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The hybrid pyrroloisoindolone-dehydropyrrolizine alkaloid (-)-chlorizidine A targets proteins within the glycolytic pathway

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Abstract

The cytotoxic activity of (-)-chlorizidine A, a marine alkaloid containing a unique fusion between a pyrroloisoindolone and dehydropyrrolizine, was explored using a combination of cellular and molecular methods. Our studies began by applying preliminary SAR evidence gathered from semisynthetic bioactivity evaluations, to prepare an active immunoaffinity fluorescent (IAF) probe. This probe was then used to identify two cytosolic proteins, GAPDH and hENO1, as the targets of (-)-chlorizidine A.

Keywords

natural products; mode of action; glycolysis; enolase; drug discovery

As part of an effort to identify bioactive natural products from marine microbes,[1-3] we recently described the isolation and structure elucidation of (-)-chlorizidine A (**1**, Scheme 1) a cytotoxic alkaloid from cultures of a *Streptomyces* sp., strain CNH-287, obtained from deep ocean sediments collected off the coast of San Clemente, California.[4] After initial optimization efforts, we were able to identify conditions to reproducibly produce (-)-

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chlorizidine A (**1**) from cultures of the strain on a 20 L scale (~1 mg/L). The isolated natural product was then used to examine the structural requirements for activity.

As described during the isolation efforts,[4] methylation of the phenols of **1** resulted in a *bis*-methyl ether, which lacked cytotoxicity in the *in vitro* HCT-116 colon carcinoma assay (IC₅₀ value > 50 µM). Acetylation, on the other hand, provide the more stable *bis*-acetate **2** (Scheme 1) that offered an increase in activity (IC₅₀ values were determined as 1.8±1.2 µM for **2** and 4.0±0.9 µM for **1**, respectively),[4] suggesting that the low activity of **1** may arise due to instability in cells or a lack of cellular penetrance. This observation was further supported by the fact that we also obtained samples of a hydrolyzed and decarboxylated product (–)-chlorizidine B (**3**, Scheme 1) during the isolation effort.[4] Further studies indicated that the C5 carbonyl was indeed subject to nucleophilic attack as demonstrated by the addition of benzylamine and cysteine-*O*-methyl ester producing **4** and **5**, respectively (Scheme 1).[4] Additional analyses also demonstrated that the congener (–)-chlorizidine B (**3**) was also inactive in HCT-116 bioassays indicating that the C5 amide was key to the activity observed in **1**. [4]

We then applied an immunoaffinity fluorescent (IAF) protocol developed in our laboratories to characterize the activity of **1** in tumor cells.[5–7] Merging our SAR data[4] with the functionally-diverse IAF tags,[8] we targeted the C6 and C8 phenols for installation of the acid-terminal immunoaffinity fluorescent (IAF) tag **6**. Using a carbodiimide coupling (Scheme 2), a *mono*-labeled probe **8** was prepared by esterification of **1** with acid **6**. [9] NMR analysis indicated that while probe **8** was pure (purity was also confirmed *via* LC/MS and HPLC analyses), it was likely a mixture of the C6 and C8 isomers (Supporting Information). We have previously observed a similar lack of selectivity in labeling polyphenolic natural products.[10] Biological analyses indicated that probe **8** retained activity in HCT-116 cells with an IC₅₀ value of 2.2±0.8 µM, while compound **7**, a control for the IAF tag, was inactive (IC₅₀ value >50 µM in HCT-116 cells). Comparable results were also observed in other cell lines (Table S2, Supporting Information).

Our target evaluation studies began by examining the uptake and subcellular localization of **8** in HCT-116 cells. Using time course fluorescence microscopy (Fig. 1A), we found that probe **8** readily entered HCT-116 cells, appeared within the cytosol and remained there for the entire imaging time course. Confocal imaging provided further support, depicting probe **8** within the cytosol (Fig. 1B–1C) after 6 h even after washing with media. The uptake of **8** was reduced by either pretreatment with **1** (Fig. 1F) or by subsequent treatment with **1** (Fig. 1G) confirming that the localization of the blue fluorescence from **8** correlated with the activity of **1**.

Over the following 10 h, probe **8** was transferred from cytosol into subcellular vesicles (Fig. 1D–1E), while the IAF control **7** was completely washed from the cells (inset, Fig. 1E). Using co-staining experiments, we were able to confirm that this subcellular localization was in the lysosomes. As shown in Fig. 1h, the blue fluorescence from **8** co-localized with the red fluorescence from LysoTracker red DND-99.

Next, we examined the affinity features of the IAF probe to screen for protein targets. We applied our immunoprecipitation (IP) strategy using Affigel 10 resin containing the covalently linked monoclonal antibody XRI-TF35, to identify proteins targeted by **8** in HCT-116 cell lysate (lane L1, Fig. 2A). Using conditions where treatment with the IAF control **7** returned no background (less than 1 ng total protein per IP),[7] we consistently obtained four bands (lane L2, Fig. 2A) with the major bands at 50 kDa (strongest) and 26 kDa and weaker bands at 90 and 45 kDa, respectively from lysates immunoprecipitated with **8**. The amount of protein returned was dependent on the concentration of **8** and the ratio of bands remained consistent over multiple repetitions.

Using trypsin-digest LC-MS/MS analyses, we examined bands from multiple repeats of the IP procedure. The major band at 48 kDa was identified as alpha-enolase, hENO1, with 12 matching peptides identified (Fig. 3A). The next band at 27 kDa, with ~ 20% of the intensity of the 48 kDa band, was identified as D-glyceraldehyde-3-phosphate dehydrogenase, GAPDH (Fig. 3B). The two minor bands at 90 kDa and 45 kDa (lane L2, Fig. 2A) were also evaluated and identified as HSP90 (Fig. 3C) and actin (Fig. 3D), respectively.

We then turned to western blot analyses to validate the presence of the identified proteins within the fractions immunoprecipitated with probe **8**. A dose dependent return of hENO1 was obtained as noted in lanes L4-L5 (Fig. 2B) treated with probe **8**, but not in experiments conducted with control **7** (lane L3, Fig. 2B). Comparable evidence was also obtained for the bands corresponding to HSP90 (Fig. 2C), actin (Fig. 2D) and GAPDH (Fig. 2E) when immunoprecipitating with **8**, therein supporting the protein ID analyses. The fact that each of the proteins was apparent in the fraction immunoprecipitated by **8** but not **7** provided strong support for their targeting by the active motif in **1**.

To further explore this activity, we further characterized the activities of **1** and its corresponding *bis*-acetate **2** in HCT-116 cells. As indicated in Fig. 4, treatment of HCT-116 cells with either **1** or **2** resulted in reduced concentrations of cells with a minor adjustment of the percentage of non-viable cells, indicating that treatment led to the inhibition of proliferation rather than cell death. We then evaluated the effects of **1** and **2** on the cell cycle. As shown in Fig. 5, compound **1** did not display statistically relevant effects on the population of cells. Treatment with the more active and stable *bis*-acetate **2** resulted in an increased population of cells in G₁ phase with a corresponding loss of cells in S phase. This observation was consistent with the fact that inhibiting the glycolytic pathway results in halting the cell cycle at G₁ phase in HCT-116 as well as HeLa cells.[11] Furthermore, these data are consistent with recent observations[12] that indicate that the levels of glycolysis are enhanced during G₁ phase.

Interested in further exploring this response, we conducted isothermal calorimetry (ITC) studies using recombinantly expressed hENO1.[13] Using nano-ITC, we obtained a K_d of 1.9 ± 0.9 μM over multiple titrations of hENO1 with **1** (Supporting Figure S2). These studies also indicated that the binding occurred with a stoichiometry of 1.4-1.6 molecules of **1** per hENO1. Comparable analyses on heat-decomposed samples of **1** or *bis*-acetate **2** did not show binding under the same conditions, as it was indistinguishable from background, indicating that while **2** displays activity it arises only after hydrolysis to **1**. Further analyses

however did indicate that probe **8** retained binding to hENO1 with a K_d of $0.69 \pm 0.01 \mu\text{M}$ (Supporting Figure S2), suggesting that while full acetylation removed activity the mono-acetylated probe was active. Combined, these data validated the interaction between **1** and hENO1.

Interestingly, the two most abundant immunoprecipitated proteins hENO1 and GAPDH share a close proximity in the glycolytic pathway (Scheme 3) with GAPDH acting on glyceraldehyde-3-phosphate (**9**) and hENO1 on 3-phospho-D-glycerate (**12**). It is quite remarkable that the top proteins isolated by IP studies with **8** are two proteins steps apart within the same pathway. This evidence clearly suggests that (-)-chlorizidine A (**1**) may play a role in signaling processes regulated by GAPDH or hENO1.

In addition, this discovery suggests that **1** or stabilized derivatives such as **2** may serve as tools to further study the role of hENO1 and GAPDH in tumor cell growth and progression. This suggestion is further supported by the immunoprecipitation of hENO1 and GAPDH by probe **8**, as well as by the fact that probe **8**, hENO1[14] and GAPDH[15] localized within the cytosol. The identification of HSP90 (Fig. 2C) and actin (Fig. 2D), observed in lesser quantities during the target identification process, could suggest further interactivity between hENO1 or GAPDH and cytoskeletal[16] or heat shock[17] proteins. Alternatively, and most likely, the isolation of HSP90 and actin, two abundant proteins in mammalian cells, could have resulted from non-specific binding events based upon the nucleophilic reactivity of the C5 carbonyl in probe **8**. [18]

The glycolytic pathway is well recognized as a primary target for therapeutic intervention, as most invasive cancers display specific modulations of cellular energy metabolism.[19] Many of the enzymes within this pathway have subsequently been identified as therapeutic targets. For instance, GAPDH has an established role in cell death and carcinogenesis.[20] Recently, it has been shown to induce cancer cell senescence.[21] This, combined with the fact that hENO1 has been linked with tumor progression in several cases of breast and lung cancer, [22] as well as serving as an auto antigen associated with Hashimoto's encephalopathy[23] and severe asthma,[24] suggests possible biomedical utility for (-)-chlorizidine A (**1**) or a suitable, more stable analogue. Initial evidence suggests that **1**, **2** and **8** display selectivity to tumor cell lines as indicated by the enhanced activity in PC-3M (prostate carcinoma) and HCT-116 (colon carcinoma) cells as compared to normal MRC-5 cells (Table S1 in the Supporting Information).

Recent evidence clearly suggests a role for anticancer agents that target proteins associated with the glycolytic pathway.[24] The fact that an active IAF probe has been developed herein also suggests that it may be adapted as a probe for confocal microscopy. This is supported by the fact that while probe **8** initially localized within the cytosol, overtime it localized into lysosome or autophagosomal compartments, as illustrated by comparing the localization of **8** after 6 h (Fig. 1B-1C) *versus* that at 16 h (Fig. 1D-1E). Efforts are now underway to develop a detailed understanding of the regulatory effects of **1**, including determining if **1**, and associated analogues such as **2**, act by blocking the activity of hENO1 and GAPDH or by other mechanisms such as regulating protein interactivity associated with these proteins.[26-28] Further efforts will be focused on determining the structural details of

these binding events and elucidating the pathways that connect the targeting of hENO1 and/or GAPDH to the observed cell death phenomenon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Subcellular localization of **8** in HCT-116 cells. **A)** Time course imaging of HCT-116 cells treated with 10 μM **8**. Confocal microscopic images depicting HCT-116 cells treated with **B,C)** 5 μM **8** for 6 h or **D,E)** 5 μM **8** for 16 h and washed with media lacking **8** prior to imaging. Images are provided (**B,D**) with and **C,E**) without overlaid bright field images. Insets in **C)** and **E)** depict the same experiments repeated with IAF control **7** showing complete removal after washing. **F)** A confocal microscopic image depicting HCT-116 cells treated 25 μM **1** for 1 h prior to staining with 5 μM **8** for 6 h. **G)** A confocal microscopic image depicting HCT-116 cells treated with 5 μM **8** for 6 h and then treated with 25 μM **1** for 1 h prior to imaging. **H)** A confocal microscopic image depicting HCT-116 cells treated

with 5 μM **8** for 16 h and then co-stained with 2.5 μM LysoTracker red DND-99 for 15 min before imaging. The appearance of violet in the combined image indicates the co-localization of blue from **8** and red from the LysoTracker red DND-99. Bars denote 10 μm .

Figure 2.

Affinity analyses. **A)** Immunoprecipitation (IP) of HCT-116 cell lysate (1 mg/mL total protein) in PBS pH 7.2 containing 10 μ M **8** returned four distinct protein bands after staining with GelCode Blue (Pierce). Lane L1-3 depicts HCT-116 cell lysate (L1), the IP fraction (L2) from probe **8**, and the IP fraction (L2) from control **7**. **B)** Western blot analysis depicting the level of hENO1 in the IP fraction of HCT-116 cells with IAF control **7** and probe **8**. **C)** Western blot analysis depicting the validation of HSP90 in fraction of HCT-116 cell lysate immunoprecipitated with probe **8**. **D)** Western blot analysis depicting the validation of actin in fraction of HCT-116 cell lysate immunoprecipitated with probe **8**. **E)** Western blot analysis depicting the validation of GAPDH in fraction of HCT-116 cell lysate immunoprecipitated with probe **8**. Ten fold more IP fraction was loaded on the gel for the experiments in **C-E)** as in **B)**, as noted by the increased blot intensity.

A) ENO1 - 12 matching queries

MSILKIHAREIFDS**RGNPTVEVDLFTSKGLFRAAVPSGASTGIYEALRLD**NDKTRYMGK
 GVSKEVEHIN**KTIAPALVSKK**LNVTQEKEIDKLMIEDGTENKS**KFGANAILGVSLAVCK**
AGAVEKGVPLYRHADLAGNSEVILPVPFNVINGGSHAGN**KLAMQEFMILPVGAANFRE**
AMRIGAEVYHNLKNVIKEKYGDATNVGDEGGFAPNILEN**KEGLELLKTAIGKAGYTDKV**
VIGMDVAASEFFRSGKYDLD**FKSPDDPSRYISPDQLADLYK**SFIKDYPVVSIEDPFDDDD
 WGAWQ**KFTASAGIQVVGDDLTVTNPKRI**AKAVNEKSCNCLLL**KVNQIGSVTESLQACKLA**
 QANGWGVMSVSHRSGEETDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLLRIIEELGSK
 AKFAGRNFNRPLAK

B) GAPDH - 5 matching queries

MGKVKVGNGFGGRIGRLVTRAAFNSGKVDIVAINDPFIDLNYMVYMFQYDSTHGKFGHTV
 KAENG**KLVINGNPITIFQERD**PSKIKWGDAGAEYVVESTGVFTTMEKAGAHQGGAK**RVI**
ISAPSADAPMFVMGVNHEKYDNSLKIIISNASCTTNCLAPLAKVIHDFGIVEGLMTTVHA
 ITATQKTVDGSPGKLWRDGR**RGALQNIIPASTGAAKA**VGKVIPELNGKLTGMAFRVPTANV
SVVDLTCLREKPAKYDDIKKVVKQASEGPLKGILGYTEHQVVSDFNSDTHSSTFDAGAG
 IALNDHFV**KLISWYDNEFGYSNRV**VDLMAHMASKE

C) HSP90 - 12 matching queries

MPEEVHHGEEVETFAQAEIAQLMSLIINTFYSNKEIFL**RELISNASDALDKI**RYESLT
 DPSKLDGSGKEL**KIDIIPNPQERTLT**LVDTGIGMT**KADLINNLGTIAK**SGTKAFMEALQAG
 ADISMIGQFVGFGYSAYLVAEKVVITKHNDDEQYAWESSAGGSFTVRADHGEPIGRGTK
 VILHL**KEDQTEYLEERR**VKEVVKKHSQFIGYPITLYLEKEREKEISDDEAEEEKGEKEEE
 DKDDEEKPIEDVGSDEEDDSGDKKKKTKKI**KYIDQEELNKT**KPIWTR**RNPDDITQEE**
YGEFYKSLTNDWEDHLAVKHFSVEGQLEF**RALLFIPRRAPFDLFENKK**KKNNIKLYVRRV
 FIMDSCELIPEYLNFI**RGVVDSEDLPLNISRE**MLQQSKILKVIRKNIVKKCLELFS
 EDKENYKFFYAFSKNLKLGIEDSTNRRRLSELLRYHTSQSGDEMTSLSEYVSRMKETQ
KSIYYITGESKEQVANSFAFVERVRKRGFEVVYMTPEIDEYCVQQLKEFDGKSLSVT**KEG**
LELPEDEEEKKMEESKAKFENLCKLMKEILDKKVEKVTISNRLVSSPCCIVTSTYGWTA
 NMERIMKAQALRDNSTMGYMMAKKHLEINPDHPIVETLRQAEADKNDKAVKDLVLLFE
 TALLSSGFSLEDPQTHSNRIYRMIKLGLGIDEDEVAAEENAAVPDEIPPLEGDESDASRM
 EEVD

D) actin - 7 matching queries

MDDIAALVDNGSGMCKAGFAGDDAPRAVFPISVGRPRHQGMVGMGQKDSYVGEAQS
 KRGILTLYPIEHGIVTNWDDMEKIWHHTFYNEL**RVAPEEHPVLLTEAPLNPKAN**REKMT
 QIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLDL
 AGRDLTDYLMKIL**TERGYSFTTTAERE**IVRDIKEKLCYVALDFEQEMATAASSSSLE**KSY**
ELPDGQVITIGNERRCPEALFQPSFLGMESCGIHETTFNSIMKCDVDIR**KDLYANTVLS**
GGTTMYPGIADRMCKEITALAPSTMKIKIIPPERKYSVWIGGSILASLSTFQQMWSIKQ
EYDESGPSIVHRKCF

Figure 3.

Protein ID analyses. **A)-D)** Protein sequences depicting the peptides (shaded) obtained for each of the protein bands immunoprecipitated in Fig. 2A. Dark shading denotes peptides observed and light shading highlights sites of cleavage.

Figure 4. Cell density and viability analyses. **A)** Cell density analyses with **1** and its *bis*-acetate **2**. **B)** Cell viability analyses with **1** and *bis*-acetate **2**. * p < 0.05, ANOVA followed by Dunnett's test.

Figure 5. Cell cycle analyses. HCT-116 cells were treated with the ascribed compound for 24 h. The cells were then collected and evaluated by flow cytometry. Levels are given as indicated by G₀/G₁ (dark grey), G₂/M (grey) and S (light grey). Histograms depicting the corresponding levels are provided in the Supporting Information.

Scheme 1

Structures of chlorizidine A (**1**), chlorizidine A *bis*-acetate (**2**), chlorizidine B (**3**) and adducts **4-5**. Conversion of **1** to an inactive congener **3** arose by hydrolysis followed by decarboxylation. In addition, nucleophiles readily added to **1** resulting in adducts such as **4** or **5** that also displayed a loss of activity in HCT-116 cells.[4]

Scheme 2

Synthesis of probe 8 by the esterification of 1 with IAF tag 6. Methyl ester 7 was prepared as a negative control for the IAF tag. NMR analyses suggested the presence of both C6 and C8 isomers as duplicate peaks were observed at proximal positions, H9/C9 and H10/C10, while single peaks at remote positions such as H1/C1 and H13/C13 (see Supporting Information).

Scheme 3

Steps 6-9 of the glycolysis of D-glucose, denoting the roles of GAPDH (step 6) and hENO1 (step 9), the most abundant immunoprecipitated proteins found in HCT-116 cell lysates when using probe 8.