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Identification of a melampomagnolide B analog as a potential lead molecule for treatment of acute myelogenous leukemia

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Abstract

A series of carbamate derivatives of the antileukemic sesquiterpene melampomagnolide B (MMB) has been synthesized utilizing a 1,2,4-triazole carbamate conjugate of MMB as an intermediate synthon. Five imidazole- and benzimidazole-carbamate analogs of MMB (**8a-8e**) were prepared and evaluated for anti-leukemic activity against cultured M9 ENL1 AML cells. All the analogs exhibited improved anti-leukemic activity ($EC_{50} = 0.90\text{--}3.93\text{ }\mu\text{M}$) when compared to parthenolide and the parent sesquiterpene, MMB ($EC_{50} = 7.0\text{ }\mu\text{M}$ and $15.5\text{ }\mu\text{M}$, respectively). The imidazole carbamate analog, **8a** ($EC_{50} = 0.9\text{ }\mu\text{M}$), was 16 times more potent than MMB. The comparative bioavailabilities of **8a** and MMB were determined in BALB/c mice following oral dosing of these compounds. It has been demonstrated that the absolute plasma bioavailabilities of MMB and **8a** were $6.7 \pm 0.8\%$, and $45.5 \pm 2\%$, respectively. These results indicate that, compared to MMB, the PK parameters for **8a** display significantly improved bioavailability and exposure after oral administration. Analog **8a** is considered to be a potential clinical candidate for treatment of acute myelogenous leukemia.

Graphical Abstract

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Keywords

Leukemia; Melampomagnolide B; Carbamates; LC/MS/MS; Pharmacokinetic properties; Bioavailability

1. Introduction

Acute myelogenous leukemia (AML) is a relatively rare cancer of the myeloid line of blood cells and is a clonal malignancy of the hematopoietic system characterized by rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells.¹ AML is the most common type of leukemia in adults but has the lowest survival rate of all leukemias, accounting for approximately 1.2% of cancer deaths in the United States.^{2,3} Treatments for AML have not substantially changed over the last 30 years, and the incidence of AML is expected to increase as the world population ages.⁴ Thus, there is a clear need for new drug therapies to treat this blood-borne cancer.

Sesquiterpene lactones have been isolated from many species of medicinal plant,⁵ and possess a wide variety of biological activities.^{6–8} One such sesquiterpene, parthenolide (PTL) (Fig. 1), originally isolated from the medicinal herb feverfew (*Tanacetum parthenium*),⁹ has been identified as an anticancer agent that is particularly effective against both hematological and solid tumors.^{10–16} PTL is a potent antileukemic agent¹⁷ but possesses poor drug-like properties, having low water-solubility and poor oral bioavailability,¹⁸ precluding its use as a clinical agent for the treatment of AML. Structural modification of the PTL molecule has been carried out in attempts to improve the drug-like properties of the molecule while retaining its antileukemic properties.¹⁸ One such PTL analog, dimethylaminoparthenolide (DMAPT) (Fig. 1), is currently in Phase I clinical studies for evaluation as a treatment for AML and other related leukemias.^{11,18}

A structurally related sesquiterpene, melampomagnolide B (Fig. 1; **1**, MMB),¹⁹ a melampolide originally isolated from *Magnolia grandiflora*,²⁰ has similar antileukemic activity to PTL.²¹ MMB is somewhat less potent than PTL, but both sesquiterpenes exhibit significantly reduced cytotoxicity against normal bone marrow stem cells when compared to their effect on AML stem cells.^{21, 22}

The presence of a C-14 primary allylic hydroxyl group in the MMB molecule allows the medicinal chemist the opportunity to examine the biological activity of a wide variety of new MMB derivatives via conjugation and functional group transformations. Utilizing this approach, C-14 carbamate conjugates of MMB have recently been shown to possess improved cytotoxicity against hematological tumor cells and solid tumor cell lines when compared to the parent compound,^{22,23} and a C-14 biotin-conjugated derivative of MMB has been prepared and used in elucidating the mechanism of action of MMB in primary human AML cells^{11,16,21}.

Antileukemic agents such as PTL and MMB cause a selective increase in reactive oxygen species (ROS) in AML stem cells through inhibition of key enzymes involved in glutathione biosynthesis and catabolism.¹¹ The mechanism of action is thought to involve promotion of

apoptosis of AML cells by inhibition of key enzymes involved in glutathione biosynthesis and catabolism. Both PTL and MMB may perturb glutathione homeostasis by a multifactorial mechanism.^{11,16} Streptavidin pull-down and amino acid sequencing studies with the C-14 biotin conjugate of MMB identified key proteins involved in glutathione biosynthesis and catabolism pathways.¹¹ MMB appears to target the glutathione-cysteine ligase enzyme, which is the rate-limiting step in the glutathione biosynthetic pathway, as well as glutathione peroxidase (GPX1). We have utilized homology modeling of GCLC (Glutamate-cysteine ligase catalytic subunit) and GCLM (Glutamate-cysteine ligase modifier subunit) to show that PTL can bind covalently to active site cysteine residues on both GCLC and GCLM via formation of Michael addition adducts.¹¹ These adducts have been suggested to result from the reaction of an appropriate exposed cysteine thiolate anion with the electron deficient sp² carbon of the α -methylene- γ -lactone moiety of PTL,^{24,25} a moiety that is also present in the MMB molecule.

PTL and MMB have also been shown to inhibit the NF κ B transcription factor complex, leading to down-regulation of anti-apoptotic genes under NF κ B control (Fig 2).^{26,27} From streptavidin pull-down and LC/MS/MS peptide sequencing studies with the MMB-biotin probe,¹¹ both IKK β and p65 proteins of the NF κ B pathway were identified as potential targets in the mechanism of action of MMB. These sesquiterpene lactones are both believed to bind to IKK β and/or p65, leading to decreased phosphorylation of I κ B and reduced DNA binding of p50/p65.

In the current study, we have evaluated several novel azaaromatic imidazole and benzimidazole carbamate analogs of MMB and compared their antileukemic properties to those of both MMB and PTL. We have also compared the oral pharmacokinetic profile of the lead compound (analog **8a**) from these studies with that of MMB in BALB/c mice to determine if this carbamate conjugate has improved oral bioavailability over MMB.

2. Synthesis of imidazole and benzimidazole carbamate analogs of MMB

Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. The synthesized compounds were fully characterized by ¹H NMR and ¹³C NMR spectral analysis. ¹H and ¹³C NMR spectra were recorded on a Varian 400 MHz spectrometer equipped with a Linux workstation running on vNMRj software. HRMS data were obtained on an Agilent 6210 LCTOF instrument operated in multimode. All synthetic reactions were carried out at ambient temperature, and products were purified by flash column chromatography (silica gel; methanol/dichloromethane), when necessary.

2.1. Synthetic procedure and analytical data for the preparation of the carbonyldi-1,2,4-triazolecarbamate conjugate of MMB (**7**)

To a stirred solution of MMB (50 mg, 0.18 mmol) in dichloromethane, was added carbonyldi-(1,2,4-triazole) (46.5 mg, 0.28 mmol) at room temperature and the reaction mixture was stirred for 1 h. After completion of the reaction (monitored by TLC), water (5 mL) was added and the mixture was extracted with dichloromethane (2x5 mL). The organic layer was washed with water (3x5 mL), dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to afford pure product **7** as a white solid (yield 93%).

¹H NMR (CDCl₃, 400 MHz): δ 8.83 (s, 1H), 8.06 (s, 1H), 6.26 (d, *J* = 3.6 Hz, 1H), 5.92 (t, *J* = 8.4 Hz, 1H), 5.55 (d, *J* = 3.2 Hz, 1H), 5.08 (d, *J* = 11.6 Hz, 1H), 4.90 (d, *J* = 12 Hz, 1H), 3.86 (t, *J* = 9.6 Hz, 1H), 2.91-2.83 (m, 2H), 2.56-2.17 (m, 6H), 1.84-1.72 (m, 1H), 1.55 (s, 3H), 1.14 (t, *J* = 12.4 Hz, 1H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 169.2, 154.0, 147.7, 145.8, 138.6, 134.4, 133.3, 120.6, 80.9, 71.5, 63.3, 59.9, 42.7, 36.5, 25.7, 24.3, 24.1, 18.1 *ppm*. MS (ESI), *m/z*: 382.10 (M + Na)⁺

2.2. General synthetic procedure and analytical data for the imidazole- and benzimidazole-carbamate derivatives of MMB (8a–8e)

To the triazole carbamate ester of MMB (7) (0.167 mmol) in dichloromethane (2 mL) was added the appropriate imidazole or benzimidazole amine (0.167 mmol) at ambient temperature, and the reaction mixture was stirred for 4–16 h. After completion of the reaction (monitored by TLC), water was added and the resulting mixture extracted with dichloromethane. The organic liquors were separated, washed with water, followed by brine solution, dried over anhydrous Na₂SO₄, filtered, and the solvent removed to afford the crude reaction product. The crude product was purified by column chromatography (silica gel, 2–5% methanol in dichloromethane) to afford the appropriate carbamate derivative of MMB as a white solid (yield: 65–75 %).

2.2.1. ((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10]cyclo deca[1,2-*b*]furan-5-yl)methyl-1*H*-imidazole-1-carboxylate (8a)—¹H NMR (CDCl₃, 400 MHz): δ 8.13 (s, 1H), 7.41 (s, 1H), 7.09 (s, 1H), 6.29 (d, *J* = 3.6 Hz, 1H), 5.84 (t, *J* = 8.4 Hz, 1H), 5.58 (d, *J* = 3.2 Hz, 1H), 5.0 (d, *J* = 12.4 Hz, 1H), 4.81 (d, *J* = 12.4 Hz, 1H), 3.87 (t, *J* = 9.6 Hz, 1H), 2.85-2.79 (m, 2H), 2.56-2.18 (m, 6H), 1.79-1.72 (m, 1H), 1.56 (s, 4H), 1.14 (t, *J* = 12 Hz, 1H). ¹³C NMR (CDCl₃, 100 MHz): δ 169.2, 148.6, 138.4, 137.1, 133.6, 132.9, 131.0, 120.6, 117.1, 80.9, 70.1, 63.3, 59.9, 42.6, 36.5, 25.5, 24.0, 18.0 *ppm*. HRMS (ESI) *m/z* calcd. for C₁₉H₂₃N₂O₅ (M + H)⁺ 359.1601, found 359.1598.

2.2.2. ((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10]-cyclodeca[1,2-*b*]furan-5-yl)methyl-2-methyl-1*H*-imidazole-1-carboxylate (8b)—¹H NMR (CDCl₃, 400 MHz): δ 7.32 (d, *J* = 2.0 Hz, 1H), 6.87 (d, *J* = 1.6 Hz, 1H), 6.28 (d, *J* = 3.6 Hz, 1H), 5.81 (t, *J* = 8.4 Hz, 1H), 5.57 (d, *J* = 3.2 Hz, 1H), 4.93 (d, *J* = 12 Hz, 1H), 4.79 (d, *J* = 12 Hz, 1H), 3.87 (t, *J* = 9.6 Hz, 1H), 2.85-2.80 (m, 2H), 2.65 (s, 3H), 2.55-2.17 (m, 6H), 1.79-1.72 (m, 1H), 1.56 (s, 3H), 1.13 (t, *J* = 12 Hz, 1H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 169.2, 149.4, 148.1, 138.5, 133.8, 132.5, 128.2, 120.6, 117.9, 80.9, 69.7, 63.3, 59.9, 42.6, 36.5, 25.5, 24.1, 23.9, 18.0, 16.8 *ppm*. HRMS (ESI) *m/z* calcd. for C₂₀H₂₅N₂O₅ (M + H)⁺ 373.1758, found 373.1758.

2.2.3. ((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10]-cyclodeca[1,2-*b*]furan-5-yl)methyl-2-ethyl-4-methyl-1*H*-imidazole-1-carboxylate (8c)—¹H NMR (CDCl₃, 400 MHz): δ 7.0 (s, 1H), 5.79 (t, *J* = 8.4 Hz, 1H), 6.28 (s, 1H), 4.90 (d, *J* = 12.4 Hz, 1H), 4.75 (d, *J* = 12.4 Hz, 1H), 3.87 (t, *J* = 9.6 Hz, 1H), 3.01 (q, *J* = 7.2, 14.8 Hz, 2H), 2.87-2.81 (m, 2H), 2.55-2.16 (m, 10H), 1.78-1.72 (m, 1H), 1.56 (s, 3H), 1.31 (t, *J* = 7.6 Hz, 3H), 1.13 (t, *J* =

12.8 Hz, 1H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 169.2, 152.7, 149.1, 138.5, 137.2, 133.9, 132.1, 120.6, 113.7, 80.9, 69.3, 63.3, 60.0, 42.7, 36.5, 25.6, 24.2, 23.9, 23.4, 18.0, 13.4, 11.9 ppm. HRMS (ESI) m/z calcd for $\text{C}_{22}\text{H}_{29}\text{N}_2\text{O}_5$ ($\text{M} + \text{H}$) $^+$ 401.2071, found 401.2068.

2.2.4. ((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10]cyc- lodeca[1,2-*b*]furan-5-yl)methyl-1*H*-benzo[*d*]imidazol-2-ylcarbamate (8d)— ^1H NMR (CDCl_3 , 400 MHz): δ 7.56 (d, J = 8.4 Hz, 1H), 7.36 (d, J = 9.2 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.06 (t, J = 7.6 Hz, 1H), 6.26 (d, J = 3.2 Hz, 2H), 5.86 (t, J = 8.0 Hz, 1H), 5.56 (d, J = 3.2 Hz, 1H), 5.06 (d, J = 12 Hz, 1H), 4.90 (d, J = 12.4 Hz, 1H), 3.86 (t, J = 9.2 Hz, 1H), 2.86 (d, J = 9.2 Hz, 2H), 2.56-2.19 (m, 7H), 1.79-1.70 (m, 1H), 1.56 (s, 3H), 1.13 (t, J = 12.4 Hz, 1H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 169.2, 153.5, 151.9, 142.4, 138.5, 133.8, 132.7, 130.0, 125.0, 121.2, 120.7, 116.9, 113.8, 80.9, 69.7, 63.4, 60.0, 42.6, 36.6, 25.6, 24.1, 24.0, 18.1 ppm. HRMS (ESI) m/z calcd. for $\text{C}_{23}\text{H}_{26}\text{N}_3\text{O}_5$ ($\text{M} + \text{H}$) $^+$ 424.1867, found 424.1827.

2.2.5. ((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10]cyc- lodeca[1,2-*b*]furan-5-yl)methyl((1*H*-benzo[*d*]imidazol-2-yl)methyl)carbamate (8e)— ^1H NMR ($\text{DMSO}-d_6$, 400 MHz): δ 12.2 (s, 1H), 7.80 (t, J = 5.2 Hz, 1H), 7.53 (d, J = 7.2 Hz, 1H), 7.44 (d, J = 7.6 Hz, 1H), 7.16-7.09 (m, 2H), 6.01 (d, J = 3.6 Hz, 1H), 5.67-5.48 (m, 2H), 4.58 (d, J = 12.4 Hz, 1H), 4.44-4.39 (m, 3H), 4.08 (t, J = 9.2 Hz, 1H), 3.02 (t, J = 3.6 Hz, 1H), 2.85 (d, J = 9.2 Hz, 1H), 2.34-2.05 (m, 6H), 1.64 (t, J = 10.8 Hz, 1H), 1.47 (s, 3H), 0.92 (t, J = 11.6 Hz, 1H). ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz): δ 169.9, 156.8, 152.8, 143.5, 140.0, 136.1, 134.6, 128.6, 122.2, 121.4, 119.7, 118.7, 111.6, 81.1, 66.7, 63.0, 60.4, 42.2, 36.8, 25.1, 24.3, 23.5, 17.9 ppm. HRMS (ESI) m/z calcd for $\text{C}_{24}\text{H}_{28}\text{N}_3\text{O}_5$ ($\text{M} + \text{H}$) $^+$ 438.2023, found 438.2016.

3. Anti-leukemic activity of imidazole- and benzimidazole-carbamate derivatives of MMB against M9-ENL1 AML cells

The imidazole and benzimidazole-carbamate derivatives of MMB (**8a–8e**) were screened for antileukemic activity utilizing the following procedure. M9-ENL1 cells obtained from transduction of human blood cells with the leukemia-derived MLL-ENL translocation gene²⁸ were plated at a density of 10^6 cells/mL in alpha-MEM culture media (Invitrogen) supplemented with 5% human plasma, 20% FBS, and the cytokines SCF, IL-3, IL-7, and FLT3 (PeproTech). Drugs were diluted from a DMSO stock into PBS such that the final concentration of DMSO did not exceed 0.5%. For each compound, *in vitro* cytotoxicity studies were performed after 24 h of drug exposure using viability labeling with trypan blue dye, as well as flow cytometric analysis by labeling with Annexin V and propidium iodide or 7-aminoactinomycin D to delineate apoptotic cell populations and to identify the percentage of non-apoptotic cells, which was defined as the population of cells with negative staining for both labels. The percentage of non-apoptotic cells observed was normalized to that of the vehicle control, and dose-response curves were analyzed using GraphPad Prism software to determine EC_{50} values. All analyses were conducted in triplicate. In all these studies, PTL and MMB were included as reference standards.

4. Pharmacokinetic profile of MMB and 8a in mice

4.1. Materials

The buffer salts, chemicals and solvents utilized were of LC/MS/MS grade or equivalent quality. Acetonitrile and formic acid were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Heparin sodium injection (10,000 USP units/ml), was purchased from Baxter Healthcare Corporation (Deerfield, IL). Nembutal sodium (pentobarbital sodium injection, USP) was obtained from Abbott Laboratories (North Chicago, IL).

4.2. Animals

All animal procedures in this study were conducted in compliance with the guidelines of the University of Arkansas for Medical Sciences (UAMS) Institutional Animal Care and Use Committee established by the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication). BALB/c male mice (25–30 g) were obtained from Harlan (Indianapolis, IN) and housed five per cage with ad libitum access to food and water in the Division of Laboratory Animal Resources, UAMS. Mice were anesthetized with pentobarbital (40 mg/kg i.p.) and surgically implanted with jugular vein cannula for blood sampling.²⁹ MMB and **8a** were administered i.v. via lateral tail vein injection. For the first 3 to 4 days after surgery, the mice were observed for signs of infection at the surgical sites, yellowing of hair and hair texture, presence of blood around the eyes or nose, indications of loss of appetite, and decreased or absent fecal activity before the start of oral or i.v. dosing.

Twenty three BALB/c male mice were assigned to one of five study groups: No treatment (n=3), MMB (n=5, i.v.), MMB (n=5, oral), **8a** (n=5, i.v.), **8a** (n=5, oral). MMB and **8a** were prepared in a solution of DMSO:PEG-400:water (5:40:55) and filtered through a 0.2- μ m filter. Doses and route of administration were chosen on the basis of results obtained from pilot experiments. Doses of 20 mg/kg (equivalent to 75.7 μ mol/kg) for MMB and 27.1 mg/kg (equivalent to 75.7 μ mol/kg) for **8a** were administered by i.v. injection; doses of 200 mg/kg (equivalent to 757.0 μ mol/kg) for MMB and 271.2 mg/kg (equivalent to 757.0 μ mol/kg) for **8a** were administered by the oral route in different groups of BALB/c mice. Blood samples (20 μ L) were obtained at 0, 15, 30, 60, 120, 240 and 300 min after administration of each dose. The withdrawn blood was replaced with heparinized plasma from a donor mouse.²⁹

4.3. Extraction and LC/MS/MS analysis of plasma samples

Blood samples were centrifuged at 1200 x g for 10 min, and the plasma was separated. Plasma samples were extracted and the drug extracts were analyzed directly for the presence of MMB and **8a** by a sensitive LC/MS/MS analytical method. To each of 20 μ L of control or drug-treated plasma samples, 10 μ L of acetonitrile (ACN) internal standard solution containing 0.1 mg/mL caffeine-d₉ (internal standard) and 600 μ L ACN were added. The mixture was vortexed for 30s and centrifuged at 10,000 x g for 10 min at room temperature. The supernatant was transferred to 5 mL glass tubes and evaporated to dryness at 37°C under nitrogen gas. This extraction procedure was repeated 2 times and the combined evaporated extracts reconstituted in 50 μ L of ACN, followed by vortex-mixing for 30s,

sonication for 1 min and centrifugation at 10,000 x g for 10 min. The supernatant was separated and 5 μ L injected onto the LC/MS/MS unit.

Chromatographic separations were performed on a 2.7 μ m, 4.6 x 50 mm Agilent EC-C18 analytical column, (Agilent technologies, Santa Clara, CA) equipped with an Altima C-18, 5 μ m, 4.6 x 7.5 mm guard column (Grace Discovery Sciences, IL, USA). MMB, **8a** and caffeine- d_9 were eluted from the analytical column utilizing isocratic elution with a mobile phase composed of LC-MS grade water containing 0.05% formic acid: acetonitrile containing 0.05% formic acid (60:40, v/v). MMB, **8a** and caffeine- d_9 were separated with retention times of 2.2, 2.9 and 1.5 min, respectively. Quantification was determined utilizing caffeine- d_9 as the internal standard.

Analyses were carried out on an Agilent LC/MS/MS Triple Quad 6410 instrument (Santa Clara, CA, USA) operating in the positive electrospray ionization (ESI) mode with optimal ion source settings determined by standards of MMB and **8a**. A curtain gas of 20 psi, an ion spray voltage of 4000 V, an ion source gas1/gas2 of 35 psi and temperature of 300 $^{\circ}$ C were also employed in the collection of chromatographic data. MRM transitions monitored were as follows: MMB m/z 265.2/145.1, m/z 265.2/105.0; **8a** m/z 359.2/315.2, m/z 359.2/91.0 and caffeine- d_9 m/z 204.1/144.1, m/z 204.1/45.1.

The method was validated for each analyte over the concentration range 17.5 nM – 10.0 μ M, and good linearity was observed ($R^2 = 0.9926$ and 0.9876 for MMB and **8a**, respectively). Limit of detection (LOD) was determined as the lowest possible concentration of analyte to be detected, taking into consideration a 1:3 baseline noise-calibration point ratio. A reproducible lowest concentration that could be quantified was considered as the limit of quantitation (LOQ). The LOD and LOQ values for MMB were 37.9 ± 5.2 nM, and 75.7 ± 3.4 nM, respectively. For **8a**, the LOD and LOQ values were 27.9 ± 4.9 nM and 55.8 ± 6.3 nM, respectively. Average percentage recovery of five spiked plasma samples was $58.8 \pm 2.7\%$ for MMB and $72.4 \pm 4.7\%$ for **8a**. The inter- and intra-day variation did not exceed 7% with an accuracy range of 96–102% for both MMB and **8a**.

4.4. Pharmacokinetic (PK) analysis

The PK parameters for MMB and **8a** were determined from mouse plasma samples collected at 0, 15, 30, 60, 120, 240 and 300 min following administration of each dose after both i.v. and oral administration. Data from individual MMB and **8a** plasma concentration-time profiles were analyzed using the program Phoenix WinNonlin (Professional, version 6.2, Pharsight, Mountain View, CA). PK parameters, including peak concentration (C_{max}), time to attain C_{max} (T_{max}), area under the curve (AUC) from 0 to infinity (AUC_{0-inf}), elimination half-life $t_{1/2}$ (β), and total body clearance (Cl_{tot}), were estimated using the computer program described above.

Data from i.v. bolus administration of MMB and **8a** versus time (Figs 4 and 5) were best fitted to a one-compartment open model and a first order elimination and weighting factor as $1/Y^2$. The goodness of fit was determined by the AIC (Akaike information criterion), the SC (Schwartz criterion), and the WSSR (weighted sum of squared residuals or chi-squared test). The lower the AIC, SC, and WSSR, the more appropriate is the selected model. The decline

in plasma MMB and **8a** concentrations (C) versus time data can be adequately described by a mono-exponential equation (see Equation 1 below):

$$C_p = C_p^0 e^{-K_{el}t} \quad \text{Equation 1}$$

where C_p and C_p^0 are plasma concentrations in $\mu\text{mol/L}$ at time t and $t = 0$, respectively, and t is time in minutes.

Following oral administration of MMB and **8a**, data were analyzed by a one-compartment extravascular model with first order elimination to determine the comparative pharmacokinetic profiles of MMB and **8a** (Figs. 4 and 5). PK parameters, including peak concentration ($C_{\max}$), maximum time to attain $C_{\max}$ ($T_{\max}$), area under the curve from 0 to infinity ($AUC_{0-\infty}$), elimination half-life $t_{1/2}(\beta)$, and total body clearance (Cl_{tot}), were estimated using the computer program described above.

Bioavailabilities of MMB and **8a** were determined using Equation 2:

$$F\% = \frac{(AUC_{p.o.})(Dose_{i.v.})}{(AUC_{i.v.})(Dose_{p.o.})} \times 100 \quad \text{Equation 2}$$

where $AUC_{p.o.}$, $AUC_{i.v.}$, $Dose_{p.o.}$, and $Dose_{i.v.}$ represent the $AUC_{0-\infty}$ and corresponding dose for the oral and i.v. injections of MMB and **8a**, respectively.

Using $F\%$ values for each compound derived from Equation 2, the $F\%$ value for **8a** divided by the $F\%$ value for MMB represents the relative amount of **8a** delivered systemically following first-pass metabolism compared to that of MMB.

The ratio of areas under the plasma drug concentration vs. time curve for **8a** and MMB are shown in Equation 3. Doses are expressed in molar terms to account for the different molecular weights of the two compounds.

$$\frac{AUC_{8a}}{AUC_{MMB}} = \frac{F_{abs,p.o.}^{8a} (Cl_{MMB}) (Dose)_{8a}}{F_{abs,p.o.}^{MMB} (Cl_{8a}) (Dose)_{MMB}} \quad \text{Equation 3}$$

Since **8a** and MMB are two different compounds with different clearances, there is no cancellation of clearances in this equation. Further inspection of this equation shows that, for

equimolar doses, the ratio $\frac{AUC_{8a}}{AUC_{MMB}}$ will be directly proportional to the two factors $\frac{F_{abs,p.o.}^{8a}}{F_{abs,p.o.}^{MMB}}$ and $\frac{(Cl_{MMB})}{(Cl_{8a})}$.

5. Results

5.1. Synthesis of imidazole- and benzimidazole-carbamate analogs of MMB

MMB was synthesized utilizing a modification of the method of Macias *et al.*¹⁹ via selenium oxide oxidation of the C-14 methyl group of parthenolide (PTL). Initially, we investigated the synthesis of the C-14 imidazole and benzimidazole carbamate derivatives of MMB utilizing the *p*-nitrophenylchloroformate ester intermediate of MMB.²² However, this conjugation procedure suffered from the drawback of low yields of the desired carbamate products and the production of unwanted C-13 Michael addition byproducts (5–10%). Also, although the *p*-nitrophenylchloroformate ester intermediate worked satisfactorily for strongly nucleophilic amines, it did not prove to be useful for poorly nucleophilic aromatic amines. We also investigated the use of the imidazole carbamate intermediate of MMB, which could be prepared by reaction of MMB with carbonyldiimidazole (CDI) (Scheme 1, 2). However, the desired product was not obtained and only product **3** was formed, which likely results from initial formation of the C-14 carbamate conjugate of MMB, generating imidazole as a by-product, which then participates in a Michael addition reaction at the electron-deficient exocyclic C-13 position to afford MMB carbamate-Michael adduct **3** (Scheme 1). In a similar manner, we also reacted MMB with thiocarbonyldiimidazole **4** (Scheme 1), and obtained two products: the desired MMB thiocarbamate **5** (70% yield) and the MMB thiocarbamate-Michael adduct **5a** (30% yield) (Scheme 1), which proved difficult to separate. As a result of these investigations we concluded that none of the above methodologies were suitable for the facile generation of either carbamate or thiocarbamate derivatives of MMB.

We then investigated the use of carbonyldi-1,2,4-triazole (Scheme 2, **6**) in the synthesis of the imidazole- and benzimidazole-carbamate derivatives of MMB. Intermediate **7** was obtained by reaction of MMB with carbonyl-di-(1,2,4-triazole) (Scheme 2). The reaction was complete within 1 h and the desired product was obtained without column purification. In this reaction, although 1,2,4-triazole is generated as a by-product, it does not participate in Michael addition at C-13 of the MMB molecule, presumably due to its lower *pK_a* value when compared to imidazole (i.e. 10.3 vs 14.4 for imidazole).³⁰ Compound **7** was obtained in good yield and isolated without further purification, and served as an excellent synthon for the preparation of the five imidazole- and benzimidazole-carbamate analogs of MMB, **8a–8e** (Scheme 3 and Table 1).

5.2. Anti-leukemic activity of imidazole- and benzimidazole-carbamate derivatives of MMB against M9-ENL1 AML cells against M9-ENL1 Cells

All 5 carbamate analogs of MMB were screened for anti-leukemic activity against the M9 ENL-1 cell line (Fig. 3 and Table 2). The majority of the carbamate analogs evaluated (**8a–8d**) exhibited improved anti-leukemic activity (EC_{50} = 0.90–2.1 μ M) compared to PTL and MMB (EC_{50} = 7.0 and 15.5 μ M, respectively). Compound **8a** showed 8-fold and 16-fold greater anti-leukemic activity when compared to PTL and MMB, respectively. Analogs **8c** and **8d** exhibited about 4- to 5-fold greater anti-leukemic activity than PTL and were about 9- to 10-fold more potent than MMB. Analog **8a** showed the most promising anti-leukemic activity (EC_{50} = 0.9 μ M) in the above *in vitro* studies, and was selected for *in vivo*

pharmacokinetic (PK) analysis in BALB/c mice to compare its oral bioavailability to the parent compound, MMB, after a dose of 757.0 $\mu\text{mol/kg}$.

5.3. Pharmacokinetic Profile and oral bioavailability of MMB and **8a** in mice

Following oral dosing of MMB and **8a** in BALB/c male mice, it was observed from the mean concentration *vs.* time profile that both MMB and **8a** appeared in blood within 15 min (Figs. 4 and 5). Plasma concentrations of **8a** increased with time to reach a maximum level within 30 min. It could also be observed that at any time point in the time-course, **8a** was present at a higher concentration compared to MMB, indicating greater absorption and/or lower hepatic extraction of **8a**. It is noteworthy that plasma concentration of MMB declined rapidly with time, resulting in a lower AUC value when compared to **8a**. This substantial difference in plasma levels of **8a** when compared to MMB after oral treatment with these compounds indicates a more prolonged exposure to **8a** than to MMB in this animal model.

Data from oral MMB and oral **8a** were compared to data from i.v. MMB and i.v. **8a**, respectively. Oral administration of MMB and **8a** in mice resulted in peak plasma concentrations of 4.5 ± 0.5 and 10.6 ± 0.9 $\mu\text{mol/L}$ that were reached at T_{max} values of 120 ± 20 min and 30.1 ± 3.2 min, respectively (Figs. 4 and 5). Apparent elimination half-lives for **8a** and MMB were 92.3 ± 18.5 and 61.8 ± 4.5 min, respectively. Area under the MMB and **8a** plasma concentration *vs.* time curves for an oral dose of 757.0 $\mu\text{mol/kg}$ were 868.3 ± 55.3 and 2137.5 ± 87.6 $\mu\text{mol/L} \cdot \text{min}$, respectively. The ratio of the latter AUC to the former AUC indicates an enhancement factor of 2.5-fold for **8a** over MMB. Clearance (Cl_{tot}) values were 0.2 and 0.07 L/min for **8a** and MMB, respectively, and absolute plasma bioavailabilities for **8a** and MMB were $45.5 \pm 2.0\%$ and $6.7 \pm 0.8\%$, respectively, yielding a ratio of 6.8 to 1.

The reason for the lower oral bioavailability of MMB can be explained in part due to saturation of absorption pathways at the administered dose level of MMB. As a consequence, the plasma concentration of MMB might not reach significant levels in mice treated with MMB compared to **8a**. Bioavailability of MMB and **8a** is based on the amount of each drug present in the plasma. Since total bioavailability of **8a** was higher compared to that of MMB after oral administration, it follows that a greater amount of **8a** is absorbed orally than is MMB.

The value for the ratio $\frac{F_{\text{abs,p.o.}}^{\text{8a}}}{F_{\text{abs,p.o.}}^{\text{MMB}}}$ in this study was 6.8; while the value for the ratio $\frac{AUC_{\text{8a}}}{AUC_{\text{MMB}}}$

was 2.5. These data predict a clearance ratio $\frac{(Cl_{\text{MMB}})}{(Cl_{\text{8a}})}$ equal to 0.35. Thus, the oral bioavailability of **8a** is 680% that of MMB; while **8a** exhibits 250% greater exposure than MMB.

6. Conclusions

Procedures have been developed for the efficient synthesis of five azaheterocyclic carbamate analogs of MMB which have improved antileukemic potencies against M9-ENL1 AML cells

when compared to the parent compound, MMB. One of these analogs, **8a**, is 16-fold more potent than MMB and 8-fold more potent than PTL as an antileukemic agent. The comparative plasma PK profiles and oral bioavailabilities of MMB and the imidazole carbamate analog **8a** in BALB/c male mice indicate that **8a** has improved exposure after oral administration when compared to an equimolar dose of MMB, and has 1.5-fold longer half-life than MMB. The significantly greater oral bioavailability of **8a** when compared to that of MMB, coupled with the 16-fold greater antileukemic potency of **8a** compared to MMB (Table 2), predict a potential therapeutic advantage of **8a** over an equimolar dose of MMB.

Analog **8a** is being investigated further as a potential clinical candidate for treatment of AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.
Structures of parthenolide (PTL), DMAPT (dimethyl- aminoparthenolide) and melampomagnolide B (MMB, **1**)

Fig 2.

Sesquiterpene lactone-mediated anticancer mechanisms. Parthenolide and MMB-mediated inhibit the mitochondrial glutathione system, causing an increase in reactive oxygen species (ROS) and oxidative stress leading to mitochondrial dysfunction and activation of intrinsic apoptosis in cancer cells. A second synergistic mechanism involves inhibition of IKKβ causing decreased phosphorylation of IκB and/or p65, resulting in down-regulation of anti-apoptotic gene transcription and sensitization of cancer cells to apoptotic signals.

Figure 3. Dose-response viability curves for PTL, MMB and imidazole- and benzimidazole-carbamate derivatives of MMB with M9-ENL1 AML cells.

Figure 4. Plasma levels of MMB after an i.v. injection of 20 mg/kg (75.7 μmol/kg) of MMB or an oral dose of 200 mg/kg (757 μmol/kg) of MMB in BALB/c mice; n=5.

Figure 5. Plasma levels of **8a** after i.v. administration of 27.1 mg/kg (equivalent to 75.7 $\mu\text{mol/kg}$) of **8a**, or oral administration of 271.2 mg/kg (equivalent to 757 $\mu\text{mol/kg}$) of **8a** in BALB/c mice; n=5.

Scheme 1.

Synthesis of imidazole-carbamate and thioimidazole-carbamate analogs of MMB

Scheme 2.

Synthesis of the 1,2,4-triazole-carbamate analog of MMB

Scheme 3.

Synthesis of imidazole and benzimidazole-carbamate analogs of MMB

Table 1

The structures, yields, reaction times and melting points for imidazole- and benzimidazole-carbamate analogs of MMB

Compound	Yield (%)	Time (hr)	Mp (°C)
8a	75	8	130–131
8b	65	8	150–152
8c	70	6	155–156

Compound	Yield (%)	Time (hr)	Mp (°C)
 8d	67	16	260–261
 8e	67	4	125–127

Table 2

EC₅₀ (95% CI) values for PTL, MMB and the imidazole- and benzimidazole-carbamate derivatives of MMB against M9-ENL1 AML cells.

Compound	EC ₅₀ value (μM)
PTL	7.0 (5.2–6.7)
MMB	15.5 (14–17)
8a	0.9 (0.8–1.0)
8b	2.1 (2.0–2.3)
8c	1.4 (1.3–1.5)
8d	1.6 (1.5–1.7)
8e	3.9 (3.5–4.4)

EC₅₀ values below 1 μM are bolded