Published in final edited form as:

Biochem Pharmacol. 2017 January 01; 123: 29–39. doi:10.1016/j.bcp.2016.10.011.

Dual activities of ritanserin and R59022 as DGK α inhibitors and serotonin receptor antagonists

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Abstract

Diacylglycerol kinase α (DGK α) catalyzes the conversion of diacylglycerol (DAG) to phosphatidic acid (PA). Recently, DGK α 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 DGK α . 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 DGK α kinase activity while increasing the enzyme's affinity for ATP *in vitro*. In addition, R59022 and ritanserin function as DGK α 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 DGK α and 5-HTRs *in vitro*.

Graphical Abstract

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Chemical compounds used in this article

Diacylglycerol kinase inhibitor I (PubChem CID:3012); Diacylglycerol kinase inhibitor II (PubChem CID:657356); ritanserin (PubChem CID:5074); ketanserin (PubChem CID:3822).

6. CONFLICT OF INTEREST

The authors state no conflict of interest

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Keywords

Diacylglycerol kinase (DGK); R59022; ritanserin; Protein Kinase C (PKC); serotonin receptor (5-HT₂R)

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 DGK α as a positive regulator of carcinogenesis; DGK α 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 DGK α caused a reduction in tumor growth [8]. This and other work strongly implicated DGK α 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 inhibitors, R59022 and R59949 (DGK inhibitor I and II, respectively) [10,11]. Very recently, another small molecule, CU-3, was identified as a novel DGK α 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, Ca²⁺-dependent DGKs, particularly DGK α , 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 ritanserlin has striking structural similarity to R59022 [20]. Ritanserlin 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 ritanserlin, these compounds, as well as R59949, were to our knowledge never grouped as being functionally similar. In this study, we present evidence that ritanserlin is a DGK α inhibitor while both R59022 and R59949 are 5-HTR antagonists [20].

2. MATERIALS AND METHODS

2.1 Materials

[γ ³²P]-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 also from Avanti Polar Lipids (Alabaster, AL). M2 FLAG beads, FLAG antibody, rabbit and mouse alkaline-conjugated secondary antibodies, R59949, R59022, and ritanserlin were from Sigma-Aldrich (St. Louis, MO). Ketanserlin, 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-DGK α [25], pcDNA3-FLAG-rat-DGK β [26], and pCMV-human-DGK δ 1-3xFLAG [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-DGK ι , was also gifted to Dr. Lynch by Dr. Matthew Topham (University of Utah) [28]. DGK ι cDNA was sub-cloned into the pCMVTag2A vector. The DNA encoding pLenti6-human-DGK θ 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 DGK α 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-DGK α for 72 h. The cells were fed daily during this period, harvested and lysed using a 22 G needle, in 500 μ l/plate of buffer A (10 mM Na₂HPO₄, 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 $\times g$ for 10 min. The supernatant was collected and incubated with 15 μ l/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-DGK α 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 DGK α 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 μ g 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 μ l/plate of 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and protease inhibitors (as above). To solubilize DGK ι , buffer A was used. The cell homogenates were cleared by centrifugation at 16,000 $\times g$ 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 CHCl₃, 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 DGK α inhibition and changes in kinetics in the presence of R59022 and ritanserin, the compounds were incorporated into the liposomes. They were first dissolved in CHCl₃ then added to and dried down with the lipids at 0.5 and 2.0 mol%. The lipids were hydrated to 19 mM in 50 mM (3-(*N*-morpholino)propanesulfonic acid) (MOPS), pH 7.5, 100 mM NaCl and 5 mM MgCl₂ (buffer B). 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 DGK α activity was modified from Epand *et al.* [30]. Briefly, the reactions contained buffer B, 1 mM CaCl₂, 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 DGK α in the absence of CaCl₂, 1 mM EGTA was also used. The reactions were initiated by the addition of 10 μ l of 10 mM [γ ³²P]-ATP to a final volume of 100 μ l and were allowed to proceed for 15 min at 25°C. The kinase assays using cell homogenate contained buffer B, 1 mM DTT, 2 mM lipids and 5 μ g of protein from homogenate. For assays with DGK α and DGK β , 1 mM CaCl₂ was also added. The reactions were initiated as described above and were allowed to

proceed for 20 min at 30°C. For measuring DGK isoenzyme inhibition in cell homogenates, indicated compounds were dissolved in DMSO, serially diluted in buffer B and added directly to kinase assays to the desired concentration. To solubilize the compounds, water bath sonication was used. The final concentration of DMSO in the reactions was less than 1% (v/v) and did not affect enzyme activity. All reactions were terminated with the addition of 0.5 ml of methanol with 0.1 N HCl, followed by 1 ml of CHCl₃. The organic phase was washed two times with 1 M MgCl₂ and thoroughly vortexed. To measure the incorporation of [³²P] into DAG, 0.5 ml of the organic phase was removed and the radioactivity was measured using a scintillation counter.

The activity of purified protein was normalized to background radioactivity measured from assays without enzyme. The activities of lysates overexpressing DGKs (signal) were normalized to activities of lysates expressing only GFP (background). The specific activities of lysates with GFP were less than 0.1 nmol/min/mg (less than 10% of signal) and were not altered by the presence of inhibitors. The signal to background ratio was calculated as follows: S/B = mean signal/mean background.

2.6 Protein kinase C (PKC) Activation

HeLa and human glioblastoma cells, U87 and U251, were treated as follows: 4 and 40 µM of R59022 and ritanserin, 40 µM ketanserin, and 100 nM PMA for 30 min with and without pre-treatment with 500 nM bis for 1 h. For additional PKC activation experiments, HeLa cells were treated with 10 µM TCB-2 for 6 h with and without co-treatment with 40 µM ketanserin, 40 µM ketanserin alone, and with 500 nM bis for 1 h. The cells were lysed in IPBB, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors and the cell lysate was cleared by centrifugation at 16,000 × *g* for 10 min. The cleared homogenate was loaded on an 8.75% SDS-PAGE gel and PKC substrate phosphorylation was detected as described in section 2.7.

2.7 Western immunoblot analysis

To verify the expression of the DGK isotypes and detect PKC substrate, 50 µg of proteins from cell homogenates were separated by 8.75% SDS-PAGE gel under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane, Immobilon (Darmstadt, Germany). The membrane was blocked by incubation in Tris-buffered saline with detergent Tween 20 (TBST) containing 10% (w/v) dried milk for 1 h at 25°C. The TBST contained the following: 50 mM Tris, 150 mM NaCl, and 0.05% (w/v) Tween 20, pH 7.4. For detection of DGKs, the membrane was incubated with monoclonal anti-FLAG antibody (1:1000) in TBST, at 25°C for 1 h with gentle agitation. For the detection of PKC substrates, the PVDF membrane was incubated with Phospho-(Ser)-PKC substrate antibody, Cell Signaling (Danvers, MA), (1:1000) in TBST overnight at 4°C with gentle agitation. The membranes were then incubated with alkaline phosphatase-conjugated mouse or rabbit secondary antibody (1:10,000) diluted in TBST with 2% (w/v) dried milk, for 1 h at 25°C. After three 15 min washes with TBST, the membrane was briefly incubated in chemiluminescent alkaline phosphatase substrate, Applied Biosystems (Foster City, CA). The immunoreactivity was detected using a Fuji LAS 4000.

2.8 qPCR

Quantitative qPCR was carried out as per the MIQE guidelines. Total RNA was isolated with TRIzol reagent, Ambion (Carlsbad, CA), according to the manufacturer's instructions. Briefly, cells were harvested and suspended in 1 ml TRIzol reagent. Isolated RNA was treated with DNase I, NEB (Ipswich MA) in a 1:10 ratio of DNase and DNase reaction buffer (10 mM Tris-HCl, pH 7.6, 2.5 mM MgCl₂, 0.5 mM CaCl₂) and incubated at 37°C for 30 min. cDNA was synthesized from 1 µg of RNA with the SensiFAST cDNA Synthesis Kit, Bioline (Taunton, MA). Real-time qPCR was performed using SensiMix SYBR and Fluorescein Kit, Bioline (Taunton, MA) according to manufacturer's instruction. All the samples were assayed in duplicates and analyzed using a CFX96 Real-Time PCR Detection System, Bio-Rad (Hercules, CA). Relative expression was calculated using 2^{-ddCt} method [31]. For the internal control, 18S rRNA was used.

2.9 Measurement of serotonin receptor antagonism

The compounds R59949 and R59022 were tested against 5-HT₂Rs by the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH PDSP) at the University of North Carolina, Chapel Hill. To identify the potency and K_i of each compound against the receptors, radioligand competition binding assays and the Cheng-Prusoff equation were performed as described in more detail in Besnard *et al.* [32,33]. To confirm whether the compounds are agonists or antagonists at the receptors of interest, functional assays were performed as described in Kroeze *et al.* [34]. The detailed protocols can also be found online (<http://pdsp.med.unc.edu/pdspw/binding.php>).

2.10 Statistical Analysis

The statistical analysis and determination of all kinetic constants were done using Graphpad Prism software. For calculation of K_m^{app} values, non-linear regression of Michaelis-Menten plots were used. The R59022 and ritanserin dose dependent curves were fitted to the data sets with linear interpolation. A Graphpad Prism function called log [inhibitor] vs. normalized response was used to calculate IC₅₀ values. For comparisons between and within more than two groups, one-way Analysis of Variance (ANOVA) and two-way ANOVA were used, followed by Dunnett's or Tukey post-hoc analysis (as indicated in the figure legends). All values are reported as the mean of triplicate values ± SEM. Data shown are representative of at least three independent experiments and when appropriate, from separate enzyme preparations. Significance was set to p < 0.05.

3. RESULTS

3.1 Purification and enzymatic characterization of DGKα

FLAG-DGKα was affinity-purified from HeLa cells (Fig. 1A). We confirmed that DGKα was active and that it was linear in a time- and concentration-dependent manner to 15 min and 3.2 µg of protein (Fig. 1B and C).

Classical biochemical assays for studying DGK activity involve the use of partially purified enzyme or cell homogenate and detergent mixed micelles [6,17,35]. Few studies have utilized liposomes, which are composed of a lipid bilayer and thus may more closely

resemble cellular membranes [30,36]. We investigated the kinetics and catalytic properties of DGK α using purified enzyme and PC:DAG:PS liposomes. The two substrates of DGKs are ATP and DAG. Fig. 2A shows the ATP-dependent activity of DGK α . The K_m^{app} of DGK α for ATP determined with this assay was 0.05 mM (Fig. 2A and Table 1). The K_m^{app} against DAG was 0.12 mM (Fig. 2B and Table 1). These data are consistent with what has been previously reported for DGK α [17,19]. Furthermore, the activity of DGK α has long been known to depend on Ca^{2+} [14,15,37,38]. This is thought to be due to the Ca^{2+} -dependent dissociation of an auto-inhibitory, intra-molecular interaction between the enzyme's C1 domains and the EF-hand motifs [13]. In the absence of exogenous Ca^{2+} , the activity of DGK α was reduced by 75%; the addition of 10 μ M Ca^{2+} was sufficient to restore the majority of the kinase activity (Fig. 2C). Some DGKs, including DGK μ , are also strongly activated by PS [16,39,40]. Titration of 10 – 40 mol% PS into PC:DAG liposomes resulted in a PS dose-dependent increase in kinase activity (Fig. 2D).

3.2 Ritanserin is an inhibitor of DGK α enzymatic activity in vitro

The structures of the known small-molecule inhibitors of DGKs as well as two 5-HTRs, ritanserin and ketanserin, are depicted in Fig. 3. We assessed the extent to which ritanserin inhibits the activity of DGK μ as compared to R59022. Since these compounds are lipophilic, they can easily be incorporated into the liposome bilayer. We added 0.5 mol% and 2.0 mol% R59022 and ritanserin into the PC:DAG:PS liposomes. The bulk concentrations of the drugs were 24 μ M and 95 μ M. The rationale for using high concentrations of inhibitors was based on the reasoning that some of the compounds would incorporate into the inner leaflet of the liposome bilayer and would thus be unable to access enzyme. The activity of DGK α was assayed in the context of the substrates ATP and DAG.

The presence of 0.5 mol% R59022 decreased the V_{max} of purified DGK α by 25% in the context of ATP and 40% in the context of DAG. With 2.0 mol% R59022, about 50% of the specific activity was inhibited for both substrates (Fig. 4A and C, Table 1). Interestingly, the apparent K_m of DGK α for ATP decreased in the presence of 2.0 mol% R59022 (Table 1). Under the same conditions, 0.5 mol% ritanserin decreased V_{max} of DGK α by greater than 50% in the context of both ATP and DAG. At 2.0 mol%, enzymatic activity was attenuated by about 70% (Fig. 4B and D, Table 1). As R59022, ritanserin significantly decreased the apparent K_m of DGK α towards ATP (Table 1). Together, these data suggest that under these *in vitro* conditions, ritanserin is an inhibitor of DGK α . Further, the increase in affinity of DGK α for ATP but not DAG in the presence of inhibitors, suggests that R59022 and ritanserin may bind an allosteric site on the enzyme and affect its interaction with each substrate differently.

3.3 R59022 and ritanserin are more potent inhibitors of DGK α than of other, selected DGKs

The compounds R59022 and R59949 were reported to be specific inhibitors of class I DGKs [2,17]. This claim has been subsequently challenged. For example, R59022 was reported to inhibit DGK ϵ and DGK θ and it was demonstrated that 30 μ M R59022 did not inhibit the two other class I isotypes, DGK β and DGK γ [18,19]. Motivated by these conflicting results, we investigated the activity of R59022 and ritanserin against various DGKs, first using liposomes containing dioleoyl DAG. The expression of FLAG-tagged-DGKs was forced in

HEK 293T cells (Fig. 5A). R59022 and ritanserin were dissolved in DMSO, diluted, and added directly to the kinase reactions, which contained cell homogenate over-expressing the indicated DGK enzyme. Fig. 5B and C show the percent of activity from control (kinase assay with DMSO only) of a representative DGK from each of the five classes. (DGK ϵ was excluded because it is an integral membrane protein [41,42]). The activity of DGK α decreased by about half with 20 μ M inhibitors (Fig. 5B and C). Under these conditions, the activities of the indicated DGKs were not detectably inhibited, including that of DGK β (another Class I DGK; Fig. 5B and C). We examined whether this pattern of inhibitor selectivity would be observed in the context of other DAG species. It was found that R59022 and ritanserin were similarly potent against DGK α when assayed using liposomes containing dioctanoyl (Fig. 5D and E) and stearoyl arachidonoyl (Fig. 5F and G) DAGs. To expand on these findings, the activities of DGK α and DGK ι were tested against a range of R59022 and ritanserin concentrations. Under the given conditions, the IC₅₀ values of R59022 and ritanserin for DGK α were approximately 25 and 15 μ M, respectively (Fig. 5H and I). On the other hand, for DGK ι the IC₅₀ values were between 55 and 65 μ M (Fig. 5J and K).

In order to verify that the concentrations of ATP and DAG used the above experiment were saturating for not just DGK α but all of the DGKs used, we performed ATP and DAG dose dependent curves. Under these conditions, we found that DGK α has the highest apparent affinity for ATP, followed by DGK β , while DGK δ has the lowest affinity for ATP, at 0.13 mM (Tables 1 and Table 2). With respect to 18:1,18:1 DAG, DGK α had the lowest apparent affinity and DGK θ had the highest, with a K_m^{app} of 0.03 mM (Table 1 and Table 2). In summary, the concentrations of DAG and ATP in the kinase experiments in Fig. 5 were saturating for the DGKs tested.

3.4 R59022 and R59949 are 5-HT_{1A} antagonists

R59022 (structure in Fig. 3A) is a close structural analog of ritanserin, with the sole difference being the substitution of the hydrogen isostere, fluorine, at a second position to generate ritanserin. Despite the similarities between the DGK inhibitors and some 5-HT_{1A} antagonists, including ritanserin, the action of R59022 and R59949 against 5-HT_{1A} receptors has not been reported. To test the potency of these DGK α inhibitors against these receptors, radioligand competition assays were used as described in [32]. Briefly, in the primary screen, 10 μ M of the compounds were used to investigate % inhibition (of binding) of a radioligand. Both molecules inhibited binding at all receptors except 5-HT₃ (Table 3). In the secondary assays, K_i values were determined at the receptors where significant inhibition was observed. The lowest K_i values of R59022 and R59949 were against 5-HT_{2A}R. Interestingly, R59022 was also potent against 5-HT_{2B/2C} receptors as well as 5-HT_{1D} and 5-HT₇ (Table 3).

These assays elucidate the potency of R59022 and R59949 for 5-HT_{1A} receptors but do not describe whether they function as antagonists or agonists. As such, functional assays were used to determine the action of these compounds at 5 receptors where low K_i values were observed for either R59022 or R59949, as described in [34]. The agonist assays showed very low to no activity (data not shown). Both compounds showed high antagonist activity at all of the

receptors, with the exception of R59949 at 5-HT_{2C} and 5-HT₇ (Table 3). Together with the low K_i values, these data support the conclusion that R59949 and R59022 are antagonists of 5-HT₂Rs and possibly other serotonin receptors.

3.5 R59022 and ritanserin stimulate PKC in HeLa and U87 cells but not in U251 cells

The ability of chemical compounds to inhibit DGKs must ideally be demonstrated by treatment of intact cells and detection of subsequent changes in various DAG and PA species. Probably the most robust method for detecting such changes is by using mass spectrometry. However, this has proven to be difficult without genetic manipulations [8,12,43]. Using LC-MS, we analyzed changes in DAG, PA and PC in cells expressing endogenous DGKs after treatment with ritanserin and R59022 but could not obtain statistically significant results. Both DAG and PA are signaling lipids and serve as effectors of many proteins [3–5]. For example, DAG activates PKC [44]. The phosphorylation of PKC downstream targets was investigated following treatment of cells with ritanserin and R59022, as an indirect measure of DGK α inhibition and DAG accumulation (Fig. 6A). Treatment with 40 μ M of either compound increased the phosphorylation of PKC downstream targets by about 2.5-fold in HeLa cells. The treatment of cells with the known PKC activator, PMA, induced substrate phosphorylation by greater than 3-fold (Fig. 6B and C). Induction of PKC activity was reversible by bis, a PKC inhibitor (Fig. 6B and C). We next tested the activity of ketanserin, another 5-HT₂R antagonist with structural similarities to ritanserin and R59022, against DGK α . The compound was dissolved in DMSO and added to kinase assays containing purified DGK α and PC:DAG:PS liposomes. Under the given *in vitro* conditions, the IC₅₀ of ketanserin was 264 μ M (Fig. 6D). Importantly, when HeLa cells were treated with ketanserin, no activation of PKC was observed (Fig. 6B and C).

These effects were also observed in the glioblastoma U87 but not U251 cells, perhaps due to low expression of DGK α (Fig. 6E and F). To test this hypothesis, the relative mRNA expression of DGKs and 5-HTRs were measured in all three cell lines. In both HeLa and U87 cells, DGK α showed the highest relative expression (Fig. 6G and H). Both cell lines also expressed 5-HT_{2A/2C}Rs but not 5-HT_{2B}R (Fig. 6G and H). On the other hand, although U251 cells had some expression of DGK α , it was low compared to expression of other DGKs such as DGK δ and DGK θ (Fig. 6I).

Ketanserin is a 5-HTR antagonist but does not inhibit DGK α (Fig. 6 and [21]). Thus, ketanserin should decrease levels of DAG and attenuate PKC. We did not observe a decrease in the phosphorylation of PKC downstream targets when we treated cells with ketanserin alone (Fig. 6B–F). One explanation is that under control conditions, 5-HTRs (particularly 5-HT_{2A}R and 5-HT_{2C}R) and/or PKCs are not detectably active. As such, we cannot detect the antagonistic effects of ketanserin. To verify this hypothesis, we treated HeLa cells with bis alone. As demonstrated in Fig. 6, bis reverses PMA, R59022, and ritanserin-stimulated phosphorylation of PKC substrates. We did not see any changes in basal PKC activity with only bis treatment (Fig. 7). Additionally, we treated HeLa cells with TCB-2, a 5-HT_{2A}R agonist, and stimulated 5-HT_{2A}R and PKC (Fig 7 and [45]). Under the conditions of active 5-HT_{2A}R, co-treatment with ketanserin and TCB-2 inhibited the ability of TCB-2 to activate PKC (Fig. 7).

4. DISCUSSION

DGK α was recently established as a therapeutic target in GBM and other cancers and the DGK α inhibitor R59022 was effective in countering cancer cell growth and progression both *in vitro* and *in vivo* [8]. In the present study, we hypothesized that the structurally related 5-HT₂R antagonist, ritanserin, would also inhibit DGK α activity. Using purified DGK α in combination with PC:DAG:PS liposomes, we show that ritanserin attenuates kinase activity *in vitro* and establish it as a third small-molecule inhibitor of DGK α . Further, we demonstrate that R59022 and R59949 are 5-HT₂R antagonists.

More specifically, both R59022 and ritanserin inhibit the activity of DGK α with respect to ATP and DAG (Fig. 4). At 2 mol%, R59022 decreased the $V_{\max}$ of DGK α by 50% (Fig. 4A and C, Table 1). The same concentration of ritanserin attenuated DGK α activity by 70% (Fig. 4B and D, Table 1). These data suggest that under the given *in vitro* conditions, ritanserin may be a better inhibitor of DGK α than R59022. Further, the inhibition of enzymatic activity was not surmountable by increasing concentrations of either substrate and a significant decrease in the K_m^{app} for ATP was observed in the presence of both inhibitors (Fig. 4 and Table 1). This complements previous findings using R59949 and suggests that R59022 and ritanserin prefer to bind to an ATP-enzyme complex [17]. One plausible explanation is that in the absence of ATP, the drug-binding pocket is less accessible to the inhibitors. A conformational change in response to the binding of ATP may allow the compounds to better interact with the enzyme. At the same time, the apparent affinity of DGK α for DAG was not significantly altered in the presence of the inhibitors (Table 1). It is possible that this is a case of mixed inhibition, where R59022 and ritanserin have higher affinity for the ATP-bound DGK α than free enzyme, but do not affect binding of DAG. In-depth structural studies are needed to know for certain where and how these compounds bind to DGK α . Little is known about how DAG and ATP bind mammalian DGKs and how the enzyme catalyzes phosphate transfer. In the case of *E. coli* DAGK, however, ATP and DAG were shown to bind the enzyme independent of each other [46]. If this is also the case with mammalian DGK α , then perhaps it is not surprising that the DGK inhibitors display mixed inhibition in the context of ATP and DAG.

Ritanserin is a potent 5-HT₂R antagonist that was in late stage clinical trials for schizophrenia and substance abuse [21–23]. It is orally bioavailable, has a half-life of approximately 40 hours in humans, and was found to have few adverse side effects [47,48]. As such, repurposing ritanserin as a DGK α inhibitor for an oncological indication might be a viable option. However, aspects of its polypharmacology, including its selectivity among the ten mammalian DGKs, need to be elucidated. While some of these studies are outside the scope of our work, it is of interest to investigate whether ritanserin attenuates the activity of other mammalian DGKs. To date, there are ten known DGK isotypes divided into 5 classes [5]. The R59949 and R59022 compounds were initially thought to be selective for class I, Ca²⁺-dependent DGKs (α , β , γ), but recent work has indicated that this may not be the case [17–19]. Under the given assay conditions, 20 μ M ritanserin and R59022 attenuated the activity of DGK α but did not significantly inhibit the other DGK isotypes (Fig. 5B and C). The liposomes used for these experiments contained dioleoyl DAG. Cellular membranes contain a variety of DAG species but *in vitro*, only DGK ϵ differentiates among them,

preferring the *sn*-2 arachidonoyl DAG [49,50] (although only a handful of the hundreds of possible DAGs are available for testing). Using dioctanoyl and stearoyl arachidonoyl DAGs, we found that R59022 and ritanserin still significantly inhibited the activity of only DGK α (Fig. 5D–G). This is also reflected in the dose-response curves, which show that the IC₅₀s of R59022 and ritanserin for DGK α are around 60 μ M (Fig. 5J and K)—about 2–3 fold higher than the IC₅₀s observed for DGK α (Fig. 5H and I). These data suggest that R59022 and ritanserin are most potent towards DGK α but at higher concentrations may attenuate other DGKs as well. The variation between our findings and those of others may be due to differences in assay conditions.

While they share obvious structural similarities to ritanserin, the activities of R59022 and R59949 against 5-HTRs have, to our knowledge, not been reported. In the present study, we show that the two known DGK inhibitors are also potent 5-HTR antagonists, with highest affinity for 5-HT₂Rs, particularly 5-HT_{2A/2C} (Table 3). Interestingly, although ritanserin has activity against various 5-HT receptors, its K_i is lowest for 5-HT_{2A/2C} receptors as well [21,32].

The 5-HT₂Rs signal through G α_q GPCR and activate protein lipase C (PLC) to generate DAG and inositol triphosphate (IP₃) [51]. Thus, antagonizing these receptors would result in a decrease in DAG (Fig. 6A; red arrows). Attenuation of DGK activity, on the other hand, would cause elevations in DAG (Fig. 6A; blue arrows). Since DAG is an activator of PKC, we wanted to elucidate whether R59022 and ritanserin reach DGK α inside the cell by detecting changes in PKC activity. Early work characterizing R59022 suggested that treatment of intact human platelets with R59022 resulted in an increase in the phosphorylation of an unknown, 40 kDa PKC substrate [10]. A more recent report studying the role of DGK δ in insulin resistance found that treatment of isolated rat muscle cells caused attenuation of total DGK activity and increased PKC activity [52]. We showed that treatment of HeLa cells with 40 μ M R59022 and ritanserin resulted in a significant increase in the phosphorylation of PKC substrates as compared to untreated cells. This was reversible with a known PKC inhibitor (Fig. 6B and C). Further, these results were recapitulated in U87 but not in U251 glioblastoma cells (Fig. 6E and F). Congruent with this data, it was found that U251 cells have a low relative mRNA expression of DGK α , compared to many of the other DGKs (Fig. 6I). On the other hand, HeLa and U87 cells express DGK α most abundantly (Fig. 6G and H). Additionally, we showed that another 5-HTR antagonist, ketanserin, does not inhibit DGK α *in vitro* and does not activate PKC in intact cells (Fig. 6B–F). These data increase our confidence that ritanserin and R59022 are functional DGK α inhibitors.

Ketanserin is a 5-HTR antagonist and as such, should attenuate PLC, decrease DAG, and PKC activity (Fig. 6). However, we did not notice an attenuation of PKC in Fig. 6. We reasoned that perhaps this is due to a lack of 5-HT₂R and/or PKC activity under basal conditions. This hypothesis was tested by treatment of cells with bis, which resulted in no difference in the phosphorylation of PKC substrates (Fig. 7). To address the question of basal 5-HTR activity, HeLa cells were treated with the 5-HT_{2A}R agonist, TCB-2, which has been shown to stimulate production of DAG [45]. TCB-2 caused a significant elevation of PKC activity and co-treatment with ketanserin reversed this effect (Fig. 7). Together, these

data suggest that in HeLa, as well as perhaps in U87 and U251 cells, PKC and 5-HTRs require exogenous stimulation for detectable activity. Additionally, these data give us further confidence that the increase in phosphorylation of PKC substrates with R59022 and ritanserin treatment is a result of DAG accumulation.

Our study has some limitations. We have demonstrated that ritanserin and R59022 can inhibit the function of more than one enzyme. Furthermore, ritanserin is reported to also have activity against dopamine receptors [21]. This data must be considered when investigating the effects of these compounds *in vivo*. Our results summarized in figures 6 and 7, however, support the conclusion that at least in cultured cells, ritanserin and R59022 cause DAG accumulation. While showing DGK attenuation, our experiments do not simultaneously demonstrate the effects of ritanserin and R59022 on 5-HTR signaling. These phenomena have been well studied by others and the ability of R59022 to antagonize 5-HTRs has now been shown by us (Table 3 and [21,22]). The purpose of the experiment in Fig. 6 was to test the hypothesis that ritanserin and R59022 can *also* attenuate DGK activity, which we showed by the increase in PKC activity (Fig. 6 and 7). We are also limited by *in vitro* assays due to the fact that cells express several DGK isotypes that are dynamically regulated and differentially expressed [1]. As such, it is difficult to say whether these compounds are selective for DGK α *in vivo*. Finally, cells contain many DAG and PA species, while we can only study only the small number of the lipid species that are commercially available.

In conclusion, we have demonstrated that the 5-HTR antagonist, ritanserin, is an inhibitor of DGK α and increases the affinity of the enzyme for ATP *in vitro* and that the two known DGK inhibitors, R59022 and R59949, are also 5-HTR antagonists. Further, ritanserin and R59022 are more potent against DGK α than against four other DGKs and have similar selectivity within these DGKs when assayed using various DAG species. Finally, we demonstrate that treatment of cells with ritanserin and R59022, but not ketanserin, activates PKC in cells that have a high relative expression of DGK α . Our data provide evidence that ritanserin may be a viable option for *in vivo* translation and an additional pharmacological tool for studying DGK biology.

Acknowledgments

We want to thank Dr. Tyler Basing at the University of Virginia for his help with statistics and Bryan Roth at the NIMH PDSP at UNC, Chapel Hill, for his collaboration. Also, we would like to thank Dr. Kevin Lynch for all of his help with editing of the manuscript and Dr. Mark Beenhakker for help with linear interpolation of data. We would also like to thank Dr. Kaoru Goto at Yamagata University, Dr. Fumio Sakane at the Chiba University, Dr. Matthew Topham at the University of Utah, and Dr. Daniel Raben at Johns Hopkins University for expression plasmids.

This work was supported by the National Institutes of Health research grants (R01 DK101946 (T.E.H), R01 CA180699 (B.W.P), R01 CA189524 (B.W.P)). S.B. was supported by an NIH training grant (T32 GM005572) and the University of Virginia Wagner Graduate Fellowship.

Abbreviations

ATP	adenosine triphosphate
K _m ^{app}	affinity

V_{max}	maximal specific kinase activity
IC₅₀	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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Fig. 1. Purification of recombinant DGK α . HeLa cells overexpressing FLAG-DGK α were harvested, the DGK α protein was affinity-purified, and activity was tested against DAG-containing liposomes. (A) The enzyme and BSA standards were separated on an SDS-PAGE and stained with Coomassie blue; a representative image is shown. Purified DGK α activity was linear in a (B) Time dependent manner with 0.5 μ g protein and (C) Linear in a protein concentration dependent manner by 15 minutes. Each data point represents the mean of triplicate $\pm$ SEM of a representative protein preparation.

Fig. 2.

Characterization of purified DGK α enzymatic properties. (A) ATP-dependent increase in DGK α activity with DAG at 5 mol%. (B) DAG-dependent increase in DGK α activity with ATP at 1 mM. (C) Effect of indicated concentrations of Ca^{2+} on the activity of DGK α . (D) The change in activity of DGK α with increasing mol% PS titrated into PC:DAG liposomes. Each point represents the mean of triplicate $\pm$ SEM of a representative experiment. One-way ANOVA was used to analyze statistical significance, followed by Tukey post-hoc analysis: *** $p < 0.0001$, ** $p < 0.0005$, * $p < 0.05$.

Fig. 3.
Chemical structures of the compounds used in this study.

Fig. 4.

Ritanserin inhibits the activity of purified DGKα. Compounds were titrated to 0.5 and 2 mol % into PC:DAG:PS liposomes containing DAG. Change in ATP-dependent DGKα activity in the presence of (A) R59022 and (B) Ritanserin with 5 mol% DAG. Change in DAG-dependent activity of DGKα in the presence of (C) R59022 and (D) Ritanserin with 1 mM ATP. Key: (●) No inhibitor, (■) 0.5 mol% drug, (▲) 2.0 mol% drug. Each point represents a mean of triplicate ± SEM of a representative experiment. Two-Way ANOVA was used to analyze statistical significance, followed by Tukey post-hoc analysis: *p < 0.0001 differences between V_{max} of control and each drug dose, †p < 0.0001 differences between V_{max} with 0.5 mol% and 2.0 mol% drugs.

Fig. 5.

R59022 and ritanserin are more potent against DGKα than other DGKs when tested using various DAG species. (A) FLAG-DGKs over-expressed in HEK 293 T cells were separated on an SDS-PAGE gel and probed with FLAG antibody. A representative image is shown. Using PC:DAG:PS liposomes with dioleoyl DAG, DGK activity was tested using cell homogenate with and without (B) 20 μM R59022 and (C) 20 μM ritanserin. Liposomes were prepared with dioctanoyl DAG and DGK activity was tested with and without (D) 20 μM R59022 and (E) 20 μM ritanserin. Liposomes were prepared with stearoyl arachidonoyl DAG and DGK activity was tested with and without (F) 20 μM R59022 and (G) 20 μM ritanserin. The values shown are percent of activity from no inhibitors, which was set to 100. The activity of lysates over-expressing only GFP was less than 10% of the lysates overexpressing DGKs and did not change in the presence of inhibitors. DGK specific activity was normalized to GFP specific activity. Each bar represents mean of triplicate ± SEM of a representative experiment. One-way ANOVA was used to test statistical significance between DGK activity with inhibitor and 100% activity - DGK activity without inhibitor, followed by Tukey's post-hoc analysis. *p<0.05. A log dose-dependent curve of DGKα activity using with (H) R59022 and (I) Ritanserin. A log dose-dependent curve of DGKι activity with (J) R59022 and (K) Ritanserin. The assays contained cell homogenate, 1 mM ATP and 10 mol% DAG. Veh represents enzyme activity with no drug. The data points on the graphs were fitted to linear interpolation.

Fig. 6.

Ritanserin and R59022 activate PKC in HeLa and U87 cells but not in U251 cells. (A) Schematic outlining the rationale and signaling pathway relevant to the experiment. (B) A representative western blot of HeLa cell extracts treated with PKC activator (PMA), PKC inhibitor (bis), R59022, ritanserin, and ketanserin probed with the phosphor-(Ser) PKC substrate antibody. (C) Quantitation of four independent experiments in HeLa cells. (D) A ketanserin dose-dependent curve of purified DGK α activity. (E) A quantitation of three independent experiments in U87 cells. (F) A quantitation of three independent experiments in U251 cells. The mRNA expression of indicated DGKs (top) and indicated 5-HTs (bottom) in (G) HeLa, (H) U87 and (I) U251 cells. Each bar represents mean $\pm$ SEM. One-way ANOVA was used to analyze statistical significance between control and each treatment, followed by Dunnett's post-hoc analysis: *** $p < 0.0001$, ** $p < 0.0005$, * $p < 0.005$, N.S = no statistical significance.

Fig. 7.

Ketanserin reverses the activation of PKC by TCB-2. (A) A representative western blot of HeLa cell extracts treated with PKC activator PMA, PKC inhibitor bis, 40 μM R59022, ritanserin, ketanserin and the 5-HTR_{2A}R agonist TCB-2 with and without 40 μM ketanserin. (B) A quantitation of four independent experiments. Each bar represents mean $\pm$ SEM. One-way ANOVA was used to determine statistical significance between control and each treatment, followed by Dunnett's post-hoc analysis. ** $p = 0.0001$, * $p < 0.005$.

Table 1

Kinetics of DGK α inhibition by R59022 and ritanserin. R59022 and ritanserin decrease $V_{\max}$ and K_m^{app} in the context of ATP and decrease $V_{\max}$ in the context of DAG. The units for $V_{\max}$ are nmol/min/mg. The K_m^{app} is indicated in mM concentration.

		R59022			Ritanserin		
		No Drug	0.5 mol%	2.0 mol%	No Drug	0.5 mol%	2.0 mol%
ATP	$V_{\max}$	43 $\pm$ 1	32 $\pm$ 1 [*]	20 $\pm$ 1 ^{*†}	41 $\pm$ 1	20 $\pm$ 1 [*]	12 $\pm$ 0.01 ^{*†}
	K_m^{app}	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.03 $\pm$ 0.01 [#]	0.05 $\pm$ 0.01	0.03 $\pm$ 0.004 [#]	0.01 $\pm$ 0.01 [#]
DAG	$V_{\max}$	37 $\pm$ 4	22 $\pm$ 1 [*]	16 $\pm$ 1.5 [*]	37 $\pm$ 4	17 $\pm$ 2 [*]	11 $\pm$ 2 [*]
	K_m^{app}	0.12 $\pm$ 0.03	0.11 $\pm$ 0.02	0.1 $\pm$ 0.02	0.12 $\pm$ 0.03	0.12 $\pm$ 0.04	0.07 $\pm$ 0.04

Two-Way ANOVA was used to analyze statistical significance:

^{*} p<0.0001 between control (no drug) and each drug dose,

[†] p<0.0001 between the two drug doses,

[#] significant difference in the K_m^{app} of control (no drug) and each drug dose as determined by 95% confidence interval.

Table 2

The affinity of various DGK isoenzymes for ATP and DAG. Graphpad Prism software was used to measure Michaelis-Menten kinetic constants and SEM.

	K_m^{app}	
	ATP (mM)	DAG (mM)
DGKβ	0.06 ± 0.04	0.09 ± 0.06
DGKδ	0.13 ± 0.04	0.05 ± 0.006
DGKι	0.09 ± 0.03	0.03 ± 0.003
DGKθ	0.08 ± 0.03	0.08 ± 0.04

Table 3

The effect of R59022 and R59949 on a panel of serotonin receptors. Data in first two rows are shown as mean % inhibition of binding (n=4) with 10 μ M test compounds using radioligand competition assays. Significant inhibition was considered to be >50% and K_i values were determined at these receptors from non-linear regression of radioligand competition binding isotherms. The K_i values are calculated from the best-fit IC_{50} values using the Cheng-Prusoff equation [33]. For experimental details, see [32]. The data in the last two rows are results from antagonist functional assays and represent percent inhibition – with 10 μ M test compounds – of the response to an EC90 concentration (empirically determined) of a reference agonist. Response elicited by reference antagonist was set to 100% and response elicited by vehicle was set to 0%. For experimental details see [34].

	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}	5-HT ₃	5-HT _{5A}	5-HT ₆	5-HT ₇
	Percent Inhibition of Binding									
R59949	61.1	20.3	76.7	94.2	96.9	72.2	0.7	71.6	71.8	82.8
R59022	82.4	83.5	90.4	98.2	100.5	81.7	10.9	92.0	96.9	98.5
	K_i (nM)									
R59949	2002.0		64.5	9.2	80.0	46.0		3248.5	3070.0	681.0
R59022	410	655.5	12.3	2.2	2.7	0.8		18.0	55.3	14.2
	Percent Inhibition of Agonist Response									
R59949			135.9	100.7	99.4	28.1				32.9
R59022			108.0	99.9	100.2	99.0				99.5