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Derivatization of Agelastatin A Leading to Bioactive Analogs and a Trifunctional Probe

Morgan Jouanneau^a, Brandon McClary^c, Jeremy C. Reyes^{b,§}, Rong Chen^d, Yuling Chen^d, William Plunkett^d, Xin Cheng^e, Andrew Z. Milinichik^e, Earl F. Albone^e, Jun O. Liu^c, and Daniel Romo^a

^aDepartment of Chemistry and Biochemistry, Baylor University, Waco, TX 76798, USA

^bDepartment of Chemistry, Texas A&M University, College Station, TX, 77842-3012

^cDepartment of Pharmacology and Molecular Sciences, John Hopkins School of Medicine, 725 North Wolfe St., Baltimore, MD 21205, USA

^dDepartment of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA

^eDepartment of Biochemistry Discovery, Morphotek, Inc, 210 Welsh Pool Road, Exton, PA 19422, USA

Abstract

(–)-Agelastatin A (AglA, **1**), a member of the pyrrole-aminoimidazole marine alkaloid (PAI) family, possesses a unique tetracyclic structure and is one of the most potent anticancer PAIs isolated to date. In efforts to expand the SAR of these agents and delineate sites that tolerate modification while retaining activity, we synthesized several derivatives and tested their anticancer activity. The cytotoxic effects of these derivatives were measured against several cancer cell lines including cervical cancer (HeLa), epidermoid carcinoma (A431), ovarian (Igrov and Ovar3), osteosarcoma (SJSA1), acute T cell leukemia (A3), epidermoid carcinoma (A431) in addition to primary human chronic lymphocytic leukemia cells. New positions for modification of AglA and new substitutions were explored leading to novel derivatives, 14-chloro AglA (**3**) and 14-methyl AglA (**12**), that retained activity toward various cancer cell lines with decreased toxicity toward B- and T-cells. The SAR data informed the synthesis of a trifunctional probe bearing an alkyne and a diazine potentially useful for cellular target identification.

Graphical Abstract

Correspondence to: Daniel Romo.

[§]Current address: The Dow Chemical Co, Freeport, TX.

Supplementary Material

Supplementary data including detailed experimental procedures and selected characterization data (¹H and ¹³C NMR) for all new compounds, and assay protocols employed can be found in the online version.

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Keywords

Agelastatin A; Photoaffinity probe; Diazirine; Pyrrole-aminoimidazole alkaloid; Structure-activity relationship

(–)-Agelastatin A (**1**, AglA) is a tetracyclic member of the pyrrole-2-aminoimidazole alkaloid (PAI) family.¹ Since its isolation in 1993 by Pietra and co-workers from the sponge *AglAs dendromorpha*,² this family has grown to include six agelastatin congeners (A through F, Figure 1a).³ Weinreb⁴ described the first total synthesis of AglA and since then several additional racemic⁵ and enantioselective⁶ syntheses of AglA have been reported.⁷ Recently, Movassaghi developed an elegant and efficient bioinspired approach to AglA and all congeners that involved a late stage C4–C8 bond formation.^{6a} Our group reported an alternative bioinspired approach that involved a final N_{pyrrole}–C7 bond formation.⁸ The reported bioactivities of this alkaloid are varied and include inhibition of β -catenin,⁹ suppression of osteopontin-mediated malignant transformation and inhibition of glycogen synthase kinase (GSK-3b).¹⁰ AglA and derivatives demonstrated good potency against leukemia¹¹ and notably displayed excellent blood-brain barrier penetration.¹² These recent studies by Molinski and Yoshimitsu revealed that substitutions at C13 on the pyrrole ring are tolerated (Figure 1c) with addition of a C13-trifluoromethyl and chloro substituent providing a marked increase in potency toward a leukemia and a breast cancer cell line, respectively. While AglA has been an attractive target for both synthetic and biological studies over two decades, a molecular level understanding of its effects in cells including cellular target(s) has not been described. Herein, we report expanded structure-activity relationship studies of agelastatin A enabling the identification of an active chloro derivative and the design and synthesis of a bioactive, trifunctional cellular probe that could assist with cellular target identification.

Our derivatization of AglA focused on functionalization at C14 and C15 of the pyrrole ring given the lack or absence of investigations of these positions. Given the efficiency and scalability of the Movassaghi synthesis of all known agelastatins, we prepared (–)-agelastatin A (**1**) for our studies as described starting from D-aspartic acid in 9 steps in ~4% overall yield (95% ee) and all derivatives prepared herein began with this material.^{6a} Iodination with *N*-iodosuccinimide (NIS) promoted by (InOTf)₃¹³ gave 14-iodo **2** in 74% yield (Scheme 1) which enabled subsequent functionalization of C14 through chemoselective Pd-mediated reactions. Initial chlorination conditions employing standard conditions (NCS) gave complex mixtures, however chlorination of AglA with

Palau'chlor[®] ¹⁴ gave a mixture of the desired 14-chloro AglA **3** (24%) and AgelB **4** (30%) along with traces of C13,C14-dichlorinated derivative detected by LCMS. Modified reaction conditions allowed access to AgelB (**4**) in 5 minutes using KBr and selectfluor[®], as brominating agent.¹⁵ Longer reaction times (1 h) with excess brominating agent gave AgelB (**4**) with 15-bromoAgelB (**5**) in 46% yield, the first C15 functionalized AglA derivative. The 15-iodoAgelB (**6**) was synthesized from AgelB (**4**) employing a large excess of iodine in the presence of Lewis acid¹³ in 71% yield over extended periods (2 days). Alternatively, treatment of AglA with 1.5 equivalent of NBS followed by iodine at 60 °C for 16 h gave iodo derivative **6** in 60% yield.

With various halogenated pyrrole derivatives in hand, we studied methylation of the pyrrole ring to differentiate steric versus electronic effects in this heterocycle beginning with C15. Methylation at C15 was studied through both Stille and Suzuki conditions (Scheme 2). Under Suzuki conditions, a mixture of the mono and dimethylated compounds **7**, **8** was obtained in favor of the dimethyl derivative. Stille conditions delivered only the monomethyl derivative **7** in 77% yield. Hydrogenolysis of bromide **7** gave 15-methyl-13,14-debromoAglA (**9**) in quantitative yield (Scheme 2).

Further substitutions on the pyrrole ring were investigated including arylation, alkynylation and acylation (Scheme 3). Suzuki coupling with phenylboronic acid gave a mixture of bis and mono-arylation products providing 13,14-diphenyl derivative **10** and 14-phenyl derivative **11** derivatives in 23% and 19% yields, respectively. Stille coupling with tetramethyltin and tributylethynylphenyltin gave the 14-methyl derivative **12** and 14-ethynylphenyl derivative **14** in 26% and 55% yield, respectively. Stille coupling with an enol ether stannane followed by mild hydrolysis gave the C-acetylated derivative **13** in 46% yield over two steps.

Modifications of the C5 and N9 positions were also studied leading to several *O*- and *N*-substituted derivatives and a stable aminor derivative. Triazole amide derivative **15** was obtained *via* acid catalyzed C5 substitution with 4-pentyn-1-ol followed by Sharpless-Hüisgen cycloaddition with α -azido-*N*-benzylamide (**A**) to provide amide **15** in 46% (Scheme 4). Inspired by Pietra's work,¹⁶ acetylation of the C5 carbinolamine enabled milder substitution conditions with nucleophiles such as 4-hydroxybutanone to provide the alkyl carbinolamine **17** or ammonia to give aminor **18** (Scheme 4). The presence of the amine was confirmed by HRMS and by changes in the ¹³C chemical shift of C5; namely, a shift from 95.7 to 82.0 ppm supports formation of an aminor at C5. This derivative exhibited excellent stability in water. No trace of AglA by HPLC was detected after 30 days in water at pH = 7 at 37 °C. To access the unknown methyl-substituted N9 derivative **19**, we took advantage of the differential acidity of protons in AglA (C5-OH > N9-H > N3-H). Addition of 1.0 equiv of LiHMDS, presumably led to initial deprotonation of the carbinolamine and addition of TMSCl gave an intermediate silylether derivative that was not isolated but directly treated with a second equivalent of LiHMDS followed by quenching with methyl iodide. The silylether was then cleaved by direct addition of TBAF to provide the monomethylated compound **19** in good yield (72%). The regioselectivity of this methylation was confirmed through HMBC correlations.

Structure-activity relationship studies: anticancer activity of AglA derivatives

AglA derivatives **2–25** were screened against several cancer cell lines and primary chronic lymphocytic leukemia (CLL) cells. HeLa cells were studied for comparison to published cytotoxicity of previously described AglA derivatives. Among the C14 and C15 AglA derivatives, only 14-chloro AglA (**3**) and the 14-methyl derivative (**12**) retained activity toward CLL albeit with only μM activity ($\text{IC}_{50} = 2.82 \mu\text{M}$ and $7.12 \mu\text{M}$, respectively vs $0.71 \mu\text{M}$ for AglA, Table 1). Likewise, toward HeLa cells, the chloro derivative had greater activity than the methyl derivative ($\text{IC}_{50} = 0.479 \mu\text{M}$ and $4.53 \mu\text{M}$, respectively). Furthermore, the 14-methyl compound (**12**) showed comparable potency when compared to AgelB (**4**) (HeLa, $\text{IC}_{50} = 4.53 \mu\text{M}$ and $7.85 \mu\text{M}$, respectively). Given that a bromine atom and a methyl group have similar steric size (VDW radii = $1.85 \text{ \AA}$ and $1.80 \text{ \AA}$, respectively)¹⁷ but different electronic properties, this result suggests that the size of the group at C14 is more important than electronic effects when considering activity toward primary HeLa cells. This was further substantiated by comparison to the C14-chloro derivative **3** for which the size decreases from $1.85 \text{ \AA}$ (Br) to $1.75 \text{ \AA}$ (Cl) and the potency significantly increased (17X toward HeLa cells). However, introduction of electron-withdrawing groups at C14 such as acetyl derivative (**13**) resulted in loss of activity ($>10 \mu\text{M}$ and $>20 \mu\text{M}$ for CLL and HeLa, respectively, Table 1). While previous reports demonstrated that a phenyl group is tolerated at C13, for example toward triple-negative breast cancer cells,¹² the introduction of a phenyl (**11**) or ethynylphenyl (**14**) at C14 led to loss of activity ($>10 \mu\text{M}$ and $>20 \mu\text{M}$ for CLL and HeLa, respectively, Table 1). Similarly, the 13,14-diphenyl derivative **10** did not show significant activity against HeLa and CLL cell lines. The 15-bromo and 15-methyl derivatives, **5** and **7**, were inactive against HeLa cells (Table 1). Additionally, no activity was observed with the *bis*-methyl compound **8**. These results suggest that substitution at C15 is not tolerated for AglA derivatives.

AglA derivatives **1, 3, 4** and **25** that displayed activity toward CLL were also assayed against various cancer cell lines (Table 2, CLL data included for comparison). The 14-chloro derivative **3** exhibited comparable activity to AglA while AgelB **4** was $\sim 10\text{X}$ less potent than AglA toward all cell lines. Notably, compound **25** showed comparable activity with parent natural product against Igrov and Ovar3 (IC_{50} 0.306 and $0.276 \mu\text{M}$, respectively) whereas against SJSA1, A3 and A431 the IC_{50} values were above $1 \mu\text{M}$.

Consistent with previously reported SAR studies,^{2b,11} *O*-alkylated compounds **15, 17** and **20** suffered from loss of activity toward HeLa and CLL cells ($\text{IC}_{50} > 10\text{--}20 \mu\text{M}$, Table 3) with the exception of derivative **17**. However, we determined that AglA was slowly regenerated from alkoxy carbinolamine **17** in water ($t_{1/2} = 16 \text{ h}$ at 25°C) likely from β -elimination. This suggests that this and related alkoxy carbinolamine derivatives of AglA could be useful as prodrugs or alternatively the carbinolamine center could serve as a point of attachment for antibody drug conjugate synthesis if an appropriate pH sensitive alkoxy carbinolamine is identified.¹⁸ On the other hand, the activity of aminal **18** was significantly reduced toward HeLa cells ($0.084 \mu\text{M}$ to $17 \mu\text{M}$, $\sim 200\text{X}$) and primary CLL cells ($>30 \mu\text{M}$) suggestive of the

stability of this amination derivative ($t_{1/2} = 7$ days at pH = 5 at 37 °C). Introduction of a N9-methyl group in derivative **19** resulted in complete loss of activity ($>20 \mu\text{M}$ against HeLa).

Protein binding studies

The propensity of AglA (**1**) and 14-Cl-AglA (**3**) to bind to plasma proteins and their stability in these media was investigated with both human plasma and fetal bovine serum (FBS, Table 4). The cytotoxicity of the compounds did not change dramatically when maintained in either human plasma or FBS suggesting that they were not highly protein bound. Further studies were performed to verify these results, and indeed full recovery of these derivatives was possible also pointing to their high stability. Low recovery is often an indication of material loss during the analysis due to compound instability, non-specific binding, or low solubility.

Differential cytotoxicity studies

The toxicity of AglA (**1**) and 14-Cl-AglA (**3**) was compared between CLL cells and normal B and T-lymphocytes (Figure 2). The study revealed that compared to CLL cells, AglA was less toxic toward B and T cells from healthy donors. The selectivity for CLL leukemia cells was even more apparent for 14-chloro derivative **3**. Although both compounds did demonstrate toxicities toward normal B lymphocytes, this is generally not a concern for future clinical application, as the B cell targeting antibodies rituximab and ofatumumab were proven safe for therapeutic treatment of B cell malignancies.²⁰

Synthesis of AglA Affinity Probes

Building on the SAR gathered for the C14-methyl and chloro derivatives **3** and **12**, we targeted a photoaffinity probe that could be useful for affinity chromatography experiments.¹⁹ Given the low potency exhibited by such derivatives ($\sim 0.5\text{--}4.5 \mu\text{M}$) however, we elected to synthesize a photoaffinity probe to improve the possibility of capturing interacting proteins. Toward this goal, a trifunctional probe **24** containing both a diazirine for covalent capture of putative cellular target(s) through photocrosslinking and an alkyne for subsequent biotin attachment to facilitate purification of covalently modified proteins was synthesized (Scheme 5). Beginning with previously prepared iodide **2**, Stille coupling with a stannyl bis-alkyne gave amine **21**. Acylation of this amine with diaziridine **22** gave amide **23** in modest yield with recovered amine **21**. Oxidation of the diaziridine with I_2 in methanol delivered the trifunctional diazirine probe **24**. We were pleased to find that both diaziridine **23** and diazirine **24** retained activity toward HeLa cells albeit greatly reduced compared to AglA.

In summary, we expanded the SAR profile of the anticancer agent, AglA through further investigations of the C14 position of the pyrrole and positions not previously investigated, C15 and N9. The 14-Cl-AglA (**3**) retained activity against various cancer cell lines including HeLa, CLL and A431. This AglA derivative also showed better selectivity toward CLL cells over B and T-cells than AglA and exhibits low serum protein binding and good stability given that recovery from serum was excellent. A photoaffinity, trifunctional probe of AglA,

albeit with low cytotoxic activity relative to AglA, was synthesized and ongoing photo-affinity chromatography studies will reveal the utility of this probe. These studies, in conjunction with previous work by others, demonstrate that few modifications of the AglA structure are tolerated while retaining high potency relative to the parent natural product. Finally, we demonstrated that alkoxyaminal derivatives of AglA may prove useful as prodrugs given their ability to regenerate AglA under mild hydrolysis conditions. Studies toward cellular target identification, using this probe and other strategies, in addition to further studies of potential prodrugs are ongoing and will be disclosed in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Agelastatin A (**1**) and congeners. b) Structure-activity relationship (SAR) overview of agelastatin A (**1**). c) Two known potent AglA derivative

Figure 2.
Effect of AglA (1) and 14-Cl-AglA (3) on B and T-cells and all plasma cells from healthy donors.

9 steps
(ref. 6a)
(overall
yield: 4%)
ee: 95-98%

KBr, Selectfluor
DMF
23 °C, 1 h

agelastatin B (**4**, 40%)

5 (46%)

Palau'chlor, DMF
23 °C, 16 h
or NIS, DTBMP
THF-H₂O (2:1)
0 °C, 3 h

2: X = I (74%)
3: X = Cl (24%)

agelastatin B (**4**, 30%)

KBr
Selectfluor
DMF
23 °C, 5 min
(90%)

NBS
DTBMP
THF-H₂O
0 °C, 2 h

then I₂, In(OTf)₃
60 °C, 16 h
(60%)

I₂
DTBMP
In(OTf)₃
THF-H₂O (2:1)
23 °C, 2 days
(71%)

agelastatin B (**4**)

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Scheme 2.
Synthesis of methylated pyrrole AglA derivatives

Scheme 3.
Synthesis of various C14 Agla derivatives **10–14**

Scheme 4.

Synthesis of *O*-substituted, N9 and aminated AglA derivatives **15**, **17–19**

Scheme 5.
Synthesis of an AglA trifunctional probe **24**

Cytotoxicity of AglA Derivatives **1-14** and **25** with Pyrrole Ring Modifications Toward CLL and HeLa Cell Lines.

Table 1

Compound	X	Y	Z	IC ₅₀ (μM)	
				CLL	HeLa
AglA (1)	Br	H	H	0.71 ± 0.10	0.084 ± 0.05
2	Br	I	H	NT	>20
3	Br	Cl	H	2.82 ± 1.07	0.479 ± 0.31
AglB (4)	Br	Br	H	>30	7.85 ± 1.12
5	Br	Br	Br	>10	>20
7	Br	Br	Me	NT	>20
8	Br	Me	Me	NT	>20
9	H	H	Me	NT	>20
10	Ph	Ph	H	>10	>20
11	Br	Ph	H	>10	>20
12	Br	Me	H	7.12 ± 1.44	4.53 ± 1.63
13	Br	Ac	H	>10	>20
14	Br	PhCC	H	>10	>20
31	Me	H	H	9.77 ± 4.48	>20

NT = Not tested.

Table 2

Antitumor Activities of AgIA Derivatives Toward Various Cancer Cell Lines with Pyrrole Ring Modifications.

Cmpd	X	Y	IC ₅₀ (μM)					
			CLL	HeLa	Igrov	SJSA1	A3	Ovar3
AgIA (1)	Br	H	0.71 ± 0.10	0.084 ± 0.05	0.17 ± 0.00	0.44 ± 0.03	0.38 ± 0.05	0.46 ± 0.13
3	Br	Cl	2.82 ± 1.07	0.479 ± 0.31	0.26 ± 0.06	0.56 ± 0.02	0.47 ± 0.03	0.34 ± 0.01
AgIB (4)	Br	Br	>30	7.85 ± 1.12	>1	>1	>1	>1
25	Me	H	9.77 ± 4.48	>20	0.31 ± 0.02	>1	>1	0.28 ± 0.00

CLL (primary human cells), HeLa, Igrov (human ovarian), SJSA1 (osteosarcoma), A3 (human acute T cell leukemia), A431 (epidermoid carcinoma) and Ovar3 (ovarian carcinoma).

Table 3Cytotoxicity of AglA Derivatives **15-20** with Modifications at C5 and N9 Positions.

Compound	R ₁	R ₂	IC ₅₀ (μM)	
			CLL	HeLa
AglA (1)	OH	H	0.71 ± 0.33	0.084 ± 0.05
15		H	>10	>20
17^a		H	5.74 ± 2.99 ^a	1.72 ± 0.53 ^a
18	NH ₂	H	>30	17 ± 2.83
19	OH	Me	NT	>20
20		H	>10	>20

^a AglA (**1**) was released (t_{1/2} = 16 h at 25 °C in water); NT: not tested. Analog **19** is a racemate.

Table 4Plasma Protein Properties of AglA (**1**) and 14-Cl-AglA (**3**)

Compound	IC ₅₀ , μ M		Human plasma protein binding	
	Plasma	FBS	Fraction Bound (%)	Recovery (%)
AglA (1)	0.71 $\pm$ 0.33	0.93 $\pm$ 0.47	23.9	99.2
3	2.82 $\pm$ 1.86	2.65 $\pm$ 1.22	46.5	102.1

FBS: fetal bovine serum; Fraction bound (%): the portion of the compound that is bound to human plasma; % recovery: the percentage of compound recovered after the incubation period for plasma protein binding analysis.