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- (54)
- Title:**
- CHEMICALLY-STABILIZED A KINASE ANCHORING PROTEIN (AKAP) PEPTIDE DISRUPTORS

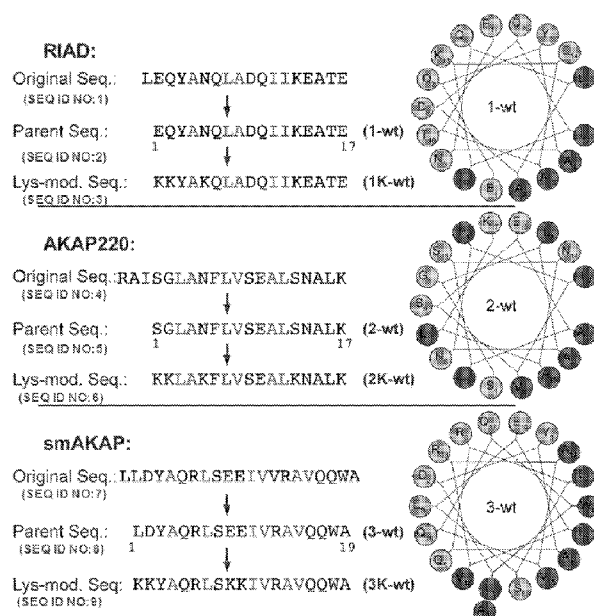


FIGURE 2

- (57)
- Abstract:**
- Disclosed are polypeptides with a chemically stabilized -helical shape that mimic an A Kinase Anchoring protein (AKAP) docking helix and bind the docking/dimerization domain of protein kinase A-R subunit (PKA-R) so as to inhibit endogenous AKAPs from binding to an endogenous PKA-R in an isoform-selective manner.



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CHEMICALLY-STABILIZED A KINASE ANCHORING PROTEIN (AKAP) PEPTIDE DISRUPTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Application No. 61/856,112, filed
5 July 19, 2013, which is hereby incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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10 has certain rights in the invention.

BACKGROUND

Understanding the mechanisms underlying breast cancer is of vital importance. Breast
cancer has an incidence rate of over 10% worldwide, making it the second most common cancer
in women (The World Cancer Report--the major findings. Cent Eur J Public Health. 2003
15 11(3):177-9). Alarming, the United States has the highest rate of incidence worldwide where it
is the most common cancer in women and over 200,000 women are diagnosed each year (Jemal
A, et al. 2010 CA Cancer J Clin. 2010 60(5):277-300). The mechanisms leading to cell growth
and activated survival pathways that ultimately leads to a cancerous state are under intense
scrutiny. Multiple studies have implicated a class of enzymes called kinases in breast cancer
20 etiology due to its fundamental role in regulation of cellular processes (Lurje G, et al. Oncology.
2009 77(6):400-10). Since altered kinase activity has been directly linked breast cancer, it is of
great importance to understand their biological roles in a cancerous state.

Protein kinase A (PKA), or cAMP-dependent protein kinase, has broad substrate
specificity and regulates a myriad of highly diverse cellular processes. Multiple mechanisms
25 exist to fine-tune the spatial and temporal regulation of PKA on subcellular signaling (Taylor, S.
S., et al. (2012) Nat. Rev. Mol. Cell Biol. 13:646-658; Taylor, S. S., et al. (2013) Biochim.
Biophys. Acta 1834:1271-1278; Welch, E. J., et al. (2010) Mol. Interventions 10:86-97). The
PKA holoenzyme complex is a tetramer composed of two catalytic subunits (PKA-C) and a

regulatory subunit dimer (PKA-R). When intracellular cAMP levels increase, the PKA-R subunits bind cAMP and undergo a conformational change to release the catalytic subunits, which then perform substrate phosphorylation (Johnson, D. A., et al. (2001) *Chem. Rev.* 101:2243–2270; Herberg, F. W., et al. (1996) *Biochemistry* 35:2934–2942). Regulation of PKA activity is partly controlled through the utilization of four distinct PKA-R subunit isoforms: PKA-RI (RI α and RI β) and PKA-RII (RII α and RII β). The PKA-R isoforms differ in many aspects including tissue expression, cAMP sensitivity, and intracellular localization (Taylor, S. S., et al. (2012) *Nat. Rev. Mol. Cell Biol.* 13:646–658).

PKA activity is further regulated by a class of proteins called A kinase-anchoring proteins (AKAPs) (Welch, E. J., et al. (2010) *Mol. Interventions* 10:86–97; Skroblin, P., et al. (2010) *Int. Rev. Cell Mol. Biol.* 283:235–330). The AKAP family is structurally diverse but shares the commonality of binding to PKA-R and compartmentalizing the PKA holoenzyme to multiple subcellular locations including the plasma membrane, endoplasmic reticulum, and mitochondria (Welch, E. J., et al. (2010) *Mol. Interventions* 10:86–97). AKAPs act as scaffolding proteins that tether PKA along with other proteins so as to integrate PKA activity into distinct multivalent signaling complexes. Other proteins tethered to these subcellular complexes include kinases, phosphatases, adenylyl cyclases, phosphodiesterases, and various substrates (Dessauer, C. W. (2009) *Mol. Pharmacol.* 76:935–941; Sanderson, J. L., et al. (2011) *Neuroscientist* 17:321–336; Diviani, D., et al. (2011) *Am. J. Physiol. Heart Circ. Physiol.* 301:H1742–1753). By confining PKA to subsets of cellular substrates within a local cAMP environment, AKAPs provide intrinsic specificity to cAMP-PKA signaling pathways and therefore act as key regulators for various cellular processes (Figure 1A) (Welch, E. J., et al. (2010) *Mol. Interventions* 10:86–97; Skroblin, P., et al. (2010) *Int. Rev. Cell Mol. Biol.* 283:235–330). While most AKAPs preferentially bind to PKA-RII, several AKAPs have been identified that have PKA-RI specificity or can bind both PKA-RI and PKA-RII (dual specific) (Gold, M. G., et al. (2006) *Mol. Cell* 24:383–395; Herberg, F. W., et al. (2000) *J. Mol. Biol.* 298:329–339). Isoformselective interactions appear to be critical for AKAP-mediated signaling since altered interactions between AKAPs and the PKA-R isoforms correlate with misregulated PKA activity and various disease states (Burgers, P. P., et al. (2012) *J. Biol. Chem.* 31:31).

The significance of AKAP regulation on PKA activity is further underscored by its correlation with various disease phenotypes. Altered AKAP activity is implicated in many pathological processes including cardiovascular disorders, immune diseases, and multiple cancer phenotypes (Troger, J., et al. (2012) *Br. J. Pharmacol.* 29:1476–5381; Carnegie, G. K., et al.

(2009) IUBMB Life 61:394–406; Blant, A., et al. (2012) Can. J. Physiol. Pharmacol. 90:1161–1170). While AKAPs are clearly important regulators of PKA, their full biological roles are largely elusive due to the complex nature of spatial and temporal regulation. In order to elucidate the role of AKAPs on localized PKA signaling, significant efforts have been put forth to block interactions between PKA and AKAP in a highly isoform-selective manner (Figure 1B). One of the first peptide disruptors, Ht31, was derived from AKAP-Lbc and was subsequently modified to contain a stearylated moiety to allow for cell permeability (Carr, D. W., et al. (1992) J. Biol. Chem. 267:13376–13382). Other peptides were also developed with improved properties including greater isoform specificity or higher binding affinities such as RIAD (RI-anchoring disruptor) (Carlson, C. R., et al. (2006) J. Biol. Chem. 281:21535–21545) and SuperAKAP-IS (Gold, M. G., et al. (2006) Mol. Cell 24:383–395). Collectively, these peptides have become valuable tools to block PKA signaling mediated by either PKA-RI or PKA-RII PKA. However, there are still limitations with the physical properties of these compounds including poor cellular uptake by intact cells, loss of the secondary structural fold in solution, and susceptibility to proteolytic degradation that is intrinsic to nonmodified peptidyl bonds. Various modifications including addition of stearic acid (Vijayaraghavan, S., et al. (1997) J. Biol. Chem. 272:4747–4752) and additions of either a poly arginine tag or HIV-1 TAT sequences (Nakase, I., et al. (2008) Adv. Drug Delivery Rev. 60:598–607; Patel, H. H., et al. (2010) J. Biol. Chem. 285:27632–27640) have been utilized to improve cellular permeability. Nevertheless, many limitations still exist using these synthetic strategies including lack of reinforced secondary structure in solution, relatively short half-life values, and potential mislocalization caused by the addition of conjugated sequences or moieties.

SUMMARY

Disclosed are polypeptides with a chemically stabilized α -helical shape that mimic the A-Kinase Binding (AKB) helix of an A Kinase Anchoring protein (AKAP) that binds the docking/dimerization (D/D) domain of a protein kinase A (PKA) so as to inhibit endogenous AKAPs from binding to an endogenous PKA-R. These polypeptides can act as universal AKAP inhibitors, and are in some cases isoform-specific. Therefore, the disclosed polypeptides can be used to disrupt the spatiotemporal regulation of PKA as it may relate to disease, such as breast cancer.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is a schematic showing RII-selective disruption of AKAP-mediated PKA anchoring using hydrocarbon-stapled peptides. Figure 1A illustrates that AKAPs regulate the phosphorylation of PKA substrates in a spatiotemporal manner by recruiting related machinery to subcellular locations for compartmentalized signaling. Figure 1B illustrates that isoform-selective peptides were designed to mimic the AKB helix from AKAP that binds PKA-R. Peptides can be engineered to have specificity toward either isoform of PKA-R, thereby blocking downstream signaling through displacement of PKA-R from the AKAP signaling complex. Figure 1C illustrates pairs of the non-natural amino acid S5 introduced into AKB or AKB-like sequences at i and $i + 4$ positions. A hydrocarbon staple is formed by ring-closing olefin metathesis to form the conformationally constrained product. The hydrophobic residues that are essential for PKA-AKAP interactions were left unchanged. The engineered stapled peptide can target the AKB-binding site on the surface of the docking/dimerization (D/D) domain of PKA-RII. Structure rendered in PyMol using PDB ID 2HWN.

Figure 2 shows sequences for design of stapled peptides. Original AKB or AKB-like sequences were slightly shortened to yield the parent sequences for the compound library. Optimization of the sequence was performed to increase the amphipathic properties and water solubility of the sequences, rendering the Lys-modified sequence libraries. Helical wheels of the parent sequences demonstrate the hydrophobic nature of the binding surface. The hydrophobic residues were left unchanged. Non-natural amino acids and Lys residues were introduced on the solvent-exposed hydrophilic surface.

Figures 3A to 3C are graphs showing stapled peptides STAD-1 (Fig. 3A), STAD-2 (Fig. 3B), and STAD-3 (Fig. 3C) are highly selective for the PKA-RII isoform. Normalized FP spectra are shown for each of the full-length PKA-R subunit isoforms. PKA-RI is represented as circles (closed = α , open = β), and PKA-RII is represented as triangles (closed = α , open = β). STAD-2 and STAD-3 show preference for PKA-RII binding by 1–2 orders of magnitude.

Figures 4A to 4H show that hydrocarbon stapled peptides selectively bind PKA-RII and disrupt AKAP-mediated PKA signaling in cells. Figure 4A is a series of fluorescent images of diverse cell lines (HeLa, MDA-MB-231, PC-3) after treatment with FITC-labeled peptides (5

μM) for 7 h, demonstrating that STAD-1, -2, and -3 are cell-permeable and have at least partial cytosolic localization. Each image is representative of three replicates. Figure 4B shows results of immunoprecipitation experiments performed in MDA-MB-231 cells. Cells were incubated with N-terminal biotin-labeled peptides (5 μM) and pulled down by avidin-coated resin, and PKA-RI and PKA-RII were detected by immunoblotting. All three peptides demonstrated interactions with PKA-RII to varying degree, while none showed any appreciable affinity for PKA-RI in cells. Figure 4C shows that the STAD peptides were found to cause dose-dependent disruption of PKA substrate phosphorylation. Cells were serum-starved, followed by stimulation with 50 μM forskolin (except the basal lane). The PKA inhibitor H89 was used as a control (50 μM). Phosphorylation of CREB was independently monitored to demonstrate that the peptides inhibit a known PKA substrate that is partly regulated by AKAP activity. The image is representative of three independent experiments. Figure 4D shows that STAD scramble peptides (8 μM) were also monitored for their effects on PKA substrate phosphorylation. The scramble control peptides had no effect on PKA substrate phosphorylation or on CREB phosphorylation after forskolin stimulation. Figures 4E and 4F show cytosolic PKA activity monitored using the AKAR4 reporter. When treated with either STAD-2 or the STAD-2 scramble control, PKA activity was still stimulated by Fsk (50 μM) and IBMX (100 μM) and inhibited by H89 (20 μM), indicating that STAD-2 does not affect non-localized PKA activity. Figure 4G and 4H show PKA activity localized near the plasma membrane monitored using the pmAKAR4 reporter. In this instance, PKA activity was not stimulated by Fsk/IBMX or inhibited by H89 in the presence of STAD-2, indicating that STAD-2 selectively inhibits localized PKA activity. This effect was not evident when the pmAKAR4 reporter was tested with the STAD-2 scramble control.

Figure 5 shows that parent sequence peptides did not gain intracellular access. HeLa cells were treated with 5 μM 5(6)-carboxyfluorescein labeled peptides of the original parent sequences in either a non-stapled or stapled format. Cells were pretreated with peptides for 6 hrs before washing, fixation and imaging by fluorescence microscopy. As expected, none of the compounds tested demonstrated notable cell permeability.

Figure 6 shows fluorescence polarization of 1K library of peptides. Fluorescence polarization of the 1K Lys-modified peptide library was measured using purified protein constructs of the D/D domains from either PKA-RI or PKA-RII. Peptides were plated at a final concentration of 10 nM and the D/D dimerization domains were tested over a concentration range of 0.1 nM to 100 μM. Data was collected in triplicates for each concentration measurement.

Figure 7 shows fluorescence polarization of 2K library of peptides. Fluorescence polarization of the 2K Lys-modified peptide library was measured using purified protein constructs of the D/D domains from either PKA-RI or PKA-RII. Peptides were plated at a final concentration of 10 nM and the D/D dimerization domains were tested over a concentration range of 0.1 nM to 100 μ M. Data was collected in triplicates for each concentration measurement.

Figure 8 shows fluorescence polarization of 3K library of peptides. Fluorescence polarization of the 3K Lys-modified peptide library was measured using purified protein constructs of the D/D domains from either PKA-RI or PKA-RII. Peptides were plated at a final concentration of 10 nM and the D/D dimerization domains were tested over a concentration range of 0.1 nM to 100 μ M. Data was collected in triplicates for each concentration measurement.

Figure 9 shows cell permeability of STAD peptides in HeLa cells. Cell permeability of STAD-1, -2, and -3 are shown using HeLa cells at 40X magnification. Cells were treated with 5 μ M of 5(6)-carboxyfluorescein-labeled peptides for 6 hrs before imaging.

Figure 10 shows cell permeability of STAD peptides in MDA-MB-231 cells. Cell permeability of STAD-1, -2, and -3 are shown using MDA-MB-231 cells at 40X magnification. Cells were treated with 5 μ M of 5(6)-carboxyfluorescein-labeled peptides for 6 hrs before imaging.

Figure 11 shows cell permeability of STAD peptides in PC-3 cells. Cell permeability of STAD-1, -2, and -3 are shown using PC-3 cells at 40X magnification. Cells were treated with 5 μ M of 5(6)-carboxyfluorescein-labeled peptides for 6 hrs before imaging.

Figure 12 shows cell permeability of STAD scramble control peptides. Cell permeability of the scramble controls for STAD-1, -2, and -3 were tested in MDAMB-231 cells. Cells were treated with 5 μ M of 5(6)-carboxyfluorescein-labeled peptides for 6 hrs before imaging. All three peptides were found to gain intracellular access to the cytoplasm.

Figure 13 shows PKA response in STAD-2-treated cells using nuclear-excluded AKAR4. In Figure 13A, the PKA response in HeLa cells pretreated with STAD-2 was measured using an AKAR4 probe that is excluded from the nucleus (by introduction of an NES sequence). This implies that the signal in diffusible AKAR4 (Fig.4E) was not due to nuclear contributions. In Figure 13B, the PKA response is shown in HeLa cells lacking peptide pretreatment using the pmAKAR4 probe. PKA activity is enhanced in response to Fsk/IBMX stimulation, and is inhibited by treatment with H89.

Figures 14A and 14B show amphipathic helical structure of RI-specific AKAP. Figure 14A is a helical wheel presentation of RIAD in DNASTAR. The hydrophobic and hydrophilic surfaces are shown. Figure 14B illustrates α -helical peptide in the left panel. The hydrocarbon stapled on the water-exposing surface is shown. The right panel shows the stapled peptide binding on the surface of the docking/dimerization (D/D) domain of PKA-RI with its hydrophobic surface. Structure rendered in PyMol using PDB ID 3IM4.

Figures 15A to 15C show normalized fluorescence polarization (FP) binding spectra for each of the full-length PKA R-subunit isoforms with the indicated fluorescently labeled peptide STRIAD-1, -2 and -3. PKA-RI is represented by circles (closed circles = α , open circles = β), and PKA-RII is represented by triangles (closed triangles = α , open triangles = β). Peptides were plated at a final concentration of 4 nM and the PKA R-subunits were tested over a concentration range of 60 pM to 10 μ M.

Figures 16A to 16C show STRIAD selectively bind PKA-RI and disrupt AKAP-mediated type I PKA signaling in cells. Figures 16A and 16B show fluorescent images of MDA-MB-231 and PC-3 cells after treatment with FITC-labeled peptides (5 μ M) for 7 h or 21 h showing that oRIAD v3, v5 and v6 are cell-permeable. Each image is representative of three replicates. Figure 16C shows STRIAD-1 and STRIAD-2 immunoprecipitate with RI but not RII. MDA-MB-231 cell were treated with 5 μ M N-terminal biotin-labeled peptides for 12 h. Lysates were pulled down by incubation with avidin-coated resin, and PKA-RI and PKA-RII were detected by immunoblotting.

Figures 17A to 17H FP spectra of hydrocarbon stapled peptides. Fluorescence polarization of the peptides were measured using purified protein constructs of the D/D domains from either PKA-RI (circles) or PKA-RII (squares). Peptides were plated at a final concentration of 10 nM and the D/D dimerization domains were tested over a concentration range of 0.1 nM to 100 μ M. Data was collected in triplicates for each concentration measurement.

Figures 18A and 18B are images showing insufficient uptake of wt and v1 peptides. MDA-MB-231 cells treated with 5 μ M FITC-labeled wt (Fig. 18A) and v1 (Fig. 18B) for 21 h. No apparent uptake was observed for wt. v1 did not have an ideal uptake either. Each image is representative of three replicates.

Figure 19 is a graph showing FP competition spectrum with the negative control peptide of Ht31 (Ht31PP) is shown for the PKA-RI α with STRIAD-2 peptide. Ht31PP is not able to compete the interaction between the PKA R-subunit and STRIAD-2.

DETAILED DESCRIPTION

Disclosed are synthetically engineered peptides that can be used to probe kinase regulation and function in signaling in normal and disease state cells. In some embodiments, the peptides have a stabilized secondary structure. For example, the peptides can have α -helices stabilized using hydrocarbon peptide “stapling” (Schafmeister CE, et al. J Am Chem Soc. 2000 122(24):5891-2). This method integrates, in one synthetic unit, two structural elements that strongly stabilize and constrain an α -helical structure through α -methylation and macrocyclic ring formation (Mansuy D, et al. Med Sci (Paris). 2005 21(11):995-6). This stabilization of the secondary structure introduces an entropically favorable pre-ordered binding state where key interacting residues are spatially poised for target binding. Further, such conformationally-locked peptides exhibit drug-like properties including greatly enhanced cell permeability, increased binding affinity and resistance to cellular degradation (Walensky LD, et al. Science. 2004;305(5689):1466-70; Walensky LD, et al. Mol Cell. 2006;24(2):199-210; Bernal F, et al. J Am Chem Soc. 2007;129(9):2456-7; Moellering RE, et al. Nature. 2009 462(7270):182-8). Stapled peptides can be used to effectively target and disrupt protein-protein interactions since they provide an elongated binding surface that can bind to shallow protein surfaces. This methodology was used to develop synthetic peptides that can selectively manipulate protein:protein interactions in signaling pathways within a cellular environment.

The protein kinase superfamily comprises one of the largest gene families encoded in the human genome. Kinases play a major regulatory role in eukaryotic cells for diverse activities including cell growth, differentiation, and cell death (Taylor SS, et al. Biochim Biophys Acta. 2008 1784(1):16-26). Altered kinase activity has been directly linked to numerous cancer phenotypes. A comprehensive understanding of kinase activity under normal and disease states is critical in order to identify targets for disease intervention. While significant efforts have been put forth to develop kinase inhibitors/activators, success has been limited due to a variety of reasons including lack of specificity.

As a new targeting strategy for kinases, chemically stabilized, cell permeable, α -helical peptides that target protein interfaces are disclosed as tools for studying kinase regulation. Numerous protein:protein interactions are mediated via α -helical interactions including intracellular targeting, regulatory:catalytic subunit interactions, and kinase inhibition, as well as surface binding pocket interactions on the catalytic subunit. Engineered helices can be developed and applied broadly to diverse kinases in order to investigate specific aspects of kinase activity.

As a paradigm for studying kinase regulation, stapled peptides were used as a tool to extend understanding of the role of isoform-specific regulatory subunit targeting as it applies to protein kinase A (PKA) regulation. PKA has broad substrate specificity so hierarchical mechanisms exist to regulate its activity. One regulatory mechanism utilizes direct interactions between A Kinase Anchoring proteins (AKAPs) and PKA regulatory subunits to promote subcellular localization near specific target subsets. The significance of AKAP-mediated localization is underscored by the occurrence of familial breast cancer when a single amino acid polymorphism (SNP) in the PKA binding region (AKB) of D-AKAP2 is observed (Wirtenberger M, et al. *Carcinogenesis*. 2007 28(2):423-6). Regulation is further controlled through the utilization of four distinct regulatory subunit isoforms: type I (RI α and RI β) and type II (RII α and RII β) that differ in tissue distribution, cAMP sensitivity and AKAP-mediated localization, thereby allowing for finely tuned regulation of PKA activity.

In order to better understand the biological role of AKAP localization on PKA activity, isoform-specific peptides were identified from peptide array screening that could inhibit dual-specific AKAP 2 (D-AKAP2), an AKAP that was found to bind both type I and type II isoforms (Burns LL, et al. *Biochemistry*. 2003 42(19):5754-63). However, non-modified peptides are not readily cell permeable and prone to loss of secondary structure, thus limiting their potential as an investigative tool for cell-based studies. Chemically stabilized AKAP peptides provide an effective research tool that can be used intracellularly and temporally to study the isoform-specific effects of AKAP localization on PKA signaling, thereby providing tremendous insight into PKA signaling in breast cancer cells. Chemically stabilized AKAP peptides can also be applied towards AKAP-specific diseases as therapeutics or for diagnostics for spatiotemporal signaling by PKA and AKAP-regulated signaling in general. Chemically stabilized AKAP peptides could potentially be used in therapeutic settings when the AKAP involved in etiology of the disease is the only or predominantly expressed AKAP that has the same PKA-R isoform preference. For example, as most of the AKAPs in cells are RII-selective, if a disease is found involved with AKAP-mediated type I PKA signaling, that particular AKAP (RI- or dual-selective) could be the only AKAP in the cells that anchors PKA-RI. In this case, RI-selective AKAP peptides can be used to disrupt the interaction between that AKAP and RI and therefore intervene the signaling pathway that leads to the disease.

The protein kinase superfamily is one of the largest protein classes in the human genome and regulates key signaling processes that have been implicated in development and disease, including cancer (Lahiry P, et al. *Nat Rev Genet*. 2010 11(1):60-74). As such, it is of paramount

importance to identify the signaling and regulatory mechanism of kinases within their cellular environment in order to develop effective strategies for therapeutic disease intervention. Small molecule kinase modulators have become important therapeutic tools and often target catalytic domains that are among the most structurally and functionally conserved regions of these enzymes (Johnson LN. Q Rev Biophys. 2009 42(1):1-40). While these tools have provided tremendous insight into kinase activity, specificity has been difficult to achieve due to the high degree of structural conservation among kinases. Further, many protein:protein interactions that regulate kinase activity are mediated through α -helical interactions that are difficult to disrupt using small molecule approaches. As a means to circumvent these limitations, synthetically modified α -helices were developed for use as investigative tools to mediate protein:protein interaction events involved in kinase regulation (Figure 1). As an alternative to small molecule modulators, the disclosed synthetic peptides mediate larger protein surface interactions within a cellular environment, thereby providing specific temporal regulation of cell signaling events. The engineered helices can be applied more broadly to different types of kinase regulatory interactions by specifically targeting distinct interacting surfaces, and can also be applied to highly diverse kinases. This study provides insight into the link between AKAP-mediated kinase regulation and breast cancer.

A strategic method to bestow drug-like properties onto α -helical peptides was developed called peptide “stapling” (Schafmeister CE, et al. J Am Chem Soc. 2000 122(24):5891-2). This strategy involves the incorporation of two non-natural amino acids within the peptide sequence that are di-substituted to contain α -methyl and α -alkenyl groups. The peptide secondary structure is conformationally locked via Grubbs I catalyzed ring closing metathesis to form a macrocyclic ring using the α -alkenyl groups (Mansuy D, et al. Med Sci (Paris). 2005 21(11):995-6). Further studies have shown that this chemical modification introduces an entropically favorable pre-ordered binding state that increases substrate binding affinity, causes resistance to proteolytic degradation, and greatly enhances cell permeability (Schafmeister CE, et al. J Am Chem Soc. 2000 122(24):5891-2; Walensky LD, et al. Science. 2004 305(5689):1466-70; Walensky LD, et al. Mol Cell. 2006 24(2):199-210; Bernal F, et al. J Am Chem Soc. 2007 129(9):2456-7; Moellering RE, et al. Nature. 2009 462(7270):182-8). By applying this chemical modification to a peptide-based scaffold, large binding areas on protein surfaces can be targeted with a high degree of specificity that would otherwise be elusive for targeting using a small molecule approach.

“Peptide stapling” is a term coined from a synthetic methodology wherein two olefin-containing side-chains present in a polypeptide chain are covalently joined (e.g., “stapled together”) using a ring-closing metathesis (RCM) reaction to form a cross-linked ring (see, the cover art for J. Org. Chem. (2001) vol. 66, issue 16 describing metathesis-based crosslinking of alpha-helical peptides; Blackwell et al.; Angew Chem. Int. Ed. (1994) 37:3281). However, the term “peptide stapling,” as used herein, encompasses the joining of two double bond-containing side-chains, two triple bond-containing side-chains, or one double bond-containing and one triple bond-containing side chain, which may be present in a polypeptide chain, using any number of reaction conditions and/or catalysts to facilitate such a reaction, to provide a singly “stapled” polypeptide. Additionally, the term “peptide stitching,” as used herein, refers to multiple and tandem “stapling” events in a single polypeptide chain to provide a “stitched” (multiply stapled) polypeptide.

Non-natural, synthetic polypeptides are disclosed that contain a chemically stabilized α -helical shape that mimics the protein kinase A (PKA) binding sequence of an A Kinase Anchoring protein (AKAP), allowing them to bind to an endogenous protein kinase A-R subunit (PKA-R) in physiological, or supraphysiological, conditions and to inhibit the PKA-R from binding to an endogenous AKAP.

The disclosed polypeptides can contain a hydrocarbon staple to chemically stabilized α -helical shape. “Peptide stapling” is a term coined from a synthetic methodology wherein two olefin-containing side-chains present in a polypeptide chain are covalently joined (e.g., “stapled together”) using a ring-closing metathesis (RCM) reaction to form a cross-linked ring. However, the term “peptide stapling,” as used herein, encompasses the joining of two double bond-containing side-chains, two triple bond-containing side-chains, or one double bond-containing and one triple bond-containing side-chain, which may be present in a polypeptide chain, using any number of reaction conditions and/or catalysts to facilitate such a reaction, to provide a singly “stapled” polypeptide.

In some embodiments, the disclosed peptides include a hydrocarbon staple. The genesis of the hydrocarbon stapling technique can be traced to the ruthenium based Grubb’s catalysis used for ring closing metathesis. The α -helix features 3.6 residues per complete turn, which places the i , $i+4$, $i+7$, and $i+11$ side chains on the same face of the folded structure. Therefore, stapling cross-links two α,α disubstituted amino acids bearing olefinic chains of variable length at positions “ i ” and “ $i+4$ ” or “ $i+7$ ” in the peptide sequence. In general, the first step in designing stapled peptides for macromolecular target is the identification of appropriate sites for

incorporating the non natural amino acids used to form the hydrocarbon cross-link. Generally, residues which are not involved in the target recognition are chosen as potential sites for incorporation of olefin-bearing building blocks. These site are subsequently used to incorporate various suitable stapling systems such as i, i+3; i, i+4 or i, i+7. The classical strategy to stabilize the α -helical conformation in peptides employs covalent bonds between the i and i+3, i and i+4 or i and i+7 side chain groups.

In some embodiments, the polypeptide comprises two non-natural amino acids on the same side of the α -helix that are crosslinked to stabilize the α -helical shape. For example, the two non-natural amino acids can be four (i and i+4) or seven (i and i+7) amino acids apart. In some cases, the non-natural amino acids can comprise olefinic side chains, such as (S)-2-(2'-propenyl)alanine ("S3"), (S)-2-(4'-pentenyl)alanine ("S5"), (S)-2-(5'-hexenyl)alanine ("S6"), (S)-2-(7'-octenyl)alanine ("S8"), (R)-2-(2'-propenyl)alanine ("R3"), (R)-2-(4'-pentenyl)alanine ("R5"), (R)-2-(5'-hexenyl)alanine ("R6"), (R)-2-(7'-octenyl) alanine ("R8").

The disclosed peptides can be stapled in any suitable pairing, including, but not limited to, pairing selected from the group consisting of an S5-S5 pairing (i.e., i, i+4), an S5-R8 pairing (i.e., i, i+7), an S8-R5 pairing (i.e., i, i+7), an R3-S6 pairing (i.e., i, i+3), an R6-S3 pairing (i.e., i, i+3), an R3-S5 pairing (i.e., i, i+3), an R5-S3 pairing (i.e., i, i+3), or combinations of pairings within the polypeptide sequence.

The hydrocarbon bridge can then be formed by a ring-closing metathesis reaction catalyzed by benzyldienebis(tricyclohexyl-phosphine)-dichlororuthenium (Grubb's catalyst).

Stapling of a peptide using all-hydrocarbon cross-link has been shown to help maintain its native conformation and/or secondary structure, particularly under physiologically relevant conditions. For example, stapling a polypeptide by an all-hydrocarbon crosslink predisposed to have an alpha-helical secondary structure can constrain the polypeptide to its native alpha-helical conformation. The constrained secondary structure may, for example, increase the peptide's resistance to proteolytic cleavage, may increase the peptide's hydrophobicity, may allow for better penetration of the peptide into the target cell's membrane (e.g., through an energy-dependent transport mechanism such as pinocytosis), and/or may lead to an improvement in the peptide's biological activity relative to the corresponding uncrosslinked (e.g., "unstapled") peptide.

Other forms of chemical stabilization may also be used in the disclosed peptides. For example, amino acids, and unstapled, partially stapled, and stapled peptides and proteins, and unstitched, partially stitched, and stitched peptides and proteins) may exist in particular

geometric or stereoisomeric forms. The disclosed peptides can include all such compounds, including *cis*- and *trans*-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof. Where an isomer/enantiomer is preferred, it may, in some embodiments, be provided substantially free of the corresponding enantiomer, and may also be referred to as "optically enriched." "Optically enriched," as used herein, means that the compound is made up of a significantly greater proportion of one enantiomer. In certain embodiments the compound of the present invention is made up of at least about 90% by weight of a preferred enantiomer. In other embodiments the compound is made up of at least about 95%, 98%, or 99% by weight of a preferred enantiomer.

The polypeptide can be a synthetic peptide containing non-natural amino acids, or a peptidomimetic. As used herein, "peptidomimetic" means a mimetic of a peptide which includes some alteration of the normal peptide chemistry. Peptidomimetics typically enhance some property of the original peptide, such as increase stability, increased efficacy, enhanced delivery, increased half life, etc. Use of peptidomimetics can involve the incorporation of a non-amino acid residue with non-amide linkages at a given position. One embodiment of the present invention is a peptidomimetic wherein the compound has a bond, a peptide backbone or an amino acid component replaced with a suitable mimic. Some non-limiting examples of non-natural amino acids which may be suitable amino acid mimics include β -alanine, L- α -amino butyric acid, L- γ -amino butyric acid, L- α -amino isobutyric acid, L- ϵ -amino caproic acid, 7-amino heptanoic acid, L-aspartic acid, L-glutamic acid, N- ϵ -Boc-N- α -CBZ-L-lysine, N- ϵ -Boc-N- α -Fmoc-L-lysine, L-methionine sulfone, L-norleucine, L-norvaline, N- α -Boc-N- δ -CBZ-L-ornithine, N- δ -Boc-N- α -CBZ-L-ornithine, Boc-p-nitro-L-phenylalanine, Boc-hydroxyproline, and Boc-L-thioproline.

The disclosed compounds may also be substituted with any number of substituents or functional moieties. In general, the term "substituted" refers to the replacement of hydrogen radicals in a given structure with the radical of a specified substituent. When more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. As used herein, the term "substituted" is contemplated to include substitution with all permissible substituents of organic compounds, any of the substituents described herein (for example, aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, oxo, imino, thiooxo, cyano, isocyano, amino, azido, nitro, hydroxyl, thiol, halo, etc.), and any combination thereof (for example, aliphaticamino, heteroaliphaticamino, alkylamino, heteroalkylamino,

arylamino, heteroarylamino, alkylaryl, arylalkyl, aliphaticoxy, heteroaliphaticoxy, alkyloxy, heteroalkyloxy, aryloxy, heteroaryloxy, aliphaticthioxy, heteroaliphaticthioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroarylthioxy, acyloxy, and the like) that results in the formation of a stable moiety. The disclosed peptides can contain any and all such combinations in order to arrive at a stable substituent/moiety. For the disclosed peptides, heteroatoms such as nitrogen may have hydrogen substituents and/or any suitable substituent as described herein which satisfy the valencies of the heteroatoms and results in the formation of a stable moiety.

Peptides and peptidomimetics can be prepared by any method, such as by synthesizing the peptide or peptidomimetic, or by expressing a nucleic acid encoding an appropriate amino acid sequence in a cell and harvesting the peptide from the cell. Of course, a combination of such methods also can be used.

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Clz-Bzl (2,6-dichlorobenzyl) for the amino groups; NO₂ (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups; and tBu (t-butyl) for the hydroxyl groups). After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Methods of *de novo* synthesizing peptides and peptidomimetics are described, for example, in Chan et al., *Fmoc Solid Phase Peptide Synthesis*, Oxford University Press, Oxford, United Kingdom, 2005; *Peptide and Protein Drug Analysis*, ed. Reid, R., Marcel Dekker, Inc., 2000.

Alternatively, the peptide may be synthesized using recombinant techniques. In this case, a nucleic acid encoding the peptide is cloned into an expression vector under the control of expression control sequences (e.g. a promoter, a terminator and/or an enhancer) allowing its expression. The expression vector is then transfected into a host cell (e.g. a human, CHO, mouse, monkey, fungal or bacterial host cell), and the transfected host cell is cultivated under conditions suitable for the expression of the peptide. Standard recombinant DNA and molecular cloning

techniques are described for example in: Sambrook, and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Silhavy et al., Experiments with Gene Fusions, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1984); and, Ausubel et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

The method of producing the peptide may optionally comprise the steps of purifying said peptide, chemically modifying said peptide, and/or formulating said peptide into a pharmaceutical composition.

The polypeptide can be isoform specific. In some embodiments, the polypeptide is selective for PKA-R1 α , PKA-R1 β , or a combination thereof. For example, the polypeptide can comprise the amino acid sequence SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:115, or SEQ ID NO:116. In some embodiments, the polypeptide is selective for PKA-R2 α , PKA-R2 β , or a combination thereof. For example, the polypeptide can comprise the amino acid sequence SEQ ID NO:18, SEQ ID NO:28, or SEQ ID NO:35. As used herein, a polypeptide is “selective” for a receptor if it specifically binds one isoform of a receptor with a binding affinity that is at least 5X higher than its affinity for the other isoform. For example, the polypeptide can have a binding affinity for one isoform that is at least 5, 6, 7, 8, 9, 10, 20, or more than that of the other isoform.

In some embodiments, the polypeptide can bind both isoforms with high affinity.

In some embodiments, the peptide is about 15 to 100 amino acids in length, including about 15 to 50 amino acids in length. In some embodiments, the peptide is less than 51 amino acids in length, including less than 50, 45, 40, 35, 30, 25, or 20 amino acids in length. Therefore, the provided polypeptide can further constitute a fusion protein or otherwise have additional N-terminal, C-terminal, or intermediate amino acid sequences.

In some cases, introduction of a hydrocarbon staple results in poor water solubility and cell permeability. To increase cell permeability and solubility of these peptides, the disclosed polypeptide can be linked to a cell permeability moiety. A “cell permeability” or a “cell-penetration” moiety refers to any molecule known in the art which is able to facilitate or enhance penetration of molecules through membranes. Non-limitative examples include: hydrophobic moieties such as lipids, fatty acids, steroids and bulky aromatic or aliphatic compounds; moieties which may have cell-membrane receptors or carriers, such as steroids, vitamins and sugars, natural and non-natural amino acids and transporter peptides. Examples for lipidic moieties which may be used according to the present invention: Lipofectamine, Transfectace, Transfectam, Cytofectin, DMR1E, DLR1E, GAP-DLR1E, DOTAP, DOPE, DMEAP, DODMP,

DOPC, DDAB, DOSPA, EDLPC, EDMPC, DPH, TMADPH, CTAB, lysyl-PE, DC-Cho, -alanyl
 cholesterol; DCGS, DPPES, DCPE, DMAP, DMPE, DOGS, DOHME, DPEPC, Pluronic,
 Tween, BRIJ, plasmalogen, phosphatidylethanolamine, phosphatidylcholine, glycerol-3-
 ethylphosphatidylcholine, dimethyl ammonium propane, trimethyl ammonium propane,
 5 diethylammonium propane, triethylammonium propane, dimethyldioctadecylammonium
 bromide, a sphingolipid, sphingomyelin, a lysolipid, a glycolipid, a sulfatide, a
 glycosphingolipid, cholesterol, cholesterol ester, cholesterol salt, oil, N-
 succinyldioleoylphosphatidylethanolamine, 1,2-dioleoyl-sn-glycerol, 1,3-dipalmitoyl-2-
 succinylglycerol, 1,2-dipalmitoyl-sn-3'-succinylglycerol, 1-hexadecyl-2-
 10 palmitoylglycerophosphatidylethanolamine, palmitoylhomocystiene, N,N'-Bis
 (dodecylaminocarbonylmethylene)-N,N'-bis((-N,N,N-trimethylammoniummethyl-amino-
 carbonylmethylene)ethylenediamine tetraiodide; N5N"-
 Bis(hexadecylaminocarbonylmethylene)-N,N', N"-tris((-N,N,N-trimethylammonium-
 ethylaminocarbonylmethylenediethylenetriamine hexaiodide; N,N-
 15 Bis(dodecylaminocarbonylmethylene)-N,NM-bis((-N,N,N-trimethylammonium
 ethylaminocarbonylmethylene)cyclohexylene-1,4-diamine tetraiodide; 1,7,7-tetra-((-N,N,N,N-
 tetramethylammoniummethylamino-carbonylmethylene)-3-hexadecylaminocarbonyl-
 methylene-1,3,7-triazaheptane heptaoidide; N5N5N', N'-tetra((-N,N,N-trimethylammonium-
 ethylaminocarbonylmethylene)-N'- (152-dioleoylglycero-3-phosphoethanolamino
 20 carbonylmethylene)diethylenetriamine tetraiodide; dioleoylphosphatidylethanolamine, a fatty
 acid, a lysolipid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine,
 phosphatidylglycerol, phosphatidylinositol, a sphingolipid, a glycolipid, a glucolipid, a sulfatide,
 a glycosphingolipid, phosphatidic acid, palmitic acid, stearic acid, arachidonic acid, oleic acid, a
 lipid bearing a polymer, a lipid bearing a sulfonated saccharide, cholesterol, tocopherol
 25 hemisuccinate, a lipid with an ether-linked fatty acid, a lipid with an ester-linked fatty acid, a
 polymerized lipid, diacetyl phosphate, stearylamine, cardiolipin, a phospholipid with a fatty acid
 of 6-8 carbons in length, a phospholipid with asymmetric acyl chains, 6-(5-cholesten-3b-yloxy)-
 1-thio-b-D-galactopyranoside, digalactosyldiglyceride, 6-(5-cholesten-3b-yloxy)hexyl-6-amino-
 6-deoxy-1-thio-b-D-galactopyranoside, 6-(5-cholesten-3b-yloxy)hexyl-6-amino-6-deoxyl-1-
 30 thio-a-D-mannopyranoside, 12-(((7'-diethylamino-coumarin-3-yl)carbonyl)methylamino)-
 octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methyl-amino)
 octadecanoyl]-2-aminopalmitic acid; cholesteryl-4'-trimethyl-ammonio)butanoate; N-
 succinyldioleoyl-phosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1'-dipalmitoyl-sn-S-

succinyl-glycerol; 1,3-dipalmitoyl-2-succinylglycerol, 1-hexadecyl-2-palmitoylglycerophosphoethanolamine, and palmitoylhomocysteine.

In some embodiments, the disclosed polypeptide can be linked to a protein transduction domain to effectively enter a cell. The protein transduction domain sequence can be any internalization sequence known or newly discovered in the art, or conservative variants thereof. Non-limiting examples of cellular internalization transporters and sequences include Polyarginine (e.g., R₉), Antennapedia sequences, TAT, HIV-Tat, Penetratin, Antp-3A (Antp mutant), Buforin II, Transportan, MAP (model amphipathic peptide), K-FGF, Ku70, Prion, pVEC, Pep-1, SynB1, Pep-7, HN-1, BGSC (Bis-Guanidinium-Spermidine-Cholesterol, and BGTC (Bis-Guanidinium-Tren-Cholesterol).

Addition of water soluble polymers or carbohydrates to polypeptide drugs has been shown to prevent their degradation and increase their half-life. For instance, "PEGylation" of polypeptide drugs protects them and improves their pharmacodynamic and pharmacokinetic profiles. The PEGylation process attaches repeating units of polyethylene glycol (PEG) to a polypeptide drug. PEGylation of molecules can lead to increased resistance of drugs to enzymatic degradation, increased half-life in vivo, reduced dosing frequency, decreased immunogenicity, increased physical and thermal stability, increased solubility, increased liquid stability, and reduced aggregation. Therefore, in some embodiments, the disclosed polypeptide is covalently linked to a water soluble polymer, such as a polyethylene glycol.

The most common route for PEG conjugation of polypeptides has been to activate the PEG with functional groups suitable for reactions with lysine and N-terminal amino acid groups. The monofunctionality of methoxyPEG makes it particularly suitable for protein and peptide modification because it yields reactive PEGs that do not produce cross-linked polypeptides, as long as diol PEG has been removed. Branched structures of PEG have also been proven to be useful for PEGylation of a protein or a peptide. For example, a branched PEG attached to a protein has properties of a much larger molecule than a corresponding linear mPEG of the same molecular weight. Branched PEGs also have the advantage of adding two PEG chains per attachment site on the protein, therefore reducing the chance of protein inactivation due to attachment. Furthermore, these structures are more effective in protecting proteins from proteolysis, in reducing antigenicity, and in reducing immunogenicity.

To increase cell permeability and solubility of these peptides, the peptides can be optimized to increase their amphipathic properties. In some cases, an overall net charge (neutral or positive) is needed for permeability. Any method that alters the overall net charge can affect

permeability. In some cases, 1, 2, 3, 4, or more hydrophilic residues can be added on the solvent-exposed face of the helix. For example, the hydrophilic residue can be a lysine, aspartic acid, glutamic acid, arginine, histidine, serine, asparagine, or glutamine. In some cases, lysine and/or arginine is used since they have positive charges that help to increase permeability. Non-natural amino acids bearing hydrophilic or charged properties can also be added.

Pharmaceutical Compositions

Also disclosed is a pharmaceutical formulations, comprising any of the polypeptides disclosed herein in a pharmaceutically acceptable carrier. The disclosed polypeptides can be incorporated in the formulations described below as neutral compounds, pharmaceutically acceptable salts, and/or prodrugs. Pharmaceutical formulations can be designed for immediate release, sustained release, delayed release and/or burst release of one or more polypeptides in a therapeutically effective amount.

The compounds described herein can be formulated for parenteral administration. Parenteral formulations can be prepared as aqueous compositions using techniques is known in the art. Typically, such compositions can be prepared as injectable formulations, for example, solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a reconstitution medium prior to injection; emulsions, such as water-in-oil (w/o) emulsions, oil-in-water (o/w) emulsions, and microemulsions thereof, liposomes, or emulsomes.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, one or more polyols (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), oils, such as vegetable oils (e.g., peanut oil, corn oil, sesame oil, etc.), and combinations thereof.

Solutions and dispersions of the active compounds as the free acid or base or pharmacologically acceptable salts thereof can be prepared in water or another solvent or dispersing medium suitably mixed with one or more pharmaceutically acceptable excipients including, but not limited to, surfactants, dispersants, emulsifiers, pH modifying agents, and combination thereof.

Suitable surfactants may be anionic, cationic, amphoteric or nonionic surface active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions. Examples of anionic surfactants include sodium, potassium, ammonium of long chain alkyl sulfonates and alkyl aryl sulfonates such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium bis-(2-ethylthioxyl)-sulfosuccinate; and alkyl sulfates such as sodium lauryl sulfate. Cationic surfactants include, but are not limited

to, quaternary ammonium compounds such as benzalkonium chloride, benzethonium chloride, cetrimonium bromide, stearyl dimethylbenzyl ammonium chloride, polyoxyethylene and coconut amine. Examples of nonionic surfactants include ethylene glycol monostearate, propylene glycol myristate, glyceryl monostearate, glyceryl stearate, polyglyceryl-4-oleate, sorbitan acylate, sucrose acylate, PEG-150 laurate, PEG-400 monolaurate, polyoxyethylene monolaurate, polysorbates, polyoxyethylene octylphenylether, PEG-1000 cetyl ether, polyoxyethylene tridecyl ether, polypropylene glycol butyl ether, Poloxamer[®] 401, stearyl monoisopropanolamide, and polyoxyethylene hydrogenated tallow amide. Examples of amphoteric surfactants include sodium N-dodecyl- β -alanine, sodium N-lauryl- β -iminodipropionate, myristoamphoacetate, lauryl betaine and lauryl sulfobetaine.

The formulation can contain a preservative to prevent the growth of microorganisms. Suitable preservatives include, but are not limited to, parabens, chlorobutanol, phenol, sorbic acid, and thimerosal. The formulation may also contain an antioxidant to prevent degradation of the active agent(s).

The formulation is typically buffered to a pH of 3-8 for parenteral administration upon reconstitution. Suitable buffers include, but are not limited to, phosphate buffers, acetate buffers, and citrate buffers.

Water soluble polymers are often used in formulations for parenteral administration. Suitable water-soluble polymers include, but are not limited to, polyvinylpyrrolidone, dextran, carboxymethylcellulose, and polyethylene glycol.

Sterile injectable solutions can be prepared by incorporating the active compounds in the required amount in the appropriate solvent or dispersion medium with one or more of the excipients listed above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those listed above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The powders can be prepared in such a manner that the particles are porous in nature, which can increase dissolution of the particles. Methods for making porous particles are well known in the art.

The parenteral formulations described herein can be formulated for controlled release including immediate release, delayed release, extended release, pulsatile release, and

combinations thereof. For parenteral administration, the compounds, and optionally one or more additional active agents, can be incorporated into microparticles, nanoparticles, or combinations thereof that provide controlled release. For example, the compounds and/or one or more additional active agents can be incorporated into polymeric microparticles which provide controlled release of the drug(s). Release of the drug(s) is controlled by diffusion of the drug(s) out of the microparticles and/or degradation of the polymeric particles by hydrolysis and/or enzymatic degradation. Suitable polymers include ethylcellulose and other natural or synthetic cellulose derivatives.

Polymers which are slowly soluble and form a gel in an aqueous environment, such as hydroxypropyl methylcellulose or polyethylene oxide may also be suitable as materials for drug containing microparticles. Other polymers include, but are not limited to, polyanhydrides, poly(ester anhydrides), polyhydroxy acids, such as polylactide (PLA), polyglycolide (PGA), poly(lactide-co-glycolide) (PLGA), poly-3-hydroxybutyrate (PHB) and copolymers thereof, poly-4-hydroxybutyrate (P4HB) and copolymers thereof, polycaprolactone and copolymers thereof, and combinations thereof.

The polypeptide can also be formulated for depot injection. In a depot injection, the active agent is formulated with one or more pharmaceutically acceptable carriers that provide for the gradual release of active agent over a period of hours or days after injection. The depot formulation can be administered by any suitable means; however, the depot formulation is typically administered via subcutaneous or intramuscular injection. A variety of carriers may be incorporated into the depot formulation to provide for the controlled release of the active agent. In some cases, depot formulations contain one or more biodegradable polymeric or oligomeric carriers. Suitable polymeric carriers include, but are not limited to poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid)-polyethyleneglycol (PLA-PEG) block copolymers, polyanhydrides, poly(ester anhydrides), polyglycolide (PGA), poly-3-hydroxybutyrate (PHB) and copolymers thereof, poly-4-hydroxybutyrate (P4HB), polycaprolactone, cellulose, hydroxypropyl methylcellulose, ethylcellulose, as well as blends, derivatives, copolymers, and combinations thereof. In depot formulations containing a polymeric or oligomeric carrier, the carrier and active agent can be formulated as a solution, an emulsion, or suspension. One or more neuroactive steroids, and optionally one or more additional active agents, can also be incorporated into polymeric or oligomeric microparticles, nanoparticles, or combinations thereof.

Formulations may also be in the form of an organogel (assuming the compound steroid is relatively water insoluble) or a hydrogel. Numerous gel formulations are known. See, for example, U.S. Patent No. 5,411,737 by Hsu, et al. Hydrogels, especially those further including nanoparticles microparticles for sustained, immediate and/or delayed release, can also be used.

5 Oral pharmaceutical dosage forms are either solid, gel or liquid. The solid dosage forms are tablets, capsules, granules, and bulk powders. Types of oral tablets include compressed, chewable lozenges and tablets which may be enteric-coated, sugar-coated or film-coated. Capsules may be hard or soft gelatin capsules, while granules and powders may be provided in non-effervescent or effervescent form with the combination of other ingredients known to those skilled in the art.

10 The compounds may be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Topical administration is contemplated for transdermal delivery and also for administration to the eyes or mucosa, or for inhalation therapies. Nasal solutions of the active compound alone or in combination with other pharmaceutically acceptable excipients can also be administered. These solutions, particularly those intended for ophthalmic use, may be formulated as 0.01% - 10% isotonic solutions, pH about 5-7, with appropriate salts.

20 Other routes of administration, such as transdermal patches, including iontophoretic and electrophoretic devices, vaginal and rectal administration, are also contemplated herein. Transdermal patches, including iontophoretic and electrophoretic devices, are well known to those of skill in the art. For example, pharmaceutical dosage forms for rectal administration are rectal suppositories, capsules and tablets for systemic effect. Rectal suppositories are used herein mean solid bodies for insertion into the rectum which melt or soften at body temperature releasing one or more pharmacologically or therapeutically active ingredients. Pharmaceutically acceptable substances utilized in rectal suppositories are bases or vehicles and agents to raise the melting point. Examples of bases include cocoa butter (theobroma oil), glycerin-gelatin, carbowax (polyoxyethylene glycol) and appropriate mixtures of mono-, di- and triglycerides of fatty acids. Combinations of the various bases may be used. Agents to raise the melting point of suppositories include spermaceti and wax. Rectal suppositories may be prepared either by the compressed method or by molding. The weight of a rectal suppository, in one embodiment, is about 2 to 3 g.

The disclosed polypeptides can also be administered adjunctively with other active compounds such as analgesics, anti-inflammatory drugs, antipyretics, antiepileptics, antihistamines, antimigraine drugs, antimuscarinics, anxiolytics, sedatives, hypnotics, antipsychotics, bronchodilators, anti asthma drugs, cardiovascular drugs, corticosteroids, dopaminergics, electrolytes, parasympathomimetics, stimulants, anorectics and anti-narcoleptics.

Methods

Also disclosed is a method for inhibiting binding of an AKAP to a PKA-R. This method can involve contacting PKA-R in physiological conditions with a polypeptide disclosed herein.

Also disclosed is a method for treating a disease associated with AKAP activity. This method can involve administering to the subject a therapeutically effective amount of a pharmaceutical composition containing a polypeptide disclosed herein. Tight spatial and temporal regulation of cAMP signaling is of fundamental importance for many physiological processes. This is particularly evident as altered cAMP signaling is associated with or causes a variety of pathological cellular responses involved in endocrinological, nephrological, neurodegenerative, cardiovascular and immune diseases and several types of cancer. In many instances, the diseases do not involve changes in global cAMP/PKA signaling, but often involve malfunctions of specific AKAP complexes. The following description of the role of AKAPs in disease is reproduced from Tröger J, et al. Br J Pharmacol. 2012 166(2): 420–433.

Direct evidence for the involvement of distinct AKAPs in cardiac diseases comes from genetic polymorphisms and knockout studies. A single nucleotide polymorphism (SNP) in Yotiao, S1570L, reduces its interaction with KCNQ1, thereby altering the repolarization of cardiac myocytes in the human heart and causes long-QT syndrome. An SNP in the PKA-binding domain of D-AKAP2 (AKAP10), I646V, can cause shortening of the PR interval of the cardiac cycle, elevated resting heart rate and diminished heart rate variability, which are markers that predict an increased risk of sudden cardiac death. In line with this, mutant mice lacking the last 51 amino acids of the D-AKAP2 gene display cardiac arrhythmia and die prematurely. Disregulation of AKAPs and their interactions are also associated with chronic heart failure. In human failing hearts there is an increase in the interaction between PKA and the AKAPs SPHKAP and AKAP2 (sixfold each), AKAP18 (more than twofold) and MAP2 (12-fold), whereas there is a decrease in the interactions of PKA with AKAP1 by 50% and with Yotiao by 15% of the normal level.

Neurological disorders including Alzheimer's disease, seizure, mental retardation and drug addiction are usually accompanied by perturbations in the plasticity of excitatory

glutamatergic synapses, that is, the inability to modulate the strength of synaptic transmission. In the hippocampus, two forms of altered synaptic strength have been intensively studied, long-term potentiation and long-term depression (LTD). Two major players involved in these changes are the glutamate receptors, NMDA and AMPA receptors. Phosphorylation by PKA and other kinases modulates the activity of glutamate receptors and thereby the depolarization of the post-synaptic neurons. The most prominent AKAP involved in regulation of synaptic plasticity is AKAP5 (AKAP79/150), which contributes to NMDA receptor-mediated LTD. AKAP5-deficient mice showed altered synaptic transmission and exhibited deficiencies in neuronal processes including motor coordination. Mice expressing an AKAP5 mutant that lacks the PKA binding domain showed even stronger defects in synaptic plasticity and learning processes.

Several AKAPs are expressed in the male and female reproductive systems. Investigations have particularly focused on the roles of D-AKAP1, WAVE1, AKAP3 and AKAP4 in reproduction. The maturation of oocytes is controlled by AKAP–PKA interactions. They participate in the maintenance of meiotic arrest. The resumption of oocyte maturation has been suggested to involve AKAP1. Knockout studies in mice revealed that the complete absence of AKAP1 leads to infertility of females with maturation defective ovaries, whereas the fertility of male animals was not affected. During fertilization of the oocyte, WAVE1 re-localizes to the nuclear envelope, which is accompanied by redistribution of PKA type II and the tyrosine kinase Abl. Alterations in the localization of WAVE1 perturb the normal progression of fertilization, which might represent a possible link to the early developmental defects in flies observed upon mutations in the WAVE/Scar gene. In males, AKAP–PKA interactions are crucial for sperm motility. AKAPs involved in this process are the sperm-specific AKAP3 and AKAP4, both located in the fibrous sheath. Evidence for a role of AKAP4 in sperm motility was provided by gene knockout in mice, which resulted in reduced motility of sperm and infertile male animals. In line, the abundance of AKAP4 correlates with sperm motility. Evidence for the involvement of AKAP3 in the regulation of sperm motility comes from studies analyzing the stimulating effect of bicarbonate. Bicarbonate activates soluble adenylyl cyclase and thereby triggers a signaling cascade, which evokes tyrosine phosphorylation of AKAP3 resulting in enhanced anchoring of PKA by AKAP3.

The activation of T cells by the T cell receptor (TCR) is an important step in cellular immune responses. PKA type I, the predominant variant in these cells, is anchored in lipid rafts by the dual-specific AKAP ezrin. Ezrin anchors PKA in close proximity to the TCR/CD3 complex. Activation of PKA by cAMP suppresses T cell replication and maintains T cells in a

resting state. The underlying mechanism involves phosphorylation of the C-terminal Src kinase (Csk) by PKA, which increases Csk activity, inhibiting activity of downstream Src kinases and ultimately preventing T cell activation. Ezrin forms a protein complex containing PKA, ezrin/radixin/ moesin binding protein of 50 kDa, phosphoprotein associated with glycosphingolipid-enriched microdomains and Csk suggesting a tight spatiotemporal control of PKA signaling in this context. HIV-1 infection has been associated with increased levels of cAMP and enhanced activation of PKA. Studies on immune responses in HIV patients revealed that increased activation of PKA type I contributes to T cell dysfunction, and inhibition of PKA had beneficial effects on T cell proliferation. In a murine AIDS model, specific disruption of AKAP-PKA type I complexes with the RI-anchoring disruptor peptide RIAD causes resistance of T cells to retrovirus-induced immunodeficiency which is most likely evoked by perturbations in the PKA/ezrin/Csk pathway. Besides ezrin, D-AKAP1 is also involved in the progression of HIV infection. D-AKAP1 binds HIV reverse transcriptase and, in the manner of a cofactor, supports reverse transcription during HIV infection.

Diabetes mellitus is a disease caused by insulin deficiency (type I diabetes) or by an initial insulin resistance and consequent insufficient insulin secretion (type II diabetes). This leads to impaired glucose metabolism and, ultimately, to diabetes mellitus, which may be associated with diabetic nephropathy, polyneuropathy, retinopathy, and cardiovascular complications including atherosclerosis and heart failure. Inhibition of AKAP-PKA interactions with the PKA anchoring disruptor Ht31 diminished insulin secretion from a rat insulinoma cell line and isolated rat pancreatic islets. AKAPs involved in insulin release are AKAP150 (AKAP5), AKAP18 α and AKAP18 γ . Their specific functions seem to be different. Overexpression of AKAP18 α in rat pancreatic β cells significantly increases glucagon-like peptide 1-mediated insulin secretion. AKAP18 γ has the opposite effect. Silencing studies confirmed a decrease in glucose-stimulated insulin release upon AKAP18 α depletion and an increase in the case of AKAP18 γ depletion. This is consistent with their respective regulations by glucose. AKAP150 interacts with PKA and calcineurin (protein phosphatase 2B), whereby it coordinates the reversible phosphorylation of PKA targets involved in insulin exocytosis. AKAP150 interacts with the GTPase IQGAP1, which is involved in the control of the cytoskeleton. This interaction may play a role in the transport of insulin-bearing vesicles. Hence, pharmacological targeting of specific AKAP-PKA complexes has the potential for the development of new medication for the treatment of diabetes mellitus. However, this example

also highlights the necessity of specifically targeting defined AKAP–PKA pools rather than global interference with these interactions.

Differential regulation of AKAPs is involved in a variety of human cancers. One example is gravin. It is a tumor suppressor protein involved in the regulation of the cell cycle and cell migration. Down-regulation of SSeCKS/gravin/AKAP12 is observed in a number of tumors including radiation-induced osteoblastoma, breast, ovary and prostate cancer. Accordingly, re-expression of SSeCKS in prostate cancer cells was shown to suppress tumorigenesis and to cause inhibition of metastasis. A likely explanation for reduced expression of gravin orthologues is hypermethylation of the promoter region of AKAP12 occurring in a variety of human cancers. In Addition, the AKAP12 gene is located in a hot spot region, which is deleted in prostate, breast and ovary cancers. AKAP4 (also termed AKAP82) is a testis-specific AKAP, with a pivotal role in sperm motility and thus male fertility. Recently, AKAP4 has been classified as a cancer testis antigen (CTA), which is strongly expressed in multiple myeloma. SNPs of several AKAPs are associated with an increased risk for the development of breast cancer [including AKAP9, AKAP-Lbc and D-AKAP2. The SNP A2073G in D-AKAP2 results in the amino acid substitution I646V, which is located in the PKA-binding domain of the protein. This substitution alters PKA binding in an isoform-specific manner: the binding of RII α or RII β seems not to be affected, whereas the valine variant exhibits a threefold stronger interaction with RI α , probably causing altered subcellular distribution of PKA type I.

The herein disclosed compositions, including pharmaceutical composition, may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. For example, the disclosed compositions can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. The compositions may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, ophthalmically, vaginally, rectally, intranasally, topically or the like, including topical intranasal administration or administration by inhalant.

In some embodiments, the disclosed polypeptide is administered in a dose equivalent to parenteral administration of about 0.1 ng to about 100 g per kg of body weight, about 10 ng to about 50 g per kg of body weight, about 100 ng to about 1 g per kg of body weight, from about 1 μ g to about 100 mg per kg of body weight, from about 1 μ g to about 50 mg per kg of body weight, from about 1 mg to about 500 mg per kg of body weight; and from about 1 mg to about 50 mg per kg of body weight. Alternatively, the amount of polypeptide administered to achieve a

therapeutic effective dose is about 0.1 ng, 1 ng, 10 ng, 100 ng, 1 µg, 10 µg, 100 µg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg, 16 mg, 17 mg, 18 mg, 19 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg, 100 mg, 500 mg per kg of body weight or greater.

Definitions

The term “subject” refers to any individual who is the target of administration or treatment. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human or veterinary patient. The term “patient” refers to a subject under the treatment of a clinician, e.g., physician.

The term “therapeutically effective” refers to the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

The term “pharmaceutically acceptable” refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

The term “inhibit” refers to a decrease in an activity, response, condition, disease, or other biological parameter. This can include but is not limited to the complete ablation of the activity, response, condition, or disease. This may also include, for example, a 10% reduction in the activity, response, condition, or disease as compared to the native or control level. Thus, the reduction can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between as compared to native or control levels.

The terms “peptide,” “protein,” “polypeptide,” “polyamino acid,” are used interchangeably to refer to a natural or synthetic molecule comprising two or more amino acids linked by the carboxyl group of one amino acid to the alpha amino group of another. In addition, as used herein, the term “polypeptide” refers to amino acids joined to each other by peptide bonds or modified peptide bonds, e.g., peptide isosteres, etc. and may contain modified amino acids other than the 20 gene-encoded amino acids. The polypeptides can be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. The same type of modification can be present in the same or varying degrees at several sites in a

given polypeptide. Also, a given polypeptide can have many types of modifications.

Modifications include, without limitation, acetylation, acylation, ADP-ribosylation, amidation, covalent cross-linking or cyclization, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphatidylinositol, disulfide bond formation, demethylation, formation of cysteine or pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pergylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. Also included in the term “polypeptides” are cis- and trans-isomers, R- and S-enantiomers, D-isomers, L-isomers, and racemic mixtures.

The term “residue” as used herein refers to an amino acid that is incorporated into a polypeptide. The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

The term “treatment” refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

EXAMPLES

Example 1: Synthesis and biophysical characterization of chemically-stabilized, isoform-specific peptides that inhibit D-AKAP2 interactions.

Panels of isoform-specific peptides were synthesized using Fmoc chemistry on Rink Amide MBHA resin (Novabiochem). Residues that are required for R-subunit interactions (denoted by arrows) were excluded from modifications. Synthesis of the required α -methyl, α -

alkenyl amino acids was previously reported (Schafmeister CE, et al. J Am Chem Soc. 2000 122(24):5891-2). This synthetic route was applied to generate the required amino acids. For the i, i+4 staples, S,S stereochemistries were used. For the i, i+7 staples, the S,R configuration was used, as this is the only synthetically accessible configuration. Sequences for the synthesized proteins are provided in Table 1 and sequences for modified peptides are shown in Table 2.

Table 1. Stapled Peptide Library

Peptide	SEQUENCE	SEQ ID NO	RI D/D	RII D/D
RI-wt	FEELAWKIAKMIWSDVF	SEQ ID NO:41		
RI-V1	FE*LAW*IAKMIWSDVF	SEQ ID NO:42		
RI-V2	FEELAW*IAK*IWSDVF	SEQ ID NO:43		
RI-V3	FEELAWKIAK*IWS*VF	SEQ ID NO:44		
RI-V4	FEELAWKIAKMIW*DVF*	SEQ ID NO:45		
RII-wt	EELLWKIAKMIVSDVM	SEQ ID NO:46		
RII-V1	E*LLW*IAKMIVSDVM	SEQ ID NO:47		
RII-V2	EELLW*IAK*IVSDVM	SEQ ID NO:48		
RII-V3	EELLWKIAK*IVS*VM	SEQ ID NO:49		
RII-V4	EELLWKIAKMIV*DVM*	SEQ ID NO:50		
SKIP-wt	DFAEELADTVVSMATEIAAI	SEQ ID NO:51	3435	18114
SKIP-V1	DFAE*LAD*VVSMATEIAAI	SEQ ID NO:52	22335	429.1
SKIP-V2	DFAEELAD*VVS*ATEIAAI	SEQ ID NO:53	15573	218.9
SKIP-V3	DFAEELADTVVS*ATE*AAI	SEQ ID NO:54	nc	22332
SKIP-V4	DFAEELADTVVSMAT*IAA*	SEQ ID NO:55	19691	9466
RIAD-wt	LEQYANQLADQIIKEATE	SEQ ID NO:56	5152	605.1
RIAD-V1	LE*YAN*LADQIIKEATE	SEQ ID NO:57	790.6	105.3
RIAD-V2	LEQYAN*LAD*IIKEATE	SEQ ID NO:58	353.1	122.8
RIAD-V3	LEQYANQLAD*IIK*ATE	SEQ ID NO:59	240.1	2.037
RIAD-V4	LEQYANQLADQIIK*ATE*	SEQ ID NO:60	14822	232.5
hsmAKAP-wt	VILEYAHRLSQDILCDALQQ	SEQ ID NO:61	13111	4352
hsmAKAP-V1	VIL*YAH*LSQDILCDALQQ	SEQ ID NO:62	1895	357.3
hsmAKAP-V2	VILEYAH*LSQ*ILCDALQQ	SEQ ID NO:63	78.74	266.6
hsmAKAP-V3	VILEYAHRLSQ*ILC*ALQQ	SEQ ID NO:64	410.5	29.7
hsmAKAP-V4	VILEYAHRLSQDIL*DAL*Q	SEQ ID NO:65	7062	3498
AKAP220-wt	RAISGLANFLVSEALSNALK	SEQ ID NO:66	130803	22.39
AKAP220-V1	RAIS*LAN*LVSEALSNALK	SEQ ID NO:67	11636	28.65
AKAP220-V2	RAISGLAN*LVS*ALSNALK	SEQ ID NO:68	16095	28.65
AKAP220-V3	RAISGLANFLVS*ALS*ALK	SEQ ID NO:69	12340	6.193
AKAP220-V4	RAISGLANFLVSEAL*NAL*	SEQ ID NO:70	ns	ns
zsmAKAP-wt	YAQRLSEEIVRAVQQWA	SEQ ID NO:71	17915	6320
zsmAKAP-V1	*YAQ*LSEEIVRAVQQWA	SEQ ID NO:72	1205	197.3
zsmAKAP-V2	YAQ*LSE*IVRAVQQWA	SEQ ID NO:73	7555	859.3
zsmAKAP-V3	YAQRLS*EIV*AVQQWA	SEQ ID NO:74	37390	355.7
zsmAKAP-V4	YAQRLSEEIV*AVQ*WA	SEQ ID NO:75	11012	819.3
zsmAKAP-V5	YAQRLSEEIVRAV*QWA*	SEQ ID NO:76	1364	2.051

* is an abbreviations for S α -methyl, α -alkenyl cross-linking amino acid.

Methionine is replaced by norleucine in actual synthesis.

ns = not soluble

Table 2. Chemically-stabilized, isoform-specific peptides

RI-wt	HHLAWKIAK&IWSDFV	SEQ ID NO:1
RI-V1	H*LAW*I AK&IWSDFV	SEQ ID NO:2
RI-V2	HHLAW*I AK*IWSDFV	SEQ ID NO:3
RI-V3	HHLAWKIAK*IWS*VF	SEQ ID NO:4
RI-V4	HHLAWKIAK&IW*DVF*	SEQ ID NO:5
RII-wt	HHLLWKIAK&IVSDV	SEQ ID NO:6
RII-V1	H*LLW*I AK&IVSDV	SEQ ID NO:7
RII-V2	HHLLW*I AK*IVSDV	SEQ ID NO:8
RII-V3	HHLLWKIAK*IVS*V	SEQ ID NO:9
SKIP-wt	KKLAKTVVS&ATKIAAIAIKK	SEQ ID NO:10
SKIP-V1	K*LAK*VVS&ATKIAAIAIKK	SEQ ID NO:11
SKIP-V2	KKLAK*VVS*ATKIAAIAIKK	SEQ ID NO:12
SKIP-V3	KKLAKTVVS*ATK*AAIAIKK	SEQ ID NO:13
SKIP-V4	KKLAKTVVS&AT*I AA*KK	SEQ ID NO:14
RIAD-wt	KKYAKQLADQIIKEATE	SEQ ID NO:15
RIAD-V1	K*YAK*LADQIIKEATE	SEQ ID NO:16
RIAD-V2	KKYAK*LAD*I I KEATE	SEQ ID NO:17
RIAD-V3	HKYANQLAD*I IK*ATE	SEQ ID NO:18
RIAD-V4	KKYAKQLADQII*I EAT*	SEQ ID NO:19
smAKAP-wt	KKYAKRLSQDILKDALQQ	SEQ ID NO:20
smAKAP-V1	K*YAK*LSQDILKDALQQ	SEQ ID NO:21
smAKAP-V2	KKYAK*LSQ*ILKDALQQ	SEQ ID NO:22
smAKAP-V3	KKYAHRLSQ*ILK*ALQQ	SEQ ID NO:23
smAKAP-V4	KKYAKRLSQDIL*DAL*Q	SEQ ID NO:24
AKAP220-wt	KKLAKFLVSEALKNALK	SEQ ID NO:25
AKAP220-V1	K*LAK*LVSEALKNALK	SEQ ID NO:26
AKAP220-V2	KKLAK*LVS*ALKNALK	SEQ ID NO:27
AKAP220-V3	HKLANFLVS*ALS*ALK	SEQ ID NO:28
AKAP220-V4	KKLAKFLVSEAL*NAL*	SEQ ID NO:29
mmAKAP-wt	KKYAQRLSKKIVRAVQQWA	SEQ ID NO:30
mmAKAP-V1	K*YAQ*LSKKIVRAVQQWA	SEQ ID NO:31
mmAKAP-V2	KKYAQ*LSK*IVRAVQQWA	SEQ ID NO:32
mmAKAP-V3	KKYAQRLS*KIV*AVQQWA	SEQ ID NO:33
mmAKAP-V4	KKYAQRLSKKIV*AVQ*WA	SEQ ID NO:34
mmAKAP-V5	KKYAQRLSKKIVRAV*QWA*	SEQ ID NO:35
oRIAD-wt	PEG-LEQYANQLADQIIKEATE	SEQ ID NO:36
oRIAD-v1	PEG-LE*YAN*LADQIIKEATE	SEQ ID NO:37
oRIAD-v1-2	PEG-LE*YAN*LADQIIKEATEK	SEQ ID NO:38
oRIAD-v2	PEG-LEQYAN*LAD*I I KEATE	SEQ ID NO:39
oRIAD-v3	PEG-LEQYANQLAD*I IK*ATE	SEQ ID NO:40

* is an abbreviations for α -methyl, α -alkenyl cross-linking amino acid.

& is an abbreviation for norleucine.

In order to identify high affinity, isoform-specific R-subunit binders, biochemical characterization was performed. Isomer specificity and K_m values were measured by

fluorescence polarization experiments using purified R subunit isomers. Peptides were plated at 10 nM concentrations and treated with either RI α or RII α (ranging from 10 μ M to 0.1 nM) and incubated for 30-60 minutes before measuring fluorescence polarization. Dissociation constants for select peptides are provided in Table 3.

5

Table 3. Chemically-stabilized, isoform-specific peptides

Peptide	SEQUENCE	SEQ ID NO	RI(nM)	RII(nM)
Modified				
SKIP-wt	KKLAKTVVS&ATKIAAIKK	SEQ ID NO:10	3435	18114
SKIP-V1	K*LAK*VVS&ATKIAAIKK	SEQ ID NO:11	22335	429.1
SKIP-V2	KKLAK*VVS*ATKIAAIKK	SEQ ID NO:12	15573	218.9
SKIP-V3	KKLAKTVVS*ATK*AAIKK	SEQ ID NO:13	-	22332
SKIP-V4	KKLAKTVVS&AT*IAA*KK	SEQ ID NO:14	19691	9466
Original				
SKIP-wt	Peg-ITDFAEELADTVVSMATEIAA	SEQ ID NO:77	-	-
SKIP-V1	Peg-I*DFA*ELADTVVSMATEIAA	SEQ ID NO:78	-	-
SKIP-V2	Peg-ITDFAE*LAD*VVSMATEIAA	SEQ ID NO:79	-	-
SKIP-V3	Peg-ITDFAEELAD*VVS*ATEIAA	SEQ ID NO:80	72.51	3.621
SKIP-V4	Peg-ITDFAEELADTVVS*ATE*AA	SEQ ID NO:81	-	-
SKIP-V5	Peg-ITDFAEELADTVVSMAT*IAA*	SEQ ID NO:82	-	-
Align 1				
SKIP-wt	Peg-ITKFAEKLAKTIVVK&ATKIAA	SEQ ID NO:83	nt	nt
SKIP-V1	Peg-IT*FAE*LAKTVVK&ATKIAA	SEQ ID NO:84	61.96	10.62
SKIP-V2	Peg-ITKFAEKLA*TVV*&ATKIAA	SEQ ID NO:85	99.99	4.106
SKIP-V3	Peg-IT*FAE*LAKTVV*&AT*IAA	SEQ ID NO:86	37.95	5.616
Align 2				
SKIP-wt	Peg-ITDFAEKLADKVVK&ATKIAAK	SEQ ID NO:87	nt	nt
SKIP-V1	Peg-ITDFAE*LAD*VVK&ATKIAAK	SEQ ID NO:88	6.707	8.461
SKIP-V2	Peg-ITDFAEKLADKVV*&AT*IAAK	SEQ ID NO:89	nt	nt
SKIP-V3	Peg-ITDFAEKLADKVVK&AT*IAA*	SEQ ID NO:90	35.9	0.233

* is an abbreviations for α -methyl, α -alkenyl cross-linking amino acid.

& is an abbreviation for norleucine.

nt = not tested

Toxicity was assessed by treating the various cell lines with a panel of peptides in a concentration range of 0.05 μ M to 5.0 μ M for 6 hours. Cytotoxicity was determined using the MTT assay using the CellTiter Non-Radioactive Cell Proliferation Assay (Promega).

10 Concentrations that cause greater than 10% cell death were eliminated from future experimental studies. Once synthesis was complete, the classes of chemically stabilized peptides were purified by RP-HPLC and structurally characterized.

Two versions of stapled peptides were synthesized for each isoform and were biophysically characterized. In all cases, the peptide secondary structure was enhanced as compared to their non-modified counterparts. Preliminary studies on a subset of peptides demonstrate that stapled versions of the AKAP peptides are cell permeable and have enhanced resistance to proteolytic degradation relative to the non-modified versions.

Helical content and thermal melting behavior was assessed by circular dichroism (CD) spectropolarimetry. Cell permeability was measured using FITC-labeled versions of each peptide and assessed using confocal microscopy.

Example 2: Isoform-Selective Disruption of AKAP-Localized PKA Using Hydrocarbon Stapled Peptides

Hydrocarbon peptide stapling was used to develop isoform-selective AKAP disruptors. This chemical modification constrains the secondary structure of α -helices through α -methylation and macrocyclic ring formation (Figure 1C) (Verdine, G. L., et al. (2012) *Methods Enzymol.* 503:3–33). Further, this modification was found to increase the proteolytic stability of the peptide while also making it more entropically favorable for binding by locking it in a prebinding state (Verdine, G. L., et al. (2007) *Clin. Can. Res.* 13:7264–7270). As a strategy to disrupt AKAP interactions, focus was on the conserved docking (AKB) helix that is shared among AKAPs. The AKB binds to the docking/dimerization (D/D) domain of PKA-R that is formed at the PKA-R dimer interface (Burns-Hamuro, L. L., et al. (2005) *Protein Sci.* 14:2982–2992). Crystallographic studies show that interactions between the amphipathic AKB peptides and the D/D domain of either PKA-RI or PKA-RII are predominantly driven by hydrophobic interactions (Kinderman, F. S., et al. (2006) *Mol. Cell* 24:397–408; Sarma, G. N., et al. (2010) *Structure* 18:155–166). Many AKB or AKB-like sequences have been previously identified; however, the majority of these sequences are highly hydrophobic and therefore are limited in their potential as biochemical tools. Three intrinsically more hydrophilic sequences that target the AKB binding were chosen and used as templates for generating hydrocarbon stapled peptide inhibitors: RIAD, AKAP220, and small membrane AKAP (smAKAP) (Table 4). In addition, the non-modified AKB sequences inherently have specificity for either PKA-RI or PKA-RII, thereby providing a basis for PKA-R isoform selectivity (Figure 1B). Non-natural olefinic amino acids ((S)-2-(4'-pentenyl)alanine), abbreviated as S5, were introduced into the peptide sequences in the i, i + 4 positions (Figure 1C). The olefinic amino acids were covalently crosslinked using ring-closing metathesis chemistry (Schafmeister, C. E., et al. (2000) *J. Am.*

Chem. Soc. 122:5891–5892; Blackwell, H. E., et al. (2001) J. Org. Chem. 66:5291–5302).

Libraries were generated where N- and C-terminal truncations were made to shorten the AKB sequence while preserving the hydrophobic residues of the binding interface (Figure 2, parent sequences). The hydrocarbon staples were introduced into various positions of the sequence by introducing the non-natural amino acids into positions on the solvent-exposed face of the helix. However, after introduction of the hydrocarbon staple into the parent sequences, these peptides had poor water solubility and therefore demonstrated minimal cell permeability (Figure 5). To remedy the limited cell permeability and solubility of these peptides, the AKB peptide mimics were optimized to increase their amphipathic properties through the addition of hydrophilic Lys residues on the solvent-exposed face of the helix (Figure 2, Lys-modified sequences). In addition, a short (PEG)₃ group was added to the N-terminus of the Lys-modified sequences to further improve water solubility.

Table 4. Library of parent sequence stapled peptides lacking the addition of Lys residues or an N-terminal PEG3 group

Template	Peptide	Parent Sequence Libraries	
RIAD	1-wt	EQYANQLADQIIKEATE	SEQ ID NO:91
	1-1	E*YAN*LADQIIKEATE	SEQ ID NO:92
	1-2	EQYAN*LAD*IIKEATE	SEQ ID NO:93
	1-3	EQYANQLAD*IIK*ATE	SEQ ID NO:94
	1-4	EQYANQLADQII*EAT*	SEQ ID NO:95
AKAP220	2-wt	SGLANFLVSEALSNALK	SEQ ID NO:96
	2-1	S*LAN*LVSEALSNALK	SEQ ID NO:97
	2-2	SGLAN*LVS*ALSNALK	SEQ ID NO:98
	2-3	SGLANFLVS*ALS*ALK	SEQ ID NO:99
smAKAP	3-wt	LYAQRLSEEIVRAVQQWA	SEQ ID NO:100
	3-1	*YAQ*LSEEIVRAVQQWA	SEQ ID NO:101
	3-2	LYAQ*LSE*IVRAVQQWA	SEQ ID NO:102
	3-3	LYAQRLS*EIV*AVQQWA	SEQ ID NO:103
	3-4	LYAQRLSEEIV*AVQ*WA	SEQ ID NO:104
	3-5	LYAQRLSEEIVRAV*QWA*	SEQ ID NO:105
* = S5 or α -methyl, α -alkenyl cross-linking amino acid.			

Next, the binding affinities of the Lys-modified stapled peptides were measured using fluorescence polarization (FP) assays. Peptides were screened against the D/D domains of either RI α or RII α (Table 5, Figures 6–10). The Lys-modified sequence lacking the addition of a hydrocarbon staple for each sequence was used as a control. Of the stapled peptides tested, none had an appreciable binding affinity for the PKA-RI subunit. Although the unmodified, original

sequence of RIAD and smAKAP both demonstrate preferential binding to PKA-RI (Burgers, P. P., et al. (2012) J. Biol. Chem. 31:31; Carlson, C. R., et al. (2006) J. Biol. Chem. 281:21535–21545), the chemically modified peptides are not as inherently flexible and therefore may have altered binding properties including their entropic and enthalpic properties.

Nevertheless, multiple candidates were found that were highly selective for PKA-RII binding. Indeed, almost all of the peptides bearing a hydrocarbon staple at various positions were found to increase the binding affinity for PKA-RII α . Among all of the Lys-modified peptides tested, three were found that demonstrated K_D values in the low nM range: 1K-3 (2 nM), 2K-3 (6.2 nM), and 3K-5 (2.1 nM). Further, 1K-3 showed weak binding of PKA-RI α in the submicromolar range, while 2K-3 and 3K-5 showed no appreciable binding affinities to PKA-RI α . These three promising candidates for highly selective disruption of PKA-RII were subsequently renamed Stapled Anchoring Disruptors (STADs; 1K-3 is STAD-1, 2K-3 is STAD-2, and 3K-5 is STAD-3). Stapled scrambled controls were also examined for each STAD peptide.

Fluorescence polarization assays of the Lys-modified peptide libraries were determined using purified protein constructs of the D/D domains from either PKA-RI or PKA-RII (Table 5). Peptides were plated at a final concentration of 10 nM, and the D/D dimerization domains were tested over a concentration range of 0.1 nM to 100 μ M. Dissociation constants were calculated using nonlinear regression and are presented as mean $\pm$ standard error of triplicates (Table 5). 1K-3 (STAD-1), 2K-3 (STAD-2), and 3K-5 (STAD-3) were identified as peptides with low K_D values for PKA-RII and were highly selective for PKA-RII over PKA-RI.

Table 5. Stapled peptides highly selective for the PKA-RII isoform.

Peptide	Sequence		K_D		
			RI α (nM)	RII α (nM)	
1K-wt	KKY AKQ LADQ II KEATE	SEQ ID NO:15	>1000	605 $\pm$ 190	STAD-1 wt
1K-1	K*Y AK *LADQ II KEATE	SEQ ID NO:16	791 $\pm$ 237	105 $\pm$ 34	
1K-2	KKY AK *LAD* II KEATE	SEQ ID NO:17	353 $\pm$ 193	123 $\pm$ 62	
1K-3	KKY AKQ LAD* IIK *ATE	SEQ ID NO:18	240 $\pm$ 56	2.0 $\pm$ 0.8	STAD-1
1K-4	KKY AKQ LADQ II *EAT*	SEQ ID NO:19	> 1000	232 $\pm$ 53	
1K-3-scr	AA EDYK IKI *LKT*Q AK	SEQ ID NO:106	> 1000	> 1000	
2K-wt	KK LAK FLVSE ALKN ALK	SEQ ID NO:25	> 1000	22.4 $\pm$ 6.1	STAD-2 wt
2K-1	K* LAK *LVSE ALKN ALK	SEQ ID NO:26	> 1000	17.6 $\pm$ 7.0	
2K-2	KK LAK *LVS* ALKN ALK	SEQ ID NO:27	> 1000	28.7 $\pm$ 9.8	
2K-3	KK LAK FLVS* ALK * ALK	SEQ ID NO:28	> 1000	6.2 $\pm$ 2.0	STAD-2
2K-3-scr	KALV KLAAL *KFK* LKS	SEQ ID NO:107	> 1000	> 1000	
3K-wt	KKY AQR LSKK IVRAV QQWA	SEQ ID NO:30	> 1000	> 1000	STAD-3 wt
3K-1	K*Y AQ *LSKK IVRAV QQWA	SEQ ID NO:31	> 1000	197 $\pm$ 110	
3K-2	KKY AQ *LSK* IVRAV QQWA	SEQ ID NO:32	> 1000	> 1000	

3K-3	KKY A QRLS*KIV* AV QQWA	SEQ ID NO:33	> 1000	356 ± 189	
3K-4	KKY A QRLSKKIV* AV Q*WA	SEQ ID NO:34	> 1000	819 ± 383	
3K-5	KKY A QRLSKKIVR AV *QWA*	SEQ ID NO:35	> 1000	2.1 ± 6	STAD-3
3K-5-scr	R V QK I VLRWKY AA SQ*K AK *	SEQ ID NO:108	> 1000	> 1000	
* = S5 or α-methyl, α-alkenyl cross-linking amino acid.					

Next, the K_D values were measured for the three STAD peptides using full-length constructs human PKA-R (RI α , RI β , RII α , and RII β) since this would provide a more relevant portrayal of binding affinities and selectivity in the context of human cells (Table 6, Figures 3A to 3C). Each of the isoforms was purified as previously described (Bertinetti, D., et al. (2009) BMC Chem. Biol. 9:3) and tested over a concentration range from 0.1 nM to 15 μ M. While all three peptides were found to have K_D values of 50 nM or less for PKA- RII α , STAD-1 also had a comparable affinity for PKARI α (93 nM). However, STAD-2 and STAD-3 interacted more weakly with PKA-RI α with STAD-2 having a K_D value of greater than 1 μ M and STAD-3 having a value of 144 nM. STAD-3 had the lowest K_D values for PKA-RII (8 nM for RII α and 16 nM for RII β). However, STAD-2 has a slightly reduced affinity compared to STAD-3 for PKA-RII (31 nm for RII α versus 64 nM for RII β) but has higher PKA-RII selectivity since STAD-2 binding to PKA-RII α is approximately 40 times more favorable than for PKA-RI α . Thus, it appears that STAD-2 and STAD-3 have the most pronounced isoform selectivity against full-length human constructs of PKA-RII by approximately 1–2 orders of magnitude as compared to PKA-RI.

Fluorescence polarization was measured using full-length human proteins for each PKA-R isoform (Table 6). Each single FP experiment was performed in triplicate. While all three peptides tested bound to PKA-RII α with a K_D value of 50 nM or less, STAD-2 and STAD-3 appear to have the greatest selectivity for PKA-RII binding over PKA-RI.

Table 6. K_D values using full-length human PKA-R constructs			
Peptide	K_D (nM)		
	1K-3 (STAD-1)	2K-3 (STAD-2)	3K-5 (STAD-3)
hRI α	93 ± 19 (n=5)	1,300 ± 122 (n=4)	144 ± 16 (n=4)
hRI β	131 ± 8 (n=4)	522 ± 52 (n=5)	97 ± 17 (n=6)
hRII α	49 ± 12 (n=4)	31 ± 5 (n=4)	8 ± 1 (n=5)
hRII β	153 ± 11 (n=3)	64 ± 4 (n=3)	16 ± 2 (n=5)

To test the cellular uptake of these compounds, three highly diverse human cell lines (HeLa, MDA-MB-231, and PC-3 cells) were treated with 5 μ M 5(6)-carboxyfluorescein-labeled

peptides for 6 h before washing and fixation (Figures 4A and 9–11). While the non-stapled wild type control peptides for each peptide class were virtually impermeable to each of the cell types, STAD-1, STAD-2, and STAD-3 showed considerable intracellular access in all three cell lines. Further, although some punctate staining is evident, likely indicating intracellular localization in vesicles, particularly for STAD-3, there is a considerable amount of peptide localized in the cytoplasm that would therefore be accessible to AKAP-PKA complexes. Of note, the original and stapled parent sequences were not cell-permeable even after addition of a hydrocarbon staple (Figure 5); however, the stapled STADs and their scramble control peptides were all cell-permeable (Figures 4A and 12). These observations indicate that addition of Lys residues on the hydrophilic face of the peptide promoted cellular uptake of the peptide sequences.

Since the peptides appeared to have appreciable cytoplasmic localization, experiments were conducted to determine whether they were physically interacting with PKA-R within the intracellular environment. In order to test this, immunoprecipitation assays were performed using MDA-MB-231 cells (Figure 4B). Biotinylated STAD peptides (1 μ M) were added to the cell media 1 h before lysis. Cells that were not peptide-treated were used as a control. Pull-downs were performed, followed by immunoblotting for either PKA-RI or PKA-RII. It is clear that both STAD-2 and STAD-3 interact with PKA-RII, while STAD-1 was found to weakly associate with PKA-RII in cells. None of the peptides appear to have any affinity for PKA-RI within cells. This experiment confirms that the STAD peptides are highly RII-selective even within the context of a cellular environment.

To determine whether the STAD peptides can effectively block PKA signaling in cells, PKA substrate phosphorylation was monitored in cells. MDA-MB-231 cells were serum-starved overnight, followed by pretreatment with different concentrations of STAD peptides for 1 h prior to stimulation with 50 μ M forskolin (Fsk) to increase cAMP levels. Serum-starvation was performed to downregulate PKA activity so that a robust activation of PKA could be detected upon stimulation of intracellular cAMP levels in the presence or absence of the STAD peptides. PKA activity was measured as a function of substrate phosphorylation using the antiphospho-(Ser/Thr) PKA substrate antibody to detect phosphorylated PKA substrates in MDA-231 cells (Figure 4C). As a control, the small molecule inhibitor H89 (50 μ M) was used to inhibit PKA-C activity. Phosphorylation of CREB was also independently monitored since this is a known AKAP-mediated substrate of PKA.²⁹ All three STAD peptides decreased phosphorylation of various PKA substrates in a dose-dependent manner as compared to the forskolin-stimulated positive control. However, STAD-2 and STAD-3 appear to be more effective at inhibiting

substrate phosphorylation as well as reducing phospho-CREB levels in these cell-based assays. Furthermore, the effect on substrate phosphorylation is not universal, but rather some substrates are more impacted than others, most notably under the lower 4 μM treatment conditions. This suggests that phosphorylation of substrates that are regulated by signaling complexes involving AKAPs and PKA-RII are disproportionately reduced. To confirm that the peptide sequences are critical for targeted disruption of AKAP signaling complexes, scrambled versions of each STAD peptide were tested using the same assay. All three scrambled peptides had no apparent inhibitory effect on PKA signaling as measured by PKA substrate phosphorylation as well as phospho-CREB levels (Figure 4D). Taken together, these results suggest that STAD-2 and STAD-3 can be effectively localized within cells and can selectively disrupt AKAP-regulated signaling involving PKA-RII.

As a means of measuring the effects of the STAD peptides on AKAP-anchored versus non-anchored PKA activity, cytosolic PKA activity was probed using the diffusible biosensor AKAR4 in HeLa cells (Depry, C., et al. (2011) *Mol. BioSyst.* 7(1):52–58) (Figures 4E and 4F). Cells treated with STAD-2 responded to Fsk (50 μM)/IBMX (100 μM) stimulation with a $15 \pm 6\%$ ($n = 3$) increase in yellow to cyan emission ratio, compared to a $39 \pm 3\%$ ($n = 9$) response from cells treated with the scramble control peptide. Using biosensor pmAKAR4 (targeted by a CAAX sequence), however, STAD-2 could completely inhibit the subpool of PKA located at plasma membrane as compared to a non-peptide-treated control (Figures 4G and 13). The scramble STAD-2 control peptide did not alter the plasma membrane (Figure 4H) PKA responses in HeLa cells. In summary, conformationally constrained, cell-permeable peptides were developed that are highly selective for disruption of the interactions between AKAPs and PKA-RII. By conformationally constraining these AKAP inhibitor peptides, the binding interface is spatially poised to interact with the D/D of PKA-RII while also decreasing susceptibility to proteolytic degradation (Verdine, G. L., et al. (2012) *Methods Enzymol.* 503:3–33). While AKAPs are important regulators of cAMP-mediated signaling in cells, there are still many unknowns regarding their roles in normal and disease-state signaling. This novel class of isoform-selective peptides targeting the AKAP binding site on PKA-R can be utilized as effective tools to selectively disrupt localized signaling complexes mediated by interactions between AKAPs and PKA-RII and block downstream signaling in normal and disease-state cells.

Methods

Materials. The N- α -Fmoc protected amino acids and Rink Amide MBHA Resin were purchased from Novabiochem. (S)-N-Fmoc-2-(4'-pentenyl)alanine was purchased from Okeanos Tech. All other reagents and organic solvents used in this study were purchased from Fisher Scientific except where noted. HPLC grade methanol, acetonitrile, and trifluoroacetic acid were used for all solutions involving preparation or analysis of samples.

Cell Culture. MDA-MB-231 and PC-3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI) Medium with L-glutamine (Lonza), 10% fetal bovine serum (Thermo Scientific), and penicillin/streptomycin (Amresco). HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with glucose and L-glutamine (Lonza), 10% fetal bovine serum (Thermo Scientific), and penicillin/streptomycin (Amresco).

Peptide Synthesis. Peptides were synthesized on Rink Amide MBHA resin using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis. Deprotection steps were performed using a 25% (v/v) solution of piperidine in 1-methyl-2-pyrrolidinone (NMP) for 30 min. For each coupling step, 10 equiv of N- α -Fmoc-protected amino acids (0.25 M final concentration in NMP) were added, followed by addition of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, 0.23 M final concentration) in NMP and 8% (v/v) N,N-diisopropyl ethylamine (DIEA).

Olefin metathesis was performed using 0.4 equiv bis-(tricyclohexylphosphine) benzylidene ruthenium(IV) dichloride (Grubbs' first generation catalyst, Sigma Aldrich) relative to resin substitution. The reaction was performed in 1,2-dichloroethane at RT for 1 h with agitation. The reaction was repeated once more using the same conditions to ensure complete conversion to the cyclized product. 11-Amino-3,6,9-trioxaundecanoic acid (NH-PEG3-CH₂COOH, ChemPep Inc.) was added to the N-terminus of all of the Lys-modified sequences and their scramble controls. The PEG₃ group was introduced using standard coupling conditions with 4 equivs before the addition of biotin or 5(6)-carboxyfluorescein. N-Terminal fluorescein labeling was performed using 2 equiv of 5(6)-carboxyfluorescein (Acros Organics) along with 0.046 M HCTU and 2% (v/v) DIEA in N,N-dimethylformamide (DMF) overnight. N-Terminal biotin labeling was performed using 10 equiv of D-biotin (Anaspec), 0.14 M HCTU, and 4% (v/v) DIEA in a 1:1 mixture of DMF and dimethyl sulfoxide (DMSO) overnight. Completed peptides were cleaved from resin using 95% trifluoroacetic acid, 2.5% water, and 2.5% of triisopropylsilane (Sigma Aldrich) for 4–5 h, precipitated in methyl-tert-butyl ether at 4 °C, and lyophilized. All peptides were purified by high-performance liquid chromatography (HPLC) and

verified by mass spectrometry (MS). Fluorescein-labeled peptides were quantified by measuring absorbance of 5(6)-carboxyfluorescein at 495 nm using a Synergy 2 microplate reader (Bio-Tek). Biotin-labeled peptides were quantified by measuring decreased absorbance of the 2-hydroxyazobenzen-4'-carboxylic acid (HABA)-avidin complex (VWR) at 500 nm.

The molecular weights of the purified peptides are as follows: 1K-wt = 2537.4 (expected mass = 2537.8); 1K-1 = 2531.4 (expected mass = 2531.9); 1K-2 = 2531.4 (expected mass = 2531.9); 1K-3 (STAD-1) = 2530.5 (expected mass = 2531.0); 1K-4 = 2530.5 (expected mass = 2530.9); 1K-3-scr (STAD-1 scr) = 2516.1 (expected mass = 2516.9); 2K-wt = 2461.2 (expected mass = 2461.9); 2K-1 = 2436.3 (expected mass = 2436.9); 2K-2 = 2435.1 (expected mass = 2436.0); 2K-3 (STAD-2) = 2454.0 (expected mass = 2455.0); 2K-3-scr (STAD-2 scr) = 2454.0 (expected mass = 2455.0); 3K-wt = 2862.0 (expected mass = 2862.3); 3K-1 = 2827.5 (expected mass = 2828.3); 3K-2 = 2827.5 (expected mass = 2828.3); 3K-3 = 2827.2 (expected mass = 2828.3); 3K-4 = 2828.4 (expected mass = 2828.4); 3K-5 (STAD-3) = 2984.1 (expected mass = 2984.5); and 3K-5-scr (STAD-3 scr) = 2983.4 (expected mass = 2984.5).

Protein Expression and Purification. The RI α docking/dimerization (D/D) domain (residues 1–61) of *Bos taurus* and the RII α D/D (1–44) of *Rattus norvegicus* were expressed as previously described (Kinderman, F. S., et al. (2006) Mol. Cell 24:397–408; Banky, P., et al. (2000) J. Biol. Chem. 275:35146–35152). RI α D/D or RII α D/D cells were suspended and lysed in buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, and 0.1 mM phenylmethanesulfonylfluoride (PMSF) before purification. The protein constructs were purified using a Talon cobalt-affinity resin (Clontech). Cobalt-purified proteins underwent further purification using a Superdex 75 (10 mm $\times$ 300 mm) size exclusion column (AKTA) on an AKTA Purifier UPC 10 (AKTA). Proteins were concentrated using Vivaspins 6 columns with a 3 kDa molecular weight cutoff (GE Healthcare). Proteins were concentrated, and 20% glycerol was added before being snap frozen in liquid nitrogen and stored at -80°C .

Expression and Purification of Recombinant PKA-R Subunits. Recombinant human PKA regulatory subunits (hRI α , hRI β , hRII α , hRII β) were expressed and purified as previously described using Sp-8-AEA-cAMPS agarose (Bertinetti, D., et al. (2009) BMC Chem. Biol. 9:3). SDS-polyacrylamide gel electrophoresis was used to monitor protein expression and purity. Typically, the recombinant proteins were purified to $\geq 95\%$ homogeneity. Fluorescence Polarization Using D/D Domain Constructs. Fluorescence polarization (FP) assays were used to measure the binding affinity of designed peptides to the D/D domain of the PKA regulatory subunit isoforms. Each fluorescein-labeled peptide (10 nM) was plated with either RI α D/D or

RII α D/D. The protein constructs were 10-fold serially diluted from 100 μ M to 0.1 nM in 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20. The plates were incubated in the dark at RT for 30 min. Fluorescence polarization was measured in triplicate using a Synergy 2 microplate reader (Biotek). Binding curves were generated, and dissociation constants (K_D) were calculated from the nonlinear regression curve using GraphPad Prism.

Fluorescence Polarization Using Full Length PKA-R. To investigate the binding affinity of AKAP peptides to the full-length regulatory subunits of PKA, FP was applied in a direct assay format. Increasing concentrations (from 0.1 nM to 15 μ M) of the four different PKA regulatory subunits were mixed with 5–10 nM fluorescently labeled AKAP peptide in 20 mM MOPS pH 7, 150 mM NaCl, 0.005% (v/v) CHAPS. Data were obtained using a FusionTM alpha-FP plate reader at RT and a data acquisition of 2 s at Ex 485 nm/Em 535 nm in a 384 well microtiterplate (Perkin-Elmer Optiplate, black). Data represent the mean $\pm$ standard error of the mean of triplicate measurements (n = 3 per data point) for a single experiment. K_D determination was performed as described above.

Cell Permeability Assays. HeLa, MDA-MB-231 or PC-3 cells per well were seeded at 100,000 cells/well on 8-well tissue culture slides (BD Biosciences). Cells were grown overnight in medium with 10% fetal bovine serum. Next, 5 μ M 5(6)-carboxyfluorescein-labeled peptides were added and incubated at 37 °C for 6 h before fixation in 2% paraformaldehyde. Slides were imaged using an Olympus X71 fluorescent microscope.

Immunoprecipitation Assays. MDA-MB-231 cells were pretreated with 1 μ M biotin-labeled peptides before being lysed in NP-40 buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA). Lysates were incubated with 50 μ L immobilized avidin resin (G-Biosciences) overnight at 4 °C. The resin was collected by centrifugation at 1000*g for 2 min, washed three times with NP-40 buffer and boiled in Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) at 95 °C for 5 min. PKA-RI (1:500, BD Biosciences) and RII (1:1500, Abcam) antibodies were used for Western blot detection. Antirabbit IRDye 800CW (1:25,000) and anti-mouse IRDye 680LT(1:30,000) secondary antibodies were used (LI-COR Biosciences). Blots were imaged using an Odyssey Fc imaging system (LI-COR Biosciences).

Detection of Phosphorylated PKA Substrates. MDA-MB-231 cells were grown on 12-well culture plate. Cells were serum-starved for 24 h in serum-free RPMI media with glutamine (0.3 g/L). Peptides were added to cell at 2, 4, or 8 μ M concentrations for 1 h, followed by stimulation with 50 μ M forskolin for 10 min. As a control, cells were treated with H89 (50 μ M)

for 30 min prior to forskolin stimulation. Cells were lysed in Laemmli sample buffer and analyzed by Western blotting. Anti-phosphoserine/threonine PKA substrate (1:1000, Cell Signaling Technology) or tubulin (1:2000, DSHB) primary antibodies were used, followed by anti-rabbit IRDye 800CW (1:25,000) or anti-mouse IRDye 680LT secondary antibodies
 5 (1:30,000) (LI-COR Biosciences). Blots were imaged using an Odyssey Fc imaging system.

AKAR Reporter Assays. The HeLa cells utilized for these experiments were between passages 60 and 61. Cells were maintained in DMEM growth media supplemented with 10% FBS and 1% penicillin and streptomycin. They were transfected with the appropriate biosensor at an approximate confluency of 70% using Lipofectamine 2000 reagent and incubated for 24 h.
 10 Prior to imaging, cells were pretreated with 5 μ M active or control peptides at 37 °C in DMEM for 6 h. They were then imaged in HBSS buffer supplemented with the corresponding peptide at RT.

Epifluorescence imaging was performed on a Zeiss Axiovert 200 M Microscope equipped with a xenon lamp and a cooled CCD, under a 40X oil immersion objective. FRET
 15 microscopy of CFP/YFP biosensors was performed using the following excitation/emission filter combinations (bandwidths in nm): CFP: Ex 420/20, Em 475/40; YFP: Ex 495/10, Em 535/25; FRET: Ex 420/20, Em 535/25. All epifluorescence experiments were subsequently analyzed using the MetaFluor software. All cells were analyzed, including those with visible blebbing or other morphological defects. Such cells typically present problems such as biosensor leakage and
 20 did not accurately reflect activity. They were therefore rejected from reporting. The reported FRET ratio is calculated as follows and normalized with respect to the first frame in the time series (I = intensity):

$$\frac{I_{FRET} - I_{FRET,background}}{I_{CFP} - I_{CFP,background}}$$

Example 2:

25 Table 7 shows sequence alignment of several RI- and RII-specific PKA-binding helix of natural AKAPs or artificial AKAP previously developed by other labs. The four hydrophobic registers shared by all sequences are bolded. The aromatic residues shared by RI-specific sequences are underlined. This is an example of RI- versus RII-specific sequences derived from the docking helix of AKAPs.

Table 7 .			
RII-specific	AKAP-Lbc	GAD L IEE AA SR IV DA V IEQ	SEQ ID NO:109
	AKAP95	PEE VA AD VL AE VI TA AV RA	SEQ ID NO:110

	AKAP79	YET LL IE TASSLV KN AI QL	SEQ ID NO:111
RI-specific	RIAD	LEQ YANQLADQII KEATE-	SEQ ID NO:112
	AKAP-CE	LYQ FADRFS EL VI SE AL NH	SEQ ID NO:113
	SKIP	ITD FAEELADTVV SMATEI	SEQ ID NO:114
	smAKAP	ILE Y AH RLS QD ILCDAL QQ	SEQ ID NO:115

Figures 14A and 14B show amphipathic helical structure of RI-specific AKAP. Figure 14A is a helical wheel presentation of RIAD in DNASTAR. The hydrophobic and hydrophilic surfaces are shown. Figure 14B illustrates α -helical peptide in the left panel. The hydrocarbon stapled on the water-exposing surface is shown. The right panel shows the stapled peptide binding on the surface of the docking/dimerization (D/D) domain of PKA-RI with its hydrophobic surface. Structure rendered in PyMol using PDB ID 3IM4.

Stapled RI anchoring disruptor (STRIAD) peptides were developed (Table 8). Fluorescence polarization was used to determine the dissociation constant of the peptides and purified protein constructs of the D/D domains from either PKA-RI or PKA-RII. Final concentration of peptides is 10 nM, and the protein concentration ranges from 0.1 nM to 100uM. Dissociation constants were calculated using nonlinear regression and are presented as mean $\pm$ standard error of triplicates (Table 8).

Table 8. Stapled RI anchoring disruptor (STRIAD) peptides					
			K_D		
			RI α	RII α	
wt:	LEQYANQLADQIIKEATE	SEQ ID NO:56	22.70 $\pm$ 5.7	105.9 $\pm$ 31.5	
v1	LE*YAN*LADQIIKEATE	SEQ ID NO:57	12.1 $\pm$ 3.1	34.6 $\pm$ 8.1	
v2	LEQYAN*LAD*IIKEATE	SEQ ID NO:58	15.6 $\pm$ 5.6	11.7 $\pm$ 6.0	
v3	LEQYANQLAD*IIK*ATE	SEQ ID NO:59	1.5 $\pm$ 0.2	2.6 $\pm$ 1.4	STRIAD-1
v4	LEQYANQLADQIIK*ATE*	SEQ ID NO:60	34.1 $\pm$ 11.0	17.78 $\pm$ 5.8	
v1-1	L*QYA*QLADQIIKEATEK	SEQ ID NO:116	0.3 $\pm$ 0.2	5.1 $\pm$ 1.5	STRIAD-2
v1-2	LE*YAN*LADQIIKEATEK	SEQ ID NO:117	22.49 $\pm$ 10.9	245.6 $\pm$ 42.1	STRIAD-3
v1-3	LEQ*ANQ*ADQIIKEATEK	SEQ ID NO:118	>1000	48.5 $\pm$ 20.8	
* = S5 or α -methyl, α -alkenyl cross-linking amino acid.					

Figures 15A to 15C show normalized fluorescence polarization (FP) binding spectra for each of the full-length PKA R-subunit isoforms with the indicated fluorescently labeled peptide STRIAD-1, -2 and -3. PKA-RI is represented by circles (closed circles = α , open circles = β), and PKA-RII is represented by triangles (closed triangles = α , open triangles = β). Peptides were plated at a final concentration of 4 nM and the PKA R-subunits were tested over a concentration range of 60 pM to 10 μ M.

Table 9 shows Comparison of K_D values of FP binding assays. STRIAD-2 and STRIAD-3 have higher selectivity for PKA-RI over PKA-RII. Figure 15D shows FP competition spectra are shown for the PKA-RI α with the three indicated STRIAD peptides. The assay was performed with a final concentration of 4 nM STRIAD and 5 nM of the RI α . The competitive peptide Ht31 was tested over a concentration range of 15 nM to 30 μ M. Table 10 shows the apparent EC_{50} values of FP competition assays with RI α . All FP-data were collected in triplicates for each concentration measurement.

Table 9. Fluorescence polarization of STRIAD peptides for each PKA-R isoform				
	hRIa	hRIb	hRIIa	hRIIb
Peptide	(mean $\pm$ SEM) [nM]	(mean $\pm$ SEM) [nM]	(mean $\pm$ SEM) [nM]	(mean $\pm$ SEM) [nM]
STRIAD-1	5.8 $\pm$ 0.5 (p=2, n=6)	14.1 $\pm$ 1.2 (p=2, n=4)	19.4 $\pm$ 2.6 (p=2, n=6)	166 $\pm$ 41 (p=2, n=6)
STRIAD-2	6.2 $\pm$ 0.6 (p=2, n=6)	12.1 $\pm$ 2.2 (p=2, n=6)	159 $\pm$ 15 (p=2, n=6)	492 $\pm$ 41 (p=2, n=6)
STRIAD-3	7.3 $\pm$ 0.5 (p=2, n=6)	34.5 $\pm$ 2.4 (p=2, n=6)	427 $\pm$ 26 (p=2, n=6)	649 $\pm$ 52 (p=2, n=5)
p= different protein batches/preparation n= total number of measurement				

Table 10. Competition experiments with RI-specific inhibitors	
Ht31 peptide	EC_{50} / [μ M] (mean $\pm$ SEM)
STRIAD 1	1.9 $\pm$ 0.1 (p=1, n=3)
STRIAD 2	2.1 $\pm$ 0.2 (p=1, n=3)
STRIAD 3	1.5 $\pm$ 0.2 (p=1, n=2)
p= different protein batches/preparation n= total number of measurement	

Figures 16A to 16C show STRIAD selectively bind PKA-RI and disrupt AKAP-mediated type I PKA signaling in cells. Figures 16A and 16B show fluorescent images of MDA-MB-231 and PC-3 cells after treatment with FITC-labeled peptides (5 μ M) for 7 h or 21 h showing that oRIAD v3, v5 and v6 are cell-permeable. Each image is representative of three replicates. Figure 16C shows STRIAD-1 and STRIAD-2 immunoprecipitate with RI but not RII. MDA-MB-231 cell were treated with 5 μ M N-terminal biotin-labeled peptides for 12 h. Lysates

were pulled down by incubation with avidin-coated resin, and PKA-RI and PKA-RII were detected by immunoblotting.

Figures 17A to 17H FP spectra of hydrocarbon stapled peptides. Fluorescence polarization of the peptides were measured using purified protein constructs of the D/D domains from either PKA-RI (circles) or PKA-RII (squares). Peptides were plated at a final concentration of 10 nM and the D/D dimerization domains were tested over a concentration range of 0.1 nM to 100 μ M. Data was collected in triplicates for each concentration measurement.

Figures 18A and 18B are images showing insufficient uptake of wt and v1 peptides. MDA-MB-231 cells treated with 5 μ M FITC-labeled wt (Fig. 18A) and v1 (Fig. 18B) for 21 h. No apparent uptake was observed for wt. v1 did not have an ideal uptake either. Each image is representative of three replicates.

Figure 19 is a graph showing FP competition spectrum with the negative control peptide of Ht31 (Ht31PP) is shown for the PKA-RI α with STRIAD-2 peptide. Ht31PP is not able to compete the interaction between the PKA R-subunit and STRIAD-2.

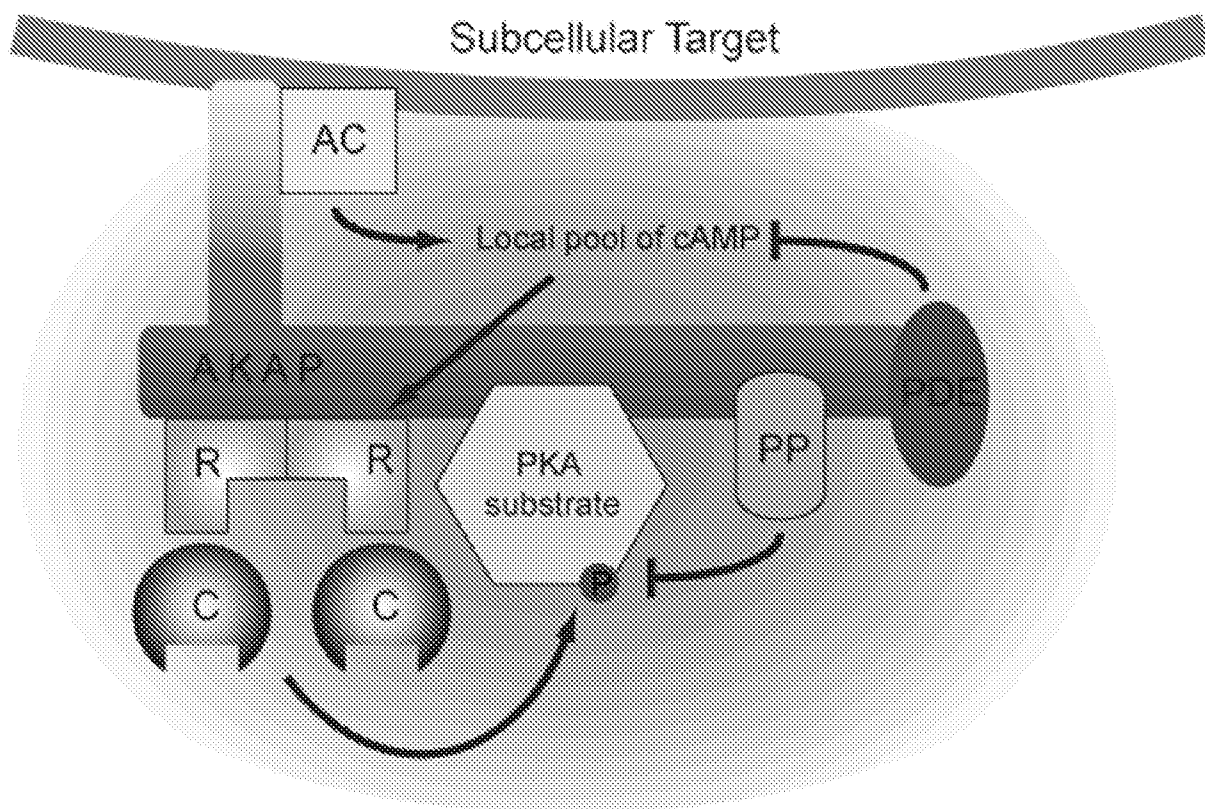
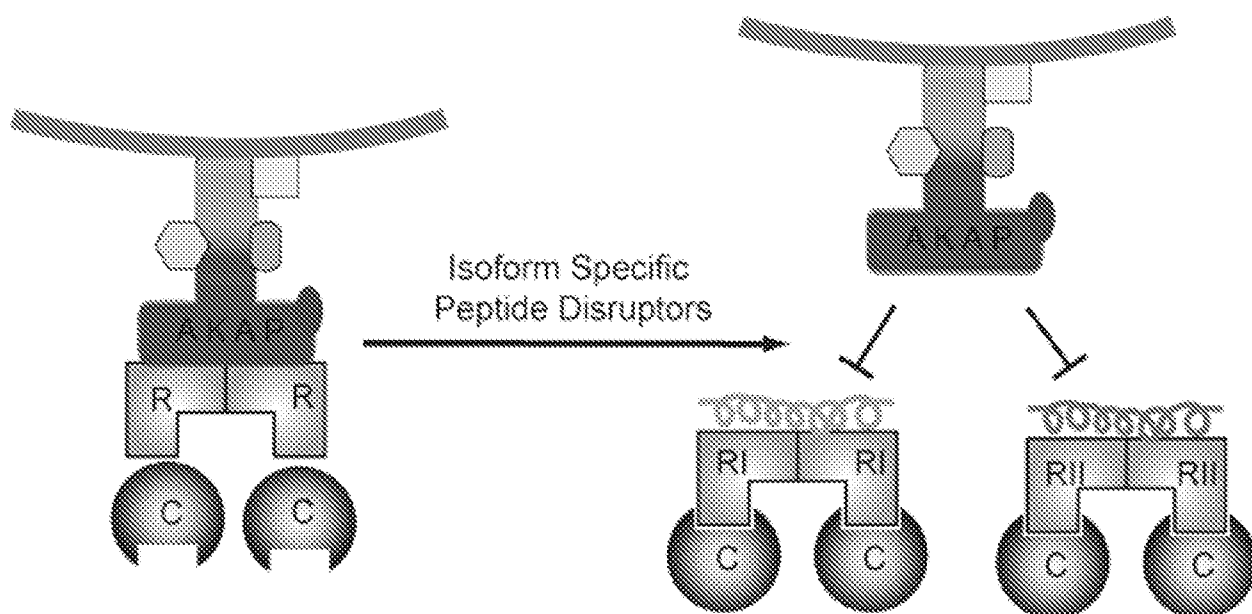
Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

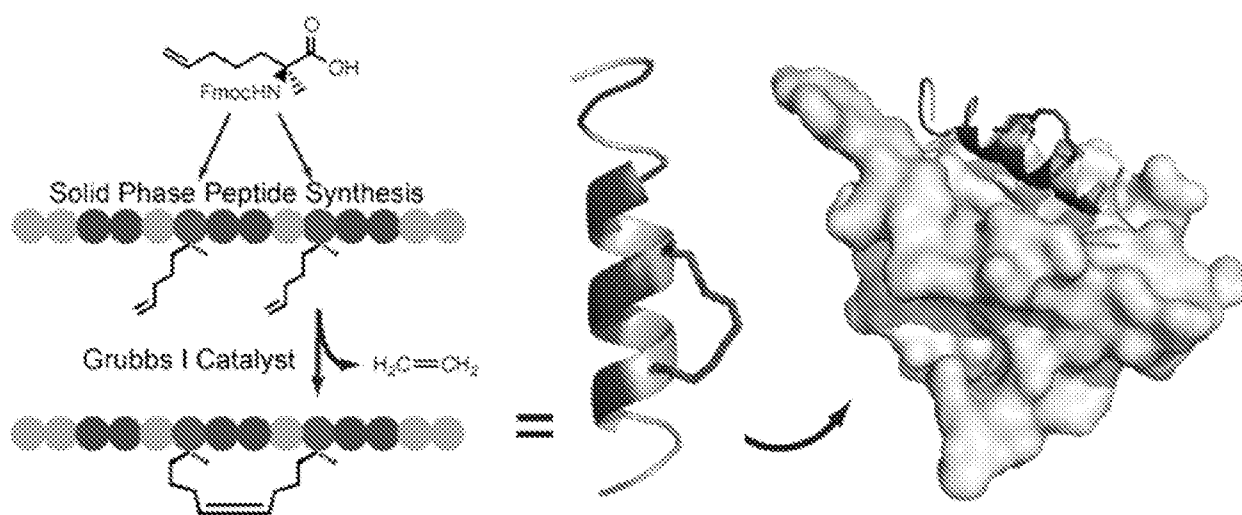
WHAT IS CLAIMED IS:

1. A synthetic polypeptide, comprising an amino acid sequence that
 - (a) mimics the A-Kinase Binding (AKB) helix of an A Kinase Anchoring protein (AKAP) that binds the docking/dimerization (D/D) domain of a protein kinase A (PKA),
 - (b) comprises at least one pair of non-natural amino acids inserted into the α -helix that are crosslinked to stabilize the α -helical shape, and
 - (c) is capable of binding to an endogenous protein kinase A-R subunit (PKA-R) in physiological conditions and inhibiting the endogenous PKA-R from binding to an endogenous AKAP in an isoform-specific manner.
2. The polypeptide of claim 1, wherein the pair of non-natural amino acids are three (i and i+3), four (i and i+4) or seven (i and i+7) amino acids apart.
3. The polypeptide of claim 1 or 2, comprising two or more pairs of the non-natural amino acids inserted into the α -helix that are crosslinked to stabilize the α -helical shape.
4. The polypeptide of any one of claims 1 to 3, wherein the polypeptide is 15 to 50 amino acids in length.
5. The polypeptide of any one of claims 1 to 4, wherein the polypeptide is isoform specific.
6. The polypeptide of claim 5, wherein the polypeptide is selective for PKA-R1 α , PKA-R1 β , or a combination thereof.
7. The polypeptide of claim 6, wherein the polypeptide comprises the amino acid sequence SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:115, or SEQ ID NO:116.
8. The polypeptide of claim 5, wherein the polypeptide is specific for PKA-R2 α , PKA-R2 β , or a combination thereof.
9. The polypeptide of claim 8, wherein the polypeptide comprises the amino acid sequence SEQ ID NO:18, SEQ ID NO:28, or SEQ ID NO:35.
10. The polypeptide of any one of claims 1 to 4, wherein the polypeptide can bind both isoforms with high affinity.
11. The polypeptide of any one of claims 1 to 10, wherein the polypeptide is from 15 to 100 amino acids in length.
12. The polypeptide of any one of claims 1 to 11, wherein the polypeptide comprises one or more hydrocarbon staples.
13. The polypeptide of any one of claims 1 to 12, wherein the polypeptide is a peptidomimetic.

14. The polypeptide of any one of claims 1 to 13, wherein the polypeptide further comprises a spacer.
15. The polypeptide of any one of claims 1 to 14, wherein the polypeptide further comprises a protein transduction domain.
16. The polypeptide of any one of claims 1 to 16, wherein the polypeptide is covalently linked to a water soluble polymer.
17. The polypeptide of claim 16, wherein the water soluble polymer is a polyethylene glycol.
18. The polypeptide of any one of claims 1 to 17, comprising two or more hydrophilic residues on the solvent-exposed face of the α -helix.
19. The polypeptide of claim 18, wherein the hydrophilic residue comprises lysine.
20. A pharmaceutical composition, comprising the polypeptide of any one claims 1 to 19 in a pharmaceutically acceptable carrier.
21. A method for inhibiting binding of an A Kinase Anchoring protein (AKAP) to a protein kinase A (PKA), comprising contacting the AKAP in physiological conditions with the polypeptide of any one claims 1 to 19.
22. A method for treating an AKAP-related disease in a subject, comprising administering to the subject a therapeutically effective amount of the pharmaceutical composition of claim 20.

**FIGURE 1A****FIGURE 1B**

2 / 21

**FIGURE 1C**

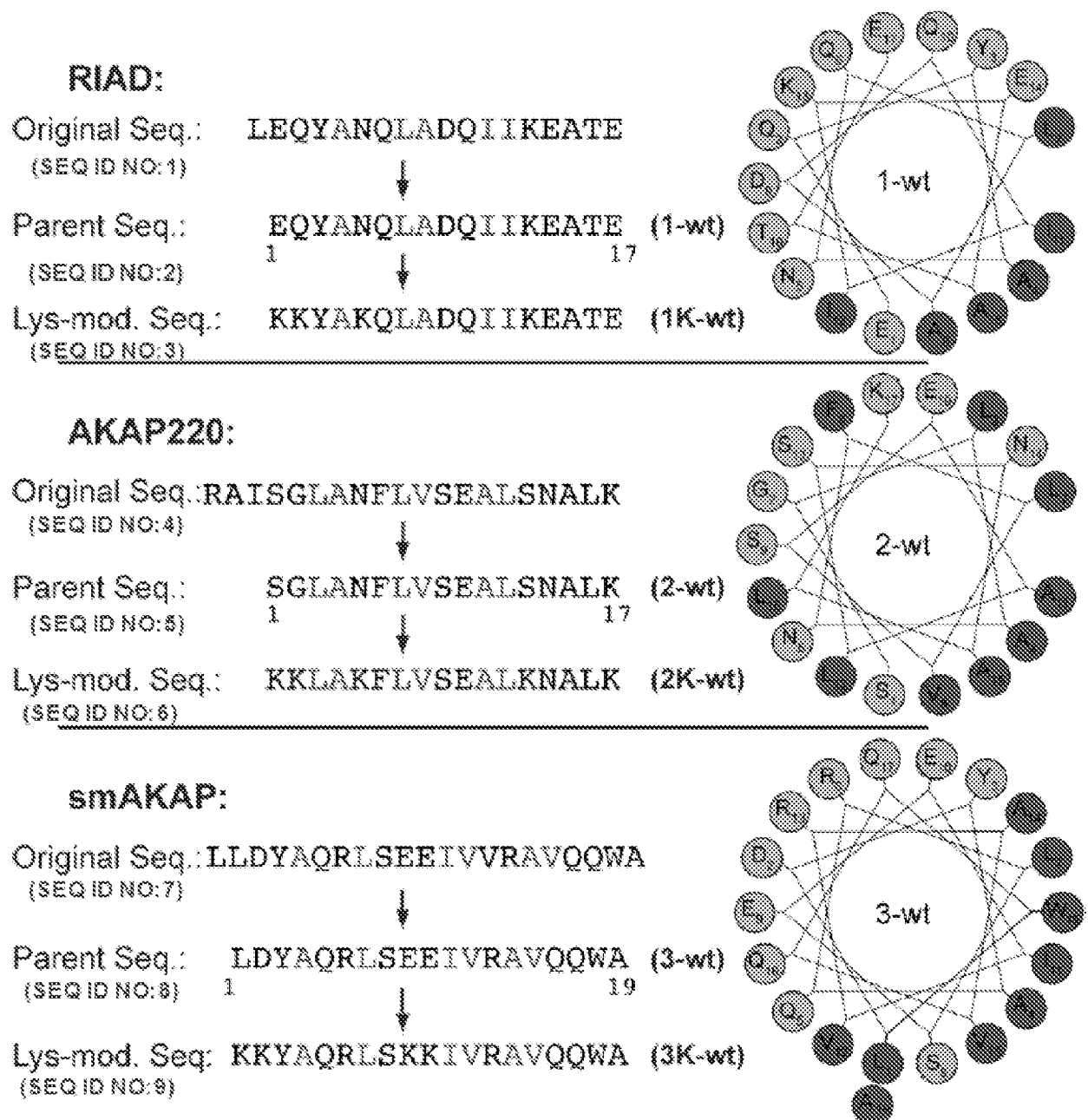


FIGURE 2

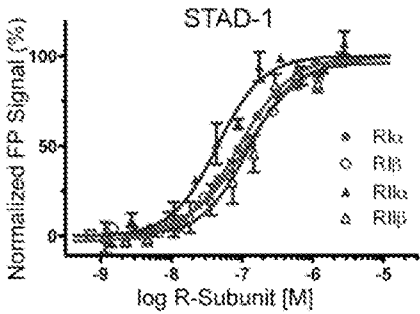


FIGURE 3A

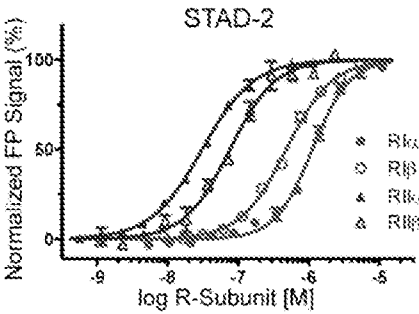


FIGURE 3B

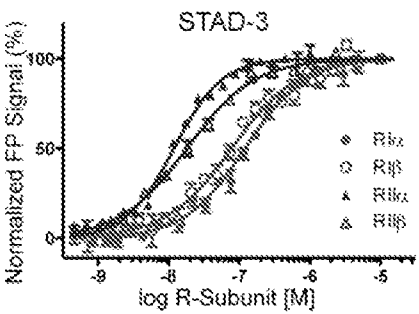


FIGURE 3C

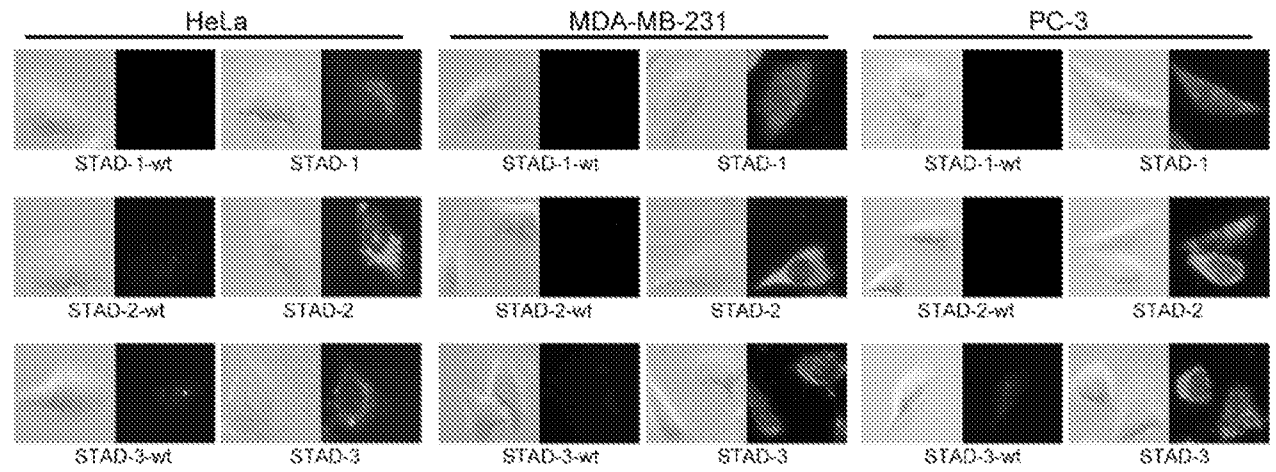


FIGURE 4A

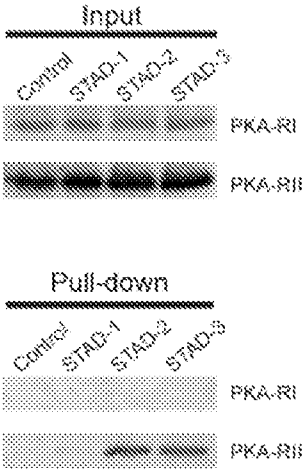


FIGURE 4B

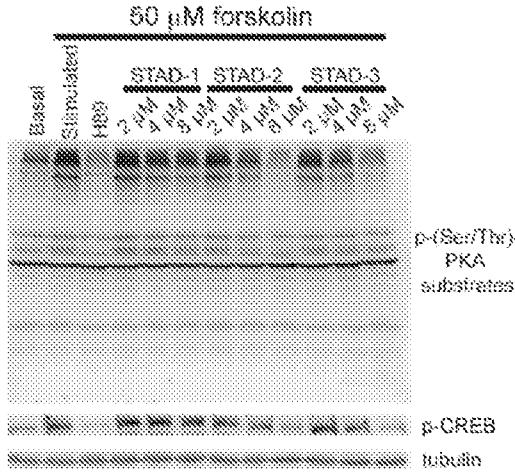


FIGURE 4C

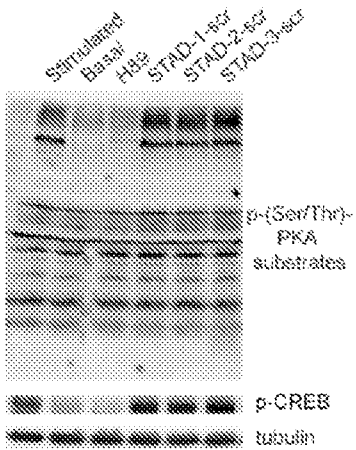


FIGURE 4D

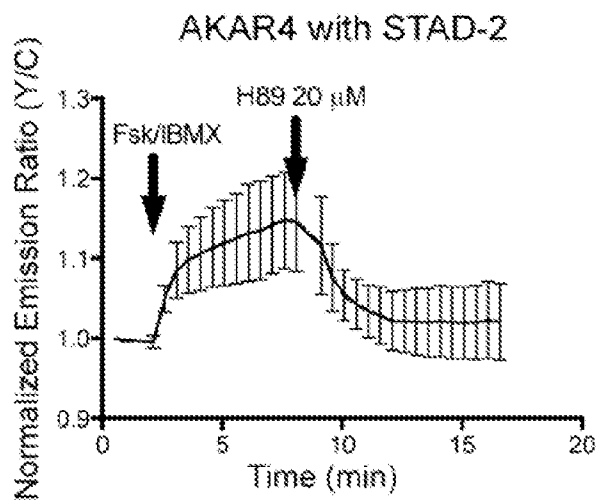


FIGURE 4E

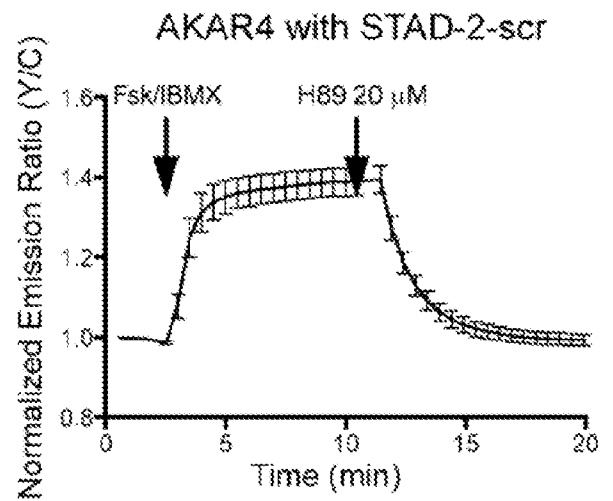


FIGURE 4F

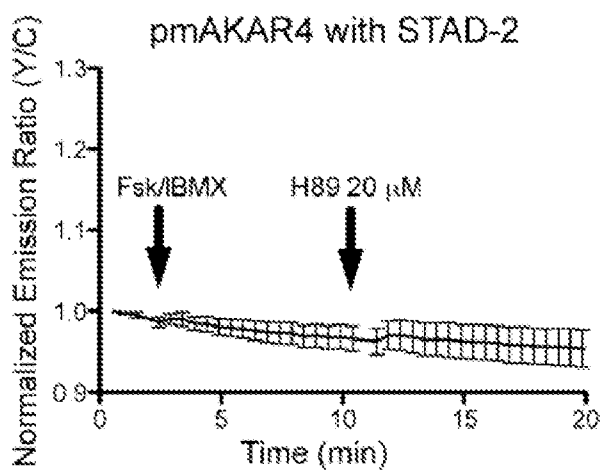


FIGURE 4G

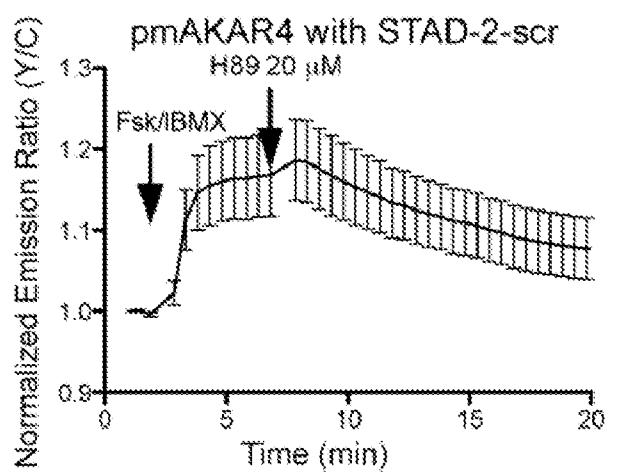
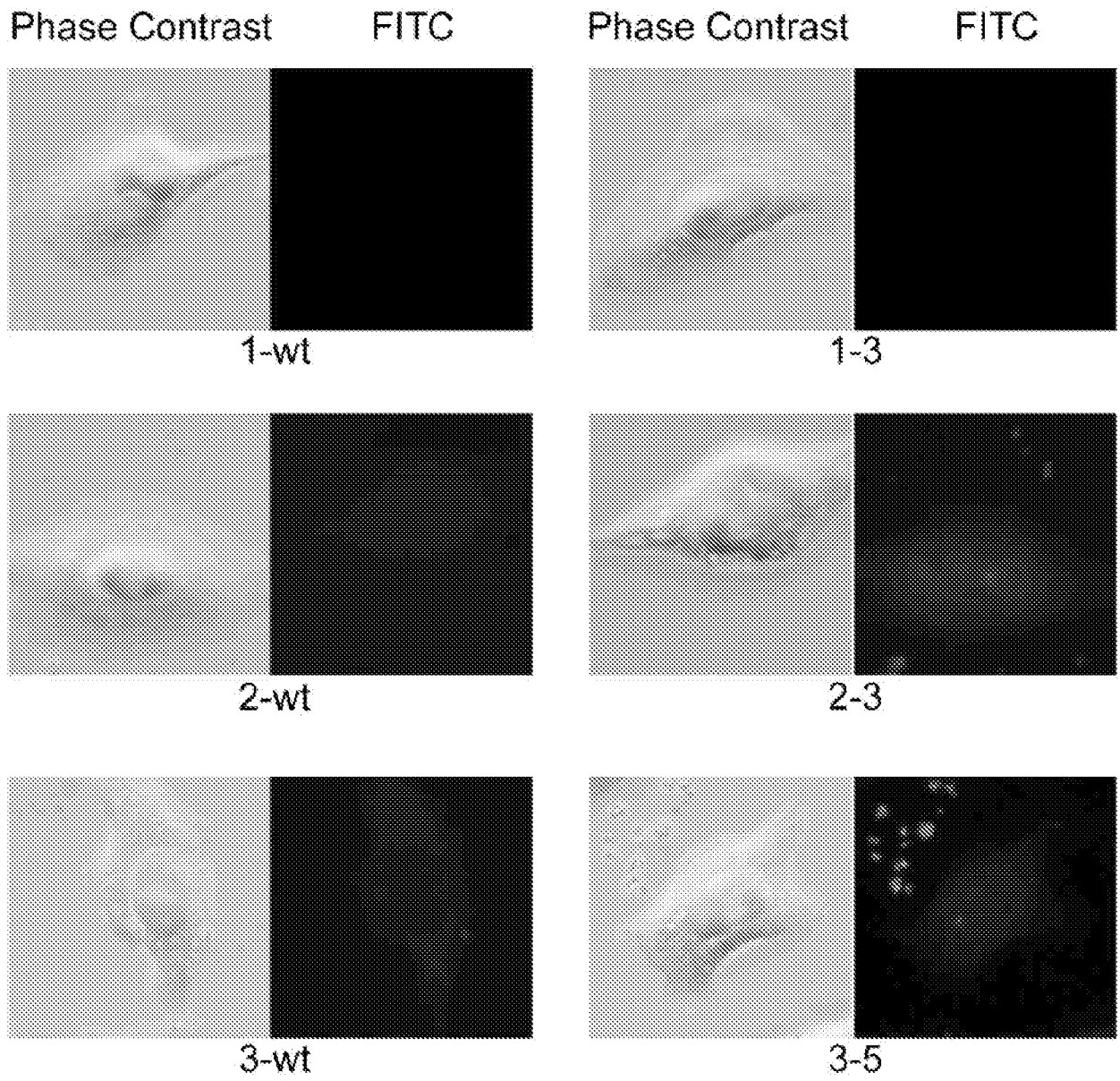


FIGURE 4H

6 / 21

**FIGURE 5**

7/21

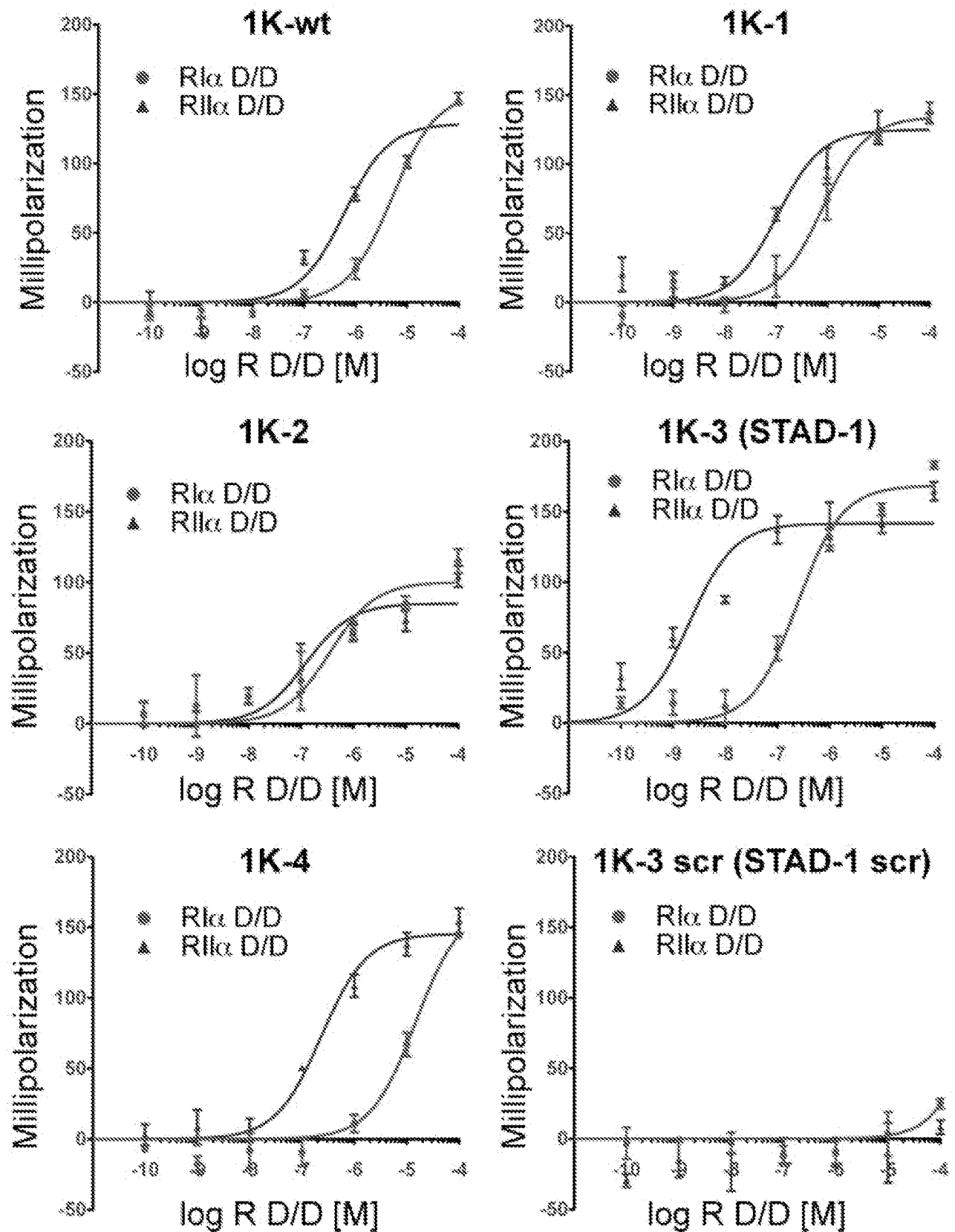


FIGURE 6

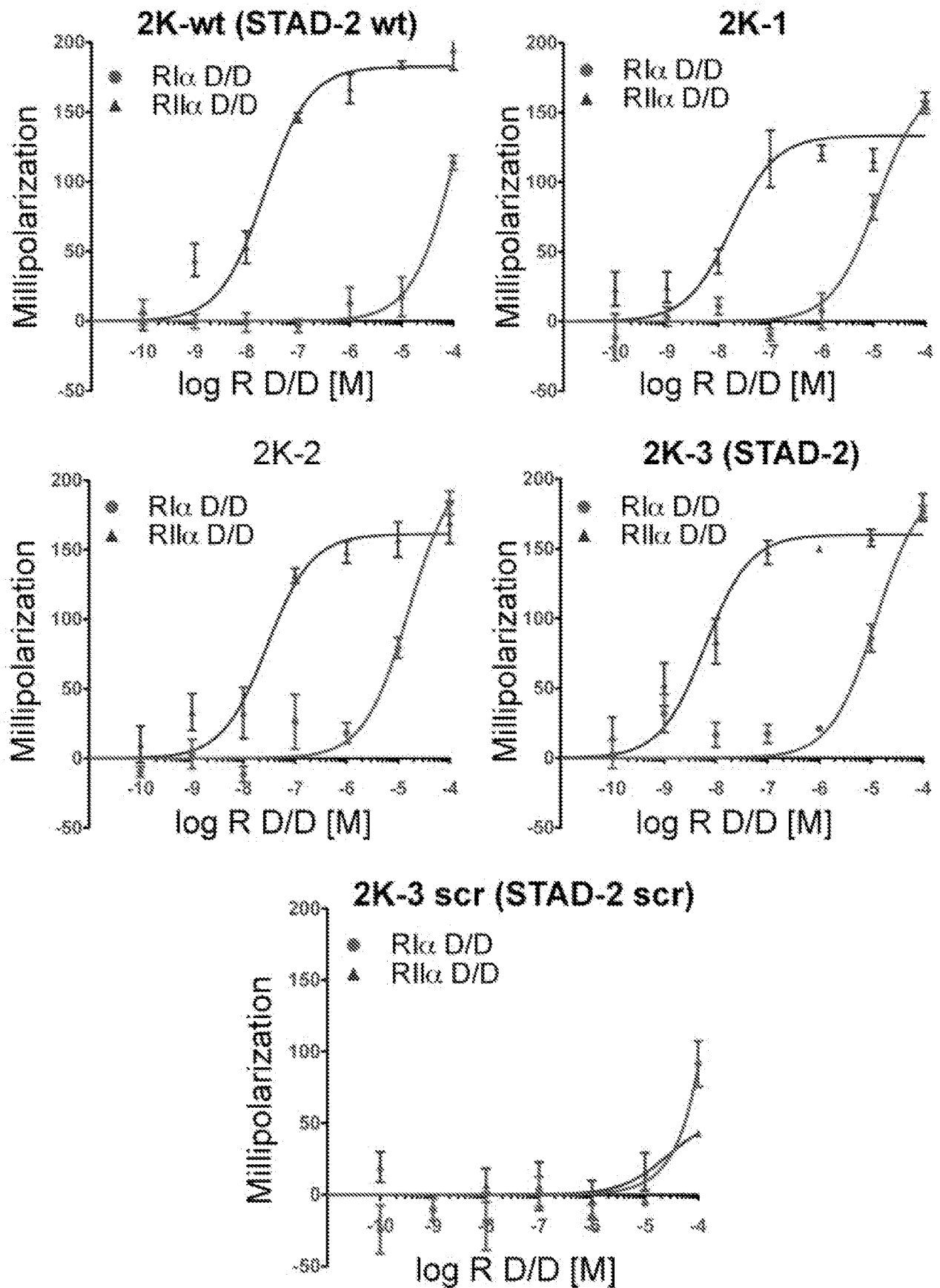


FIGURE 7

9 / 21

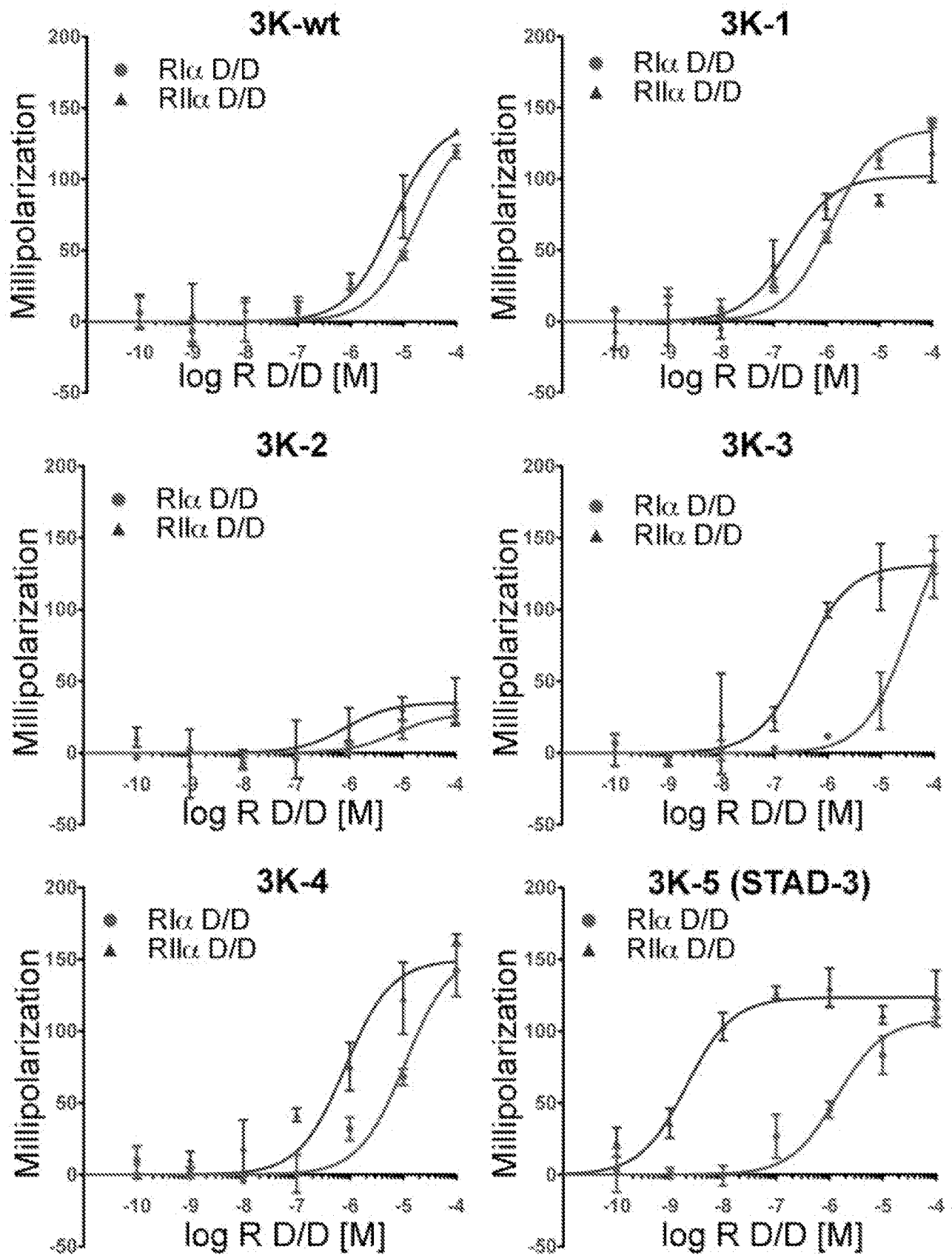
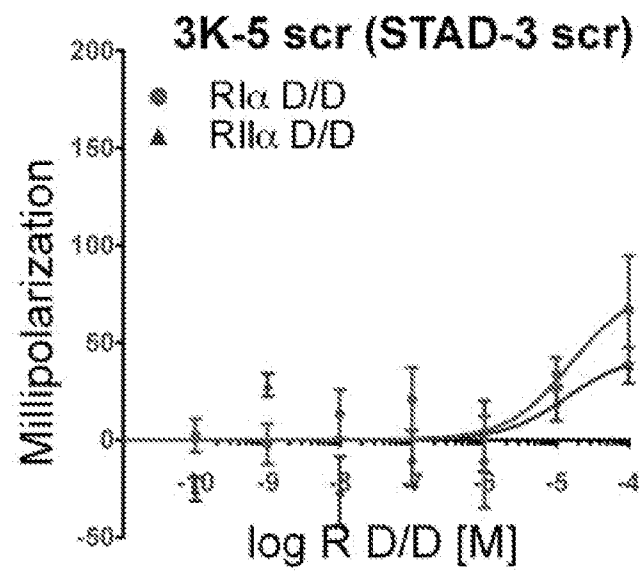


FIGURE 8

**FIGURE 8 (CONT...)**

11 / 21
HeLa

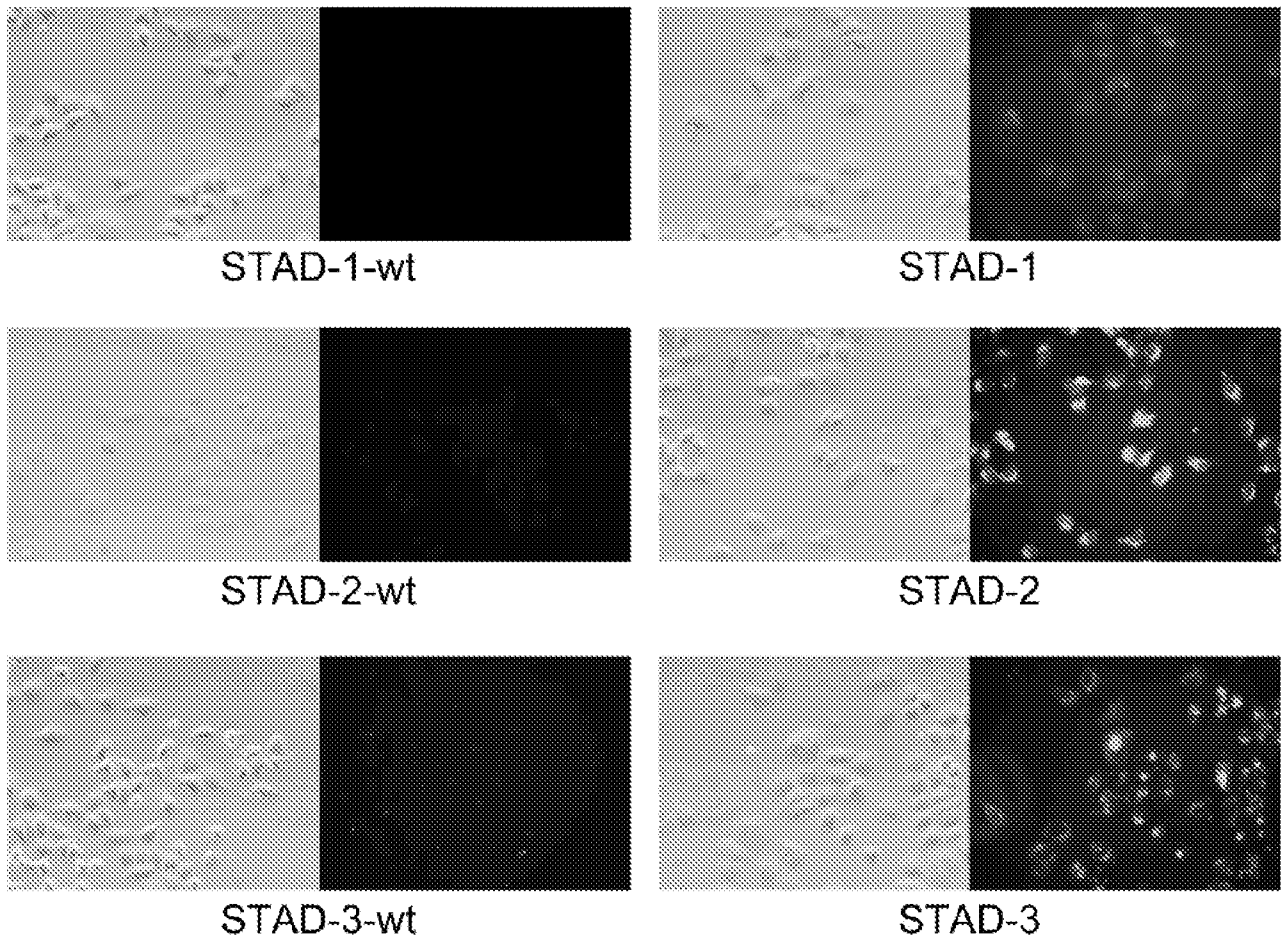


FIGURE 9

12 / 21
MDA-MB-231

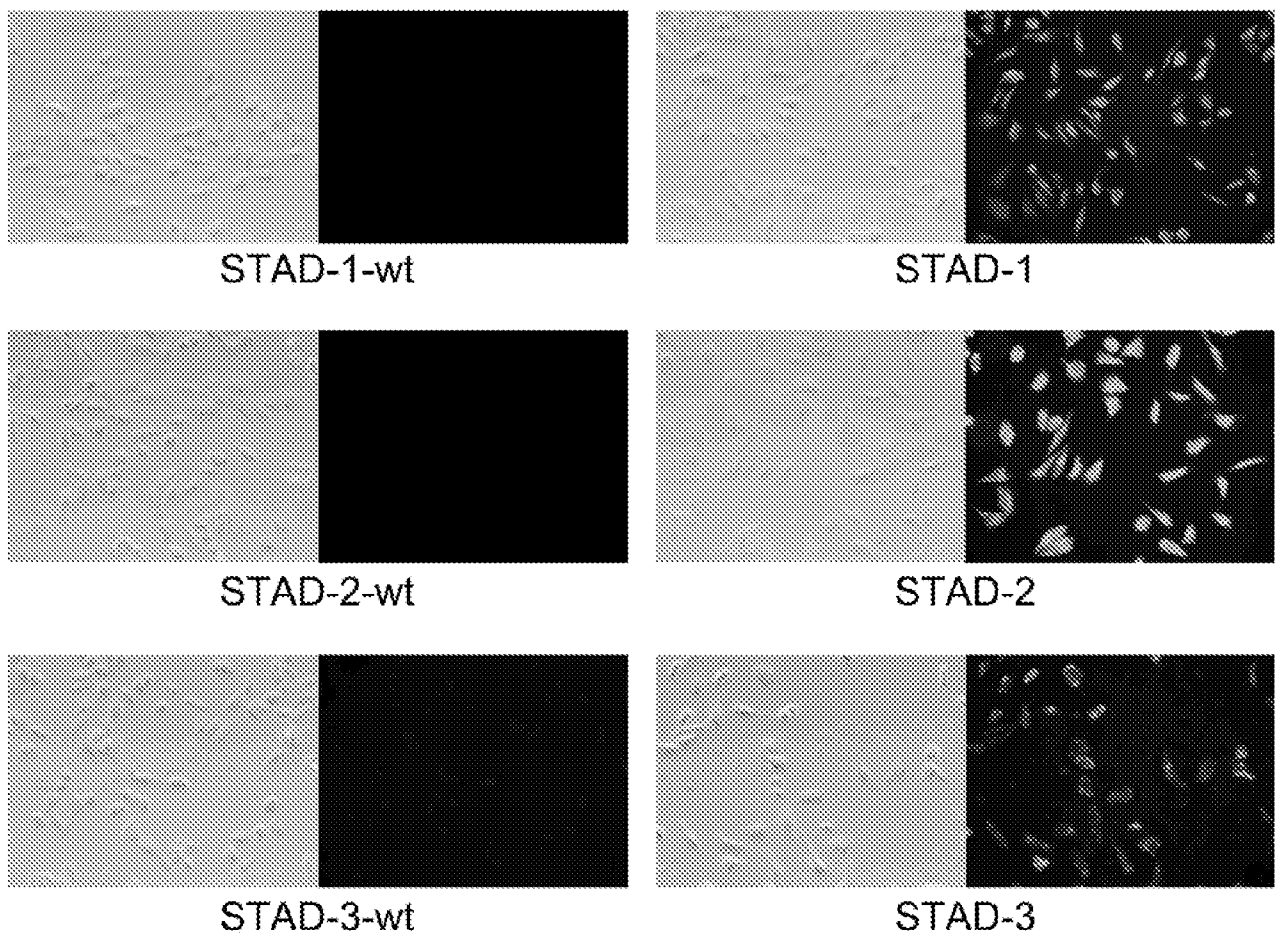


FIGURE 10

13 / 21
PC-3

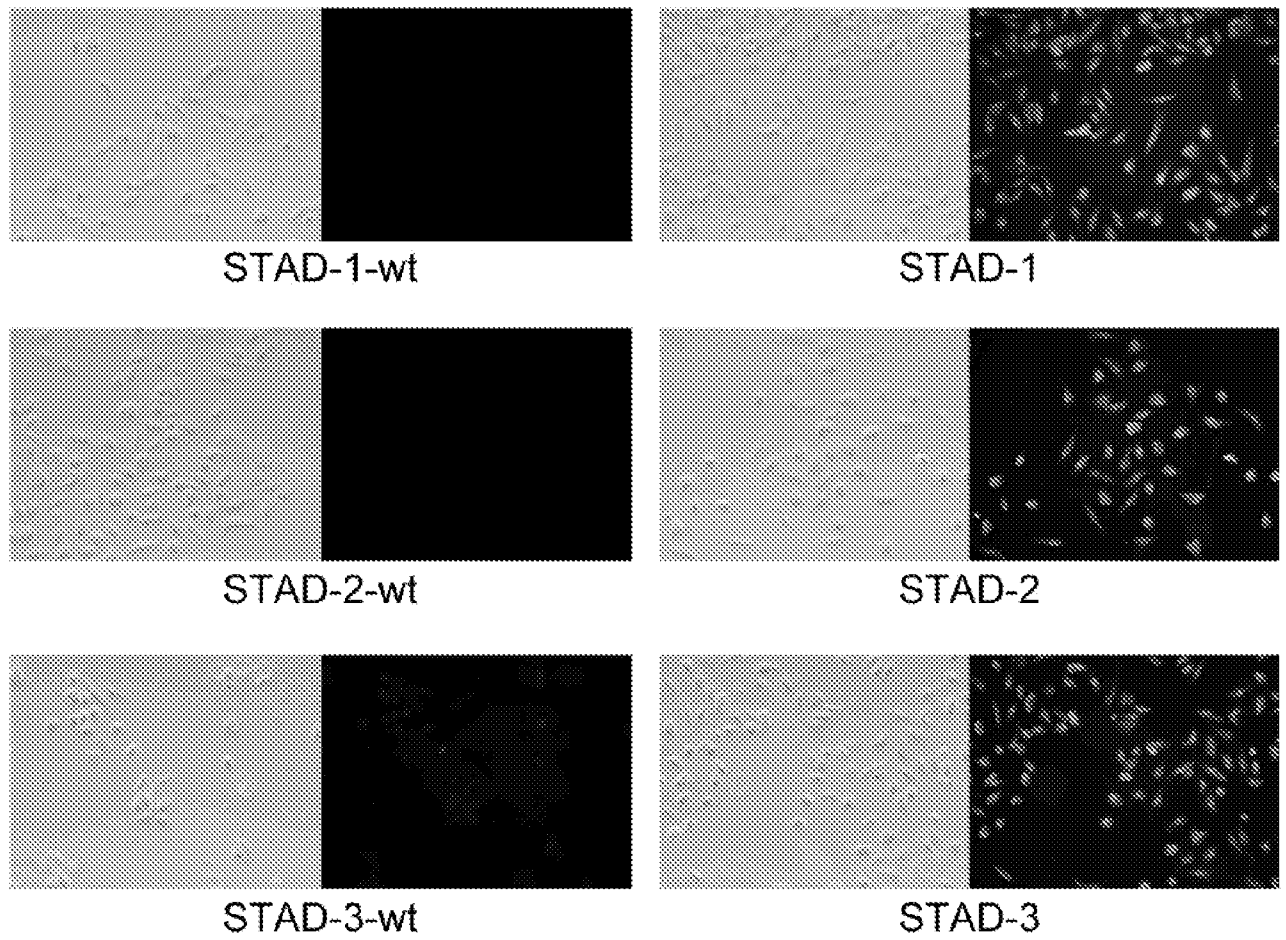
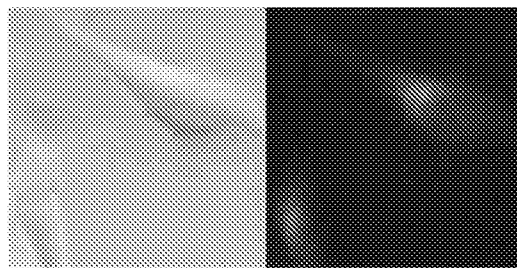


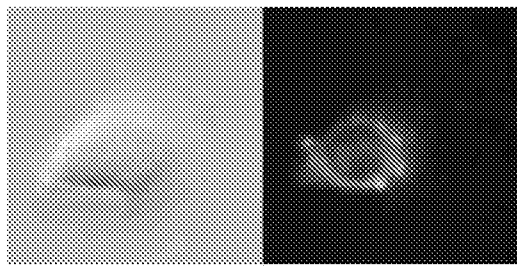
FIGURE 11

14 / 21

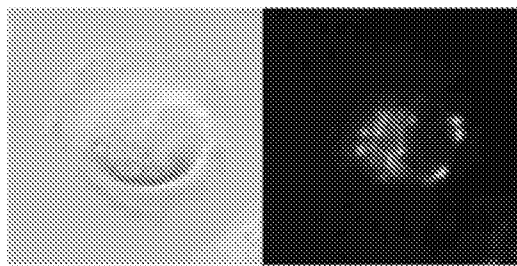
Phase Contrast FITC



STAD-1-scr



STAD-2-scr



STAD-3-scr

FIGURE 12

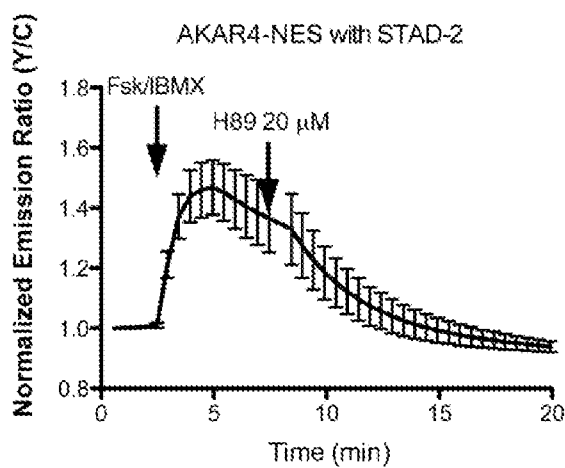


FIGURE 13A

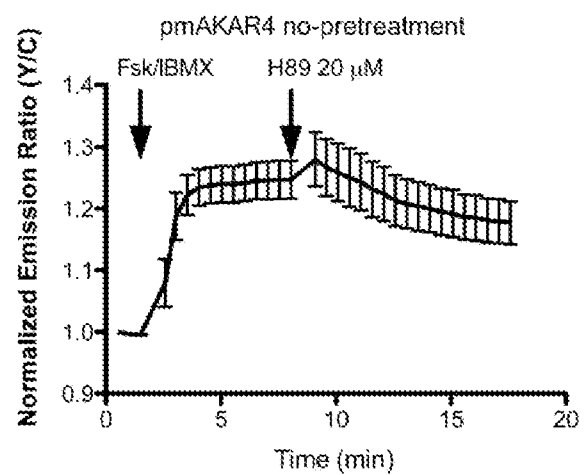


FIGURE 13B

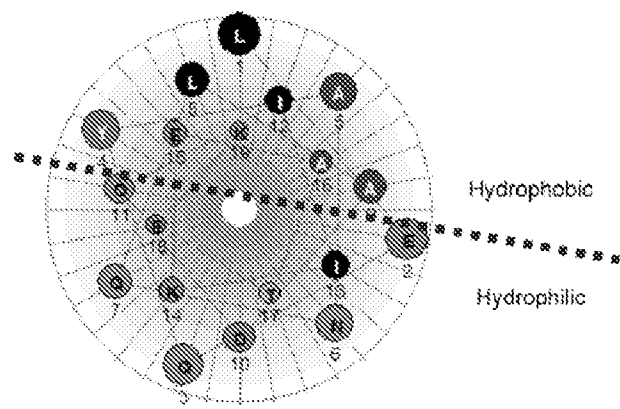


FIGURE 14A

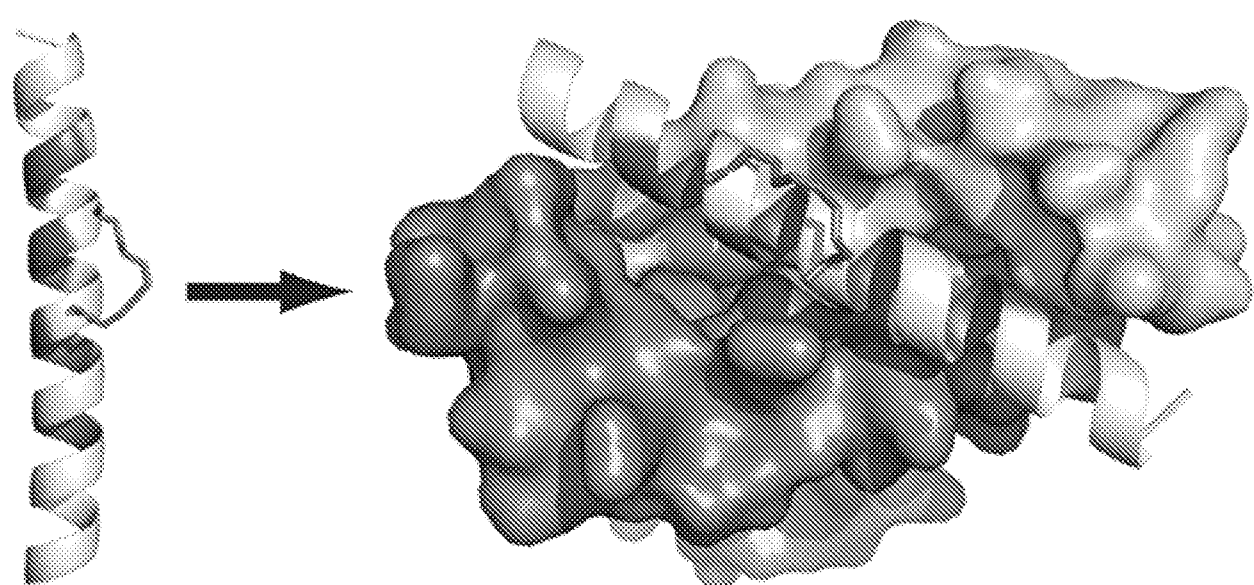


FIGURE 14B

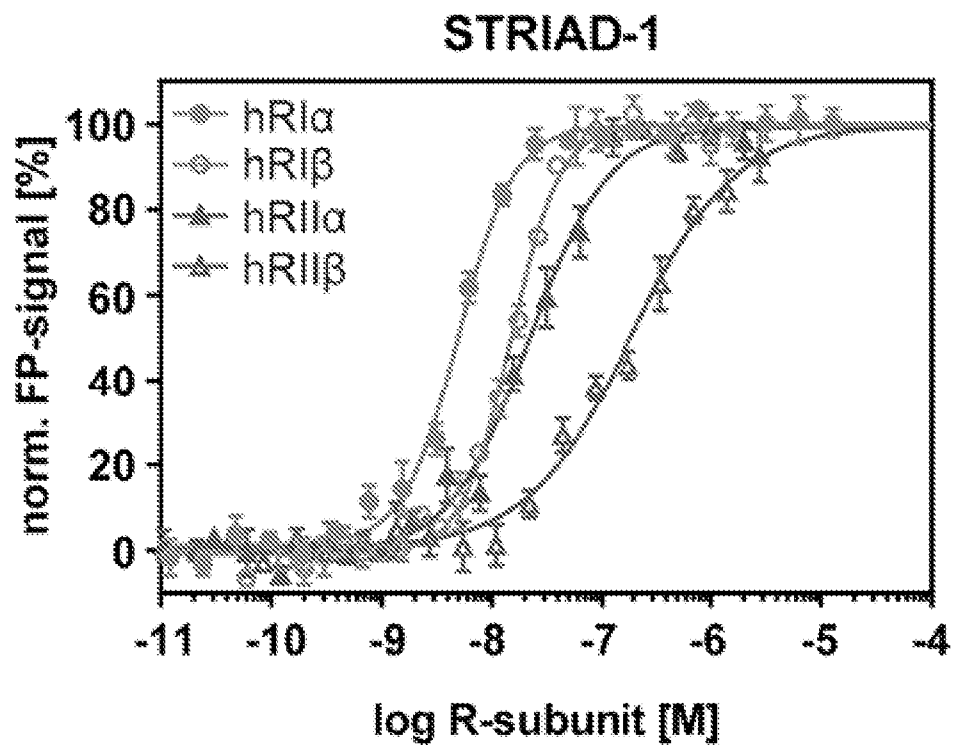


FIGURE 15A

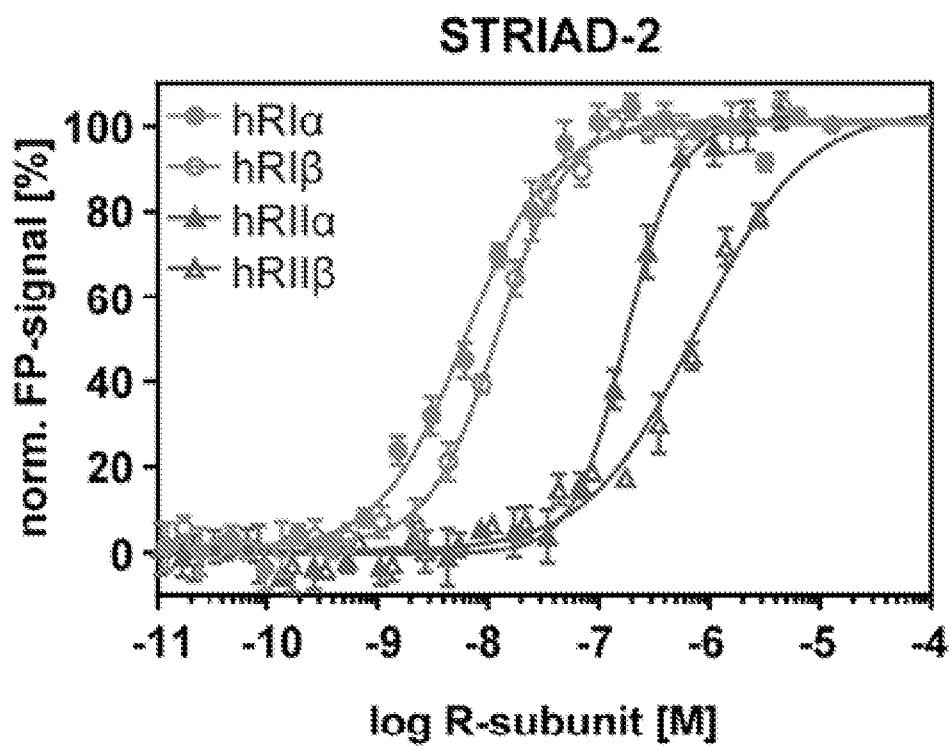


FIGURE 15B

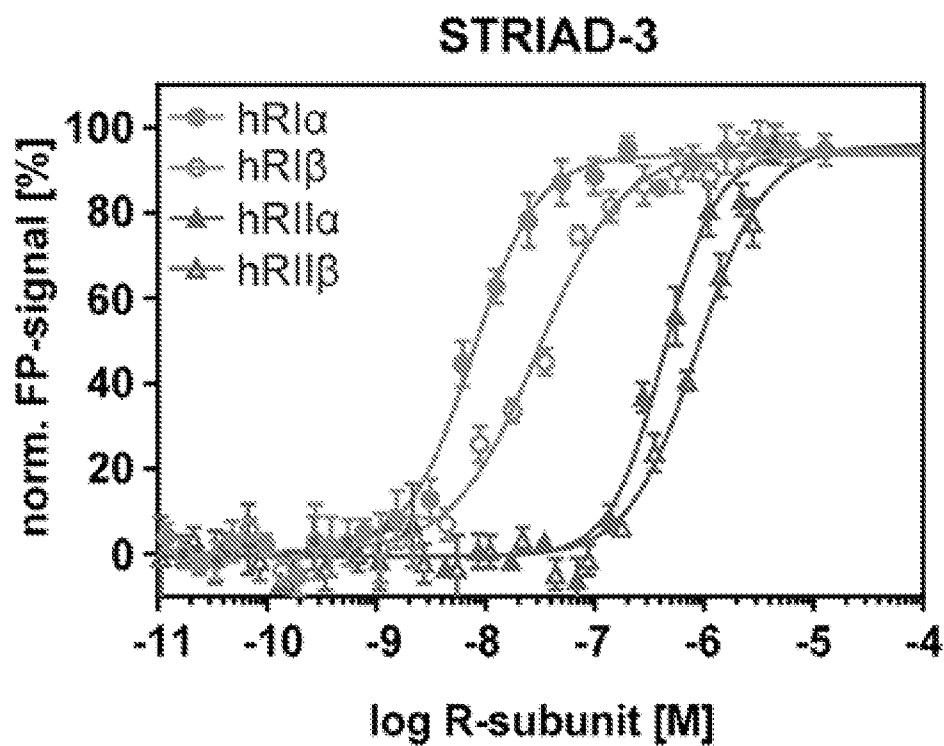


FIGURE 15C

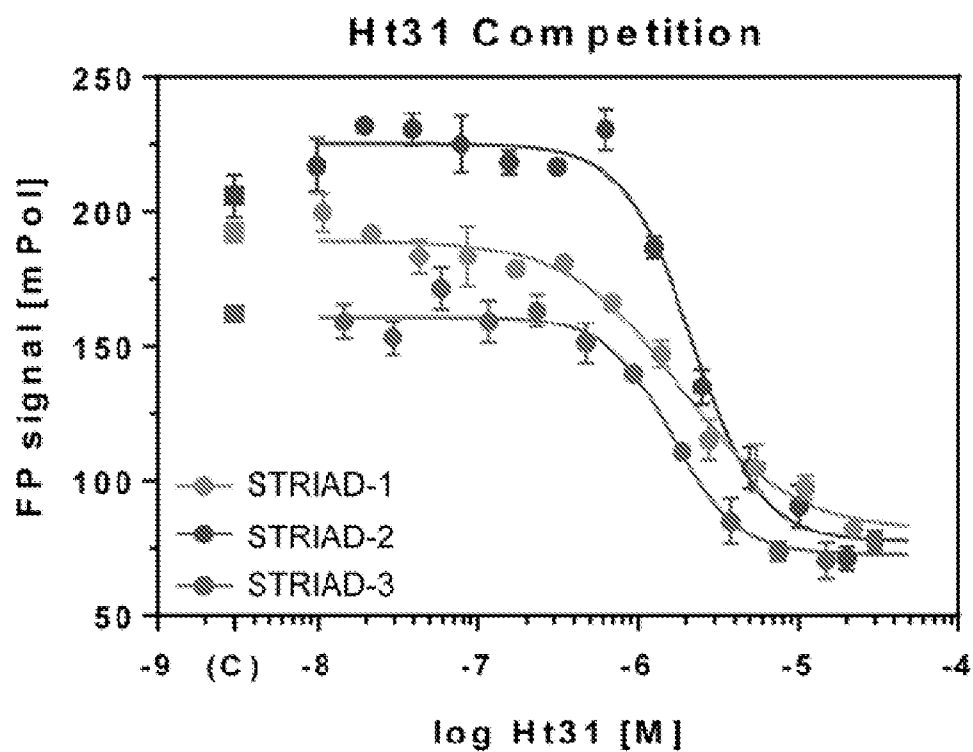


FIGURE 15D

MDA-MB-231

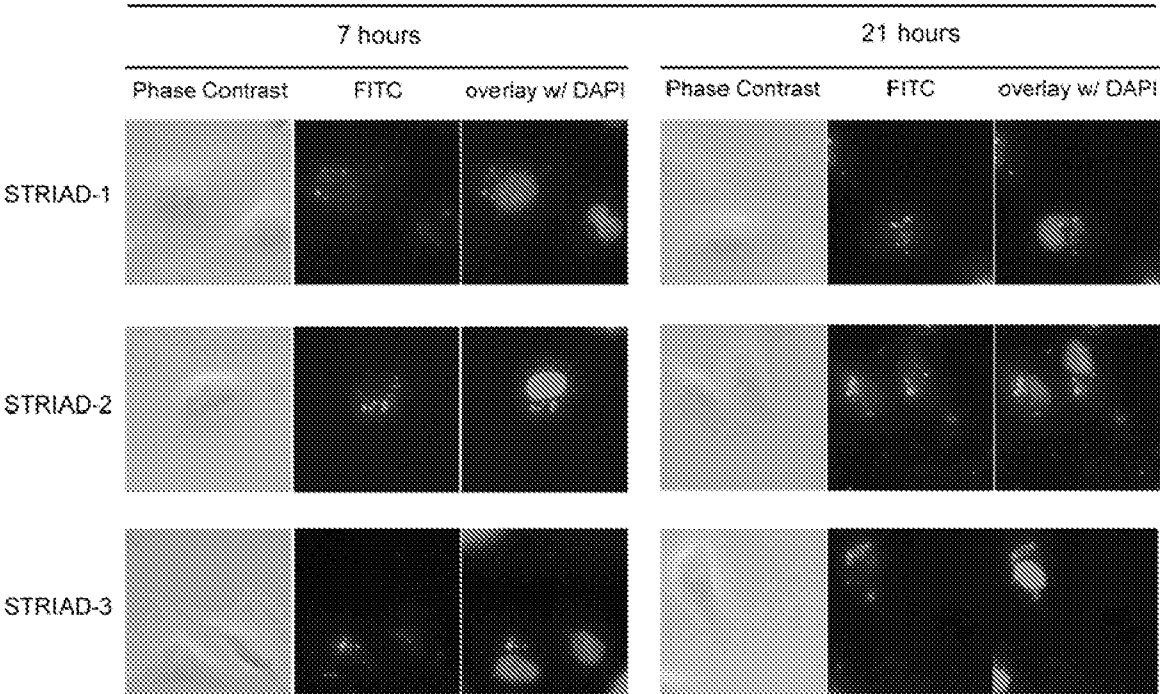


FIGURE 16A

PC-3

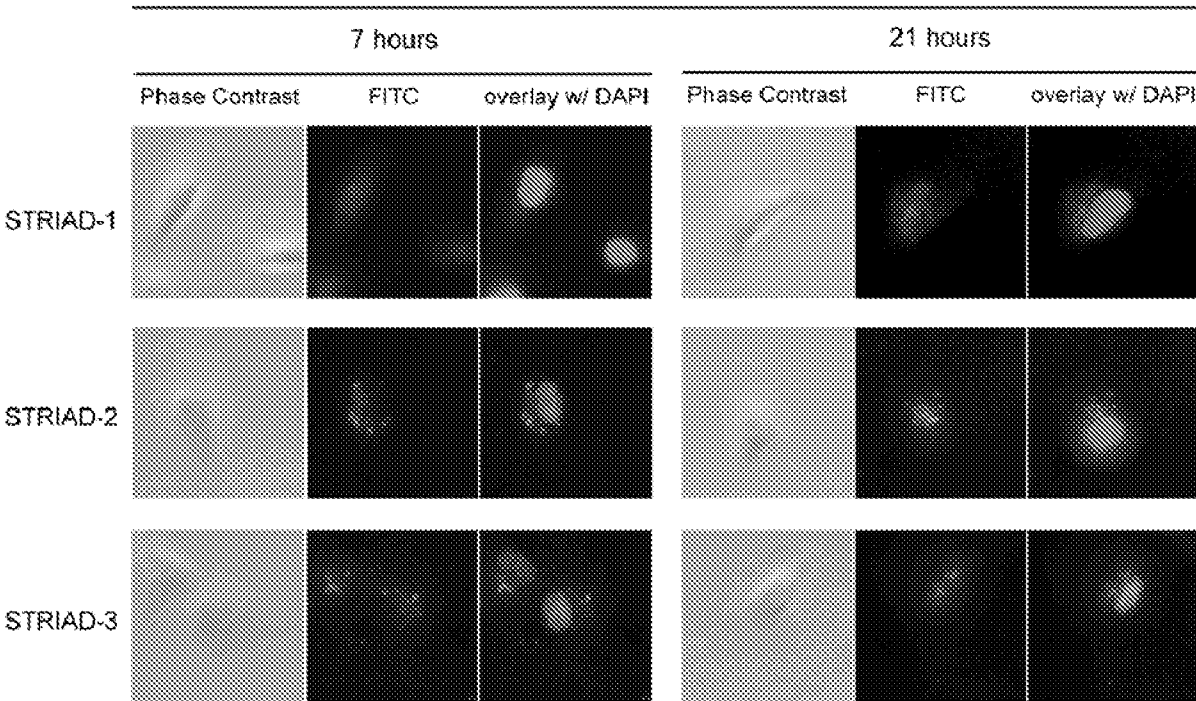


FIGURE 16B

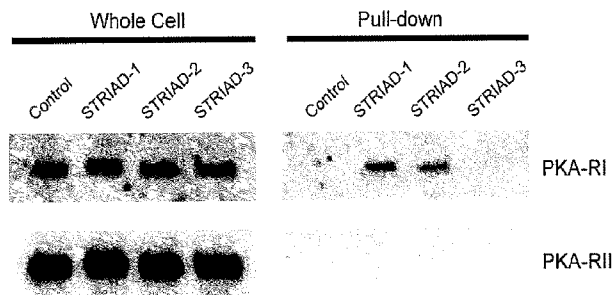


FIGURE 16C

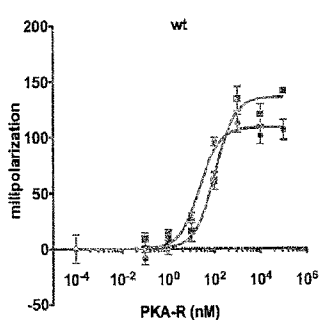


FIGURE 17A

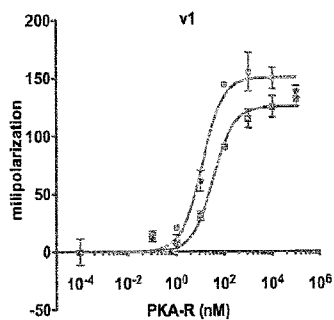


FIGURE 17B

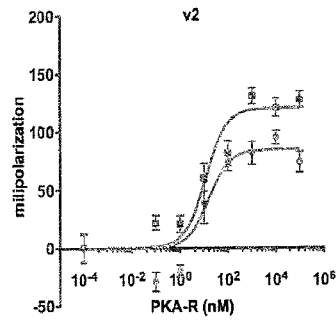


FIGURE 17C

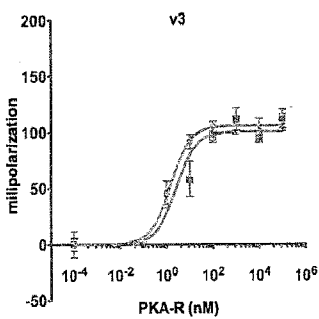


FIGURE 17D

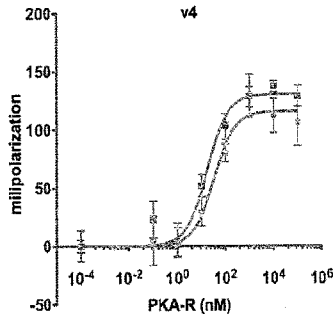


FIGURE 17E

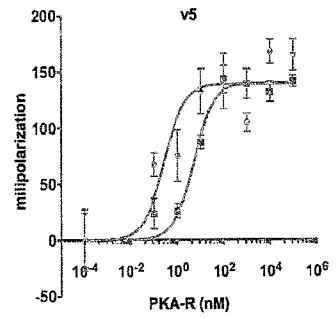


FIGURE 17F

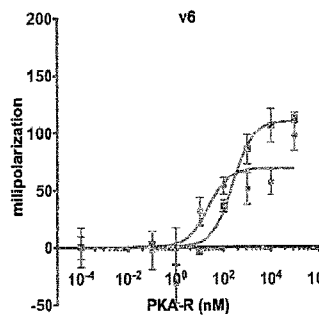


FIGURE 17G

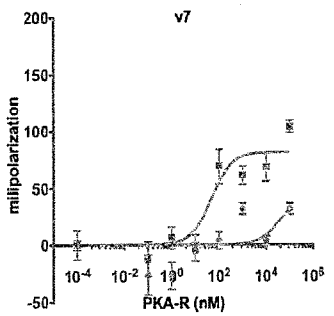
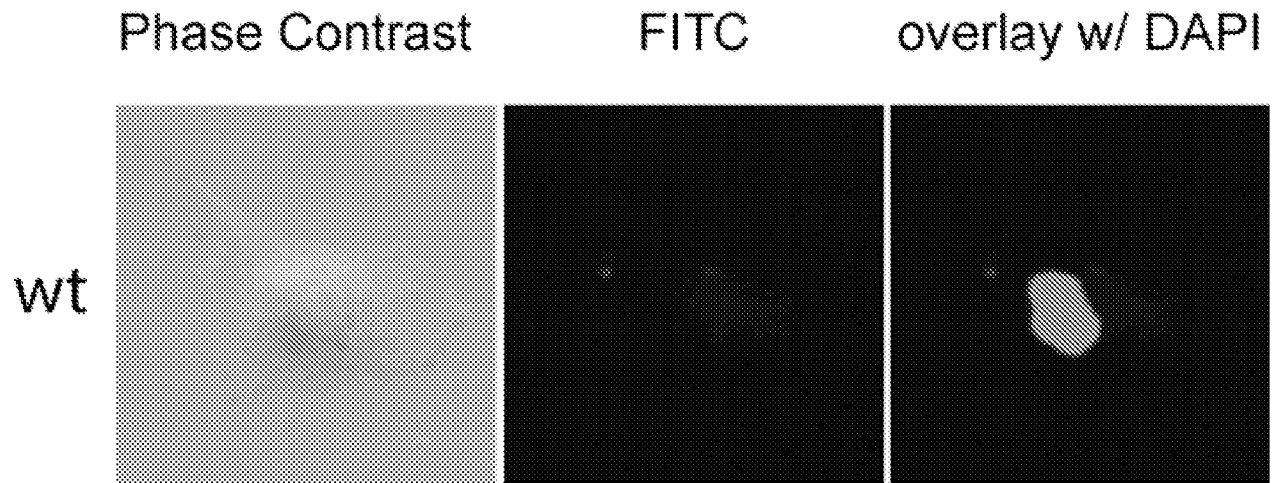
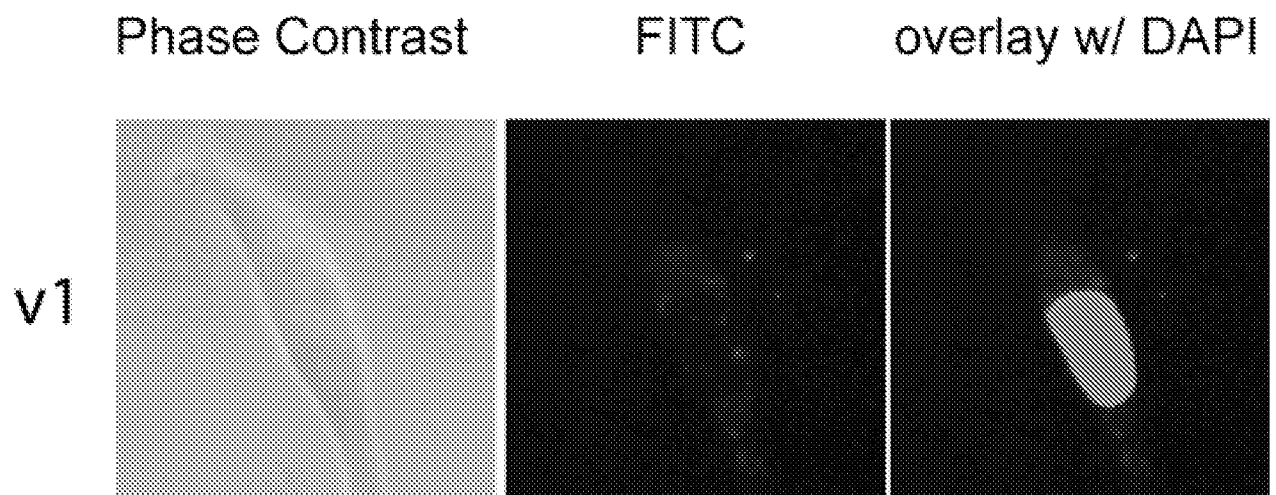


FIGURE 17H

**FIGURE 18A****FIGURE 18B**

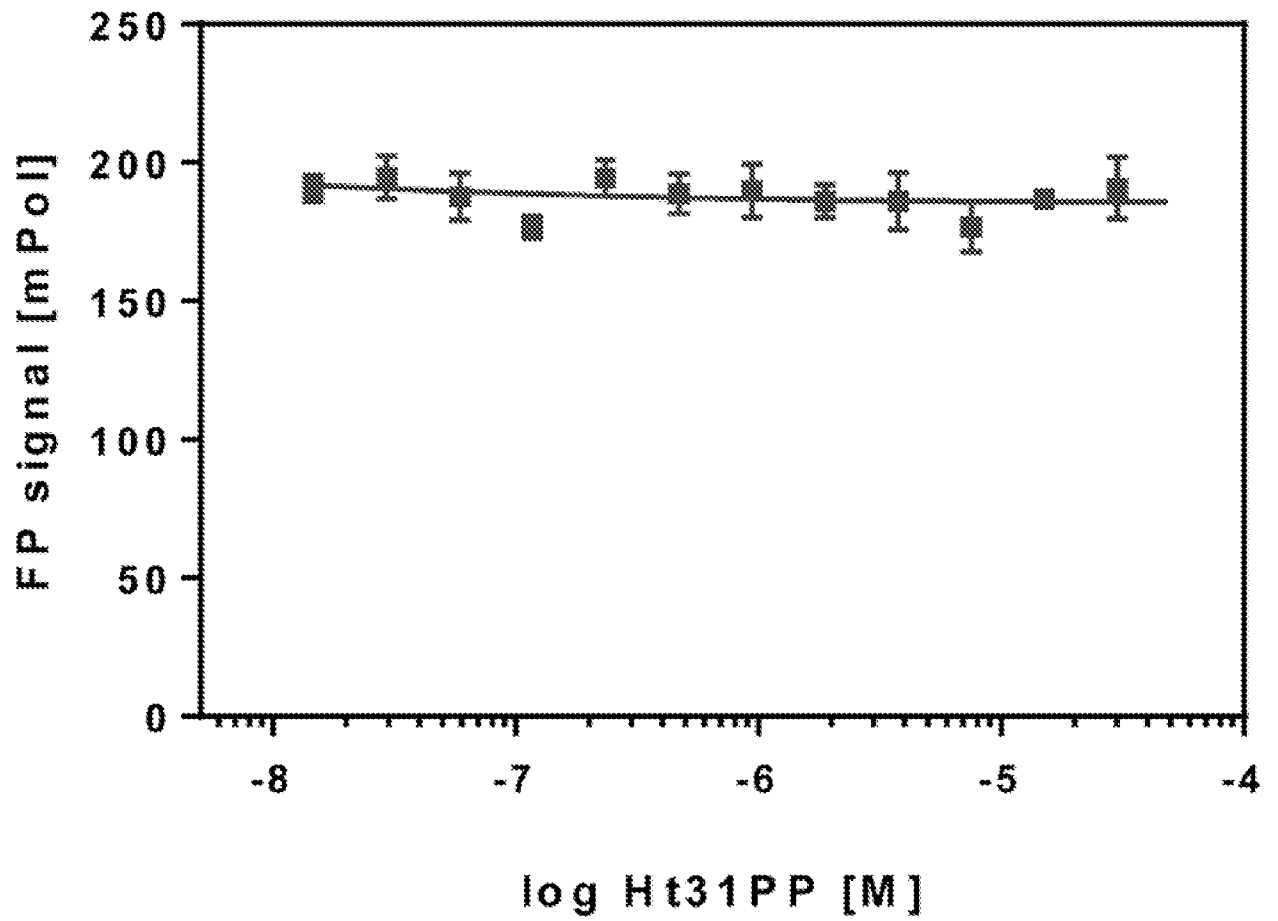


FIGURE 19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/047236

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☒

in the international application as filed

☐

together with the international application in electronic form

☐

subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOS: 57, 59, 115, and 116 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/047236

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest☐

The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/047236

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/10 (2014.01)

CPC - A61K 38/10 (2014.09)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 38/10; C07K 7/00, 14/47; C12N 9/12 (2014.01)

USPC - 424/185.1; 435/375; 514/2, 21.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 38/00, 38/04, 38/10, 47/48338; C07K 14/47, 14/4702, 14/4703 (2014.09) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Pub Med

Search term used: protein kinase A A-kinase anchoring protein peptide helix helical alkenyl isoform

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0248008 A1 (CARLSON et al) 09 October 2008 (09.10.2008) entire document	1-3
Y	US 2011/0144306 A1 (VERDINE et al) 16 June 2011 (16.06.2011) entire document	1-3
A	WO 2006/032909 A2 (LYGREN et al) 30 March 2006 (30.03.2006) entire document	1-3
A	BURNS et al. "Isoform specific differences in binding of a dual-specificity A-kinase anchoring protein to type I and type II regulatory subunits of PKA," Biochemistry. 20 May 200 (20.05.2003), Vol. 42, Pgs. 5754-5763. entire document	1-3
A	HERBERG et al. "Analysis of A-kinase anchoring protein (AKAP) interaction with protein kinase A (PKA) regulatory subunits: PKA isoform specificity in AKAP binding," J Mol Biol. 28 April 2000 (28.04.2000), Vol. 298, Pgs. 329-339. entire document	1-3

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 November 2014

Date of mailing of the international search report

15 DEC 2014

Name and mailing address of the ISA/US

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Blaine R. Copenheaver

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