RESEARCH PAPER

Synthesis and Biological Evaluation of Piperic Acid and 4-Ethylpiperic Acid β-Amino Acid Conjugates as Anticancer Agents Targeting PI3K/Akt/NF-κB Signaling in Pancreatic Cancer Cells

Preeti Shrivastava[1], Pranjal Upadhyay[2], Maila Swapna[3], B. Madhavilatha[4], Yasmin Khatoon[5], Mounica Peela[6], Siddharth Sharma[7], Sonu[8*]

[1]Faculty, Department of Biochemistry, Mata Jijabai Govt PG Girls College, Indore, Madhya Pradesh 452001 India

[2]Intern, Datta Meghe Medical College (DMIHER), Wanadongri, Hingna, Nagpur, 441110 India

[3]Assistant Professor, Nalla Narsimha Reddy Education Society's Group of Institutions- Integrated Campus, Medchal Malkajgiri, Telangana 500088 India

[4]Associate Professor, Nalla Narsimha Reddy Education Society's Group of Institutions- Integrated Campus, Medchal Malkajgiri, Telangana 500088 India

[5]Professor, Institute of Pharmacy, Shri Ramswaroop Memorial University, Barabanki, Lucknow, Uttar Pradesh 225003 India

[6]Assistant Professor, Vignan Institute of Pharmaceutical Technology (A), Duvvada, Kapujaggarajupeta, Visakhapatnam, Andhra Pradesh 530049 India

[7]Assistant Professor, Chandigarh Pharmacy College, CGC University, Mohali, Punjab 140307 India

[8*]Assistant Professor, School of Pharmaceutical Sciences, Rayat Bahra University, Village - Sahauran, Tehsil-Kharar, Mohali, Punjab, 140104 India

Email: sonu.19177@rayatbahrauniversity.edu.in

(Corresponding Author)

Abstract

Background: Pancreatic cancer is one of the most aggressive malignancies with limited therapeutic options, primarily due to late detection, invasive behavior, and resistance to chemotherapy. The phosphatidylinositol 3-kinase (PI3K)/Akt/NF-κB signaling axis is constitutively activated in pancreatic tumors, driving proliferation, survival, and metastasis. Natural product scaffolds such as piperic acid (PA) and 4-ethylpiperic acid (EPA) have shown anticancer activity, and incorporation of β-amino acids has been proposed to enhance stability and potency. **Methods:** In this study, PA and EPA were conjugated with β-amino acids via carbodiimide-mediated coupling. The resulting conjugates were structurally confirmed using FTIR, NMR, and mass spectrometry. Biological evaluation was performed against PC-3 (prostate), PANC-1 (pancreatic), and HCT-116 (colorectal) cell lines using cytotoxicity (MTT assay), clonogenic, wound-healing, Matrigel invasion, 3D collagen invasion, cell scattering, and fluorescent gelatin degradation assays. Apoptosis was assessed by DAPI staining, caspase-3/7 activity, and PARP cleavage. Western blotting was used to evaluate PI3K/Akt/NF-κB, MAPK/ERK, MMP-2/9, TIMP-1, and E-cadherin expression, while cell cycle effects were analyzed using FUCCI sensor technology. **Results:** Among all derivatives, conjugates 5 (PA-based) and 20 (EPA-based) exhibited the most potent activity. Conjugate 5 induced apoptosis in PANC-1 cells (IC_{50} ~7.0 μM) via PARP and caspase-3 cleavage, BAX upregulation, BCL2/XIAP downregulation, G2 arrest, and inhibition of MAPK/ERK phosphorylation. Conjugate 20 strongly inhibited migration, clonogenicity, and invasion (IC_{50} ~4.0 μM in PANC-1), suppressed invadopodia-mediated ECM degradation, downregulated MMP-2/9, induced TIMP-1, restored E-cadherin, and inhibited PI3K/Akt/NF-κB and mTOR/S6K signaling cascades. **Conclusion:** PA and EPA β-amino acid conjugates display distinct but complementary anticancer mechanisms, with conjugate 5 acting as an apoptosis inducer and conjugate 20 as an anti-metastatic agent. Their ability to modulate PI3K/Akt/NF-κB signaling underscores their potential as promising leads for targeted pancreatic cancer therapy.

Keywords: Piperic acid conjugates; β-amino acids; Pancreatic cancer; PI3K/Akt/NF-κB signaling; Anticancer and anti-metastatic agents

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1. Introduction

Pancreatic cancer remains one of the most lethal malignancies, characterized by rapid progression, late diagnosis, and poor survival outcomes. Globally, it ranks among the leading causes of cancer-related mortality, with a five-year survival rate of less than 10%, highlighting its highly aggressive nature [1]. The major challenge in treating pancreatic cancer lies in its asymptomatic progression during early stages and its strong resistance to conventional chemotherapeutic regimens. Tumor recurrence, invasion of surrounding tissues, and metastasis to distant organs often occur before clinical detection, thereby reducing the efficacy of existing therapeutic strategies [2]. Moreover, the dense stromal microenvironment of pancreatic tumors poses an additional barrier to drug penetration, making this cancer particularly difficult to treat [3]. Thus, there is a critical need for the development of novel therapeutic agents that not only inhibit proliferation but also target the molecular mechanisms responsible for invasion and metastasis. At the molecular level, pancreatic cancer is driven by aberrant signaling cascades that sustain uncontrolled growth, resist apoptosis, and promote metastatic dissemination. Among these, the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling axis plays a central role in regulating cell survival, proliferation, angiogenesis, and motility [4]. Hyperactivation of the PI3K/Akt pathway is frequently observed in pancreatic tumors and has been associated with tumor progression, therapy resistance, and poor prognosis [5]. Akt, a serine/threonine kinase, regulates downstream effectors that control cell cycle progression and prevent apoptosis, thereby conferring a survival advantage to cancer cells [6]. Furthermore, Akt signaling is intricately linked to the transcription factor nuclear factor-kappa B (NF-κB), which is constitutively activated in pancreatic cancer [7]. NF-κB enhances tumor aggressiveness by upregulating anti-apoptotic genes, inflammatory mediators, and matrix metalloproteinases (MMPs) that facilitate invasion and metastasis [8]. Together, the PI3K/Akt/mTOR and NF-κB pathways establish a pro-oncogenic environment that supports tumor progression and metastasis, making them attractive therapeutic targets for pancreatic cancer intervention [9]. In recent years, natural products have emerged as valuable sources for anticancer drug discovery due to their structural diversity, ability to modulate multiple targets, and relatively favorable safety profiles [10]. Several plant-derived compounds have shown promising activity against pancreatic cancer, either as single agents or in combination therapies. Piperine, an alkaloid derived from black pepper (*Piper nigrum*), has attracted attention for its broad spectrum of biological activities, including anti-inflammatory, antioxidant, and anticancer effects [11]. Studies have shown that piperine inhibits tumor cell proliferation, induces apoptosis, and suppresses metastasis in multiple cancer models, including breast, colon, and prostate cancer [12]. Structural analogs of piperine, particularly piperic acid and its derivatives, have also demonstrated notable cytotoxicity and tumor-selective properties [13]. Piperic acid amides have been reported to inhibit tumor growth, suppress metastatic spread, and modulate apoptotic pathways, positioning them as potential lead compounds for anticancer drug development [14]. Importantly, these molecules exert their effects partly through modulation of the PI3K/Akt/NF-κB signaling cascade, aligning with the key therapeutic targets in pancreatic cancer [15]. Despite these promising attributes, limitations such as metabolic instability, suboptimal pharmacokinetics, and incomplete tumor selectivity have restricted the clinical translation of piperic acid derivatives. To overcome these challenges, structural modifications and conjugation strategies have been employed to enhance potency, stability, and selectivity [16]. One such strategy involves the incorporation of β-amino acids into bioactive conjugates. Unlike the naturally occurring α-amino acids that form the building blocks of proteins, β-amino acids possess an additional methylene group, which confers unique chemical and biological properties. Incorporation of β-amino acids into natural product scaffolds improves metabolic stability, resists enzymatic degradation, and often enhances receptor binding affinity [17]. Furthermore, β-amino acids are recognized as structural motifs in several bioactive natural products and therapeutic agents, including anticancer, antimicrobial, and immunomodulatory compounds. Their integration into piperic acid derivatives is thus expected to yield novel conjugates with improved bioavailability, enhanced biological activity, and greater therapeutic potential. The concept of designing piperic acid and 4-ethylpiperic acid β-amino acid conjugates stems from the dual need to improve the pharmacological properties of natural scaffolds and to develop agents that can selectively inhibit key signaling pathways in pancreatic cancer. By modifying the parent structures with β-amino acids,

these conjugates are hypothesized to achieve increased potency against cancer cells, with specific emphasis on inhibiting metastasis and invasion through suppression of the PI3K/Akt/NF-κB pathway. Previous studies have demonstrated that β-amino acid–containing conjugates of natural compounds display improved cytotoxicity profiles and selective activity against aggressive cancer cell lines [14,16]. Building upon this rationale, the present study aims to synthesize and biologically evaluate piperic acid and 4-ethylpiperic acid β-amino acid conjugates for their anticancer efficacy, particularly focusing on pancreatic cancer cells. The primary objective of this investigation is to establish a mechanistic link between the structural modifications of piperic acid derivatives and their functional ability to disrupt oncogenic signaling pathways in pancreatic cancer. Specifically, the study seeks to determine whether these β-amino acid conjugates can effectively reduce cancer cell viability, inhibit migration and invasion, and induce apoptosis by targeting the PI3K/Akt/NF-κB axis. Through a systematic synthesis and bioevaluation approach, the research also intends to compare the relative activities of different conjugates, thereby identifying lead candidates with superior anticancer profiles. Ultimately, the goal is to provide preclinical evidence that these structurally modified conjugates hold promise as therapeutic agents capable of counteracting the invasive and resistant nature of pancreatic cancer.

2. Materials and Methods

Materials

Piperic acid, 4-ethylpiperic acid, and β-amino acids were procured from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media (DMEM, RPMI-1640), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco (Thermo Fisher Scientific, USA). Antibodies against PI3K, Akt, NF-κB, MMP-2, MMP-9, PARP, and caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA). All other solvents and reagents used were of analytical grade and purchased from Merck, India.

2.1. Synthesis of Conjugates

Step 1: Starting acids (PA/EPA)

Piperic acid (PA): Piperine (1.0 equiv) was refluxed with ethanolic NaOH (2 M, 5–6 equiv) for 2–3 h. The solvent was removed and the aqueous layer was acidified to pH ~2 with ice-cold 2 M HCl. The resulting yellow solid was filtered, washed, and recrystallized from ethanol to obtain PA with ≥95% purity. 4-Ethylpiperic acid (EPA): EPA was obtained from a commercial source (analytical grade) or from a previously prepared batch (≥95% purity confirmed by HPLC/LC-MS).

Step 2: Acid activation (EDCI/NMM)

PA/EPA (1.0 equiv) was dissolved in dry DCM (0.05–0.1 M) under an ice-bath (0–5 °C). EDCI·HCl (1.2–1.5 equiv) and N-methylmorpholine (NMM) or DIPEA (2.0–3.0 equiv) were added. Optionally, HOBt (0.1–0.2 equiv) was added to suppress O→N acyl transfer. The mixture was stirred for 10–15 min for pre-activation under inert N_2.

Step 3: Coupling with β-amino acid

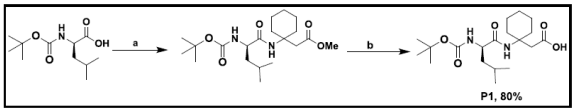
The chosen β-amino acid (free base; if HCl salt, 1.0 equiv extra base was added) was introduced into the same solvent (1.05–1.2 equiv). The reaction mixture was allowed to warm from 0–5 °C to room temperature (22–25 °C) and was stirred for 12–18 h. Progress was monitored by TLC/LC-MS until disappearance of the acid spot.

Step 4: Work-up

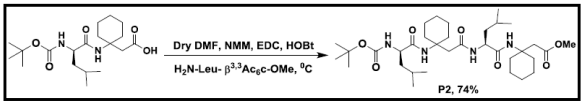
The reaction was quenched with saturated aq. NH_4Cl (or 1 M HCl), and the organic layer was separated. The organic fraction was washed sequentially with saturated $NaHCO_3$ followed by brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure at ≤35 °C.

Step 5: Purification

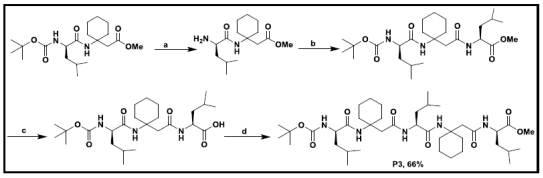
The crude products were purified by column chromatography over silica gel (100–200 mesh). For non-polar conjugates, a Hexane:EtOAc gradient (7:3 → 1:1) was employed, while for polar/basic conjugates, a DCM:MeOH gradient (100:0 → 95:5) containing 0.1% Et_3N was used to avoid tailing. The appropriate fractions were pooled, solvents were removed under reduced pressure, and the products were dried under vacuum to constant weight. Typical isolated yields ranged between 55–80% [17,18].



Scheme 1. Stepwise synthesis of compound P1 via peptide coupling (a) and ester hydrolysis (b), yielding P1 in 80%.



Scheme 2. Coupling reaction of intermediate with H_2N-Leu-β³,³Ac₆c-OMe using EDC/NMM/HOBt in dry DMF at 0 °C to yield compound P2 (74%).



Scheme 3. Multistep synthesis of compound P3 (66%) via sequential transformations (a–d).

2.2. Cell Culture and Reagents

Human pancreatic carcinoma (PANC-1), prostate carcinoma (PC-3), and colorectal carcinoma (HCT-116) cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). PANC-1 and HCT-116 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), while PC-3 cells were maintained in RPMI-1640 medium, each supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Primary antibodies against PI3K (p85α, p110α), phospho-Akt (Ser473), NF-κB (p65), MMP-2, MMP-9, TIMP-1, and E-cadherin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for caspase-3, PARP, BCL2, BAX, XIAP, and β-actin (loading control) were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All culture media, sera, and antibiotics were of cell-culture grade, while other reagents used were of analytical grade (Merck, India) [19].

2.3. Biological Assays

Cytotoxicity (MTT assay): The cytotoxic activity of synthesized conjugates was assessed using the standard MTT dye uptake method. Briefly, PANC-1, PC-3, and HCT-116 cells were seeded in 96-well plates (2.5×10^3 cells/well) and treated with varying concentrations of test compounds for 48 h. Cell viability was quantified by measuring absorbance at 570 nm (TECAN Infinite M200 Pro, Switzerland), and IC_{50} values were calculated from dose–response curves [20].

Clonogenic assay: To evaluate the long-term proliferative potential, PANC-1 cells (1×10^3 cells/well) were seeded in 6-well plates, treated with conjugates for 5 days, and then cultured in drug-free medium for 10–14 days. Colonies were fixed with methanol, stained with crystal violet (0.5%), and counted under an inverted microscope [21].

Wound healing assay: Cell migration was studied by creating a linear scratch on a confluent PANC-1 monolayer with a sterile pipette tip. Cells were treated with conjugates in low-serum medium (1% FBS), and images were captured at 0 and 24 h using a Nikon D3100 inverted microscope. Migration rates were quantified by ImageJ software [22].

Matrigel and 3D invasion assays: Anti-invasive potential was determined using a Matrigel-coated Boyden chamber system (BD Biosciences, USA). PANC-1 cells (1.2×10^6) were seeded in the upper chamber, treated with test compounds, and allowed to invade for 24 h. Invaded cells were fixed, stained, and quantified microscopically. Additionally, a 3D collagen invasion assay was performed, where PANC-1 spheroids were embedded in rat tail collagen matrix and invasion into the surrounding gel was monitored microscopically [23].

Cell scattering assay: To examine epithelial-to-mesenchymal transition (EMT)-like behavior, PANC-1 colonies were stimulated with VEGF (20 ng/mL) in the presence of test compounds. Morphological changes and scattering patterns were observed under an inverted microscope, with suppression indicating inhibition of mesenchymal transition.

Fluorescent gelatin degradation assay: Invadopodia activity was measured by culturing PANC-1 cells on FITC-conjugated gelatin-coated coverslips. After 24 h of treatment, cells were fixed, stained with DAPI, and imaged on a fluorescence microscope. Gelatin degradation zones were quantified using ImageJ, indicating invadopodia-mediated ECM degradation [24].

Apoptosis detection: Nuclear morphological changes were detected by DAPI staining after compound exposure. Caspase-3/7 activity was quantified using the Caspase-Glo® 3/7 assay kit (Promega, USA), while PARP cleavage was analyzed by western blotting as a hallmark of apoptosis.

Cell cycle analysis: The effect of conjugates on cell cycle progression was examined using the Premo-FUCCI cell cycle sensor (Invitrogen, USA). Following treatment, cells were analyzed under a fluorescence microscope, and distribution across G0/G1 and G2/M phases was determined [25,26].

Western blotting: Protein expression studies were performed to elucidate molecular mechanisms. Whole-cell lysates from treated and control cells were resolved on SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against PI3K, phospho-Akt (Ser473), NF-κB, MMP-2, MMP-9, TIMP-1, E-cadherin, PARP, caspase-3, BCL2, BAX, and XIAP. β-actin served as a loading control, and protein bands were visualized using chemiluminescence [27].

2.4. Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical comparisons between treated and control groups were carried out using Student's *t*-test. A *p*-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Cytotoxicity of Conjugates

The cytotoxic potential of piperic acid (PA), 4-ethylpiperic acid (EPA), and their β-amino acid conjugates was evaluated against PC-3 (prostate), PANC-1 (pancreatic), and HCT-116 (colorectal) cell lines using the MTT assay. Comparative IC_{50} analysis demonstrated that both parent acids (PA and EPA) exhibited relatively weak activity, with IC_{50} values above 80 µM across all tested cell lines. In contrast, several β-amino acid conjugates showed markedly enhanced cytotoxicity, particularly against the PANC-1 cells. Among these, conjugate **5** (a piperic acid derivative) displayed strong antiproliferative activity with an IC_{50} of ~7.0 µM, while conjugate **20** (an EPA-based derivative) showed the most potent effect, with an IC_{50} of ~4.0 µM in PANC-1 cells. Notably, conjugate 20 also reduced viability in PC-3 cells (IC_{50} ~15 µM) but had limited cytotoxicity against HCT-116 (>100 µM), indicating selective action towards pancreatic cancer cells. These findings identify conjugates 5 and 20 as the lead compounds, with conjugate 20 emerging as the most promising candidate for further anti-metastatic studies.

Table 1. Cytotoxic effects of piperic acid (PA), 4-ethylpiperic acid (EPA), and their conjugates against PC-3, PANC-1, and HCT-116 cells (MTT assay)

Compound	PC-3 (µM)	PANC-1 (µM)	HCT-116 (µM)
PA (2)	84.3 ± 0.5	93.4 ± 0.2	91.1 ± 0.5
EPA (7)	79.2 ± 0.2	81.1 ± 0.2	87.7 ± 0.3
Conjugate 2	88.4 ± 0.2	92.3 ± 0.5	76.4 ± 0.5
Conjugate 4	73.1 ± 0.3	89.4 ± 0.2	78.9 ± 0.3
Conjugate 5	**36.8 ± 0.2**	**7.0 ± 0.5**	68.8 ± 0.5
Conjugate 6	81.2 ± 0.2	49.6 ± 0.2	87.8 ± 0.2
Conjugate 7	43.5 ± 0.4	6.7 ± 0.1	47.1 ± 0.3
Conjugate 10	80.0 ± 0.2	48.7 ± 0.2	82.8 ± 0.2
Conjugate 11	41.9 ± 0.3	>100 ± 0.6	48.3 ± 0.5
Conjugate 12	16.0 ± 0.3	42.2 ± 0.5	49.8 ± 0.4
Conjugate 14	>100 ± 0.4	>100 ± 0.4	>100 ± 0.3
Conjugate 15	77.6 ± 0.2	98.1 ± 0.4	92.5 ± 0.2
Conjugate 17	43.2 ± 0.2	49.1 ± 0.2	39.5 ± 0.5
Conjugate 18	45.2 ± 0.3	10.0 ± 0.2	47.6 ± 0.2
Conjugate 20	**15.0 ± 0.1**	**4.0 ± 0.5**	>100 ± 0.1[1]

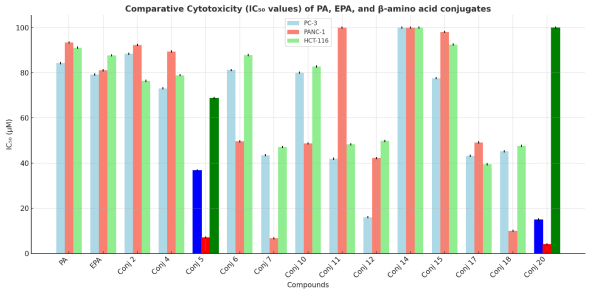


Figure 1. Comparative cytotoxicity (IC_{50} values) of piperic acid (PA), 4-ethylpiperic acid (EPA), and their β-amino acid conjugates against PC-3, PANC-1, and HCT-116 cells (MTT assay, mean ± SD, n = 3).

3.2. Anti-Proliferative and Apoptotic Effects

Conjugate 5, a piperic acid–β-amino acid derivative, demonstrated strong anti-proliferative activity against PANC-1 cells, with an IC_{50} value of ~7.0 µM, indicating selective cytotoxicity. Western blot analysis confirmed its ability to induce apoptosis through cleavage of PARP-1 and procaspase-3, consistent with activation of the caspase cascade. This apoptotic induction was further validated by the Caspase-Glo® 3/7 assay, which showed a dose-dependent increase in caspase-3/7 activity, and by DAPI nuclear staining, where treated cells exhibited chromatin condensation and nuclear fragmentation characteristic of apoptosis. In addition to caspase activation, conjugate 5 modulated key survival and apoptotic regulators. Expression of the anti-apoptotic proteins BCL2 and XIAP was markedly downregulated, while the pro-

[1] IC_{50} values are expressed as mean ± SD (µM) of three independent experiments.

apoptotic protein BAX was significantly upregulated, shifting the balance towards programmed cell death. Cell cycle analysis revealed that exposure to conjugate 5 led to G2 phase arrest, thereby halting cell division and further potentiating its cytostatic effect. Mechanistically, conjugate 5 was found to inhibit the p38-MAPK/ERK1/2 pathway, a critical signaling axis that promotes survival and proliferation in pancreatic cancer cells. Western blotting demonstrated reduced phosphorylation of both p38-MAPK and ERK1/2 following treatment, confirming pathway suppression. Collectively, these findings establish conjugate 5 as a potent apoptosis-inducing agent that not only disrupts survival signaling but also enforces G2 arrest, making it a strong candidate for targeted therapy in pancreatic cancer.

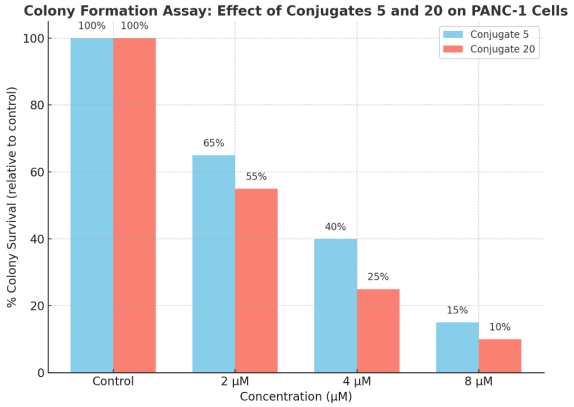


Figure 2. Effect of β-amino acid conjugates 5 and 20 on colony formation ability of PANC-1 cells. Data are expressed as % survival relative to control (mean ± SD, n = 3).

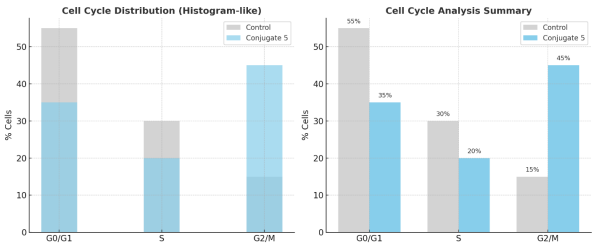


Figure 3. Cell cycle analysis of PANC-1 cells following treatment with Conjugate 5. Histograms illustrate the distribution of cells across G0/G1, S, and G2/M phases. Bar graph summarizes percentage of cells in each phase, showing significant G2/M arrest compared to control.

Table 2. Summary of Anti-Proliferative and Apoptotic Effects of Conjugate 5 in PANC-1 Cells

Parameter	Observed Effect	Interpretation
PARP cleavage	↑	Activation of apoptosis via DNA repair inhibition
Caspase-3 cleavage/activity	↑	Execution of apoptotic cell death
Caspase-3/7 activity assay	↑ (dose-dependent)	Significant induction of apoptosis ($p < 0.05$)
DAPI nuclear staining	Chromatin condensation, nuclear fragmentation	Morphological hallmark of apoptosis
BAX (pro-apoptotic)	↑	Promotion of mitochondrial apoptosis
BCL2 (anti-apoptotic)	↓	Loss of survival signaling
XIAP (anti-apoptotic)	↓	Reduced inhibition of caspases
Cell cycle analysis	Arrest at G2 phase	Inhibition of proliferation
MAPK/ERK phosphorylation	↓	Suppression of survival/proliferation pathway

3.3. Anti-Invasion and Anti-Metastatic Activity

Conjugate 20, a β-amino acid derivative of 4-ethylpiperic acid (EPA), demonstrated remarkable anti-metastatic potential in PANC-1 pancreatic cancer cells. In wound-healing assays, treatment with 2–4 μM of conjugate 20 significantly inhibited cell migration, whereas control cells treated with vehicle (DMSO) completely closed the scratch gap within 48 h. Consistent with these findings, colony formation assays revealed a dose-dependent reduction in clonogenic growth, with marked suppression of crystal violet–stained colonies compared to untreated controls ($p < 0.05$). The Boyden chamber Matrigel invasion assay further confirmed that conjugate 20 effectively blocked invasion through extracellular matrix barriers, reducing invasive cell counts in a concentration-dependent manner. Complementary 3D collagen invasion assays showed that spheroids embedded in collagen failed to extend invasive protrusions following treatment with conjugate 20, while control cells exhibited prominent invasive structures. These results indicate that conjugate 20 impairs both motility and invasion of PANC-1 cells. Mechanistic evaluation revealed that conjugate 20 suppressed invadopodia formation and extracellular matrix degradation, as demonstrated by the fluorescent gelatin degradation

assay. FITC-gelatin substrates showed large degradation zones in untreated PANC-1 cells, whereas treatment with conjugate 20 markedly reduced the degraded areas, indicating impaired invadopodia activity. Western blot analysis supported these phenotypic findings, showing a dose-dependent inhibition of MMP-2 and MMP-9 expression, accompanied by upregulation of TIMP-1, a natural inhibitor of matrix metalloproteinases. Importantly, conjugate 20 also restored expression of E-cadherin, a cell adhesion molecule typically downregulated during metastasis and epithelial-to-mesenchymal transition (EMT).

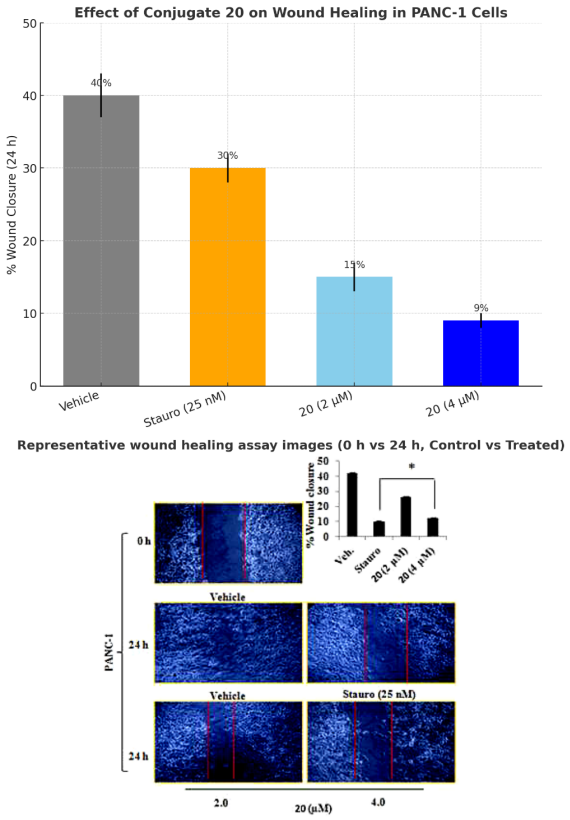


Figure 4. Effect of Conjugate 20 on wound healing in PANC-1 cells. (Top) Quantitative analysis showing significant dose-dependent inhibition of wound closure. (Bottom) Representative microscopy images of wound healing at 0 h and 24 h in vehicle- and conjugate-treated cells.

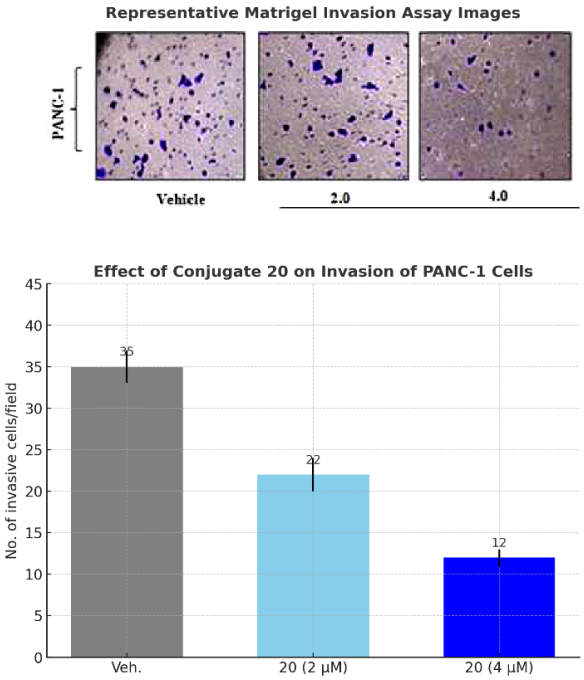


Figure 5. Conjugate 20 significantly inhibited invasion of PANC-1 cells in a dose-dependent manner. (Top) Representative Matrigel invasion assay images. (Bottom) Quantitative analysis of invasive cell numbers per field (mean ± SD, n = 3).

3.4. Mechanistic Insights

To delineate the molecular basis of the anticancer activity, the effect of β-amino acid conjugates, particularly conjugate 20, on major oncogenic signaling pathways was evaluated in PANC-1 cells. Western blot analysis revealed that conjugate 20 treatment caused a clear inhibition of PI3K/Akt phosphorylation in a dose-dependent manner. Both PI3K (p85α and p110α subunits) and phospho-Akt (Ser473) levels were significantly reduced compared to untreated controls, indicating direct suppression of the PI3K/Akt survival pathway. Further investigation demonstrated marked downregulation of NF-κB (p65) activation, a transcription factor critically involved in invasion and metastasis. This inhibition correlated with reduced expression of its downstream effectors, including MMP-2 and MMP-9, and was accompanied by the upregulation of TIMP-1 and restoration of E-cadherin expression, further supporting its role in blocking epithelial-to-mesenchymal transition. Additionally, conjugate 20 also exerted effects on the mTOR/S6K signaling cascade, a downstream branch of Akt involved in cell growth and protein synthesis. Treatment reduced phosphorylation of mTOR and its effector S6 kinase (S6K), while total mTOR expression remained unchanged. This demonstrates that conjugate 20 selectively inhibited signaling activity without

affecting overall protein levels. Taken together, these results suggest that β-amino acid conjugates act through dual mechanisms: (i) suppression of invasion and metastasis via downregulation of PI3K/Akt/NF-κB signaling, and (ii) induction of apoptosis and cytotoxicity through caspase activation and pro-apoptotic protein modulation. Overall, conjugate 20 emerges as a potent anti-metastatic agent capable of targeting multiple oncogenic pathways, thereby providing a mechanistic rationale for its strong activity against pancreatic cancer cells.

4. Discussion

Pancreatic cancer continues to represent one of the most formidable challenges in oncology due to its aggressive biology, high metastatic potential, and resistance to conventional chemotherapies. The results of the present study highlight the therapeutic promise of structurally modified natural products, specifically piperic acid (PA) and 4-ethylpiperic acid (EPA) β-amino acid conjugates, in targeting key molecular pathways that drive pancreatic cancer progression. By systematically evaluating their cytotoxic, anti-proliferative, apoptotic, and anti-invasive properties, we demonstrate that these conjugates exert potent activity against PANC-1 cells, thereby providing a mechanistic rationale for their development as lead candidates for novel anticancer therapy. A central finding of this work is the distinct functional profile of conjugates 5 and 20, which emerge as the most promising derivatives. Conjugate 5, a PA-based β-amino acid derivative, primarily functions as an apoptosis inducer. Its activity was validated through multiple assays, including PARP and caspase-3 cleavage, elevated caspase-3/7 activity, and nuclear fragmentation observed in DAPI staining. These hallmarks clearly establish the compound's ability to activate intrinsic apoptotic pathways. Moreover, the observed downregulation of anti-apoptotic proteins such as BCL2 and XIAP, coupled with upregulation of pro-apoptotic BAX, further underscores its role in shifting the balance toward programmed cell death. Importantly, conjugate 5 also induced G2 phase cell cycle arrest, thereby halting cellular proliferation. Mechanistic interrogation revealed inhibition of p38-MAPK and ERK1/2 phosphorylation, two critical signaling nodes that sustain cancer cell growth. Taken together, these findings position conjugate 5 as a cytotoxic and pro-apoptotic agent with significant potential for tackling the proliferative arm of pancreatic cancer biology. By contrast, conjugate 20, an EPA-based derivative, demonstrates its strength in suppressing invasion and metastasis, processes that ultimately determine pancreatic cancer lethality. Functional assays including wound healing, clonogenic survival, Matrigel invasion, and 3D collagen assays consistently showed a concentration-dependent inhibition of migratory and invasive behaviors. The ability of conjugate 20 to impair invadopodia formation and reduce FITC-gelatin matrix degradation provides compelling evidence of its capacity to block extracellular matrix remodeling, a prerequisite for metastatic dissemination. At the molecular level, conjugate 20 downregulated expression of MMP-2 and MMP-9, enzymes strongly implicated in pancreatic cancer invasion, while concomitantly upregulating TIMP-1, a natural MMP inhibitor. Restoration of E-cadherin expression further indicates that conjugate 20 reverses epithelial-to-mesenchymal transition (EMT), reinforcing its role as a suppressor of metastatic potential. These findings are particularly relevant because invasion and metastasis remain the chief determinants of pancreatic cancer prognosis and are often unresponsive to standard chemotherapeutics. A mechanistic comparison between conjugates 5 and 20 highlights how structural modifications of the piperic acid scaffold can generate compounds with divergent but complementary anticancer functions. Conjugate 5 preferentially activates apoptotic pathways and halts proliferation, whereas conjugate 20 disrupts invasive and metastatic traits. Both activities converge on the suppression of survival signaling, albeit through different nodes. Conjugate 5 inhibits MAPK/ERK signaling, thereby triggering apoptosis, while conjugate 20 suppresses the PI3K/Akt/NF-κB axis, resulting in reduced MMP activity and EMT reversal. This divergence underscores the potential of combining such derivatives or developing dual-functional molecules that simultaneously induce apoptosis and block metastasis. A key innovation of this study lies in the incorporation of β-amino acids into PA and EPA conjugates. Unlike conventional α-amino acids, β-amino acids confer enhanced metabolic stability and resistance to proteolytic degradation due to their altered backbone structure. Their presence improves pharmacokinetic properties and receptor interactions, allowing for stronger and more durable biological effects. Indeed, β-amino acid–containing conjugates are increasingly recognized in medicinal chemistry for their role in enhancing potency and selectivity of natural product derivatives. In the context of this study, β-amino acid incorporation not only strengthened the cytotoxicity of PA and EPA derivatives but also provided a structural basis for selective activity against pancreatic cancer cells, as

seen with conjugate 20's limited toxicity toward HCT-116 cells. Such selectivity is crucial for minimizing off-target effects and improving therapeutic windows in clinical settings. The broader implication of these findings is that PA and EPA conjugates may serve as valuable lead compounds for the rational design of targeted anticancer agents. Their ability to modulate the PI3K/Akt/NF-κB pathway is particularly significant, given that this axis is constitutively activated in the majority of pancreatic cancers and underlies both survival and metastasis. Current clinical inhibitors of this pathway often suffer from toxicity and limited efficacy due to redundancy and crosstalk within signaling networks. Natural product–derived conjugates such as those described here may offer a balanced approach, attenuating key survival pathways while simultaneously restoring tumor-suppressive proteins such as E-cadherin. In addition to their individual therapeutic potential, these conjugates also provide valuable chemical biology tools to dissect the interplay between apoptotic and metastatic signaling networks in pancreatic cancer. Their differential mechanisms of action enable the study of how pathway inhibition translates into phenotypic changes, such as apoptosis induction versus invasion blockade. This dual perspective enriches our understanding of pancreatic cancer biology and highlights potential combination strategies, where apoptosis inducers like conjugate 5 can be paired with anti-invasive agents like conjugate 20 to achieve synergistic control of tumor progression.

5. Conclusion

The present study establishes that structural modification of piperic acid (PA) and 4-ethylpiperic acid (EPA) through β-amino acid conjugation results in potent derivatives with selective anticancer activity against pancreatic cancer cells. Among the synthesized series, conjugates 5 and 20 demonstrated the most promising profiles, albeit through distinct mechanisms. Conjugate 5 acted predominantly as an apoptosis inducer, triggering caspase activation, PARP cleavage, BAX upregulation, and G2 phase cell cycle arrest, while simultaneously inhibiting the MAPK/ERK survival pathway. In contrast, conjugate 20 exhibited strong anti-invasive and anti-metastatic properties, markedly suppressing cell migration, clonogenicity, invadopodia activity, and extracellular matrix degradation. These effects were mechanistically linked to inhibition of PI3K/Akt phosphorylation, downregulation of NF-κB signaling, and modulation of metastatic markers, including suppression of MMP-2/9 and induction of TIMP-1, alongside restoration of E-cadherin expression. The incorporation of β-amino acids proved to be a valuable strategy, enhancing stability, bioactivity, and selectivity of the conjugates, particularly against pancreatic cancer cells. Collectively, these findings highlight PA and EPA β-amino acid conjugates as promising scaffolds for the development of novel therapeutics. By targeting both survival and metastatic pathways, these compounds provide a dual approach to combat pancreatic cancer, a malignancy characterized by poor prognosis and high resistance. Future in vivo studies and pharmacokinetic evaluations will be essential to validate their translational potential and advance them toward preclinical and clinical development.

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