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Adenosine analogue–oligo-arginine conjugates (ARCs) serve as high-affinity inhibitors and fluorescence probes of type I cGMP-dependent protein kinase (PKGI α)

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Abstract

Introduction—Type I cGMP-dependent protein kinase (PKGI α) belongs to the family of cyclic nucleotide-dependent protein kinases and is one of the main effectors of cGMP. PKGI α is involved in regulation of cardiac contractility, vasorelaxation, and blood pressure; hence, the development of potent modulators of PKGI α would lead to advances in the treatment of a variety of cardiovascular diseases.

Aim—Representatives of ARC-type compounds previously characterized as potent inhibitors and high-affinity fluorescent probes of PKA catalytic subunit (PKAc) were tested towards PKGI α to determine that ARCs could serve as activity regulators and sensors for the latter protein kinase both *in vitro* and in complex biological systems.

Results—Structure–activity profiling of ARCs with PKGI α *in vitro* demonstrated both similarities as well as differences to corresponding profiling with PKAc, whereas ARC-903 and ARC-668 revealed low nanomolar displacement constants and inhibition IC₅₀ values with both cyclic nucleotide-dependent kinases. The ability of ARC-based fluorescent probes to penetrate cell plasma membrane was demonstrated in the smooth muscle tissue of rat cerebellum isolated arteries, and the compound with the highest affinity *in vitro* (ARC-903) showed also potential for *in vivo* applications, fully abolishing the PKGI α -induced vasodilation.

Keywords

Protein kinase inhibitor; ARC; cGMP-dependent protein kinase (PKG); cAMP-dependent protein kinase (PKA); Fluorescence anisotropy; Pressure myography

1. Introduction

PKGI α belongs to the family of cyclic nucleotide-dependent protein kinases, and is one of the main effectors of the NO/cGMP pathway [1,2]. The physiological functions of PKGI α involve relaxation of vascular smooth muscle tone [3–5] and prevention of platelet aggregation [6,7]. The potent modulators of PKGI α activity may therefore enable

elucidation of dilation and constriction mechanisms of blood vessels and thus facilitate focussed treatment of cardiovascular disorders.

The significant sequence homology of catalytic cores of PKGI α and the closely related cAMP-dependent protein kinase (PKA), and the preference of cyclic nucleotide-dependent protein kinases towards the similar substrate consensus sequences [8–11] have been a concern from the aspect of design of selective PKGI α inhibitors. The PKGI α inhibitors reported so far might be classified into three types according to their binding site in the kinase: cGMP analogues [12,13] [*e.g.*, (Rp)-8-Br-PET-cGMPS, (Rp)-8-pCPT-cGMPS] that bind to the cGMP-sites in the regulatory domain of PKGI α , compounds that bind to the ATP-binding cleft in the catalytic domain of PKGI α (*e.g.*, Hidaka's inhibitors [14,15], KT5823 [16]), and peptidic inhibitors that presumably bind to the protein/peptide substrate pocket of PKGI α (*e.g.*, TQAKRKALAMA [17], LRK₅H, DT-2 and its analogues [18,19], *etc.* [20,21]). Within this set, the best characteristics from the aspect of inhibition potential (K_i = 0.8 nM in the presence of cGMP [19]) and selectivity towards PKGI α *versus* PKA, are exposed by D-DT-2 (although D-DT-2 has not been fully characterized in terms of broad selectivity profiling).

On the other hand, despite several differences of PKA and PKGI α considering their localization within the cell and tissue [2,22,23] and their roles in some pathways [24–27], there is a growing evidence of extensive cross-talk and confluence of cAMP and cGMP cascades in various tissues [4,28–32]. In the latter cases, a semi-selective inhibitor possessing high affinity towards both cyclic nucleotide-dependent protein kinases might be of great value. The additional advantage of such semi-selective compound would be its applicability for the broad-profile *in vitro* assays for the determination of an active kinase concentration, or for the screening of more selective PKGI α or PKA inhibitors *in vitro* and *in vivo*.

The conjugates of adenosine analogue and oligo-arginine (ARCs) have been developed as the bisubstrate-type inhibitors of PKA catalytic subunit (PKAc) [33–35]. The most potent representatives of ARC-inhibitors possess subnanomolar K_i and K_d values towards PKAc and some other basophilic protein kinases [34,36–38]; moreover, ARCs tolerate several structural modifications (*e.g.*, the conjugation with a fluorescent dye) without loss of affinity towards the target kinase, which has enabled the application of ARCs in effective reagent-saving *in vitro* assays [35,39]. The *in vivo* potential of ARCs and ARC-based assays has not been previously explored, although the cell plasma membrane-penetrative properties of ARCs and ARC-mediated kinase inhibition effects have been confirmed in cell cultures [40–41, A. Vaasa (manuscript in preparation)].

In this study, the potential of ARCs as PKGI α inhibitors and fluorescent probes was revealed. ARC-903 and its fluorescent derivative ARC-1059 exhibited high affinity towards both PKAc and PKGI α according to the inhibition and binding studies *in vitro*, and successfully penetrated into smooth muscle tissue of intact arteries where ARC-903 abolished both PKGI α - and PKAc-induced vasodilation, demonstrating the applicability of ARCs and ARC-based assays both *in vitro* and in complex biological systems.

2. Materials and methods

2.1. Materials

All chemicals were obtained from commercial sources unless otherwise noted. PKAc type α^1 was obtained from BIAFFIN. PKGI α (human) for fluorescence polarization-based assay

¹PKAc type α is further referred to as PKAc in the manuscript text.

was obtained from Millipore, and PKGI α (bovine) for other assays was expressed as previously reported [18]. The catalytic properties of both PKGI α preparations were identical according to the extent of substrate phosphorylation in the kinetic assay (data not shown). Cygnet 2.1 was expressed and purified as described previously [42]. The fraction of the active kinase in the stock solution was determined with direct binding assay [39] with ARC-1059 ($c=20$ nM, for human PKGI α and for bovine PKGI α ; $c=5$ nM, for Cygnet 2.1).

Fmoc Rink Amide MBHA resin and other chemicals for the synthesis of ARC-type inhibitors were from Neosystem, Novabiochem, Advanced ChemTech, and AnaSpec. Solvents were from Rathburn and Fluka. H89 and staurosporine were from Biaffin, TAT-peptide from AnaSpec. The synthesized products were purified with Gilson HPLC system using the C18 reverse-phase column (GL Sciences) Inertsil ODS-3 (5 μ m, 25 cm $\times$ 0.46 cm) as previously described [36]. Mass spectra of all synthesized compounds were measured with MALDI-TOF mass spectrometer Voyager DE-Pro (Applied Biosystems). Unicam UV 300 (ThermoSpectronic) spectrometer was used for measuring UV-vis spectra and quantification of the products. Concentrations of compounds for biological testing were measured by UV spectroscopy (based on molar extinction coefficient of 15,000 M⁻¹ cm⁻¹ at 260 nm for adenosine-containing compounds and for 5-(2-aminopyrimidin-4-yl) thiophene-containing compounds, 4400 M⁻¹ cm⁻¹ at 323 nm for isoquinoline-sulfonamide derivatives, 80,000 M⁻¹ cm⁻¹ at 558 nm for 5-TAMRA-containing compounds, and 30,000 M⁻¹ cm⁻¹ at 260 nm for H89).

[γ -³²P]ATP (4500 Ci/mmol) for the radioactive kinetic assay with PKGI α was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, USA and Eschwege, Germany) and phospho-cellulose paper filters (P-81) from Whatman (Whatman House, UK). For FRET measurements, gene construct Cygnet 2.1 [42] was used (consists of cDNA of ECFP, PKGI α with N-terminal deletion Δ 1–77 and mutation T516A, and Citrine [43]).

2.2. Equipment

Fluorescence anisotropy readings were taken on a PHERAstar microplate reader (BMG Labtech) with FP optic module [excitation 540(20) nm, emission 590(20) nm]. Corning black low volume 384-well NBS plates (Code 3676) were used for fluorescence anisotropy readings. FRET measurements were performed with fluorescence spectrometer F-4500; Hitachi, Tokyo [excitation 480(20) nm, emission 535(20)/580 (20) nm]. Fluorescence intensity measurements were performed either with PHERAstar microplate reader in parallel to fluorescence anisotropy measurements, or with fluorescence spectrometer F-4500; Hitachi, Tokyo [excitation 540(10) nm, emission 580(10) nm].

Isolated arteries were examined with a Bio-Rad MRC 1024ES confocal scanning laser microscopy system. All images were captured with a 100 $\times$ oil immersion objective lense (NA=1.3) mounted on an Olympus BX50 (New Hyde Park, NJ) upright microscope. Optical thickness was approximately 1.5 μ m. Single optical sections were acquired with seven Kalman averages.

2.3. Software

The data from the assays were fitted with the aid of GraphPad Prism Version 5. Anisotropy titration equations [39] were used for the binding curves, and sigmoidal dose-response variable slope regression functions for the displacement, FRET and inhibition curves.

2.4. Synthesis of ARCs

Synthesis of ARC-type inhibitors and chemical labelling of ARCs with 5-TAMRA N-hydroxysuccinimide (NHS) ester were performed according to the previously described

procedures [34,37–39]. All compounds except ARC-661, ARC-668, ARC-669, ARC-905, ARC-1059, ARC-1067, ARC-1068, and ARC-1069 have been previously described. The structures of all ARCs are presented in the Supplementary data. All compounds used in biological tests were >95% pure by HPLC and possessed predicted m/z values according to mass spectrometric measurements.

2.5. Fluorescence anisotropy/polarization-based binding/displacement assay (FA-assay)

FA measurements were carried out according to the previously described protocol [39]. For the determination of binding characteristics towards PKGI α or Cygnet 2.1, titration of fluorescent ARCs (2 nM or 5 nM, respectively) was performed with the kinase in the presence (1 μ M) or absence of cGMP. For the determination of binding proportion of PKGI α , the compound with the best binding affinity ARC-1059 at 20 nM concentration was titrated with PKGI α solution. To assess the effect of Mg²⁺, magnesium acetate (10 mM) was added to the buffer. For the determination of dissociation/displacement IC₅₀ values, fixed concentrations of PKGI α and ARC-1059 (4 nM and 2 nM, respectively) in the presence of cGMP (1 μ M) were added to different concentrations of the displacing compound.

2.6. Fluorescence intensity-based binding/displacement assay (FI-assay)

Fluorescence intensity was first monitored in parallel to fluorescence anisotropy in binding assay with PHERAstar microplate reader, and the further experiments were performed with fluorescence spectrometer F-4500. In the first case, the conditions were the same as in FA-assay. In the second case, the binding of ARC-1059 (2 nM) to PKGI α was performed in a quartz cuvette in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) in the presence of cGMP (40 μ M) and glycerol (1% v/v) to reduce the non-specific binding to the cuvette surface. The total solution volume in the cuvette was 500 μ L. The starting concentration of PKGI α was 42 nM (3.4 μ g/mL), then 250 μ L of the reaction mixture was taken out of the cuvette and 250 μ L of ARC-1059 (2 nM) and cGMP (40 μ M) solution in PBS was added. The concentration of PKGI α was hence 2-fold reduced, whereas the concentration of the other components was kept constant. The dilution steps were repeated until the concentration of PKGI α was below 0.5 nM. The displacement of ARC-1059 (2 nM) from its complex with PKGI α (4.2 nM, 0.34 μ g/mL) in the presence of cGMP (40 μ M) was performed by addition of increasing concentration of the displacing compounds.

2.7. Inhibition assay

Inhibition IC₅₀ values for compounds were measured with the phospho-cellulose assay [18] at 100 μ M [γ -32P]ATP (specific activity 300–400 cpm/pmol) and 11 μ M substrate peptide (amino acid sequence TQAKRKSLAMA). The inhibition mechanism of ARC-903 and ARC-668 towards ATP was established as follows. To the eppendorf tubes containing 3-fold dilution series of [γ -32P]ATP (with the fixed specific activity of 50–100 cpm/pmol), inhibitor ARC-903 or ARC-668 and the reaction mixture containing peptide substrate solution was added. The reaction was started by the addition of PKGI α solution. The final solution in each eppendorf tube contained cGMP (10 μ M), substrate peptide (11 μ M), PKGI α (0.4 nM), varying concentration of [γ -32P]ATP, and fixed concentration of the inhibitor (1 nM, 2 nM and 5 nM in case of ARC-903; 2 nM, 5 nM and 10 nM in case of ARC-668; H₂O in case of non-inhibited curve). After incubation for 5 min at 30 °C, aliquots were spotted on phospho-cellulose paper filters, extensively washed in 75 mM phosphoric acid, and the dried filters were subjected to scintillation counting. The curves were plotted on Schild plot and analyzed according to the suitable Schild equation. The inhibition mechanism of ARC-903 and ARC-668 towards substrate peptide was determined analogically to the previous, except that the substrate dilution series were used in the first

step to which ARC-903 or ARC-668 and the reaction mixture containing [γ - 32 P]ATP (1 mM, specific activity 50–100 cpm/pmol) were subsequently added.

2.8. Measurements of FRET between Citrine of Cygnet 2.1 and TAMRA of ARC

The construct of PKGI α (Cygnet 2.1) that has Citrine (excitation maximum at 510 nm, emission maximum at 530 nm [43]) fused to the C-terminus of the kinase fragment was used as the FRET donor, and ARC-1059 incorporating fluorescent dye TAMRA (excitation maximum at 558 nm, emission maximum at 585 nm) as the FRET acceptor. The measurements of the emission spectra in the wavelength range from 500 nm to 700 nm were performed with fluorescence spectrometer F-4500 in a cuvette in PBS buffer (the total solution volume 500 μ L). First of all, Cygnet 2.1 (24 nM, 2.9 μ g/mL) was titrated in the absence of cGMP with increasing concentration of ARC-1059; next, the same titration was carried out in the presence of the saturating concentration (12 μ M) of non-fluorescent competing compound ARC-903. The difference of the values of the emission ratio 580 nm/530 nm between these two curves was defined as the maximum FRET signal at the given concentration of Cygnet 2.1 and ARC-1059. Subsequently, the same titration was performed in the presence of cGMP (40 μ M). From the data, the concentration of ARC-1059 was determined (100 nM) where the emission ratio 580 nm/530 nm was 2-fold higher for the curve measured without the disrupting compound ARC-903 as compared to the curve measured with the disrupting compound. Next, displacement of ARC-1059 (100 nM) from its complex with Cygnet 2.1 (24 nM, 2.9 μ g/mL) in the presence (40 μ M) or absence of cGMP was performed by addition of increasing concentration of the disrupting compounds.

2.9. Fluorescence imaging of vascular smooth muscle cells in intact arteries

Isolated intact cerebral arteries were incubated with ARC-1059 (100 nM, 1 μ M or 10 μ M for 20 min, 40 min or 1 h) or ARC-669 (10 μ M for 1 h) in Hepes buffer; fluorescein-labelled DT-2 (2 μ M) was used as a reference. For artery incubated with FITC-DT-2, nuclear staining was performed using TOTO-3 (red) as previously described [18]. Arteries were washed 3 times and examined with a confocal scanning laser microscopy system at 20 $\times$ and 40 $\times$ magnification. TAMRA was imaged with 568 nm excitation, fluorescein with the 488 nm excitation, and nuclear stain with 647 nm excitation line of a krypton–argon laser.

2.10. Diameter measurements in isolated pressurized cerebral arteries

The diameter of segments of small posterior cerebral arteries was measured by using a video dimension analyzer as previously described [44]. The vessels were pressurized to 80 mm Hg, which induced sustained myogenic tone; vasodilation mediated by 50 μ M 8-Br-cGMP or 25 nM MAHMA-NONOate was used for monitoring ARC-903 (4 μ M) inhibitory effects on PKGI α , and vasodilation mediated by 0.5 μ M forskolin or 100 μ M 8-CPT-cAMP for monitoring ARC-903 (4 μ M) inhibitory effects on PKAc. Before the application of every vasodilator, the artery was subjected to PSS wash. Finally, to confirm the functionality of the artery after the second part of experiment the arteries were treated with Ca $^{2+}$ -channel blocker nisoldipine, which induced the maximal vasodilation.

3. Results

3.1. Binding assays in vitro

During the course of ARC design and optimization towards PKAc, four main structural subtypes of ARCs were established [34,38,39] and developed into the fluorescent probes ARC-583, ARC-1059, ARC-1042, and ARC-669 (see structures in Supplementary data); on the basis of these ligands, a swift homogeneous assay with fluorescence anisotropy/polarization detection (FA) was elaborated [39]. FA allows determination of the dissociation

constants (K_D) of the fluorescently labelled compounds from the direct binding assay by titration with the kinase (Fig. 1A), and the displacement constant values (K_d) from the displacement assay format, where the fluorescent probe is displaced from its complex with the kinase by the increasing concentration of the competing compound (Fig. 1B).

Additionally, the previous studies revealed that upon binding of the fluorescent probe to the kinase not only increase in anisotropy but also change of the emission intensity of chromophore might be monitored. Hence, a constant Q was introduced to the mathematical model for FA-assay [39], representing the ratio between the emission intensity of bound form and non-bound form of a fluorescent ARC; the value of Q was dependent on the structure of fluorescent ARC, the conjugated chromophore, and the protein kinase used in the assay. In case of sufficiently high Q value ($Q > 2$), the binding or displacement of a fluorescent ARC might be monitored not only *via* change of fluorescence anisotropy but also *via* change of fluorescence intensity (Fig. 1); this phenomenon was attributed to the partial quenching of the chromophore in solution as compared to the kinase-bound state.

As no preliminary data existed about affinity of ARCs towards PKGI α , the first step was performance of FA direct binding assay with all four probes representing main structural subtypes of ARCs. The binding curves were first measured in the absence of both, cGMP and Mg²⁺ (Table 1). Out of four compounds, ARC-583 and ARC-1042 had very low affinity towards PKGI α , whereas ARC-1059 and ARC-669 bound to kinase with nanomolar K_D values, and the effect of cGMP and Mg²⁺ on their binding to PKGI α could be assessed. In case of ARC-669, the addition of cGMP (in the absence of Mg²⁺) increased the affinity of probe by 5-fold, and in case of ARC-1059 the affinity was increased 8-fold resulting in subnanomolar K_D value. The latter result is in good agreement with the fact that upon activation by cGMP, the catalytic activity of PKGI α increases by 8–10-fold.

In case of ARC-1059 and ARC-669, not only increase in anisotropy but also increase in the emission intensity of chromophore (Q values of 3.5 and 1.5, respectively) upon binding of the fluorescent probe to PKGI α could be monitored (Fig. 2A and B), whereas the K_D values calculated from the binding curves from fluorescence intensity measurements were in good agreement with the K_D values calculated from FA (Table 1). This observation led to the development of an alternative assay with the detection of fluorescence intensity (FI), whereas FI could be also successfully performed in a cuvette with fluorescence spectrometer (Table 1 and Fig. 2C) and thus required neither complicated equipment for the detection of polarization nor microplate reader format, which is a substantial advantage over FA. Consequently, spectrometer-format was chosen for the further FI measurements to demonstrate the broad applicability of the novel assay.

3.2. Displacement and inhibition assays *in vitro*

In order to perform wide-scale screening of various ARCs with PKGI α , the displacement of fluorescent probe ARC-1059 from its complex with PKGI α (in the presence of cGMP) by non-fluorescent ARCs was carried out. ARC-1059 was chosen as the fluorescent probe, as it revealed the lowest K_D value in both FA (0.36 nM) and FI (0.63 nM) binding assays. In parallel, the inhibitory properties of non-fluorescent ARCs were assessed by the 'classical' kinetic assay applying radioactive ATP. The phosphorylation of substrate peptide TQAKRKSLAMA with [γ -32P]ATP catalyzed by PKGI α was monitored in the presence of cGMP at the different concentrations of the inhibitors; H9 and H89 were used as reference compounds. Table 2 presents the results of the displacement and inhibition experiments.

The displacement and inhibition studies *in vitro* (Table 2) revealed the most potent ARCs that could further be applied for the detailed *in vitro* studies and determination of

physiological effects of ARCs in more complex biological systems rich in PKAc and PKGI α . The best displacement and inhibition characteristics (displacement IC₅₀ value of 5.4 nM in FA-assay, inhibition IC₅₀ below 1 nM in inhibition assay) were possessed by ARC-903 (the non-fluorescent analogue of ARC-1059). The displacement and inhibition IC₅₀ values of compounds ARC-663 and ARC-664 were also in the low nanomolar range, which correlated well with high degree of structural similarity of both compounds to the fluorescent probe ARC-669.

There was a good correlation between structure–activity relationship data from the FA and FI displacement assays, as well as inhibition assay, although it was clear that in case of the compounds with best inhibitory properties (*e.g.*, ARC-903), the kinetic assay was performed in the tight-binding conditions [46], meaning that the active enzyme concentration was close to the obtained IC₅₀ values. There were only two major deviations between the data from different assays: in FI, the relatively high displacement IC₅₀ value (89.1 nM) for ARC-668, the compound derived from ARC-664 and possessing low nanomolar IC₅₀ in inhibition measurements; and in inhibition assay, the surprisingly high inhibition IC₅₀ value of compound ARC-349 as compared to the displacement data from FA-assay. In general, both FI and especially inhibition assay tended to overestimate the affinity of competing compounds (*i.e.*, slightly underestimate IC₅₀ values), as also confirmed by the relatively low inhibition IC₅₀ values of the reference compounds H9 and H89 (141 nM and 55 nM, respectively, whereas K_i values of 870 nM and 480 nM had been reported in the literature for H9 and H89, accordingly [14,15]).

ARC-903 and ARC-668 were then further characterized to determine their inhibition mechanism *versus* ATP or *versus* substrate peptide. In the first case, the concentration of ATP (K_m = 20 μ M) was varied (but the specific activity preserved) and the concentration of the substrate peptide was fixed (10 μ M); in the second case, the concentration of the substrate peptide (K_m = 0.26 μ M) was varied and the concentration of ATP was fixed (1 mM concentration of ATP was used to achieve distinguishable effects in the presence of high-affinity ARCs). In both cases, the phosphorylation extent was then measured at different concentrations of the inhibitor and the inhibition pattern assessed on the basis of the shift of the parameters of the inhibited kinetic curves *versus* the non-inhibited kinetic curve, applying Schild equation. The data for ARC-903 is presented in Fig. 3.

Both inhibitors were competitive *versus* ATP, in accordance with the results of the previously reported inhibition studies with PKAc [36]. On the other hand, both inhibitors were clearly not competitive *versus* substrate peptide; in fact, the obtained Schild plot resembled uncompetitive inhibition pattern, although in case of tight-binding conditions and steep Hill slope, it would be difficult to distinguish between uncompetitive and non-competitive inhibition.

The values of inhibition constant K_i (calculated from the Schild competitive logarithmic plot *versus* ATP for ARC-903 and ARC-668) were 1.33 nM and 2.27 nM, respectively, which was in good agreement with inhibition IC₅₀ values from the previously described assay (0.982 nM and 1.45 nM, respectively). These results showed that even at 1 mM concentration of ATP, PKGI α is fully inhibited already at 10 nM concentration of ARC; hence, ATP-competitive mechanism should not be an obstacle for the intracellular application of ARCs.

3.3. FRET measurements

The FA and FI-assays possess several advantages, such as low detection limit and low consumption of reagents; nevertheless, those assays are sensitive towards the sample matrix effects, and thus have limited applicability in biologically complex systems such as cell

lysates and living cells. Currently, a large number of semi-quantitative *in vitro* and intracellular assays are performed in FRET format, as it allows considerable reduction of background noise and off-target signals; in case of ARCs, the FRET-format assays have previously been performed between PKAc catalytic subunit tagged to a fluorescent protein (FRET donor) and a fluorescently labelled ARC (FRET acceptor) [35, A. Vaasa (manuscript in preparation)]. The same experimental design could be applied for FRET measurements between a fragment of PKGI α tagged to a fluorescent protein (Citrine, FRET donor) and a ARC-1059 (labelled with TAMRA, FRET acceptor). If FRET occurs upon addition of ARC-1059 to the Cygnet 2.1, the fluorescence intensity of Citrine emission at 530 nm should decrease and the fluorescence intensity of TAMRA emission at 580 nm should increase due to the Förster resonance energy transfer from Citrine to TAMRA (Fig. 4A); this FRET signal should be disrupted when the displacement of fluorescent ARC-1059 occurs by its non-fluorescent analogue (Fig. 4B).

As FRET donor, previously reported construct Cygnet 2.1 [42] was used which contains a fluorescent protein Citrine fused to the C-terminus of the PKGI α fragment. The initial experiment showed indeed that in the mixture of Cygnet 2.1 and ARC-1059, the ratio of emission intensities at 580 nm and 530 nm is higher than in pure Cygnet 2.1 or pure ARC-1059 solutions (excited at 480 nm), indicating occurrence of the FRET phenomenon. Next, the disruption of FRET between Cygnet 2.1 and ARC-1059 by non-fluorescent compounds ARC-903 and ARC-668 in the presence or absence of cGMP was monitored. In all cases, the excitation of Citrine was performed at 480 nm (local absorption maximum in Citrine spectrum) to avoid the straight excitation of TAMRA by photons with the higher wavelength.

The FRET disruption data by ARC-903 is presented in Fig. 5; as fluorescence intensity of ARC-1059 decreases upon dissociation from the complex with PKGI α , both the ratio of emission intensities at 580 and 530 nm and the change of emission intensity at 530 nm alone (Citrine emission channel) were considered.

Interestingly, the FRET disruption 'window' (the difference between MAX and MIN in Fig. 5) was smaller in the presence of cGMP, but the disruption potency of ARC-903 and ARC-668 was independent on the absence or presence of cGMP. Overall, these results demonstrate that ARCs might be successfully applied in FRET-format assays with cGMP *in vitro*, and provide basis for intracellular FRET-based assays for the studies of localization of fluorescently tagged PKGI α and screening of PKGI α -targeted compounds.

3.4. Fluorescence imaging of vascular smooth muscle cells in intact arteries

ARCs contain an oligo-D-arginine motif that is a well-known cell-penetrating peptide (CPP) sequence [47]; moreover, the D-amino acids provide ARCs with proteolytic stability. Hence, despite highly charged nature, ARCs are potential candidates for the intracellular and *in vivo* assays. Previously, the internalization of ARCs into cultured HEK-293 and HeLa cells [40] and their intracellular activity [41] has been demonstrated; this time, cell-penetrative properties of fluorescent ARCs were monitored in vascular smooth muscle tissue, which is rich in PKGI α and PKAc.

The rat isolated cerebral arteries were incubated for 20, 40 or 60 min at room temperature with ARC-1059 (100 nM, 1 μ M or 10 μ M) or ARC-669 (10 μ M). As a reference, fluorescein-labelled DT-2 (2 μ M) was used together with a nuclear stain. Both ARCs internalized into vascular smooth muscle cells within intact pial arteries from rat brain (Fig. 6) and demonstrated nuclear translocation that allowed spotting of the different shapes of the nuclei (straight long nucleus in rest phase and sickle-like shape during contraction), the phenomenon characteristic for vascular smooth muscle cells.

The internalization of ARC-1059 in smooth muscle tissue was not well observable at concentrations lower than 1 μM . Importantly, at 10 μM concentration of ARC-1059 the internalization into tissue cells was observable already after 20 min incubation although the nucleus was not yet stained (Fig. 6D). The former effect is similar to the previously reported characteristics of FITC-labelled DT-2 [48] that also relies upon highly positively charged CPP for transport into the cells.

On the whole, the tissue staining studies demonstrated that despite their high charge and molecular weight, fluorescent ARCs are able to penetrate through the cell plasma membrane, which encouraged performance of the further experiments in order to examine whether ARC induce physiological effects by inhibition of PKGI α and/or PKAc in isolated arteries. Additionally, the cell-penetrative properties of ARCs pointed to the possibility of conversion of assays based on fluorescent ARCs (with FA-, FI- or FRET-detection) from *in vitro* biochemical format (assays with purified kinase solution) to cellular format (assays with living cells).

3.5. Diameter measurements in isolated pressurized cerebral arteries

Both PKGI α and PKAc induce vasodilation by targeting the components of contractile signalling pathways by both relaxation of the initial Ca^{2+} -dependent contraction and the sustained (Ca^{2+} -independent) muscle contraction [4,49]. Thus, pressure myography where the change in diameter of a pre-constricted artery is monitored as a response to the externally applied vasodilating and/or -constricting compounds offers a unique possibility to assess the physiological properties of PKGI α /PKAc-targeted inhibitors by blockage of PKGI α /PKAc-triggered vasodilation [18,19,44,50].

ARC-903, the inhibitor with subnanomolar K_d towards PKAc and the best inhibition and affinity characteristics of the studied ARCs towards PKGI α , was chosen for the pressure myography experiments. Diameter measurements were carried out in rat isolated pressurized cerebral arteries (Fig. 7). Vasodilation mediated by 50 μM 8-Br-cGMP or 25 nM NO donor MAHMA-NONOate was used for monitoring ARC-903 (4 μM) inhibitory effects on PKGI α , and vasodilation mediated by 0.5 μM forskolin or 100 μM 8-CPT-cAMP for monitoring ARC-903 (4 μM) inhibitory effects on PKAc. In all cases, after addition of ARC-903 (4 μM) a substantial constriction (below the basal level) was observed (Fig. 8). This effect has been previously reported for DT-2 in case of PKGI α -caused vasodilation [50]; however, DT-2 as a selective inhibitor of PKGI α over PKAc did not diminish the PKAc-induced vasodilation [50]. Moreover, ARC-903 caused constriction of arteries not stimulated by vasodilators (Fig. 8, last column), pointing to the ability of ARC-903 to inhibit the basal activity of protein kinases.

4. Discussion

ARCs consist of two structural fragments: the nucleosidic moiety (adenosine analogue) and the peptidic moiety (oligo-D-arginine), which are joined by a flexible linker [34,36]; in the ARCs of the later generations, a chiral spacer was additionally introduced into the linker structure (Fig. 9). Variation of any of the aforementioned structural fragments results in changes of affinity and selectivity of ARCs towards their biological targets, as has been demonstrated in previous studies [34,36–38,51].

The current study revealed that the structure–activity (SA) profile of ARCs with PKGI α is considerably different from that with PKAc. Out of four fluorescent probes, ARC-1059 and ARC-669 containing respectively an 5-isoquinoline-sulfonamide derivative (Hidaka's protein kinase inhibitor H9) or an aminopyrimidine derivative as the nucleosidic fragment of the probe, bound to kinase with nanomolar K_D values, whereas ARC-583 and ARC-1042

(both contain an adenosine derivative as the nucleosidic fragment) had very low affinity towards PKGI α . The same tendencies were confirmed in the displacement and inhibition studies with PKGI α : ARCs containing 5-isoquinoline-sulfonamide or aminopyrimidine derivatives (*e.g.*, ARC-903, ARC-663, and ARC-668) possessed considerably higher affinity towards PKGI α than their adenosine derivative-containing counterparts (*e.g.*, ARC-902 and ARC-1028). These results reveal clearly different patterns of compound binding to PKGI α *versus* PKAc, as ARC-583 and especially ARC-1042 are high-affinity fluorescent probes of PKAc (K_D = 0.48 nM and K_D below 0.3 nM, respectively) [34,39], and the corresponding non-fluorescent compounds (ARC-902 and ARC-1028, respectively) possess subnanomolar displacement constant and inhibition IC_{50} values. On the other hand, it has been previously demonstrated with the protein kinase panel testing that the H9-containing compound ARC-903 and aminopyrimidine-containing compound ARC-664 were more generic inhibitors than the adenosine-containing compounds [34,36,38], which fits with the current findings, as well as the extremely weak binding to PKGI α exhibited by the compound ARC-1044 (incorporating carbocyclic 3'-deoxyadenosine analogue in the nucleosidic moiety) that has shown remarkable PKAc-selectivity in binding assays with different representatives of AGC-group kinases [37].

Nevertheless, there were also similar tendencies in the SA profile of ARCs with PKGI α as previously reported for ARCs with PKAc. Firstly, PKGI α was able to bind inhibitors that contain either L- or D-amino acids, whereas conjugates containing D-amino acids (ARC-903 and ARC-902) possessed even higher affinity and inhibition potential than their counterparts containing L-amino acids (ARC-349 and ARC-341, respectively); the similar results have already been demonstrated in assays with compounds derived from the substrates of PKAc and PKGI α [8], and with DT-2 family representatives [19]. Secondly, PKGI α did not tolerate well the reduction of the number of arginine residues in the peptidic fragment of ARCs, as can be seen from comparison of displacement IC_{50} values for ARC-664 and ARC-1102 (10.7 nM and 229 nM, respectively), or ARC-903 and ARC-1069 (5.4 nM and 457 nM, respectively), although in the latter case the introduction of amide bond into the linker chain should also be taken into account. Thirdly, PKGI α demonstrated preference for elongated linker structures, as affinities of compounds increased in the row ARC-1067 < ARC-1068 < ARC-1069. Finally, the low displacement IC_{50} and inhibition IC_{50} values of both ARC-663 (incorporates D-Ala residue as the chiral spacer) and ARC-664 (incorporates D-Lys residue in the corresponding position) showed that the basic chiral spacer is not crucial for binding to PKGI α , similarly to PKAc and differently from the ROCK-II and PKB γ , according to the previous studies [34].

Previously, it has been demonstrated that ARCs inhibit PKAc by the bisubstrate-analogue mechanism; that is, upon binding of an ARC to PKAc, the nucleosidic fragment occupies the ATP-binding site and the peptidic fragment occupies the protein/peptide substrate-binding site of the kinase (Figs. 1 and 9). The bisubstrate character of ARCs in respect to PKAc was confirmed by displacement of a fluorescent ARC from its complex with PKAc by either the ATP-competitive or the substrate peptide-competitive inhibitors [34,39,52], by crystallographic data [A. Pflug (manuscript in preparation)], and by kinetic studies of PKAc inhibition by a non-fluorescent ARC [36]. In assays with PKGI α , the fluorescent probe ARC-1059 could be successfully displaced from its complex with PKGI α by ATP, MgATP and ATP-competitive inhibitors H9, H89 and staurosporine. KT-5823 failed to displace ARC-1059 from its complex with PKGI α , but several recent reports [53–55] have also claimed that KT-5823 failed to inhibit PKGI α , raising doubts considering its affinity. In kinetic studies of PKGI α inhibition mechanism, both ARC-903 and ARC-668 were competitive *versus* ATP, in accordance with the results of the previously reported inhibition studies with PKAc [36]. All of the aforementioned results indicate that the nucleosidic moiety of ARC-1059 indeed binds to the ATP-pocket of PKGI α .

However, the displacement by the compounds targeted to the peptide-site of PKGI α (substrate peptide TQAKRKKSLAMA-NH₂, results not shown, and its non-phosphorylatable analogue TQAKRKKALAMA-NH₂) was inconclusive, as the displacement of the fluorescent probe was apparent only at very high (over 100 μ M) concentration of the peptides, and could not be completed. Another unexpected observation was poor displacement of ARC-1059 from its complex with PKGI α by D-DT-2, as the displacement seemed to be only partial and the calculated K_d value for D-DT-2 was irrelevantly high (nearly micromolar, results not shown). On the other hand, the comparison of displacement and inhibition IC₅₀ values of compounds with (D-Arg)₆ and (D-Arg)₂ moieties reveals that the peptidic moiety of ARC-compounds develops interactions with some region PKGI α , as the formers show in any case several powers of magnitude better binding affinity than the latters. Furthermore, the measurement of FRET from Cygnet 2.1 to ARC-1059 was successful. Cygnet 2.1 has Citrine-tag at the C-terminus of the kinase fragment, and the C-terminus of ARC-1059 is labelled with TAMRA, thus the efficient Förster resonance energy transfer from Citrine to TAMRA is only possible if TAMRA dye is spatially positioned relatively close to the Citrine fluorophore, meaning that the peptidic moiety of ARCs still binds to a domain situated near the C-terminus of the PKGI α .

In kinetic studies with PKGI α , both ARC-903 and ARC-668 were clearly not competitive *versus* the substrate peptide; in fact, the obtained Schild plot resembled uncompetitive inhibition pattern, although in case of tight-binding conditions and steep Hill slope, it would be difficult to distinguish between uncompetitive and non-competitive inhibition. The previously reported studies with PKAc revealed non-competitive mechanism of ARC-inhibition *versus* substrate peptide [36]. To sum it up, it is possible that ARC-type compounds are not the bisubstrate-analogue inhibitors of PKGI α but rather express a different (biligand) binding pattern where the nucleosidic moiety predictably binds to the ATP-pocket but the peptidic moiety interacts with amino acids of enzyme not positioned in the protein/peptide substrate pocket. However, the recent investigation of binding of DT-2 peptide to PKGI α by means of mass spectrometry [56], and the unusually steep Hill slopes (values from -1.5 to -2.0) of the ARC displacement and inhibition curves suggest that other binding modes are possible (*e.g.*, association of an ARC molecule with both polypeptides of the dimeric PKGI α apoenzyme), and allosteric modulation and cooperative effects should also be considered.

Overall, the *in vitro* biochemical experiments proved that: (A) several ARCs possessed low nanomolar affinity towards PKGI α ; (B) SA profiling of ARCs with PKGI α demonstrated both similarities as well as differences to corresponding profiling with PKAc; (C) FA-assay based on application of fluorescent ARCs and developed for PKAc and ROCK-II could be successfully customized for another important biological target, PKGI α ; and (D) fluorescent probe ARC-1059 exhibited substantial increase of brightness upon binding to PKGI α and thus enabled design of FI-assay, which is applicable in the microplate reader format as well as in the alternative spectrometer cuvette-format.

The unique properties of ARCs, however, allow spreading the field of ARC application out of the limits of *in vitro* biochemical assays. ARCs contain an oligo-D-arginine motif that is a well-known cell-penetrating peptide (CPP) sequence [47]; the mechanism of internalization of arginine-rich peptides is still under dispute and probably combines several modes of penetration [57–60], although endocytosis has been suggested as the main variant. From the studies of the effect of time and concentration on ARC-1059 internalization into smooth muscle tissue of intact arteries, it was established that internalization is not well observable at concentrations of the compound lower than 1 μ M, which has been previously discovered in cultured HEK-293 cells; probably, the internalization mechanism of ARCs requires a ‘critical’ concentration of the compounds to penetrate through the cell plasma membrane

[61]. Another common feature of studies in HEK-293 cells and in smooth muscle tissue was observance of nuclear inclusion phenomenon; previously, the localization of ARCs into nuclei of cultured HEK-293 cells has also been observed whereas the staining of certain regions of the nucleus (probably nucleoli) was evident [A. Vaasa (manuscript in preparation)].

The application of non-fluorescent ARC-903 in the pressure myography studies with the intact cerebellum arteries showed that ARCs not only internalize into the smooth muscle tissue, but also induce physiological responses by diminishing the PKG1 α - and PKAc-induced vasodilation; moreover, ARC-903 caused constriction of non-dilated artery, indicating that ARCs are capable of inhibition of the basal kinase activity. All of the aforementioned results confirm the fact that the inhibitor with high affinity towards several basophilic kinases is not only able to successfully influence physiological cascades of its target molecules but also yield remarkable responses. ARC-903 is a generic inhibitor of basophilic protein kinases (especially from the AGC-group); in addition to PKG1 α , it is also highly potent inhibitor of PKAc, PKB isoforms, and ROCK-II. As both kinases, PKG1 α and PKAc induce vasodilation, the inhibition of PKG1 α (activity stimulated by 8-Br-cGMP or MAHMA-NONOate) and PKAc (activity stimulated by Forskolin or 8-CPT-cAMP) should logically result in muscle contraction to basal level. However, ROCK-II mediates inhibition of MLC phosphatase; hence, inhibition of ROCK-II (normal level) should result in increase of MLC phosphatase activity, dephosphorylation of MLC₂₀ and relaxation of smooth muscle above basal level [4,49,62]. Overall, the concomitant inhibition of all the aforementioned kinases (PKAc, PKG1 α and ROCK-II) should result only in partial constriction of the diameter of the arteries. However, the pressure myography assay showed that upon addition of ARC-903, the contraction occurred to the basal level or even below. Our explanation to the phenomenon is that the effects resulting from the inhibition of PKG1 α and PKAc exceed the effects resulting from the inhibition of ROCK-II. To sum up, the effects of ARC-903 should be more closely studied in arteries with stimulated activity of ROCK-II, and the future design of inhibitors should be focussed on compounds with high affinity towards both PKG1 α and PKAc, and low affinity towards ROCK-II to achieve even higher levels of arterial constriction.

5. Conclusion

In conclusion, ARC-type compounds that had been initially developed and optimized for PKAc inhibition were evaluated as inhibitors and fluorescent probes of PKG1 α . Two of these compounds, ARC-903 and ARC-668 revealed low nanomolar displacement constants and inhibition IC₅₀ values with PKG1 α . The binding assay with fluorescence anisotropy detection that utilizes ARC-based fluorescent probes and has recently been introduced for closely related kinases PKAc and ROCK-II, could be successfully applied for the determination of the affinity of PKG1 α -targeted compounds. Moreover, based on the substantial increase of the brightness of fluorescent probe ARC-1059 as a result of its binding to the target kinase, new variant of the binding/displacement assay with the detection of fluorescence intensity change was developed. Finally, the ability of ARC-based fluorescent probes to penetrate cell plasma membrane was demonstrated in the smooth muscle tissue of rat cerebellum isolated arteries, and the compound with highest affinity *in vitro* (ARC-903) showed also potential for *in vivo* applications, fully abolishing the PKG1 α -induced vasodilation. To the best of our knowledge, this was the first demonstration of the tissue-level physiological effect of ARC-type inhibitors and bisubstrate-analogue inhibitors of protein kinases in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ac	ethanoic acid moiety
Adc	adenosine-4'-dehydroxymethyl-4'-carboxylic acid
Ahx	6-aminohexanoic acid moiety
AMTH	5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid
ARC	conjugate of adenosine analogue and oligo-D-arginine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCDA	carbocyclic 3'-deoxyadenosine
cGMP	cyclic guanosine monophosphate
CPP	cell-penetrating peptide
DTT	dithiothreitol
FA	fluorescence polarization/anisotropy
FI	fluorescence intensity
Fmoc	N-(fluorenyl-9-methoxycarbonyl) moiety
FRET	Förster resonance energy transfer
Hex	n-hexanoic acid moiety
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MLC₂₀	myosin regulatory light chain
PBS	phosphate-buffered saline
PKA	cAMP-dependent protein kinase (protein kinase A)
PKAc	cAMP-dependent protein kinase catalytic subunit type α
PKG1	cGMP-dependent protein kinase (protein kinase G) isoform I
PKI	thermostable protein kinase inhibitor peptide
PSS	physiological salt solution
PUPI	1-(9H-purin-6-yl)piperidin-4-amine
PUTR	1-(9H-purin-6-yl)-1H-1,2,3-triazole-4-carboxylic acid
R-II	cAMP-dependent protein kinase catalytic subunit type II
ROCK-II	Rho kinase type II

(Rp)-8-Br-PET-cGMPS	8-bromo- β -phenyl-1,N ² -ethenoguanosine-3',5'-cyclic monophosphorothioate, Rp-isomer
(Rp)-8-Br-cGMPS	8-bromoguanosine-3',5'-cyclic monophosphorothioate, Rp-isomer
SA profile	structure–activity profile
TAMRA	5'-carboxy tetramethylrhodamine

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbapap.2010.04.007.

Fig. 1.

Assays based on measurement of fluorescence of labelled ARCs (ARC-Photos): binding of ARC-Photo to a protein kinase leads to change of both anisotropy and emission intensity of the chromophore. A, Binding format; B, displacement format.

Fig. 2. Titration of 2 nM ARC-1059 with PKG1 α in the presence of cGMP. A, FA-assay with PHERAstar microplate reader (BMG Labtech); B, FI-assay with PHERAstar microplate reader (BMG Labtech); C, FI-assay with fluorescence spectrometer (Hitachi F-4500).

Fig. 3. Determination of mechanism of inhibition of PKGI α with ARC-903 in the presence of cGMP. A, Inhibition pattern *versus* ATP; B, inhibition pattern *versus* substrate peptide. The insets show the corresponding logarithmic Schild plots (A, competitive inhibition; B, uncompetitive inhibition).

Fig. 4. Fluorescent assays based on fluorescently labelled ARCs (ARC-Photos): application of FRET phenomenon. A, Binding format; B, displacement format.

Fig. 5.

Disruption of FRET between Cygnet 2.1 and ARC-1059 by ARC-903. A, Change of ratio of emission intensities at 580 and 530 nm; B, change of emission intensity depicted separately for both channels (with or without cGMP, as indicated in the legend). MAX, measured in the absence of ARC-903; MIN, measured in the absence of ARC-1059.

Fig. 6. Internalization of compounds into vascular smooth muscle cells in intact arteries. A, FITC-labelled DT-2 (2 μ M, green colour) with nuclear stain (blue colour), 20 $\times$ magnification, incubation time 60 min; B, ARC-669 (10 μ M) 40 $\times$ magnification, incubation time 60 min; C, ARC-1059 (10 μ M) 40 $\times$ magnification, incubation time 60 min; D, ARC-1059 (10 μ M) 40 $\times$ magnification, incubation time 20 min.

Fig. 8.

Comparison of diameter change of isolated pressurized cerebral arteries after application of vasodilators and ARC-903, or ARC-903 alone.

Fig. 9. Schematic representation of an ARC bound to a protein kinase. The fragments of inhibitor are represented as coloured shapes; flashes indicate positions where fluorescent dyes or other entities may be conjugated. The black curves represent parts of the protein kinase interacting with the ARC.

Table 1

Binding constants (standard errors in parentheses) of fluorescent ARCs with PKGI α and with PKAc as determined by FA- and FI-assays.

Code	Structure	K_D PKGI α , nM		K_D PKAc, nM
		FA	FI	FA
ARC-583	Adc-Ahx-(D-Arg) $_6$ -[D-Lys(TAMRA)]-NH $_2$	<i>ca</i> 390	nd	0.48 [39]
ARC-669	pre6-Ahx-(D-Arg)-Ahx-(D-Arg) $_6$ -[D-Lys(TAMRA)]-NH $_2$	12.7 (1.3)	nd	Below 0.3
+Mg $^{2+}$		7.73 (2.3)	nd	
+cGMP		2.70 (0.29)	3.86 (1.23) ^P	
+cGMP, +Mg $^{2+}$		12.0 (0.9)	nd	
ARC-1042	Adc-Ahx-(D-Arg)-Ahx-(D-Arg) $_6$ -[D-Lys(TAMRA)]-NH $_2$	<i>ca</i> 250	nd	Below 0.3 [34]
ARC-1059	H9-Hex-(D-Arg) $_6$ -[D-Lys(TAMRA)]-NH $_2$	3.18 (0.17)	nd	0.58
+Mg $^{2+}$		2.18 (0.15)	nd	
+cGMP		Below 1 (assessed 0.36)	Below 1 (assessed 0.44 ^P , 0.63 ^s)	
+cGMP, +Mg $^{2+}$		1.04 (0.11)	nd	

^P Determined by FI-assay with PHERAstar microplate reader (BMG Labtech);

^s determined by FI-assay with fluorescence spectrometer (Hitachi F-4500);

nd not determined.

Table 2

Logarithms of displacement or inhibition IC₅₀ values (standard errors in parentheses) of compounds with PKGI α as determined by FA-assay, FI-assay, or radioactive inhibition assay, and with PKAc with non-radioactive inhibition assay [45].

Code	Structure	log IC ₅₀ , PKGI α			log IC ₅₀ , PKAc
		FA	FI	Inhibition ^a	Inhibition
ARC-341	Adc-Ahx-(L-Arg) ₆ -NH ₂	-5.59 (0.08)	nd	-6.48 (0.05)	-6.77 ^b [36]
ARC-349	H9-Hex-(L-Arg) ₄ -NH ₂	-7.83 (0.03)	-7.91 (0.04)	-7.80 (0.02)	-7.52 ^b [36]
ARC-661	PUPI-Hex-(D-Arg) ₆ -NH ₂	-7.47 (0.03)	nd	nd	-6.86 ^b
ARC-663	AMTH-Ahx-(D-Ala)-Ahx-(D-Arg) ₆ -(D-Lys)-NH ₂	-7.58 (0.05)	-7.19 (0.14)	-8.60 (0.01)	-8.31 ^c [38]
ARC-664	AMTH-Ahx-(D-Lys)-Ahx-(D-Arg) ₆ -NH ₂	-7.97 (0.01)	-7.77 (0.09)	-8.63 (0.02)	-8.26 ^c [38]
ARC-666	PUTR-Ahx-(D-Lys)-Ahx-(D-Arg) ₂ -NH ₂	No displacement	nd	nd	-5.49 ^b [38]
ARC-668	AMTH-Ahx-(D-Arg)-Ahx-(D-Arg) ₆ -(D-Lys)-NH ₂	nd	-7.05 (0.02)	-8.84 (0.02)	nd
ARC-902	Adc-Ahx-(D-Arg) ₆ -NH ₂	-6.70 (0.09)	nd	-7.06 (0.03)	-8.08 ^b [36]
ARC-903	H9-Hex-(D-Arg) ₆ -NH ₂	-8.27 (0.04)	-8.20 (0.10)	-9.01 (0.01)	-8.28 ^b [36]
ARC-905	H9(Fmoc)-Hex-(D-Arg) ₆ -(D-Lys)-NH ₂	-6.10 (0.07)	nd	nd	-5.95 ^b
ARC-1028	Adc-Ahx-(D-Lys)-Ahx-(D-Arg) ₆ -NH ₂	-6.33 (0.05)	nd	nd	-8.28 ^c [34]
ARC-1044	CCDA-Ahx-(D-Ala)-Ahx-(D-Arg) ₂ -NH ₂	-4.90 (0.12)	nd	nd	-7.68 ^b [34]
ARC-1102	AMTH-Ahx-(D-Lys)-Ahx-(D-Arg) ₂ -NH ₂	-6.64 (0.37)	nd	nd	-7.85 ^b [38]
ARC-1067	H9-Ac-Gly-(D-Arg) ₂ -NH ₂	-5.98 (0.03)	nd	nd	nd
ARC-1068	H9-Ac- β Ala-(D-Arg) ₂ -NH ₂	-5.90 (0.05)	nd	nd	nd
ARC-1069	H9-Ac-Abu-(D-Arg) ₂ -NH ₂	-6.34 (0.05)	nd	nd	nd
	H89	-5.52 (0.03)	-5.96 (0.10)	-7.26 (0.03)	-7.00 ^b [36]
	H9	nd	-5.67 (0.02)	-6.85 (0.02)	-5.43 ^b [36]
	Staurosporine	-7.43 (0.08)	nd	nd	nd
	ATP	-2.02 (0.33)	-2.75 (0.22)	nd	–
	MgATP	-2.35 (0.08)	-3.07 (0.12)	nd	–
WW21	TQARKKALAMA-NH ₂	-1.93 (0.42)	-3.60 (0.12)	nd	nd
	+MgATP	-2.01 (0.14)			

nd^d Not determined.

^a At 100 μ M ATP and 11 μ M TQAKRKKSLAMA substrate.

^b At 100 μ M ATP and 30 μ M TAMRA-Kemptide substrate.

^c At 1 mM ATP and 30 μ M TAMRA-Kemptide substrate.