothecins*

Twenty-four h after transfection with LPA alone, MS2 or AS2 (3 μM), cells were treated with increasing doses of TPT or CPT-11 for 24 h (0.1-10 μM TPT or 10-40 μM CPT-11). Cells were then harvested and PARP

cleavage was examined by Western blot. Optimal results were observed with 1  $\mu$ M TPT and 40  $\mu$ M CPT-11. Figure 5 shows ~8-24% PARP cleavage after treatment with 1  $\mu$ M TPT for 24 h and 13-33% increase in PARP cleavage after treatment with 40  $\mu$ M CPT-11 for 24 h in AS2 treated cells compared with control cells treated only with TPT (2-3% PARP cleavage), CPT-11 (2-4% PARP cleavage) or AS2 (1-5% PARP cleavage). Control cells treated with LPA or MS2 alone also showed trace levels of PARP cleavage. Furthermore, treatment with AS2 followed by CPT-11 induced about 2-3 times the levels of PARP cleavage compared to the corresponding values for the MS2/CPT-11 treated cells. Similarly, AS2 pretreatment increased the sensitivity of the cells to TPT at least 3 fold as compared to the level observed for the MS2 pretreated cells (Fig. 5).

## Discussion

It is well established that most solid tumors are intrinsically resistant to chemotherapy. Furthermore, after chemotherapeutic treatments the residual tumor cells become increasingly resistant (Glinsky, 1998). Overcoming this drug resistance phenotype in solid tumors is a major challenge during drug development. We have previously observed that interference with integrin-signaling sensitizes cervical cancer cells to treatment with TPT (Whitacre and Berger, 1997). Since the strong correlation between the percentage of PCD measured by the PARP cleavage assay and by acridine orange staining is well recognized (Whitacre and Berger, 1997), we have utilized both methods to assess PCD in this study. Here we further demonstrate an association between cell anchorage status and sensitivity to TPT or CPT-11 in isogenic human breast cancer cell lines. Our results show that adherent cells (ZR-75-1 and 9D3S-A) are more resistant to TPT or CPT-11 than suspension cells (9D3S-S). Because FAK is overexpressed in metastatic breast cancer and in breast cancer cell-lines and is a transducer in the  $\beta$ 1-integrin signaling pathway, we have hypoth-

esized a potential role for this protein in the resistance phenotype to camptothecins. To directly test the relationship between cell attachment and sensitivity to Topo I inhibitors we have treated the anchorage-dependent ZR-75-1, MDA-MB-231 and MCF7 breast cancer cells with a phosphorothioated antisense oligonucleotide against FAK previously shown to induce melanoma cell detachment. In this study we demonstrate that attenuation of FAK sensitizes breast cancer cells to the camptothecin analogs TPT and CPT-11. Several pro-survival mechanisms have been suggested for FAK including a regulatory role on the conformation of the Bax BH3 epitope (Gilmore *et al.*, 2000), with consequences on p53-mediated PCD (Ilic *et al.*, 1998). FAK is known (Sonoda *et al.*, 2000) to promote activation of the anti-apoptotic factor NF $\kappa$ B by phosphorylation of I $\kappa$ B $\alpha$ . In addition, it is possible that changes in cell adhesion status may impact cell cycle progression, therefore affecting the sensitivity of cells to subsequent treatment with Topo I inhibitors. Because FAK is required for tumor cell adhesion (Maung *et al.*, 1999), the use of antisense to FAK along with TPT or CPT-11 may be useful for *in vivo* studies in animals. FAK is the center of an intricate mesh of signaling pathways controlling cell proliferation, adhesion, survival and migration, all related to tumor growth, suggesting that attenuation of FAK may also affect these pathways *in vivo*. There is the concern that interference with cell adhesion *in vivo* may favor metastasis. Nonetheless, since FAK is also involved in angiogenesis, attenuation of FAK could also impair cancer progression. Hence, our results point to FAK as potential target for the development of novel anticancer treatments.

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