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Synthesis and antileukemic activities of C1–C10-modified parthenolide analogues

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ABSTRACT

Parthenolide (**PTL**) is a sesquiterpene lactone natural product with anti-proliferative activity to cancer cells. Selective eradication of leukemic stem cells (LSCs) over healthy hematopoietic stem cells (HSCs) by **PTL** has been demonstrated in previous studies, which suggests **PTL** and related molecules may be useful for targeting LSCs. Eradication of LSCs is required for curative therapy. Chemical optimizations of **PTL** to improve potency and pharmacokinetic parameters have focused largely on the α-methylene-γ-butyrolactone, which is essential for activity. Conversely, we evaluated modifications to the C1–C10 olefin and benchmarked new inhibitors to **PTL** with respect to inhibitory potency across a panel of cancer cell lines, ability to target drug-resistant acute myeloid leukemia (AML) cells, efficacy for inhibiting clonal growth of AML cells, toxicity to healthy bone marrow cells, and efficiency for promoting intracellular reactive oxygen species (ROS) levels. Cyclopropane **4** was found to possess less toxicity to healthy bone marrow cells, enhanced potency for the induction of cellular ROS, and similar broad-spectrum anti-proliferative activity to cancer cells in comparison to **PTL**.

1. Introduction

Sesquiterpene lactones (SL) are a diverse family of plant-derived natural products with utilities in treating inflammatory diseases and cancer.[1,2] Parthenolide (**PTL**) is a well-studied SL derived from the feverfew plant *Tanacetum parthenium*,[3] bearing broad-spectrum anti-proliferative activities to a variety of cancer types through multiple mechanisms of inhibition.[4,5] The seminal discovery that **PTL** induces apoptosis in acute myeloid leukemia (AML) stem and progenitor cells without exhibiting comparable toxicity to healthy hematopoietic stem cells (HSCs) has anointed **PTL** as the prototypical member of next-generation therapies for eradicating leukemic stem cells (LSCs).[6] AML growth is hierarchical and originates from LSCs.[7,8] Therefore, small molecules that eliminate LSCs are expected to confer more durable and potentially curative therapies.[9–13] In addition to its anti-leukemic activity, **PTL** has been explored as a potential therapeutic for a spectrum of indications.[1,4,14]

Chemical optimizations of **PTL** have been required to optimize the natural product for in vivo applications. A Phase I dose escalation trial of feverfew extract failed to achieve measurable levels of **PTL** in serum and oral dosing (40 mg/kg) of **PTL** in mice yielded approximately 200 nM concentrations in serum, which is not sufficient to confer anti-proliferative activity.[15,16] Conversion of **PTL** to prodrug dimethylamino-parthenolide fumarate, **DMAPT** (or **LC-1**), increased water solubility by ~1000-fold and yielded an analogue with substantially improved pharmacokinetic parameters (mice: C_{max} = 25 μM, $t_{1/2}$ = 0.6 h; canine: C_{max} = 61 μM, $t_{1/2}$ = 1.9 h) with oral dosing (100 mg/kg).[17,18] Hetero-Michael

Abbreviations: **PTL**, parthenolide; LSC, leukemic stem cell; AML, acute myeloid leukemia; SL, sesquiterpene lactone; HSC, hematopoietic stem cell; DMAPT, dimethylamino-parthenolide; **MelB**, melampomagnolide B; ROS, reactive oxygen species; PtO_2, platinum(IV) oxide; DME, dimethoxyethane; Log*D*, distribution coefficient; CSC, cancer stem cell; **CTL**, costunolide; BM, bone marrow; DOX, doxorubicin; AraC, cytarabine; MEM, minimum essential medium; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; HEPES, (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid); IMDM, Iscove's modified Dulbecco's medium; MTS, 3-(4,5-dimethylthiaol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; SFEM, serum-free expansion medium.

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addition of aliphatic amines to natural products and synthetic analogues bearing α-methylene-γ-butyrolactones has constituted a modular strategy to enhance water solubilities through prodrug formation.[19–25] Semisynthetic modifications to **PTL** outside of the α-methylene-γ-butyrolactone warhead, however, are significantly less developed. Acid-catalyzed conversion of **PTL** to 5-7-5 guaianolide, Micheliolide (**1**), has been achieved, yielding a derivative with anti-proliferative activity comparable to its predecessor.[21,26,27] Photochemical isomerization of the C1–C10 olefin of **PTL** has also been reported, yielding *cis*-olefin **2**.[28–31] Allylic oxidation of the C1–C10 vinyl methyl group of **PTL** results in formation of another natural product, Melampomagnolide B (**MelB**), which exhibits comparable anti-proliferative activity as **PTL**, but contains an allylic alcohol, which is useful for further transformations (e.g., synthesis of affinity pulldown reagents and O-functionalized analogues).[32–36] Recently, the Fasan laboratory has utilized P450 enzymes to oxygenate proximal to the C1–C10 olefin of **PTL**, yielding alcohols at C9 and C14, which were subsequently esterified with substituted benzoic acids to yield **PTL** analogues with anti-leukemic activities.[37]

In this study, we examined the necessity of the **PTL** C1–C10 olefin and its tolerance to structural modification with respect to sustained anti-proliferative activities to cancer cells through the synthesis and biochemical screening of C1–C10 modified **PTL** analogues. Included among our small library of compounds are established **PTL** analogues, such as Micheliolide (**1**), *cis*-**PTL** (**2**), and **MelB**, as well as additional mechanistic probes, such as **3** (reduced C1–C10 olefin) and **4** (cyclopropanated C1–C10 olefin). Interestingly, cyclopropanated analogue **4** was found to exhibit similar anti-proliferative activity to cancer cells as **PTL**, but conferred less toxicity to healthy bone marrow and more potently induced cellular reactive oxygen species (ROS), which is known to promote cell death to AML stem cells and other cancer cells.[38–47]

2. Results and discussion

2.1. Design and synthesis of PTL analogues

A small library of C1–C10 olefin modified **PTL** analogues and control compounds were synthesized or purchased commercially (Fig. 1). Previous studies have found that the C1–C10 olefin of **PTL** can participate in electrophilic transannular cyclizations with the C4–C5 epoxide under Brønsted or Lewis acid conditions to yield guaianolide analogues.[28,29] Therefore, we synthesized reduced analogue **3** to eliminate any potential for acid instability. The exocyclic methylene on the α-methylene-γ-butyrolactone of **PTL** was transiently protected by dimethylamine addition to yield dimethylamino-**PTL**, which was not converted to the fumarate salt for this application (Scheme 1). Hydrogenation of dimethylamino-**PTL** free base with PtO_2 catalyst at 50 psi H_2 resulted in facial selective reduction (~15:1), yielding 10*R*-diastereomer **3**. The exocyclic methylene was deprotected under Hofmann elimination conditions (excess MeI, THF, H_2O)[20,48,49] to yield **3** in 32% yield (over three steps). The stereochemistry of the methyl group was assigned by X-ray crystallography (**SI**).

Cyclopropanes are unique ring systems with significant sp^2-character, thereby mimicking the electronics of double bonds.[50] Such modifications can be valuable for increasing the stability of a drug candidate. In the case of **PTL**, replacement of the C4–C5 epoxide with a cyclopropane significantly enhanced plasma half-life ($t_{1/2}$ = 13.9 h vs 1.6 h for **PTL**; testing in mouse plasma).[51] To further probe the role of the C1–C10 olefin in **PTL** with a structurally analogous mimetic, we synthesized C1–C10-cyclopropanated **4**. Utilizing the Furukawa modification ($ZnEt_2$) to the classical Simmons–Smith reaction,[52–54] **PTL** was treated with pre-formed $Zn(CH_2I)_2$ in a solution of DME and CH_2Cl_2, which yielded (1*S*,10*R*) **4** in 41% yield following silica gel chromatography (Scheme 1). Interestingly, no attack to the exocyclic olefin was observed, with the remaining mass balance consisting of mostly unreacted **PTL**. The structure of **4** was assigned by X-ray crystallography (Fig. 2 and SI). As expected, the solid-state structure of cyclopropane **4** was highly similar to a recently reported **PTL** X-ray structure[51] with a root-mean-square deviation of 0.167 Å (Fig. 2; alignment of structures provided in the **SI**). Recognizing that **4** may suffer from poor aqueous solubility akin to **PTL**, we synthesized dimethylamine congener **5**, which was converted to the fumarate salt for consistency with **DMAPT**.[18] **PTL** and Costunolide (**CTL**) were purchased from commercial vendors and the remaining analogues in our library (**1**, **2**, **MelB**, and **6**) were synthesized as previously reported.[18,21–26,28,29,32–35] The structure of synthesized Micheliolide (**1**) was verified by X-ray crystallography (**SI**) and compared to a previous report.[55]

2.2. Lipophilicity analyses

The distribution coefficients (Log*D*) of select C1–C10 **PTL** analogues were assessed through calculated and measured analyses (Table 1). Calculated Log*D* values were less predictive in comparison to experimentally derived measurements for **PTL**

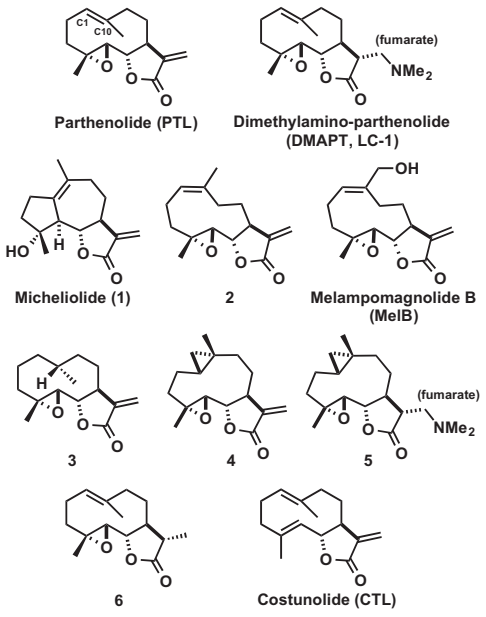
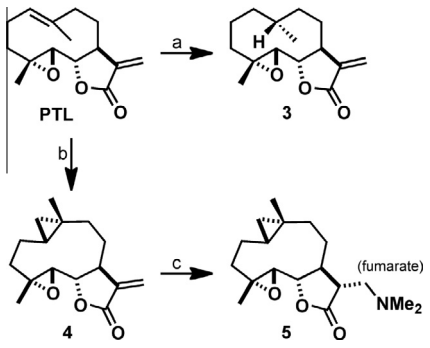


Figure 1. Structures of **PTL** and **DMAPT**, C1–C10 modified **PTL** analogues (**1**–**5** and **MelB**), and control compounds for biochemical assays (**6** and **CTL**).



Scheme 1. (a) $NHMe_2$, MeOH; Pt_2O, H_2 (50 psi), EtOAc; MeI, THF, H_2O, 45 °C, 32% (3 steps); (b) $Zn(CH_2I)_2$, DME, CH_2Cl_2, 41%; (c) $NHMe_2$, MeOH; fumaric acid, 85%.

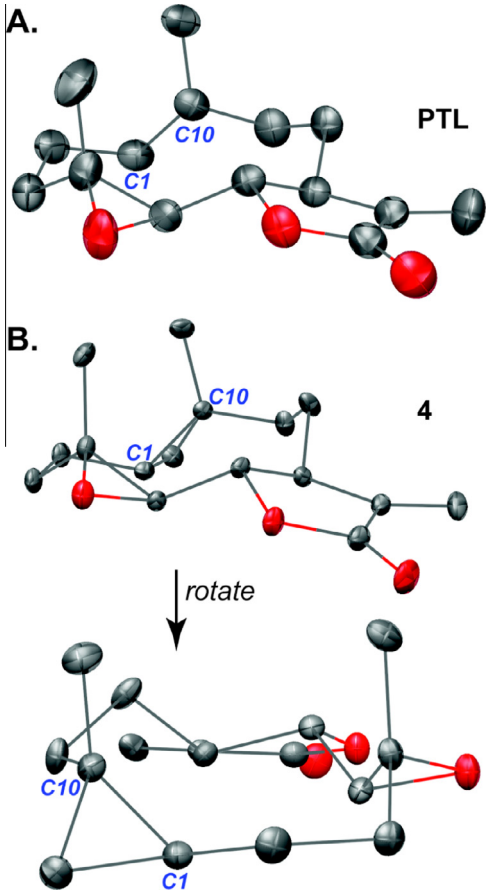


Figure 2. X-ray crystal structures. (A) **PTL**[18] and (B) cyclopropane **4** adopt similar conformations in the solid-state. Root-mean-square deviation between the two structures is 0.167 Å.

Table 1
Calculated Log*D* values ($c\text{Log}D_{7.4}$; MarvinSketch) and measured Log*D* values ($\text{Log}D_{7.4}$; Sirius Analytical, average of two measurements) at pH 7.4 for select C1–C10 **PTL** analogues

Compound	$c\text{Log}D_{7.4}$	$\text{Log}D_{7.4}$
PTL	3.07	1.79
1	1.97	2.18
2	3.07	2.00
3	3.48	2.30
4	3.16	2.29
CTL	4.22	2.90

($c\text{Log}D_{7.4}$ = 3.07 vs $\text{Log}D_{7.4}$ = 1.79) and *cis*-**PTL** isomer **2** ($c\text{Log}D_{7.4}$ = 3.07 vs $\text{Log}D_{7.4}$ = 2.00), whereas calculated and measured values were generally consistent for the remaining analogues (Table 1). Both reduction and cyclopropanation of the C1–C10 olefin increased the overall lipophilicity of the **PTL** skeleton ($\text{Log}D_{7.4}$ = 2.30 for **3** and $\text{Log}D_{7.4}$ = 2.29 for **4**). **CTL**, which contains a C4–C5 olefin in place of the epoxide in **PTL**, was substantially more lipophilic ($\text{Log}D_{7.4}$ = 2.90 for **CTL** vs $\text{Log}D_{7.4}$ = 1.79 for **PTL**). The distribution coefficients of dimethylamine fumarate salts of **PTL** and **4**, analogues **DMAPT** and **5**, respectively, were not measured because their calculated Log*D* values ($c\text{Log}D_{7.4}$ = 0.50, **DMAPT**; $c\text{Log}D_{7.4}$ = 0.56, **5**) were outside the measurable range of the assay (1–5 units).

2.3. Cellular cytotoxicity screening

All compounds were screened for anti-proliferative activity against 7 cell lines representing blood lineage cancers (HL-60 and CCRF-CEM) and solid tumors (U-87 MG, GBM6, MCF-7, DU-145, and NCI/ADR-RES). An established drug-resistant tumor cell line, NCI/ADR-RES, was included in our screen to determine if **PTL** and related analogues possess activity against cancer cell lines with high P-glycoprotein expression.[56,57] Clinically used chemotherapeutic drugs gemcitabine and doxorubicin are inactive (IC_{50} >500 μM) against NCI/ADR-RES cells,[58] and cancer stem cells (CSCs) are known to have high expression levels of drug efflux machinery.[59–61] Additionally, we included the GBM6 glioblastoma multiforme (GBM) cell line in our primary screen because it possesses a $CD133^+$ population of cells,[62] which is a frequently used marker of GBM stem cells.[63]

Screening of **PTL** and related analogues revealed broad-spectrum, low-micromolar IC_{50} growth inhibitory activity to all cancer cell lines regardless of modification to the C1–C10 olefin (Table 2). In contrast, **6**, which bears a reduced exocyclic methylene on the α-methylene-γ-butyrolactone, was found to be completely inactive against all cell lines examined (IC_{50} >500 μM). These data are consistent with previous reports,[18,32,64] and reinforce the necessity of the α-methylene-γ-butyrolactone for anti-proliferative activity of molecules of this class.[1,20,23,65] **CTL** was found to be equipotent to the C1–C10 modified analogues, suggesting the C4–C5 epoxide is non-essential for activity, which is also consistent with a previous report.[51] All compounds except for exomethylene-reduced **6** were active against drug-resistant NCI/ADR-RES cells (IC_{50} range: 9.4–22.0 μM).

2.4. Bone marrow toxicity studies

The $CD34^+CD38^-$ bone marrow (BM) immunophenotype is enriched for self-renewing stem cells.[66,67] Previous studies have demonstrated that **PTL** is non-toxic to total BM and $CD34^+CD38^-$ BM cells when dosed at 5 μM for 18 h.[6] To assess BM toxicity of the synthesized C1–C10 **PTL** analogues in comparison to **PTL**, we performed flow cytometry assays with human BM cells and measured cellular viability by flow cytometry using markers for apoptosis (Annexin V) and necrosis (7-AAD). Since **PTL** has been shown to elicit some overall BM toxicity at a dose of 7.5 μM for 18 h,[6] we elected to utilize a slightly higher dose to exacerbate the toxicity of **PTL** so that analogues with less toxicity to BM in comparison to **PTL** could be measured. Doxorubicin (**DOX**) was included as a positive control since it is known to elicit BM toxicity.[68] The mean overall viability of the BM specimen utilized in our study was 78% (Fig. 3A) and 94% for the $CD34^+CD38^-$ population (Fig. 3B). Treatment with 0.5 μM **DOX** for 12 h resulted in a 56% reduction in total BM viability and an 85% reduction in the primitive $CD34^+CD38^-$ BM population (Fig. 3). **PTL** treatment at 25 μM resulted in a 48% reduction in total BM viability, whereas C1–C10 modified **PTL** analogues **1**, **MelB**, and **4**, as well as control analogue, **CTL**, were less toxic (range: 22–26% average reduction of total BM viable cells). **PTL** was found to elicit no significant toxicity to primitive $CD34^+CD38^-$ BM cells at 25 μM dose and the C1–C10 **PTL** analogues were similarly non-toxic at the same concentration (Fig. 3B). Therefore, modification to the C1–C10 olefin of **PTL** significantly lowers its overall toxicity to BM cells. However, the observed toxicity of **PTL** to total BM would be expected to be transient since little cell death was measured in the $CD34^+CD38^-$ BM population upon **PTL** treatment, which is responsible for BM clonal growth.[66,67] Studies in our group using **PTL** prodrug, **DMAPT**, have revealed no measurable toxicity to mice upon continuous oral dosing (100 mg/kg, daily) for over four weeks.[64]

2.5. Inhibition of drug-resistant AML and toxicity to LSCs

Given the selectivity of our compounds for inhibiting growth of blood lineage cancer cells (e.g., HL-60 and CCRF-CEM, Table 2) and

Table 2
Growth inhibitory activities (IC_{50}) of **PTL**, C1–C10-modified **PTL** analogues, and related probes to cell lines: HL-60 (acute promyelocytic leukemia), CCRF-CEM (acute lymphoblastic leukemia), U-87 MG (glioblastoma multiforme), GBM6 (glioblastoma multiforme), MCF-7 (breast adenocarcinoma), DU-145 (prostate cancer), and NCI/ADR-RES (ovarian cancer; adriamycin-resistant), IC_{50} values are mean ± SD in μM ($n \geqslant 3$ analyses)

Compound	HL-60	CCRF-CEM	U-87 MG	GBM6	MCF-7	DU-145	NCI/ADR-RES
PTL	9.3 ± 3.8[a]	4.7 ± 1.6	8.8 ± 2.1[a]	3.4 ± 1.1[a]	9.7 ± 2.8	8.9 ± 4.6[a]	11.4 ± 2.4[b]
LC-1	7.1 ± 0.4	1.9 ± 0.4	8.8 ± 1.9[a]	3.5 ± 1.1[a]	10.4 ± 1.2	8.4 ± 4.5	12.3 ± 2.7
1	9.2 ± 2.2	2.7 ± 1.1	17.8 ± 5.0	8.5 ± 0.7	9.6 ± 1.0	14.8 ± 4.0	22.0 ± 5.3
2	8.0 ± 1.1	2.2 ± 1.0	9.1 ± 4.4	3.3 ± 0.8	7.5 ± 0.8	6.0 ± 4.2	9.4 ± 2.1
MelB	7.5 ± 3.0	5.5 ± 1.2	16.3 ± 6.8	4.5 ± 1.9	9.5 ± 1.9	14.3 ± 5.9	15.0 ± 4.9
3	9.0 ± 2.1	2.5 ± 2.1	7.5 ± 0.4	2.0 ± 0.3	20.1 ± 1.3	5.5 ± 1.5	15.6 ± 3.1
4	4.4 ± 1.3	2.0 ± 0.6	11.6 ± 1.4	2.3 ± 0.5	14.1 ± 1.0	14.8 ± 6.7	12.0 ± 2.5
5	6.5 ± 2.7	2.9 ± 0.6	10.5 ± 0.9	3.2 ± 0.7	16.9 ± 2.0	13.3 ± 2.2	11.7 ± 3.1
6	>500	>500	>500	>500	>500	>500	>500
CTL	13.0 ± 0.2	2.3 ± 0.2	9.6 ± 0.8	7.0 ± 1.8	17.5 ± 3.7	7.7 ± 2.8	17.1 ± 0.9

[a] Obtained previously.[58,64]
[b] Slightly lower than previously reported (57.6 μM).[58]

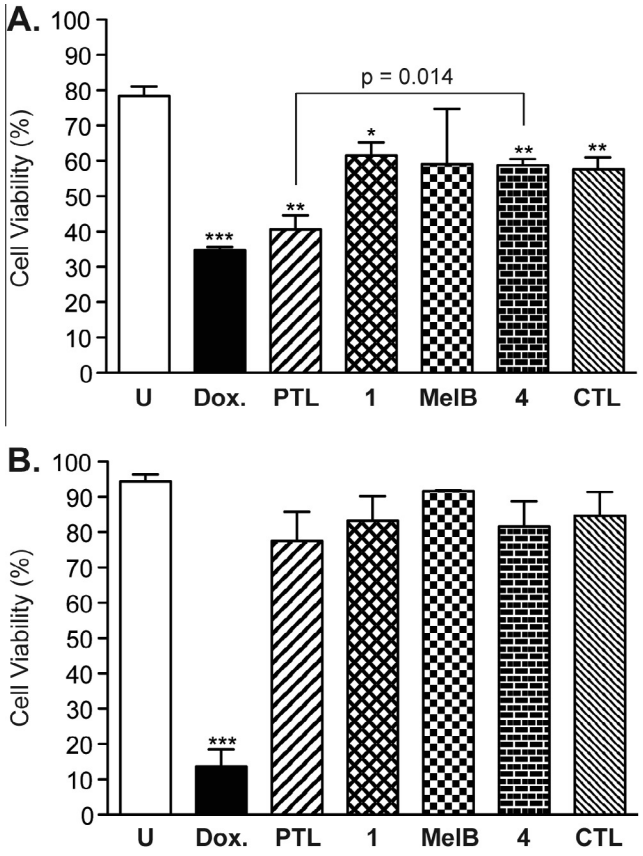


Figure 3. Toxicity of **PTL** and analogues to human bone marrow cells. BM was dosed with **DOX** (0.5 μM), and **PTL**, **1**, **MelB**, **4**, and **CTL** (25 μM) for 12 h. Cellular viability was then measured by flow cytometry and viable cells (%) were those not stained by Annexin V (apoptosis) and 7-AAD (necrosis) reagents. (A) Viability of the total bone marrow cell population and (B) viability of the $CD34^+CD38^-$ population. Data is the mean ($n \geqslant 3$ analyses) ± SD. $^*p = 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ in comparison to untreated control (U).

their lack of toxicity to healthy BM (Fig. 3), we focused subsequent efforts on characterizing the anti-leukemic activities of our molecules. Four murine AML cell lines were utilized for our initial screen (Table 3). B117P and B140P are murine cell lines isolated from the BXH-2 mice strain that spontaneously develops AML due to the presence of a murine leukemia virus.[69] These cells are sensitive to cytarabine (**AraC**), which is used in standard-of-care AML therapy. Continual low dosing of B117P and B140P with cytarabine resulted in cytarabine-resistant cell lines B117H and B140H, respectively.[70,71] Cytarabine resistance is conferred in B117H and B140H by loss-of-function mutations in the deoxycytidine kinase gene *Dck*, which inhibits intracellular metabolism of cytarabine to its 5′-monophosphate.[72] **PTL** and C1–C10-modified analogues **1**, **MelB**, and **4**, and control analogue **CTL** all inhibited the growth of this panel of cell lines with low micromolar activity (IC_{50} range: 1.1–13.5 μM). No loss in potency was observed between cytarabine-sensitive (parental) cell lines B117P and B140P and cytarabine-resistant lines B117H and B140H for any of the molecules tested. These data suggest that **PTL** analogues have the potential to sustain anti-proliferative activities to AML cell lines that become sensitive to cytarabine.

PTL is known to eradicate LSCs,[6] and therefore, we investigated if the C1–C10-modified **PTL** analogues could inhibit LSCs with similar potency as the parent natural product. We utilized an engineered leukemia cell line, TEX, for these assays. TEX cells are derived from lineage depleted (Lin^-) human cord blood cells transduced with the fusion gene *TLS-ERG*. TEX cells effectively mimic human AML by maintaining the potential for multi-lineage differentiation through their heterogeneous population of cells with hierarchical growth properties. A large population of primitive $CD34^+$ cells are present in the TEX model system, which has also been utilized in high-throughput screening for small molecule inhibitors of LSCs.[73,74] Screening of **PTL** and related analogues (**1**, **MelB**, **4**, and **CTL**) revealed broad-spectrum inhibitory activity (IC_{50} range: 2.7–6.8 μM) by metabolic viability staining following 48 h treatment (Table 4). Analysis of the LSC-enriched $CD34^+CD38^-$ population of TEX cells treated with 25 μM **PTL**, **1**, **MelB**, and **CTL** for 12 h revealed a nearly complete reduction in cellular viability by flow cytometry analysis (cell viability range: 1–10%, Fig. 4), with the majority of cells staining positive for 7-AAD, indicating necrotic cell death. Treatment of cells with 15 μM **PTL** analogues yielded slightly higher amounts of viable cells (cell viability range: 10–25%) with no statistical significant differences in potencies between the analogues tested. A relatively low dose of **DOX** (0.5 μM) was sufficient to reduce the viability of $CD34^+CD38^-$ TEX cells to 6%. Consequently, all of the **PTL** analogues tested were able to induce cell death in the LSC-enriched

Table 3
Growth inhibitory activities (IC_{50}) of **PTL**, C1–C10-modified **PTL** analogues, and related probes to murine AML cell lines B117P, B117H, B140P, and B140H. IC_{50} values are mean ± SD in μM ($n \geqslant 3$ analyses)

Compound	B117P	B117H	B140P	B140H
PTL	1.1 ± 0.2	4.7 ± 1.6	5.8 ± 2.3	3.4 ± 1.1
1	6.4 ± 1.0	8.8 ± 1.7	10.0 ± 0.4	9.3 ± 1.5
MelB	2.1 ± 0.8	2.5 ± 1.0	2.9 ± 1.0	2.2 ± 0.7
4	2.9 ± 0.6	6.4 ± 2.1	13.5 ± 2.3	5.9 ± 2.4
CTL	3.5 ± 0.4	4.8 ± 0.7	3.6 ± 1.2	2.8 ± 0.7

Table 4
Growth inhibitory activities (IC_{50}) of **PTL**, C1–C10-modified **PTL** analogues, and related probes to TEX cells. IC_{50} values are mean ± SD in μM ($n \geqslant 3$ analyses)

Compound	TEX
PTL	2.8 ± 0.2
1	2.7 ± 0.4
MelB	6.8 ± 2.3
4	3.8 ± 0.2
CTL	4.9 ± 0.4

$CD34^+CD38^-$ population of TEX cells. To corroborate that the observed inhibition of primitive $CD34^+CD38^-$ TEX cells also affects the LSC population, we performed methylcellulose clonal growth assays of TEX cells in the presence of our compounds. As previously mentioned, AML is characterized by hierarchical growth properties that originate from LSCs,[7,8] and therefore, small molecule inhibition of LSCs will prevent clonal growth of cells in methylcellulose. Control (DMSO) treated TEX cells yield on average 20.8 clones per assay (Table 5). Treatment with **PTL** or analogues **1**, **MelB**, **4**, or **CTL** all potently inhibit clonal outgrowth of TEX cells at 15 μM dose, with **PTL** and **1** yielding no measurable clones. Decreasing the dosage to 2.5 μM with the same compounds also elicits inhibition of TEX clonal growth (range: 2.6–7.0 clone average). **DOX** and **AraC** were both able to inhibit TEX clonal growth in our assay, with 0.5 μM treatment of both compounds completely inhibiting cell growth. Taken together, these data demonstrate **PTL** and related analogues are proficient at inhibiting LSCs, which is likely mediated through their common pharmacophore, the α-methylene-γ-butyrolactone.

2.6. Induction of reactive oxygen species

The mechanism by which **PTL** eradicates cancer cell viability is an area of substantial debate. **PTL** has been shown to affect a variety of cellular processes, including (among many others) inhibition of NF-κB signaling and microtubule detyrosination, reduction in DNA methylation, and induction of cellular ROS (reviewed in Refs. 1,2,4,5,75,76). Multiple studies have implicated ROS induction as a mechanism of **PTL**-mediated cancer cell death.[40,43,46,47] Consequently, we measured changes in intracellular ROS in TEX cells resulting from treatment with **PTL**, **1**, **MelB**, **4**, and **CTL** to rank order the pharmacological utility of our compounds. Treatment of

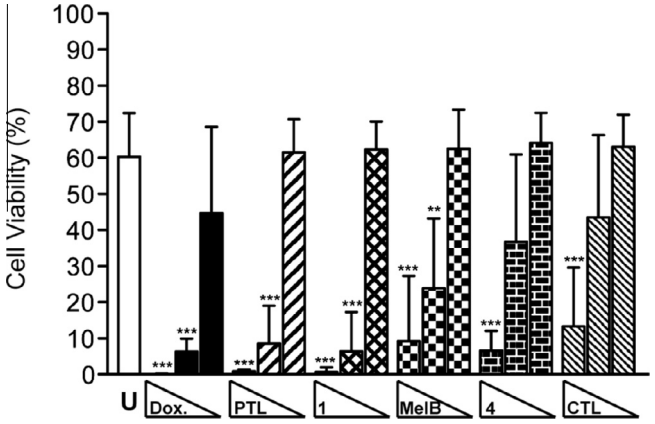


Figure 4. Cellular viability (%) of $CD34^+CD38^-$ TEX cells treated with **DOX** (2.5, 0.5, 0.05 μM) and **PTL**, **1**, **MelB**, **4**, **CTL** (25, 15, 2.5 μM). Viable cells were those not stained by Annexin V (apoptosis) and 7-AAD (necrosis) markers. Values are mean ± SD ($n \geqslant 5$ analyses). $^{*}p = 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ in comparison to untreated control (U).

Table 5
Clonal growth assay with TEX cells

Compound	Colonies	
	2.5 μM	15 μM
PTL	2.6 ± 2.7	N.D.
1	4.2 ± 2.5	N.D.
MelB	5.3 ± 1.6	0.1 ± 0.3
4	7.0 ± 5.1	0.6 ± 0.9
CTL	6.9 ± 3.6	5.6 ± 4.7
	0.1 μM	0.5 μM
DOX	0.7 ± 1.3	N.D.
AraC	2.6 ± 1.0	N.D.
DMSO	20.8 ± 9.0	

TEX cells were plated 6000 cells/well and dosed with DMSO (0.05%, control wells) or compounds at the concentrations noted. Values are the mean number of colonies ± SD (3 biological replicates) observed after 11 d growth on methylcellulose. N.D., clonal growth not detected. $p \leqslant 0.001$ in comparison to DMSO control for all samples, except **4** at 2.5 μM ($p \leqslant 0.01$).

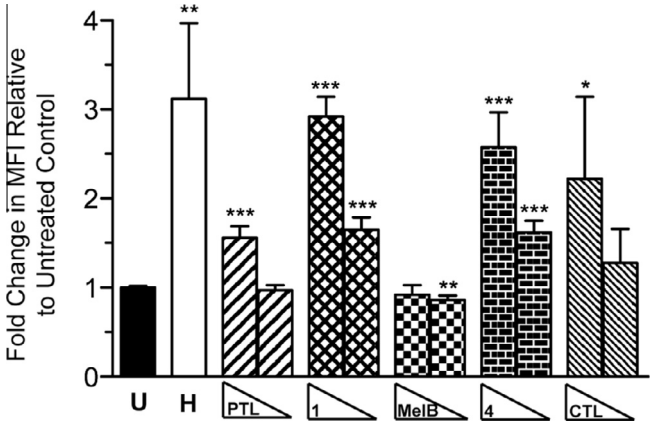


Figure 5. Intracellular ROS induction by **PTL** and analogues. TEX cells were treated with hydrogen peroxide **H** (100 μM) and **PTL**, **1**, **MelB**, **4**, **CTL** (100, 25 μM) and ROS activity was measured by flow cytometry using CellROX Green reagent. The median fluorescence intensity (MFI) of each sample was normalized to the untreated control and averaged. Values are mean MFI ± SD ($n \geqslant 3$ analyses). $^{*}p = 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ in comparison to untreated control.

TEX cells with hydrogen peroxide results in a 3.1-fold induction of intracellular ROS after 30 min, which is consistent with a previous study where a similar induction of ROS was measured by flow cytometry in HL-60 cells.[77] Dosage of TEX cells with **1** (2.9-fold), **4** (2.6-fold), and **CTL** (2.2-fold) all substantially induced ROS levels at 100 μM dose in comparison to untreated control (Fig. 5). **PTL** induced ROS as well, but to a lower level (1.6-fold). No observable induction of ROS was detected with **MelB** at either concentration tested. Decreasing the concentration of compounds to 25 μM also resulted in a significant induction of ROS levels for **1** (1.7-fold) and **4** (1.6-fold). Consequently, these data suggest that **1** and **4** more potently induce ROS than parent natural product, **PTL**.

3. Conclusions

A small library of C1–C10 **PTL** analogues was synthesized and evaluated for anti-proliferative activity to cancer cells, toxicity to healthy BM, ability to inhibit drug-resistant AML and target LSCs, and proficiency at inducing intracellular ROS. All compounds with the exception of **6** were capable of inducing cancer cell death with low micromolar potency. However, Micheliolide (**1**) and cyclopropane **4** were found to inhibit the growth of drug-resistant

AML and eliminate LSCs similarly to **PTL**, but offer the advantages of being less toxic to healthy BM and more potently activating ROS in AML cells than **PTL**. Additionally, elaboration of **4** to its dimethylamine congener **5** provided an analogous prodrug to **DMAPT** (**LC-1**). Given the continued interest in **PTL**, highlighted by its first total synthesis,[14] and the rekindled popularity of covalent drugs in general,[78,79] C1–C10 modifications such as cyclopropanation may be useful for optimizing **PTL** and related germacranolides for therapeutic applications.

4. Material and methods

4.1. Log*D* measurements

Calculated Log*D* values were obtained using MarvinSketch (ChemAxon). *c*Log$D_{7.4}$ was calculated using 0.1 mol/dm^3 electrolyte concentrations (Cl^-, Na^+, K^+) at pH 7.4. Experimental Log$D_{7.4}$ measurements were performed by Sirius Analytical. The Log$D_{7.4}$ of each sample was determined using the LDA (liquid–liquid distribution chromatography) method. The data is the average of two measurements.

4.2. Preparation of stock solutions

Compound stock solutions were prepared in DMSO (20–100 mM concentrations) and stored at −20 °C when not in use. Compound purities were assessed frequently by analytical reverse-phase HPLC analysis and fresh solutions were prepared as needed.

4.3. Cell culture

All cell lines were maintained in a humidified 5% CO_2 environment at 37 °C in tissue culture flasks (Corning) under normoxic conditions. Adherent cells were dissociated using either Trypsin-EDTA solution (0.25%, Gibco) or TrypLE Express solution (Invitrogen). HL-60, CCRF-CEM, U-87 MG, GBM6, DU-145, and NCI/ADR-RES cells were cultured as described previously.[58,64,80] MCF-7 cells (ATCC, HTB-22) were cultured in MEM media (Cellgro) supplemented with 10% FBS (Gibco), bovine insulin (0.01 mg/mL, Sigma), penicillin (100 I.U./mL, ATCC), and streptomycin (100 μg/mL, ATCC). TEX cells[73,74] were cultured in IMDM containing L-glutamine (Cellgro) supplemented with 15% FBS (Gibco), Stem Cell Factor (20 ng/mL, PeproTech), Interleukin-3 (2 ng/mL, PeproTech), penicillin (100 I.U./mL, ATCC), and streptomycin (100 μg/mL, ATCC).

4.4. Human cancer cell line cytotoxicity assays

Alamar blue cellular cytotoxicity assays and data analyses were performed as previously described.[58,64,80] Suspension cell lines (HL-60, CCRF-CEM and TEX[73,74]) were seeded at a density of 10,000 cells/well in media (50 μL) and adherent cell lines (U-87 MG, GBM6, MCF-7, DU-145, and NCI/ADR-RES) were seeded at a density of 5,000 cells/well in media (50 μL) 24 h prior to treatment with compounds in 96-well plates (Costar 3595, Corning, Inc.). IC_{50} values ($n \geqslant 3$ biological replicates) are the mean ± SD.

4.5. Murine cytotoxicity assay

Cell culture and cytotoxicity assays with murine cell lines B117P, B117H, B140P, and B140H were performed as previously described.[71,72] Cells were seeded at a density of 25,000, 28,000, 36,000 and 44,000 cells/well for B117P, B117H, B140P, and B140H cell lines, respectively, in media (200 μL) in 96-well plates (Costar 3596, Corning, Inc.). Assays were conducted in biological triplicate and IC_{50} values are the mean ± SD.

4.6. Bone marrow cell culture

Frozen human mononuclear bone marrow cells were purchased from AllCells (Cat. #ABM011F). These bone marrow cells were from two donors (#5630 [Lot #BM4565] and #4887 [Lot #BM4118]). The cells were thawed according to vendor instructions and then cultured in StemSpan SFEM (STEMCELL Technologies, Inc.) media supplemented with StemSpan CC100 cytokine cocktail (STEMCELL Technologies, Inc.) in a humidified 5% CO_2 environment at 37 °C in tissue culture flasks (Corning) under normoxic conditions.

4.7. Flow cytometry analysis of cytotoxicity in bone marrow and TEX cells

Human bone marrow or TEX cells were plated in their respective media at 1×10^6 cells/mL (1 mL/well) in a 24-well plate format (Corning). Cells were dosed with compounds or 1% DMSO/media and incubated for 12 h at 37 °C and 5% CO_2 under normoxic conditions. The final DMSO concentration was 0.03% (v/v) per well. After 12 h of incubation, each sample was transferred into FACS tubes and centrifuged for 5 min at 800 rpm. The supernatant was decanted and each sample was washed with cold 1X PBS (1 mL) and centrifuged again. After centrifugation, the supernatant was decanted and the samples were stained with Brilliant Violet 421 mouse anti-human CD34 (BD Biosciences [Cat. #562577]; 5 μL/sample) and APC mouse anti-human CD38 (BD Biosciences [Cat. #555462]; 20 μL/sample) antibodies in FACS buffer (1X PBS, 2% FBS, 0.1% sodium azide; 100 μL total volume/sample) for 10 minutes at 4 °C. The cells were then diluted with FACS buffer (1 mL) and centrifuged. The supernatant was decanted and stained with Annexin V-FITC (BD Biosciences [Cat. #556420]; 5 μL/sample) and 7-AAD (eBioscience [Cat. #00-6993-50]; 5 μL/sample) in FACS buffer (100 μL total volume/sample) for 10 minutes at room temperature in the dark. The samples were diluted with FACS buffer (300 μL), and kept on ice during analysis by flow cytometry using a BD Biosciences LSR II flow cytometer. Greater than 5×10^4 events were measured for each sample during analysis. All antibodies and stains were stored at 4 °C in the dark. After data collection, each sample was processed using FlowJo (Tree Star; v 7.6.5). The cell viability is expressed as a mean of 3–5 biological replicates ± SD. Statistical significance was determined using unpaired t-tests (GraphPad Prism v. 5.0). An example of the data processing is shown in Supporting information.

4.8. Colony growth assay

TEX cells were added to Methocult H4230 (STEMCELL Technologies Inc.) supplemented with penicillin (100 I.U./mL, ATCC) and streptomycin (100 μg/mL, ATCC) at a final cell density of 1.2×10^4 cells/mL. Compounds were diluted in TEX cell media and dosed to each cell suspension to obtain the respective concentration. Each sample was vortexed vigorously to evenly distribute the cells before and after compound dosing. Each sample (1.5 mL) was plated into three wells (0.5 mL/well, three technical replicates) of a 24-well plate (Corning) and incubated under normoxic conditions at 37 °C, 5% CO_2 for 11 days before scoring colonies. The final DMSO concentration was 0.05% (v/v) per well. Colonies were counted for each well at 10× magnification with a light microscope by two people independently and averaged for each sample. The data is the mean number of colonies for three biological replicate ± SD. Statistical significance was determined using unpaired t-tests (GraphPad Prism v. 5.0).

4.9. ROS Assay

TEX cells were seeded in 24-well plates at 5×10^5 cells/mL (1 mL per well) and incubated overnight at 37 °C and 5% CO_2. The cells were then treated with compounds (25 and 100 μM), including H_2O_2 (100 μM; positive control). The final DMSO concentration was 0.25% (v/v) per well. Immediately after treatment with compounds, CellROX Green (Invitrogen) reagent was added to the appropriate samples at a final concentration of 5 μM. The cells were then incubated for 30 min at 37 °C and 5% CO_2 under normoxic conditions. Following incubation, the samples were transferred to 5 mL FACS tubes and washed twice with FACS buffer (3mL; FACS buffer: 1X PBS, 2% FBS, 0.1% sodium azide). The samples were run using a BD Biosciences LSR II flow cytometer and 5×10^4 events were recorded for each sample. Flow cytometry data was analyzed using FlowJo software (Tree Star; version 7.6.5). Samples were run in quadruplicate with the exception of H_2O_2 (triplicate data). Median fluorescence intensity (MFI) values were obtained for each sample and were normalized to the untreated control. Data are shown as mean MFI value ± SD. Statistical significance was determined using unpaired t-tests (GraphPad Prism v. 5.0).

4.10. General synthesis information

Chemical reagents were typically purchased from Sigma-Aldrich and used without additional purification unless noted. Bulk solvents were from Fisher Scientific. **PTL** was purchased from Enzo Life Sciences and **CTL** was purchased from Santa Cruz Biotechnology. Previously reported analogues **DMAPT**, **MelB**, **1**, **2** and **6** were synthesized as described.[18,21–26,28,29,32–35] The structure of **1** was further confirmed by small molecule X-ray crystallography (SI; CCDC 1033012) and compared to the previous report.[55] Tetrahydrofuran (THF) was rendered anhydrous by passing through the resin column of a solvent purification system (MBraun). Reactions were performed under an atmosphere of dry N_2 unless noted. Silica gel chromatography was performed on a Teledyne-Isco Combiflash Rf-200 instrument utilizing Redisep R_f Gold High Performance silica gel columns (Teledyne-Isco). Analytical HPLC analysis was performed on an Agilent 1200 series instrument equipped with a diode array detector and a Zorbax SB-C18 column (4.6 × 150 mm, 3.5 μm, Agilent Technologies). The method started with 10% CH_3CN (with 0.1% trifluoroacetic acid (TFA)) in H_2O (0.1% TFA). The 10% CH_3CN (with 0.1% TFA) was increased to 85% over 22 minutes, and then increased to 95% CH_3CN (with 0.1% TFA) over 2 more minutes. Nuclear magnetic resonance (NMR) spectroscopy was employed by using either a Bruker Avance (400 MHz for ^1H; 100 MHz for ^{13}C) or Bruker Ascend (500 MHz for ^1H; 125 MHz for ^{13}C) NMR operating at ambient temperature. Chemical shifts are reported in parts per million and normalized to internal solvent peaks or tetramethylsilane. High-resolution masses were obtained from the University of Minnesota Department of Chemistry Mass Spectrometry lab, employing a Bruker BioTOF II instrument.

4.10.1. C1–C10 Reduced 3

To a stirred solution of **PTL** (0.050 g, 0.201 mmol) in MeOH (2 mL) was added dimethylamine (2.0 M in MeOH, 1 mL). The reaction was allowed to stir at RT overnight and then concentrated in vacuo. The crude material was used without further purification. The residual material was dissolved in EtOAc (3 mL) and PtO_2 (0.005 g, 0.022 mmol) was added. The reaction mixture was degassed, then shaken for 8 h in a Parr shaker under an atmosphere of H_2 (50 psi). The mixture was then degassed, filtered through celite, and concentrated in vacuo. The crude material was taken on to the next step without further purification. The reaction mixture was dissolved in THF (3 mL) and iodomethane was added in excess (0.10 mL, 1.60 mmol). The reaction was allowed to stir at RT for 2 h. The solvent and excess iodomethane were removed in vacuo resulting in a white solid. Water (10 mL) was added and the reaction was heated to 45 °C. Complete solvation of the yellowish material resulted within minutes of heating. The reaction was allowed to stir with heating for 3 h, and then solvent was removed in vacuo. Aqueous $NaHCO_3$ (sat'd, 5 mL) was added to the reaction mixture, and the product was extracted with DCM (3 × 20 mL). The combined organic layers were washed with brine (20 mL), and dried with Na_2SO_4. The reaction was purified by flash chromatography over SiO_2 (10–50% ethyl acetate in hexanes gradient) to yield **3** as a white solid (0.014 g, 32%). ^1H NMR ($CDCl_3$, 500 MHz): δ 6.24 (d, J = 2.8 Hz 1H), 5.53 (d, J = 2.4 Hz, 1H), 3.84 (t, J = 7.6 Hz, 1H), 3.10 (d, J = 7.6 Hz, 1H), 2.99–2.94 (m, 1H), 2.20–2.14 (m, 2H), 1.81–1.75 (m, 2H), 1.75–1.56 (m, 2H), 1.51 (s, 3H), 1.51–1.40 (m, 2H), 1.26 (m, 2H), 1.17–1.14 (m, 2H), 0.93 (d, J = 4.8 Hz, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): 169.7, 139.5, 119.7, 81.0, 66.4, 61.3, 43.9, 36.7, 36.1, 30.1, 27.9, 24.7, 21.3, 20.6, 19.2. HRMS (ESI^+) m/z calc'd for $[C_{15}H_{22}O_3+Na]^+$ 273.1461; found 273.1470. The structure of **3** was further confirmed by small molecule X-ray crystallography (SI; CCDC 1033013).

4.10.2. Cyclopropane 4

A 0.20 M solution of $Zn(CH_2I)_2$·DME complex was made in the following manner: To a stirred solution of diethyl zinc (1.0 M solution in hexanes, 4.0 mL, 4.00 mmol) in CH_2Cl_2 (20 mL) and DME (0.50 mL) at 0 °C was added diiodomethane (0.80 mL, 9.92 mmol) under N_2. The mixture was stirred for 10 minutes. **PTL** (0.090 g, 0.36 mmol) in CH_2Cl_2 (3 mL) was added dropwise over 10 min to the $Zn(CH_2I)_2$·DME complex at 0 °C. The reaction was allowed to warm to rt over 12 h. The reaction was quenched with aqueous NH_4Cl (sat'd, 5 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with aqueous $NaHCO_3$ (sat'd, 20 mL), brine (20 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude mixture was purified using silica gel chromatography (gradient 10–30% EtOAc in hexanes over 15 min) to yield **4** (0.036 g, 40%) as a colorless oil and recovered **PTL** (0.037 g, 41%). ^1H NMR (400 MHz, $CDCl_3$) δ: 6.28 (d, J = 3.7 Hz, 1H), 5.57 (d, J = 3.3 Hz, 1H), 3.96 (t, J = 9.1 Hz, 1H), 2.98 (d, J = 9.0 Hz, 1H), 2.67 (m, 1H), 2.39 (dd, J = 8.0 Hz, J = 14.7 Hz, 1H), 2.19 (dd, J = 2.3 Hz, J = 8.3 Hz, 1H), 1.95 (m, 2H), 1.70 (m, 1H), 1.40 (s, 3H), 1.28 (m, 2H), 1.09 (s, 3H), 0.85 (dd, J = 11.1 Hz, J = 14.7, 1H), 0.64 (td, J = 6.0 Hz, J = 9.5 Hz, 1H), 0.39 (dd, J = 4.3 Hz, J = 9.4 Hz, 1H), -0.08 (dd, J = 4.6 Hz, J = 5.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 169.4, 139.9, 120.5, 82.7, 65.5, 60.6, 48.0, 42.3, 38.4, 25.7, 24.5, 22.3, 20.4, 18.8, 18.5, 17.1. HRMS (ESI^+) m/z calc'd for $[C_{16}H_{22}O_3+Na]^+$ 285.1461; found 285.1470. The structure of **4** was further confirmed by small molecule X-ray crystallography (SI; CCDC 1033014).

4.10.3. Cyclopropyl-PTL Dimethylamine Fumarate 5

To a stirred solution of **4** (0.009 g, 0.034 mmol) in MeOH (2 mL) was added dimethylamine (2.0 M in MeOH, 1.5 mL). The reaction was stirred for 12 h at rt. The reaction mixture was concentrated in vacuo purified by silica gel chromatography (gradient 0–50% EtOAc in hexanes over 10 min, then gradient 0–25% MeOH in CH_2Cl_2 over 10 min) to yield the dimethylamino product as a white solid (0.009 g). To a stirred solution of this product in THF (5 mL) was added fumaric acid (0.0034 g, 0.029 mmol). A white precipitate was observed after stirring overnight at rt. The reaction mixture was concentrated in vacuo to give the fumarate salt **5** as white solid (0.0124 g, 85%). ^1H NMR (DMSO-d_6, 400 MHz): δ 6.61 (s, 2H), 4.09 (t, J = 9.5 Hz, 1H), 3.04 (d, J = 9.2 Hz, 1H), 2.64 (m, 3H), 2.24 (s, 6H), 2.14 (m, 2H), 2.05 (m, 1H), 1.78 (m, 2H), 1.59 (m, 1H), 1.31 (s, 3H), 1.19 (m, 2H), 1.02 (s, 3H), 0.74 (m, 2H),

0.28 (dd, J = 3.8 Hz, J = 9.2 Hz, 1H), -0.18 (t, J = 4.8 Hz, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz): 176.6, 166.1, 134.1, 81.8, 64.3, 60.3, 57.2, 47.4, 45.6, 45.3, 41.7, 38.0, 24.2, 24.0, 21.6, 20.1, 18.4, 18.3, 16.7. HRMS (ESI$^+$) m/z calc'd for $[C_{18}H_{30}NO_3+H]^+$ 308.2220; found 308.2216.

4.11. Analysis of X-ray structures

The X-ray structures of **4** and **PTL**[51] were analyzed for root-mean-square deviation (RMSD) and graphics were rendered using UCSF Chimera.[81,82] Thermal ellipsoids were drawn at the 50% probability level. RMSD was calculated for all non-hydrogen atoms.

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Supplementary data

CCDC (1033012–1033014) contains the supplementary crystallographic data for compounds **1**, **3**, and **4** in this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Supplementary data (NMR spectra, HPLC characterization data, and additional details of biochemical assays and X-ray crystallographic structures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.05.037.

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