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Biemamides A–E, Inhibitors of the TGF- β Pathway that Block the Epithelial to Mesenchymal Transition

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Abstract

Screening of a marine natural products library for inhibitors of TGF- β revealed five pyrimidinedione derivatives, biemamides A–E (**1–5**). The structures were determined by 2D NMR and HRMS experiments; absolute configurations were established by advanced Marfey's analysis and ECD calculations. Biemamides A–E specifically inhibited *in vitro* TGF- β induced epithelial-to-mesenchymal transition in NMuMG cells. Additionally, using *Caenorhabditis elegans*, selected biemamides were found to influence *in vivo* developmental processes related to body size regulation in a dose-dependent manner.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b01871.

General information for all experimental procedures, characterization data, biological activity assays (including 48 initial hit screening data), and NMR spectra for compounds **1–5** (PDF).

The authors declare no competing financial interest.

The transforming growth factor- β (TGF- β) superfamily of proteins consists of more than 30 members, including TGF- β , activins, inhibins, growth differentiation factors, and bone morphogenetic proteins (BMPs). It regulates many biological processes, such as cellular proliferation, differentiation, morphogenesis, matrix formation, extracellular proteolysis, angiogenesis, and inflammation.¹ In mammals, three different TGF- β isoforms (TGF- β 1, β 2, and β 3) encoded by different genes have been identified, and they exhibit similar effects in a variety of biological assays.²

TGF- β plays prominent roles in human disease, especially cancer,³ where it functions as a tumor suppressor in normal and early neoplastic cells. However, in established cancers, TGF- β is a central player in tumor growth, invasion and subsequent metastasis. TGF- β also coordinately upregulates the expression of many matrix proteins, and has been shown to play a causative role in the progression of many fibrotic disorders.⁴ Consequently, the development of TGF- β inhibitors has become a high priority; inhibitors promise to serve as useful biochemical tools as well as prospective therapeutics for fibrotic and oncological diseases.⁵ Indeed, several small molecule TGF- β inhibitors have been reported, and some have been advanced into clinical trials including Galunisertib (LY2157299 monohydrate),⁶ an oral small molecule inhibitor of the TGF- β receptor I kinase, and to date, the most advanced TGF- β signaling inhibitor under clinical development.

Inspired by recent advances in TGF- β and cancer biology, we, like many others, have incorporated a TGF- β modulating assay into screening strategies to identify new bioactive species from novel sources. Natural products have been, and remain, rich sources of clinically important compounds as reflected by the fact that 83% of small molecule anticancer drugs developed between 1981 and 2014 were either natural products or were inspired by them.⁷ We report here the discovery and characterization of five pyrimidinedione derivatives, biemamides A–E (**1–5**; Figure 1), from a marine-derived *Streptomyces* sp. These discoveries were enabled by a mink lung cell-based luciferase assay for TGF- β pathway inhibition; **1–5** were found to be potent TGF- β inhibitors.

In focusing on new natural product discoveries from marine-derived organisms, we have previously reported a highly productive metabolomics-based drug discovery platform. Microbial producer strain prioritization employs metabolomics and an LC/MS fractionation system to generate high-purity natural product libraries for high-throughput screening.⁸ In addition, low-temperature evaporative light scattering detectors (ELSD-LT) are generally used to quantitate high-purity natural product libraries in our approach.⁹ In this specific case, mink lung epithelial cells (MLEC) were stably transfected with an expression construct containing a truncated plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reported gene.¹⁰ Since PAI-1 is strongly induced by TGF- β , addition of TGF- β to the transfectants induces a dose-dependent increase in luciferase activity in the cell lysates. Hence, correlated luciferase activity to TGF- β pathway signaling represented a quantifiable means of monitoring TGF- β signaling efficiencies and the resultant mink cell line enabled a facile means of screening for small molecule TGF- β inhibitors. A total of 3840 fractions from 48 strains were screened, and, of these, 48 active fractions were identified. Dose-response TGF- β data and cytotoxicity assays using fibroblasts were

employed to more rigorously evaluate the 48 initial hits. Three active wells (G8, G9, G10) from the strain WMMA2266 inhibited TGF- β signaling with IC₅₀ values of 38.0, 45.7 and 49.5 ng/mL; none displayed significant cytotoxicity at the highest concentration 1 μ g/mL. Chemical analysis of the secondary metabolites in the active wells/extract fractions of WMMA2266 led to the discovery of five pyrimidinedione derivatives, biemamides A–E (**1**–**5**). Subsequent application of the MLEC-based TGF- β inhibition assay revealed that purified **1**–**5** inhibited TGF- β with IC₅₀s of 51.5, 18.7, 51.5, 101.7, and 83.2 nM, respectively, whereas the positive control SB431532 showed an IC₅₀ value of 411 nM [see Supporting Information (SI)].

Notably, as we were identifying new TGF- β inhibitors **1**–**5**, Goto and co-workers patented compounds **1** and **2**, as antitumor agents from an alternative *Streptomyces* strain; no stereochemical assignments were publicized at the time.¹¹ The 2D structures of WMMA2266-derived **1** and **2** were elucidated on the basis of NMR and MS data (Tables S1 and S2; SI) which were also in good agreement with Goto's report.¹¹

The absolute configurations of **1** and **2** were elucidated via application of Marfey's advanced method.¹² The acid hydrolysate of **1** was split into two equal portions and derivatized with L-FDLA and DL-FDLA, respectively. LCMS analysis of the L-FDLA and DL-FDLA products supported the assignments of D-(2,3-diaminopropanoyl)methylcarbamic acid (DAPMA) (Figure S51; SI).

The absolute configurational assignment of **1** was supported by comparing experimental and calculated electronic circular dichroism (ECD) spectra generated by time-dependent functional theory (TDDFT).¹³ A simplified structure **6** was used for *R*- and *S*-**1** to limit conformational flexibility associated with the pendant lipid. A conformational analysis was performed using Spartan 14 software and MMFF molecular mechanics calculations. The MMFF conformational search was further optimized using TDDFT at the B3LYP/6–31G(d) basis set level, affording the eight lowest energy conformers.

The overall calculated ECD spectra of (*R*)-**6** and (*S*)-**6** were generated by Boltzmann distribution of the eight lowest energy conformers with 94.5%, 1.4%, 1.4%, 1.1%, 1.0%, 0.4%, 0.2% and 0.1%. The overall experimental CD spectra of **1** compared well only to that of the calculated ECD spectrum of (*R*)-**6**; both showed negative Cotton effects (CE) in the regions of 230–260 nm (Figure 2). Therefore, the absolute configuration of **1** was deduced to be 3*R*.

HRMS data indicated that both **3** and **4** possess the molecular formula C₂₄H₄₂N₄O₄. Additionally, ¹H and ¹³C NMR data (Tables S3 and S4; SI) for **3** and **4** revealed a high degree of structural similarity to **1** and **2**, although both **3** and **4** appeared to contain an olefin within each respective lipid chain. Both olefins were assigned as *Z* on the basis of ¹³C NMR chemical shifts and the realization that allylic carbons of linear olefins of the *Z*-isomers resonate at higher field than those of *E*-isomers (*Z* ~27 ppm; *E* ~32 ppm).¹⁴ Olefin positions for **3** and **4** were then determined on the basis of HRMS for semi-synthetic oxidatively cleaved variants of **3** and **4**. Subjection of both **3** and **4** to NaIO₄/OsO₄ led, predictably, to aldehyde installation – both reactions afforded **7** (Figure S31; SI).¹⁵ HRMS supporting the

molecular formula $C_{17}H_{28}N_4O_5$ for **7** along with all other spectral data revealed the Z olefin to reside between C-17 and C-16 of aldehyde precursors **3** and **4**.

The elemental composition of **5** was established as $C_{26}H_{44}N_4O_4$ on the basis of HRESIMS and NMR data revealed that **5** possessed a core scaffold almost identical to that of **4**. Clear however in spectra of both **4** and **5** was the presence of an additional olefinic signal within the lipid chain of **5** relative to **4**. Analysis of 2D NMR (Table S5; SI), enabled us to identify the regiochemistry of both olefins within the pendant lipid of **5** and this assignment was readily validated by HRMS analyses of semi-synthetically oxidized **5**. The ^{13}C NMR chemical shifts of the allylic methylene carbons C-15 (δ_C 26.7), C-18 (δ_C 25.3), and C-21 (δ_C 26.7) suggested that the geometric conformation of the two olefins were Z.

Structural characterization of **1–5** was completed upon assigning stereochemistry to **2–5**. On the strength of our C-3 assignment for **1** and the structural/biosynthetic relatedness of all species herein, we tentatively assigned **2–5** as 3*R*. Not surprisingly, this absolute configuration was confirmed on the basis of CD spectra and comparisons to data achieved for compound **1**.

TGF- β plays a central role in processes related to tumor metastasis and consequently, its inhibition by small molecules has become a high profile pursuit in both industrial and academic settings. Epithelial to mesenchymal transitions (EMTs) are paramount to a variety of developmental processes and are largely orchestrated by TGF- β .¹⁶ Significantly, EMT dictates, to a large degree, the lethality of human cancers; primary tumor cells that have undergone EMT pose a tremendous threat by virtue of their capacity to trigger tumor metastasis. In addition, aberrant EMTs are a critical feature of other diseases, such as end-stage organ failure by fibrosis. Given this, and initial bioactivities noted for **1–5** in our early mink cell screens, we next sought to assess the ability of **1–5** to modulate EMTs via the agency of TGF- β attenuation.

The ability of biemamides A–E to modulate downstream TGF- β signaling (as reflected by EMT), was assessed using a NMuMG cell line in vitro. Notably, NMuMG is a non-transformed mouse mammary epithelial cell line that exhibits both a growth inhibitory and EMT response to TGF- β .¹⁹ In the absence of TGF- β , NMuMG cells form an epithelial shape; incubation with 50 pM TGF- β for 72 h induces a more fibroblast-like, spindle shaped morphology indicative of mesenchymal cells (Figure 3). As a positive control 1 μ M of SB431542,²⁰ known as a TGF- β Type I receptor kinase inhibitor, blocked TGF- β -induced transition of the NMuMG epithelial cells into mesenchymal cells. Nine concentrations (160–0.625 nM) of **1–5** were tested for their ability to impact TGF- β signaling in NMuMG cells. The imaging results suggest that **1–5** effectively inhibited EMT induction; cells maintained their epithelial phenotype as reflected by data for **1** which is shown in Figure 3 below.

Having established *in vitro* TGF- β inhibiting efficacy of **1–5**, we next sought to evaluate possible *in vivo* activities. In *C. elegans*, two TGF- β -related pathways regulating dauer formation or body length have been identified, the Dauer pathway and the Sma/Mab pathway.²² In nutrient-rich environments, worms rapidly develop from embryo through four larval stages (L1–L4) to adult in 3.5 d (20 °C) in a process termed reproductive

development. If exposed to nutrient limited conditions early in larval life, animals divert their development to a specialized third larval stage called the dauer diapause (L3d).²³ *C. elegans* use TGF- β related signaling to regulate dauer formation. In addition to the dauer/TGF- β pathway, a second TGF- β related pathway Sma/Mab has been identified in *C. elegans*.²⁴ Mutations in this pathway are characterized by small body size (Sma) and male tail defects (Mab). These two homologous pathways are genetically distinct, utilizing different ligands, type I receptors, and Smads. Only the type II receptor DAF-4 is shared between the two. Therefore, a TGF- β inhibitor may target the Dauer or Sma/Mab pathways individually or in tandem; specific pathway differentiation as a target of **1–5** was deemed early on as being beyond the scope of this report.

C. elegans were grown (in triplicate) in four concentrations of **1–5** ranging from 10 nM–10 μ M in a liquid 96-well plate at room temperature; *Escherichia coli* served as a food source. After 3 d incubation at room temperature, all *C. elegans* were microscopically imaged and body length assessments were carried out (Figures 4, 5 and S45–S49 in SI). *C. elegans* subjected only to solvent (control) had grown to adulthood, as evidenced by the onset of egg production as well as attainment of a typical adult size. Worms subjected to the highest concentration (10 μ M) of **1–5** clearly displayed signs of late development and small body type individuals were easily detected. Unlike the *in vitro* system, no good TGF- β inhibitory positive controls were available for *C. elegans* studies. However, at reduced biemamide concentrations, a dose-dependent effect on development and body size regulation was clearly observed. These *in vivo* results suggest biemamides can inhibit TGF- β related pathways with effective concentrations spanning 100 nM and 1 μ M and with likely IC₅₀ values in the realm of ~300 nM. SB431542, which had been shown to inhibit TGF- β induced EMT in NMuMG cells *in vitro*, did not alter *C. elegans* size under three concentrations spanning 1 μ M $\rightarrow$ 100 μ M (Figure S50; SI).

In conclusion, potent TGF- β inhibitors have tremendous promise as therapeutics for pulmonary fibrosis and assorted cancers, especially those that become metastatic. In contrast to many known TGF- β inhibitors based on five-membered heterocyclic chemotypes (imidazoles, pyrazoles, or thiazoles), such as SB431542, SB505124,²⁵ Galunisertib (LY2157299), LDN-193189,²⁶ LY2109761,²⁷ LY364947,²⁸ GW788388,²⁹ EW-7197,³⁰ and SM-16,³¹ the natural product biemamides have a completely different scaffold. Highly significant is that these compounds potently suppress TGF- β signaling both *in vitro* and *in vivo*, suggesting that neither membrane permeability nor metabolic liabilities represent obvious future roadblocks to their continued development. Taken together, these findings highlight the importance of marine-derived microbes as sources of new natural products with biological activities likely to beneficially impact human health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of 1–5.

Figure 2. Experimental CD spectrum of compound **1** in $\text{CH}_3\text{OH}/\text{CHCl}_3$ (1:1) and calculated ECD spectra of two enantiomers, (3*R*)-**6** and (3*S*)-**6**.

Figure 3.

Effect of **1** on 50 pM TGF- β induced EMT in NMuMG cells (scale bars: 400 μ m). Each experiment was repeated three times with similar results. (A) Untreated cells. (B) 50 pM TGF- β treated cells. (C) 160 nM compound **1** was incubated with 50 pM TGF- β treated cells for 72 h. (D) 1 μ M SB431542 (positive control) was incubated with 50 pM TGF- β treated cells for 72 h.

Figure 4.
Alterations in *C. elegans* development by 1–5.

Figure 5. Phenotypes of *C. elegans* treated with **1**. All panels are identically scaled [200 μm-see **A** red bar]. (**A**) Solvent only control. (**B**) 10 μM **1**. (**C**) 1 μM **1**. (**D**) 100 nM **1**. (**E**) 10 nM **1**.