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Indole Diterpene Alkaloids as Novel Inhibitors of the Wnt/ β -catenin Pathway in Breast Cancer Cells

Asmaa A. Sallam^a, Nehad M. Ayoub^{a,b}, Ahmed I. Foudah^a, Chris R. Gissendanner^a, Sharon A. Meyer^c, and Khalid A. El Sayed^{a,*}

^aDepartment of Basic Pharmaceutical Sciences, College of Pharmacy, University of Louisiana at Monroe, USA

^bDepartment of Clinical Pharmacy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan

^cDepartment of Toxicology, College of Pharmacy, University of Louisiana at Monroe, USA

Abstract

Penitrems are indole diterpene alkaloids best known for their BK channel inhibition and tremorgenic effects in mammals. In a previous study, penitrems A–F (**1–5**), their biosynthetic precursors, paspaline (**6**) and emindole SB (**7**), and two brominated penitrem analogs **8** and **9** demonstrated promising *in vitro* antiproliferative, antimigratory, and anti-invasive effects in the MTT (MCF-7 and MDA-MB-231), wound-healing, and Cultrex[®] BME cell invasion (MDA-MB-231) assays, respectively. The study herein reports the novel ability of penitrem A to suppress total β -catenin levels in MDA-MB-231 mammary cancer cells. Nine new penitrem analogs (**10–18**) were semisynthetically prepared, in an attempt to identify pharmacophores correlated with BK channel inhibition and tremorgenicity of penitrems and decrease their toxicity. The degree of BK channel inhibition was assessed using the nematode *Caenorhabditis elegans*, and *in vivo* tremorgenic EC₅₀ was calculated using CD-1 male mice following an Up-and-Down-Procedure (UDP). Although new analogs were generally less active than parent compound **1**, some showed no BK channel inhibition or tremorgenicity and retained the ability of penitrem A (**1**) to suppress total β -catenin levels in MDA-MB-231 cells. Paspaline (**6**) and emindole SB (**7**), both lacking BK channel inhibition and tremorgenicity, represent the simplest indole diterpene skeleton that retains the antiproliferative, antimigratory and total β -catenin suppressing effects shown by the more complex penitrem A (**1**).

Keywords

Antimigratory; Antiproliferative; BK channel inhibition; Breast cancer; *Caenorhabditis elegans*; Invasion; Penitrems; Wnt/ β catenin Pathway

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*To whom Correspondence should be addressed. Telephone 318-342-1725. Fax: 318-342-1737. elsayed@ulm.edu.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:. These include: Figures SI1–SI10, ¹H NMR and ¹³C data of **10**, **13**, and **15–17**.

1. Introduction

Breast cancer is the most common neoplasm in women worldwide [1,2]. While only approximately 5% of all newly diagnosed breast cancer patients in the U.S. present with metastatic disease at the time of initial diagnosis, up to one third of patients with early stage disease will subsequently develop metastasis. Despite the many advances in therapeutic regimens, metastatic breast cancer (MBC) remains incurable, with an estimated 5-year overall survival rate of only 23% [3]. Resistance to chemotherapy is also a major problem in the management of breast cancer, where many of the initially responsive tumors relapse and develop resistance to diverse chemotherapeutic agents [2]. Therefore, novel therapies and targets are still in demand for the management and treatment of various forms of breast cancer.

Wnt/ β -catenin signaling has been shown to regulate cell fate decisions in development and affect cell proliferation, morphology, migration, apoptosis, or differentiation in a variety of tissue settings [4,5]. Several types of human cancers, such as colorectal, hepatocellular and breast cancers, carry mutations in at least one component of the canonical Wnt/ β -catenin pathway, maintaining its upregulation in a ligand-independent manner [4,6,7]. Consequently, disruption of Wnt/ β -catenin signaling represents a valid target to develop novel anticancer drugs [5].

A hallmark of the Wnt/ β -catenin signaling activation is the stabilization of cytosolic β -catenin, which enters the nucleus to activate Wnt target genes by binding transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family [5]. In the absence of Wnt ligands, β -catenin levels are efficiently regulated by a supramolecular complex containing the scaffolding protein axin, the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinases 3 (GSK3) [8]. This complex promotes phosphorylation of β -catenin by casein kinase 1 (CK1) and GSK3 β . Phosphorylated β -catenin becomes multiubiquitinated (Ub) and subsequently undergoes proteasomal degradation [5,8]. The action of this complex is inhibited upon the binding of Wnt to its receptors on the cell surface [5].

In breast cancer, the Wnt pathway may be de-regulated by autocrine mechanisms [9,10]. Autocrine activation involves the co-expression of multiple Wnt ligands and their receptor, Frizzled (FZD) receptor, in primary human breast tumors and in breast cancer cell lines. In addition, most breast tumors (~80%) show hypermethylation of the promoter region of secreted Frizzled-related protein 1 (sFRP1), a known extracellular inhibitor of Wnt signaling, which competes with FZD receptor for ligand binding. Hypermethylation leads to the downregulation of sFRP1 protein expression and loss of its regulatory role in Wnt signaling. Overall, the best evidence to date that implicates Wnt signaling in human breast cancer is the observation that elevated levels of nuclear and/or cytoplasmic β -catenin are detectable by immunohistochemical staining in a majority (approximately 60%) of breast tumor tissue samples, but not in normal breast tissues, and this has been associated with poor prognosis [4,9,10]. Taken together, these observations strongly suggest that Wnt signaling may frequently be de-regulated and enhanced in breast cancer, and may contribute to its proliferation, survival, migration and invasion [9]. Interference with autocrine Wnt signaling has been shown to block *in vitro* proliferation as well as both *in vitro* and *in vivo* migration of many human breast cancer cell lines, providing further evidence to support approaches to target Wnt pathway activity in metastatic breast cancer [9,10].

Penitrems belong to a large class of fungal secondary metabolites known as indole diterpene alkaloids [11]. These metabolites are associated with an impressive biological activity profile, including insect feeding deterrence, modulation of insect and mammalian ion

channels and inhibition of mammalian acyl-CoA:cholesterol *O*-acyltransferase (ACAT) and mitotic kinesin [12,13]. Penitrems are potent BK channel inhibitors and well-known tremorgens [11]. In a previous study, we described the isolation and characterization of penitrems A, B, D, E, and F (**1–5**), two of their biosynthetic precursors, paspaline (**6**) and emindole SB (**7**), as well as two brominated penitrem analogs (**8** and **9**) from a marine-derived *Penicillium commune* isolate GS20 and reported their antiproliferative, antimigratory, and anti-invasive activities against breast cancer cells [14]. Breast cancer is a heterogeneous disease that progresses to the critical hallmark of metastasis. Wnt/ β -catenin pathway is a key contributor to the migratory and invasive potential of breast cancer cells. This study reports, for the first time, the effect of penitrems and related compounds on the Wnt/ β -catenin pathway in MDA-MB-231 breast cancer cells using immunocytochemical fluorescence staining assay. Our study also describes semisynthetic attempts at modifying penitrem A (**1**) structure to minimize its toxicity and improve, or at least maintain, its favorable activities. The nematode *C. elegans* was successfully employed as a model for measuring BK channel inhibition and an Up-and-Down Procedure (UDP) using CD-1 mice was used for assessing the *in vivo* toxicity (tremorgenicity) of tested compounds.

2. Results and Discussion

2.1. Chemistry

Semisynthetic attempts initially aimed at targeting those structural features, such as the C-25 hydroxyl group, the C-23/C-24 epoxide and the C-11/C-33 and C-37/C-38 exomethylene groups, previously shown to be implicated in or contributing to the BK activity profile of these compounds (Figure 1) [11,12,15]. The structural complexity as well as extreme acid sensitivity of the parent penitrem A (**1**) rendered the semisynthetic attempts challenging and in some cases unsuccessful. This challenge is commonly encountered with complex natural products [16]. Nevertheless, nine new ester, ether, peroxide and lead tetraacetate-mediated ring opening and recyclization products were obtained (Scheme 1).

The esterification reaction was accomplished using benzoyl chloride and *N,N*-dimethylaminopyridine (DMAP) as a catalyst to give compound **10** (Scheme 1a) [17,18]. The HREIMS of **10** showed an $[M+H]^+$ peak at m/z 738.3210, suggesting the molecular formula $C_{44}H_{48}ClNO_7$ and a possible C-25 benzoate analog of **1**. Tables 1 and 2 show the 1H and ^{13}C NMR data of **10** as well as parent compound **1**. As expected, esterification of the secondary alcohol C-25-OH caused a downfield shift at C-25 ($\Delta\delta_C +2.1$ and $\Delta\delta_H +1.6$). Esterification also induced an upfield shift for carbons C-24 ($\Delta\delta_C -3.1$) and C-26 ($\Delta\delta_C -1.3$) whose corresponding protons showed slight downfield shift, $\Delta\delta_H +0.29$ and $\Delta\delta_H +0.18$, respectively. 2D HMBC experiments were used to verify the presence of an intact benzoyl moiety as follows: the new aromatic methine protons H-3' and H-7' (δ_H 7.97) showed 3J -HMBC correlations with each other (δ_C 129.4), the ester carbonyl C-1' (δ_C 165.2), and the aromatic methine C-5' (δ_C 133.2). Similarly, the aromatic methine protons H-4' and H-6' showed 3J -HMBC correlations with each other (δ_C 128.6) and with the aromatic quaternary carbon C-2' (δ_C 130.1). Thus, compound **10** was confirmed to be the C-25 benzoate ester of **1**.

Ether analogs (**11–15**) were obtained by reacting **1** with the corresponding alkyl iodide or aryl bromide in the presence of NaH (Scheme 1b) [19–21]. The reaction with CH_3I yielded three products (**11–13**): *N*-1,*O*-25-dimethylpenitrem A (**11**), *N*-1-methylpenitrem A (**12**), and the *O*-25-methylpenitrem A (**13**). Tables 1 and 2 show the 1H and ^{13}C NMR data of **11** compared to parent compound **1**. The HREIMS of **11** showed an $[M+H]^+$ peak at m/z 662.3250, suggesting the molecular formula $C_{39}H_{48}ClNO_6$ and a possible dimethylether analog of **1**. The methylation at N-1 was evident from the following observations:

replacement of indolic NH proton signal, which appears at δ_H 10.01 in **1** (Table 2), with an *N*-methyl moiety C-1'' (δ_C 33.1, δ_H 3.88), whose protons showed 3J -HMBC correlations with quaternary carbons C-2 and C-9; as well as the downfield shift of the quaternary carbons C-2 ($\Delta\delta_C$ +1.6), C-3 ($\Delta\delta_C$ +0.4), and C-9 ($\Delta\delta_C$ +1.1). Methylation of the C-25 secondary alcohol resulted in a methoxy group C-1' (δ_C 58.4, δ_H 3.44), which showed a 3J -HMBC correlation with the oxygenated methine C-25. The upfield shift of the methine C-24 ($\Delta\delta_C$ -2.9) and the downfield shift of the oxygenated methine C-25 ($\Delta\delta_C$ +10.4) were also observed.

Monomethylation of **12** and **13** at N-1 or O-25, respectively, was also confirmed using their MS and NMR data as described for compound **11**. The HREIMS of **12** showed an $[M+H]^+$ peak at m/z 648.3090. Moreover, 1H NMR showed an additional methyl singlet at δ_H 3.87 and ^{13}C NMR showed a new methyl signal at δ_C 33.1, both of which are typical of an N-CH₃ group, suggesting the molecular formula C₃₈H₄₆ClNO₆ and a possible monomethyl analog of **1**. This methyl group replaced the indole NH proton signal (δ_H 10.01) in **1**. The ESIMS of compound **13** showed an $[M-H]^-$ peak at m/z 646.2 and 1H and ^{13}C NMR suggested C-25 methyl ether analog of **1** (δ_C 54.2, δ_H 3.44).

The HREIMS of **14** showed an $[M+H]^+$ peak at m/z 724.3397, suggesting the molecular formula C₄₄H₅₀ClNO₆ and a possible C-25 benzyl ether analog of **1** (Scheme 1b). The 1H and ^{13}C NMR data of **14** (Tables 1 and 2) further confirmed the identity of **14** as the C-25 benzyl ether of penitrem A (**1**).

The HREIMS of **15** showed an $[M+H]^+$ peak at m/z 874.4094, suggesting the molecular formula C₅₃H₆₀ClNO₈ and a possible N-1 and C-25 di-3-methoxybenzyl ether analog of **1** (Scheme 1b). Comparing the 1H and ^{13}C NMR data of **15** to that of parent compound **1** (Tables 1 and 2) confirmed its identity as the 1,25-di-3-methoxybenzyl ether of penitrem A (**1**).

m-Chloroperoxybenzoic (*m*-CPBA) is an oxidizing agent reported to be superior to hydrogen peroxide or other peracids in terms of reactivity, stereoselectivity, as well as in purity and yield of products [22,23]. It was reacted with **1** in an attempt to oxidize the C-25 secondary alcohol into a ketone and possibly induce epoxidation of the exomethylene $\Delta^{11,33}$ and/or $\Delta^{37,38}$ [13]. Despite several attempts to change reaction time, temperature, solvents and catalysts, none of the expected products were obtained and **16** was the only obtained product (Scheme 1c). It showed a molecular ion peak at m/z 650.2872 $[M+H]^+$, suggesting the molecular formula C₃₇H₄₄ClNO₇ and a possible hydroperoxy analog of **1**. Evidence of this peroxidation was based on the 1H and ^{13}C NMR data (Tables 1 and 2), which showed a downfield shift of the quaternary carbon C-15 ($\Delta\delta_C$ +4.8) and an upfield shift of the methine C-14 ($\Delta\delta_C$ -4.8) with a corresponding downfield shift of its proton H-14 ($\Delta\delta_H$ +0.66). The 2J -HMBC correlations between the methines H-12 and H-14 with the quaternary hydroperoxy-bearing carbon C-15 (δ_C 84.9) and the 3J -HMBC correlation of the methylene protons H₂-13 with the same carbon confirmed these assignments. Therefore, **16** was confirmed to be the 15-hydroperoxy analog of penitrem A (**1**).

Lead tetraacetate (LTA) has been one of the most important oxidants in organic synthesis. Representative reactions with a variety of functionalities mediated by LTA are dehydrogenation, oxidative-decarboxylation, oxidative-demethylation, oxidative fragmentation, and cyclic ether and bridged aziridine formation [24,25]. Although the procedure was simple and only called for reacting the starting material **1** with an equimolar quantity of LTA [26], the reaction was degradative and several attempts were made to optimize reaction time, conditions, solvent and temperature to obtain products **17** and **18** (Scheme 1d).

The ^1H and ^{13}C NMR data for **17** were nearly identical to those of penitrem A (Tables 1 and 2 for **1** and Table 3 for **17**) except for carbons within ring H (Figure 2a). As evident from NMR data, the reaction resulted in the loss of the isobutenyl segment C-26-C-36-C-37-C-38, with subsequent cyclization. This resulted in the replacement of the six-membered pyran ring H of **1** with a five-membered tetrahydrofuran in **17**. This was further confirmed by the HREIMS of **17**, which showed an $[\text{M}+\text{H}]^+$ peak at m/z 580.2413, suggesting the molecular formula $\text{C}_{33}\text{H}_{38}\text{ClNO}_6$. Extensive analysis of NMR, HMBC and NOESY data led to the final structure **17**. The epoxide methine proton H-24 and the methine H-27 (δ_{H} 3.66 and 4.24, respectively) showed ^2J - and ^3J -HMBC correlations with the downfield dioxygenated methine carbon C-25 (δ_{C} 97.2), respectively. The methine proton singlet H-25 (δ_{H} 5.30) showed a ^3J -HMBC correlation with the epoxide quaternary carbon C-23 (δ_{C} 71.2) and the oxygenated methine C-27 (δ_{C} 76.3). To establish the relative stereochemistry of C-25, NOESY and molecular modeling experiments were conducted (Figures 2a and b) [27,28]. The NOESY correlations between the epoxide methine singlet H-24 (δ_{H} 3.66) and the β -oriented methyl singlet H₃-35 (δ_{H} 1.31) suggested that both share a similar stereo-orientation. The methyl singlet H₃-35 also showed a NOESY correlation with the β -oriented methine proton H-19 (δ_{H} 2.69). These correlations confirmed the fact that the epoxide system at C-23-C-24 maintained the same parent original stereochemistry [29]. The β -oriented H-24 also showed a strong NOESY correlation with the methine singlet H-25 (δ_{H} 5.30), confirming similar stereo-orientation. These assignments were further supported by molecular modeling study (Figure 2b), which estimated a distance of 2.535 Å between the methines H-24 and H-25, and 4.065 Å between the methine H-24 and the methyl H₃-35, justifying the observed NOESY correlations. Thus, it can be postulated that C-24 has retained its *R* stereochemical orientation and C-25 also assumes the *R* configuration.

The ^1H and ^{13}C NMR data of **18** were almost identical to those for penitrem A (**1**) except for ring H, which was the site of the LTA-mediated ring opening, rearrangement, and alkoxylation (Tables 1 and 2 for **1** and Table 3 for **18**). The HREIMS of **18** showed an $[\text{M}+\text{H}]^+$ peak at m/z 692.2993, suggesting the molecular formula $\text{C}_{39}\text{H}_{47}\text{ClNO}_8$. The ^1H and ^{13}C NMR data suggest the same five-membered tetrahydrofuran ring H observed in **17**. However, in **18** there was evidence of double bond migration and allylic acetoxylation of the isobutenyl segment C-26-C-36-C-37-C-38. Figure 2c shows the most important HMBC correlations that further supported the proposed structure. The methyl singlet H₃-4' (δ_{H} 1.58) showed ^2J -HMBC correlation with the quaternary olefinic carbon C-2' and ^3J -HMBC correlations with the olefinic methine carbon C-1' and the oxygenated methylene carbon C-3'. The latter methylene protons showed a ^3J -HMBC correlation with the acetate carbonyl C-1''. The acetate methyl singlet H₃-2'' also showed a ^2J -HMBC correlation with the carbonyl carbon C-1'', connecting the acetoxy group to the isobutene system. The *Z*-geometry of the $\Delta^{1,2'}$ system was based on the NOESY correlation of H-1' with the methyl singlet H₃-4' (Figure 2c). The splitting pattern and coupling constants of H-27 (δ_{H} 4.78, J = 11.5 and 6.4, axial-axial and axial-equatorial couplings, respectively) with the adjacent H₂-28 methylene protons suggested its axial orientation. Thus, axial orientation of H-27 proton mandates the α -orientation and accordingly, an *S* configuration (similar to H-28 of the parent compound **1**) was assigned.

2.2. Biological evaluation and structure-activity relationship (SAR)

Penitrems A–E (**1–5**), their biosynthetic precursors **6** and **7**, and the brominated penitrem analogs (obtained by precursor-directed biosynthesis (PDB), **8** and **9**) were previously evaluated for their effect on proliferation, migration and invasion of breast cancer cells. Penitrem A proved to be the most active of all tested compounds [14].

Despite their interesting *in vitro* activity against breast cancer cells, penitrems suffer from potent BK channel inhibition and subsequent tremorgenic effects [11,38]. Consequently, studies aiming at investigating their structure-activity relationship (SAR) profile were necessary. The ultimate goal is to make structural modifications that would result in reduced toxicity (no inhibition of BK channels or tremorgenicity) while enhancing, or at least maintaining, the desirable biological properties. Analysis of the structure of penitrems and penitrem-related compounds suggest a number of structural pharmacophoric features mostly implicated in influencing the biological activity (Figure 1). Previously, we established the effects of substitution at C-6 and C-15 as well as the type of halogen at C-6 on the antiproliferative, antimigratory and anti-invasive properties of penitrems against breast cancer cells [14], and their influence on BK channel inhibition and tremorgenicity has already been reported in literature [11,12]. Moreover, it was shown that modifications affecting the oxidation status at C-10 and C-11 (as in penitremones **19** and **20**) and those masking the C-25 hydroxyl group (for example, within the oxetane ring of pennigretrem, **21**) greatly influenced the BK channel inhibitory activity as well as tremorgenicity [11,12,15].

2.2.1. Penitrem A affects canonical Wnt signaling—Penitrems A, B and E were screened in Eli Lilly's assay panel which is part of their Phenotypic Drug Discovery (PD²) initiative [37]. The first five phenotypic screening assays available via PD² are insulin secretion assay, apoE secretion assay, Wnt pathway assay, angiogenesis assay and G2M cell cycle assay [37]. Interestingly, **1** was able to enhance β -catenin nuclear translocation at an EC₅₀ value of 0.98 μ M (Figure 3) in C2C12 cells, suggesting a potential effect for penitrem A on components of the Wnt signaling pathway. This finding encouraged further studies to investigate its effects on this pathway in MDA-MB-231 cells.

Figure 4b shows the effect of penitrem A (**1**) treatment on the total levels of β -catenin in MDA-MB-231 breast cancer cells using immunocytochemical fluorescence staining. In these cells, autocrine mechanisms are responsible for the activation of the canonical Wnt/ β -catenin signaling pathway which is evident from the presence of β -catenin in the nucleus and cytoplasm (treatment-free control, red staining, Figure 4a). It is generally accepted that the detection of β -catenin in the nucleus or cytoplasm is considered a hallmark of active Wnt signaling [4,5]. Penitrem A treatment remarkably decreased the expression of total β -catenin levels in MDA-MB-231 mammary cancer cells (Figure 4b): decreased intensity of red fluorescence reflects decreased levels of total β -catenin, compared to cells in the treatment-free control (Figure 4a). The reduction of intracellular levels of total β -catenin may explain, in part, one of the potential molecular mechanisms through which penitrem A exerts its *in vitro* effects in MDA-MB-231 breast cancer cells. Targeting components of autocrine Wnt signaling has been shown to be an effective approach to block the *in vitro* proliferation as well as both *in vitro* and *in vivo* migration of many human breast cancer cell lines [9,10].

2.2.2. In vitro activity of new analogs—All penitrem analogs were screened in the MTT, wound-healing and Cultrex[®] BME Cell Invasion assays [30–33]. The human mammary carcinoma cell lines MCF-7 and MDA-MB-231 were both used in the MTT assay whereas MDA-MB-231 cell line was used in the wound-healing and cell invasion assays. To evaluate the effect of penitrem analogs on the Wnt/ β -catenin pathway in MDA-MB-231 cells, an immunocytochemical fluorescence staining assay was conducted, in which the levels of total β -catenin in response to treatment were evaluated. The nematode *C. elegans* was used as a model to assess the BK channel inhibitory potential of new analogs [34,35]. In order to determine tremorgenicity *in vivo*, male CD-1 mice were used in an Up-and-Down Procedure (UDP) and an EC₅₀ for each tested analog was calculated [36].

With a few exceptions, where a few new analogs exhibited comparable activity to parent compound **1** in some assays, analogs were generally associated with a weakened biological

activity profile (Table 4, Figure 5). Apparently, substitution at the C-25 hydroxyl group (hydrogen bond donor) and/or the indolic NH-1 as well as rearrangement at ring H compromised the antiproliferative, antimigratory and anti-invasive potential of penitrem A (**1**). These findings clearly indicate the importance of the free C-25 hydroxy and NH groups as well as the pyran ring for *in vitro* activity.

To test whether new analogs still retained the effect of **1** in suppressing total β -catenin levels in MDA-MB-231 cells, two compounds were selected for the immunocytochemical fluorescence staining assay: the previously obtained bromo analog **9** (to show the effect of halogen type), and the semisynthetic analog **17** (to show the effect of masking the C-25 hydroxyl group and modifying ring H) (Figures 4c and d). Compounds were tested at their IC₅₀ value in the WHA. Activity levels were determined by comparing the intensity of red fluorescence, which reflects the amount of total β -catenin. Compound **9** showed a comparatively better activity than parent compound **1**, suggesting a superior role for the bromo substitution in suppressing total β -catenin levels. Though **17** is ~6 fold less active than penitrem A (**1**) in the WHA, it was only slightly less effective in suppressing total β -catenin at the tested concentration. Although, an intact ring H and a free C-25-OH group are both essential for overall *in vitro* activity (proliferation, migration and invasion assays), their modification does not necessarily abolish molecular mechanisms. These results point both to the complex structural requirement and molecular mechanisms through which penitrems exert their effects, which is the case with most natural products [39,40].

The biosynthetic precursors paspaline (**6**) and emindole SB (**7**) also potently suppressed total β -catenin levels in MDA-MB-231 cells, with paspaline showing better activity than emindole SB (red fluorescence intensity, Figures 4e and f). These two compounds are the simplest stable indole diterpene alkaloids isolated thus far [11,13]. They shed light on the minimal structural requirements for *in vitro* activity of this class of secondary metabolites. Their activity and structural simplicity make them promising starting hits for future studies.

2.2.3. Caenorhabditis elegans for BK channel inhibition assessment—The nematode *Caenorhabditis elegans* was used to assess BK channel inhibition *in vivo* [35,36]. BK channel inhibition in *C. elegans* is associated with an abnormal behavior of locomotion of worms in terms of increased reversals, i.e., the number of times a worm stops and reverses its direction, which can be easily assessed and quantified [35]. For the assay, 30–40 synchronized L1 stage larvae of the wild-type N2 strain were added to *E. coli* OP50 preseeded NGM plates containing 1 μ M of each test compound [35]. Plates were incubated at 20°C and the number of reversals was counted for at least 10 worms per treatment 42 hours (L4 larval stage) post exposure. The *C. elegans slo-1* knockout strain NM1968 *slo-1(js379)V*, which shows the phenotype of increased reversals [35], was used as a positive control. Compound **7** (for which no BK channel data have been reported) and the new analogs **9–11** and **17** were tested (Figure 6). BK channel inhibition by bromo analog **9** was comparable to that of penitrem A, suggesting that halogen type is not a major determinant of BK channel inhibition. Unlike penitrem A, however, none of the semisynthetic analogs **10**, **11** and **17** showed BK inhibitory activity as can be seen from the number of reversals exhibited by treated worms compared to that of vehicle (DMSO) control (Figure 6). Emindole SB (**7**), like paspaline (**6**) [11], did not cause BK channel inhibition, further highlighting the importance of these biosynthetic precursors in representing the minimal structural features maintaining activity and, at the same time, devoid of the toxicity associated with this class of natural products.

2.2.4. In vivo assessment of toxicity and tremorgenic activity—There are conflicting reports in literature regarding BK channel inhibition and its association with

tremorgenic potential of indole diterpene alkaloids [11,38]. This necessitated the use of an animal model as a conclusive evidence for the tremorgenic or lack thereof for new analogs. We opted for *in vivo* Up-and-Down Procedure (UDP) using male CD-1 mice. It is worth mentioning that no sex differences in responsiveness have been reported for these tremorgens when administered experimentally [41].

The Up-and-Down Procedure (UDP) is generally used for determining acute toxicity and calculating LD₅₀, the dose causing death in 50% of dosed animals [42]. In our procedure, however, the end point monitored was the presence or absence of tremors upon dosing, therefore, an EC₅₀ was calculated instead. We followed the experimental settings described by Meyer *et al.*, which were conducted according to the 2002 EPA acute oral toxicity guideline 870.1100 [36,42].

Parent compound **1** as well as compounds **8–11** and **17** were selected for the study. In summary, the starting dose was a half-log increment of the reported tremorgenic dose for penitrem A (**1**), i.e. $3.2 \times 0.6 \text{ mg/kg} = 1.92 \text{ mg/kg}$. Half-log increments or reductions were then applied for subsequent doses based on animal response: if the starting dose caused tremors, the next dose was reduced by a factor of 3.2. If the starting dose, however, caused no response (absence of tremors), the next dose was then raised by an increment of 3.2, and so on. Table 5 shows the results of the study. A dose higher than 19.6 mg/kg was not attainable for analogs **10**, **11** and **17** because of poor solubility and dosing was stopped accordingly. Tremorgenic EC₅₀ for penitrem A calculated at the end of the experiment (Table 5) was within the range reported in literature (0.25–0.6 mg/Kg) [11]. Compound **9** (EC₅₀ 0.32 mg/Kg) showed a tremorgenic potential comparable to that reported for its chlorinated counterpart **1**; suggesting that the type of halogen does not profoundly influence tremorgenicity (Table 5). Compound **8**, however, was almost 12-times less tremorgenic than **9**, suggesting the important influence of C-15 hydroxyl group on the tremorgenic potential of this class of compounds. None of the tested semisynthetic analogs **10**, **11**, and **17** caused tremors in mice at doses up to 10-times the tremorgenic dose of penitrem A (Table 5). These results are consistent with the assumption that blocking the BK channel inhibitory potential will result in the absence of tremors [38].

3. Conclusions

Penitrems are an important class of secondary metabolites with a plethora of reported biological activities. The ability of penitrem A to interfere with the Wnt/ β -catenin pathway in MDA-MB-231 cells is a significant addition to their activity profile. Inhibition of aberrant Wnt pathway activity in breast cancer cell lines efficiently blocks their growth and migration, highlighting the great potential of therapeutics targeting this pathway. The major drawback of penitrems, however, is tremorgenicity. A number of semisynthetic modifications were conducted throughout the study in an attempt to generate new analogs devoid of the tremorgenic profile of penitrem A. Although mostly detrimental to *in vitro* activity, these modifications confirmed previously-reported pharmacophoric features on the penitrem A skeleton that are major determinants of its BK channel inhibitory activity and tremorgenicity. Moreover, analog **17** showed that loss of BK channel inhibition was associated with loss of tremorgenicity without affecting the ability to suppress total β -catenin. This indicates that these compounds indeed act on the Wnt/ β -catenin pathway through BK channel-independent mechanisms. Further studies are required to better understand the SAR profile of penitrems and hopefully make a distinction between structural features required for *in vitro* activity and those responsible for tremorgenicity. Paspaline (**6**) and emindole SB (**7**), with the least complex indole diterpene alkaloid structure, still retain the activity of penitrem A without being tremorgenic, making them interesting hits for future studies.

4. Materials and methods

4.1. General experimental procedures

IR spectra were recorded on a Varian 800 FT-IR spectrophotometer. TLC analysis was carried on precoated Si gel 60 F₂₅₄ 500 μ m TLC plates (EMD Chemicals), using *n*-hexane-EtOAc (7:3) as a developing system. For column chromatography, Si gel 60 (Natland International Corporation, 230–400 μ m) was used, and gradient *n*-hexane-EtOAc solvent system was used as mobile phase. ¹H and ¹³C NMR spectra were recorded in CD₃OD on a JEOL Eclipse-ECS NMR spectrometer operating at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR. The HREIMS experiments were conducted at Louisiana State University (LSU) on a 6200-TOF LCMS (Agilent) equipped with multimode source (mixed source that can ionize the compounds alternatively by ESI and APCI). The ESIMS experiments were conducted using a 3200 Q-trap LC/MS/MS system (Applied Biosystems, Foster City, CA) using Analyst version 1.4.1 software (MDS Sciex; Toronto, Canada) where analytes were ionized using electrospray ionization (ESI) interface operated in the negative mode. The analysis was conducted using Q₁ scan and mass scan range was *m/z* 50–700 (0.15 s/scan).

4.2. Chemical reactions

(A) Esterification of **1**. To a solution of **1** (0.040 mmol) in CH₂Cl₂ (2.0 mL), *N,N*-dimethylaminopyridine (2 equivalents) and benzoyl chloride (2 equivalents) were added and refluxed overnight (Scheme 1a) [18,19]. Reaction mixture was worked out first by dilution with CH₂Cl₂ followed by the addition of water (10.0 mL). The organic layer was then collected and the aqueous layer further extracted with CH₂Cl₂. Organic layers were then dried over anhydrous Mg₂SO₄ and the solvent removed *in vacuo*. Residue was collected and purified on Si gel 60 using gradient *n*-hexane-EtOAc system to afford the benzoyl ester **10** in approximately 45–60% yield.

4.2.1. Penitrem A-25-O-benzoate (10)—White powder; IR $\nu_{\max}$ (CH₂Cl₂) 3746, 3568, 3458, 3056, 2926, 1720, 1421, 1113, 894 cm^{−1}; ¹H and ¹³C NMR, see Tables 1 and 2, and Figures SI1 and SI2 in Supplementary Information; HRESIMS *m/z* 738.3210 [M+H]⁺ (calcd for C₄₄H₄₉ClNO₇, 738.3198).

(B) Etherification of **1**. To an ice-cold solution of **1** (0.079 mmol) in dry DMF (1.0 mL), cooled (5°C) suspension of NaH (0.316 mmole) in 1.0 mL dry DMF was added and stirred over an ice bath for 30 minutes (Scheme 1b). Different alkyl or aryl halides (1–2 equivalents; 0.079–0.158 mmol) were then added drop-wise to the mixture [20–2]. After H₂ evolution ceased, solutions were warmed to room temperature and stirred overnight. Each solution was then poured into 1N NaOH and the mixture was extracted with CH₂Cl₂ (3 × 10 mL). Organic layer was then dried over anhydrous Na₂SO₄ and crude mixtures purified on Si gel 60 using gradient *n*-hexane-EtOAc system, which afforded **11–14** in 47–70% yields.

4.2.2. N-1-25-O-dimethylpenitrem A (11), N-1-methylpenitrem A (12), and 25-O-methylpenitrem A (13)—Reaction of 50 mg of **1** with 9.8 μ L of iodomethane was carried out to give **11** (74% yield), **12** (17% yield) and **13** (11% yield): all as white powders; IR $\nu_{\max}$ for **11** (CH₂Cl₂) 3746, 3571, 3054, 2944, 1456, 1070, 928, 903 cm^{−1}; ¹H- and ¹³C-NMR, see Tables 1 and 2; HRESIMS of **11** *m/z* 662.3250 [M+H]⁺ (calcd for C₃₉H₄₉ClNO₆, 662.3248); HRESIMS *m/z* of **12** 648.3090 [M+H]⁺ (calcd for C₃₈H₄₇ClNO₆, 648.3092); ESIMS *m/z* of **13** 646.2 [M-H][−] (calcd for C₃₈H₄₅ClNO₆, 646.2935). For ¹H- and ¹³C-NMR spectra of **13**, see Figures SI3 and SI4 in Supplementary Information.

4.2.3. 25-O-Benzylpenitrem A (14)—Reaction of 25 mg of **1** with 9.4 μ L benzyl bromide was carried out to give **14** in 32% yield: white powder, IR $\nu_{\max}$ (CH₂Cl₂) 3746,

3056, 2926, 1517, 893 cm^{-1} ; ^1H and ^{13}C NMR, see Tables 1 and 2; HRESIMS m/z 724.3397 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{44}\text{H}_{51}\text{ClNO}_6$, 724.3405).

4.2.4. N-1-25-O-di-(3-methoxy)benzylpenitrem A (15)—Reaction of 25 mg of **1** with 11.1 μL 3-methoxybenzyl bromide was carried out to give **15**, 47% yield: white powder; IR ν_{max} (CH_2Cl_2) 3746, 3056, 2926, 1451, 894 cm^{-1} ; ^1H and ^{13}C NMR, see Tables 1 and 2, and Figures SI5 and SI6 in Supplementary Information; HRESIMS m/z 874.4094 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{53}\text{H}_{61}\text{ClNO}_8$, 874.4086).

(C) Reaction of **1** with *m*-chloroperoxybenzoic acid (*m*-CPBA): To a solution of 25 mg of **1** in 2.0 mL CH_2Cl_2 , 11.7 mg *m*-CPBA in 1.0 mL CH_2Cl_2 containing barium carbonate (as an acid quencher) was added and stirred initially over ice bath and eventually at room temperature overnight (Scheme 1c) [43,44]. The reaction was stopped by the addition of 10% Na_2SO_4 and the aqueous layer was extracted with CH_2Cl_2 . Organic layers were then combined and dried over anhydrous MgSO_4 before evaporation [44]. The residue was chromatographed over Si gel 60 using a gradient *n*-hexane-EtOAc mobile phase to give **16**, 32% yield.

4.2.5. 15-hydroperoxypenitrem A (16)—White powder; IR ν_{max} (CH_2Cl_2) 3746, 3056, 2926, 1517, 893 cm^{-1} ; ^1H and ^{13}C NMR, see Tables 1 and 2, and Figures SI7 and SI8 in Supplementary Information; HRESIMS m/z 650.2872 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{37}\text{H}_{45}\text{ClNO}_7$, 650.2885).

(D) Reaction of **1** with lead tetraacetate (LTA) [27]: To a solution of 25.0 mg of **1** (0.039 mmol) in 2.0 mL CH_2Cl_2 , 17.5 mg (0.039 mmol) of LTA solution in CH_2Cl_2 was added after filtration over sodium bicarbonate (to get rid of any residual acetic acid). The reaction was stirred at room temperature for 6–8 h and quenched with water (Scheme 1d). Extraction with CH_2Cl_2 (3×5.0 mL) followed and the organic layers were dried over MgSO_4 . The residue obtained upon drying the solvent was subjected to column chromatography on Si gel 60 using gradient *n*-hexane-EtOAc elution system, which afforded **17** and **18** in 36 and 42% yields, respectively. Both were obtained as white powders. For **17**: IR ν_{max} (CH_2Cl_2) 3746, 3056, 2926, 1517, 1420, 893 cm^{-1} ; ^1H and ^{13}C NMR, see Table 3, and Figures SI9 and SI10 in Supplementary Information; HRESIMS m/z 580.2413 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{33}\text{H}_{39}\text{ClNO}_6$, 580.2466). For **18**: IR ν_{max} (CH_2Cl_2) 3746, 3056, 2926, 1739, 893 cm^{-1} ; ^1H and ^{13}C NMR, see Table 3; HRESIMS m/z 692.2993 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{39}\text{H}_{47}\text{ClNO}_8$, 692.2990).

4.3. In vitro activities

Breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from ATCC (Manassas, VA). Both cell lines were grown in RPMI 1640 medium (GIBCO-Invitrogen, NY) supplemented with 10% fetal bovine serum (FBS) and glutamine (2 mmol/L), and containing penicillin G (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). Cells were incubated at 37°C in a humidified incubator under 5% CO_2 .

A stock solution of each compound was prepared in DMSO at a concentration of 25 mM for all assays. Appropriate media (serum-free, 0.5% FBS or 5% FBS) were used to prepare compounds at their final concentrations for each assay. The vehicle (DMSO) control was prepared by adding the maximum volume of DMSO used in preparing test compounds to the appropriate media type such that the final DMSO concentration never exceeded 0.2%.

4.3.1. MTT (proliferation assay)—The antiproliferative effect in this study was tested on the human breast cancer cell lines, MCF-7 and MDA-MB-231 following the procedure described previously [31,32]. Briefly, cells in exponential growth were plated in a 96-well

plate at a density of 10×10^3 (MCF-7) and 8×10^3 (MDA-MB-231) cells per well, and allowed to attach overnight at 37°C under 5% CO₂ in a humidified incubator. Complete growth medium was then replaced with 100 µL of either RPMI serum-free medium (GIBCO-Invitrogen, NY) for MCF-7 cells or RPMI media supplemented with 5% FBS for MDA-MB-231 cells, containing various doses of the specific test compound and incubation resumed at 37°C under 5% CO₂ for 72 h. Control and treatment media were then removed, replaced with fresh media, and 50 µL MTT solution (at 1 mg mL⁻¹) were added to each well and plates were re-incubated for 4 h. At the end of the incubation period, the color reaction was stopped by removing the media and adding 100 µL DMSO to dissolve the formazan crystals formed. Incubation at 37°C was resumed for up to 20 minutes to ensure complete dissolution of crystals. Absorbance was determined at λ 570 nm using an ELISA plate reader (BioTek, VT, USA). The number of cells per well was calculated against a standard curve prepared at the start of each experiment by plating various numbers of cells (in the range 1,000–60,000 cells per well), as counted by a hemocytometer. The IC₅₀ value for each compound was calculated by nonlinear regression (curve fit) of log (concentration) versus the % survival, implemented in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA) [43]. The number of cells at the end of the 72-hour incubation period in the vehicle (DMSO) control was 33×10^3 for MCF-7 and 28×10^3 for MDA-MB-231. The % cell survival was calculated as follows: % cell survival = (Cell No._{treatment}/Cell No._{DMSO}) × 100%.

4.3.2. Wound-healing assay (WHA)—The WHA is a simple method for evaluating directional cell migration *in vitro* [33]. MDA-MB-231 cells were plated in sterile 24-well plates and allowed to form a confluent cell monolayer per well (>95% confluence) overnight. Wounds were then inflicted in each cell monolayer using a sterile 200 µL pipette tip. Media was removed and cells were washed twice with PBS and once with fresh RPMI media. Test compounds at the desired concentrations were prepared in fresh media (0.5% FBS) and were added to wells in triplicate. The incubation was carried out for 24 h, after which media was removed and cells were washed, fixed and stained using Diff-Quick™ staining (Dade Behring Diagnostics, Aguada, Puerto Rico). Cells which migrated across the inflicted wound were counted under the microscope in at least five randomly selected fields (magnification: 400X).

4.3.3. Cultrex® BME cell invasion assay—This assay was conducted according to the protocol provided with the kit [34]. Briefly, about 50 µL of basement membrane extract (BME) coat (1X) was added per well. After overnight incubation at 37°C in a 5% CO₂, 50,000/50 µL of MDA-MB-231 cells in 0.5% FBS RPMI medium was added per well to the top chamber. Test compounds were prepared at 6X the desired concentrations (45 and 90 µM) and 10 µL of each compound was added in triplicate to achieve the final test concentrations (7.5 and 15 µM). 150 µL of RPMI medium was then added to the lower chamber. Media contained 10% FBS and penicillin/streptomycin as well as fibronectin (1 µL/mL) and *N*-formyl-met-leu-phe (10 nM) as chemoattractants. Plates were re-incubated at 37°C in 5% CO₂ for 24 h after which the top and bottom chambers were aspirated and washed with wash buffer supplemented with the kit. 100 µL of cell dissociation/calcein-AM solution was added to the bottom chamber and incubated at 37°C in 5% CO₂ for 1 h. The cells internalize calcein-AM, and the intracellular esterases cleave the acetomethylester (AM) moiety to generate free calcein. Fluorescence of the samples was determined at $\lambda_{\text{excitation}}$ 485 nm and $\lambda_{\text{emission}}$ 528 nm using an ELISA plate reader (BioTek, VT, USA). The numbers of cells that invaded through the BME coat were calculated using a standard curve. For the vehicle (DMSO) control, the total number of cells which actually invaded the BME coat was 17512. % invasion of different treatments were compared using One-Way-ANOVA and individual treatments were then compared to the DMSO control using

Dunnett's test implemented in PASW Statistics 18. A *p*-value <0.05 was considered statistically significant.

4.3.4. Immunocytochemical fluorescent staining assay—For immunocytochemical fluorescent studies, MDA-MB-231 mammary cancer cells were seeded on 8-chamber culture slides (Becton Dickinson and Company, NJ, USA) at a density of 5×10^4 cells/chamber (2 replicates/group) and allowed to attach overnight in complete growth media supplemented with 10% fetal bovine serum (FBS). Cells were then washed with Ca^{2+} and Mg^{2+} -free phosphate buffered saline (PBS) and incubated with vehicle control or treatment media containing 0.5% FBS for 48 h in culture. At the end of treatments, cells were washed with pre-cooled PBS, fixed with methanol pre-cooled to -20°C for 2 min. Fixed cells were then washed with PBS and blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) for 1 h at room temperature. Cells were then incubated with specific primary antibody for total β -catenin (1:300) overnight at 4°C in 2% BSA-TBST. At the end of incubation time, cells were washed five times with pre-cooled PBS followed by incubation with Alexa Fluor 594-conjugated secondary antibody (1:5000) in 2% BSA-TBST for 1 hr at room temperature. After final washing for five times with pre-cooled PBS, cells were embedded in Vectashield Mounting Medium with DAPI (Vector Laboratories IN., Burlingame, CA, USA). Fluorescent images were obtained by using confocal laser scanning microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA). The red color indicates the positive fluorescence staining for β -catenin and the blue color represents MDA-MB-231 cell nuclei counter-stained with DAPI. Each experiment was repeated at least three times and multiple images for each chamber were captured. Magnification of each photomicrograph is 200X.

4.4. In vivo activities

4.4.1 *Caenorhabditis elegans*

4.4.1.1. Strains: The wild-type N2 Bristol strain of *C. elegans* and the *slo-1* knockout strain NM1968 *slo-1(js379)V* (*Caenorhabditis* Genetics Center, University of Minnesota) were used. Worm stocks were maintained at 20°C on nematode growth medium (NGM) plates preseeded with OP50 *E. coli* (*Caenorhabditis* Genetics Center) as a food source [46,47].

Stock solutions for treatment compounds were prepared in DMSO. Proper dilutions to achieve final test concentrations were conducted in NGM agar after autoclaving and cooling to $\sim 50^\circ\text{C}$. Final DMSO concentration was 28 mM [35]. This DMSO concentration does not have any effects on *C. elegans* locomotion. All plates were seeded with $\sim 100 \mu\text{l}$ *E. coli* OP50.

4.4.1.2. Number of reversals count: The setup for the reversal assay was as follows [35,36]: 30–40 synchronized L1 stage larvae of N2 and NM1968 *slo-1(js379)V* strains were transferred to NGM plates seeded with *E. coli* OP50 and containing $1.0 \mu\text{M}$ of each compound or DMSO (vehicle control). Plates were incubated for 42 h at 20°C . At the end of the incubation period, L4 larvae were individually transferred to a plain un-seeded NGM plate and allowed to crawl for one minute to acclimatize and assist in the removal of any residual bacteria that may be adherent to the worm. The reversals (defined as the number of times a worm stops and reverses direction) were then counted for a period of 3 minutes. Numbers of reversals in different treatments were compared using One-Way-ANOVA and individual treatments were then compared to the DMSO control using Dunnett's test implemented in PASW Statistics 18. A *p*-value <0.05 was considered statistically significant.

4.4.2. Animal model

4.4.2.1. Animals and treatment: Male CD-1 mice were obtained from Harlan Laboratories (www.harlan.com; Madison, WI) and maintained in accordance with the Guide for Use and Care of Animals (National Research Council, 1996). Mice were housed in a 12 h light/dark cycle and had free access to tap water and pelleted rodent chow (no.7012, Harlan/Teklad, Madison, WI). Three days prior to trials, groups of mice (18–22 g) were housed individually in polycarbonate cages on hardwood bedding (Sani-Chips- Harlan Teklad, Madison, WI). Study protocol had prior approval by the ULM Animal Care and Use Committee.

Stock solutions of test compounds were prepared in DMSO and diluted in sterile normal saline (total volume 200 μ L) just before intraperitoneal (i.p.) administration. DMSO concentration did not exceed 2.0 μ L/g (LD₅₀ for DMSO when administered i.p. is 11.0 μ L/g) [48]. Control mice received the vehicle (DMSO/normal saline) only.

4.4.2.2. Up-and-Down Procedure (UDP) study: The UDP study was conducted according to the 2002 EPA acute oral toxicity guideline 870.1100 [42] with a slope factor sigma = 0.33 and a starting dose of 1.92 mg/kg (3.2 X reported tremorgenic dose for penitrem A (~ 0.25–0.60 mg/kg) [11]). Each subsequent dose was determined based on the response (tremors or no tremors) observed from the previous dose. Dose range utilized throughout the study was 0.19–19.6 mg/kg. Dose progression was stopped when one of the three stopping rules of the AOT425StatPgm program (USEPA, 2003) was satisfied. Since our endpoint was the occurrence of tremors rather than animal death, an EC₅₀ instead of LD₅₀ was calculated. Qualitative assessments of appearance, righting reflex, forelimb strength and incidence of tremors and/or convulsions were made at 10–15 min intervals during the first hour after dosing, hourly over the next 8 h and daily thereafter over the 14-day observation period. Mice were weighed at the beginning of the study and then at days 7 and 14 post dosing. At the conclusion of the study, mice were euthanized by cervical dislocation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Nine new penitrem analogs were semisynthesized
- They showed antiproliferative, anti-invasive and antimigratory activities
- Penitrems suppress total β -catenin levels in MDA-MB-231 breast cancer cells
- Important pharmacophores associated with BK channel inhibition identified

Figure 1. Highlighted are structural features (pharmacophores) proven to have an important influence on penitrem A activities [11].

Figure 2.

Proposed structure of compounds **17** and **18** obtained from the reaction of **1** with LTA.

(a) 2J - and 3J - HMBC (black arrows) as well as NOESY (maroon lines) correlations used to assign the structure of ring H. The rest of the molecule is identical to parent compound **1**. (b) Local minimum energy confirmation of **17** generated by SYBYL 8.1. The distances 2.535 Å between H-24 and H-25 and 4.065 Å between H-24 and H₃-35 support the observed NOESY correlations. (c) Main 2J - and 3J - HMBC correlations, which confirmed the structural modification of ring H. Maroon line indicates NOESY correlations between H-1' and H₃-4' confirming the Z geometry of $\Delta^{1',2'}$ system.

Figure 3.

Dose-response curve of penitrem A in inducing the translocation of β -catenin in C2C12 cells. Figure was provided by Eli Lilly [37].

Figure 4.

Visualization of the effect of different compound treatments on total β -catenin levels in MDA-MB-231 breast cancer cells using immunocytochemical fluorescence staining. Red color indicates positive fluorescent staining for total β -catenin (nuclear and cytoplasmic) and blue color represents cell nuclei counterstained with DAPI. (a) Treatment-free control; (b) Penitrem A (**1**) 8.0 μ M treatment; (c) 6-bromo analog of penitrem A (**9**) 9.6 μ M treatment; (d) LTA product (**17**) 48.2 μ M treatment; (e) Paspaline (**6**) 7.6 μ M treatment; (f) Emindole SB (**7**) 19.0 μ M treatment. Compounds were tested at their IC_{50} values in the wound-healing assay (WHA).

Figure 5.

Anti-invasive activities of compounds **1**, **10–13** and **15–17** against the human breast cancer cell line MDA-MB-231 using the Cultrex[®] BME Cell Invasion assay kit. Error bars indicate the SEM of $n=3$ per compound. * $P < 0.05$ compared to the vehicle (DMSO)-treated control group.

Figure 6.

C. elegans as an *in vivo* model to assess the BK channel inhibitory effect of new analogs **7**, **9–11** and **17** as compared to the parent **1**. Wild type N2 strain treated with 1 μ M of each compound is compared with DMSO (vehicle control) and the *slo-1(js379)V* mutant strain NM1968 (positive control). Error bars indicate SEM for at least $n=10$. * $P < 0.05$ compared to their respective vehicle (DMSO)-treated control group.

	R ₁	R ₂	R ₃	R ₄
1	Cl	OH	H	H
8	Br	H	H	H
9	Br	OH	H	H
10	Cl	OH		H
11	Cl	OH	CH ₃	CH ₃
12	Cl	OH	H	CH ₃
13	Cl	OH	CH ₃	H
14	Cl	OH		H
15	Cl	OH		
16	Cl	OOH	H	H

R = H; Penitremone A (**19**)
R = OH; Penitremone B (**20**)

Paspaline (**6**)

Emindole SB (**7**)

Pennigretrem (**21**)

Figure 7.

Scheme 1.

Semisynthetic modifications of penitrem A (**1**). (a) Benzoyl chloride, *N,N*-DMAP, CH₂Cl₂, reflux. (b) Alkyl or aryl halide, NaH, dry DMF, room temperature, overnight. (c) *m*-Chloroperoxybenzoic acid (*m*-CPBA), CH₂Cl₂, room temperature, overnight. (d) Lead tetraacetate (LTA), CH₂Cl₂, room temperature, 6–8 hours.

Table 1

¹³C NMR Spectroscopic Data of Compounds **1**, **10**, **11**, and **14–16**^a

Position	δ_c					
	1	10	11	14	15	16
2	153.5, qC	153.3, qC	155.1, qC	153.3, qC	155.2, qC	153.7, qC
3	119.7, qC	119.8, qC	120.1, qC	119.7, qC	120.4, qC	120.9, qC
4	132.4, qC	132.5, qC	132.6, qC	132.4, qC	133.0, qC	132.5, qC
5	124.9, qC	124.9, qC	124.9, qC	124.8, qC	125.5, qC	125.3, qC
6	123.7, qC	123.7, qC	124.3, qC	123.7, qC	124.7, qC	124.1, qC
7	110.9, CH	111.0, CH	109.4, CH	111.0, CH	109.9, CH	119.7, CH
8	121.2, qC	121.1, qC	121.0, qC	121.1, qC	121.7, qC	121.3, qC
9	138.8, qC	138.9, qC	139.9, qC	138.9, qC	140.6, qC	139.3, qC
10	34.2, CH ₂	34.2, CH ₂	34.2, CH ₂	34.2, CH ₂	34.3, CH ₂	34.7, CH ₂
11	148.6, qC	148.6, qC	148.4, qC	148.6, qC	148.3, CH ₂	147.7, qC
12	46.2, CH	46.1, CH	46.2, CH	46.1, CH	46.2, CH	46.3, CH
13	23.8, CH ₂	23.8, CH ₂	23.6, CH ₂	23.8, CH ₂	23.6, CH ₂	23.5, CH ₂
14	51.8, CH	51.8, CH	51.8, CH	51.8, CH	51.8, CH	47.0, CH
15	80.1, qC	80.1, qC	79.9, qC	80.1, qC	79.9, CH	84.9, qC
16	75.2, qC	75.2, qC	74.9, qC	75.2, qC	75.0, qC	79.9, qC
18	71.6, CH	71.5, CH	70.6, CH	71.6, CH	70.7, CH	71.4, CH
19	57.9, CH	57.9, CH	58.1, CH	57.9, CH ₂	58.5, CH	51.4, CH
20	17.7, CH ₂	17.7, CH ₂	17.4, CH ₂	17.7, CH ₂	17.5, CH ₂	17.9, CH ₂
21	29.7, CH ₂	29.7, CH ₂	29.6, CH ₂	29.7, CH ₂	29.3, CH ₂	29.7, CH ₂
22	77.4, qC	77.1, qC	78.2, qC	78.0, qC	78.1, qC	78.1, qC
23	65.3, qC	65.2, qC	65.1, qC	65.3, qC	65.1, qC	65.5, qC
24	61.0, CH	57.9, CH	58.1, CH	58.4, CH	58.0, CH	61.5, CH
25	65.4, CH	67.5, CH	75.8, CH	73.3, CH	73.8, CH	65.9, CH
26	73.8, CH	72.5, CH	73.3, CH	73.7, CH	73.4, CH	74.2, CH
28	71.1, CH	70.9, CH	70.4, CH	70.4, CH	70.4, CH	71.4, CH
29	28.0, CH ₂	29.7, CH ₂	28.1, CH ₂	28.3, CH ₂	28.0, CH ₂	28.3, CH ₂
30	26.0, CH ₂	25.9, CH ₂	26.3, CH ₂	26.0, CH ₂	26.0, CH ₂	25.6, CH ₂

Position	δ_c							
	1	10	11	14	15	16		
31	42.7, CH ₂	42.9, CH ₂	43.6, qC	42.9, qC	43.5, qC	43.4, qC		
32	49.2, qC	49.1, qC	52.4, qC	49.1, qC	52.9, qC	52.5, qC		
33	106.3, CH ₂	106.2, CH ₂	106.3, CH ₂	106.3, CH ₂	106.5, CH ₂	108.0, CH ₂		
34	19.5, CH ₃	19.6, CH ₃	19.6, CH ₃	19.5, CH ₃	19.6, CH ₃	20.4, CH ₃		
35	30.2, CH ₃	30.1, CH ₃	30.3, CH ₃	30.3, CH ₃	30.3, CH ₂	30.1, CH ₃		
36	18.9, CH ₃	18.9, CH ₃	19.0, CH ₃	19.0, CH ₃	19.0, CH ₃	19.3, CH ₃		
37	142.4, qC	141.6, qC	143.2, qC	143.2, qC	143.1, qC	142.8, qC		
38	110.8, CH ₂	111.4, CH ₂	110.4, CH ₂	110.6, CH ₂	110.6, CH ₂	111.3, CH ₂		
39	18.1, CH ₃	17.8, CH ₃	17.5, CH ₃	18.0, CH ₃	17.4, CH ₃	19.2, CH ₃		
40	20.6, CH ₃	20.5, CH ₃	20.1, CH ₃	20.6, CH ₃	20.7, CH ₃	20.1, CH ₃		
1'	-	165.2, qC	58.4, CH ₃	72.6, CH ₂	72.6, CH ₂	-		
2'	-	130.1, qC	-	132.7, qC	140.6, CH	-		
3'	-	129.4, CH	-	127.4, CH	120.4, CH	-		
4'	-	128.5, CH	-	128.2, CH	160.2, qC	-		
5'	-	133.2, CH	-	127.8, CH	113.3, CH	-		
6'	-	128.5, CH	-	128.2, CH	129.7, CH	-		
7'	-	129.4, CH	-	127.4, CH	118.0, CH	-		
8'	-	-	-	-	54.6, CH	-		
1''	-	-	33.1, CH ₃	-	48.8, CH ₂	-		
2''	-	-	-	-	139.7, CH	-		
3''	-	-	-	-	112.2, CH	-		
4''	-	-	-	-	159.8, qC	-		
5''	-	-	-	-	112.0, CH	-		
6''	-	-	-	-	129.2, CH	-		
7''	-	-	-	-	112.8, CH	-		
8''	-	-	-	-	54.6, CH	-		

^aIn (CD₃)₂CO, *J* in Hz. 100 MHz for ¹³C NMR. Carbon multiplicities were determined by APT or PENDANT experiments, C = quaternary, CH = methine, CH₂ = methylene, CH₃ = methyl carbons.

Table 2

¹H NMR Spectroscopic Data of Compounds **1**, **10**, **11**, and **14–16**^a

Position	δ_H , mult. (<i>J</i> in Hz)					
	1	10	11	14	15	16
1	10.01, brs	10.02, brs	-	10.03, brs	-	10.03, brs
7	7.18, s	7.22, s	7.35, s	7.22, brs	7.18, s	7.06, s
	3.58, d (15.6)	3.60, d (15.6)	3.63, d (16.0)	3.61, d (15.6)	3.63, d (16.0)	3.44, d (16.5)
10	3.21, dd (16.0, 1.4)	3.22, dd (15.6, 1.4)	3.24, dd (15.6, 1.4)	3.23, dd (16.0, 1.4)	3.23, dd (16.0, 1.4)	3.10, dd (16.5, 1.4)
12	2.92, m	2.95, m	2.95, m	2.96, m	2.98, m	2.93, m
13	2.34, m	2.35, m	2.37, m	2.39, m	2.42, m	2.30, m
	2.19, m	2.14, m	2.22, m	2.19, m	2.26, m	2.25, m
14	2.42, m	2.41, m	2.45, m	2.46, m	2.46, m	3.08, m
18	4.87, d (8.2)	4.89, d (8.2)	4.86, m	4.91, d (8.2)	4.88, d (8.3)	4.53, d (10.5)
19	2.56, m	2.55, m	2.57, m	2.62, m	2.59, m	2.99, m
20	1.88, m	1.90, m	1.90, m	1.89, m	1.85, m	1.79, m
	1.75, m	1.77, m	1.78, m	1.78, m	1.79, m	1.57, m
21	1.72, m	1.71, m	1.76, m	1.68, m	1.66, m	2.66, m
	1.45, m	1.52, m	1.51, m	1.50, m	1.46, m	1.66, m
24	3.52, brs	3.81, m	3.74, m	3.76, m	3.67, brs	3.53, m
25	3.98, brs	5.58, dd (3.2, 1.4)	3.70, m	3.95, m	3.89, m	4.00, brs
26	3.98, brs	4.25, brs	4.02, brs	4.07, brs	4.02, brs	4.00, brs
28	4.24, brd (8.7)	4.42, brd (8.2)	4.28, brd (9.4)	4.31, brd (8.2)	4.20, brd (8.7)	4.23, brd (8.7)
29	2.21, m	2.21, m	2.24, m	2.24, m	1.89, m	2.18, m
	2.04, m	2.07, m	2.06, m	1.97, m	1.89, m	2.05, m
30	2.56, m	2.67, m	2.69, m	2.57, m	2.51, m	2.56, m
	1.52, m	1.63, dd (13.3, 5.0)	1.86, m	1.55, m	1.52, m	1.72, m
33	4.96, brs	5.07, m	5.02, m	5.00, m	5.03, m	5.01, m
	4.82, brs	4.83, m	4.84, m	4.86, m	4.88, m	4.82, m
34	1.01, s	1.00, s	1.04, brs	1.06, brs	1.02, s	1.65, s
35	1.70, s	1.71, s	1.74, brs	1.73, brs	1.75, s	1.16, brs

Position	δ_H , mult. (<i>J</i> in Hz)							
	1	10	11	14	15	16		
36	1.66, s	1.71, s	1.66, brs	1.72, brs	1.71, s	1.67, brs		
38	5.02, brs 4.82, brs	4.98, m 4.79, m	5.02, m 4.83, m	5.05, m 4.86, m	5.03, m 4.86, m	5.04, m 4.84, m		
39	1.18, brs	1.23, s	1.25, brs	1.23, brs	1.18, s	1.26, brs		
40	1.35, brs	1.40, s	1.35, brs	1.38, brs	1.31, s	1.31, brs		
1'	-	-	3.44, brs	4.62, d (11.4) 4.78, d (11.4)	4.59, d (11.9) 4.70, d (11.9)	-		
3'	-	7.97, m	-	7.33, m	6.90, m	-		
4'	-	7.44, m	-	7.27, m	-	-		
5'	-	7.56, m	-	7.22, m	6.81, m	-		
6'	-	7.44, m	-	7.27, m	7.23, m	-		
7'	-	7.97, m	-	7.33, m	6.54, d (8.2)	-		
8'	-	-	-	-	3.71, s	-		
1''	-	-	3.88, brs	-	5.53, d (17.4) 5.60 d (17.9)	-		
3''	-	-	-	-	6.59, m	-		
5''	-	-	-	-	6.79, m	-		
6''	-	-	-	-	7.19, m	-		
7''	-	-	-	-	6.88, d (7.8)	-		
8''	-	-	-	-	3.73, s	-		

^a_{In} (CD₃)₂CO, *J* in Hz. 400 MHz for ¹H NMR.

Table 3

¹³C and ¹H NMR Spectroscopic Data of Compounds **17** and **18**

Position	17		18	
	δ_C^a	δ_H , mult. (J in Hz) ^b	δ_C^a	δ_H , mult. (J in Hz) ^b
1		9.96, brs -	-	10.03, brs
2	153.9, qC	-	153.8, qC	-
3	119.6, qC	-	118.3, qC	-
4	132.4, qC	-	131.5, qC	-
5	125.0, qC	-	125.0, qC	-
6	123.6, qC	-	124.0, qC	-
7	111.0, CH	7.20, s	110.9, CH	7.22, s
8	121.1, qC	-	120.4, qC	-
9	138.8, qC	-	138.9, qC	-
10	34.2, CH ₂	3.60, d (16.6) 3.21, dd (16.0, 1.4)	34.0, CH ₂	3.66, d (15.1) 3.29, m
11	148.6, CH ₂		148.3, qC	-
12	46.2, CH	2.94, brd (8.7)	45.9, CH	2.92, brd (8.3)
13	23.8, CH ₂	2.36, m 2.21, m	23.6, CH ₂	2.37, m 2.24, m
14	51.8, CH	2.43, m	52.0, CH	2.50, m
15	80.2, qC	-	80.1, qC	-
16	75.2, qC	-	76.1, qC	-
18	71.6, CH	4.90, d (8.3)	72.2, CH	4.92, d (5.0)
19	57.6, CH	2.69, m	57.4, CH	2.63, m
20	17.9, CH ₂	1.95, m 1.81, m	17.7, CH ₂	1.81, m 1.78, m
21	30.6, CH ₂	1.76, m 1.49, m	29.4, CH ₂	1.69, m 1.55, m
22	76.4, qC	-	78.3, qC	-
23	71.2, qC	-	67.8, qC	-
24	63.1, CH	3.66, s	64.0, CH	3.01, d (7.3)
25	97.2, CH	5.30, brd (3.7)	95.8, CH	5.04, d (7.3)
27	76.3, CH	4.24, dd (9.2, 9.2)	77.7, CH	4.78, dd (11.5, 6.4)
28	28.9, CH ₂	2.04, m 2.09, m	27.4, CH ₂	2.09, m 1.96, m
29	26.9, CH ₂	2.53, m 1.56, m	27.2, CH ₂	2.53, m 1.66, m
30	44.1, qC	-	43.9, qC	-
31	49.5, qC	-	49.8, qC	-
32	106.3, CH ₂	4.98, m 4.84, m	105.6, CH ₂	5.00, m 4.88, m
33	19.5, CH ₃	1.03, s	18.9, CH ₃	1.71, brs
34	30.2, CH ₃	1.71, s	29.3, CH ₃	1.09, brs
35	18.8, CH ₃	1.31, s	18.2, CH ₃	1.26, brs
36	20.4, CH ₃	1.42, s	19.9, CH ₃	1.39, brs

Position	17		18	
	δ_C^a	$\delta_H, \text{mult. (J in Hz)}^b$	δ_C^a	$\delta_H, \text{mult. (J in Hz)}^b$
Position	17		18	
	$\delta_C(100 \text{ MHz})^a$	$\delta_H, \text{mult. (J in Hz)}^b$	$\delta_C(100 \text{ MHz})^c$	$\delta_H, \text{mult. (J in Hz)}^d$
1`	-	-	145.2, CH	6.09, brs
2`	-	-	106.3, qC	-
3`	-	-	61.5, CH ₂	4.65, brs
4`	-	-	14.2, CH ₃	1.58, d (1.4)
1``	-	-	170.3, qC	-
2``	-	-	19.5, CH ₃	2.02, brs

^a In (CD₃)₂CO, *J* in Hz. 100 MHz for ¹³C NMR. Carbon multiplicities were determined by APT or PENDANT experiments, C = quaternary, CH = methine, CH₂ = methylene, CH₃ = methyl carbons.

^b In (CD₃)₂CO, *J* in Hz. 400 MHz for ¹H NMR.

Table 4

Antiproliferative and Antimigratory Activities of Compounds **1** and **10–18** Using Human Breast Cancer Cell Lines, MCF-7 and MDA-MB-231.

Compound	Antiproliferative activity (IC ₅₀ , μ M)		Antimigratory activity (IC ₅₀ , μ M)
	MCF-7	MDA-MB-231	MDA-MB-231
1	11.9	9.8	8.7
10	> 50	13.8	> 50
11	> 50	> 50	> 50
12	NA	> 50	> 50
13	22.7	> 50	> 50
14	> 50	> 50	> 50
15	> 50	> 50	> 50
16	> 50	> 50	> 50
17	13.3	43.8	48.2
18	32.3	10.0	> 50

Table 5
Results of the Up-and-Down Procedure (UDP) used for testing the tremorgenic potential of compounds **1**, **8–11** and **17**

Compound	Dose (mg/Kg)	Animal								Incidence of tremors	Calculated EC ₅₀ (mg/kg) ^a
		1	2	3	4	5	6	7	8		
1	1.92	x			x	x				3/3	0.60 ^b
	0.60		x		o		o		o	1/4	
	0.19			o						0/1	
8	19.6						x			1/1	3.43
	6.14		x				o		x	2/3	
	1.92	o		x		o				1/3	
	0.60				o					0/1	
9	1.92	x								1/1	
	0.60		x		x		x			3/3	0.32
	0.19			o	o	o	o			0/3	
10	19.6			o							
11	6.14		o							Study terminated because of solubility issues.	
17	1.92	o									

x: caused tremors

o: no tremors

^a: AOT425StatPgm program was used for calculating EC₅₀.

^b: Tremorgenic dose of **1**: 250–600 mg/kg, ip in mice [10].