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Phorboxazole Analogues Induce Association of cdk4 with Extranuclear Cytokeratin Intermediate Filaments

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Phorboxazoles A and B (**1** and **2**, Figure 1) were isolated from sponges *Phorbas* sp.[1] and *Raspailia* sp.[2] collected off Western Australia in 1993 and 1996, respectively. These scarce natural products arrest cancer cell growth in S phase at low to subnanomolar concentrations,[1,3] making them promising candidates for therapeutic development.[1a,4] Although substantial efforts have yielded several total syntheses,[5] the cellular biology of **1** and **2** has remained undefined. Summarized here is a fluorescent label-based study[6] reliant upon total synthesis[5a,7] that elucidates the cellular uptake, localization, and biomolecular association of the phorboxazoles. This work provides the first detailed insight into a potential mode of the phorboxazoles' unique cytostatic activity.

An abiotic *N*,*N*-dialkyl-7-aminocoumarin derivative was selected as the label because this class of blue fluorescent dye does not interfere with protein association or inhibit cellular uptake.[6] It was previously determined that modification of the C33 hemiketal (**3**, Figure 1) or the C45,46-vinyl bromide (**4**) of phorboxazole A did not substantially diminish cytostatic activity against a panel of human cancer cells.[3,8] Therefore, fluorescent analogues **5** and **6** (Figure 1) were prepared via Sonogashira couplings between the corresponding vinyl iodides and C46 terminal alkynes (see Supporting Information).[9]

The cellular uptake of **5** and **6** was examined in HeLa cells by LED fluorescence microscopy.[10] The resulting images were compared with those obtained using control fluorophores **7**−**9**[11] and established probes **10**−**12** (Figure 2).[6] The uptake of **5** and **6** (Figure 2) was comparable to that of protein phosphatase ligand **10**, membrane intercalator **11**, and DNA binder **12**. Probes **5** and **6** localized on intermediate-sized filaments (IF) and led to a dramatic restructuring of the IF to form a large aggregate (IFC) adjacent to the nucleus (Figure 2).

Uptake and localization studies were accompanied by microscopic stop-flow cell cycle staging using a fluorescent staging cocktail to determine the phase of HeLa cells.[12] Probes **5** and **6** induced complete HeLa cell cycle arrest at S phase at 10.2 nM **5** and 8.2 nM **6**. Next, HeLa cells were separately treated with **5** and **6** for 4 h, then fractionated into nuclear, cytosolic, endoplasmic reticulum, Golgi, and membrane partitions.[10] Fractions that displayed visual fluorescence ([label] > ∼1 μM) were obtained from the cytosolic partitions derived from each probe. Native PAGE analyses of the pooled fluorescent fractions yielded fluorescent bands at ∼32 and ∼54 kDa (Figure 3). However, only the band near 54 kDa persisted after an affinity purification step using an amine-reactive agarose gel, Affigel 10, displaying an anti-*N*,*N*-dimethyl-7-aminocoumarin-4-acetamide antibody.

Samples of the 54 kDa band in the blot obtained using **6** (Figure 3a) were submitted to sequence analysis.[13] Among three replicates,

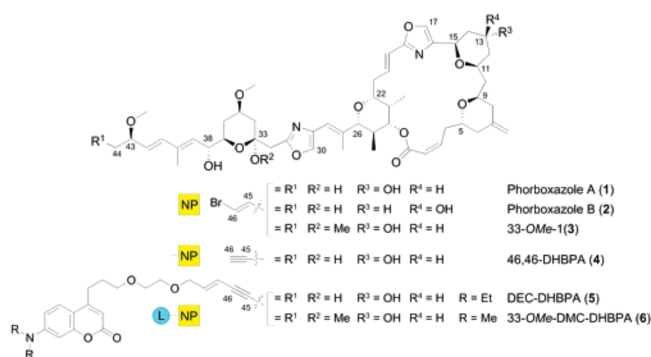

Figure 1. Structures of phorboxazoles and synthetic variants.

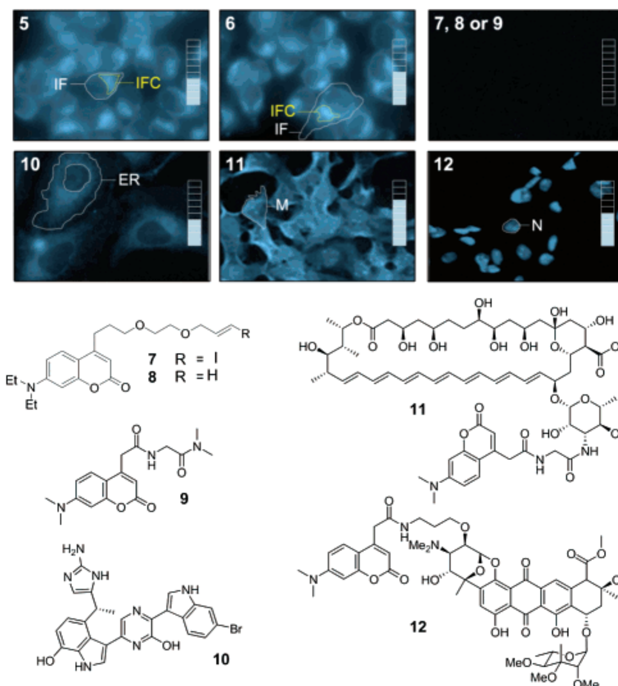

Figure 2. Comparative analysis of cellular uptake, localization, and response of phorboxazole probes **5** and **6**, controls **7**, **8**, or **9**, and organelle labels **10**−**12**. Images depict the localization of each probe 10 min after exposure of a 0.34 cm^2 well containing ∼10^6 HeLa cells/cm with 350 μL of a 10 nM solution of probe, followed by washing the cells twice with media. Bars denote the relative intensity of fluorescence obtained per cell. Localization on the IF was confirmed by immunofluorescent staining with a green fluorescent anti-cytokeratin mAb (see Supporting Information).

the strongest correlations were to human cytokeratins KRT1, KRT9, and KRT10.[14] This proteomic identification of cytokeratin targeting was consistent with visualized localization of **5** and **6** on IF (Figure 2). KRT10 and the cancer biomarker KRT18[15] were then cloned from a HeLa cDNA library, expressed, and gel purified.[14] The

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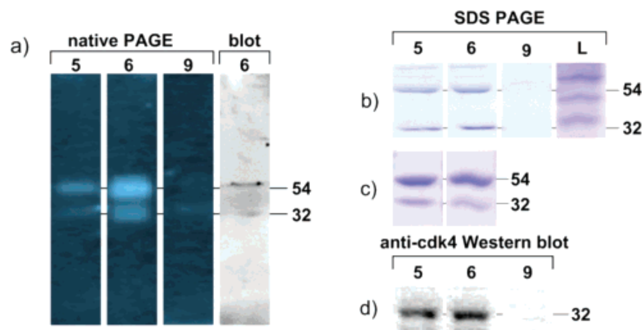

Figure 3. Affinity analyses of **5**, **6**, and **9** in HeLa cell cytosolic fractions. (a) Fluorescent gel analysis depicting fluorescence from **5** and **6** bound to protein targets, including a native blot analysis using anti-DYE antibody denoting bound complex. (b) Coomassie-stained SDS−PAGE gel depicting the pull down of a protein with mass of 32 kDa with an anti-KRT10 affinity resin in the presence of **5** and **6**. (c) Repetition of the pull-down assay in (b) using purified cdk4. (d) Blot analysis depicting cdk4 isolated using a KRT10 affinity resin.

affinities of **5** and **6** were determined by equilibrium dialysis, yielding association constants (K_a) of 1.2 and 3.2 nM to KRT10, and 0.2 and 0.3 nM to KRT18, respectively. Probe **6** was also assayed against cloned and expressed KRT1 (K_a = 32 nM) and KRT9 (K_a = 2.3 nM). Control experiments using **7**, **8**, and **9** (Figure 2) showed insignificant affinity (K_a > 100 μM) to either KRT10 or KRT18.

We then screened for proteins whose association with KRT10 was affected by **5** and **6**. First, resins coated with an anti-KRT10 antibody were used to isolate protein complexes from HeLa cell lysates exposed to **5** and **6**. SDS−PAGE analysis of the proteins returned from these experiments revealed a second major protein (ca. 32 kDa) that was associated with KRT10 in the presence of probes **5** and **6** (Figure 3b). LC−MS/MS analysis indicated that this protein was cyclin-dependent kinase 4 (cdk4), an essential component of G1-S phase cell cycle progression[16] and a validated anticancer drug target.[17] This assignment was confirmed by repetition of the binding experiments in vitro using purified cdk4 (Figure 3c). Next, HeLa cell lysates were screened using a KRT10 affinity gel. Cdk4 was repetitively returned after extraction of cell lysates exposed to **5** and **6**, but not **9** (Figure 3d). These experiments provide conclusive evidence that the phorboxazole probes **5** and **6** induce an association between cdk4 and KRT10. Associations were confirmed by determination of in vitro binding affinities of **5**, **6**, and **9** to cdk4 by equilibrium dialysis. Analogues **5** (K_a = 350 nM) and **6** (K_a = 120 nM) bound tightly to cdk4, while control **9** (K_a > 5 μM) did not.

Perturbation of cdk4 structure and function is well-known to inhibit cell cycle progression.[18] The sequestration of cdk4 upon cytosolic IF proteins[19] induced by **1**−**6** would reduce the amount of free cellular cdk4 and prevent nuclear translocation of active cdk4-cyclin D1 complexes required for phosphorylation of the retinoblastoma (Rb) protein and consequent cell cycle progression.[16] In a similar model, the human papillomavirus type 16 protein E1−E4 is believed to cause G_2 cell cycle arrest by retention of cdk1/cyclin B1 upon cytoplasmic keratin filaments.[20] In contrast, sequestration of PKB/PKCς upon KRT10 apparently occurs without induction by xenobiotic small molecules.[21] This results in inhibition of cyclin D1 expression and Rb phosphorylation via the phosphoinositide 3-kinase signal transduction pathway.[21] Several endogenous proteins are known to inhibit cdk4 activity via direct binding.[22]

The small-molecule-mediated anchoring of cdk4 upon IF described here represents an unexploited paradigm for altering the activity of cellular proteins. Studies to elucidate the details of the molecular associations of phorboxazole derivatives and their cellular receptors and the effects of these interactions upon cell cycle progression are continuing.

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Supporting Information Available: Synthetic procedures and characterization data, and protocols for cellular studies and protein analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Searle, P. A.; Molinski, T. F. *J. Am. Chem. Soc*. **1995**, *117*, 8126. (b) Searle, P. A.; Molinski, T. F.; Brzezinski, L. J.; Leahy, J. W. *J. Am. Chem. Soc*. **1996**, *118*, 9422. (c) Molinski, T. F. *Tetrahedron Lett.* **1996**, *37*, 7879.
(2) Capon, R. J.; Skene, C.; Liu, E. H.; Lacey, E.; Gill, J. H.; Heiland, K.; Friedel, T. *Nat. Prod. Res*. **2004**, *18*, 305.
(3) Uckun, F. M.; Forsyth, C. J. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 1181.
(4) Uckun, F. M.; Narla, R. M.; Forsyth, C. J.; Lee, C. S.; Ahmed, F.; Cink, R. D. U.S. Patent 6797721, 2004; WO 01/36394, 2001; Priority: US 99-165452 19991115.
(5) (a) Forsyth, C. J.; Ahmed, F.; Cink, R. D.; Lee, C. S. *J. Am. Chem. Soc.* **1998**, *120*, 5597. (b) Evans, D. A.; Fitch, D. M.; Smith, T. E.; Cee, V. J. *J. Am. Chem.* Soc. **2000**, *122*, 10033. (c) Smith, A. B.; Minbiole, K. P.; Verhoest, P. R.; Schelhaas, M. *J. Am. Chem. Soc.* **2001**, *123*, 10942. (d) Pattenden, G.; González, M. A.; Little, P. B.; Millan, D. S.; Plowright, A. T.; Tornos, J. A.; Ye, T. *Org. Biomol. Chem.* **2003**, *1*, 4173. (e) Williams, D. R.; Kiryanov, A. A.; Emde, U.; Clark, M. P.; Berliner, M. A.; Reeves, J. T. *Angew. Chem., Int. Ed.* **2003**, *42*, 1258. (f) Li, D.-R.; et al. *Chem.*−*Eur. J.* **2006**, *12*, 1185.
(6) (a) Sandler, J. S.; Fenical, W.; Gulledge, B. M.; Chamberlin, A. R.; La Clair, J. J. *J. Am. Chem. Soc*. **2005**, *127*, 9320. (b) La Clair, J. J.; et al. *ChemBioChem* **2006**, in press.
(7) Synthesis and characterization of **3** and **4** are documented in: Lee, C. S. Ph.D. Thesis, University of Minnesota, Minneapolis, MN, 1999.
(8) Smith, A. B., III; Razler, T. M.; Pettit, G. R.; Chapuis, J.-C. *Org. Lett.* **2005**, *7*, 4403.
(9) Chen, J.; Ying, L.; Hansen, T. M.; Engler, M. M.; Lee, C. S.; La Clair, J. J.; Forsyth, C. J. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 901.
(10) (a) Huber, L. A.; Pfaller, K.; Vietor, I. *Circ. Res*. **2003**, *92*, 962. (b) Chen, X.; Murphy, R. F. *J. Biomed. Biotechnol*. **2005**, *4*, 87.
(11) Images were collected at 100 ms/image at 30 min intervals over 24 h for each compound. (a) Herman, P.; Maliwal, B. P.; Lin, H. J.; Lakowicz, J. R. *J. Microsc.* **2001**, *203*, 176. (b) Repetto, L.; Piano, E.; Pontiggia, C. *Opt. Lett.* **2004**, *29*, 1132.
(12) Böcker, W.; Gantenberg, H.-W.; Müller, W.-U.; Streffer, C. *Phys. Med. Biol.* **1996**, *41*, 523.
(13) Sequencing analyses via trypsin digestion, nanoLC-MS/MS, and Swiss protein database search were performed at the Mayo Proteomics Research Center, Mayo Clinic College of Medicine, Rochester, MN.
(14) Franke, W. W.; Schmid, E.; Weber, K.; Osborn M. *Exp. Cell. Res.* **1979**, *118*, 95.
(15) (a) Linder, S.; Havelka, A. M.; Ueno, T.; Shoshan, M. C. *Cancer Lett.* **2004**, *214*, 1. (b) Scheuemann, P.; Hosch, S. B.; Izbicki, J. R. *Dis. Esophagus* **2001**, *14*, 85. (c) Chen, P. H.; Ornelles, D. A.; Shenk, T. *J. Virol*. **1993**, *67*, 3508.
(16) (a) Kato, J.; Matsushime, H.; Hiebert, S. W.; Ewen, M. E.; Sherr, C. J. *Genes Dev.* **1993**, *7*, 331. (b) Sherr, C. J. *Science* **1996**, *274*, 1672.
(17) Grillo, M.; Bott, M. J.; Khandke, N.; McGinnis, J. P.; Miranda, M.; Meyyappan, M.; Rosfjord, E. C.; Rabindran, S. K. *Breast Cancer Res. Treat.* **2005**, *Dec 1*, 1.
(18) (a) Paramio, J. M.; Casanova, M. L.; Segrelles, C.; Mittnacht, S.; Lane, E. B.; Jorcano, J. L. *Mol. Cell Biol*. **1999**, *19*, 3086. (b) Mettus, R. V.; Rane, S. G. *Oncogene* **2003**, *22*, 8413. (c) Lee, H. Z.; Leung, H. W.; Lai, M. Y.; Wu, C. H. *Anticancer Res*. **2005**, *25*, 959. (d) Tao, G. Z.; Rott, L. S.; Lowe, A. W.; Omary, M. B. *J. Biol. Chem*. **2002**, *277*, 19295.
(19) Coulombe, P. A.; Wong, P. *Nat. Cell Biol.* **2004**, *6*, 699.
(20) Davy, C. E.; et al. *J. Virol.* **2005**, *79*, 3998.
(21) Paramio, J. M.; Segrelles, C.; Ruiz, S.; Jorcano, J. L. *Mol. Cell Biol*. **2001**, *21*, 7449.
(22) Li, J.; Joo, H. S.; Tsai, M.-D. *Mol. Biochem.* **2003**, *42*, 13476.

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