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Chlamydocin analogs bearing carbonyl group as possible ligand toward zinc atom in histone deacetylases

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Abstract—A series of chlamydocin analogs with various carbonyl functionalities were designed and synthesized as histone deacetylase (HDAC) inhibitors. Chlamydocin is a cyclic tetrapeptide containing an epoxyketone surrogate in the side chain which makes it irreversible inhibitor of HDACs, whereas apicidins are a class of cyclic tetrapeptides that contain an ethylketone moiety as zinc ligand. We replaced the epoxyketone moiety of chlamydocin with several ketones and aldehyde to synthesize potent reversible and selective HDAC inhibitors. The inhibitory activity of the cyclic tetrapeptides against histone deacetylase enzymes were evaluated and the result showed most of them are potent inhibitors. Some of them have remarkable selectivity among the HDACs.

1. Introduction

Dynamic acetylation and deacetylation of the ε-amino groups of lysines by histone acetyl transferase (HAT) and histone deacetylase (HDAC) enzymes play a fundamental role in the regulation of gene expression by altering the chromatin architecture and controlling the accessibility of DNA to transcriptional regulators.[1–4] Growing evidences support that the imbalance in histone acetylation can lead to transcriptional deregulation of genes that are involved in the control of cell cycle progression, differentiation, and/or apoptosis. Aberrant histone acetylation caused by the disrupted HAT activity or abnormal recruitment of HDACs has been related to carcinogenesis.[5–8] Inappropriate recruitment of HDACs provides a molecular mechanism by which genes necessary for proper differentiation or growth arrest can be silenced leading to excessive proliferation. Owing to the link between transcriptional repression and the recruitment of HDACs, inhibitors of this enzymatic activity are expected to reverse repression and to induce re-expression of differentiation-inducing genes. Therefore design and synthesis of HDAC inhibitors represent exciting opportunity for the development of therapeutics for treatment of cancers.

A wide variety of structurally unrelated natural and synthetic compounds have so far been reported exhibiting HDAC inhibiting activity. Among them trichostatin A (TSA),[9] depsipeptide FK228,[10–12] and the cyclic tetrapeptide family including trapoxin (TPX),[13] chlamydocin,[14] TAN-1746,[15] FR-235222,[16] 9,10-desepoxy-9-hydroxy-chlamydocin,[17] HC toxins,[18–22] Cyl-1, Cyl-2,[23–25] WF-3161,[26] apicidin,[27–30] FR-225497[31] are examples of naturally occurring HDAC inhibitors. Inhibitors like suberoylanilide hydroxamic acid (SAHA),[32] straight chain TSA and SAHA analogs,[33–35] scriptaid,[36] and the benzamide MS-275[37,38] have also been designed and synthesized.

The epoxyketone functionality of 2-amino-8-oxo-9,10-epoxydecanoic acids (Aoe) in trapoxin and other related cyclic tetrapeptides is supposed to react irreversibly to the functional nucleophile near the Zn atom of HDAC's active site.[13] Among the Aoe-containing cyclic tetrapeptides, chlamydocin is found to be a highly potent histone deacetylase (HDAC) inhibitor, inhibiting HDA activity in vitro with an IC_{50} value of 1.3 nM.[39] Though the activity is mainly due to the epoxyketone moiety, there are evidences which support that the cyclic tetrapeptide framework also has a significant structural role.

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The macrocycle provides specific hydrophobic interaction with the rim of HDAC enzymes.[40]

On the other hand, apicidins are a class of cyclic tetrapeptides that do not contain the classical electrophilic epoxyketone moiety yet are potent inhibitors of HDACs. SAR showed that the tryptophan residue in the cyclic tetrapeptide and C-8 ketone in the side chain are key constituents for HDACs' inhibitory activity.[28] Recently, we have reported a series of cyclic tetrapeptide-based HDAC inhibitors, CHAPs[41,42] and SCOPs,[43] by hybridizing the natural trapoxin, Cyl-1, Cyl-2, WF3161, chlamydocin, and HC-toxins with trichostatin and FK228, respectively.

In this paper, we proposed to synthesize conceptual hybrids of apicidin and chlamydocin in which epoxyketone moiety of chlamydocin is replaced by simple aldehyde and ketone groups as seen in apicidin. From the SAR of naturally occurring cyclic tetrapeptide-based inhibitors, it has been confirmed that a carbonyl group at C-8 position is essential for the HDAC inhibitory activity.[14,29] Based on this fact, we expect that the chlamydocin framework equipped with ketones and aldehyde can show better inhibitory activity in terms of reversibility and selectivity. For that, our strategy was to replace the Aoe of chlamydocin with a terminal double bond containing amino acid, 2-amino-8-nonenoic acid (Ae9),[44] and convert the olefin into aldehyde and various ketones. The zinc binding functional groups, with the proposed inhibitory mechanism, selected for this study is shown in Figure 1.

2. Results and discussion

Our aim was to synthesize potent inhibitors of HDACs with the chlamydocin-cyclic tetrapeptide scaffold. Therefore, we intended to synthesize a chlamydocin analog containing L-Ae9 using a solution-phase peptide synthesis strategy as shown in Scheme 1. *t*-Butyl-protected D-proline hydrochloride salt was coupled with Z-L-Phe to give the protected dipeptide Z-L-Phe-D-Pro-O^{t}Bu in quantitative yield. Z protection of the resulting dipeptide was then removed quantitatively by catalytic hydrogenation using H_2/Pd-C. The deprotected dipeptide was

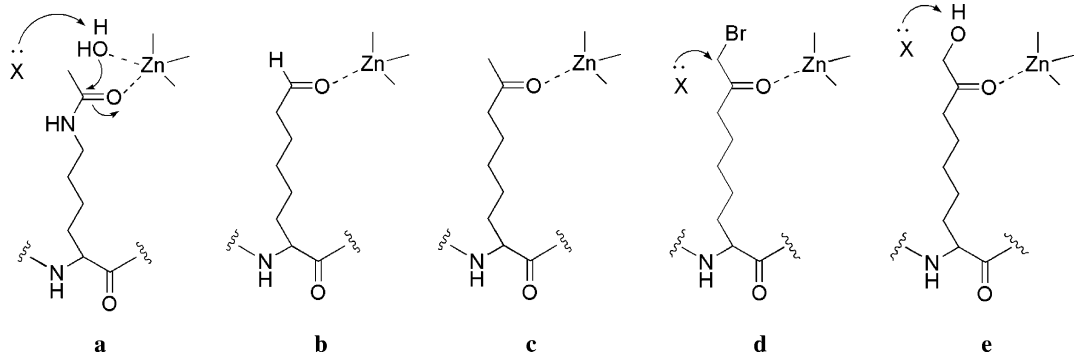
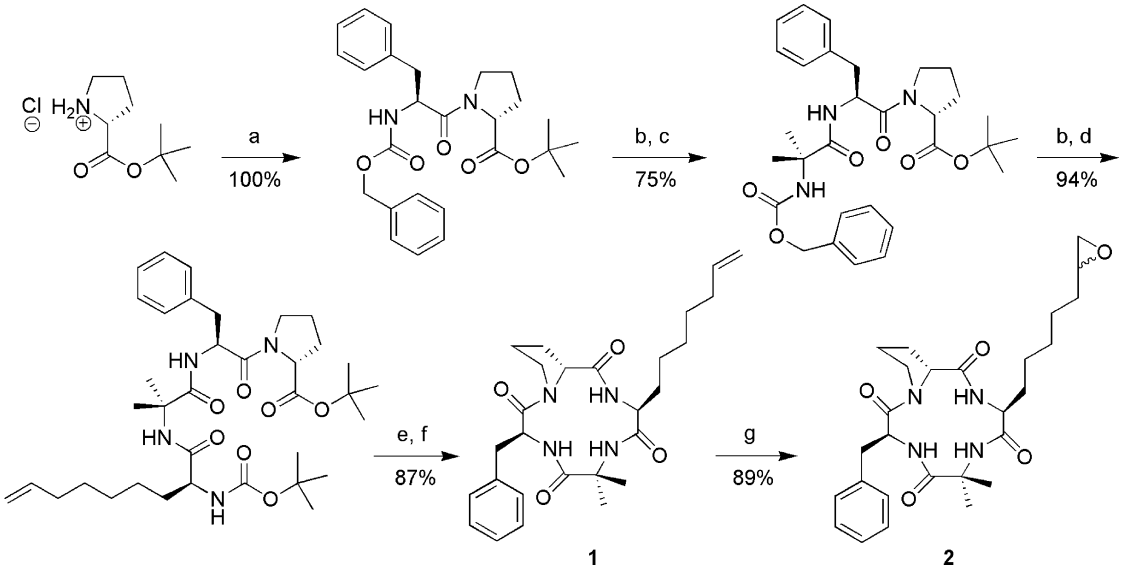


Figure 1. Proposed HDAC hydrolysis mechanism (a) and possible interaction of aldehyde and ketones (b–e) with Zn of HDAC. (a) Acetylated lysine, (b) aldehyde, (c) methylketone, (d) bromomethylketone, and (e) hydroxymethylketone.



Scheme 1. Reagents: (a) Z-L-Phe, DCC, HOBt, Et_3N; (b) H_2/Pd-C; (c) Z-Aib, DCC, HOBt; (d) Boc-L-Ae9, DCC, HOBt; 4) (e) TFA; (f) HATU, DIEA; (g) *m*-CPBA.

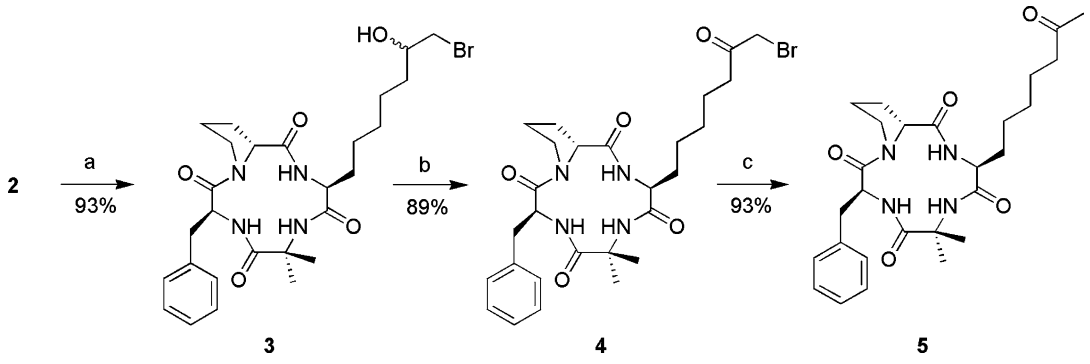
again coupled with Z-Aib to yield the tripeptide Z-Aib-L-Phe-D-Pro-O^{t}Bu in 87% yield. The N-terminal of the tripeptide was deprotected and coupled with a Boc-L-Aen9 to yield the linear tetrapeptide Boc-L-Ae9-Aib-L-Phe-D-Pro-O^{t}Bu in excellent yields. After deprotection of both the terminals by trifluoroacetic acid, the linear tetrapeptide TFA salt was then cyclized under high dilution conditions in dimethylformamide using *O*-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) to give the cyclic tetrapeptide cyclo(-L-Ae9-Aib-L-Phe-D-Pro-) in 87% yields (Scheme 1). Except for the cyclization step the coupling reagents used were DCC and HOBt throughout the synthesis.

As the epoxides are fairly reactive groups and convenient precursors, we planned to use the chlamydocin-epoxide analog as an intermediate for ketone functionalization. Accordingly, the side-chain double bond of **1** was oxidized to the epoxide (**2**) using *m*-chloroperoxybenzoic acid (Scheme 1). Initially, we checked the effect of halogen substitution on the C-9 methyl group by synthesizing a bromomethylketone from the epoxide **2**. Regioselective ring opening of the unsymmetrically substituted epoxide was successfully carried out with lithium bromide to yield the vicinal bromohydrin (**3**), which was subsequently employed for oxidation using Dess–Martin periodinane reagent to obtain the chlamydocin-bromomethylketone analog (**4**) (Scheme 2). To synthesize a C-8 methylketone, analogous to the apicidin's side-chain functionality, we reduced the terminal bromomethyl group of **4** by Zn-AcOH to derive the chlamydocin-ketone analog (**5**) (Scheme 2).

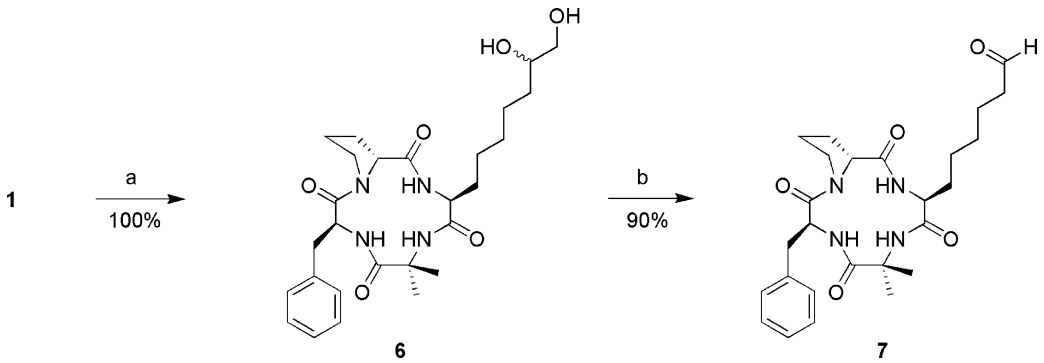
To know the influence of C-9 methyl group on HDAC inhibitory activity, we synthesized a terminal aldehyde. The synthesis was initiated by oxidizing the side-chain double bond of **1** to the chlamydocin-diol analog (**6**) using microencapsulated osmium tetroxide (MC-OsO_4)[45] and then oxidative cleavage of the diol **6** by sodium periodate led to yield the chlamydocin-aldehyde analog **7** (Scheme 3).

In order to explore the effect of a hydroxyl substituent on the C-9 methyl group, the diol **6** was employed for further modification to get a hydroxymethylketone functionality. In addition, hydroxymethylketone is the functional group in the chlamydocin analogs 9,10-desepoxy-9-hydroxy-chlamydocin, TAN-1746, and FR-235222.[15–17] These compounds with a C-10 methyl group could be compared with the synthesized chlamydocin-hydroxymethylketone analog to evaluate an effect of the terminal methyl group on the inhibitory activity. First, the primary alcohol group was selectively protected by *t*-butyldimethylsilyl chloride (TBDMSCl) to get the corresponding **8**. The protected cyclic tetrapeptide was then oxidized to the ketone **9** using Dess–Martin periodinane. TBDMS group of the ketone was deprotected by the action of tetrabutylammonium fluoride (TBAF) to get the desired hydroxymethylketone **10** (Scheme 4).

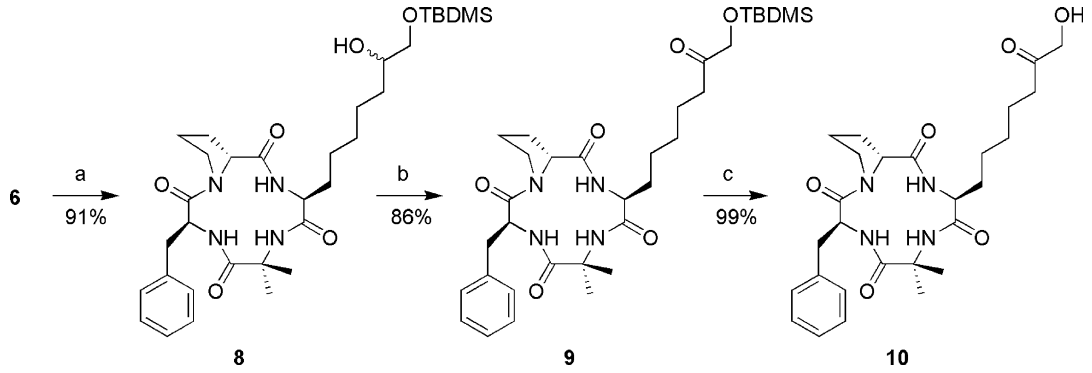
The synthesized ketones, aldehyde, and intermediate epoxide were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 prepared from 293T cells.[12] Detailed experimental procedure for the enzyme preparation and assay methods are given in the



Scheme 2. Reagents: (a) LiBr, AcOH; (b) Dess–Martin periodinane; (c) Zn, AcOH.



Scheme 3. Reagents: (a) MC-OsO4, *N*-methylmorpholine-*N*-oxide; (b) $NaIO_4$.



Scheme 4. Reagents: (a) TBDMSCl, DIEA, DMAP; (b) Dess–Martin periodinane; (c) TBAF.

experimental section. As a control experiment, the chlamydocin analogs with double bond and diol were also examined for HDAC inhibitory activity. The results of the HDAC inhibitory activity and the p21 promoter assay of the compounds are shown in Table 1.

The chlamydocin-epoxide analog **2** was active in micromolar concentrations, whereas dehydro-chlamydocin in which the C-8 carbonyl group of chlamydocin is replaced by hydroxyl group was inactive.[14] This result indicates that a C-8 carbonyl as well as a C-8,9 epoxide can inhibit HDACs.

The chlamydocin-aldehyde (**7**), which lacks the C-9 methyl group, shows moderate HDAC inhibitory activity. A higher activity of chlamydocin-bromomethylketone (**4**) than that of the corresponding methylketone (**5**) reveals that the presence of a $-I$ group with high polarizability (soft basicity) and nucleophilicity on C-9 methylene increases the inhibitory activity.

The chlamydocin-hydroxymethylketone analog **10** in which a hydrogen of the C-9 methyl group of **5** was replaced by a hydroxyl group was about fivefold more active than the parent methylketone (**5**) (Table 1). Whereas the corresponding diol **6** in which the C-8 position had a hydroxyl group instead of the carbonyl was inactive. This observation further supports the fact that a C-8 carbonyl group is essential and an atom carrying a lone pair of electrons, β to the C-8 carbonyl group, has positive effects on the activity. It should be noted that the hydroxymethylketone moiety found in 9,10-desepoxy-9-hydroxy-chlamydocin, TAN-1746, and FR-235222[15–17] as possible metabolic products from the epoxyketone moiety also showed potent HDAC inhibitory activity. The hydroxymethylketone **10** was specific toward HDAC4 compared with HDAC1 and 6. It is about four times more active toward HDAC4 than HDAC1 and 15 times more active than HDAC6. The compound was most selective among the inhibitors listed in Table 1.

Table 1. HDAC inhibitory activity and p21 promoter activity data for the chlamydoc in analogs

Compound	IC_{50} (μM)			EC_{1000} (μM)
	HDAC1	HDAC4	HDAC6	
TSA[46]	0.0190	0.0200	0.0280	0.190
1	>100	>100	>100	>250
2	4.69	4.05	>100	22.7
4	0.0619	0.0595	0.621	0.344
5	1.08	0.290	3.70	1.20
6	>100	>100	>100	NT
7	4.33	1.29	14.1	20.9
10	0.223	0.0604	0.887	0.234

NT, not tested.

The chlamydocin analogs bearing carbonyl groups were examined for hyperacetylation of H3 and H4 histone proteins as a result of HDAC inhibition. We observed a concentration-dependent inhibition profile in the Western blot analysis of H3 and H4 acetylation. The efficiencies were in order of compounds **10** ≈ **4** > **5**.

3. Conclusion

In order to develop novel ketone-based HDAC inhibitors, design and synthesis of a series of chlamydocin analogs equipped with various carbonyl functionalities have been performed successfully by using unique amino acid L-2-amino-8-nonenoic acid. Our synthetic strategy allowed the convenient preparation of the simple functional group for potent HDAC inhibition. These inhibitors have displayed potent HDAC inhibitory activity under cell free and cell-based conditions. Some of the synthesized inhibitors have selectivity among the HDAC1, HDAC4, and HDAC6. Considering the activity and selectivity together, the hydroxymethylketone group as a zinc ligand is most promising among the compounds synthesized.

4. Experimental

4.1. General

Unless otherwise noted, all solvents and reagents were of reagent grade and used without purification. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60

F_{254}) with spots visualized by UV light or charring. Analytical HPLC was performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6 · 50 mm, Merck). The mobile phases used were A: H_2O with 10% CH_3CN and 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A to B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm. FAB-mass spectra and high-resolution mass spectra (HRMS) were measured on a JEOL JMS-SX 102A instrument. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer. Unless otherwise stated, all NMR spectra were measured in $CDCl_3$ solutions with reference to TMS. All ^1H shifts are given in parts per million (s, singlet; d, doublet; t, triplet; m, multiplet). Assignments of proton resonances were confirmed, when possible, by selective homonuclear decoupling experiments or by correlated spectroscopy. Amino acids were coupled using standard solution-phase chemistry with dicyclohexyl-carbodimide (DCC) or *O*-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).

4.1.1. Synthesis of *cyclo*(-L-Ae9-Aib-L-Phe-D-Pro-) (1). To a cooled solution of HCl · H-D-Pro-O^{t}Bu (12.5 g, 60.0 mmol) and Z-L-Phe-OH (18.0 g, 60.0 mmol) in dimethylformamide (DMF) (120 mL), HOBt · H_2O (9.19 g, 60.0 mmol), DCC (14.9 g, 72.0 mmol) and triethylamine (8.52 mL, 60.0 mmol) were added and the mixture was stirred at room temperature for 8 h. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and washed with 10% citric acid, 4% sodium bicarbonate, and brine, respectively, and then dried over anhydrous $MgSO_4$. After evaporation of the ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Z-L-Phe-D-Pro-O^{t}Bu (27.2 g, 100%, HPLC, rt 9.66 min) as a colorless oil. The protected dipeptide (27.2 g, 60.0 mmol) was dissolved in acetic acid (120 mL). Pd-C (3.50 g) was added and the mixture was stirred under hydrogen atmosphere for 12 h. The reaction was monitored by TLC and HPLC. After completion of the reaction, Pd-C was filtered off and the acetic acid was evaporated. The residue was dissolved in ethyl acetate and the organic phase was washed with saturated sodium bicarbonate solution and dried over anhydrous $MgSO_4$. Evaporation of ethyl acetate gave H-L-Phe-D-Pro-O^{t}Bu (16.4 g, 86%, HPLC, rt 5.60 min.), which was coupled with Z-Aib-OH (13.1 g, 55.0 mmol) following the same procedure described above and purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to obtain Z-Aib-L-Phe-D-Pro-O^{t}Bu (24.1 g, 87%, HPLC, rt 8.95 min) as a white foam. The tripeptide Z-Aib-L-Phe-D-Pro-O^{t}Bu (24.1 g, 44.8 mmol) was dissolved in acetic acid (90 mL) and Pd-C (2.50 g) was added. The mixture was stirred under hydrogen for 12 h. After completion of the reaction, Pd-C was filtered off and the acetic acid was evaporated. The residue was dissolved in ethyl acetate, washed with 4% sodium bicarbonate, and dried over anhydrous $MgSO_4$. Evaporation of ethyl acetate gave H-Aib-L-Phe-D-Pro-O^{t}Bu (18.1 g, 100%, HPLC, rt 5.68 min). The N-terminal free tripeptide H-Aib-L-Phe-D-Pro-O^{t}Bu (8.07 g, 20.0 mmol) was coupled with Boc-L-Ae9-OH (5.43 g, 20.0 mmol) according to the method described earlier and the fully protected crude linear tetrapeptide was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae9-Aib-L-Phe-D-Pro-O^{t}Bu (12.3 g, 94%) as a white foam. Boc-L-Ae9-Aib-L-Phe-D-Pro-O^{t}Bu (12.3 g, 18.8 mmol) was dissolved in TFA (50 mL) at 0 °C and kept for 3 h. After evaporation of TFA, the residue was solidified by titration with ether to yield H-L-H-L-Ae9-Aib-L-Phe-D-Pro-OH as TFA salt (11.6 g, 100%). HPLC, rt 5.96 min. HR-FAB MS $[M+H]^+$ 501.3050 for $C_{27}H_{41}O_5N_4$ (calcd 501.3077). To a volume of DMF (800 mL) H-L-Ae9-Aib-L-Phe-D-Pro-OH · TFA (8.00 mmol, 4.92 g), HATU (3.64 g, 9.60 mmol), and DIEA (4.45 mL, 25.6 mmol) were added in five aliquots with 30 min time interval while the solution was stirred vigorously. After the final addition, the reaction mixture was allowed to stir for an additional hour. Completion of the cyclization reaction was monitored by HPLC and then DMF was evaporated under reduced pressure. The crude cyclic tetrapeptide was dissolved in ethyl acetate and the solution was washed successively by 10% citric acid, 4% sodium bicarbonate, and brine. Finally the ethyl acetate solution was dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield *cyclo*(-L-Ae9-Aib-L-Phe-D-Pro-) (**1**) (3.36 g, 87%) as a white foam after drying in vacuo. HPLC, rt 9.34 min, HR-FAB MS $[M+H]^+$ 483.2971 for $C_{27}H_{39}O_4N_4$ (calcd 483.2971), $[\alpha]_D^{25}$ −73.6 (*c* 0.1, MeOH), ^1H NMR (500 MHz, $CDCl_3$): δ_H 1.28 (m, 2H), 1.32 (m, 2H), 1.34 (s, 3H), 1.38 (m, 2H), 1.63 (m, 1H), 1.73 (m, 1H), 1.76 (m, 1H), 1.77 (s, 3H), 1.80 (m, 1H), 2.03 (m, 2H), 2.18 (m, 1H), 2.32 (m, 1H), 2.95 (dd, J = 13.3, 5.7 Hz, 1H), 3.23 (m, 1H), 3.26 (dd, J = 13.5, 10.0 Hz, 1H), 3.86 (m, 1H), 4.18 (m, 1H), 4.66 (m, 1H), 4.97 (m, 2H), 5.16 (ddd, J = 10.1, 10.1, 5.9 Hz, 1H), 5.79 (ddt, J = 17.2, 10.2, 6.5, 1H), 5.91 (s, 1H), 7.08 (d, J = 10.5 Hz, 1H), 7.21 (m, 3H), 7.27 (m, 2H), 7.52 (d, J = 10.5 Hz, 1H).

4.1.2. Synthesis of chlamydocin-epoxide analog (2). To a chilled solution of the cyclic tetrapeptide **1** (4.73 g, 9.80 mmol) in anhydrous dichloromethane (200 mL) in a solution of purified *m*-chloroperoxybenzoic acid[†] (*m*-CPBA) (3.38 g, 19.6 mmol) in 100 mL of anhydrous dichloromethane was added in aliquots for over 30 min with stirring. The reaction mixture was allowed to stir for 18 h at room temperature. TLC and HPLC showed almost complete conversion of the olefin into epoxide. The solution was then washed with 4% sodium bicarbonate and brine followed by drying over anhydrous magnesium sulfate and filtration. After evaporation of dichloromethane, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the white foam **2** (4.33 g, 89%). HPLC, rt 7.13 min. HR-FAB MS $[M+H]^+$

[†] Commercially available *m*-CPBA (75–85%) was washed thoroughly with phosphate buffer of pH 7.5 and the residue dried under reduced pressure.

499.2891 for $C_{27}H_{39}O_5N_4$ (calcd 499.2920), $[\alpha]_D^{25}$ −66.9 (*c* 0.1, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.28 (m, 2H), 1.32 (m, 2H), 1.34 (s, 3H), 1.38 (m, 1H), 1.46 (m, 1H), 1.52 (m, 2H) 1.64 (m, 1H), 1.74 (m, 1H), 1.76 (m, 1H), 1.77 (s, 3H), 1.80 (m, 1H), 2.18 (m, 1H), 2.32 (m, 1H), 2.46 (m, 1H), 2.74 (m, 1H), 2.89 (m, 1H), 2.95 (dd, *J* = 13.5, 6.0 Hz, 1H), 3.23 (m, 1H), 3.26 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.86 (m, 1H), 4.19 (m, 1H), 4.66 (m, 1H), 5.16 (ddd, *J* = 10.2, 10.2, 5.8 Hz, 1H), 5.94 (s, 1H), 7.10 (d, *J* = 10.0 Hz, 1H), 7.21 (m, 3H), 7.27 (m, 2H), 7.51 (d, *J* = 10.5 Hz, 1H).

4.1.3. Synthesis of chlamydocin-bromohydrin analog (3). To a solution of the epoxide **2** (2.49 g, 5.00 mmol) in anhydrous tetrahydrofuran (50 mL), glacial acetic (99.99+%) and anhydrous lithium bromide (695 mg, 8.00 mmol) were added. The reaction solution was stirred for 5 h at room temperature. After completion, the reaction was quenched by adding water. Ethyl acetate was added to the reaction mixture and the organic layer was washed with 4% sodium bicarbonate followed by brine. The organic phase was dried over anhydrous $MgSO_4$, filtered, and evaporated. The crude bromohydrin **3** was then purified by silica gel chromatography using a mixture of chloroform and methanol (99:2) to yield the corresponding pure **3** (2.69 g, 93%) as a white foam. HPLC, rt 6.68 min. HR-FAB MS $[M+H]^+$ 579.2156 for $C_{27}H_{40}O_5N_4{}^{79}Br$ (calcd 579.2182) and 581.2098 for $C_{27}H_{40}O_5N_4{}^{81}Br$ (calcd 581.2162), $[\alpha]_D^{25}$ −56.8 (*c* 0.1, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.30 (m, 2H), 1.31 (m, 2H), 1.34 (s, 3H), 1.35 (m, 1H), 1.46 (m, 1H), 1.54 (m, 2H), 1.63 (m, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 1H), 1.80 (m, 1H), 2.09 (s, 1H), 2.17 (m, 1H), 2.32 (m, 1H), 2.95 (dd, *J* = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.38 (m, 1H), 3.53 (m, 1H), 3.77 (m, 1H), 3.85 (m, 1H), 4.20 (m, 1H), 4.67 (m, 1H), 5.16 (ddd, *J* = 10.2, 10.2, 5.5 Hz, 1H), 6.07 (d, *J* = 8.5 Hz, 1H), 7.14 (d, *J* = 10.5 Hz, 1H), 7.22 (m, 3H), 7.27 (m, 2H), 7.52 (d, *J* = 10.5 Hz, 1H).

4.1.4. Synthesis of chlamydocin-bromomethylketone analog (4). To a solution of the bromohydrin **3** (209 mg, 0.360 mmol) in anhydrous dichloromethane (4.0 mL) Dess–Martin periodinane (458 mg, 1.08 mmol) was added. The reaction mixture was stirred at 25 °C for 3 h. TLC and HPLC showed complete conversion of the starting bromohydrin. The reaction solution was then diluted by adding diethyl ether (4.0 mL) followed by careful addition of a saturated solution of sodium bicarbonate containing 804 mg of sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$). After stirring for 10 min. the suspension became a clear solution. The organic layer was separated from the aqueous layer and was washed with brine, and dried over anhydrous $MgSO_4$. The filtered organic phase was evaporated to get a crude bromomethylketone which was then purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the corresponding white foamy ketone **4** (185 mg, 89%). HPLC, rt 7.33 min. HR-FAB MS $[M+H]^+$ 577.2051 for $C_{27}H_{38}O_5N_4{}^{79}Br$ (calcd 577.2026) and 579.1997 for $C_{27}H_{38}O_5N_4{}^{81}Br$ (calcd 579.2005), $[\alpha]_D^{25}$ −57.4 (*c* 0.1, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.31 (m, 2H), 1.32 (m, 3H), 1.34 (s, 3H), 1.62 (m, 2H), 1.75 (m, 1H), 1.77 (s, 3H), 1.79 (m, 1H), 1.80 (m, 1H), 2.17 (m, 1H), 2.32 (m, 1H), 2.65 (t, *J* = 7.5 Hz, 2H), 2.95 (dd, *J* = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.86 (m, 1H), 3.87 (s, 2H), 4.18 (m, 1H), 4.66 (m, 1H), 5.16 (ddd, *J* = 10.2, 10.2, 5.5 Hz, 1H), 5.93 (s, 1H), 7.10 (d, *J* = 10.5 Hz, 1H), 7.22 (m, 3H), 7.27 (m, 2H), 7.50 (d, *J* = 10.5 Hz, 1H).

4.1.5. Synthesis of chlamydocin-methylketone analog (5). To a stirred mixture of activated zinc powder (1.00 g) in glacial acetic acid (3.0 mL), a solution of the bromomethylketone **4** (112 mg, 0.194 mmol) in 1.0 mL of glacial acetic acid was added dropwise. After 1 h, diethyl ether was added to dilute the reaction mixture and zinc powder was filtered off. The organic phase was washed with 4% sodium bicarbonate and brine, respectively, and finally dried over anhydrous $MgSO_4$ and filtered. Ether was evaporated to get the crude methylketone, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the corresponding pure **5** (91.0 mg, 93%) as a white foam. HPLC, rt 7.24 min. HR-FAB MS $[M+H]^+$ 499.2897 for $C_{27}H_{39}O_5N_4$ (calcd 499.2920), $[\alpha]_D^{25}$ −68.1 (*c* 0.1, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.31 (m, 3H), 1.32 (m, 2H), 1.34 (s, 3H), 1.57 (m, 1H), 1.66 (m, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 2H), 2.18 (m, 1H), 2.13 (s, 3H), 2.32 (m, 1H), 2.41 (t, *J* = 7.5 Hz, 2H), 2.95 (dd, *J* = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.86 (m, 1H), 4.18 (m, 1H), 4.66 (m, 1H), 5.16 (ddd, *J* = 10.2, 10.2, 5.5 Hz, 1H), 5.90 (s, 1H), 7.09 (d, *J* = 10.5 Hz, 1H), 7.21 (m, 3H), 7.27 (m, 2H), 7.50 (d, *J* = 10.5 Hz, 1H).

4.1.6. Synthesis of chlamydocin-diol analog (6). The cyclic tetrapeptide *cyclo*(-L-Ae9-Aib-L-Phe-D-Pro-) (**1**) (1.79 g, 3.70 mmol) was dissolved in a mixture of acetone, acetonitrile, and water (3:1:1, 30 mL) to which 4-methylmorpholine-*N*-oxide (NMO) (900 mg, 7.70 mmol) and microencapsulated osmium tetroxide (MC-OsO_4, Wako) (760 mg, ≡0.300 mmol, 10 mol%) were added. The reaction mixture was stirred for 18 h and HPLC monitoring showed complete conversion of the double bond into vicinal diol. The catalyst was removed by filtration and washed thoroughly with methanol. The methanol washings were combined with the filtrate and the mixture was evaporated. The crude diol was purified by silica gel chromatography using a mixture of chloroform and methanol (95:5) to yield the corresponding pure diol **6** (1.94 g, 100%) as a white foam. HPLC, rt 5.37 min. HR-FAB MS $[M+H]^+$ 517.3034 for $C_{27}H_{41}O_6N_4$ (calcd 517.3026), $[\alpha]_D^{25}$ −65.2 (*c* 0.1, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.31 (m, 2H), 1.32 (m, 2H), 1.34 (s, 3H), 1.35 (m, 1H), 1.41 (m, 1H), 1.44 (m, 2H), 1.63 (m, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 1H), 1.81 (m, 1H), 2.17 (m, 1H), 2.32 (m, 1H), 2.95 (dd, *J* = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.44 (m, 1H), 3.65 (m, 1H), 3.70 (m, 1H), 3.85 (m, 1H), 4.19 (m, 1H), 4.67 (m, 1H), 5.16 (ddd, *J* = 10.2, 10.2, 5.5 Hz, 1H), 6.14 (d, *J* = 5 Hz, 1H), 7.15 (d, *J* = 10.0 Hz, 1H), 7.22 (m, 3H), 7.27 (m, 2H), 7.51 (d, *J* = 10.0 Hz, 1H).

4.1.7. Synthesis of chlamydocin-aldehyde analog (7). To a stirred solution of the diol **6** (32.0 mg, 0.0620 mmol) in a mixture of tetrahydrofuran (1.0 mL) and water (0.5 mL), sodium periodate ($NaIO_4$) (40.0 mg, 0.186 mmol) was added. Stirring was continued for 1 h, while HPLC showed the almost complete conversion of the starting diol into product. The reaction solution was dissolved into ethyl acetate, washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. The crude product was purified by silica gel chromatography using a mixture of chloroform and methanol (98:2) to yield the corresponding pure aldehyde **7** (27.0 mg, 90%) as a white foam. HPLC, rt 5.66 min. HR-FAB MS $[M+H]^+$ 485.2747 for $C_{26}H_{37}O_5N_4$ (calcd 485.2764), $[\alpha]_D^{25}$ −71.6 (*c* 0.1, MeOH), ^1H NMR (500 MHz, $CDCl_3$): δ_H 1.29 (m, 2H), 1.32 (m, 2H), 1.34 (s, 3H), 1.36 (m, 1H), 1.63 (m, 1H), 1.65 (m, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 2H), 2.18 (m, 1H), 2.32 (m, 1H), 2.43 (m, 2H), 2.95 (dd, J = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, J = 13.5, 10.0 Hz, 1H), 3.86 (m, 1H), 4.18 (m, 1H), 4.66 (m, 1H), 5.16 (ddd, J = 10.2, 10.2, 5.5 Hz, 1H), 5.92 (s, 1H), 7.11 (d, J = 10.5 Hz, 1H), 7.22 (m, 3H), 7.27 (m, 2H), 7.49 (d, J = 10.5 Hz, 1H), 9.76 (m, 1H).

4.1.8. Synthesis of TBDMS protected chlamydocin-diol analog (8). To a stirred solution of the diol **6** (1.94 g, 3.73 mmol) in anhydrous dichloromethane (30 mL) diisopropylethylamine (DIEA) (5.60 mL, 32.2 mmol), 4-dimethylaminopyridine (DMAP) (56.0 mg, 0.460 mmol), and *t*-butyldimethylsilyl chloride (TBDMSCl) (793 mg, 5.22 mmol) were added. Stirring was continued for 8 h, while HPLC showed the almost complete conversion of the starting diol into product. The reaction solution was washed with water and brine, dried over anhydrous $MgSO_4$, filtered and evaporated to get the crude primary hydroxyl-protected diol. The residue was purified by silica gel chromatography using a mixture of chloroform and methanol (98:2) to yield the corresponding pure monoprotected diol **8** (2.15 g, 91%) as a white foam. HPLC, rt 10.74 min. HR-FAB MS $[M+H]^+$ 631.3895 for $C_{33}H_{55}O_6N_4Si$ (calcd 631.3891), ^1H NMR (500 MHz, $CDCl_3$): δ_H 0.08 (m, 6H), 0.91 (m, 6H), 1.31 (m, 2H), 1.32 (m, 2H) 1.34 (s, 3H), 1.36 (m, 2H), 1.45 (m, 2H), 1.64 (m, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 1H), 1.81 (m, 1H), 1.84 (br, 1H), 2.17 (m, 1H), 2.32 (m, 1H), 2.95 (dd, J = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, J = 13.5, 10.0 Hz, 1H), 3.38 (m, 1H), 3.61 (m, 2H), 3.85 (m, 1H), 4.18 (m, 1H), 4.66 (m, 1H), 5.16 (ddd, J = 10.2, 10.2, 5.5 Hz, 1H), 5.93 (d, J = 6.0 Hz, 1H), 7.08 (d, J = 10.5 Hz, 1H), 7.22 (m, 3H), 7.27 (m, 2H), 7.52 (d, J = 10.5 Hz, 1H).

4.1.9. Synthesis of TBDMS protected chlamydocin-hydroxymethylketone analog (9). To a solution of the protected diol **8** (2.07 g, 3.29 mmol) in anhydrous dichloromethane (65 mL), Dess–Martin periodinane (8.15 g, 19.3 mmol) was added. The reaction mixture was stirred at 25 °C for 3 h. TLC and HPLC showed complete conversion of the starting compound. The reaction solution was then diluted by adding diethyl ether (65 mL) followed by careful addition of a saturated solution of sodium bicarbonate (50 mL) containing 14.4 g of sodium thiosulfate pentahydrate ($Na_2S_2O_3{\cdot}5H_2O$). After stirring for 10 min. the suspension became a clear solution. The organic layer was separated from the aqueous layer and was washed with brine, and dried over anhydrous $MgSO_4$. The filtered organic phase was evaporated to get a crude TBDMS protected hydroxymethylketone, which was then purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the purified ketone **9** (1.77 g, 86%) as a white foam. HPLC, rt 11.06 min. HR-FAB MS $[M+H]^+$ 629.3763 for $C_{33}H_{53}O_6N_4Si$ (calcd 629.3734), $[\alpha]_D^{25}$ −54.4 (*c* 0.1, MeOH), ^1H NMR (500 MHz, $CDCl_3$): δ_H 0.09 (m, 6H), 0.92 (m, 6H), 1.31 (m, 2H), 1.32 (m, 2H), 1.34 (s, 3H), 1.58 (m, 2H), 1.64 (m, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 1H), 1.81 (m, 1H), 2.17 (m, 1H), 2.32 (m, 1H), 2.48 (t, J = 7.0 Hz, 2H), 2.95 (dd, J = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.26 (dd, J = 13.5, 10.0 Hz, 1H), 3.85 (m, 1H), 4.15 (s, 2H), 4.18 (m, 1H), 4.66 (m, 1H), 5.16 (ddd, J = 10.2, 10.2, 5.5 Hz, 1H), 5.94 (s, 1H), 7.09 (d, J = 10.5 Hz, 1H), 7.22 (m, 3H), 7.27 (m, 2H), 7.51 (d, J = 10.0 Hz, 1H).

4.1.10. Synthesis of chlamydocin-hydroxymethylketone analog (10). To a cooled stirred solution of the TBDMS protected ketone **9** (1.27 g, 2.02 mmol) in tetrahydrofuran (THF) (50 mL), tetrabutylammonium fluoride (TBAF) (3.10 mL, 3.10 mmol) was added and stirring was continued for 25 min. The reaction was quenched by adding a solution of ammonium chloride (1.27 g in 25 mL water) with continuous stirring followed by addition of 50 mL of water and stirring. The aqueous layer was washed thoroughly with dichloromethane and the combined dichloromethane layer was washed with brine followed by drying over anhydrous $MgSO_4$, filtration, and evaporation. The crude residue was purified by silica gel chromatography using a mixture of chloroform and methanol (98:2) to yield the corresponding pure hydroxymethylketone **10** (1.03 g, 99%) as a white foam after drying in vacuo. HPLC, rt 5.41 min. HR-FAB MS $[M+H]^+$ 515.2835 for $C_{27}H_{39}O_6N_4$ (calcd 515.2870), $[\alpha]_D^{25}$ −65.1 (*c* 0.1, MeOH), ^1H NMR (500 MHz, $CDCl_3$): δ_H 1.31 (m, 2H), 1.32 (m, 3H), 1.34 (s, 3H), 1.64 (m, 1H), 1.65 (br, 1H), 1.74 (m, 1H), 1.77 (s, 3H), 1.79 (m, 1H), 1.81 (m, 1H), 1.82 (m, 1H), 2.17 (m, 1H), 2.32 (m, 1H), 2.40 (t, J = 7.5 Hz, 2H), 2.95 (dd, J = 13.5, 6.0 Hz, 1H), 3.21 (m, 1H), 3.27 (dd, J = 13.5, 10.0 Hz, 1H), 3.86 (m, 1H), 4.18 (m, 1H), 4.23 (s, 2H), 4.67 (m, 1H), 5.16 (ddd, J = 10.2, 10.2, 5.5 Hz, 1H), 6.03 (s, 1H), 7.14 (d, J = 10.0 Hz, 1H), 7.21 (m, 3H), 7.27 (m, 2H), 7.50 (d, J = 10.0 Hz, 1H).

4.2. Preparation of HDACs and assay for enzyme activity

293T cells ($1–2 \times 10^6$) were grown in a 100-mm dish for 24 h and transiently transfected with 10 μg each of the vector pcDNA3-HDAC1 for human HDAC1, pcDNA3-HDAC4 for human HDAC4, or pcDNA3-mHDA2/HDAC6 for mouse HDAC6, using the LipofectAMINE2000 reagent (Invitrogen). After successive cultivation in DMEM for 24 h, the cells were washed with PBS and lysed by sonication in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 5 mM

EDTA, and 0.5% NP40. The soluble fraction collected by microcentrifugation was precleared by incubation with protein A/G plus agarose beads (Santa Cruz Biotechnologies, Inc.). After the cleared supernatant had been incubated for 1 h at 4 °C with 4 μg of an anti-FLAG M2 antibody (Sigma–Aldrich Inc.) for HDAC1, HDAC4, and HDAC6, the agarose beads were washed three times with lysis buffer and once with histone deacetylase buffer consisting of 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 10% glycerol. The bound proteins were released from the immune complex by incubation for 1 h at 4 °C with 40 μg of the FLAG peptide (Sigma-Aldrich Inc.) in histone deacetylase buffer (200 μL). The supernatant was collected by centrifugation. For the enzyme assay, 10 μL of the enzyme fraction was added to 1 μL of fluorescent substrate (2 mM Ac-KGLGK(Ac)-MCA) and 9 μL of histone deacetylase buffer, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 30 μL of trypsin (20 mg/ml) and incubated at 37 °C for 15 min. The released aminomethyl coumarin (AMC) was measured using a fluorescence plate reader. The 50% inhibitory concentrations (IC_{50}) were determined as means with SD calculated from at least three independent dose response curves.

4.3. Stable transfection and p21 promoter assay

The human wild-type p21 promoter luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein. A luciferase reporter plasmid (pGW-FL) was constructed by cloning the 2.4 kb genomic fragment containing the transcription start site into *Hin*dIII and *Sma*I sites of the pGL3-Basic plasmid (Promega Co., Madison, WI). Mv1Lu (mink lung epithelial cell line) cells were transfected with the pGW-FL and a phagemid expressing neomycin/kanamycin resistance gene (pBK-CMV, Stratagene, La Jolla, CA) with the Lipofectamine reagent (Life Technology, Rockville, MD, USA). After the transfected cells had been selected by 400 μg/mL Geneticin (G418, Life Technology), colonies formed were isolated. One of the clones was selected and named MFLL-9. MFLL-9 expressed a low level of luciferase, whose activity was enhanced by TSA in a dose-dependent manner. MFLL-9 cells (1×105) cultured in a 96-well multi-well plate for 6 h were incubated for 18 h in the medium containing various concentrations of drugs. The luciferase activity of each cell lysate was measured with a LucLite luciferase Reporter Gene Assay Kit (Packard Instrument Co., Meriden, CT) and recorded with a Luminescencer-JNR luminometer (ATTO, Tokyo, Japan). Data were normalized to the protein concentration in cell lysates. Concentrations at which a drug induces the luciferase activity 10-fold higher than the basal level are presented as the 1000% effective concentration 1000% (EC_{1000}).

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