Effect of triptolide on focal adhesion kinase and survival in MCF-7 breast cancer cells

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Abstract. Triptolide, a diterpene from Tripterygium wilfordii, has been shown to have potent anticancer activity, exerting its effects through multiple molecular targets and signaling pathways. Yet, its effect on focal adhesion kinase (FAK), a non-receptor tyrosine kinase overexpressed in breast cancer that regulates cellular adhesion and survival, has not been reported. The current study is the first to report on the effect of triptolide on FAK expression, cell adhesion and survival using MCF-7 breast cancer cells. Triptolide significantly reduced MCF-7 anchorage-independent growth in a concentration-dependent manner. Cell rounding and detachment from culture plates were observed as early as 8 h, with significant cell detachment observed after 24 h of triptolide treatment. The adhesion potential of triptolide-treated MCF-7 cells to Matrigel was also compromised. Triptolide induced concentration- and time-dependent cleavage of FAK and PARP, which was dependent on caspase activation. The pan-caspase inhibitor, zVAD-fmk, was the only inhibitor that could significantly reduce FAK and PARP cleavage and cell detachment. However, the presence of zVAD-fmk failed to significantly reverse triptolide-induced cell death. Finally, triptolide-induced FAK cleavage was specific to MCF-7 cells, as no cleaved FAK was observed in MDA-MB-231 cells. In conclusion, our data present the first evidence of triptolide-mediated induction of FAK cleavage that correlates with cell detachment and loss of adhesion potential to the extracellular matrix.

Introduction

Triptolide is a diterpene triepoxide isolated from a traditional Chinese herb, Tripterygium wilfordii, which has been used for centuries in China. In addition to its anti-inflammatory and immunosuppressive activities (1), triptolide also exhibits potent anti-cancer activity, often with an IC₅₀ in the nanomolar range, demonstrated through a broad range of solid tumors and hematological malignancies in vitro and in vivo (1-3). Synergism in cancer cell killing could be observed when triptolide was combined with other DNA-damaging chemotherapeutic drugs (4). While the anti-cancer activity of triptolide appears promising, translation of this small molecular bioactivity is hampered by its poor water solubility and toxicity. Analogues have been developed over the years to overcome the above problems, and PG490-88 Na is one such analogue that shows improved solubility and toxicity profile. Recently, PG490-88 Na has gained entry into a Phase I clinical trial for treatment of prostate cancer in the USA (1).

Research into the mechanism of action of triptolide demonstrated that this small molecule acts through a number of molecular pathways that result in its anti-cancer activity. Triptolide has been shown to inhibit NF-κB activation and downstream gene targets, inhibit TNF-α-mediated induction of c-IAP1 and c-IAP2 (5), induce Bel-2 cleavage (6), reduce XIAP and Mcl-1 (2) and induce caspase activation (2,6,7). More recently, triptolide has been shown to cause global transcriptional arrest as evidenced by the inhibition of RNA polymerase I and II activities with subsequent alteration in nuclear substructure (8). Cellular targets including MKP-1, HSP-70, 5-Lox, histone methyltransferases, and a disintegrin and metalloproteinase 10 (ADAM10) have also been shown in various in vitro models (1,9). Among the various functional groups present in the chemical structure of triptolide (Fig. 1), the 9,11-epoxide group was shown to contribute to its anti-cancer activity (10). More recently, the C-14 hydroxyl group has been shown to modulate triptolide-induced HeLa cell death, and the 12,13-epoxide group was important for NF-κB transcriptional inhibition (11).

In view of the potency and the ability to target multiple molecular pathways, triptolide could be a potential drug candidate in the treatment of breast cancer which remains to be the main cause of cancer deaths in women worldwide (12). Among the various in vitro cell line models available for breast cancer, the MCF-7 cell line presents distinctive properties that may help shed new light into the mechanism of action of triptolide. In particular, MCF-7 is an estrogen receptor-positive cell line that lacks caspase-3 and beclin-1 (13,14), thus representing a cell model with compromised apoptotic machinery and low autophagic activity that might influence cellular response to anti-cancer drug treatment. Thus far, very little is known about
the effect of triptolide on MCF-7 breast cancer cells except that triptolide exposure results in an increase in p53 expression (6,15), a decrease in estrogen receptor-α expression (15) and a decrease in ADAM10 expression (9).

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase which plays an important role in cancer cell signaling (16). It has been shown to be overexpressed in breast cancer tumors at early stages of tumorigenesis (17), as evidenced by its high expression levels in ductal carcinoma in situ that occurs before tumor cell invasion and metastasis (18). According to a study of human tumor samples (19), up-regulation of FAK is observed in 88% of invasive and metastatic breast tumors, and FAK expression level is correlated with the invasive potential of tumors (16). Owing to the importance of FAK in breast cancer, this study aims to investigate the effect of triptolide on FAK expression, cell adhesion and survival using MCF-7 breast cancer cells, and is the first to elucidate the effect of triptolide on this important breast cancer target.

Materials and methods

Reagents. The MCF-7 and MDA-MB-231 breast carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). Antibodies against FAK and cleaved PARP (Asp 214) were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were from Pierce Biotechnology (Rockford, IL). Triptolide (98% pure) was purchased from Sigma-Aldrich. zVAD-fmk (benzoyl-valine-alanine-aspartate-O-methyl-fluoromethylketone) was purchased from R&D Systems (Minneapolis, MN). zVAD-fmk was used at a dose of 20 μM in all experiments with the exception in cell viability assay where 50 μM was used. BD Matrigel™ basement membrane matrix was purchased from BD Biosciences (San Jose, CA). Premixed WST-1 cell proliferation reagent was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Cell culture. Both MCF-7 and MDA-MB-231 breast cancer cell lines were maintained in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell lines were maintained at 5% humidified CO₂ atmosphere at 37°C and sub-cultured according to the supplier's recommendations twice a week. Only cells from <20 passages were used for experiments. Triptolide and zVAD-fmk were dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml and 20 mM stock concentrations, respectively, and subsequently stored at -20°C. Both were freshly diluted to the indicated concentrations with culture medium prior to experiments. DMSO concentrations in test conditions did not exceed 0.1%.

Soft agar colony formation assay. Cells were plated in a 6-well plate at a density of 1x10⁴ cells per well. The well was first layered with culture media supplemented with 10% FBS containing 0.5% agarose (Seakem LE agar, Cambrex). Cells were subsequently plated over the base layer in culture media supplemented with 10% FBS containing 0.3% agarose together with various concentrations of triptolide. When the agar had set, the growth medium was added to the top to prevent drying of the agar. The culture plate was incubated at 37°C for 14 days to allow for colony formation. For counting, colonies were fixed with 70% ethanol for 10 min, and subsequently stained with 0.01% crystal violet for 1 h before extensive washing with distilled water. Colonies >30 μm were counted.

Immunoblotting for FAK and PARP. Cells were lysed in ice cold lysis buffer A (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% glycerol, 1X protease inhibitor cocktail). The protein concentration in the extracts was determined using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). The protein extracts (50 μg total protein) were electrophoresed on 1.5-mm thick, 10% SDS-polyacrylamide gels using Bio-Rad Protean-III gel apparatus (Bio-Rad Laboratories, Singapore) in the presence of electrode buffer (25 mM Tris base, pH 8.3, 0.1% SDS). The gel contents were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Singapore) in ice-cold transfer buffer [39 mM glycine, 192 mM Tris base, 20% methanol (v/v)] in ice-cold lysis buffer A (20 mM Tris base, 20% methanol [v/v]). The membranes were subsequently blocked in wash buffer (10 mM Tris, pH 8.0; 150 mM NaCl, and 0.1% Tween-20) plus 5% skim milk for 1 h. The membranes were immobilized with primary antibody to FAK followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody. All antibodies were prepared in wash buffer plus 1% skim milk. The membranes were then stripped and re-probed with primary antibody to cleaved-PARP followed by HRP-conjugated goat anti-rabbit secondary antibody. Finally, the membranes were probed with actin antibody which acted as loading control. SuperSignal West Dura ECL Enhance Chemiluminescence (Pierce Biotechnology) was added prior to detection of chemiluminescence by FluorChem9000 (Alpha Innotech Corp, San Leandro, CA).

Cell detachment assay. Floating (detached cells in the supernatant) and adherent cells (obtained by trypsinization) were harvested separately after 24 and 48 h. A combination of elastase (MP Biomedicals, Illkirch, France) and trypsin was used to harvest cells. Cell counts were determined using a hemocytometer (Bright-Line, Haussser Scientific, USA) and trypsin blue exclusion method. All viable (unstained) and dead (stained) cells were counted, followed by determination of the percentage of cell detachment using the following equation:
% of detached cells = (number of floating cells/total number of cells) x 100%.

Cell adhesion assay. Cells were serum-starved for 2 h prior to treatment with 4 or 40 ng/ml triptolide or combination of 40 ng/ml triptolide and 20 µM zVAD-fmk. Adherent cells were harvested by trypsinization. Viable cells (8x10^5, as determined by trypan blue exclusion method) were transferred to a 24-well plate pre-coated with Matrigel, and allowed to adhere for 24 h at 37°C in a CO₂ incubator. Non-adherent cells were washed away using PBS for three times, and adherent cells were quantified by adding 200 µl pre-mixed WST-1 cell viability reagent (Clontech Lab Inc., Mountain View, CA) followed by a 30-min incubation at 37°C. Subsequently, absorbance was read using a Sunrise microtiter plate reader (Tecan Austria Gmbh, Grodig, Austria) with a wavelength of 440 nm. The percentage of adherent cells relative to that of vehicle control (% adherent cells = 100%) was determined.

Cell viability assay. MCF-7 cells were plated in 96-well plates at a density of 5000 cells per well. The culture medium was removed the following day, and cells were treated with fresh media or triptolide with or without 20 or 50 µM zVAD-fmk. Cells were subsequently incubated at 37°C in CO₂ incubator for 72 h. At the end of the incubation, 50 µl of MTT working solution (1 mg/ml in culture media) was added to each well. The plate was then further incubated at 37°C for 4 h to allow the formation of formazan. After 4 h, the MTT-containing medium was removed, and 150 µl DMSO was added to each well. The plate was shaken at 200 rpm until homogeneous purple coloration was obtained across the wells. Absorbance at 570 nm was measured using a Tecan Sunrise plate reader. Viability was calculated as follows: viability = (Abs_{test} - Abs_{blank})/(Abs_{vehicle control} - Abs_{blank}) x 100%, where Abs_{test}, Abs_{blank}, Abs_{vehicle control} represent the absorbance readings from the drug-treated wells, medium-only wells and vehicle control wells, respectively.

Statistical analysis. Statistical differences were analyzed using one-way ANOVA followed by the post-hoc Dunnett's test. Statistical significance was set at p<0.05. All results were expressed as the mean value of at least three independent experiments ± the standard error of the mean (SEM).

Results

Triptolide inhibits MCF-7 anchorage-independent growth and induces cell detachment. MCF-7 cells were exposed continuously to various concentrations of triptolide present in soft agar for a period of 14 days, and the formed colonies were counted and expressed as a percentage of the untreated control (Fig. 2A and B). Significant reduction in colony formation could be observed at 1.25 ng/ml (equivalent to 3.5 nM) triptolide, which is in good agreement with a previous report (20). Triptolide at 5 and 10 ng/ml, completely abolished MCF-7 colony formation. Morphological changes upon triptolide exposure were investigated under shorter exposure times of up to 48 h. As early as 8 h, 40 ng/ml triptolide induced a substantial loss of cell-cell contact and cell rounding. From 24 to 48 h, significant cell detachment from the culture plate was evident (Fig. 2C).

Concentration- and time-dependent FAK and PARP cleavage by triptolide was caspase-dependent. Cell detachment is mainly regulated by integrins, growth factor receptors and SRC-family kinases, whereas FAK has been demonstrated to be a key component in mediating the molecular signals involved in these pathways (16). As such, the role of FAK in
Triptolide-induced cell detachment was investigated. Triptolide exposure induced a concentration-dependent increase in the 77-kDa fragment of FAK, indicating that FAK cleavage was induced (Fig. 3A). It has been demonstrated that FAK cleavage is mediated by caspases; thus, triptolide-induced FAK cleavage was investigated in the presence of the pan-caspase inhibitor, zVAD-fmk, and the established caspase substrate, poly(ADP-ribose) polymerase (PARP), was used as the positive control. As shown in Fig. 3B, both FAK and PARP cleavage could be prevented by zVAD-fmk, which is an observation consistent with the notion that caspase activation mediates these two processes. Furthermore, through a time course study on triptolide-induced FAK and PARP cleavage, it was found that FAK cleavage preceded PARP cleavage, whereby FAK cleavage occurred as early as 2 h after triptolide exposure while PARP cleavage started only from 8 h (Fig. 3C). Of note, dephosphorylation of FAK at tyrosine397, which is important for FAK full enzyme activity and cell adhesion (21), occurred after 8 h of triptolide exposure (Fig. 3C).

To further elucidate the role of caspases in triptolide-induced FAK cleavage, various specific caspase inhibitors were used to probe the caspase specificity of FAK cleavage induced by triptolide. The caspase inhibitors used in the current study were selected based on previous reports describing caspase specificity of FAK cleavage (22,23). As shown in Fig. 3D, the presence of caspase-4, -6, -8 and -10 inhibitors in triptolide-treated MCF-7 cells induced a modest reduction in the level of cleaved FAK; however, the cleaved FAK levels in cells treated with these inhibitors were not statistically different from that in cells treated with triptolide alone (p>0.05). Only the pan-caspase inhibitor zVAD-fmk could produce a significant reduction in the level of cleaved FAK.

In addition to the expression of cleaved FAK, MCF-7 cell morphology and the ability to attach to the extracellular matrix were investigated to further correlate the role of FAK in mediating MCF-7 cell detachment and adhesion. As shown in Fig. 4A and B, the use of zVAD-fmk significantly reduced the percentage of detached cells from the culture plate upon triptolide exposure. In addition to culture plates, the adhesion potential of triptolide-treated MCF-7 cells was investigated through the use of Matrigel to simulate the extracellular matrix of the tumor microenvironment. Cells were exposed to triptolide with or without zVAD-fmk for 4 h, and the viability was ensured before the adhesion study to rule out the possibility that reduced adhesion was due to cell death. Consistent with the detachment studies, MCF-7 cell adhesion to the Matrigel was significantly compromised with triptolide exposure (Fig. 4C and D). Only in the presence of the pan-caspase inhibitor could triptolide-treated cells adhere to the Matrigel to an extent similar to the untreated control. Taken together, the results from Figs. 2-4 suggest an association between triptolide-induced FAK cleavage and impaired cell adhesion.

Triptolide-induced cell death is not reversed by the pan-caspase inhibitor zVAD-fmk. According to the results illustrated in Fig. 3D, the pan-caspase inhibitor zVAD-fmk was the only inhibitor that resulted in a significant preven-
tion of FAK cleavage. Thus, it was of interest to examine if zVAD-fmk could reverse triptolide-induced cell death in addition to its ability to inhibit triptolide-induced FAK cleavage. As shown in Fig. 5, zVAD-fmk did not significantly improve the viability of triptolide-treated MCF-7 cells (p>0.05). This observation suggests that the role of caspases in FAK cleavage, cell detachment and cell viability may be more complicated than anticipated in MCF-7 cells.

Figure 5. The pan-caspase inhibitor zVAD-fmk does not improve the viability of triptolide (Trp)-treated MCF-7 cells. MCF-7 cells were exposed to triptolide with or without zVAD-fmk for 48 h, and the viability was determined by the MTT assay as described in Materials and methods. Data presented are the means ± SEM from three independent studies.

Triptolide-induced FAK cleavage is specific to MCF-7 cells. To examine if FAK cleavage is a general phenomenon in response to triptolide exposure in breast cancer cells, another cell line, MDA-MB-231, was exposed to triptolide at a concentration similar to that used for MCF-7 cells over a time course of 48 h. Interestingly, no cleaved FAK was observed despite the presence of cleaved PARP (Fig. 6), suggesting that caspases are activated but may not be involved in FAK cleavage. Furthermore, the pan-caspase inhibitor zVAD-fmk was able to reverse triptolide-induced MDA-MB-231 cell death, whereby viability

Figure 4. Triptolide (Trp)-treated MCF-7 cell detachment (A and B) and reduced adhesion to Matrigel-based extracellular matrix (C and D). For cell detachment, MCF-7 cells were exposed to triptolide for 24 and 48 h with or without zVAD-fmk, and representative phase-contrast images are presented in (A). Floating and adherent cells were harvested and counted to determine the percentage of detached cells as described in Materials and methods, and data are presented as mean ± SEM from three independent studies in (B). For adhesion to Matrigel, MCF-7 cells were exposed to various treatment conditions for 4 h, followed by transfer and plating onto culture plates pre-coated with Matrigel. Cells were subsequently incubated for 24 h before determining the extent of cell adhesion. Representative phase-contrast images are presented in (C), and the extent of cell adhesion was expressed as a percentage of untreated control, with each data value representing mean ± SEM from three independent studies in (D). *p<0.05 as compared to vehicle control.
of cells was significantly increased from 43±5% (triptolide alone) to 70±12% (tritolide + zVAD-fmk) as determined by the MTT assay (p<0.05). Taken together, our results suggest that caspase-dependent FAK cleavage as a result of triptolide exposure is specific to MCF-7 cells.

Discussion

Triptolide in the herbal form of Tripterygium wilfordii has been widely used in China for the treatment of inflammatory and autoimmune diseases for centuries. In addition to its anti-inflammatory and immunosuppressive effects, the anti-cancer effect of triptolide was first reported by Kupchan in 1972 using a leukemia model (10), which has subsequently attracted much research effort in demonstrating the anti-cancer potential of triptolide in vitro and in vivo. As reviewed by Liu (1), the anti-cancer effect of triptolide could be mediated through multiple molecular targets and signaling pathways. These include apoptosis-related genes (such as caspase-3, -8 and -9), Bel-2 family proteins, tumor suppressors (such as p53 and p21), NF-κB signaling, MAPK signaling and heat shock proteins. Yet, FAK which is an important molecular target in cancer has never been examined upon triptolide treatment. As such, it is the intention of the current study to elucidate the effect of triptolide on FAK and cell survival, using MCF-7 cells which have distinctive properties of compromised apoptotic machinery (caspase-3-deficient) and low autophagic activity. Several interesting implications could be drawn from the results presented here.

FAK is a non-receptor tyrosine kinase that localizes to focal adhesions. It associates temporally and spatially with integrins (24,25), and also with other components of focal adhesions such as paxillin, p130Cas, GRB2, pp60src, pp59fyn, talin, and phosphatidylinositol 3’-kinase (22). Phosphorylation at tyrosine397 of FAK is crucial to dynamic adhesions (26), and also with other components of focal adhesions (24,25). Phosphorylation at tyrosine397 of FAK is crucial to dynamic adhesions (26), and indicates FAK activation (21). Loss of focal adhesion sites, followed by cell detachment and death are preceded by FAK dephosphorylation and degradation (27). FAK is expressed at low levels in normal cells but is overexpressed in 88% of invasive and metastatic breast tumors (16). In light of the importance of FAK in tumorigenesis and progression, small-molecule inhibitors of FAK are actively being developed and have entered into early-phase clinical trials with promising results (28). According to the results in Fig. 3, triptolide was demonstrated to induce concentration- and time-dependent cleavage of FAK and dephosphorylation, and these results further corroborated with those presented in Figs. 2 and 4 in which triptolide-treated MCF-7 cells showed significant detachment from culture plates and disruption in adhering to Matrigel-based extracellular matrix. These observations provide strong evidence that triptolide could be a potential inhibitor of FAK with potency in the nanomolar range.

It has been demonstrated that FAK could be cleaved by caspasas and that FAK showed unique sensitivity to different caspases (22). According to the results in Fig. 3, it is clear that FAK cleavage could be significantly reduced in the presence of the pan-caspase inhibitor, zVAD-fmk. Further probing with various caspase-specific inhibitors, selected based on previous reports on caspase-dependent FAK cleavage (22,23), showed that zVAD-fmk was the only inhibitor effective in reducing FAK cleavage upon triptolide treatment. zVAD-fmk is an inhibitor of caspase-1, -3, -4 and -7. It is likely that FAK cleavage in MCF-7 cells is mediated through caspase-7, based on the following notions: i) MCF-7 cells are deficient in caspase-3; ii) the caspase-4-specific inhibitor was not effective in preventing FAK cleavage (Fig. 3D) and caspase-1 is acting downstream of caspase-4; and iii) FAK cleavage has been reported to be more sensitive to caspase-7 in comparison to caspase-3. Nevertheless, other molecular regulations of FAK cleavage may be involved in MCF-7 cells. FAK expression has been shown to be regulated by p53 (29). It is possible that p53 could also contribute to triptolide-induced FAK dephosphorylation and degradation, as triptolide could increase p53 expression in wild-type MCF-7 cells as previously reported (6).

Caspase activation has been a well-established hallmark of apoptosis. As caspase activation is involved in triptolide-induced FAK cleavage, it was thus of interest to examine the role of caspase activation in triptolide-induced cell death, and in particular, in the caspase-3-deficient MCF-7 cells. Surprisingly, the use of zVAD-fmk could prevent FAK cleavage but did not improve cell viability upon exposure to triptolide (Fig. 5), despite the clear evidence of PARP cleavage which could be prevented by zVAD-fmk (Fig. 3). This result suggests that the role of caspase in triptolide-induced FAK cleavage and cell death might be more complicated than expected in MCF-7 cells. It is possible that FAK cleavage may be mediated by a specific caspase (potentially, caspase-7) that is not directly involved in triptolide-mediated cell death. Alternatively, other caspasas might be involved in mediating triptolide-induced cell death that is independent of PARP cleavage. Yet, FAK cleavage and cell death may be regulated by different molecular mechanisms with caspasas playing differential roles in these two processes. Taken together, further studies that attempt to correlate p53 expression, activation of specific caspasas, FAK cleavage and cell death would shed new light on the molecular mechanism of triptolide.

In summary, the current study presented novel evidence on the ability of triptolide to induce concentration- and time-dependent FAK cleavage that is correlated with MCF-7 cell detachment and inability to adhere to the extracellular matrix. The current findings suggest that triptolide could be a potential inhibitor to FAK and could modulate the metastatic potential of MCF-7 cells. Further development of triptolide derivatives with improved toxicity profiles and specificity may produce promising anticancer drug candidates that could benefit not only breast cancer but also other types of malignancies.
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