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Efficient photocaging of a tight-binding bisubstrate inhibitor of cAMP-dependent protein kinase†

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Photocaging of a tight-binding bisubstrate inhibitor of cAMP-dependent protein kinase (PKA) with a nitrodibenzofuran-based group fully abolished its inhibitory potency. The affinity difference between the photocaged and the active inhibitor was over 5 orders of magnitude. The photocaged inhibitor disrupted the PKA holoenzyme in cell lysates upon photolysis under a 398 nm LED.

External spatial and temporal control over the bioactivity of compounds is required in clinical as well as biomedical research applications. Such control can be achieved *via* photocaging technology that is based on temporal masking of the bioactivity of compounds with photo-removable groups. Photocaging has been implemented on bioactive substances of different sizes from protons and metal ions to small molecules and proteins.[1] During the past 40 years, a variety of photocages has been introduced with the action spectrum covering the full UV-Vis spectral range.[2] The most extensively implemented photocage types are nitrobenzyl and coumarin that can be photolyzed by exposure to long-wavelength UV radiation. To overcome the phototoxicity to and limited transparency of tissues, a lot of effort has been put into the development of photocages removable upon one- or two-photon excitation (2PE) with red and NIR light.[3] In addition to blocking interacting functional groups of ligands, photo-removable groups may carry other useful properties, *e.g.*, the ability to emit or quench fluorescence,[4] change the hydrophobicity of the compounds, *etc.*

Protein kinases (PKs) affect almost all cell functions.[5] Since many PKs participate in cell development, reproduction, survival, and migration, aberrant regulation of the amount and/or activity of PKs is associated with cancers and neurodegenerative disorders.[6] The number of PK inhibitors (PKIs) approved by the FDA had reached 49 by June 2019.[7] Most of the approved PKIs are reversible ATP-competitive inhibitors with a low molecular mass (300–550 Da).[8] Photocaged PKIs have been reported for many PKs, *e.g.*, VEGRF-2, RET, Rho, PKA, and tyrosine PKs and up to a hundred-fold affinity difference has been demonstrated for the caged and non-caged compounds.[9]

Previously, we have developed highly potent (K_D values in picomolar range) and selective bisubstrate inhibitors for acidophilic[10] and basophilic PKs[11] (ARC series). In the current work, the proof of principle of photocaging bisubstrate PKIs is presented. ARCs are composed of an ATP-mimicking moiety and a phosphoacceptor protein mimicking peptide analogue, which are covalently bound together with a flexible linker.[12] Thus, ARCs are heterobidentate (heterobivalent, bisite) inhibitors with two structural fragments that simultaneously occupy distinct binding sites on the same protein – the ATP-binding pocket and the protein substrate-binding region of the catalytic subunit of a PK. The conjugation of two fragments boosts the binding energy of the inhibitor due to the avidity effect.[13] Consequently, blocking the access of just one part of the ARC to its target site could drastically reduce the overall affinity of the inhibitor. Efficient reduction of inhibitory potency by installing only a single photocage would be useful because of the structural and synthetic simplicity of the approach.

A previously reported conjugate ARC-1408 was chosen as the molecule to be photocaged since it binds to the catalytic subunit of cAMP-dependent PK (PKAcα; K_D = 0.18 nM) and to other basophilic PKs (*e.g.*, AKT3, ROCKII) with an outstanding affinity, whereas it possesses a relatively low molecular weight for an inhibitor of the ARC-series (798 Da).[11] ARC-1408 (Table 1) is composed of a 4-(piperazin-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (7DP-Pip) moiety and two DArg residues, all three separated by two hydrophobic linkers: nonanedioic acid (Nda) and 6-aminohexanoic acid (Ahx). Three positions were considered for attachment of the photocage: *N*7 of the 7DP-Pip heterocycle and the nitrogen atoms of the guanidino groups on the side chains of the DArg-s. As it is evident from the previously published crystal structure,[11] the named functional groups are directly involved in the complex formation with PKAcα (Fig. S5, ESI†).

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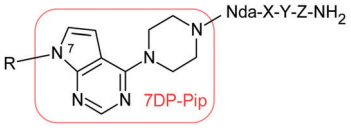
† Electronic supplementary information (ESI) available: Experimental details, full structures of compounds, binding curves, UV-Vis, HPLC-MS, and ^{1}H and ^{13}C NMR data. See DOI: 10.1039/c9cc04978a

Table 1 Structures of ARCs and their K_D values in complex with PKAcα as determined by the FA-based[a] or TGL-based assay[b]. Full structures are given in the ESI. (7DP-Pip, 4-(piperazin-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidine; Ahx, 6-aminohexanoic acid; Nda, nonanedioic acid; NDBF, nitrodibenzofuran; IvDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl)

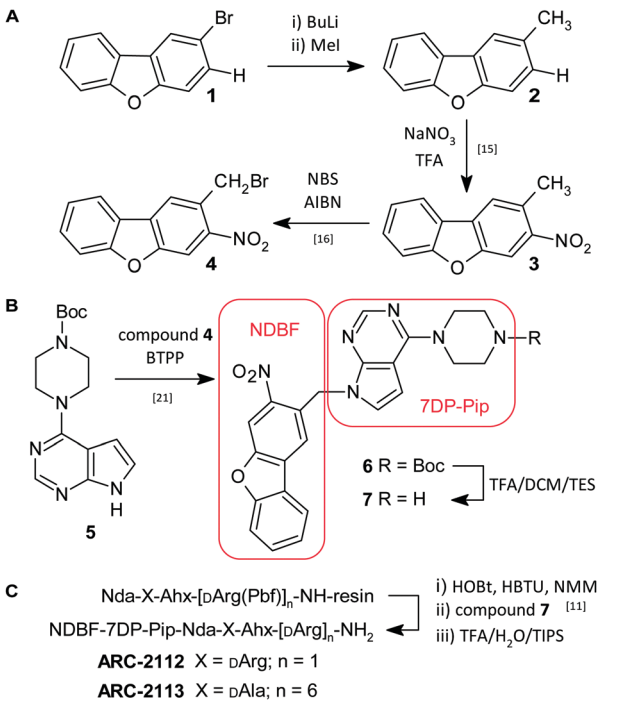


ARC-code	R	X	Y	Z	K_D/nM	Irradiated K_D^{app}/nM
2114	H	DArg	Ahx	DLys(IvDde)	12 ± 1^a	
2115	H	DLys(IvDde)	Ahx	DArg	2.9 ± 0.2^a	
2116	$EtOCOCH_2$	DArg	Ahx	DArg	1700 ± 230^a	
1408	H	DArg	Ahx	DArg	0.18[11]	
2112	NDBF	DArg	Ahx	DArg	$>15\,000^a$	0.76 ± 0.25^a
1411	H	—	—	$[DArg]_6$-DLys	0.003[11]	
2123	H	DAla	Ahx	$[DArg]_6$	0.005 ± 0.001^b	
2113	NDBF	DAla	Ahx	$[DArg]_6$	1900 ± 500^a	$<0.1^a$

A significant fraction of ARC-1408's binding energy comes from strong interactions between the 7DP-Pip moiety and the ATP-binding pocket. The DArg residues of ARC-1408 reach out from the catalytic pocket and interact with amino acids on the surface of the PK. Attachment of a single photocage to one of the DArg residues would still not be effective because the arginine chain is flexible and prone to adopt different conformations which might, to some extent, compensate for losing an interaction with the protein. At the same time, photocaging the 7DP not only blocks the functional groups interacting with the protein but adds steric bulk that cripples the access of the strongly interacting ATP-mimicking fragment to its binding pocket. To confirm these considerations on a quantitative level, three model compounds were made with light-stable bulky groups attached one at a time to the aforementioned positions (Table 1). In ARC-2114 and ARC-2115, the C-terminal DArg (ARC-2114) and the middle DArg (ARC-2115) residues, respectively, were replaced with IvDde-protected D-lysines. In ARC-2116, *N*7 of 7DP was alkylated with ethyl bromoacetate.

The binding affinity of the constructed compounds to PKAcα was evaluated by the equilibrium competitive binding assay based on detection of fluorescence anisotropy (FA).[14] As expected, the affinity of the compounds was most affected when the ATP-pocket targeting moiety 7DP was derivatized, resulting in a K_D increase of four orders of magnitude if compared to the complex with the parent compound ARC-1408 (Table 1). Substitutions in the oligoarginine part of the inhibitor provided a much smaller, less than 2 orders of magnitude K_D increase. This proves that the aromatic structure targeting ATP-binding pocket is the fragment of choice for attaching the photocage.

2-Methyl-3-nitrodibenzo[*b*,*d*]furan (NDBF) was selected as the photocage because it has several suitable qualities: high quantum yield (Φ = 0.7) and extinction coefficient ($\varepsilon_{330\,nm}$ = 18 400 M^{-1} cm^{-1}), resulting in a great photolysis efficiency; fast cleavage; absorption spectra reaching up to 420 nm as well as a possibility for 2PE ($\delta \approx 0.6$ GM).[16a] The NDBF photocage has been mostly assembled from benzene derivatives by multistep transformations and by the involvement of transition metal catalysis.[15–19] We developed a short 3-step transition metal free route to compound **4**, an electrophilic precursor of NDBF (Scheme 1A). Commercially available 2-bromodibenzo[*b*,*d*]-furan **1** was converted to **2** *via* lithium-halogen exchange reaction.[20] The synthesis of **4** was continued according to the published procedures.[15,16] 7DP-Pip-Boc **5** was alkylated with **4** in the presence of a strong organic phosphazene base P1-*t*-Bu-tris(tetramethyl) (BTPP).[21] After removal of Boc, **7** was coupled to the resin-bound peptide as described previously,[11] followed by liberation of the target compound ARC-2112 (photocaged ARC-1408, Table 1) from the resin (Scheme 1C).



Scheme 1 (A) Synthesis of the NDBF precursor, (B) its attachment to the ATP-pocket targeting moiety 7DP-Pip, and (C) solid phase synthesis of the final compounds ARC-2112 and ARC-2113; (7DP-Pip, 4-(piperazin-1-yl)-7*H*-deazapurine; Ahx, 6-aminohexanoic acid; Nda, nonanedioic acid; BTPP, P1-*t*-Bu-tris(tetramethyl); NDBF, nitrodibenzofuran).

The inhibitory potency of a photocaged derivative of tight-binding inhibitor is sensitive even to a minute content of the photolyzed compound. In an ideal case, the photocaged inhibitor is not able to associate with its target. However, such caged inhibitor would still reveal submicromolar apparent affinity (K_D^{app}) if it is contaminated with only 0.1% of the uncaged inhibitor with a sub-nanomolar affinity. Therefore, special measures were taken to protect the developed photocaged ARC from premature photolysis: the procedures with light sensitive compounds were carried out under red LED (580–660 nm), and the purification by HPLC was conducted with a switched-off UV-Vis detector. The fraction containing the compound was collected after chromatographic separation based on its previously established retention time and the purity of the compounds was later evaluated by HPLC-MS (Fig. S5, ESI†).

According to the results of biochemical assay with purified recombinant PKAc, photocaging decreased the affinity of ARC-1408 over five orders of magnitude, more than it was observed with the model compound ARC-2116. Even at the highest concentration used (100 μM), ARC-2112 was not able to displace the fluorescent probe ARC-583[14] from its complex with PKAcα (Table 1). Irradiation of ARC-2112 under Hg vapour lamp gradually liberated ARC-1408 as monitored by HPLC with UV-Vis and MS detectors (Fig. S6 and S7, ESI†). Upon 8 min irradiation, low-nanomolar K_D^{app} was attained for the reaction mixture (Table S3, ESI†) and after 1 h irradiation, over 90% of ARC-2112 had been photolyzed, resulting in a subnanomolar K_D^{app}, close to the K_D value of ARC-1408 (Table 1). Under the same conditions of photolysis, the inhibitors not bearing a photocage were stable (data not shown). It should be noted that quantitative release of the active inhibitor is not mandatory for efficient restoration of bioactivity. Upon 50% liberation of the active compound, the IC_{50} values of the photolyzed and intrinsically active inhibitor would only have a two-fold difference. Thus, it is not practical to extend the time of irradiation to urge the photolysis to completion.

Next, it was tempting to see whether the photocaging strategy presented here would work on a bisubstrate inhibitor that possessed an even stronger interaction with the protein substrate-binding region of the catalytic subunit of PK. Previously, we have shown that ARCs that incorporate more DArg residues in the peptide chain bind more tightly to basophilic PKs due to a higher number of interactions with the amino acid residues in the protein substrate-binding site of PKs. This can be illustrated by comparing the affinities of two previously reported ARC-inhibitors comprising the 7DP-Pip fragment: ARC-1408 (with 2 DArg-s) and ARC-1411 (with 6 DArg-s). ARC-1411, comprising 4 extra DArg residues, is endowed with a nearly hundred-fold lower K_D value of 3 pM with PKAcα (Table 1). In addition, it has been shown that the affinity of ARCs towards PKAcα increases when the linker fragment is elongated.[22] Thus, a hexaarginine inhibitor ARC-2123 was prepared, which incorporated an extra DAla-Ahx fragment in the linker region compared to ARC-1411 (Table 1).

The affinity of ARC-2123 was higher than the limit of K_D-determination capability of the used FA assay. Instead, another type of equilibrium competitive binding assay was used, based on the measurement of TGL signal in the presence of high concentration of the TGL-probe.[23] This assay allowed us to establish 5 pM K_D for the complex of ARC-2123 with PKAcα, close to the affinity of ARC-1411. Next, a photocaged variant of ARC-2123 was synthesised – ARC-2113 (Scheme 1C). ARC-2113 showed a modest residual affinity towards PKAcα (Table 1). The close to 2 μM K_D of ARC-2113 is about 100-fold lower if compared to the unmodified $DArg_6$.[24] Although premature photolysis cannot be ruled out, it is possible that the hydrophobic NDBF fragment has weak association with the ATP-binding pocket of PK, therefore ARC-2113 is a very weak bisubstrate inhibitor of PKAcα. However, the affinity decline of 380 000-fold upon photocaging (*i.e.*, ratio of K_D values of ARC-2123 *vs.* ARC-2113) is remarkable and demonstrates that a bisubstrate PKI possessing picomolar affinity could still be very efficiently deactivated by attaching a single photocage to the ATP-pocket-targeting fragment of the inhibitor.

Having obtained the photocaged bisubstrate inhibitor, we proceeded to test whether it could be used to target PKAcα in the presence of other intracellular components. In cells, the activity of PKAcα is blocked by the formation of heterotetrameric holoenzyme until an increase in the intracellular cAMP concentration induces a conformational change in the regulatory dimer (PKAr) and unleashes the catalytically active subunits (PKAc).[5] Recently, we have shown that the dissociation of the holoenzyme can be induced by a bisubstrate inhibitor ARC-1411, which outcompetes PKAr in binding to PKAc.[11] Hence, experiments in PKA-rich cell lysates were performed to see whether ARC-2113 could disrupt the PKA holoenzyme in a photolysis-triggered manner. A lysate of CHO cells (C9H6) expressing genetically modified fusion proteins PKArIIβ-CFP and PKAcα-YFP was used.[11,25] The dissociation of the holoenzyme was monitored by a decrease of Förster resonance energy transfer (FRET) between the donor CFP and the acceptor YFP.

The experiments with ARC-1411 and cAMP reproduced our previous results[11] as shown by time- and concentration-dependent FRET decrease upon addition of the effectors (Fig. 1A and Fig. S9, ESI†). The photolysis experiments were conducted with a more efficient irradiation system containing LED lamps with a well-defined irradiation wavelength of 398 nm to obtain faster photolysis and herewith to minimize bleaching and evaporation of the solution. Irradiation of the neat cell lysate demonstrated no remarkable bleaching of the fluorescent proteins during the photolysis (Fig. S8, ESI†). Moreover, irradiation did not affect the dissociation of the holoenzyme in the presence of photostable compounds ARC-1411 or cAMP (Fig. 1B). Before photolysis, ARC-2113 did not induce FRET change at the highest used concentration of 10 μM (Fig. 1A). Upon irradiation for 1 min, the FRET signal started to decrease, in the same way as it was observed in the non-irradiated ARC-1411-treated cell lysate (Fig. S9, ESI†). After an equilibrium had been established, the dose-response curves of photolyzed ARC-2113 and ARC-1411 overlapped (EC_{50} = 7.2 nM and 10.5 nM, respectively; Fig. 1B), which proved that the photocage had been efficiently removed in the conditions used and the liberated ARC-2123 worked as efficiently as ARC-1411 in the cell lysate.

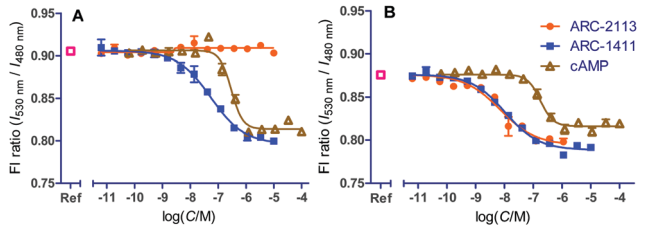


Fig. 1 Disruption of PKA holoenzyme by PKAcα inhibitors and by cAMP before (A) and after (B) irradiation with 398 nm LED. Lysates of C9H6 cells (diluted to 16%) expressing PKArIIβ-CFP and PKAcα-YFP were treated with dilution series of cAMP (brown △), ARC-2113 (orange ●) and ARC-1411 (blue ■). Reference points (Ref) indicate neat 16% lysate. FRET was measured 1 h after the addition of the effector to the cell lysate (A and B) and irradiation (B).

In conclusion, we have demonstrated that bisubstrate tight-binding inhibitors could be effectively photocaged at the ATP-pocket targeting fragment that provides spatial and temporal control over the functionality of this type of compound. The observed 10^5 times affinity change upon irradiation highlights the importance of finding the best energetic 'hot spots' for blocking the interaction of the ligand with the protein by the photocage, but also points to the importance of finding the most suitable photocaging group and avoiding premature photolysis of the photocaged ligand. The photocaged inhibitors ARC-2112 and ARC-2113 possess no effect on the activity of PKAcα at micromolar concentration range and the inhibitory potency of the uncaged compounds can be quickly (within 1 min) restored with irradiation at 400 nm. This result encourages applications of photocaged bisubstrate inhibitors in cellular studies.

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Conflicts of interest

There are no conflicts to declare.

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