Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 569–574

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

1-METHOXY-AGROCLAVINE FROM *PENICILLIUM* SP. WC75209, A NOVEL INHIBITOR OF THE LCK TYROSINE KINASE

Ramesh Padmanabha,* Yue-Zhong Shu,*[a] Lynda S. Cook, Judy A. Veitch, Marcella Donovan, Susan Lowe, Stella Huang,[b] Dolores Pirnik, and Susan P. Manly

Biomolecular Screening,[a]Chemistry,[b]Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT U.S.A.

Received 26 November 1997; accepted 27 January 1998

Abstract: A high-throughput screen was developed and implemented to identify inhibitors of the Lck tyrosine kinase. This report describes the identification of a specific inhibitor of this enzyme from the solid fermentation culture of the *Penicillium* sp., WC75209. The active compound was isolated and structurally characterized as 1-methoxy-5*R*,10*S*-agroclavine, a new member of the ergot alkaloid family.

Introduction

Uncontrolled signalling from tyrosine kinases is responsible for many disease states. These include proliferative diseases such as cancer as well as atherosclerosis and psoriasis. The enhanced activity can arise by several different mechanisms such as persistent activation by ligands, overexpression of the kinase or mutations. In normal cells, tyrosine kinases play very important roles in signal transduction. In the immune system, for example, cytoplasmic tyrosine kinases are essential for the signal transduction events that control T- and B-cell proliferation. For these reasons, inhibitors of these kinases would have wide ranging utility in the treatment and control of diseases.[1-3]

One of the earliest detectable events in the activation of T-and B-cell receptors is an increase in the nonreceptor tyrosine kinase activity. The activation of these kinases is critical to the signal transduction events that result in T-cell activation.[4-6] $p56^{lck}$, $p59^{fyn}$, ZAP-70, and $p72^{syk}$ are the tyrosine kinases that have been implicated as being critical to T-cell activation.[7-9] As part of our effort to identify inhibitors of T-cell activation, a high-throughput screen of natural product extracts was run, and several extracts exhibiting $p56^{lck}$ inhibitory activity were identified. This report describes the identification and isolation of a novel inhibitor of $p56^{lck}$.

Materials and Methods

Protein Kinase Assays: Standard filter binding assays were used to monitor the state of autophosphorylation of the $p56^{lck}$ using γ-^{33}P-ATP as the phosphate donor. All reactions were carried out at room temperature in microtiter plates. The reactions were stopped by the addition of 20% TCA and the proteins were allowed to precipitate. The precipitated proteins were trapped on the glass fiber filters of a Packard Unifilter GF/B plate using a Packard Harvester. The plates were washed extensively with water and allowed to air dry before 30 μL of scintillation fluid was added per well. The plates were sealed and the trapped radioactivity was monitored in a

0960-894X/98/$19.00
PII: S0960-894X(98)00072-9

Packard Topcount scintillation counter. Assays measuring the effect of compounds on Fyn, EGF Receptor, Her2, Ca^{2+}/Calmodulin-dependant Type II, PKA, and PKC kinases were performed using a commercial service under standard assay conditions as detailed by the suppliers of the enzymes.

Media and Culture Conditions: *Penicillium* sp., WC75209, was grown on a PDA slant for 7 days at 28 °C, and 3 mL of 20% (w/v) glycerol was added and used to prepare a spore suspension, which was divided into aliquots, frozen in a dry ice-acetone bath, and stored at -80 °C. From the frozen stock, 0.1 mL was used to inoculate a PDA slant which was incubated at 28 °C for 7 days. The culture was transferred onto 20 Nunc plates each containing 250 mL of medium, by adding 6 ml of saline to each slant, agitating the surface with a cotton swab, and pouring 3 mL of the resulting solution onto the surface of one plate. The medium contained the following per liter of distilled water: glucose, 10 g; lactose, 20 g; Pharmamedia, 20 g; L-asparagine, 1 g; NaCl, 2.5 g; $CaCO_3$, 3 g, and the pH was adjusted to 7.0 before 10 g agar was added. The medium was sterilized at 121 °C for 20 min. The culture was incubated for 6 days at 28 °C.

Chemical and Spectral Analyses: HPLC/UV analyses were carried out on a Hewlett-Packard HP-1090 Liquid Chromatograph by using Microsorb Short One C18 column in a modified solvent system described by Hook and coworkers.[10] NMR data, including COSY, NOE, DEPT, HETCOR, and HMBC (increment delay, 0.06 seconds) were taken at a Brucker AM-500 spectrometer (^1H, 500 MHz; ^{13}C, 125 MHz). Electrospray mass spectra (MS) were obtained on a Finnigan TSQ7000 triple quadruple mass spectrometer using argon as collision gas and in positive ion mode. High-resolution mass spectra (HRMS) were obtained at a Kratos MS50 mass spectrometer with FAB ionization mode.

Extraction and isolation: Two hundred milliliters of methanol was added to each of 20 Nunc plates containing the culture grown on solid media. The plates were soaked at the room temperature for 2 hours with gentle reciprocating shaking. The liquid layer was combined and concentrated (to ca. 1 L) under a nitrogen stream to remove most of the methanol. Water was then added to the final volume of approximately 1 L. The aqueous layer was partitioned with nBuOH to give dark brown extracts (1.2 g). The extracts were repeatedly chromatographed on Sephadex LH-20 with 70% aqueous CH_3CN to give an active mixture (25 mg). The mixture was subjected to HPLC, with the fractions collected in microtiter plates. The Lck assay was used to locate the wells containing enzyme inhibitory activity in the microtiter plates and corresponding peaks in the HPLC. The active wells corresponded mainly to a HPLC peak with the retention time of 9.1-9.5 min. The compound was separable from other impurities on TLC ($CHCl_3$/MeOH, 6:1), and pure compound **1** (4 mg) was obtained by preparative silica gel TLC.

Compound 1: HRMS: found 269.1650, calculated for MH^+, $C_{17}H_{21}N_2O$ 269.1636; Electrospray MS: 269 (MH^+, 100%), 239 (5%); $[\alpha]_D$ -63° (*c*=0.08, MeOH); UV λ max (MeOH): 228, 282 nm; ^1H and ^{13}C NMR: Table 1.

Results and Discussion

A high-throughput screen for the identification of inhibitors of $p56^{lck}$ was developed and implemented (unpublished results). One fungal culture, WC75209 (*Penicillium* sp), was identified as having inhibitory activity. This activity was confirmed in both primary and secondary fermentations. In addition, equilibrium dialysis of the extract revealed that the activity responsible for the inhibition of the enzyme was of low molecular weight.[11] Testing against other tyrosine kinases suggested that the extract produced compounds that were specific to inhibition of $p56^{lck}$. These criteria were part of the selection of the extract for further study.

Early steps of the bioassay-guided fractionation of the extract were conducted by solvent extraction and chromatography on a Sephadex LH-20 column. In order to rapidly and precisely locate the enzyme inhibitory compound(s) present in the advanced fraction, the enriched active fraction was subjected to HPLC with a gradient solvent system consisting of acetonitrile and phosphate buffer (pH 3.5), and the fractions were collected in microtiter plates. These fractions were tested for their Lck inhibitory activity. The active wells were located in the retention time window of 9.1-9.5 min. and they corresponded mainly to a HPLC peak having an indole-like chromophore with maximal UV absorptions at 228 and 282 nm as observed by photodiode array detection. The compound (**1**) was isolated by preparative silica gel TLC.

Compound **1** has the molecular weight of 268 and molecular formula of $C_{17}H_{20}N_2O$ from MS and HRMS measurements. This formula, along with the ^1H and ^{13}C NMR data (Table 1) and the early indication of the presence of indole chromophore, suggested that **1** may belong to the class of ergot alkaloids. The HMBC data of **1** further demonstrated the connnectivity of most of the functional groups, and led to the assignment of the 8,9 unstaturated ergoline skeleton for **1** (Fig. 1).

Thus, compound **1** is a methoxy derivative of agroclavine; the localization of the methoxy group was further determined at the indole nitrogen (N-1) since a strong NOE was observed between the methoxy protons and H-2 and H-14 (Fig. 2). Naturally occurring agroclavine predominantly has the *trans* (5*R*,10*R*) C/D ring juncture (compound **2**). Agroclavine having the *cis*-C/D ring juncture was reported only rarely as a *Penicillium* metabolite.[12] The small coupling constant (J = 1.5 Hz) and the significant NOE between H-5 and H-10 clearly indicated that **1** has the *cis* C/D ring jucture. Compound **1** therefore was finally characterized as 1-methoxy-5*R*,10*S*-agroclavine, a new member of the ergot alkaloid family.

Table 1. ^{1}H and ^{13}C NMR Data of Compound **1** in $CDCl_3$

	δ_C (mult)	δ_H (mult, J = Hz)
1-OCH_3	66.3 (q)	4.05 (s)
2	119.8 (d)	7.15 (s)
3	105.0 (s)	
4	31.2 (t)	3.08 (m), 3.15 (dd, 15.3, 4.4)
5	60.6 (d)	3.76 (m)
6-NCH_3	41.4 (q)	2.84 (s)
7	55.1 (t)	3.51 (br s)
8	129.2 (s)	
9	124.5 (d)	5.79 (br s)
10	38.6 (d)	4.08 (d, 1.5)
11	124.1 (s)	
12	117.7 (d)	6.93 (d, 6.6)
13	125.1 (d)	7.23 (overlapped)
14	107.6 (d)	7.23 (overlapped)
15	131.8 (s)	
16	123.0 (s)	
17	20.0 (q)	1.23 (s)

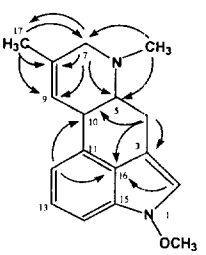


Figure 1: Key long range correlations observed in HMBC (H---C) of Compound **1**

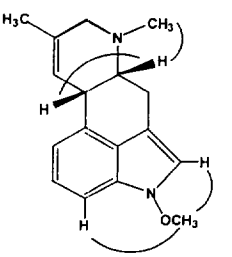
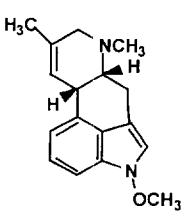


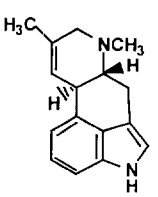
Figure2: Key NOE of Compound **1**

Two closely related compounds to **1**, namely, 5*R*, 10*R*-agroclavine (**2**), which has the *trans* C/D ring juncture, and lysergol (**3**) were also tested for their abilty to inhibit the autophosphorylation of $p56^{lck}$.

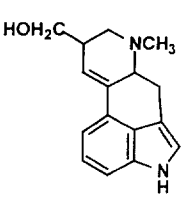
Figure 3



1



5*R*,10*R* agroclavine (**2**)



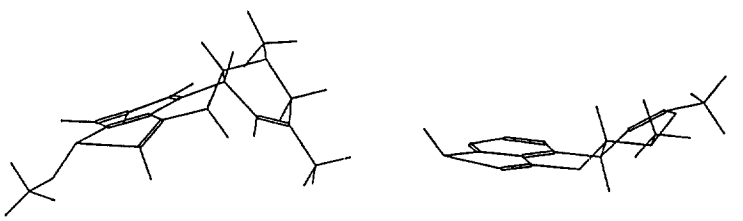
lysergol (**3**)

As shown in Table 2, both **2** and **3** did not inhibit the activity of $p56^{lck}$, nor did they inhibit the autophosphorylation of $p72^{syk}$ or $p74^{Blk}$. The compounds were tested at concentrations up to 1 mg/mL and did not show greater than 20% inhibition of kinase activity. The reason for the lack of inhibitory activity of **2**, despite its close structural similarity to the active **1** is not clear at present. The drastic 3-D strucutural difference (Fig. 4) because of the opposite C/D ring junctures of **1** and **2** probably contributes to the observed enzyme specificities of these compounds.

Table 2

	1	2	3
IC_{50} in Lck	8.5 μM	inactive	inactive
IC_{50} in Blk	285.0 μM	inactive	-
IC_{50} in Syk	inactive	inactive	-

Fig. 4. Chem 3-D Structures of Compounds **1** and **2**



1 **2**

Table 3

Protein Kinase	Substrate	% Inhibition
Lck	Poly(glu:tyr)	42
Fyn	Poly(glu:tyr)	19
EGF Receptor	Poly(glu:tyr)	14
Her2	Poly(glu:tyr)	9
Ca2+/Calmodulin-dependant Type II	BB40	inactive
cAMP-dependant	Histone H1 (HH1)	inactive
Protein Kinase C; non-selective	Histone H1 (HH1)	inactive
Protein Kinase C-α	Histone H1 (HH1)	inactive
Protein Kinase C-β (I &II)	Histone H1 (HH1)	inactive
Protein Kinase C-γ	Histone H1 (HH1)	inactive

The ability of 1 to inhibit other protein kinases was also examined. The kinases tested included other members of the Src family, as well as more distantly related enzymes, and the results are summarized in Table 3.

As is clear from the table, the only other enzymes that showed any inhibition at the test concentration of 10 μM, were Fyn and the EGF receptor kinase. Fyn is a closely related tyrosine kinase that functions in the same T-cell activation signal transduction pathway that involves Lck.[13] There is essentially no effect of the compound on the other protein kinases tested. In addition, the cytotoxicity of **1** was determined using Alomar Blue staining of M109 mouse lung carcinoma cells. The compound was not cytotoxic to this cell line when tested at concentration of greater than 40 μM.

In summary, we have described the identification and isolation of a novel protein kinase inhibitor from a fungal extract. This compound specifically inhibits the Lck tyrosine kinase with very little effect on even closely related members of the Src family of kinases. The compound was characterized as 1-methoxy-5*R*,10*S* agroclavine, a new member of the ergot alkaloid family. The unique stereochemistry of the compound appears to be important for the observed activity of the compound.

References:

1. Chang, C. J.; Geahlen, R. L. *J Nat Prod* **1992**, *55*, 1529-60.
2. Coombes, R. C. *Mol Aspects Med* **1993**, *14*, 373-449.
3. Levitzki, A. *Eur J Biochem* **1994**, *226*, 1-13.
4. Chan, A. C.; Desai, D. M.; Weiss, A. *Annu Rev Immunol* **1994**, *12*, 555-92.
5. Sefton, B. M.; Taddie, J. A. *Curr Opin Immunol* **1994**, *6*, 372-9.
6. Weiss, A. *Cell* **1993**, *73*, 209-12.
7. Sefton, B. M. *Oncogene* **1991**, *6*, 683-6.
8. Bolen, J. B.; Thompson, P. A.; Eiseman, E.; Horak, I. D. *Adv Cancer Res* **1991**, *57*, 103-49.
9. Yamamoto, T.; Yamanashi, Y.; Takeuchi, M.; Fusaki, N.; Uchiumi, F.; Katagiri, T.; Semba, K.; Toyoshima, K. *Princess Takamatsu Symp* **1991**, *22*, 293-305.
10. Hook, D. J.; More, C. F.; Yacubucci, J. J.; Dubay, G.; O'Conner, S. *J. Chromatogr.* **1987**, *385*, 99-108.
11. Padmanabha, R.; Cook, L. S.; Manly, S. P. *J. Biomol. Screening* **1996**, *1*, 131-133.
12. Sakharovsky, V. G.; Kozlovsky, A. G. *Tetrahedron Lett.* **1984**, *25*, 109-112.
13. Samelson, L. E.; Phillips, A. F.; Luong, E. T.; Klausner, R. D. *Proc Natl Acad Sci U S A* **1990**, *87*, 4358-62.