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Dual activities of ritanserin and R59022 as DGKα inhibitors and serotonin receptor antagonists

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ABSTRACT

Diacylglycerol kinase alpha (DGKα) catalyzes the conversion of diacylglycerol (DAG) to phosphatidic acid (PA). Recently, DGKα was identified as a therapeutic target in various cancers, as well as in immunotherapy. Application of small-molecule DGK inhibitors, R59022 and R59949, induces cancer cell death *in vitro* and *in vivo*. The pharmacokinetics of these compounds in mice, however, are poor. Thus, there is a need to discover additional DGK inhibitors not only to validate these enzymes as targets in oncology, but also to achieve a better understanding of their biology. In the present study, we investigate the activity of ritanserin, a compound structurally similar to R59022, against DGKα. Ritanserin, originally characterized as a serotonin (5-HT) receptor (5-HTR) antagonist, underwent clinical trials as a potential medicine for the treatment of schizophrenia and substance dependence. We document herein that ritanserin attenuates DGKα kinase activity while increasing the enzyme's affinity for ATP *in vitro*. In addition, R59022 and ritanserin function as DGKα inhibitors in cultured cells and activate protein kinase C (PKC). While recognizing that ritanserin attenuates DGK activity, we also find that R59022 and R59949 are 5-HTR antagonists. In conclusion, ritanserin, R59022 and R59949 are combined pharmacological inhibitors of DGKα and 5-HTRs *in vitro*.

1. Introduction

Diacylglycerol kinases (DGKs) phosphorylate diacylglycerol (DAG) species to yield the corresponding phosphatidic acid (PA) [1]. To date, ten DGK isotypes have been identified, characterized, and divided into 5 classes based on amino acid sequence similarities [2–6]. While much remains to be learned about the function of these enzymes, there is an increasing body of evidence highlighting their critical role in many pathological states (reviewed in [5]). In particular, recent work has implicated DGKα as a positive regulator of carcinogenesis; DGKα was shown to induce T-cell anergy, a hypo-activation of T-cells that suppresses their immunological response [7]. Additionally, Dominguez and colleagues demonstrated that pharmacological attenuation of DGK activity in glioblastoma multiforme (GBM) cells resulted in apoptosis and the genetic knockdown of DGKα caused a reduction in tumor growth [8]. This and other work strongly implicated DGKα as a novel therapeutic target in the most common and malignant primary brain tumor and possibly other cancers as well [5,8,9].

Despite advances in linking the function of DGKs to the development of cancer and other diseases, the study of their biology is challenging. Genetic and pharmacological manipulations of DGKs have been informative but with significant caveats. This is due to the large number of mammalian DGKs, the diversity of DAG and PA species in cells, and a lack of isotype-selective inhibitors. Currently available chemical tools for understanding the role of DGKs in biology and disease are confined to the two long-known inhibi-

Abbreviations: ATP, adenosine triphosphate; K_m^{app}, affinity; V_{max}, maximal specific kinase activity; IC_{50}, concentration of drug that inhibits half enzyme activity; GFP, green fluorescent protein; $t_{1/2}$, half-life; PMA, Phorbol 12-myristate 13-acetate; DMEM, Dulbecco's Modified Eagle Medium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NEB, New England Biolabs; LC–MS, liquid chromatography mass spectrometry; ATCC, American Type Culture Collection.

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tors, R59022 and R59949 (DGK inhibitor I and II, respectively) [10,11]. Very recently, another small molecule, CU-3, was identified as a novel DGKα inhibitor *in vitro* [12]. The selectivity of R59022 and R59949 for the different DGK isotypes has been debated. Some studies suggest that they are selective for class-I, Ca^{2+}-dependent DGKs, particularly DGKα, while some have reported inhibition of other DGKs [13–19]. The *in vivo* application of these compounds has been difficult due to their poor pharmacokinetics and limited ability to cross the blood–brain barrier [8]. The emerging role of DGKs in pathological states and the current limitations that exist in the study of these enzymes increase the need for the discovery of novel and perhaps more potent inhibitors, not only for translation to the clinic but also as effective probes for understanding DGK function on a cellular and physiological level.

We recently noted that ritanserin has striking structural similarity to R59022 [20]. Ritanserin was first identified as a serotonin (5-HT) receptor (5-HTR) antagonist and was shown to have drug-like properties [21]. Its use as a treatment of schizophrenia and substance dependence advanced to clinical trials but development was eventually discontinued [22–24]. Despite the obvious structural similarities between R59022 and ritanserin, these compounds, as well as R59949, were to our knowledge never grouped as being functionally similar. In this study, we present evidence that ritanserin is a DGKα inhibitor while both R59022 and R59949 are 5-HTR antagonists [20].

2. Materials and methods

2.1. Materials

[γ^{32}P]-ATP was from Perkin Elmer (Boston, MA). The diacylglycerol (DAG) species used in this study are as follows: 1,2-dioleoyl-*sn*-glycerol (dioleoyl; 18:1, 18:1), 1,2-octanoyl-*sn*-glycerol (dioctanoyl; 8:0, 8:0) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (stearoyl arachidonoyl; 18:0 20:4). These DAG species as well as 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), and all materials for the preparation of liposomes were from Avanti Polar Lipids (Alabaster, AL). M2 FLAG beads, FLAG antibody, rabbit and mouse alkaline-conjugated secondary antibodies, R59949, R59022, and ritanserin were from Sigma-Aldrich (St. Louis, MO). Ketanserin, bisindolylmaleimide II (bis), PMA, and TCB-2 were from Tocris Bioscience (Avonmouth, Bristol, UK). All other commonly used reagents were from Sigma-Aldrich, unless otherwise indicated. All cell lines were obtained from ATCC (Rockville, MD).

2.2. Construction of expression plasmids

The expression plasmids, pcDNA3-FLAG-rat-DGKα [25], pcDNA3-FLAG-rat-DGKβ [26], and pCMV-human-DGKδ1-3xFLAG [27] were gifted to Dr. Kevin Lynch (University of Virginia, School of Medicine) by Dr. Kaoru Goto (Yamagata University, School of Medicine) and Dr. Fumio Sakane (Chiba University) and were kindly shared with us by Dr. Lynch. The expression plasmid, pCMV-HA-human-DGKι, was also gifted to Dr. Lynch by Dr. Matthew Topham (University of Utah) [28]. DGKι cDNA was sub-cloned into the pCMVTag2A vector. The DNA encoding pLenti6-human-DGKθ was from the laboratory of Dr. Daniel Raben (Johns Hopkins University School of Medicine) and was sub-cloned into the pCMVtag2 vector.

2.3. Purification of DGKα and overexpression of DGK isoenzymes

Human cervical cancer (HeLa) cells (30–40 15 cm plates) were cultured in DMEM with 5% fetal bovine serum (FBS), VMR Life Science Seradigm, (Radnor, PA) and 1% penicillin/streptomycin, Fisher Scientific, (Waltham, MA). The cells were infected with an adenoviral vector, expressing rat FLAG-DGKα for 72 h. The cells were fed daily during this period, harvested and lysed using a 22 G needle, in 500 μl/plate of buffer A (10 mM Na_2HPO_4, pH 7.4, 50 mM Octyl β-D-glucopyranoside, 50 mM NaF (IPBB), 1 mM EDTA, 1 mM EGTA, 0.02% Triton X-100, and the protease inhibitors: phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin). The cell lysate was cleared by centrifugation at 16,000*g* for 10 min. The supernatant was collected and incubated with 15 μl/-plate of FLAG (M2) beads for 2 h at 4 °C. Following the incubation, the beads were loaded on an affinity screening column, Fisher Scientific (Waltham, MA) and washed 10 times with buffer A. The FLAG-DGKα was eluted with five successive additions of equal volume of 0.5 mg/ml of FLAG peptide. The fractions were collected and dialyzed against buffer A without detergent or protease inhibitors. The purified DGKα was visualized on an SDS-PAGE gel stained with Coomassie-blue dye. The protein yield was quantified by comparison to bovine serum albumin (BSA) standards. HeLa cells were chosen for the purification because we have optimized the purification of proteins at high yield from this cell line.

To study the activity and inhibition of various DGK isoenzymes (α, β, δ, ι, θ), human embryonic kidney (HEK 293T) cells (10 cm plates) were cultured in DMEM with 5% FBS and 1% penicillin/streptomycin. The cells were transiently transfected with 15 μg of FLAG-DGK plasmid DNA using Lipofectamine 2000, Invitrogen (Carlsbad, CA). Forty-eight hours following the transfection, the cells were harvested and homogenized with a 22 G needle using 250 μl/plate of 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors (as above). To solubilize DGKι, buffer A was used. The cell homogenates were cleared by centrifugation at 16,000*g* for 10 min. The supernatant was collected and used immediately or stored at −80 °C. We chose to use HEK 293T cells because they are amenable to liposome-mediated transformations.

2.4. Preparation of liposomes

The preparation of liposomes generally followed a previously reported protocol from MacDonald et al. [29]. Briefly, PC, DAG, and PS were dissolved in $CHCl_3$, combined, and dried *in vacuo* to remove all solvent. All liposomes contained dioleoyl DAG unless otherwise indicated. For assays using purified enzyme, the total liposomal concentration of lipids was as follows: 5 mol% DAG, 40 mol% PS, and 55 mol% PC. For measurement of purified DGKα inhibition and changes in kinetics in the presence of R59022 and ritanserin, the compounds were incorporated into the liposomes. They were first dissolved in $CHCl_3$ then added to and dried down with the lipids at 0.5 and 2.0 mol%. The lipids were hydrated to 19 mM in buffer B (50 mM (3-(*N*-morpholino)propanesulfonic acid) (MOPS), pH 7.5, 100 mM NaCl and 5 mM $MgCl_2$). For assays using cell homogenate, 10 mol% DAG, 40 mol% PS, and 50 mol% PC were used. The lipids were hydrated to 10 mM in buffer B. In both cases, the lipids were subjected to five freeze–thaw cycles in liquid nitrogen, followed by extrusion through a 100 nm polycarbonate filter 11 times.

2.5. Kinase assays

The protocol for measurement of purified DGKα activity was modified from Epand et al. [30]. Briefly, the reactions contained buffer B, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), purified enzyme, and 4.75 mM lipids, with and without indicated total liposomal concentrations of R59022 and ritanserin. For reactions testing the activity of DGKα in the absence of $CaCl_2$, 1 mM EGTA was also used. The reactions were initiated by the addition of 10 μl of 10 mM