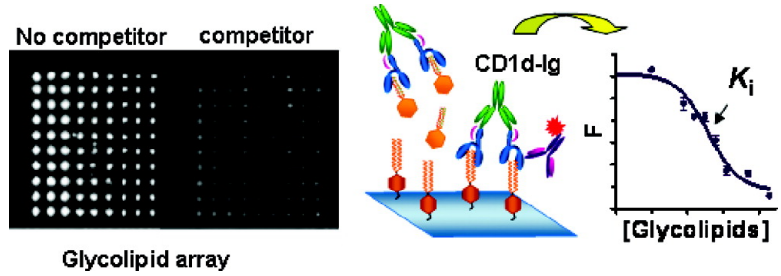
Article

Quantitative Microarray Analysis of Intact Glycolipid#CD1d Interaction and Correlation with Cell-Based Cytokine Production

Pi-Hui Liang, Masakazu Imamura, Xiangming Li, Douglass Wu, Masakazu Fujio, Richard T. Guy, Bing-Ching Wu, Moriya Tsuji, and Chi-Huey Wong

J. Am. Chem. Soc., **2008**, 130 (37), 12348-12354 • DOI: 10.1021/ja8012787 • Publication Date (Web): 20 August 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES

Published on Web 08/20/2008

Quantitative Microarray Analysis of Intact Glycolipid−CD1d Interaction and Correlation with Cell-Based Cytokine Production

Pi-Hui Liang,[†,‡] Masakazu Imamura,[§] Xiangming Li,[||] Douglass Wu,[§] Masakazu Fujio,[§] Richard T. Guy,[§] Bing-Ching Wu,[†] Moriya Tsuji,[*,||] and Chi-Huey Wong[*,§,†]

The Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang Dist., Taipei, Taiwan 11529, Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, School of Pharmacy, National Taiwan University, Taipei, Taiwan 100, and Aaron Diamond AIDS Research Center, 455 First Avenue, The Rockefeller University, New York, New York 10016

Received February 20, 2008; E-mail: wong@scripps.edu; chwong@gate.sinica.edu.tw; mtsuji@adarc.org

Abstract: The protein CD1d binds self and foreign glycolipids for presentation to CD1-restricted T cells by means of TCR recognition and activates T_H1 and T_H2 chemokine release. In this study, a variety of glycolipid ligands were attached to a microarray surface and their binding with dimeric CD1d was investigated. An α-galactosyl ceramide (α-GalCer) bearing a carbamate group at the 6′-OH position was tethered to the surface, and the dissociation constant on surface with CD1d was determined to reflect the multivalent interaction. Competition assays were then used to determine the dissociation constants (K_i) of new and intact glycolipids in solution. The 4-fluorophenyloctanoyl-modified α-GalCer (**18**) was found to bind most strongly with CD1d (K_i 0.21 μM), 2 orders of magnitude stronger than α-GalCer and more than three times more selective than α-GalCer for IFN-γ release from NKT cells. Various α-GalCer analogues were analyzed, and the results showed that the binding affinity of glycolipids to CD1d correlates well with IFN-γ production but poorly with IL-4 secretion by NKT cells, suggesting that tighter binding ligands could bias cytokine release through the T_H1 pathway.

Introduction

CD1molecules are heterodimers, composed of a heavy polypeptide chain noncovalently associated with a 2-microglobulin and have substantial structural similarity to major histocompatibility complex (MHC) class I proteins.[1] There are four members of the CD1 family, denoted group I (CD1a, b, c, and e) and group II (CD1d) on the basis of sequence identity and chromosomal location.[2,3] Each CD1 mediates T-cell responses through the presentation of self and foreign lipids, glycolipids, lipopeptides, or amphipathic small molecules to T-cell receptors (TCR).[4−6]

α-GalCer (Figure 1), a glycolipid found in the marine sponge *Agelas mauritianus*, is the most extensively studied ligand for CD1d.[7] When bound to CD1d, α-GalCer stimulates rapid T_H1 (such as IFN-γ) and T_H2 (IL-4) cytokine production by Vα14i natural killer T (Vα14i NKT) cells in mice and the human

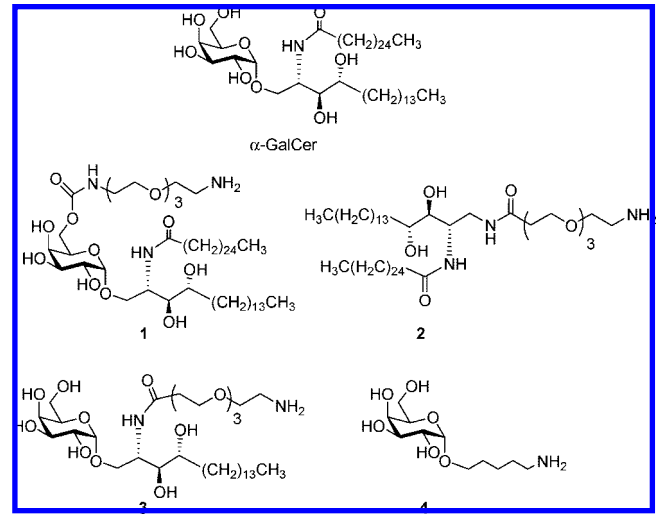


Figure 1. α-GC and its derivatives **1**−**4**.

[†] Academia Sinica.
[‡] National Taiwan University.
[§] The Scripps Research Institute.
[||] Aaron Diamond AIDS Research Center.

(1) Zeng, Z.; Castano, A. R.; Segelke, B. W.; Stura, E. A.; Peterson, P. A.; Wilson, I. A. *Science* **1997**, *277*, 339–345.
(2) Calabi, F.; Jarvis, J. M.; Martin, L.; Milstein, C. *Eur. J. Immunol.* **1989**, *19*, 285–292.
(3) Brigl, M.; Brenner, M. B. *Annu. Rev. Immunol.* **2004**, *22*, 817–890.
(4) Porcelli, S. A.; Modlin, R. L. *Annu. Rev. Immunol.* **1999**, *17*, 297–329.
(5) Jayawardena-Wolf, J.; Bendelac, A. *Curr. Opin. Immunol.* **2001**, *13*, 109–113.
(6) Porcelli, S. A. *Adv. Immunol.* **1995**, *59*, 1–98.
(7) Tsuji, M. *Cell. Mol. Life Sci.* **2006**, *63*, 1889–1898.

10.1021/ja8012787 CCC: $40.75

homologue Vα24i NKT cells by means of TCR recognition.[8−12] The production of T_H1 cytokine is thought to be responsible for the antitumor, antiviral/bacterial/parasitic, and adjuvant effects of α-GalCer, while T_H2 cytokine production is thought to correlate with the amelioration of certain autoimmune diseases (e.g., type 1 diabetes and multiple sclerosis).[13−15] α-GalCer has been the subject of several clinical trials for its anticancer potential but was found to be ineffective, possibly because the therapeutic effects of IFN-γ were hindered by IL-4.[16] Therefore, compounds which increase the selectivity of either the T_H1 or T_H2 cytokine response may be more therapeutically useful.[17,18]

Among the factors that could cause a cytokine profile shift, the stability of the CD1d/glycolipid complex may play a significant role. A less stable association between the glycolipid and CD1d, for example, could result in a shorter half-life for NKT cell stimulation. For IFN-γ production to occur, a longer TCR stimulation is required. IL-4 production occurs after only 2 h of stimulation, while IFN-γ production by NKT cells requires an additional stimulation period, at least 1−2 h.[19,20] Thus, improving the stability of the α-GalCer/CD1d complex could potentially enhance the T_H1 response by prolonging stimulation of NKT cells. The first α-GalCer analogue known to enhance the T_H1 response is a stable C-glycoside analogue (α-C-GalCer).[21] Recently, we have synthesized a series of glycolipids bearing aromatic groups on the acyl side chain and found these molecules to skew the cytokine release profile toward a T_H1 response.[22]

While the designed glycolipids were evaluated by functional assay, examination of the binding affinity between these glycolipids and CD1d was less easily addressed. Progress toward an understanding of the binding properties between glycolipids and CD1d has been slow. A major problem is the lack of a method for measuring the binding constant of the intact glycolipid to CD1d. In addition, the physical properties of lipids, e.g., critical micelle concentration (CMC), solubility, or the slow association and dissociation of binding, may make the study difficult. Because lipid antigens presented by CD1d molecules can trigger and regulate a wide variety of immune responses, a sensitive, accurate, and reproducible high-throughput assay to probe the antigen binding properties to CD1d would be very useful. Several previous studies have examined the lipid binding properties of CD1d, using surface plasmon resonance (SPR),[23] isoelectric focusing (IEF),[24] and isothermal calorimetry (ITC).[24] The SPR method suffered from a low signal-to-noise ratio and the ITC assay required a large amount of protein for each assay. The utility of fluorescent lipid probes in the study of ligand binding by recombinant soluble single chain CD1 proteins has also been evaluated,[25] but this method is only sufficiently sensitive for the study of group I CD1 proteins. One possible reason is the binding and kinetics of association of lipid probes are too slow for detection, because all spectra were obtained immediately after combining the probes with the CD1 proteins.[25] The fluorescent modification of the probe at the lipid tail may also affect the interaction with CD1d.

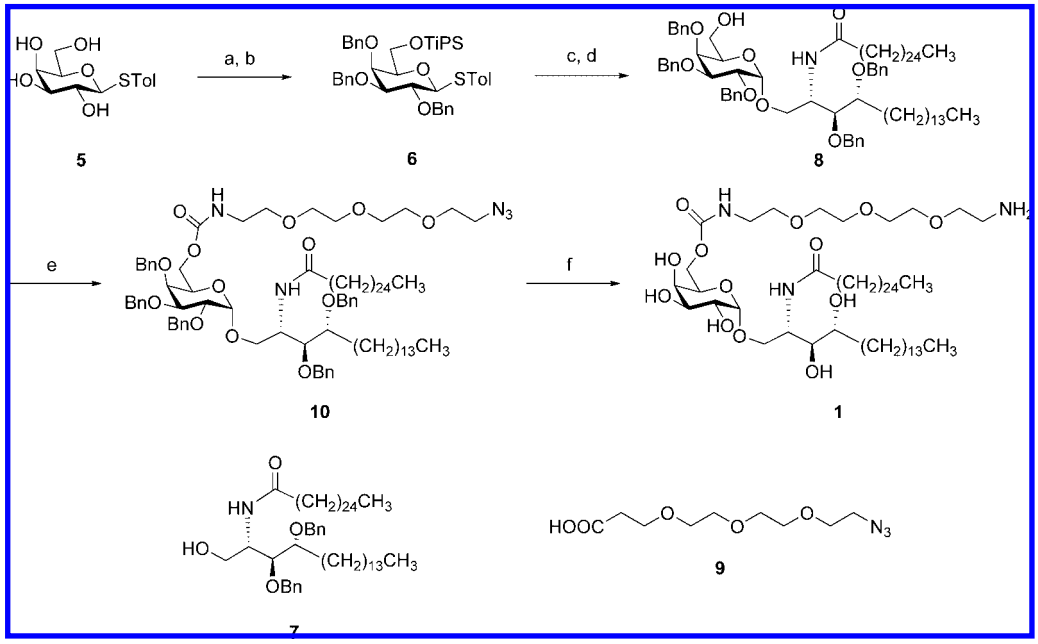
Carbohydrate microarrays allow the rapid screening of interactions between glycans and other molecules.[26−30] Recently, we have developed a quantitative glycan microarray method to determine the dissociation constants of lectins/antibodies and carbohydrate interactions on the surface at the atto-mol level.[31] Here, we report a new method for the quantitative analysis of glycolipid−receptor interactions. In this method, α-GalCer derivatives are covalently bound to a glass slide and incubated with CD1d, and their binding properties were examined (e.g., dissociation constant on the surface). Competition experiments, in which an intact glycolipid antigen and CD1d were mixed in solution and allowed to interact with surface α-GalCer, were used to determine the dissociation constants of new glycolipids in solution. As part of our ongoing search for potent CD1d agonists, this microarray platform was used to quickly determine the dissociation constants of intact α-GalCer derivatives bearing different alkylphenyl chains at either the acyl or phytosphingosine positions.

Result and Discussion

Preparation of Ligands on Array Surface. In order to minimize the amount of α-GalCer derivatives required for the microarray while maintaining a high signal/noise ratio, a ligand with higher affinity for CD1d was sought. X-ray crystallography studies[32−35] of CD1d and glycolipid showed that the 1′-anomeric O and 2′-

(8) Singh, N.; Hong, S.; Scherer, D. C.; Serizawa, I.; Burdin, N.; Kronenberg, M.; Koezuka, Y.; Van Kaer, L. *J. Immunol.* **1999**, *163*, 2373–2377.
(9) Hong, S.; Wilson, M. T.; Serizawa, I.; Wu, L.; Singh, N.; Naidenko, O. V.; Miura, T.; Haba, T.; Scherer, D. C.; Wei, J.; Kronenberg, M.; Koezuka, Y.; Van Kaer, L. *Nature Med.* **2001**, *7*, 1052–1056.
(10) Burdin, N.; Brossay, L.; Kronenberg, M. *Eur. J. Immunol.* **1999**, *29*, 2014–2025.
(11) Kronenberg, M.; Gapin, L. *Nat. Rev. Immunol.* **2002**, *2*, 557–568.
(12) Brossay, L.; Chioda, M.; Burdin, N.; Koezuka, Y.; Casorati, G.; Dellabona, P.; Kronenberg, M. *J. Exp. Med.* **1998**, *188*, 1521–1528.
(13) Taniguchi, M.; Harada, M.; Kojo, S.; Nakayama, T.; Wakao, H. *Annu. Rev. Immunol.* **2003**, *21*, 483–513.
(14) Gonzalez-Aseguinolaza, G.; Van Kaer, L.; Bergmann, C. C.; Wilson, J. M.; Schmieg, J.; Kronenberg, M.; Nakayama, T.; Taniguchi, M.; Koezuka, Y.; Tsuji, M. *J. Exp. Med.* **2002**, *195*, 617–624.
(15) Chang, Y.-J.; Huang, J.-R.; Tsai, Y.-C.; Hung, J.-T.; Wu, D.; Fujio, M.; Wong, C.-H.; Yu, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10299–10304.
(16) Giaccone, G.; Punt, C. J.; Ando, Y.; Ruijter, R.; Nishi, N.; Peters, M.; von Blomberg, B. M.; Scheper, R. J.; van der Vliet, H. J.; van den Eertwegh, A. J.; Roelvink, M.; Beijnen, J.; Zwierzina, H.; Pinedo, H. M. *Clin. Cancer Res.* **2002**, *8*, 3702–3709.
(17) Smyth, M. J.; Godfrey, D. I. *Nat. Immunol.* **2000**, *1*, 459–460.
(18) Berkers, C. R.; Ovaa, H. *Trends Pharmacol. Sci.* **2005**, *26*, 252–257.
(19) Schmieg, J.; Yang, G.; Franck, R. W.; Tsuji, M. *J. Exp. Med.* **2003**, *198*, 1631–1641.
(20) Yu, K. O.; Im, J. S.; Molano, A.; Dutronc, Y.; Illarionov, P. A.; Forestier, C.; Fujiwara, N.; Arias, I.; Miyake, S.; Yamamura, T.; Chang, Y. T.; Besra, G. S.; Porcelli, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3383–3388.
(21) Yang, G.; Schmieg, J.; Tsuji, M.; Franck, R. W. *Angew. Chem., Int. Ed.* **2004**, *43*, 3818–3822.
(22) Fujio, M.; Wu, D.; Garcia-Navarro, R.; Ho, D. D.; Tsuji, M.; Wong, C.-H. *J. Am. Chem. Soc.* **2006**, *128*, 9022–9023.
(23) Naidenko, O. V.; Maher, J. K.; Ernst, W. A.; Sakai, T.; Modlin, R. L.; Kronenberg, M. *J. Exp. Med.* **1999**, *190*, 1069–1080.
(24) Cantu III, C.; Benlagha, K.; Savage, P. B.; Bendelac, A.; Teyton, L. *J. Immunol.* **2003**, *170*, 4673–4682.
(25) Im, J. S.; Yu, K. O. A.; Illarionov, P. A.; LeClair, K. P.; Storey, J. R.; Kennedy, M. W.; Besra, G. S.; Porcelli, S. A. *J. Biol. Chem.* **2004**, *279*, 299–310.
(26) Willats, W. G.; Rasmussen, S. E.; Kristensen, T.; Mikkelsen, J. D.; Knox, J. P. *Proteomics* **2002**, *2*, 1666–1671.
(27) Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. *Nat. Biotechnol.* **2002**, *20*, 275–281.
(28) Liang, P.-H.; Wu, C.-Y.; Greenberg, W.; Wong, C.-H. *Curr. Opin. Chem. Biol.* **2008**, *12*, 86–92.
(29) Feizi, T.; Fazio, F.; Chai, W.; Wong, C.-H. *Curr. Opin. Struct. Biol.* **2003**, *13*, 637–645.
(30) Hirabayashi, J. *Trends Biotechnol.* **2003**, *21*, 141–143.
(31) Liang, P.-H.; Wang, S.-K.; Wong, C.-H. *J. Am. Chem. Soc.* **2007**, *129*, 11177–11184.
(32) Zajonc, D. M.; Maricic, I.; Wu, D.; Halder, R.; Roy, K.; Wong, C.-H.; Kumar, V.; Wilson, I. A. *J. Exp. Med.* **2005**, *202*, 1517–1526.
(33) Koch, M.; Stronge, V. S.; Shepherd, D.; Gadola, S. D.; Mathew, B.; Ritter, G.; Fersht, A. R.; Besra, G. S.; Schmidt, R. R.; Jones, E. Y.; Cerundolo, V. *Nat. Immunol.* **2005**, *6*, 819–826.

Scheme 1. Synthesis of Compound **1**[a]



[a] Reagents and conditions: (a) TIPSCl, imidazol, THF. (b) BnBr, NaH, DMF, 0 °C to rt, 55%. (c) **7**, BSP, Tf_2O, DCM, −78 °C. (d) TBAF, THF, 15% (2 steps). (e) **9**, DPPA, Et_3N, Tol, reflux, 77%. (f) 10% Pd/C, H_2, TFA, EtOH, 95%.

equatorial OH of the galactose moiety and a ceramide moiety bearing two lipid tails are essential for binding. Modification at the 6′-OH position of the sugar moiety of α-GalCer (compound **1**, Figure 1) for the attachment to the glass slide was therefore pursued. All derivatives synthesized incorporated a linker bearing a primary amine for attachment of the derivative to the glass slides. In the comparative studies, compounds **2** (comprising only a lipid part), **3** (modified at the acyl tail), and **4** (comprising only a galactose part), all truncated structures of α-GalCer, were used to study the binding with CD1d. Amine **1** was synthesized from thiocresol galactose (**5**) as shown in Scheme 1. First, the hydroxyl group of galactose **5** was protected using TIPSCl and benzyl bromide to give **6**, which was glycosylated with lipid acceptor **7** in the presence of a catalytic amount of 1-benzenesulfinylpiperidine (BSP) and Tf_2O to afford compound **8**. Silyl deprotection was achieved using TBAF/THF, and the resulting primary alcohol was coupled with an isocyanate intermediate (prepared from linker **9** and diphenylphosphoryl azide (DPPA)) to afford the carbamate **10**. Global deprotection of compound **10** was achieved by hydrogenolysis to give compound **1**. Compounds **2** and **3** were synthesized using a similar strategy (see the Supporting Information).

Derivatives **1**−**4** were printed at different concentrations (between 1 mM to 0.2 μM) on the slide coated with *N*-hydroxysuccinimide (NHS)-activated ester hydrogel. The binding was visualized in a two-step procedure (Figure 2a): first, commercially available recombinant dimeric mouse CD1d (mCD1d) was bound to the surface molecules and then detected using a biotin-labeled rat anti-mCD1d antibody, precomplexed with Cy3 labeled streptavidin. Compound **1** showed the best binding with CD1d, with a detection limit of 1 μM printing

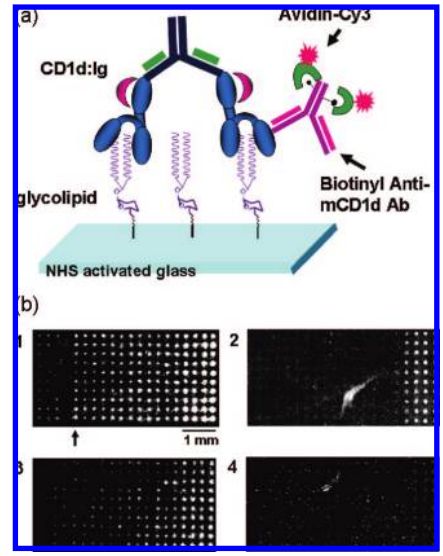


Figure 2. (a) Array fabrication and detection. The glycolipid was printed onto the slide, and its presence was detected using recombinant dimeric CD1d protein. The spot image was generated from the precomplex of biotin-anti-mCD1d antibody and Cy3 labeled streptavidin. (b) Images from the array scanner, each derivative (**1**−**4**) was printed on the slide at concentrations of 1000 (right-most column), 800, 600, 400, 200, 100, 80, 60, 40, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2 μM (left-most column) and incubated with CD1d. The arrow indicates the limit of detection for compound **1** (1 μM). The bar indicates 1 mm in length.

concentration (the lowest concentration of **1** used in the printing process); this corresponds to approximately 1 fmol of **1** per spot (Figure 2b). As expected, modification at the 6′-OH position of the sugar had a smaller effect on binding with CD1d than modification at other positions.[24] Compounds **2** and **3** also interacted with CD1d with low affinity. This result shows that the galactose moiety also participates in binding and that the two hydrophobic tails are necessary to fit into the two hydrophobic pockets of CD1d, a conclusion consistent with the crystal studies.[32−35] Compound **4**, which lacked the lipid moiety,

(34) Zajonc, D. M.; Cantu, C, III; Mattner, J.; Zhou, D.; Savage, P. B.; Bendelac, A.; Wilson, I. A.; Teyton, L. *Nat. Immunol.* **2005**, *6*, 810–818.
(35) Borg, N. A.; Wun, K. S.; Kjer-Nielsen, L.; Wilce, M. C.; Pellicci, D. G.; Koh, R.; Besra, G. S.; Bharadwaj, M.; Godfrey, D. I.; McCluskey, J.; Rossjohn, J. *Nature* **2007**, *488*, 44–49.

did not bind CD1d. This glycolipid-bearing, CD1d-bound array (Figure 2a) is unique in structure, as most of the previously reported microarrays (e.g., ganglosides microarray) tether the lipid moiety to the solid support and expose the sugar moiety for binding.[36] The new glycolipid array was more sensitive than the conventional ELISA for detecting the CD1d−glycolipid interaction.[36]

Printing and Incubation Conditions. The optimal printing condition was next sought. Due to the low solubility of α-GalCer in aqueous solution, 100% DMSO was used as the solvent for printing and found to give the best morphology of spots. After being assayed with Cy3-streptavidin, the fluorescent microspots were found to have an average diameter of 220 ± 30 μm (n = 50, printing concentration = 500 μM), about 80% larger than carbohydrates fabricated with a CMP2B pin (120 ± 20 μm, n = 100, printing concentration = 500 μM), suggesting that there was a significant spreading of lipid molecules within the microspots during the array fabrication and postfabrication processes (Figure 2b). The spot size became smaller at lower printing concentration. The fluorescence pattern also indicated that the glycolipid is randomly and almost evenly distributed within each microspot.

In the incubation step, parameters thought to affect binding were studied: pH, detergent concentrations, DMSO concentrations, immobilization time, and lipid transporter proteins. Previous studies of the pH dependence on ligand association with CD1d proteins have yielded conflicting results. Although a number of studies showed that lipid binding to CD1d may occur preferentially in acidic endocytic compartments,[37,38] in vitro studies using the surface plasmon resonance method indicated that recombinant CD1d proteins could interact with α-GalCer at neutral pH.[23] Using compound **1**, the current study confirmed the intrinsic ability of recombinant CD1d to bind ligands at neutral pH.

In addition, the effect of Tween 20, the most commonly used detergent for the binding and cell-based assays, was evaluated. Binding of CD1d with surface α-GalCer was found to be inhibited by Tween 20 at concentrations above 0.01%. Therefore, the assay was performed in 0.005% PBS buffer. Due to the low water solubility of glycolipids (less than 100 μM), the stock solutions were prepared as 1 mM (for α-GalCer) and 10 mM (other glycolipids) in DMSO solution. In the assay, DMSO was found to inhibit the binding at concentrations greater than 2.0%. In order to mitigate the influence of DMSO when diluting the glycolipids for assay, 1.0% DMSO was used in the assay systems.

The incubation time was also varied from 1 to 6 h; it was found that the interaction of surface α-GalCer and CD1d reached an equilibrium after 1 h.

In vivo, glycolipids are bound to the endosomal system with lysosomal localization by lipid transfer proteins prior to delivery to CD1 proteins.[39] Recently, saposin B was discovered to be the dominant saposin.[40] To investigate the effect of saposin binding on the interactions described in this study, glycolipid **1** was incubated with various saposins (A, B, C, D) in the presence

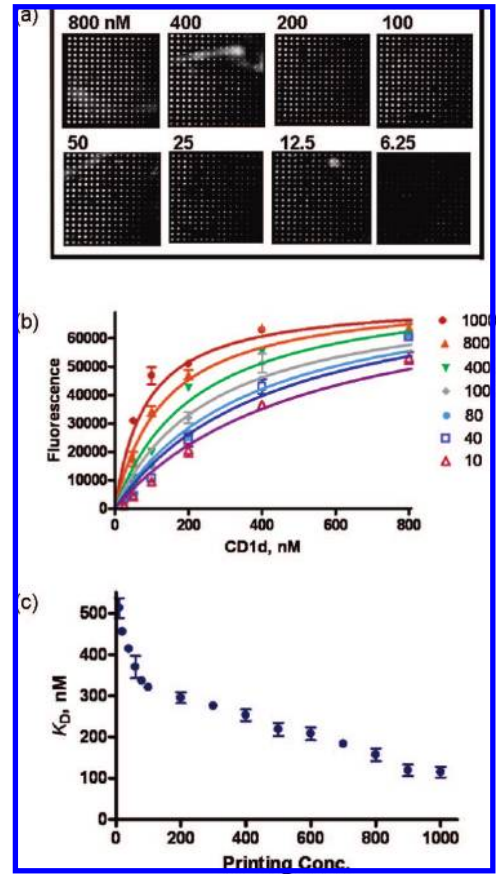


Figure 3. (a) Compound **1** was printed with concentrations of 1000 (left-most column), 900, 800, 700, 600, 500, 400, 300, 200, 100, 80, 60, 40, 20, 10, 5 μM (right-most column). The images were obtained from the slides after incubation with different concentrations of CD1d (from 800 nM to 6.25 nM as indicated above each square) followed by detection with a precomplex of biotin-labeled anti-CD1d and Cy3-labeled streptavidin. (b) Binding curves for compound **1** printed at different concentrations are shown. (c) $K_{D,surf}$ values vs printing concentrations were obtained.

of CD1d at their preference pH (pH 5.0 for saposin A, C and pH 6.0 for saposin B, D). No significant alteration was observed.

Determination of $K_{D,surf}$. To determine the dissociation constant on the surface, protein concentration was plotted against fluorescence intensity at different concentrations of compound **1** (ranging from 1 mM to 1 μM) printed on the slide. Figure 3a depicts photographs of glass slides printed at 16 different concentrations with a 16 × 16 pattern of compound **1** from 1 mM (left-most column) to 5 μM (right-most column). The arrays were incubated with eight concentrations of CD1d, ranging from 800 nM to 6.25 nM. CD1d concentrations were plotted against median fluorescence intensities of replicate spots to give a set of curves for each printed concentration (Figure 3b). Under equilibrium conditions, the curves were analyzed as Langmuir isotherms,

$$F = \frac{F_{max}[P]}{[P] + K_{D,surf}} \quad (1)$$

F_{max} is the maximum fluorescence intensity; [P] is the total CD1d concentration; and $K_{D,surf}$ is the equilibrium dissociation constant for the surface compound and CD1d. $K_{D,surf}^{-1}$ is the

(36) Kanter, J. L.; Narayana, S.; Ho, P. P.; Catz, I.; Warren, K. G.; Sobel, R. A.; Steinman, L.; Robinson, W. H. *Nature Med.* **2006**, *12*, 138–143.
(37) Spada, F. M.; Koezuka, Y.; Porcelli, S. A. *J. Exp. Med.* **1998**, *188*, 1529–1534.
(38) Gumperz, J. E.; Roy, C.; Makowska, A.; Lum, D.; Sugita, M.; Podrebarac, T.; Koezuka, Y.; Porcelli, S. A.; Cardell, S.; Brenner, M. B.; Behar, S. M. *Immunity* **2000**, *12*, 211–221.
(39) Kang, S. J.; Cresswell, P. *Nat. Immunol* **2004**, *5*, 175–181.
(40) Yuan, W.; Qi, X.; Tsang, P.; Kang, S. J.; Illarionov, P. A.; Besra, G. S.; Gumperz, J.; Cresswell, P. *Proc. Natl. Acad. Sci. U.S A.* **2007**, *104*, 5551–5556.

protein concentration at which one-half of the surface binding sites are occupied with protein. The $K_{D,\ surf}$ values are plotted in Figure 3c as a function of printing concentration. The data show that $K_{D,surf}$ (= ~100 nM at 1 mM printing concentration and = 500 nM at 5 μM printing concentration) decreased by a factor of approximately 5 as the ligand density increased, reflecting tighter binding at the higher printing concentration. In a previous glycan microarray study,[31] we found that the fluorescence intensities became saturated when the ligand was printed at concentrations over 100 μM. At printing concentrations greater than 100 μM, the surface density is calculated to be ~10^{14} molecules/cm^2, this value being close to the maximum molecular surface density of the slide. When the surface density is saturated, values of $K_{D,\ surf}$ and F_{max} would become constant. In this assay, the graph of $K_{D,\ surf}$ against printing concentrations (Figure 3c) shows an inflection point at the 100 μM printing concentration, indicating the surface saturation. However, the $K_{D,surf}$ values are gradually decreased when the printing concentration is over 100 μM, and a printing concentration of 1 mM resulted in the highest fluorescence intensity. This may be due to the noncovalent interaction of compound **1**, due to the lipid nature of the glycolipid. The "real" surface density cannot be directly ascertained using current technology. One proposed method is to print a fluorescence labeled ligand directly onto the surface and use fluorescence intensity to analyze surface density. Such experiments are currently underway in our laboratory.

Determination of K_i. In the previous section, the surface dissociation constant ($K_{D,surf}$), a measure of functional affinity, was obtained by attaching compound **1** to a glass slide and allowing molecules of CD1d to bind to it. The solution equilibrium dissociation constant (K_i), a measure of the intrinsic affinity of glycolipid for CD1d, can also be determined using microarrays, this time in a competitive assay. Here, glass slides bearing compound **1** are exposed to a solution containing both CD1d and one of compounds **11**−**26**. The equation that describes the binding of the two ligands (from solution and surface) to the same site on the protein is identical to that for the competitive inhibition of an enzyme-catalyzed reaction.[41] The final forms of the relationship can be expressed as

$$F = \frac{F_{max}[P_o]}{[P_o] + K_{D,surf}\left(1 + \frac{[I_o]}{K_i}\right)} \quad (2)$$

where $[I_o]$ is the concentration of inhibitor (the newly added glycolipids), $[P_o]$ is the concentration of protein (CD1d) used in the system; and K_i is the concentration of ligand at which one-half of protein binding sites are occupied with ligand in solution. In this assay, the concentration of the protein is kept constant at or near the $K_{D,\ surf}$ (300−500 nM of CD1d), and the printing concentration of compound **1** is at 100 μM where the surface density is close to saturation and this can avoid nonspecific binding interference of compound **1** with surface. The following four assumptions were made: (1) Only one ligand (compound **1**) on the surface can bind to one binding site of the protein at a time; no aggregation is assumed to take place. This is thought to be reasonable because steric hindrance should preclude any further interaction between a ligand and a binding site to which another ligand is already bound. (2) All CD1d binding sites act independently and have identical binding properties. (3) The initial concentration of ligand ([I]) is much greater than the initial concentration of protein so that the concentration of unbound ligand is approximately equal to the total concentration of ligand (i.e., $[I_o] = [I]$). (4) There is no monomeric CD1d interaction for surface compound **1** because sufficient ligand is attached to the slide. The glass slide was incubated with solutions containing varying concentrations of the glycolipid (covering ~2 orders of magnitude) and the protein-CD1d. Because of the slow association and slow dissociation of CD1d with α-GalCer (demonstrated by IEF assay[24]), the compounds were incubated with CD1d in a microtiter plate for 5−8 h prior to being applied onto the glass surface.

Two types of glycolipids, the acyl modified, phenyl-alkanoyl chain analogues **11**−**25**, and the phytosphingosine truncated analogue **26** were examined (Table 1). α-GalCer has a K_i value of 16 μM, similar to the values obtained from IEF (5.9 μM) and ITC (9.7 μM).[24] The value is 30−140-fold less than the $K_{D,surf}$ value from the interaction of CD1d with surface α-GalCer, a reflection of the multivalent effects which take place when **1** is attached to a surface.[42] This effect can be understood using the simple model proposed by Gargano et al., which correlates the strength of multivalent association with that of monovalent association[43] and allows the bivalent association ($K_{A,surf}$) to be expressed as

$$K_{A,surf} = F(S*10^{-2})^{n-1}(K_A^{mono})^n$$

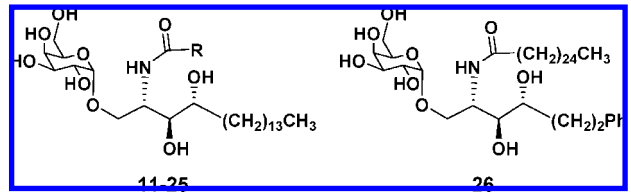
where n is the valency number, F is a statistical factor defined by the system, and s is equal to 30/[inter-receptor distance (Å)]. In this system, n is 2 and the distance between two binding site is expected to be more than 120 Å (for mouse IgG, the distance of two Fab domain is ~120 Å); $K_{A,\ surf}$ is calculated to be 9.7 × 10^6 M^{-1}, a value similar to the experimental value of the binding association constant, $K_{A,surf}$ (= $K_{D,\ surf}{}^{-1}$, 1 × 10^7 M^{-1} to 2 × 10^6 M^{-1}). That the calculated value of $K_{A,surf}$ is close to that predicted by the equation constitutes confirmation that the interaction between surface compound **1** and dimeric CD1d is indeed a bivalent one (i.e., n does equal 2). This theoretical equation for calculating $K_{A,surf}$ indicates that the strength of the multivalent association increases exponentially as a function of valency. In such a bivalent protein case, the intrinsic affinity ($K_A{}^{mono}$) is especially important for the contribution of binding enhancement for multivalent association.

Phenylalkanoyl analogues **12**, **13**, **14**, and **16** were found to bind ~1 order of magnitude more strongly with CD1d than α-GalCer. Compounds **18**−**25** were designed to have different electronic properties by varying the substitutions at the *para* positions of the phenyloctanoyl (**18**−**21**) and phenyldecanoyl (**22**−**25**) groups. Substitution with fluorine (**18**) was found to enhance the binding by approximately 1 order of magnitude, compared with the parent compound **13** (K_i = 1.3 μM). The lower binding activity of the *para*-phenyl group (compounds **21** and **25**) could be due to the lack of space in the binding pocket. The phytosphingosine analogue (**26**) was found to be a weaker agonist of mCD1d.

Cytokine Releasing Profile for New Glycolipids and Their Correlation with Array Results. The ability of these glycolipids to activate human Vαi24NKT cells was then evaluated by

(41) Copeland, R. A. *Enzymes: a practical introduction to structure, mechanism, and data analysis.* Wiley-VCH, Inc.: New York, 1996.
(42) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794.
(43) Gargano, J. M.; Ngo, T.; Kim, J. Y.; Acheson, D. W.; Lees, W. J. *J. Am. Chem. Soc.* **2001**, *123*, 12909–12910.

Table 1. K_i (μM) Values of Glycolipids Obtained Using Microarray and NKT Cell-Based Cytokine Secretions by Glycolipid Stimulation[a]



		microarray	NKT cell cytokine secretion		
compd	structure	K_i (μM)[a]	IFN-γ secretion[b]	IL-4 secretion[b]	IFN-γ/IL-4 ratio[c]
α-GalCer	R = $((CH_2)_{24}CH_3$	16 ± 2.1	1.00	1.00	1.00
11	R = $(CH_2)_5$Ph	2.1 ± 0.8	1.12 ± 0.08	0.73 ± 0.16	1.53 ± 0.15
12	R = $(CH_2)_6$Ph	1.7 ± 1.1	1.28 ± 0.08	0.74 ± 0.16	1.73 ± 0.17
13	R = $(CH_2)_7$Ph	1.3 ± 0.4	1.80 ± 0.15	0.87 ± 0.27	2.07 ± 0.26
14	R = $(CH_2)_8$Ph	3.1 ± 0.3	1.56 ± 0.11	0.72 ± 0.19	2.17 ± 0.24
15	R = $(CH_2)_9$Ph	4.3 ± 1.2	0.86 ± 0.06	0.59 ± 0.15	1.46 ± 0.15
16	R = $(CH_2)_{10}$Ph	1.6 ± 0.9	1.22 ± 0.08	0.87 ± 0.28	1.40 ± 0.15
17	R = $(CH_2)_{14}$Ph	16 ± 3.2	0.17 ± 0.01	0.71 ± 0.10	0.24 ± 0.03
18	R = $(CH_2)_7$Ph(*p*-F)	0.21 ± 0.05	2.39 ± 0.13	0.80 ± 0.09	3.00 ± 0.16
19	R = $(CH_2)_7$Ph(*p*-OMe)	0.45 ± 0.15	2.25 ± 0.06	0.79 ± 0.06	2.85 ± 0.10
20	R = $(CH_2)_7$Ph(*p*-CF_3)	1.2 ± 0.6	1.43 ± 0.07	0.76 ± 0.08	1.88 ± 0.08
21	R = $(CH_2)_7$Ph(*p*-Ph)	13 ± 4.8	0.17 ± 0.003	0.56 ± 0.04	0.30 ± 0.01
22	R = $(CH_2)_{10}$Ph(*p*-F)	0.27 ± 0.08	2.13 ± 0.003	0.81 ± 0.13	2.62 ± 0.18
23	R = $(CH_2)_{10}$Ph(*p*-OMe)	0.66 ± 0.13	1.91 ± 0.19	0.87 ± 0.07	2.19 ± 0.12
24	R = $(CH_2)_{10}$Ph(*p*-CF_3)	4.4 ± 1.3	1.65 ± 0.20	0.87 ± 0.21	1.89 ± 0.22
25	R = $(CH_2)_{10}$Ph(*p*-Ph)	>100	0.33 ± 0.001	0.61 ± 0.06	0.55 ± 0.04
26	-	58 ± 6.3	0.12 ± 0.006	0.59 ± 0.15	0.20 ± 0.02

[a] All the experiments were performed in triplicate, and the data were presented as mean ± SD. [b] Values were obtained from the cytokine (IFN-γ and IL-4) productions of glycolipids normalized to the cytokine production of α-GalCer. [c] Ratios were obtained from the IFN-γ ratio/IL-4 ratio.

comparing their proinflammatory (IFN-γ) and immunomodulatory (IL-4) responses relative to α-GalCer (Table 1). Glycolipids (**11**−**26**) induce a similar or lower level of IL-4 cytokine secretion compared with α-GalCer. In the IFN-γ secretions, modification of the acyl chain resulted in a higher production compared with α-GalCer and phytospingosine modification (**26**). Truncation of the phytosphingosine chain has been previously reported to diminish IFN-γ production.[44,45] Compound **13**, a phenyloctanoyl modification, had a peak value of IFN-γ secretion among the spacer chain elongation in acyl modification (**11**−**17**). *para*-Fluoro and methoxy substitutions (**18**, **19**) resulted in the highest IFN-γ cytokine production, more than double that of the α-GalCer induced IFN-γ secretion. The values of IFN-γ secretion, IL-4 secretion, and IFN-γ/IL-4 ratios were plotted against the K_i values, and the relationships were obtained by fitting the lines to the data points (Figure 4). The resulting trend is interesting: compounds having a lower K_i induced higher IFN-γ production. Those with weaker affinities to CD1d such as compounds **17**, **21**, and **25** induced less IFN-γ production, and this resulted in a low selectivity between IFN-γ and IL-4. IFN-γ secretion and IFN-γ/IL-4 ratios correlated well with the K_i values with $R^2 = 0.83$. This observation supports the earlier assumption that compounds which had tighter binding with CD1d could bias the cytokine releasing through the T_H1 pathway.[22] However, almost all glycolipids, regardless of their affinity to CD1d, induce similar levels of IL-4 secretion by NKT cells; the correlation of IL-4 secretion and the binding affinity is poor ($R^2 = 0.22$). It was found that NKT cells are preactivated and ready to secrete IL-4 through Notch-regulated conserved noncoding sequence-2 (CNS-2) enhancer activation.[46] Thus, even a weak signal through their invariant TCRs can induce a high level of IL-4 secretion by NKT cells.

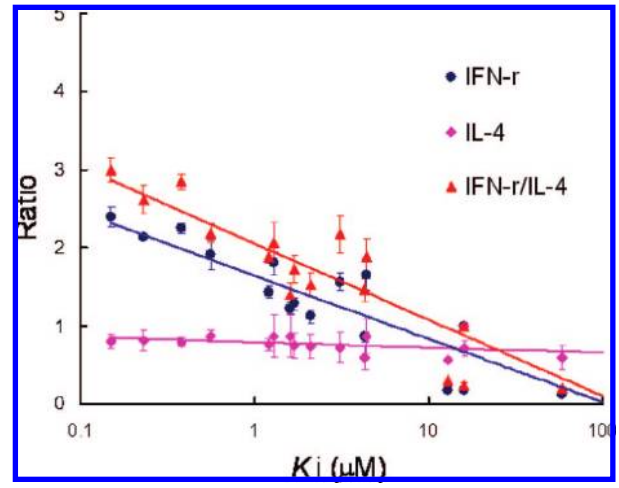


Figure 4. K_i values against IFN-γ secretion (blue), IL-4 secretion (pink), and IFN-γ/IL-4 ratios (red) were plotted. The relationships were obtained by fitting the lines between them.

Conclusion

The interaction between a glycolipid and the protein CD1d has been analyzed using a microarray-based method. The 6′-OH derivative of α-GalCer was covalently bound to a glass surface and shown to have a $K_{D,surf}$ of 100 to 500 nM with recombinant dimeric mCD1d at a variety of different printing concentrations. The solution dissociation constants (K_i) of intact

(44) Goff, R. D.; Gao, Y.; Mattner, J.; Zhou, D.; Yin, N.; Cantu, C., III; Teyton, L.; Bendelac, A.; Savage, P. B. *J. Am. Chem. Soc.* **2004**, *126*, 13602–13603.
(45) Oki, S.; Chiba, A.; Yamamura, T.; Miyake, S. *J. Clin. Invest.* **2004**, *113*, 1631–1640.
(46) Tanaka, S.; Tsukada, J.; Suzuki, W.; Hayashi, K.; Tanigaki, K.; Tsuji, M.; Inoue, H.; Honjo, T.; Kubo, M. *Immunity* **2006**, *24*, 689–701.

α-GalCer and its derivatives were obtained by performing competitive binding experiments. The *para*-F derivative **18** was found to bind most strongly to CD1d with a K_i of 0.21 μM, 2 orders of magnitude higher than α-GalCer, and induce the highest IFN-γ secretion. Using various α-GalCer analogues, the binding affinity of glycolipids to CD1d was found to correlate well with IFN-γ production by NKT cells, but less so with IL-4 secretion, consistent with our assumption that tighter binding compounds could bias cytokine release through the T_H1 pathway. This microarray method should facilitate the analysis of lipid binding and constitutes a relatively simple *in vitro* system for probing the physiological function of proteins and elucidating their role in the immune response.

Experimental Section

Materials. NHS-coated glass slides (Nexterion H slide, SCHOTT North America), mCD1d:Ig fusion protein (BD Bioscience), biotin-labeled rat anti-mCD1d IgG2b (BD Bioscience), Cy3 labeled streptavidin (Jackson ImmunoResearch), and other standard chemicals were purchased from commercial suppliers and used as received.

Generation of Vα24+ Human NKT Cell Lines. Human NKT cell lines, expressing the Vα24+ T cell receptor (TCR), were generated as follows: Vα24+ T cells and CD14+ cells were isolated from leukopaks using magnetic beads (Miltenyi biotec, Auburn, CA) coupled to an anti-Vα24+ TCR monoclonal antibody and an anti-CD14 monoclonal antibody, respectively. Immature dendritic cells were generated from the CD14+ cells after a 3-day incubation in the presence of 300 U/mL GM-CSF (R&D systems, Minneapolis, MN) and 100 U/mL IL-4 (R&D systems, Minneapolis, MN). Following irradiation with 3000 rads, the immature dendritic cells were cocultured with syngeneic Vα24+ T cells in the presence of 100 ng/mL of α-galactosylceramide and 10 IU/mL of IL-2 (R&D systems, Minneapolis, MN) for 10 days. After stimulating the Vα24+ T cells a second time with alpha-galactosylceramide-pulsed, irradiated immature dendritic cells, NKT cell lines were shown to express Vα24+ TCR (99% purity) by a flow cytometric assay.

Cytokine Secretion Assay Using Human NKT Cell Lines. 20 000 cells of a human NKT cell line and 2×10^4 Hela cells transfected with human CD1d that had been irradiated for 10 000 rads were cocultured in the presence of 10 ng/mL of each glycolipid in a 96-well plate. After culture for 24 h, the concentration of IFN-γ or IL-4 in the culture supernatants was determined by ELISA (BD Pharmingen, San Diego, CA).

Microarray Fabrication. Microarrays were printed (Genomic Solutions, Gene Machine) by robotic pin (SMP2B, TeleChem International Inc.). Approximately 0.7 nL of various concentrations of amine-containing compounds **1**−**4** in DMSO were deposited onto slides from a 384 well plate. (1) The slide for the scope of printing concentration studies: NHS-coated glass slides were printed with compounds **1**−**4** at 20 different concentrations (1000, 800, 600, 400, 200, 100, 80, 60, 40, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2 μM) from right to left with 10 replicates vertically placed in each subarray. Six identical subarrays were fabricated in a 1×6 pattern, and each subarray consisted of a 20×10 pattern of spots, with a 0.3 mm pitch. After 2 h, the surface of the slide was divided by permanent marker to avoid contamination for later protein incubation; (2) the slide for Figure 2 was printed with compound **1** with concentrations of 1000 (left most column), 900, 800, 700, 600, 500, 400, 300, 200, 100, 80, 60, 40, 20, 10, 5 μM (right-most column, 16 different concns) with 16 replicates vertically placed in each grid, and in total 16 replicate (8×2 pattern) subarrays in one slide; (3) the slides for K_i determinations: the slides were printed of compound **1** with a concentration of 100 μM and 81 replicates placed in a grid (9×9 pattern) and in total 44 subarrays replicates (4×11 pattern, each subarray is approximately 5 mm (width) $\times$ 6 mm (length) area) in one slide. After 24 h of reaction, the slides were washed with PBST buffer (0.05% Tween 20) for 30 min and then blocked with blocking solution (superblock blocking buffer in PBS, Pierce) for another 1 h. The slides were dried under a flow of argon gas and stored at room temperature in a desiccator. The slides were washed with PBS buffer (pH 7.4) before use.

Direct Binding Assay. mCD1d:Ig fusion protein was diluted in PBS buffer and then directly applied to the subarray of glass slides. Humidifying incubation was performed under foil for 1 h at room temperature. The slide was followed by a wash procedure. The wash procedure is (1) washing three times with incubation buffer, (2) washing three times with distilled water, and (3) drying under a flow of argon gas. A solution of precomplexed biotinylated rat anti-mCD1d antibody (20 μg/mL) and Cy3-labeled streptavidin (10 μg/mL) was incubated on the slide for 1 h. The slide was again washed following the above-mentioned wash procedure, and the fluorescence was visualized at a resolution of 5 Å m with a 595 nm laser using an ArrayWorx microarray reader (Applied Precision).

Competitive Binding Assay. Solutions of the competitors at different concentrations (400 to 0.1 μM) were prepared, and 5 μL aliquots were incubated with protein (5 μL, 500 nM). The mixture was then placed in a 384 well microtiter plate, covered, and left for 5 to 8 h. An aliquot of this solution (8 μL) was loaded onto the slides and incubated for 1 h under a humidifying container at room temperature. Then, the slide was treated with the wash procedure, and secondary antibodies were applied. To obtain the data, each inhibitor was assayed under this system at least three times and the average K_i values are reported.

Data Analysis

Extraction of spot intensity data was performed using ArrayVision 8.0 (Applied Precision). The background, calculated as the median of pixel intensities from the local area around each spot, was subtracted from the mean pixel intensity within each spot. The mean pixel intensity of each spot was extracted from the circle of each spot fixed to 0.1 mm and automatically made changes to the spot outline from 75% to 125%. To graphically represent the data, the values of the background-subtracted signal intensities were plotted against the known concentration of the protein spotted in the array, using the commercial nonlinear regression program GraphPad PRISM 4 (GraphPad, San Diego). The values obtained from the competition assay were plotted against the known concentrations of the competitors applied on the slide. The error bars indicated in the figures show the average percentage error for all data points reported in the figures. Sensitivity of detection for each spot was defined as a signal-to-noise ratio (*S*/*N*) of 2-fold above background. *S*/*N* was calculated as *S*/*N* = (background-subtracted median signal intensity)/(standard deviation of background signal intensity).

Acknowledgment. This work was supported by TSRI, Academia Sinica, and the Gates Foundation. We thank Dr. Takayuki Shiratsuchi for providing saposin proteins. P.-H.L. is a recipient of the NHRI postdoctoral award.

Supporting Information Available: Synthesis and spectral information for compounds **1**−**3**, **6**−**25**. This information is available free of charge via the Internet at http://pubs.acs.org/.

JA8012787