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Identification of the Ability of Highly Charged Nanomolar Inhibitors of Protein Kinases to Cross Plasma Membranes and Carry a Protein into Cells

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Abstract—A fluorescently labeled adenosine–oligoarginine conjugate (ARC), nanomolar bisubstrate analogue-type inhibitor of basophilic protein kinases PKA and PKC, readily enters cells of different origin and localizes into cytoplasm and nucleus. Moreover, the biotinylated derivative of ARC is able to deliver avidin, a non-covalently attached protein cargo, into cells.

We have recently developed[1,2] a series of novel inhibitors for basophilic protein serine/threonine kinases with activities in nanomolar region towards cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). The design of the conjugates was based on the understanding of the ternary complex kinetic mechanism of the phosphorylation reaction with direct transfer of phosphate group from ATP to protein/peptide substrate.[3] These inhibitors comprise moieties of both analogues of substrates of protein kinases: ATP binding site directed adenosine-5′-carboxylic acid (AdoC) and the protein substrate binding site directed oligoarginine. The design of the latter fragment was based on the knowledge that phosphorylation sites of basophilic protein kinases (PKA, PKC, PKB-Akt, PKG, etc.) are flanked with regions rich in arginine or/and lysine residues. Two active fragments in these bisubstrate analogue-type inhibitors were connected via a linker which structure was optimized in QSAR studies.[1] Amidation of the C-terminal of the tetra-arginine moiety of the conjugate AdoCAocArg$_4$ with 1,6-diaminohexane yielded a potent inhibitor of PKA [AdoCAocArg$_4$NH$(CH_2)_6NH_2$, $K_i = 83$ nM].[2] Successful application of the ligand for affinity chromatographic purification of PKA revealed that coupling of a bulky group to the free amino group of the conjugate does not impair the binding ability.

Adenosine–oligoarginine conjugates (ARC) are voluminous hydrophilic compounds with high positive charge. The plasma membrane surrounding a cell is generally considered to be impermeable to such molecules. However, several positively charged peptides, especially with multiple arginine residues, have been shown to penetrate into living cells even at low temperatures and in the presence of endocytosis inhibitors (for a review see ref 4). Therefore in the present study the cellular uptake of a fluorescently labeled ARC derivative and an ARC complex with the globular protein avidin was assessed in order to estimate the applicability of these protein kinase inhibitors in the cellular context.

Two new compounds (Fig. 1) possessing a fluorescent reporter group or biotin residue were designed and synthesized to study ARC uptake by the living cells.[5] ARC–BODIPY(FL), a fluorescent derivative of protein kinase inhibitor AdoCAhxArg$_4$NH$(CH_2)_6NH_2$, was synthesized in a simple one-step reaction.[5] ARC–BODIPY(FL) is a chemically stable, highly charged (+4 at pH = 7) compound with up to millimolar solubility. The choice of the fluorescent reporter group proceeded from the following characteristics of BODIPY(FL) dye.

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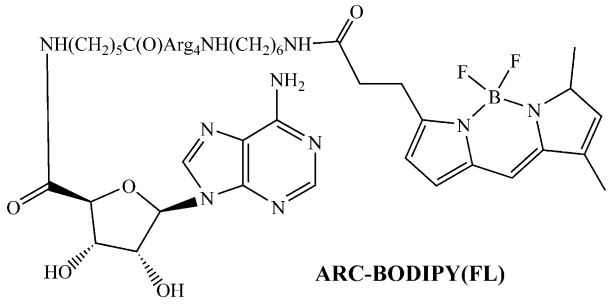
Firstly, the dye with no ionic charge was used to reduce its effect—either negative or positive—on penetration characteristics of the conjugate. Secondly, the dye has excellent optical properties, possessing both high fluorescence sensitivity and photostability, and being detectable by the same filter set as fluorescein derivatives. Thirdly, the dye is available as a single position isomer, facilitating the synthesis and purification of a spectrally homogenous product. ARC–biotin (Fig. 1) was prepared by the derivatization of the conjugate $AdoCAocArg_4NH(CH_2)_6NH_2$ ($K_i = 83$ nM for PKA[2]) and purified with C18 reversed phase HPLC.[5] ARC–biotin is a chemically stable compound with amide bonds connecting AdoC, different amino acid and biotin residues. Long hydrophobic linker was chosen to couple the biotin group to $AdoCAocArg_4$ fragment in order to enable simultaneous recognition of both pharmacophores by their binding proteins, avidin and kinase, respectively.

The cellular uptake of adenosine-polyarginine conjugates was studied with BODIPY(FL)™ labeled ARC.[6] Regardless of the high positive charge and hydrophilic nature of ARC–BODIPY, addition of the compound to the cell culture medium leads to its rapid accumulation in cells (Fig. 2A–C).[7] The cell entry of ARC–BODIPY seems to be cell type independent as penetration of the compound into cells of all tested cell-lines, originating from different cell types (e.g., HeLa, Hep G2, Bowes melanoma, Jurkat), was observed. Inside HeLa and Bowes cells ARC–BODIPY distributes diffusely over the cytoplasm with some preference for reticular and vesicular structures, which is more pronounced in HepG2 cells (Fig. 2A). Further translocation of ARC–BODIPY from cytoplasm into nuclei is less efficient in all used cell lines. However, the conjugate is easily detectable also in nuclei, where it accumulates more strongly in specific subdomains, probably nucleoli.



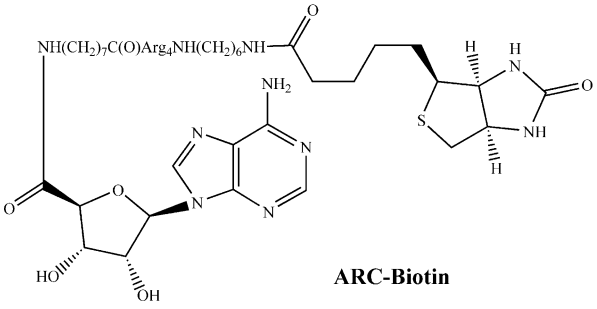


Figure 1. Structures of BODIPY-FL and biotin tagged ARC derivatives used in cellular uptake experiments.

Next, we assessed whether ARC–BODIPY uptake occurs via absorptive endocytosis by using a membrane specific dye FM 1–43.[8] Co-application of FM 1–43 and ARC–BODIPY into the medium over HeLa cells leads to internalization of both compounds, but with different cellular localization patterns (Fig. 2B). FM 1–43 is detectable mainly in vesicular-tubular structures of endosomal-lysosomal origin, while ARC–BODIPY is distributed diffusely and more evenly. Some granular–vesicular structures with higher concentration of ARC–BODIPY do not show accumulation of FM 1–43 and vice versa, that suggests different uptake pathways for

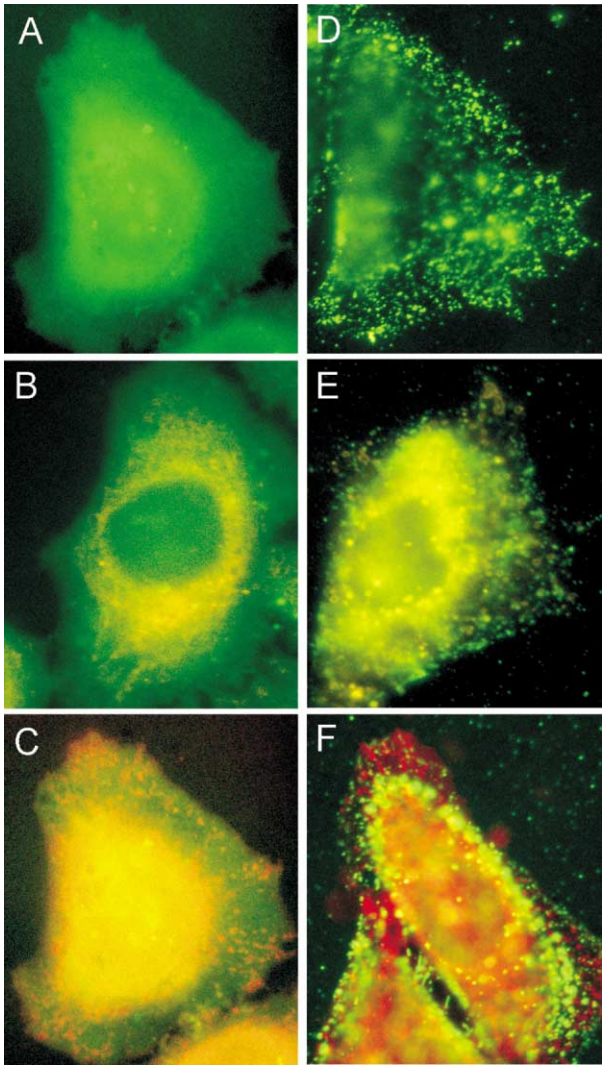


Figure 2. Uptake of adenosine oligoarginine conjugates by cultured cells. A–C Translocation of ARC–BODIPY(Fl) into cells. Localization of ARC in HeLa cells after 1 h incubation with 3 μM ARC–BODIPY(Fl) (green) at 37 °C (A–C) in relation to endosomal-lysosomal structures (B, yellow, visualized by FM 1–43) and mitochondria (C, red, labeled with Mitotracker). D–F Cellular uptake of ARC–protein complexes. Distribution of ARC–biotin complexes with avidin–FITC (green) in HepG2 (D, E) and HeLa cells (F) after 1 h incubation with 2 μM ARC–biotin and avidin–FITC (5 μg/mL) at 37 °C. The mitochondria were revealed with Mitotracker (F, red) and endosomal–lysosomal structures with FM 1–43 (E, yellow).

these compounds. The translocation of ARC–BODIPY can hardly be explained by the classical endocytosis mechanism only, as the lowering of incubation temperature to 4 °C does not abolish its internalization. The cell-introduced molecules carrying positive charge have been reported to concentrate into mitochondria and interfere with their function.[9] However, ARC–BODIPY does not concentrate in mitochondria, harm their integrity or induce changes in their morphology (Fig. 2C).

The accumulation of ARC–BODIPY from culture medium into growing cells prompted us to assess, whether adenosine-oligoarginine conjugates could also serve as transporters for cellular delivery of proteins. Instead of direct coupling of ARC to a protein, a non-covalent complex of its biotinyl derivative (ARC–biotin) with avidin-FITC was used to assess the protein uptake.[10] The respective complexes are detectable in the plasma membrane already in 5–10 min after their application and spread later over the cytoplasm. In contrary to uniformly distributed ARC–BODIPY, ARC–avidin complexes are localized in vesicular structures (Fig. 2D).[11] Similarity of distribution patterns of avidin complexes with ARC and various cell penetrating peptides[12,13] points to analogous uptake mechanisms for these transport systems.

The ability of ARC to convey proteins into cells is strongly impaired at low temperatures. Incubation of HeLa cells with ARC–biotin–avidin–FITC complexes on ice leads to insertion of complexes into the plasma membrane, but not to the efficient uptake into cells and targeting into perinuclear area. However, some complexes seem to be internalized even at low temperatures with retaining exclusively peripheral localization and are not transported towards the cell center. Dramatic decrease of internalization of ARC–avidin complexes at low temperatures suggests the endocytotic mechanism of uptake or the necessity for high membrane fluidity. Following the uptake of ARC–avidin–FITC complexes at physiological temperature in the presence of membrane specific marker FM 1–43 in HepG2 cells reveals that regardless of the similar localization pattern different populations of vesicles mediate their uptake. Despite the major overlaps of preferential mutual localization regions at the cell periphery and in perinuclear area, FM 1–43 and ARC–avidin–FITC are residing in different vesicular and tubular structures (Fig. 2E). Analogously to ARC–BODIPY, the internalized ARC–avidin–FITC complexes are not transported into the mitochondria nor disturb the mitochondrial integrity or morphology (Fig. 2F).

We can conclude that cells efficiently take up adenosine-oligoarginine conjugates. Their uptake is not significantly impaired at lower temperature, suggesting partially energy independent type of internalization, which is similar to the uptake of cell penetrating peptides (CPPs). Thereby ARC–BODYPY(FL), a compound with only four arginine residues, seems to enter cells more efficiently than fluorescein-labeled tetra-arginine.[14] The limited cellular uptake of the latter compound[15,16] points to the contribution of AdoC moiety in facilitated transport of ARC-type compounds. It has been suggested that internalization efficiency of penetratin is dependent on the presence tryptophane residues (bulky and planar) in CPP.[17] The similar structural motif of AdoC can analogously contribute to elevated internalization.

The ability of ARC-type protein kinase inhibitors to cross cell membranes and evenly distribute in the cytoplasm makes them potential candidates for family-selective regulation of protein kinases in vivo. The remarkable overlap of substrate specificity between different protein kinases makes the modulation of phosphorylation status of a protein in cells by targeting a particular kinase with a specific inhibitor very problematic. Moreover, recent experiments with putatively selective protein kinase inhibitors directed to ATP site only (e.g., H-89, a selective and potent inhibitor of PKA) have revealed that such small membrane-permeable compounds inhibit other classes of protein kinases with comparable potency[18] and even interfere with intracellular signal transduction processes by affecting the G protein coupled receptors on the cell surface.[19] The application of the strategy of bisubstrate analogue-type protein kinase inhibitors enables easier prediction of targeted enzymes and specific or family-selective regulation of their activity (e.g., inhibition of basophilic kinases with ARC). Until now the effects of bisubstrate analogue-type PK inhibitors have been characterized by biochemical means using cell free systems (ref 3 and references therein). The observation that adenosine-oligoarginine conjugates are able to enter the living cells in culture is to our knowledge the first indication that such highly charged PK inhibitors could be applied for the regulation of intracellular processes. Thereby, ARC-based inhibitors are more potent than membrane impermeable adenosine phosphate derived bisubstrate inhibitors of protein kinases with negatively charged and labile multiple phosphoryl anhydride groups.[3,20]

Rapid progress in the development of drugs of peptide and protein nature necessitates the prolongation of their activity and cellular half-lives. Therefore, the transduction of peptides and proteins into cells by transport systems that in addition to the delivery function can also inhibit cellular protein kinases (e.g., ARC-type kinase inhibitors capable to convey into cells large hydrophilic molecules, described in the present letter) might be of advantage in cases when the potency of cell transduced cargoes is reduced by phosphorylation.

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References and Notes

1. Loog, M.; Uri, A.; Raidaru, G.; Jarv, J.; Ek, P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1447.
2. Loog, M.; Uri, A.; Jarv, J.; Ek, P. *FEBS Lett.* **2000**, *480*, 244.

3. Parang, K.; Till, J. H.; Ablooglu, A. J.; Kohanski, R. A.; Hubbard, S. R.; Cole, P. A. *Nat. Struct. Biol.* **2001**, *8*, 37.
4. Lindgren, M.; Hällbrink, M.; Prochiantz, A.; Langel, Ü. *Trends Pharmacol. Sci.* **2000**, *21*, 99.
5. AdoCAhxArg$_4$NH(CH$_2$)$_6$NH$_2$ (**A**) and AdoCAocArg$_4$NH(CH$_2$)$_6$NH$_2$ (**B**) were prepared by using conventional Fmoc solid-phase peptide synthesis methods on diaminohexane trityl resin (Novabiochem) as previously described.[2] *ARC–BODIPY(FL)*. **A** (2.7 mg) was dissolved in DMF (100 μL) and DIEA (10 μL). 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, succinimidyl ester (BODIPY® FL, SE; Molecular Probes; 0.79 mg) was dissolved in DMF (30 μL) and the solution added to the solution of **A**. After 2 h reaction, the product was purified by HPLC on a C18 column (water–acetonitrile gradient, 0.1% TFA). The structure of the product was verified with MALDI TOF MS (M_r = 1392) and UV–vis spectroscopy (λ_{max} = 505 nm). *ARC–biotin*. DIEA (8 μL) and (+)-biotin 4-nitrophenyl ester (Aldrich; 3.7 mg) were added to the solution of **B** (2.0 mg) in DMF (100 μL). After 3 h reaction, the product was purified on a C18 HPLC column (water–acetonitrile gradient, 0.1% TFA). MALDI TOF MS revealed correct molecular weight of the product (M_r = 1372).
6. Human cell lines HeLa, HepG2 and Bowes melanoma were cultivated in DMEM as reported.[12] The cells on round coverslips were incubated with 1–10 μM ARC–BODIPY(FL) in serum-containing DMEM for 10–60 min in 5% CO_2 enriched air at 37 °C or on ice. Cellular organelles were visualized by supplementing the medium with 20 nM MitoTracker Red CMXRos™ (Molecular Probes) for labeling mitochondria or 1.5 μM FM 1–43 for labeling endosomal–lysosomal structures, respectively. The cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice and mounted onto preparate glasses with Slow Fade medium (Molecular Probes) as described.[12] The images were obtained by Zeiss Axioskop microscope (Carl Zeiss Inc.), equipped with a cooled digital CCD camera SensiCam (PCO Computer Optics), using the Sensicam software. Images were processed with Corel Draw 9.0 software (Corel Co.).
7. At 10 μM extracellular concentration ARC–BODIPY is detectable in HeLa cells already in 5 min. Prolongation of incubation causes the increase of intracellular fluorescence and even 0.5 μM extracellular concentration of the compound yields an easily detectable signal in 1 h.
8. Hao, M.; Maxfield, F. R. *J. Biol. Chem.* **2000**, *275*, 15279.
9. Chen, L. B. *Annu. Rev. Cell Biol.* **1988**, *4*, 155.
10. The cells on coverslips were incubated with 0.5 μg/mL avidin–FITC (Sigma) and 0.5–5 μM ARC–biotin in serum-containing DMEM for 60 min in 5% CO_2 enriched air at 37 °C and treated analogously to how described above.
11. Initial insertion of ARC–avidin complex into plasma membrane is followed by its sliding into the cell cytoplasm. In cells ARC–avidin complex does not dissociate to yield a uniform diffuse staining, but retains the vesicular/granular localization pattern. The prevalent peripheral localization of internalized complexes observed at the beginning of process is changing into more perinuclear one in 1–2 h at physiological temperature. The initial and persisting localization of the complex in the plasma membrane and the cell periphery indicates that the internalization of ARC–avidin complexes is a continuous process and that the uptake machinery is not saturated at micromolar concentrations.
12. Pooga, M.; Kut, C.; Kihlmark, M.; Hällbrink, M.; Fernaeus, S.; Raid, R.; Land, T.; Hallberg, E.; Bartfai, T.; Langel, Ü. *FASEB J.* **2001**, *15*, 1451.
13. Kilk, K.; Magzoub, M.; Pooga, M.; Eriksson, L. E.; Langel, U. U.; Graslund, A. *Bioconjugate Chem.* **2001**, *12*, 911.
14. Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000**, *56*, 318.
15. Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003.
16. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836.
17. Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiantz, A. *J. Biol. Chem.* **1994**, *269*, 10444.
18. Davies, S. P.; Reddy, H.; Caivano, M.; Cohen, P. *Biochem. J.* **2000**, *351*, 95.
19. Penn, R. B.; Parent, J. L.; Pronin, A. N.; Panettieri, R. A.; Benovic, J. L., Jr. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 428.
20. Medzihradszky, D.; Chen, S. L.; Kenyon, G. L.; Gibson, B. W. *J. Am. Chem. Soc.* **1994**, *116*, 9413.