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Inhibition of Invasion in Pancreatic Cancer Cells by Conjugate of EPA with $\beta^{3,3}$-Pip-OH via PI3K/Akt/NF-kB Pathway

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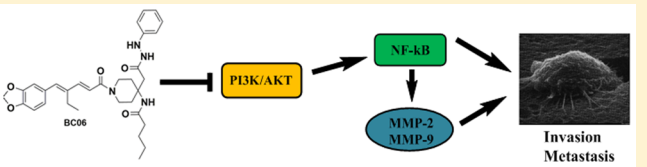
Supporting Information

ABSTRACT: The present work describes the anti-invasive effect of conjugate BC06, a novel conjugate of EPA, (2*E*,4*E*)-4-(benzo[*d*][1,3]dioxol-5-ylmethylene) hex-2-enoic acid with *β*,*β*-disubstituted-*β*-amino acid, $\beta^{3,3}$-Pip-OH (2-(4-aminopiperidin-4-yl)acetic acid), in human pancreatic carcinoma. The conjugate BC06 inhibited invasion and migration of PANC-1 cells in wound healing, matrigel invasion, and gelatin degradation assays. Apart from suppressing PI3K/Akt/NF-kB signaling, which is involved in the up-regulation of matrix metalloproteinases, our study also demonstrated that dose-dependent treatment of BC06 results in the upregulation of TIMP-1 and E-cadherin expression. Further, BC06 was found to be inhibiting the metastatic ability of PANC-1 cells by reducing MMP-2 and MMP-9 expression. These findings suggest that EPA conjugate with $\beta^{3,3}$-Pip-OH, BC06, may be used as an anti-invasive agent against human pancreatic carcinoma.



KEYWORDS: *β,β-disubstituted-β-amino acid, piperic acid, metastasis, AKT, invasion*

Pancreatic cancer remains the fourth leading cause of all cancer deaths in most developed countries including United States.[1,2] Phosphatidylinositol 3-kinase (PI3K)/AKT pathway is constitutively activated in most pancreatic cancers and offers effective targets for therapeutic development.[3−5] Akt is involved in the progression of metastasis through a wide variety of signaling molecules and mechanisms.[5] Further, transcription factor nuclear factor-kB (NF-kB), which is a direct target of Akt, has been correlated with increased metastatic potential in pancreatic cancer and has been associated with metastasis and invasion in many tumors.[6,7] Recent literatures demonstrate that NF-kB is constitutively activated in most pancreatic cancer cells,[8] in animal models for pancreatic cancer,[9] and in human pancreatic cancer tissues as well.[8] NF-kB promotes the migration and invasion of pancreatic cancer cells by a number of NF-kB-regulated gene products.[8] Unfortunately, the tolerable and specific inhibitors of the PI3K/Akt pathway are lacking until date to target pancreatic cancer. The present study unveils the identification of novel inhibitors of the PI3K/Akt pathway based on conjugation of EPA, (2*E*,4*E*)-4-(benzo[*d*][1,3]dioxol-5-ylmethylene)hex-2-enoic acid with *α*- and nonprotein amino acids shown in Figure 1.

EPA is 4-ethyl substituted piperic acid.[10] Piperic acid is obtained from the hydrolysis of piperine, an alkaloid present in black pepper (*Piper nigrum-L/Piper longum*).[11] It has been reported that piperine suppresses tumor growth and metastasis in 4T1 murine breast cancer model.[12] The inhibition of proliferation of human osteosarcoma by piperine via G2/M phase arrest and metastasis by repressing MMP-2/-9 expression has also been reported.[13]

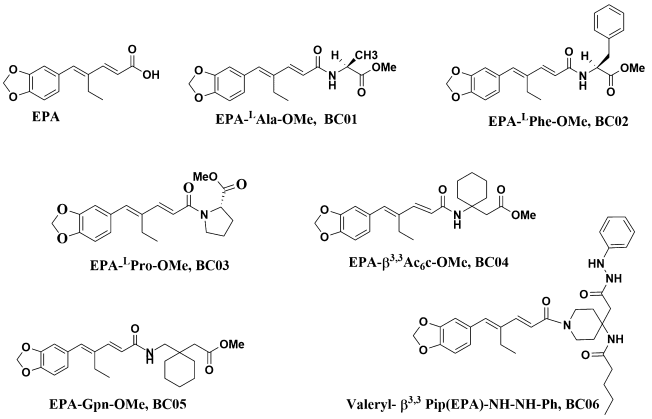


Figure 1. Chemical structure of conjugates of EPA with [L]Ala (BC01), [L]Phe (BC02), [L]Pro (BC03), $\beta^{3,3}$-Ac_6c (BC04), Gpn (BC05), and $\beta^{3,3}$, Pip-OH (BC06).

EPA was synthesized using the procedure described earlier.[14] *β*,*β*-disubstituted-*β*-amino acids, $\beta^{3,3}$-Ac_6c and $\beta^{3,3}$-Pip-OH, were synthesized using a procedure reported in the literature.[15] Further, the conjugates of EPA, BC01−BC05, were synthesized by treatment of EPA with ester hydrochloride of amino acids in the presence of 1-(3-(dimethylamino)propyl)-3-ethyle carbodiimide hydrochloride (EDCI.HCl) and 4-methylmorpholine

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(NMM) in dry dichloromethane (Scheme S2, see Supporting Information). The conjugate BC06 was synthesized by coupling of valeryl-$\beta^{3,3}$Pip(NH)-NH-NH-Ph with EPA using EDCI·HCl and NMM in dry dichloromethane (Scheme S3, see Supporting Information).

The cell viability assay of all the conjugates was performed on a panel of moderate to highly aggressive cancer cell lines. The respective compounds were exposed to the cells with concentration range (100 nM to 100 μM) for a period of 48 h. The conjugate BC06 exhibited its promising and consistent cytotoxic activity in Panc-1 cell line with an IC_{50} concentration of 4 μM, the lowest among all the conjugates, tested in all the cell lines (Table 1).

Table 1. Cytotoxic Effects of Synthesized Compounds (BC01−BC06) on a Panel of Three Different Cancer Cell Lines, *viz.*, PANC-1, PC-3, and HCT-116 through Cell Viability (MTT) Assay[a]

compd	PANC-1 IC_{50} (μM) ± SD	PC-3 IC_{50} (μM) ± SD	HCT- 116 IC_{50} (μM) ± SD
EPA	>100	>100	>100
BC01	6.7 ± 0.1	43.5 ± 0.4	47.1 ± 0.3
BC02	>100	41.9 ± 0.3	48.3 ± 0.5
BC03	42.2 ± 0.5	16 ± 0.3	49.8 ± 0.4
BC04	>100	20 ± 0.2	>100
BC05	10 ± 0.2	45.2 ± 0.3	47.6 ± 0.2
BC06	4 ± 0.5	15 ± 0.1	>100

[a]Data were compared with untreated control, and IC_{50} values were expressed as mean ± SD of three independent experiments.

As pancreatic cancer invasion and metastasis remains the most critical determinant of resectability and hence survival, so we aimed to study the effect of conjugate BC06 on the invasive potential of PANC-1 cells *in vitro*. Wound healing assay was performed to conclude whether conjugate BC06 could inhibit motility of Panc-1 cells. After 48 h of incubation with 2.0 and 4.0 μM of conjugate BC06, motility of PANC-1 cells inhibited significantly ($p < 0.05$), similar to staurosporine (Figure 2A), whereas the cells treated with vehicle DMSO essentially migrated through the wounded area to close the wound (Figure 2A). Also, the colony formation ability of Panc-1 cells was decreased by conjugate BC06 in a statistically significant manner ($P < 0.05$) (Figure 2B).

The critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the limits of primary tumor environment.[16] Boyden chamber invasion assay was carried out to study the effect of conjugate BC06 on cell invasion. As shown in Figure 2C, treatment with BC06 (2.0 and 4.0 μM) inhibited cell invasion ($P < 0.05$). Further, the effect of conjugate BC06 on cell invasion was observed using 3D invasion assay. DMSO treated cells showed increased number of invasive cells protruding beyond the original cell collagen boundary, whereas indicated treatment of conjugate BC06 suppressed the cell invasion in a dose-dependent manner (Figure 3A). These collectively demonstrate that EPA conjugate of $\beta^{3,3}$-Pip-OH, BC06, alters the invasion and metastatic potential of PANC-1 cells.

In cancer, metastasis and invasion are the two processes that MMPs are thought to mediate. Type IV collagen acts as a substrate for MMP-2 and MMP-9, and reports have connected the overexpression of these enzymes in invasion and metastasis.[17] Cells were cultured on a cross-linked fluorophore (FITC)-conjugated-gelatin matrix-coated coverslips for 24 h in

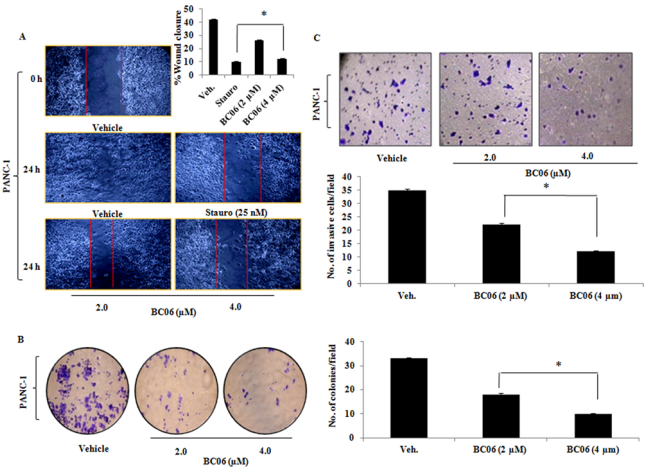

Figure 2. Effect of conjugate BC06 on motility, colony formation and invasive capability of pancreatic cancer cells. (A) Wound healing assay was performed by treating PANC-1 cells with different concentrations of conjugate BC06 to assess the degree of wound healing. Scratched areas were photographed (20× magnification) at zero hour and at 24 h. (B) Colony formation assay was carried out against PANC-1 cells (1×10^3 cells/well) and treated with various concentrations of conjugate BC06 along with DMSO for 5 days. The number of crystal violet stained colonies were counted randomly and quantified, and images were captured under inverted microscope at 20× magnification. (C) Cells were treated with various concentrations of BC06 for 24 h and analyzed for the invasive ability through matrigel invasion assay. The invaded cells from five random fields in each treatment group were counted and photographed under an inverted microscope (20× magnifications). Data from three independent experiments were subjected to statistical analysis. ($n = 3$, $^*p < 0.05$).

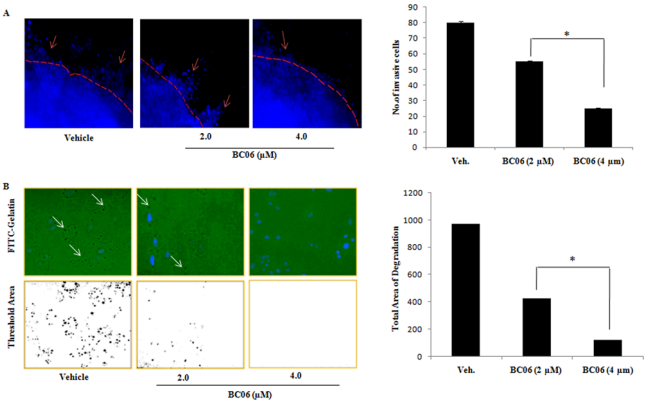

Figure 3. Effect of conjugate BC06 on invasion and matrix degradation in PANC-1 cells. (A) PANC-1 cells were combined with neutralized collagen, placed into the center of each well of a 96-well plate, and allowed to solidify, forming a cell-collagen hemisphere with a distinct boundary as described in materials and methods (Supporting Information). Fresh media was then added to each well, and the cells were allowed to invade into the surrounding matrix in the presence or absence of indicated concentrations of conjugate BC06. (B) The antimetastatic effect of conjugate BC06 was assessed by culturing PANC-1 cells on FITC conjugated gelatin matrix. Image-J software was used to process and analyze the threshold areas of degradation. Degradation zone was indicated by arrows. Statistical analysis was performed on data obtained from three independent experiments. ($n = 3$, $^*p < 0.05$).

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order to examine the ability of pancreatic cancer cells to degrade the matrix. Figure 3B clearly shows that higher concentrations of conjugate BC06 inhibited the matrix gelatin degradation by aggressive Panc-1 cells (indicated by arrows).

Image-J software highlights the degraded area, which supports the spots of gelatin matrix degradation. Further, we ought to investigate the effect of conjugate BC06 on MMP-2 and MMP-9 expression in PANC-1 cells. The immunoblot experiments demonstrated that treatment of PANC-1 cells with conjugate BC06 negatively regulated both MMP-2 and MMP-9 expressions. As TIMP-1 is known to inhibit matrix metalloproteinases and seen to suppress metastasis,[18] we further studied the effect of conjugate BC06 on TIMP-1 expression. The Western blot experiments demonstrated that the treatment of conjugate BC06 resulted in upregulation of TIMP-1 expression in a dose-dependent manner (Figure 4A).

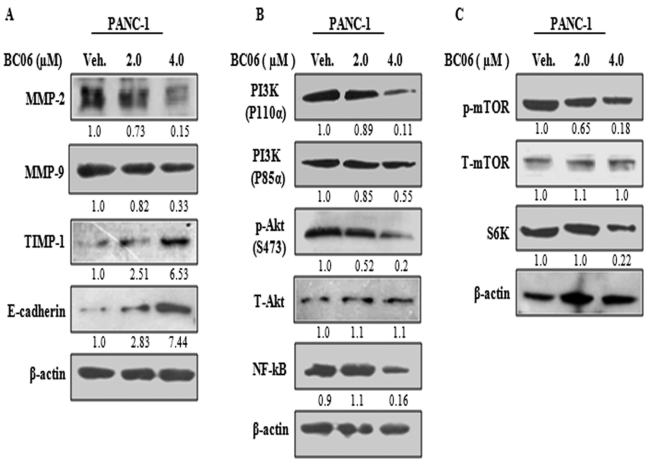

Figure 4. Conjugate BC06 inhibits MMP-2 and MMP-9 expression through the inhibition of PI3K/Akt/NF-KB pathway. PANC-1 cells were seeded in six well plates and treated with indicated concentrations of conjugate BC06. Whole cell lysates were checked for the expressions of indicated proteins by Western blotting. Values below the Western blots indicate the relative protein expression obtained by densitometric analysis of the bands.

The expression of E-cadherin or its cell surface localization is often lost in advanced tumors and has been linked to a higher incidence of metastasis and tumor recurrence.[19,20] As both loss of E-cadherin and the overexpression of MMPs are common features of an invasive phenotype, we further studied the effect of conjugate BC06 on the expression of E-cadherin. The Western blot experiments showed that the treatment of conjugate BC06 caused a robust amplification of E-cadherin in PANC-1 cells (Figure 4A).

Akt is regarded as one of the strong promoters of tumorigenecity in pancreatic cancer.[21] Also in majority of pancreatic tumors, NF-kB is constitutively activated through a phosphatidylinositol 3-kinase (PI3K)-dependent activation of IKK.[4−6] Moreover, MMPs are regulated primarily through nuclear factor-κB (NF-κB) at the level of transcription through PI3K/Akt pathway; their cell surface localization as well as activation/inhibition are regulated at the post-transcriptional level.[22,23] Rationally, we sought to study the effect of conjugate BC06 on phosphorylation of Akt and expression of PI3K and NF-kB. The immunoblot results showed that conjugate BC06 treatment tremendously impaired phosphorylation of Akt (S473) and also decreased PI3K (P85α), PI3K (P110α), and NF-KB in a dose-dependent manner (Figure 4B). As Akt is known to regulate NF-KB via mTOR,[24] we studied the effect of conjugate BC06 on mTOR and S6K. The immunoblot results demonstrated that conjugate BC06 treatment caused a sharp decrease in phosphorylation of mTOR along with down-regulation of S6K. There was no effect on total mTOR expression following BC06 treatment (Figure 4C).

Collectively, we have shown here that the conjugate of EPA with $\beta^{3,3}$-Pip-OH, BC06, significantly decreases invasion and metastasis in PANC-1 cells by downregulating MMP-2 and MMP-9 via suppression of PI3K/Akt/NF-kB pathway. Hence, we strongly suggest that EPA conjugate with $\beta^{3,3}$-Pip-OH can be used as an effective antimetastatic agent against advanced pancreatic cancer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00257.

Detailed procedure for the synthesis of EPA and conjugates BC01-06, analytical data, and biological assays (PDF)

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Notes

The authors declare no competing financial interest.

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