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Review

(D)-Amino acid analogues of DT-2 as highly selective and superior inhibitors of cGMP-dependent protein kinase Iα

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ABSTRACT

The cGMP-dependent protein kinase type I (PKG I) is an essential regulator of cellular function in blood vessels throughout the body. DT-2, a peptidic inhibitor of PKG, has played a central role in determining the molecular mechanisms of vascular control involving PKG and its signaling partners. Here, we report the development of (D)-amino acid DT-2 derivatives, namely the retro-inverso ri-(D)-DT-2 and the all (D)-amino acid analog, (D)-DT-2. Both peptide analogs were potent PKG Iα inhibitors with K_i values of 5.5 nM (ri-(D)-DT-2) and 0.8 nM ((D)-DT-2) as determined using a hyperbolic mixed-type inhibition model. Also, both analogs were proteolytically stable *in vivo*, showed elevated selectivity, and displayed enhanced membrane translocation properties. Studies on isolated arteries from the resistance vasculature demonstrated that intraluminally perfused (D)-DT-2 significantly inhibited vasodilation induced by 8-Br-cGMP. Furthermore, *in vivo* application of (D)-DT-2 established a uniform translocation pattern in the resistance vasculature, with exception of the brain. Thus, (D)-DT-2 caused significant increases in mean arterial blood pressure in unrestrained, awake mice. Further, mesenteric arteries isolated from (D)-DT-2 treated animals showed a markedly reduced dilator response to 8-Br-cGMP *in vitro*. Our results clearly demonstrate that (D)-DT-2 is a superior inhibitor of PKG Iα and its application *in vivo* leads to sustained inhibition of PKG in vascular smooth muscle cells. The discovery of (D)-DT-2 may help our understanding of how blood vessels constrict and dilate and may also aid the development of new strategies and therapeutic agents targeted to the prevention and treatment of vascular disorders such as hypertension, stroke and coronary artery disease.

1. Introduction

Inhibitors of cyclic nucleotide-dependent protein kinases have served as valuable tools in identifying the fundamental roles of cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) in intracellular signaling. Both kinases have served as Rosetta stones in our understanding of a vast number of intracellular signaling mechanisms ranging from smooth muscle cell relaxation to neuronal synaptic plasticity [1–6]. Therefore, the search for potent inhibitors of these kinases has been extensively investigated. However, the structural similarities of PKG and PKA have posed a formidable obstacle in the design of selective inhibitors that specifically target cyclic nucleotide-dependent protein kinases and show little inhibitory potency to other basophilic Ser/Thr-kinases.

The multi-domain structure of PKG dictates the target sites for putative inhibitors. (Rp)-phosphorothioate analogs of cGMP are the only known inhibitors that bind to the cyclic nucleotide binding sites [7–10]. Although their mode of action is still not completely understood, studies have indicated that the binding of these derivatives fails to induce the conformational changes essential for releasing catalytic activity [11,12]. A diverse pool of derivatives, moderate selectivity and cell membrane permeability are regarded as the major advantages of Rp-cGMPS analogs as tools in intact cell studies [10,13,14]. However, partial antagonism and limited potencies restrict their versatility [9,15]. The catalytic domain of PKG contains two target sites for inhibitors: the ATP-binding site and the substrate-binding site. In general, compounds mimicking ATP represent a diverse class of inhibitors, as has been known for all other major families of protein kinases [16–18]. Peptide inhibitors designed to block the substrate-binding site of PKG have long remained elusive, partly because the sequence requirements for PKG inhibition do not follow a classic consensus sequence and the kinase appears to nonspecifically favor positively charged amino acids [19–21].

Abbreviations: cGMP, cyclic guanosine 3′,5′-mono-phosphate; i.p., intraperitoneal; i.v., intravenous; K_i, inhibition constant; MAP, mean arterial blood pressure; K_m, Michaelis–Menten constant; MPP, membrane-permeable peptides; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; V_{max}, maximal (enzyme) velocity

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Recently, we developed a new class of potent and cell membrane-permeable PKG peptide inhibitors [19]. We utilized SPOT-based combinatorial peptide libraries [22–26] to identify PKG selective inhibitor peptides of which DT-2 was the first example [27]. DT-2 shows PKG specificity and due to its membrane-permeable segment from HIV-1 tat$^{(47-59)}$ remarkable cellular translocation characteristics [28]. In addition, only DT-2 is able to inhibit basal kinase activity, suggesting that DT-2 competes with the N-terminal domain of PKG for the catalytic center of the enzyme [27]. Although DT-2 is a potent PKG inhibitor ($K_i = 13$ nM), its *in vivo* applications are likely limited due to proteolysis. To overcome this obstacle we here report the development of a proteolytically stable derivative of DT-2, namely (D)-DT-2. This (D)-amino acid derivative was more potent against PKG and showed fast and reliable translocation in smooth muscle cells in a variety of vascular beds. Consequently, (D)-DT-2 increased blood pressure in mice and arteries removed from animals treated with (D)-DT-2 were resistant against cGMP-mediated relaxation. We propose that our novel PKG inhibitors have the potential to shed new light on the central role of PKG in vascular biology.

2. Experimental procedures

2.1. Peptide synthesis

Solid-phase synthesis of the peptides (D)-DT-2 and retro-inverso-DT-2 (ri-DT-2) in the form of C-terminal carboxamides was carried out on TentaGel S RAM resin (Rapp Polymere, Tübingen, Germany) with a Pioneer automatic peptide synthesizer (Applied Biosystems) employing Fmoc chemistry with TBTU-activation and a fourfold excess of amino acids. Side chain protections were as follows: Tyr: t-Bu; Gln and His: Trt; Arg: Pbf; Lys: Boc. Coupling time was 1 h. ri-DT-2 was acetylated N-terminally with acetic acid anhydride (5% in DMF) prior to cleavage. Peptides were cleaved from the resin and deprotected by a 3 h treatment with TFA containing 3% triisopropylsilane and 2% water (10 ml/g resin). After precipitation with *t*-butylmethyl ether, the resulting crude peptides were purified by preparative HPLC on a 250×40 mm Nucleosil 100-7 C18 column (Macherey-Nagel, Düren, Germany) with water/acetonitrile gradients containing 0.1% TFA and characterized by analytical HPLC in the same solvent system on a 50×2 mm Gemini 5 μ C18 column (Phenomenex) and MALDI-MS.

Fluorescein peptide labeling was carried out by incubating 10 mg peptide, containing an extra Cys followed by β-Ala at the N-terminus, in 1 ml of 1 M phosphate buffer, pH 7.4, with 60 μl of a 0.1 M stock solution of fluorescein-5-maleimide (Molecular Probes, Eugene, OR, USA) in dimethylsulfoxide at 4 °C overnight in the dark. Determination of the exact peptide concentrations of stock solutions (approximately 6–10 mM in water) and further characterization of the products were carried out by amino acid analysis.

(D)-DT-2 has been made commercially available through BioLog (www.biolog.de).

2.2. Kinetics

PKG Iα was expressed using SF9 insect cells (Invitrogen) in suspension as reported previously [27]. Kinetic constants were determined using a [γ-^{32}P]ATP transfer assay as previously reported [25,29] with the following modifications. PKG Iα at a concentration of 2 nM was incubated with the PKG specific substrate TQAKRKKSLAMA [22] at 16 μM.

Inhibition constants were calculated using a variety of graphical and mathematical methods. First, IC_{50} values were determined by keeping the concentration of substrate (TQAKRKKSLAMA) constant and varying the amount of inhibitor. The obtained data were plotted as v *vs.* log([*I*]) and the K_i was calculated from the IC_{50} using the Cheng–Prusoff equation [30]. Second, Dixon plot analysis was employed as an alternative to obtain K_i values and to determine the mode of kinase inhibition. The change in velocity for three increasing substrate concentrations was measured under varying inhibitor amounts. The resulting data were plotted as $1/v$ *vs.* [*I*] and the initial slopes for each substrate were replotted as slope *vs.* $1/[S]$ [30]. K_i values were then calculated from the slope of the linear regression using Eq. (1).

$$K_i = \frac{K_m}{V_{max} \times \text{slope}} \quad (1)$$

To ascertain αK_i and βV_{max} values, we measured the change in *v* from several constant inhibitor concentrations and varied the amount

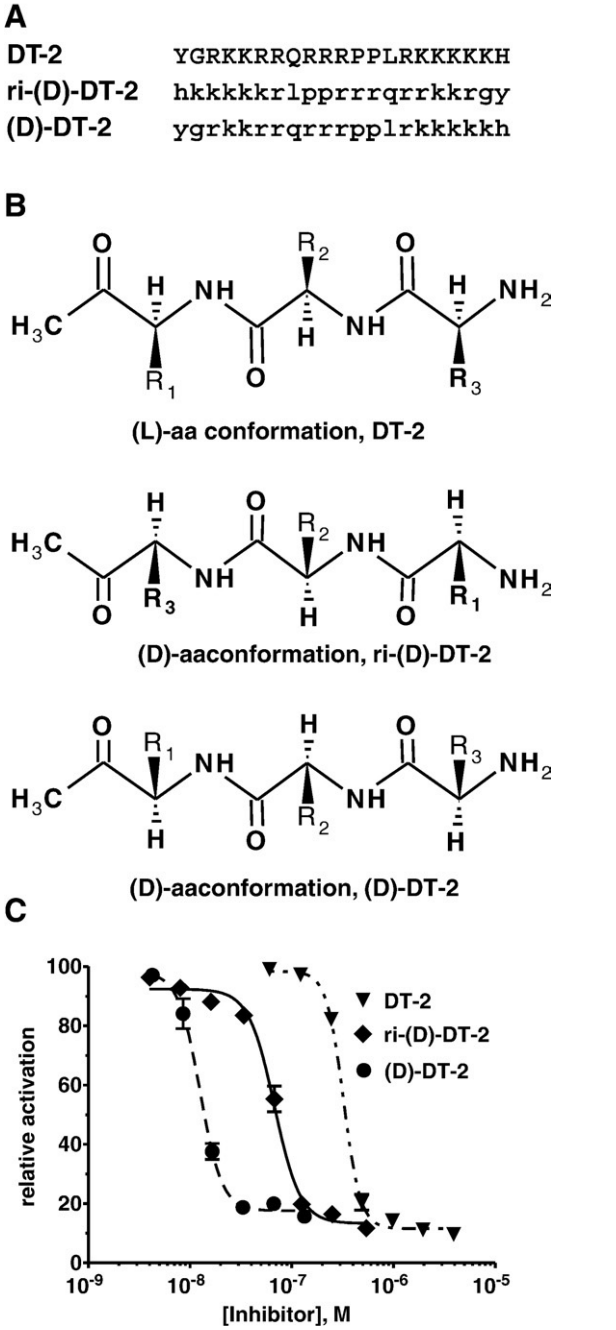

Fig. 1. Stereochemistry and IC_{50} profiles of the DT-2 family of PKG inhibitors. (A) (L)- and (D)-amino acid sequences of DT-2, ri-(D)-DT-2 and (D)-DT-2 are shown in capital and small letter formats. (B) DT-2 and (D)-DT-2 differ in the side chain orientations relative to the plane of the peptide backbones. (C) The IC_{50} curves using the substrate TQAKRKKSLAMA [19].