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Design and synthesis of AX4697, a bisindolylmaleimide exo-affinity probe that labels protein kinase C alpha and beta

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ABSTRACT

The synthesis and biochemical characterization of **AX4697**, a fluorescent, bisindolylmaleimide-derived probe for PKCα and β, is described. **AX4697** was able to quantify changes in PKC expression in drug-treated Jurkat cells and was shown to covalently label PKCα on C619, a residue that sits just outside the active site.

Interrogation of the 500+ member kinase family using probes capable of measuring changes in kinase activity is a growing area of interest. Protein kinases have been implicated in a wide array of complex cellular functions and pathways, ranging from metabolic regulation to tumorigenesis. As a result, kinase specific chemical probes can be useful tools to deconvolute the physiological role of kinases and to profile inhibitors against kinases in their native state. To date, kinase probes fall into two classes: those based on nucleotides and those based on synthetic kinase inhibitors. Recently, we described examples from the first class, ADP and ATP based probes containing an *O*-biotinoyl group linked to the terminal nucleotide phosphate via a reactive acyl phosphate moeity.[1] These probes were shown to selectively biotinylate lysines in the ATP binding sites of at least 75% of the known human protein kinases. We and others have reported probes from the second class based on kinase inhibitors such as wortmannin,[2] anilinoquinazolines,[3] pyridinyl imidazoles,[4] 5′-*p*-fluorosulfonylbenzoyl adenosine,[5] and fmk.[6]

Herein, we describe the synthesis and biochemical characterization of **AX4697**, a bisindolylmaleimide-derived probe which covalently labels both recombinant and endogenous PKCα and PKCβ (Fig. 1).[7] Similar to the design of the fluorophosphonate serine hydrolase probes pioneered by Cravatt et al.,[8] **AX4697** incorporates

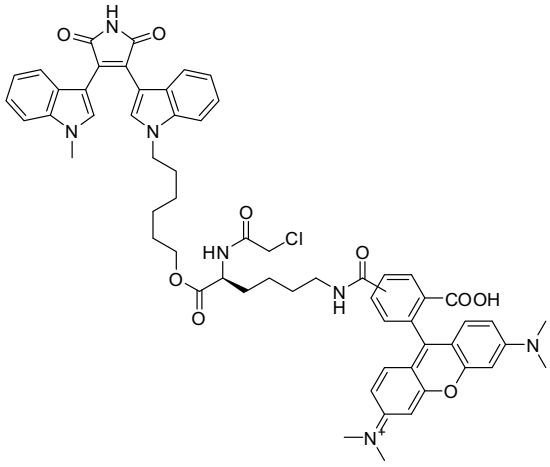

Figure 1. Structure of **AX4697**, a 5(6)-TAMRA conjugate.

the three structural elements present in all activity-based probes (ABPs): an enzyme specific recognition group containing a protein reactive functionality, a linker, and a tag that can be used for quantitation and/or capture of the covalently modified enzyme.[9] Because bisindolylmaleimides have been identified as potent inhibitors of various PKC family members,[10] this structure was chosen for the design of a probe to target PKC kinases. However,

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because such inhibitors are reversible, a reactive α-chloroacetamide group was incorporated into the probe to covalently label residues in or around the active site. Finally, carboxytetramethylrhodamine (TAMRA) was used as a tag because of its previous demonstration as a sensitive fluorescent tag for ABPs. TAMRA derived ABPs can be conveniently used in SDS–PAGE analyses, and probe-labeled proteins/peptides can be captured using an anti-TAMRA antibody affinity column.[11]

AX4697 was synthesized incorporating these design elements as shown in Scheme 1. Deprotonation of indole using sodium hydride followed by N-alkylation with $MsO(CH_2)_6OTHP$ gave the indole **1** in excellent yield. Subsequent treatment of this THP protected compound with oxalyl chloride followed by esterification using sodium methoxide in MeOH provided compound **2**. Following a methodology described by Faul et al. to synthesize bisindolylmaleimides,[12] the indolyl-3-glyoxylyl ester **2** was condensed with 1-methylindole-3-acetamide under basic conditions followed by an acidic workup. This process not only created the requisite bisindolylmaleimide core but also resulted in the deprotection of the THP group to give the alcohol **3**. Esterification of Fmoc-Lys(Boc)-OH with compound **3** followed by deprotection of the Fmoc group using diethylamine yielded compound **4** in a two-step yield of 48%. The α-chloroacetamide **5** was obtained in 70% yield by treatment of the amine **4** with chloroacetyl chloride under basic conditions. Deprotection of the Boc group of compound **5** with TFA followed by amidation of the resulting amine with the succinimidyl ester of 5(6)-carboxytetramethylrhodamine (TAMRA) yielded the mixed TAMRA conjugate **AX4697**. **AX4697** was purified using C18 reverse phase chromatography and gave satisfactory ^1H NMR, ESI-MS, and HPLC (>98% purity) analyses.

To test the reactivity of **AX4697** with PKC, we added recombinant PKCα (0.7 pmol) to MDA-MB-435 soluble proteome (25 μl, 0.3 μg of protein per μL) and incubated the proteome with **AX4697** (50 nM) for 60 min with or without pre-incubation with staurosporine (500 nM), a potent inhibitor of various PKC family members (e.g., PKCα IC_{50} = 45 nM, PKCβI IC_{50} = 23 nM, PKCβII IC_{50} = 19 nM).[10] As seen in Figure 2A, **AX4697** specifically labeled PKCα. This labeling of PKCα was blocked when PKCα was pre-incubated with 500 nM staurosporine for 10 min prior to treatment with the probe (Fig. 2A). When the enzymatic activity of PKCα was abolished by heat denaturation, **AX4697** did not label the protein (data not shown). **AX4697** was also found to label PKCβ but not staurosporine-inhibited PKCβ (data not shown). Next, the

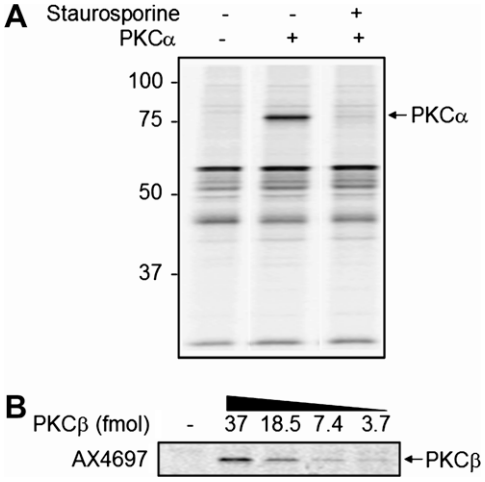
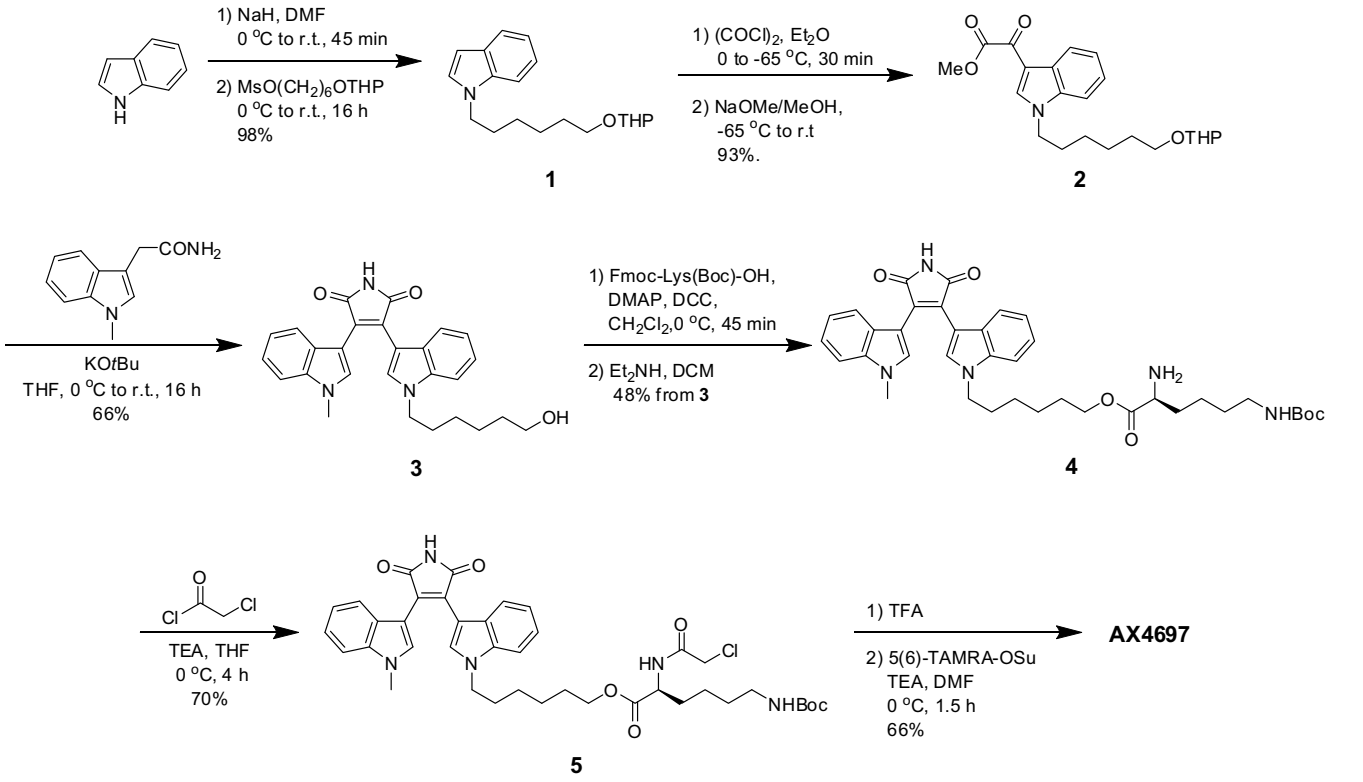


Figure 2. AX4697 specifically labels PKC. (A) Recombinant PKCα (0.7 pmol) was added to MDA-MB-435 soluble proteome (25 μl, 0.3 μg of protein per μL) and incubated with or without staurosporine (500 nM) for 10 min and then treated with **AX4697** (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio). (B) **AX4697** labeling of serially diluted recombinant PKCβ added to MDA-MB-435 soluble proteome.



Scheme 1. Synthesis of **AX4697**.

sensitivity of **AX4697** was examined by incubating this reagent with serially diluted recombinant PKCβ added to MDA-MB-435 soluble proteome. Under these conditions **AX4697** could detect as few as 3.7 fmol of PKCβ (Fig. 2B). This quantity of PKCβ, which corresponded to about 5600 copies per cell, approximates the sensitivity of other ABPs that target other enzyme families and is in line with the requirements of profiling low-abundance proteins in complex proteomes.[13,14] These results indicated that **AX4697** serves as an effective functional proteomic probe for at least two members of the PKC enzyme family.

To determine whether **AX4697** could inhibit PKC substrate phosphorylation, we performed an in vitro PKCα assay using protamine as a substrate.[15,16] When **AX4697** was added simultaneously with substrate (i.e., at time zero), an IC_{50} value of 200 nM was obtained. When **AX4697** was pre-incubated with PKCα prior to substrate addition, increased inhibition of protamine phosphorylation was observed with longer pre-incubation times (data not shown). This time-dependent inhibition was taken as evidence of the irreversible nature of **AX4697** binding.

We next examined whether **AX4697** could detect PKC derived from cells and tissues. Soluble proteomes of mouse brain or human white blood cells were incubated with this probe (50 nM) for 60 min with or without pre-incubation with staurosporine. As seen in Figure 3A and B, **AX4697**-labeled PKC from mouse brain and human white blood cells, respectively. The labeling of PKC by **AX4697** was completely inhibited by staurosporine. Western blot analysis using a monoclonal anti-PKC antibody showed the presence of equivalent levels of PKC in both staurosporine-treated and untreated samples. These results indicated that **AX4697** could detect PKC directly from a complex proteome and that **AX4697** was an effective activity-based probe for PKC.

To examine whether **AX4697** could record changes in PKC expression in cells treated with drugs, we treated Jurkat cells with staurosporine and calyculin A, a serine/threonine phosphatase PP1 and PP2A inhibitor. As shown in Figure 3C I, as expected, the **AX4697**-labeled PKC was strongly inhibited in Jurkat cells treated with staurosporine compared with control Jurkat cells, whereas **AX4697**-labeled more PKC in Jurkat cells treated with calyculin A compared with control Jurkat cells. PKC expression, as quantified by integrated fluorescent band intensity, was 1.8-fold higher in calyculin A-treated Jurkat cells relative to control Jurkat cells (Fig. 3D). Western blot analysis using a monoclonal anti-PKC antibody confirmed these observations (data not shown). These results indicate that PKC expression was dramatically decreased in staurosporine-treated cells and was significantly increased in calyculin A-stimulated cells and that **AX4697** could detect these changes. Affinity isolation of the probe-labeled protein band (I) by virtue of the TAMRA tag of **AX4697** and mass spectrometric analysis identified the 75 kDa protein (I) as PKCα and PKCβ, further confirming that **AX4697** performed as designed to covalently label the PKC family. Additionally, **AX4697** was found to label a 73 kDa enzyme that was present only in calyculin A-treated Jurkat cells (Fig. 3C, band II). This enzyme was not detected in control Jurkat cells. To determine whether this calyculin A-stimulated enzyme belongs to the PKC family, we did a Western blot against a PKC antibody. The 73-kDa protein band was neither detected by the western blot nor was **AX4697** labeling of this band inhibited by GF 109203X (30 μM, data not shown), a potent inhibitor of PKC α, β, γ, and ε, which suggested this enzyme was not a PKC.[17] Collectively these data demonstrated that **AX4697** can identify both unanticipated and PKC activity differentially expressed in drug-treated cells.

In order to determine the site of labeling of PKCα, mass spectrometry was employed. When PKCα (1.4 μM in 0.1% Triton X-100, 50 mM Tris, pH 8) was incubated with **AX4697** (1 μM), gel filtered to remove unreacted probe, and the resulting filtrate digested with trypsin, a HPLC analysis indicated the presence of two fluorescent peaks in a 1:1 ratio. LC/MS analyses indicated both peaks had identical masses and fragmentation patterns. Thus, both peaks were assigned to the same species labeled with the 5′-TAMRA and 6′-TAMRA isomers of **AX4697**. The mass of these species (m/z 714.58, doubly charged) was consistent with that of the probe-labeled tryptic peptide $VC_{619}GK$ (calculated m/z for **AX4697**+VCGK-HCl, doubly charged: 714.49). To determine the specific residue that was labeled, MS/MS analysis of this ion was undertaken. Fragmentation yielded two critical ions that pinpointed the labeled residue as C619: 665.0 and 1224.4. The former ion was assigned to a doubly charged y_3 ion (relative abundance 100%, calculated m/z for C(**AX4697**-labeled)-G-K: 664.9) and the latter to a b_2 ion (relative abundance 34%, calculated m/z for V-C(**AX4697**-labeled): 1224.5). Additional ions were consistent with

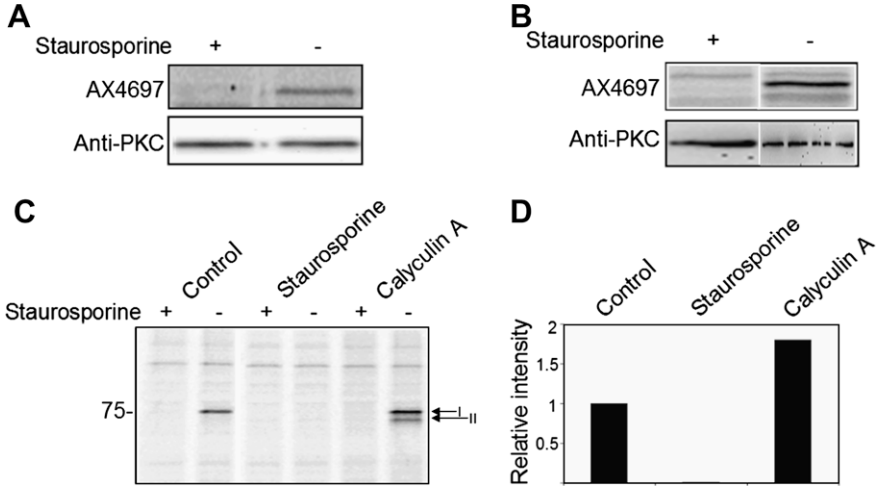


Figure 3. AX4697 can detect PKC in proteomes and can detect changes in PKC expression in cells treated with drugs. Soluble proteome from mouse brain (A) or white blood cells (B) was pre-incubated with or without staurosporine (500 nM) for 10 min and then treated with **AX4697** (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio). The scanned gels were transferred to nitrocellulose and immunoblotted with anti-PKC antibody. (C) Jurkat cells were treated with staurosporine (1 μM), calyculin A (25 nM) or control DMSO for 30 min, and soluble proteome derived from these cells were treated with **AX4697** (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio). Protein band II has not been identified. (D) Fluorescently labeled PKC (band I) was quantified by measuring integrated band intensities (normalized for volume).

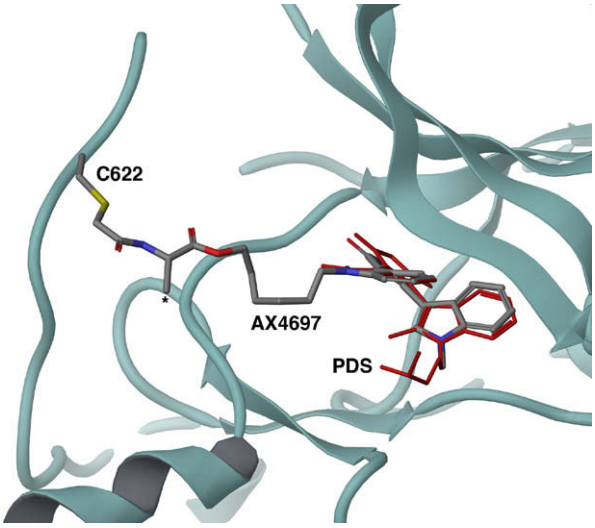


Figure 4. A model of a simplified version of **AX4697** covalently bound to PKCβII (PDB 2I0E) is shown in comparison with the pose of the bisindolylmaleimide inhibitor PDS (shown in red) present in the original crystal structure. Backbone atoms of PKCβII are shown in a ribbon representation and colored in turquoise. The side chain of the covalently bound cysteine residue C622 as well as the atoms for **AX4697** is shown in a CPK coloration and tube representation. The TAMRA moiety including its 3-carbon linker was omitted as described in Ref. 18 and the linker attachment point is labeled with an asterisk (*).

probe containing fragments of the y_3 ion, for example, 1056.5 (relative abundance 37%, calculated *m*/*z* for **AX4697**-Cl+SH: 1056.4). No y or b ions were seen that collectively supported probe labeling of K621.

To visualize the residue-specific **AX4697** labeling of PKCα, a model of **AX4697** bound to PKCβII was constructed (Fig. 4).[18] Overall, the PKCβII sequence is 84% identical to PKCα with 93% conserved residues between the two sequences. The active site residues are 100% identical between both proteins. In addition, this sequence variant of PKCβ contains a cysteine residue (C622) homologous to the **AX4697**-labeled cysteine residue in PKCα (C619).[19] Furthermore, the crystal structure of PKCβII, available from the Protein Data Bank[20] (accession number 2I0E), contains the bound bisindolylmaleimide inhibitor PDS which was used as a template to orientate **AX4697** into the active site for modeling purposes. After linking **AX4697** to C622, a full force-field based energy minimization of the model indicated a favorable pose of **AX4697** in the active site of PKCβII with very little shift of the bisindolylmaleimide atoms compared to the original crystal structure. Importantly, this modeling result supports the idea that the linker of the **AX4697** is of an appropriate length to place the electrophilic α-chloroacetamide group within striking distance of the C619 thiol group of PKCα. Interestingly, because C619 sits outside the active site, **AX4697** can be classified as an exo-affinity label, a type of affinity label in which the protein reactive group is not part of or directly attached to the ligand but rather is separated by a linker.[21]

In summary, **AX4697** has been shown to be a sensitive probe for PKCα and PKCβ, and able to quantify PKC activity directly in complex proteomes. PKCs have been linked to carcinogenesis and, in particular, the PKCα and PKCβ isozymes have been linked to malignant phenotypes. As a result, PKC inhibitors have been developed to treat cancer and selected examples have reached the clinic.[22] **AX4697** provides a unique tool for the discovery of inhibitors that selectively target these kinases as well as study the role PKCs play in tumor biology.

References and notes

1. Patricelli, M. P.; Szardenings, A. K.; Liyanage, M.; Nomanbhoy, T. K.; Wu, M.; Weissig, H.; Aban, A.; Chun, D.; Tanner, S.; Kozarich, J. W. *Biochemistry* **2007**, *46*, 350.
2. (a) Liu, Y.; Shreder, K. R.; Gai, W.; Corral, S.; Ferris, D. K.; Rosenblum, J. S. *Chem. Biol.* **2005**, *12*, 99; (b) Yee, M. C.; Fas, S. C.; Stohlmeyer, M. M.; Wandless, T. J.; Cimprich, K. A. *J. Biol. Chem.* **2005**, *280*, 29053.
3. (a) Shreder, K. R.; Wong, M. S.; Nomanbhoy, T.; Leventhal, P. S.; Fuller, S. R. *Org. Lett.* **2004**, *6*, 3715; (b) Blair, J. A.; Rauh, D.; Kung, C.; Yun, C. H.; Fan, Q. W.; Rode, H.; Zhang, C.; Eck, M. J.; Weiss, W. A.; Shokat, K. M. *Nat. Chem. Biol.* **2007**, *3*, 229.
4. Young, P. R.; McLaughlin, M. M.; Kumar, S.; Kassis, S.; Doyle, M. L.; McNulty, D.; Gallagher, T. F.; Fisher, S.; McDonnell, P. C.; Carr, S. A.; Huddleston, M. J.; Seibel, G.; Porter, T. G.; Livi, G. P.; Adams, J. L.; Lee, J. C. *J. Biol. Chem.* **1997**, *272*, 12116.
5. Ratcliffe, S. J.; Yi, T.; Khandekar, S. S. *J. Biomol. Screen.* **2007**, *12*, 126.
6. Cohen, M. S.; Hadjivassiliou, H.; Taunton, J. *Nat. Chem. Biol.* **2007**, *3*, 156.
7. Fluorescent, bisindolylmaleimide-based PKC inhibitors have been described as cytological stains, see: Chen, C. S.; Poenie, M. *J. Biol. Chem.* **1993**, *268*, 15812. However, because these compounds lack a means to covalently tag their target proteins, they cannot be used as activity based probes in proteomic profiling experiments.
8. Liu, Y.; Patricelli, M. P.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14694.
9. Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Ann. Rev. Biochem.* **2008**, *77*, 383.
10. Jirousek, M. R.; Gillig, J. R.; Gonzalez, C. M.; Heath, W. F.; McDonald, J. H., III; Neel, D. A.; Rito, C. J.; Singh, U.; Stramm, L. E.; Melikian-Badalian, A.; Baevsky, M.; Ballas, L. M.; Hall, S. E.; Winneroski, L. L.; Faul, M. M. *J. Med. Chem.* **1996**, *39*, 2664.
11. Patricelli, M. P. *Briefings in Functional Genomics and Proteomics* **2002**, *1*, 151.
12. Faul, M. M.; Winneroski, L. L.; Krumrich, C. A. *J. Org. Chem.* **1998**, *63*, 6053.
13. Jessani, N.; Liu, Y.; Humphrey, M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10335.
14. Speers, A. E.; Cravatt, B. F. *Chem. Biol.* **2004**, *11*, 535.
15. Leventhal, P. S.; Bertics, P. J. *J. Biol. Chem.* **1993**, *268*, 13906.
16. Nishizuka, Y. *Science* **1986**, *233*, 305.
17. (a) Roberts, N. A.; Haworth, R. S.; Avkiran, M. *Br. J. Pharmacol.* **2005**, *145*, 477; (b) Toullec, D.; Pianetti, P.; Coste, H.; Bellevergue, P.; Grand-Perret, T.; Ajakane, M.; Baudet, V.; Boissin, P.; Boursier, E.; Loriolle, F.; Duhamelll, L.; Charon, D.; Kirilovsky, J. *J. Biol. Chem.* **1991**, *266*, 15771.
18. The coordinates for the PDB entry 2I0E (Grodsky et al., see below) were imported into the Maestro molecular modeling environment (http://www.schroedinger.com). Hydrogen atom addition and bond-valence corrections were performed using default parameters. A model of the covalently bound **AX4697** was constructed using the build tools of Maestro and energy-minimized using the MacroModel implementation of the OPLS2005 force field: Kaminski, G. A.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. J. J. *Phys. Chem. B* **2001**, *105*, 6474. In order to reduce artifacts during the minimization calculations, a simplified version of **AX4697** lacking the TAMRA tag was used throughout. Both indole nitrogen atoms of PDS were considered as attachment points for the **AX4697** linker. Calculated energy values for both assemblies were nearly identical, and the presented model was chosen based on the minimal shift of the bisindolylmaleimide atoms compared to the crystallographic pose..
19. Grodsky, N.; Li, Y.; Bouzida, D.; Love, R.; Jensen, J.; Nodes, B.; Nonomiya, J.; Grant, S. *Biochemistry* **2006**, *45*, 13970.
20. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235.
21. Baker, B. R. *J. Pharm. Sci.* **1964**, *53*, 347.
22. Koivunen, J.; Aaltonen, V.; Peltonen, J. *Cancer Lett.* **2006**, *235*, 1.