The Psammaplysenes, Specific Inhibitors of FOXO1a Nuclear Export

Frank C. Schroeder,[†] Tweeny R. Kau,[‡] Pamela A. Silver,[‡] and Jon Clardy*[,†]

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Cancer Biology, Dana Farber Cancer Institute, Boston, Massachusetts 02115

Received November 20, 2004

A small collection of marine natural product extracts was screened for compounds that would compensate lost tumor suppressor functionality in PTEN-deficient cells. From the most active extract, the previously unreported bromotyrosine derivative, psammaplysene A (**1**), was identified. Psammaplysene A compensates for PTEN loss by relocalizing the transcription factor FOXO1a to the nucleus.

Cancer cells have gain-of-function or loss-of-function mutations, or both, that lead to unchecked cell proliferation. Small molecules can modulate gain-of-function mutations by inhibiting the mutated gene product,[1] but small molecule modulation of loss-of-function mutations has been quite difficult. Finding targets downstream of the loss-of-function mutation, which are amenable to small molecule modulation, is likely to be a more productive, if still unproven, approach. This paper describes the discovery of two previously unreported natural products from the marine sponge *Psammaplysilla* sp. These compounds were identified from a high-content screen for small molecules that restore the function of FOXO1a, a downstream target of the PTEN tumor suppressor.[2]

The path linking PTEN with FOXO1a involves several steps, not all of which are well understood (Figure 1). Loss of PTEN phosphatase activity has been noted in Cowden's disease, a hereditary disease with a marked predisposition for breast and thyroid cancers, and PTEN phosphatase deficiencies have been observed in many other cancers.[3] FOXO1a, a member of the Forkhead family of transcription factors, which negatively regulates cell cycle progression and cell survival, is an attractive downstream target for small molecule modulation of loss of PTEN function. As a result of loss of PTEN phosphatase activity, phosphorylated FOXO1a remains inappropriately localized in the cytoplasm and unable to restrain cell cycle progression.[4] Small molecules that would enforce the nuclear re-localization of FOXO1a would be, at a minimum, useful tools to investigate FOXO1a regulation and cell growth.

A cell-based screen to identify such small molecules used the subcellular localization of FOXO1a as a readout, and libraries of ~18 000 synthetic molecules (NCI Structural Diversity Set, Chembridge DiverSetE) and 352 uncharacterized extracts from the NCI collection were assayed in this primary screen.[2] Among the strongest screening positives was a dichloromethane/methanol extract from a marine sponge, *Psammaplysilla* sp., which was collected in the Indian Ocean.

For isolation of the active component, 200 mg of this extract was subjected to activity-guided fractionation. A simplified Kupchan solvent-partitioning scheme yielded hexane, dichloromethane, and methanol/water fractions, of which the dichloromethane fraction was most active. Further fractionation via silica gel chromatography followed by reversed-phase HPLC yielded two pure active compounds, which were named psammaplysene A (**1**, 7.2 mg) and psammaplysene B (**2**, 0.7 mg). Positive-ion electrospray ionization MS indicated formula weights of 765 and 751, respectively, whereby the isotopic pattern suggested the presence of four bromine atoms in both compounds. High-resolution electrospray MS gave $C_{27}H_{35}Br_4N_3O_3$ as the molecular formula for psammaplysene A. Further structural characterization using a standard set of 2D NMR experiments including dqf-COSY, NOESY, HMQC, and HMBC was straightforward. Psammaplysene A (**1**) and psammaplysene B (**2**) were characterized as previously unreported dimeric bromotyrosine alkaloids (Chart 1), each consisting of two modified dibromotyrosine units combined with fragments most likely derived from aliphatic amino acids.

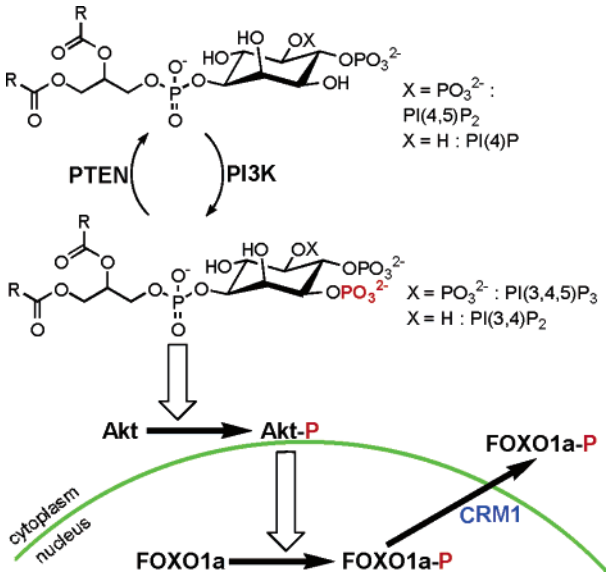

Figure 1. PTEN/PI3K/Akt/FOXO1a signaling pathway.[2] Akt phosphorylates FOXO1a, inducing FOXO1a nuclear export via CRM1. PTEN counteracts the kinase PI3K by dephosphorylating the lipid phosphates $PI(3,4)P_2$ and $PI(3,4,5)P_3$, thereby down-regulating phosphorylation of Akt and thus FOXO1a. In PTEN-null cells, FOXO1a remains constitutively phosphorylated and localized in the cytoplasm, which leads to cellular proliferation.

Psammaplysene A was among the most active inhibitors in this assay ($IC_{50} = 5\ \mu M$), whereas psammaplysene B was somewhat less active ($IC_{50} = 20\ \mu M$) (Figure 2). Among all of the compounds screened (>18 000), only five have IC_{50} values $\leq 5\ \mu M$.[2] Interestingly, the known dimeric bromotyrosine purpuramine-1 (**3**),[5] which was among the minor

* To whom corrrespondence should be addressed. E-mail: jon_clardy@hms.harvard.edu.
[†] Harvard Medical School.
[‡] Dana Farber Cancer Institute.

10.1021/np049624z CCC: $30.25
Published on Web 03/11/2005

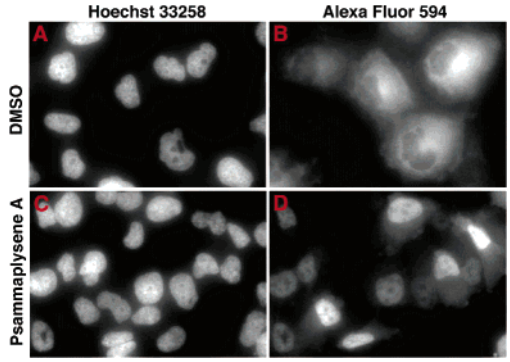

Figure 2. FOXO1a nuclear export inhibition by psammaplysene A (**1**). (A) PTEN-deficient cells[2] treated with DMSO (control) and stained with Hoechst 33258 to visualize nuclei and (B) stained with Alexa Fluor 594 to visualize localization of FOXO1a. Here, FOXO1a is predominantly in the cytoplasm. (C) cells treated with 5 μM psammaplysene A in DMSO stained with Hoechst 33258 and (D) stained with Alexa Fluor 594, showing re-localization of FOXO1a to the nuclei.

Chart 1. Structures of Psammaplysenes A (**1**) and B (**2**), Purpuramine-1 (**3**), Bastadin-5 (**4**), and Psammaplin A (**5**)

components in the extract, was not active in our assay. Purpuramine-1 and several homologous compounds, originally isolated from *Psammaplysilla purpurea*, were shown to have antibacterial properties.[6]

Sponges of the order Verongida to which the genus *Psammaplysilla* belongs are known to produce a wide range of structurally diverse bromotyrosine derivatives,[7] most prominent among them the sulfide-bridged psammaplins, such as psammaplin A (**5**), a histone deacetylase inhibitor,[8] and the macrocyclic bastadins, for example bastadin-5 (**4**),[9] a potent agonist of the RyR1 calcium channel.[10] The psammaplysenes (**1**, **2**) differ from related structures such as purpuramine-1 (**3**) and bastadin-5 (**4**) by having an α,β-unsaturated amide linkage and are distinguished by the way the two bromotyrosine subunits are connected.

There are multiple mechanisms to keep FOXO1a in the nucleus, and a series of secondary assays was used to assign the screening positives (42 including psammaplysene A) to distinct mechanistic classes. CRM1 is a general nuclear export receptor whose inhibition would localize FOXO1a and many other proteins in the nucleus.[11] A screen to assess whether compounds targeted CRM1 identified roughly half of the screening positives, 19 compounds, as CRM1 inhibitors and thus not specific inhibitors of the PI3K/Akt/FOXO1a signaling pathway. Psammaplysene A was not active in this secondary assay at 5 μM and thus considered pathway specific.[2]

Compounds specific for the PI3K/Akt/FOXO1a pathway could have targets upstream or downstream of Akt (Figure 1). By measuring levels of phosphorylated Akt by immunoblotting, the 23 pathway-specific compounds were assigned to these two classes. A total of 21 compounds led to decreased Akt phosphorylation, and two, including psammaplysene A, led to no change in Akt phosphorylation compared to untreated controls.[2] Psammaplysene A must have a target, which is as yet unidentified, downstream of Akt.

Among the five most potent positives in our primary screen, psammaplysene A (**1**) is the only compound that neither inhibited CRM1 nor reduced Akt phosphorylation. The target of psammaplysene A is not known, but small alterations of the basic structure, the removal of a methyl group to give psammaplysene B (**2**) for example, significantly diminish activity. The discovery of psammaplysene A's activity in this set of assays demonstrates that crude natural product extracts can be used to find potent and specific inhibitors in high-content, cell-based assays such as the one described. The highly modular psammaplysenes contain several easily accessible subunits and should therefore be amenable to synthesis-based exploration of structure−activity relations.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 25 °C using Varian INOVA500 (500 MHz proton, 126 MHz carbon) and Varian INOVA600 (600 MHz proton, 151 MHz carbon) spectrometers with CD_3OD or CD_2Cl_2 as the solvent. Double quantum filtered COSY (DQF-COSY) spectra were acquired using the standard pulse sequences and phase cycling. Phase-sensitive NOESY spectra were acquired with a mixing time of 600 ms. HMQC spectra were acquired in the phase-sensitive mode without gradients using phase-cycling for coherence selection. In some cases additional gradient HMQC and HSQC spectra were acquired. Magnitude-mode HMBC spectra were acquired without gradients and using phase-cycling for coherence selection. HMQC and HMBC spectra for psammaplysene B were acquired using Shigemi NMR tubes. Mass spectra were acquired using a Micromass Quattro II (positive-ion electrospray ionization), while high-resolution MS were obtained on a Micromass Autospec (positive-ion electrospray ionization). HPLC employed an Agilent 1100 series HPLC system with diode-array detector (190−900 nm) using a Supleco Discovery HS C-18 column (25 cm × 10 mm, 5 μm particle diameter).

Isolation of Psammaplysene A (1) and Psammaplysene B (2) by Activity-Guided Fractionation. Marine extract NCI-C013823-F3 (200 mg) was dissolved in 10 mL of a 9:1 mixture of methanol and water. The solution was extracted with two 10 mL portions of hexanes. Subsequently, the water content of the methanol phase was adjusted to 33%, followed by extraction with two 5 mL portions of dichloromethane. The resulting hexanes, dichloromethane, and methanol/water fractions were concentrated in vacuo and tested in

Table 1. ^{1}H and ^{13}C NMR Data of Psammaplysene A (**1**) (solvent CD_3OD, spectra referenced to 3.31 ppm for CD_2HOD, and 49.05 ppm for CD_3OD, coupling constants in Hz)

position	δ C	δ H	relevant HMBC correlations
1	56.6	3.48 (t, $J = 7$)	C-26/27
2	26.2	2.32 (quintet, $J = 7$)	
3	71.2	4.16 (t, $J = 7$)	C-4
4	154.24		
5	119.26		
6	132.8	7.82	C-4, C-10
7	135.8		
8	132.8	7.82	C-4, C-10
9	119.3		
10	137.6	7.39 ($J = 15.7$ Hz)	C-6/8, C-7, C-12
11	124.1	6.64 ($J = 15.7$ Hz)	C-12
12	167.5		
N−H		6.6 (br. t)	
13	36.6	3.60 (t, $J = 7$)	C-12
14	30.5	2.13 (quintet, $J = 7$)	
15	71.7	4.07 (t, $J = 7$)	C-16
16	153.2		
17	119.1		
18	134.1	7.59	C-16, C-19
19	136.8		
20	134.1	7.59	C-16, C-19
21	119.1		
22	30.3	3.00 (t, $J = 7$)	C-18/20, C-19
23	59.0	3.27 (t, $J = 7$)	C-19, C-24/25
24, 25	43.3	2.87 (s)	C-23, C-24/25
26, 27	43.4	2.94 (s)	C-1, C-26/27

our assay, which showed the dichloromethane fraction to be most active. For further purification, the dichloromethane fraction was chromatographed over silica using dichloromethane/methanol mixtures containing 0.4% concentrated aqueous ammonia as the solvent. Starting with 10% methanol, the methanol content of the solvent was gradually increase to 40%. Fractions eluting between 20 and 30% methanol were active and thus combined, concentrated, and subsequently rechromatographed using the same solvent system. This separation yielded two distinct fractions containing bromotyrosine-derived alkaloids. The earlier-eluting fraction contained almost pure purpuramine-1 (**3**), while the second fraction contained a mixture of psammplysenes A and B. The mixture of the two psammaplysenes was separated by reversed-phase HPLC using methanol/water mixtures with methanol contents of 35−100% as solvent, which yielded 7.2 mg of psammaplysene A (**1**, purity by NMR > 98%) and 700 μg of psammaplysene B (**2**, purity by NMR > 90%).

Psammaplysene A (1): ^{1}H and ^{13}C NMR data, see Table 1; positive-ion ESIMS *m/z* 775 (4), 774 (15), 773 (15), 772 (60), 771 (25), 770 (87), 769 (16), 768 (56), 767 (5), 766 (14) (ion cluster corresponding to M + H^+), 387.5 (20), 386.5 (68), 385.5 (100), 384.5 (70), 383.5 (18) (ion cluster corresponding to M + $2H^+$); positive-ion HRESIMS *m/z* 767.9443 (calcd for $C_{27}H_{36}{}^{79}Br_3{}^{80}BrN_3O_3$ 767.9470).

Psammaplysene B (2): ^{1}H and ^{13}C NMR data, see Table 2; positive-ion ESIMS *m/z* 761 (3), 760 (13), 759 (14), 758 (56), 757 (20), 756 (80), 765 (14), 754 (53), 753 (5), 752 (12) (ion cluster corresponding to M + H^+), 380.5 (19), 379.5 (69), 378.5 (100), 377.5 (69), 376.5 (17) (ion cluster corresponding to M + $2H^+$); positive-ion HRESIMS *m/z* 753.9247 (calcd for $C_{26}H_{34}{}^{79}Br_3{}^{80}BrN_3O_3$ 753.9314).

Table 2. ^{1}H and ^{13}C NMR Data of Psammaplysene B (**2**) (solvent CD_3OD, spectra referenced to 3.31 ppm for CD_2HOD, and 49.05 ppm for CD_3OD, coupling constants in Hz)

position	δ C	δ H	relevant HMBC correlations
1	49.0	2.83 (t, $J = 7$)	C-26
2	30.2	2.02 (quintet, $J = 7$)	
3	72.5	4.07 (t, $J = 7$)	C-4
4	154.1		
5	118.8		
6	131.9	7.66	C-4, C-10
7	133.9		
8	131.8	7.66	C-4, C-10
9	118.8		
10	137.0	7.40 ($J = 16$ Hz)	C-6/8, C-12
11	123.2	6.36 ($J = 16$ Hz)	C-12
12	164.8		
N-H		6.31 (br t)	
13	38.0	3.67 (t, $J = 7$)	C-12
14	29.8	2.10 (quintet, $J = 7$)	
15	72.3	4.10 (t, $J = 7$)	C-16
16	151.1		
17	117.9		
18	133.1	7.39	C-16, C-19
19	140.31		
20	133.1	7.39	C-16, C-19
21	117.9		
22	33.0	2.68 (t, $J = 7$)	C-18/20
23	60.8	2.47 (t, $J = 7$)	C-19, C-24/25
24, 25	45.2	2.21 (s)	C-23, C-24/25
26	36.2	2.43 (s)	C-1

Acknowledgment. This work was supported by grants from NIH (CA24487 to J.C. and GM36763 to P.S.). P.S. also had support from the Barr Investigator Program, and T.R.K. was supported by NCI (5T32CA09361). We thank Gordon Cragg at NCI for helpful comments and for facilitating access to the NCI natural products collection.

Supporting Information Available: ^{1}H NMR, DQF-COSY, coupled gHSQC, and HMBC spectra of psammaplysene A, as well as ^{1}H NMR, DQF-COSY, NOESY, HMQC, and HMBC spectra of psammaplysene B.

References and Notes

(1) (a) Druker, B. J. *Trends Mol. Med.* **2002**, *8*, S14−S18. (b) Shawer, L. K.; Slamon, D.; Ullrich, A. *Cancer Cell* **2002**, *1*, 117−123.
(2) Kau, T. R.; Schroeder, F.; Ramaswamy, S.; Wojciechowski, C. L.; Zhao, J. J.; Roberts, T. M.; Clardy, J.; Sellers, W. R.; Silver, P. R. *Cancer Cell* **2003**, *4*, 463−476.
(3) (a) Vazques, F.; Sellers, W. R. *Biochim. Biophys. Acta* **2000**, *1470*, M21−M35. (b) Kondo, D.; Yao, M.; Kobayashi, K.; Ota, S.; Yoshida, M.; Kaneko, S.; Baba, M.; Sakai, N.; Kishida, T.; Kawakami, S. *Int. J. Cancer* **2001**, *91*, 219−224.
(4) Burgering, B. M.; Medema, R. H. *J. Leukoc. Biol.* **2003**, *73*, 689−701.
(5) Jaspars, M.; Crews, P. *Tetrahedron Lett.* **1994**, *35*, 7501−7504.
(6) Yagi, H.; Matsunaga, S.; Fusetani, N. *Tetrahedron* **1993**, *49*, 3749−3754.
(7) Saeki, B. M.; Granato, A. C.; Berlinck, R. G. S.; Magalaes, A.; Schefer, A. B.; Ferreira, A. G.; Pinheiro, U. S.; Hadju, E. *J. Nat. Prod.* **2002**, *65*, 796−799, and references therein.
(8) Piña, I. C.; Gautschi, J. T.; Wang, G.-Y.-S.; Sanders, M. L.; Schmitz, F. J.; France, D.; Cornell-Kennon, S.; Sambucetti, L. C.; Remiszewski, S. W.; Perez, L. B.; Bair, K. W.; Crews, P. *J. Org. Chem.* **2003**, *68*, 3866−3873.
(9) (a) Kazlauskas, R.; Lidgard, R. O.; Murphy, P. T.; Wells, R. J.; Blount, J. F. *Aust. J. Chem.* **1981**, *34*, 765−786. (b) Coll, J. C.; Kearns, P. S.; Rideout, J. A.; Sankar, V. *J. Nat. Prod.* **2002**, *65*, 753−756.
(10) Mack, M.; Molinski, T. F.; Buck, E. D.; Pessah, I. N. *J. Biol. Chem.* **1994**, *269*, 23236−23349.
(11) Stade, K.; Ford, C. S.; Guthrie, C.; Weis, K. *Cell* **1997**, *90*, 1041−1050.

NP049624Z