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Discovery and Preliminary SAR of Bisbenzylisoquinoline Alkaloids as Inducers of C/EBP α

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Abstract

A high throughput in vitro screen has been developed to identify substances that induce expression of C/EBP α in tumor cells. An extract of the fruit of *Gyrocarpus jacquini* showed induction of C/EBP α activity that was attributed by dereplication analysis to the presence of bisbenzylisoquinoline (BBIQ) alkaloids, including the known compound pheanthine. (13). The research project was broadened to assess the effect of other natural BBIQ structural types occurring outside the genus *Gyrocarpus*. Several of the 28 compounds assayed showed enhancement of C/EBP α induction in U937 cells. The results of this study should encourage future efforts toward obtaining and screening a larger set of both natural and synthetic analogs of this interesting group of alkaloids.

Keywords

bisbenzylisoquinoline alkaloids; C/EBP α ; *Gyrocarpus jacquini*; structure-activity relationships

1. Introduction

The CCAAT/enhancer-binding proteins (C/EBP) belong to a family of transcription factors that play a key role in cell-cycle arrest and differentiation. Loss of C/EBP α function has been detected in acute myelogenous leukemias and other cancers.^{1, 2} As a tool for drug discovery, a U937 human histiocytic lymphoma cell line with a multimerized C/EBP α binding site driving a luciferase reporter gene has been engineered.³ Induction of luciferase is indicative of turning on the C/EBP α transcription factor in the cell. Increases in C/EBP α

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Supplementary data

Experimental details on plant extraction, the HPLC dereplication procedure, and the isolation and identification of BBIQ alkaloids from the crude plant extract can be found @ publishers website?...

levels are associated with granulocytic differentiation which is necessary for normal hematopoietic development.³ For compounds assayed in this screen, all activities are expressed in units normalized to the activity of luciferase induced by all trans retinoic acid (ATRA). Thus, a 2 fold C/EBP α induction value corresponds to twice the activity of ATRA. Myeloid cell lines U937 and HL-60 treated with 1 μ M ATRA have shown 2 fold increases in endogenous C/EBP α levels and granulocytic differentiation after 24 hours.³

In our laboratories, 96,000 crude natural products extracts prepared in 384 well microtiter plate format were screened at a single dose of 20 μ g/mL. Those extracts in which induction of a luciferase reporter gene was detected ($\geq$ 1.5 fold induction (FI) of C/EBP α relative to cells treated with retinoic acid) were retested in ten $\frac{1}{2}$ log doses in U937 cells to determine cytotoxicity and in vitro therapeutic index. Monitoring cytotoxicity is important since this allows recognition of extracts or pure compounds that induce C/EBP α as a stress response as well as those that inhibit the luciferase signal due to toxicity. A MeOH-CH₂Cl₂ extract of the fruits of *Gyrocarpus jacquini* (Gaertn.) produced an FI of 1.6 relative to ATRA, and the subsequent retest performed in multiple doses confirmed the activity.

A dereplication method for bioactive natural products extracts, amenable to use with high throughput screens, has been developed by our laboratory. We have previously demonstrated the efficacy of the system for the discovery of antimicrobial agents,^{4–6} and have now expanded our application to molecular target-based screening for antitumor agents.^{7, 8} The key to the success of our multi-detector HPLC dereplication technique is that we work on a preparative, rather than analytical scale, enabling us to collect higher mass fractions while maintaining good chromatographic resolution. The first round of biological assay consumes less than 5% of each HPLC fraction, allowing us to perform secondary chromatographic separations and biological assays in cases where active fractions are mixtures. Vital to the process is the quick assessment of the therapeutic index of each HPLC fraction. To this end, a counter screen that reflects toxicity is run concurrently with the targeted screen. Each dereplication fraction is assayed in 4 $\times$ $\frac{1}{2}$ log doses in both the molecular target screen and the toxicity counterscreen. From this initial round of chromatography and bioassays, we frequently can identify the active compound from the UV spectrum, the molecular weight (MS), and the taxonomy of the source organism.

2. Results and discussion

The dereplication fingerprint (Fig. 1) of *G. jacquini* revealed a zone of C/EBP α induction in HPLC fractions 15–27, with maximum FI's of 2.8 and 3.3 (relative to ATRA) seen in fractions 17 and 25, respectively. The likely presence of elevated levels of mildly toxic components in fractions 20–24, as reflected by the inhibited growth of the U937 control cell line, may explain the drop seen in FI in this region of the chromatogram. Fraction 22 showed the greatest toxicity to U937 cells (73% growth relative to the untreated control), which resulted in a negative value of C/EBP α FI of -2.6. HPLC analysis of fractions 15–27 showed a compound having UV maxima at 240 and 281 nm and a molecular ion signal at m/z 623.5 in ESI⁺ mode, which led to the presumptive identification of the known bisbenzylisoquinoline (BBIQ) alkaloid pheanthine (13), previously reported from *G. americanus*.⁹ Selective ion peaks of 621.5 and 639.5 daltons (Fig. 1) fell within the zone of induction, and suggested the presence of other BBIQ alkaloids in the extract. The data indicate that the BBIQ alkaloids present in the *G. americanus* extract induce expression of C/EBP α protein, but were toxic at slightly higher concentrations. For this reason, an SAR of an existing BBIQ alkaloid library was conducted to seek compounds possessing a greater differential between activity and toxicity. Included in the BBIQ alkaloid class of compounds is the natural product CBT-1[®], which has been shown to inhibit the action of multidrug resistance-associated protein 1 and P-glycoprotein.¹⁰

In order to efficiently uncover structural features of the BBIQ class affecting C/EBP α expression, 33 BBIQ analogs were obtained from the NCI Repository and analyzed by HPLC-MS. Only those compounds showing the correct molecular ion by ESI-MS and purity of greater than 85% by HPLC-ELSD were considered for SAR studies. Of the 33 samples, 28 met the purity/identity criteria, and so were tested in multiple 2 fold doses in triplicate side by side assays (Fig. 2).

To better visualize structural differences within this large, complex group of compounds (at least 430 known naturally occurring structures), each BBIQ is classified by type using the scheme of Shamma and Moniot (Table 1).¹¹

The monomeric isoquinoline alkaloid, laudanosine (1), and the singly-bridged BBIQ alkaloids (2–5), elicited no expression of C/EBP α greater than that induced by ATRA, and were excluded from further consideration.

Compounds that were considered to be inducers of C/EBP α showed dose-dependent response curves at non-toxic concentrations. Thus, some inducers of C/EBP α showed negative FI at cytotoxic concentrations and positive FI at lower concentrations. Ten of the fourteen doubly-bridged BBIQ alkaloids possessed dose-dependent C/EBP α activity at below-toxic concentrations of drug. Those having the R/S stereochemistry at C-1/C-1' (6–9, 11–12, 14) induced C/EBP α expression, with the exception of 8–9. Comparison of results for the type VI BBIQ alkaloids (6–10) suggested that a hydroxyl group at C-7' enhances activity, while methylation of C-12 appears to be of lesser importance. Compound 10, which differed from other two-bridged compounds by possessing an sp²-hybridized nitrogen at the 2' position, was inactive. In the type VIII group (11–16), the highest C/EBP α activity (FI = 2.6 @ 12.5 μ M) was seen for 11, which has R/S stereochemistry at C-1/C-1' and a hydroxyl substituent at C-7. However, 11 also had the greatest toxicity of all the doubly-bridged structures (Growth = 35% @ 50 μ M). Comparison of 15 and 16, both having the S/S stereochemistry, also suggested the preference for hydroxyl over methoxyl at C-7 for higher C/EBP α expression. Interestingly, the lead compound pheanthine (13), having the R/R' stereochemistry and full O-methylation, was one of the weaker inducers among the doubly-bridged BBIQ alkaloids. This finding suggests that the *G. jacquini* extract contains C/EBP α inducers of significantly greater potency. Other doubly-bridged BBIQ alkaloids not of the type VI or VIII class also exhibited activity (17–19). Compounds 18 and 19, each possessing one hydroxyl moiety, had better activity than the fully O-methylated 17. Funiferine (19), which differs from the type VIII group only in the substitution of a diphenyl bridge linking C-11 to C-11' for the diaryl ether linkage, possessed C/EBP α inducing activity greater than fangchinoline (15), while also exhibiting no detectable toxicity as reflected by the growth of the treated U937 control cells. Norpanurensine (18), the only doubly-bridged compound tested containing a non-methylated sp³-hybridized nitrogen, showed good activity and low toxicity. A consistent pattern is that for the doubly-bridged BBIQ alkaloids tested, greater C/EBP α activity was seen for compounds possessing one non-methylated oxygen in the isoquinoline portion of the molecule. This trend holds for compounds 6, 7, 11, 15, and 18. Among all of the doubly-bridged compounds tested, structures 6, 7, 15, 18, and 19 produced the optimal combination of good activity and low cytotoxicity.

Of the four BBIQ alkaloids having three connecting bridges, three produced maximum FI of 1.5 or greater. Telobine (20) yielded a C/EBP α FI of 1.9 at 3.1 μ M, making it the most potent compound that was tested. However, the toxicity of telobine was greater than any other compound of the set. Structurally, telobine has the R/R stereochemistry, is fully O-methylated, and is one of only two compounds assayed possessing an NH moiety (the other was funiferine (19)). Tiliacorinine (22), the other triply-bridged compound showing activity, has structural features in common with the active compound funiferine (19), but, whereas 19

showed no toxicity to the control cell line, 22 greatly inhibited cell growth of the control cell line. These results indicate that the presence of a third bridge does not lend a therapeutic advantage to BBIQ alkaloids of the types that were assayed and generally increases cytotoxicity.

Of the five head-tail linked BBIQ alkaloids assayed, only two of these, 24 and 27, elicited a C/EBP α response greater than 1 fold. The presence of free hydroxyl groups attached to C-7 and C-13 of 24 may contribute to the observed activity, as lack of hydroxyl groups in 27 corresponds to a decrease in potency. It appears that the effect of quaternary nitrogens in 25 and 26 and the presence of an sp² nitrogen in 28 is to reduce activity.

3. Conclusion

The results of this study show that the C/EBP α activity of BBIQ alkaloids is genuine; however, in most cases, there is only a small differential between toxic concentrations and that which produces the maximum induction of C/EBP α . Of the compounds tested, 6, 7, 15, 18, 19, and 24 can be considered for further biological evaluation including testing in cell-based assays to confirm that the C/EBP α effect is specific and that it leads to induction of differentiation. Other doubly-bridged BBIQ types with different linkages and oxygenation patterns, such as types XI, XII, XIII, XIV, XVI, would be of interest to obtain in order to complete a broader SAR study of this compound class.

4. Experimental

4.1. Plant Material

The fruit of *G. jacquini* (Gaertn) (Hernandiaceae) was collected in Saraburi, Thailand, March 31, 1987 by D. D. Soejarto of the University at Illinois at Chicago. Herbarium specimens are maintained at the U.S. National Herbarium of the Smithsonian Institution in Washington, DC, as well as in Thailand, and a representative sample is maintained in the DTP Repository in Frederick, MD as Q66O0954.

4.2. C/EBP α bioassay

A U937 human histiocytic lymphoma cell line was engineered with a multimerized C/EBP α binding site driving a luciferase reporter gene.³ Treatment of these cells with ATRA results in the concomitant induction of C/EBP α and luciferase. Assays were performed in 384 well plate format in which U937 4X C/EBP α cells were incubated for 24 hr in the presence or absence of experimental sample in a total volume of 40 μ L; after addition of Britelite reagent (PerkinElmer), luciferase induction was measured in a PerkinElmer Wallac Victor 2 microplate luminometer. Induction of luciferase is indicative of turning on the C/EBP α transcription factor in the cell. ATRA was included on each plate as a positive control. C/EBP α induction values for test compounds are normalized to induction by ATRA. Compound toxicity was assayed utilizing the Alamar Blue fluorescence assay.¹²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. HPLC-bioactivity fingerprint for *G. jacquinii*

The crude organic extract (50 mg) of the fruits of *Gyrocarpus jacquinii* was dissolved in MeCN-H₂O (1:1), filtered and loaded onto a polyhydroxyethylaspartamide hydrophilic interaction HPLC column (PHEA HILIC, PolyLC Inc., Columbia, MD, USA) eluted with an increasing amount of aqueous 20 mM ammonium acetate pH4 in MeCN. Fractions (84 × 8 mL) were collected, and each was assayed at 4 × ½ log concentrations (200, 63, 20, 6.3 μL of column eluent). ELSD, UV, and electrospray mass spectrometric data in negative and positive mode are presented with the corresponding biological data for the 84 HPLC fractions. Zones of C/EBPα induction were observed in HPLC fractions 17–19 and 25–27, activity attributed to BBIQ alkaloids by UV and MS analysis of fractions. An apparent loss

of C/EBP α induction occurs in wells 20–24, an effect which may result from cellular stress encountered at elevated doses of test sample.

Fig. 2. C/EBP α activity and cytotoxicity data for 28 BBIQ alkaloids

Pheanthine (13), first detected as the active component in the *G. jacquini* extract, produced a maximum C/EBP α FI of 0.80 at 12 μ M, although negative induction and control cell toxicity was observed at higher concentrations. This pattern is consistent with the activity/toxicity profile seen in the C/EBP α active region of the fingerprint (Fig. 1). Further biological examination of 28 standard BBIQ alkaloids, assayed in triplicate, revealed several superior agents, including structures 6, 7, 15, 18, 19, and 24. These compounds were considered candidates for further development based on the criteria of C/EBP α FI > 1 over several test concentrations while having minimal toxicity to U937 cells.

1 : 1'S **2** : 1S, 1'S; R=CH₃**3** : 1S, 1'S; R=(CH₃)₂ **4** : 1S, 1'S **5** : 1R, 3R, 1R' **6** : 1R, 1'S; R₁=H; R₂=H**7** : 1R, 1'S; R₁=Me; R₂=H**8** : 1R, 1'S; R₁=Me; R₂'=-CH₂-**9** : 1R, 1'S; R₁=H; R₂'=Me **10** : 1R **11** : 1R, 1'S; R₁=H; R₂=H**12** : 1R, 1'S; R₁=Me; R₂=Me**13** : 1R, 1'R; R₁=Me; R₂=Me**14** : 1R, 1'S; R₁=Me; R₂=H**15** : 1S, 1'S; R₁=H; R₂=Me**16** : 1S, 1'S; R₁=Me; R₂=Me **17** : 1S, 1'S

18 : 1*R*, 1'*R* **19** : 1*S*, 1'*R* **20** : 1*R*, 1'*R*; R₁=H, R₂=Me**21** : 1*S*, 1'*S*; R₁=Me, R₂=H **22** : 1*S*, 1'*S* **23** : 1*S*, 1'*S* **24** : 1*R*, 1'*R*; R₁=Me; R₂=H; R₃=H; R'₁=Me**25** : 1*R*, 1'*S*; R₁=(Me)₂; R₂=H; R₃=H; R'₁=Me**26** : 1*R*, 1'*S*; R₁=(Me)₂; R₂=Me; R₃=Me; R'₁=(Me)₂ **27** : 1*R*, 1'*R* **28** : 1*R*

Table 1

C/EBP α induction and cytotoxicity of BBIQ alkaloids in U937 cells

Compound#	NSC #	Compound Name	BBIQ Type	Min. Conc. (μ M), FI>1 ^a	Max FI, (Dose, μ M) ^b	U937 cell growth IC ₅₀ (μ M) ^c	% Cell Growth @ Max FI Dose ^d
1	35045	Laudanosine	III	>50	0.0 (25)	na	na
2	269186	Thalystiline	III	>50	0.9 (0.78)	>50	133
3	269187	Thalystiline dimethiodide	III	>50	0.4 (3.1)	>50	116
4	274893	Thalicarpine transformation product		>50	0.9 (25)	>50	61
5	676002	Herveline O		>50	0.46 (25)	>50	108
6	626657	Aromoline	VI	3.1	1.5 (25)	>50	98
7	251213	Thalugosamine	VI	3.1	1.9 (50)	>50	80
8	623442	Cepharanthine	VI	12	1.0 (12)	>50	87
9	645315	Oxyacanthine	VI	>50	0.8 (3.1)	>50	81
10	121392	Epistephanine	VI	>50	0.9 (6.2)	>50	99
11	251534	Stepholine	VIII	3	2.6 (12)	25	89
12	97338	Isotetrandrine	VIII	12	1.2 (25)	>50	69
13	105130	Pheanthine	VIII	>50	0.75 (12)	50	104
14	369310	Berbamine	VIII	25	1.2 (50)	>50	70
15	77036	Fangchinoline	VIII	25	2.0 (50)	>50	102
16	77037	Tetrandrine	VIII	>50	0.9 (25)	>50	69
17	626655	Tenuipine	X	25	1.4 (25)	>50	105
18	626652	Norpanurensine	XV	12	2.5 (50)	>50	113
19	189487	Funiferine	IV	12	2.2 (50)	>50	94
20	626656	Telobine	XXIII	1.5	1.9 (3.1)	12	82
21	181486	Trigilletine	XXIII	>50	0.8 (3.1)	50	108
22	379957	Tiliacrine	XVIII	12	1.9 (12)	25	63
23	107088	Repanduline	XXV	50	1.5 (50)	>50	67
24	P7118	Chondodendrine	XXI	12	1.9 (25)	>50	na

Compound#	NSC #	Compound Name	BBIQ Type	Min. Conc. (μM), FI>1 ^a	Max FI, (Dose, μM) ^b	U937 cell growth IC ₅₀ (μM) ^c	% Cell Growth @ Max FI Dose ^d
25	226458	Tubocurarine, HCl	XXI	>50	<0 (50)	>50	100
26	36388	Tubocurarine, O,O'-dimethyl-, diiodide	XXI	>50	0.2 (6.2)	>50	na
27	615580	Cycleamine	XX	25	1.2 (25)	>50	111
28	79640	Cissampareine	XXII	>50	0.5 (3.1)	>50	78

^aMinimum concentration of test compound invoking induction of C/EBPα equal to or greater than the response produced by the positive control all trans retinoic acid.

^bMaximum fold induction produced and the corresponding concentration of drug.

^cConcentration of test compound that inhibits 50% growth of U937 cells relative to untreated U937 cells.

^dCell growth at the concentration producing the maximum C/EBPα fold induction.