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Structure activity relationship studies on Amb639752: toward the identification of a common pharmacophoric structure for DGKα inhibitors

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1. Introduction

Diacylglycerol kinases (DGKs) are a large family of enzymes that share a common catalytic activity: the phosphorylation of diacylglycerol (DAG) to phosphatidic acid (PA). Remarkably, both the substrate (DAG) and the product (PA) of the DGK-catalysed reaction, are bioactive lipids that can act as second messengers1. DGK activity consequently serves as a switch to simultaneously dampen DAG-mediated signals and boost PA-mediated signals2. Ten mammalian DGK isozymes (α, β, γ, δ, ε, ζ, η, θ, τ, and κ) have been identified and divided into five groups (type I–V) according to their structural features3,4. The expression of these isoforms varies depending on the cell type. Among the 10 isoforms, the α isoform is among the most studied and characterised. This kinase is highly expressed in the brain, spleen, and thymus and, along with ζ isoform, in the bone marrow. This enzyme is also highly expressed in T-lymphocytes, where it acts together with DGKζ as negative regulator of the T-cell receptor (TCR) response, and a mediator of IL-2 mediated proliferation5,6. The biological relevance of DGKα is best demonstrated in patients with X-linked lymphoproliferative disease (XLP-1), who experience life-threatening, uncontrolled accumulation of CD8+ T cells in response to the Epstein–Barr virus (EBV) infection7. In those patients, germline mutations of the adaptor protein SAP (SH2D1A) perturb TCR signalling and render DGKα constitutively active8. Deregulated DGKα activity renders patient-derived lymphocytes resistant to reactivation-induced cell death (RICD). Thus, antigen-activated lymphocytes accumulate in lymphonodes and liver, resulting in severe immunopathology8. Importantly, DGKα inhibitors restore RICD sensitivity in vitro and in vivo, thus avoiding immunopathology and suggesting a putative therapeutic use of those molecules in XLP-19.

Apart from T-cell regulation, DGKα also plays a role in cancer, mediating numerous aspects of cancer cell progression including survival10,11, migration and invasion of cancer cells12–14. In particular, it has been reported that DGKα is over expressed in hepatocellular carcinoma15, and melanoma cells11 while other reports suggested that the growth of colon and breast cancer cell lines was significantly inhibited by DGKα-siRNA16 and DGKα/typical PKC/β1 integrin signalling pathway was crucial for matrix invasion of breast carcinoma cells17. In addition, expression is also higher in lymphonodal metastasis than in breast and gastric original tumour18,19. Finally, knock down of DGKα impairs glioblastoma tumorigenesis20,21.

For all these reasons, the identification of strong and selective DGKα inhibitors, it is an important field of research. To date, only a handful of two-digit micromolar inhibitors of DGKα have been identified, but only three were the most characterised, namely, R59949, R59022, and ritanserin (Figure 1). In our assay system, R59949 and R59022 have an IC50 of 11 and 20 μM, respectively22. Their efficacy has been evaluated in vivo

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studies on mice, and is limited by their rapid clearance \((t_{1/2}=\sim 2 \text{ h})\)\(^2\). Furthermore, these two inhibitors are also able to target different isofoms of DGK, in particular R59022 acts on type III and V (\(\gamma \varepsilon \theta \varphi \delta \varepsilon \lambda \gamma \)), while R59949 on type I and II (\(\gamma \varepsilon \delta \varepsilon \kappa \lambda \eta \))\(^3,24\) and a study conducted by Boroda et al. recently demonstrated their strong antagonistic activity on 5-HT\(_2\) receptors (R59022 IC\(_{50}\) SHT\(_{2A}\)=2.2 nM; R5994 IC\(_{50}\) SHT\(_{2A}\)=9.2 nM)\(^25\).

A search on ChEMBL database\(^26\) (https://www.ebi.ac.uk/chembl) show how these two molecules have activity at the same range of concentration with other biological targets, behaving like a sort of promiscuous ligands. Ritanserin, a well-known serotoninergic antagonist, is structurally similar to R59022, differing for an H-F isosteric substitution on a phenyl ring. Despite this small modification, Boroda et al. showed that ritanserin was a DGK\(\alpha\) inhibitor (IC\(_{50}\)=15 \(\mu\)M) more potent than R59022 and R59949 and with a better pharmacokinetic profile \((t_{1/2}=40 \text{ h in human})\)\(^25\). However, the comparison of ritanserin IC\(_{50}\) as serotonin antagonist and as DGK\(\alpha\) inhibitor, 0.9 nM and 15,000 nM, respectively, reveal that ritanserin is a much a powerful serotonin antagonist than a DGK inhibitor. In addition, ritanserin is also a potent inhibitor on dopaminergic receptors with an IC\(_{50}\) of 69 nM\(^27\).

Due to these drawbacks, at the beginning, in order to eliminate the strong serotoninergic activity of R59949, we reasoned to replace its protonable nitrogen atom, which at physiologically pH mimics the amino group of serotonin, with a carbon atom. We decided therefore to synthesise compound 1 (Figure 2) (see supporting information for its synthesis and a complete characterisation) and to test it as DGK\(\alpha\) inhibitor.

Interestingly, the compound was totally devoid of inhibitory activity on the enzyme, showing the importance of the basic nitrogen atom not only for the anti-serotoninergic activity, but also for the interaction with the kinase. With this in mind, we recently used an in-silico approach based on chemical homology with the two commercially available DGK\(\alpha\) inhibitors R59022 and R59949 using the programmes ROCS\(^28\) and EON\(^29\). From this study, we identified a compound, Amb639752 (Figure 2), featuring a lower IC\(_{50}\) for DGK\(\alpha\) than ritanserin (IC\(_{50}\)=17 \(\mu\)M), a better selectivity for the \(\alpha\)-isoform and devoid of anti-serotonin activity. Along with CU-3, which features an IC\(_{50}\) of 0.6 \(\mu\)M on DGK\(\alpha\)\(^30\) but contains a reactive Michael acceptor\(^31\), Amb639752 is the most effective pharmacological tool available to study DGK\(\alpha\). In this manuscript, we report the structure-activity studies on Amb639752 and, in combination with data on ritanserin, the generation of a pharmacophore model for this class of compounds, which could be useful for the identification of other potential DGK\(\alpha\) inhibitors.

2. Methods

2.1. Chemistry procedures

Commercially available reagents and solvents were used without further purification. Toluene were distilled immediately before use. Commercially available reagents and solvents were used without further purification. Toluene were distilled immediately before use. Toluene were distilled immediately before use.

2.1.1. Preparation of 2-chloro-1-(2,6-dimethyl-1H-indol-3-yl)ethanone (5)

In a Schlenk tube, under nitrogen, 2,6-dimethyl-1H-indole (3) (0.20 g, 1.38 mmol, 1 eq) was dissolved in 4 mL of dichloroethane dry and 0.25 mL of DBU (1.66 mmol, 1.2 eq) were added. The resulting solution was heated at 90 °C. When needed they were developed with KMN04 reagent. Purity of tested compounds was established by elemental analysis. Elemental analysis (C, H, N) of the target compounds is within ±0.4% of the calculated values, confirming ≥95% purity.

2.1.2. Preparation of tert-butyl 4-(2,6-dimethyl-1H-indol-3-yl)-2-oxoethyl)piperazine-1-carboxylate (6)

Under nitrogen, 200 mg of 5 (0.90 mmol, 1 eq) was dissolved in toluene dry, then N-Boc-piperazine (0.17 g, 0.90 mmol, 1 eq), K\(_2\)CO\(_3\) (0.32 g, 2.25 mmol, 2.5 eq), and KI (0.015 g, 0.09 mmol, 0.99 mmol, K\(_2\)CO\(_3\) (0.32 g, 2.25 mmol, 2.5 eq), and KI (0.015 g, 0.09 mmol, 0.99 mmol, 0.99 mmol, 0.99 mmol, 0.99 mmol, 0.99 mmol, 0.99 mmol, 0.99 mmol) m/z: 222 [M + H]\(^+\).
13.15, 129.5, 129.1, 125.1, 123.5, 121.2, 112.7, 111.6, 53.14, 53.14, 52.9, 21.7, 15.7. IR (KBr): 3225, 2793, 1658, 1409, 1261, 864 \nu_{max}/cm^{-1}. MS (ESI) m/z: 410 [M + H]^+; Anal. Calcd. for C_{23}H_{24}ClNNO_{2}C: 76.39; H: 6.90; N: 10.25; found C: 76.11; H: 6.12; N: 10.54.

2.1.7. 1-[(2,6-Dimethyl-1H-indol-3-yl)-2-(4-(4-methoxybenzoyl)piperazin-1-yl)ethan-1-one (12)
Yellow solid; yield 53%; column eluants: EtOAc, EtOAc/MeOH 9:1; m.p.: 219.9–220.8°C; 1H NMR (300 MHz, DMSO-d_6) \( \delta \) 11.70 (br s, NH), 7.85 (d, \( J = 8.2Hz, 1H \)), 7.38 (br d, AA'XX, 2H), 7.14 (s, 1H), 6.99–6.94 (m, 3H), 3.79 (br d, 3H), 3.67 (br s, 3H), 3.51 (br s, 4H) 2.67 (s, 3H), 2.58 (br s, 4H), 2.38 (s, 3H); 13C NMR (75 MHz, DMSO-d_6) \( \delta \) 192.8, 169.5, 144.6, 136.5, 135.7, 131.5, 130.0, 129.0, 127.5, 124.1, 121.2, 112.8, 111.6, 66.9, 55.8, 53.2, 53.1, 21.7, 15.7; IR (KBr): 3235, 3003, 2807, 1613, 1463, 1253, 977 \nu_{max}/cm^{-1}; MS (ESI) m/z: 406 [M + H]^+; Anal. Calcd. for C_{29}H_{28}N_{2}O_{2}C: 71.09; H: 6.71; N: 10.36; found C: 71.10; H: 6.75; N: 10.32.

2.1.8. 4-(4-[(2,6-Dimethyl-1H-indol-3-yl)-2-oxoethyl]piperazin-1-carboxylibenzonitrile (13)
Yellow solid; yield 23%; column eluants: EtOAc, EtOAc/MeOH 9:1; m.p.: 243.9–244.8°C; 1H NMR (300 MHz, DMSO-d_6) \( \delta \) 11.70 (br s, NH), 7.92–7.84 (m, 3H), 7.58 (br d, AA'XX, 2H), 7.15 (s, 1H), 6.96 (br d, 1H), 3.68 (br s, 4H), 3.29 (br s, 2H), 2.67 (s, 3H), 2.51 (br s, 4H), 2.38 (s, 3H); 13C NMR (75 MHz, DMSO-d_6) \( \delta \) 192.5, 166.3, 143.2, 139.4, 131.9, 131.9, 129.9, 126.8, 123.5, 121.9, 119.6, 117.4, 111.2, 111.1, 110.5, 51.7, 51.2, 20.1, 14.1; IR (KBr): 3410, 3254, 2816, 2790, 2233, 1609, 1454, 1291, 979 \nu_{max}/cm^{-1}; MS (ESI) m/z: 401[M + H]^+; Anal. Calcd. for C_{35}H_{36}N_{2}O_{2}C: 71.98; H: 6.04; N: 13.99; found C: 72.13; H: 6.23; N: 14.08.

2.1.9. 1-[(2,6-Dimethyl-1H-indol-3-yl)-2-(4-thiophene-2-carboxylibenzoxy)piperazin-1-yl]ethan-1-one (14)
Yellow solid; yield 29%; column eluants: EtOAc, EtOAc/MeOH 9:1; m.p.: 200.3–201.2°C; 1H NMR (300 MHz, DMSO-d_6) \( \delta \) 11.74 (br s, NH), 7.86 (d, \( J = 8.2Hz, 1H \)), 7.76 (br d, 1H), 7.41–7.40 (m, 1H), 7.15–7.10 (m, 2H), 6.96 (d, \( J = 8.0Hz, 1H \)), 3.75–3.67 (m, 4H), 3.37 (br s, 4H), 2.68 (s, 3H), 2.62 (s, 3H), 2.38 (s, 3H); 13C NMR (75 MHz, DMSO-d_6) \( \delta \) 192.8, 167.8, 144.7, 137.8, 135.7, 131.5, 130.0, 129.6, 127.6, 127.5, 124.1, 123.4, 121.2, 112.9, 111.6, 66.9, 53.2, 53.1, 21.7, 15.7; IR (KBr): 3270, 2927, 2793, 1642, 1454, 1261, 809 \nu_{max}/cm^{-1}; MS (ESI) m/z: 382[M + H]^+; Anal. Calcd. for C_{34}H_{35}N_{2}O_{2}S: C, 66.12; H, 6.08; N: 11.01; found C, 66.23; H, 6.26; N: 10.93.

2.1.10. 1-(2,6-Dimethyl-1H-indol-3-yl)-2-(4-nicotinyl)piperazin-1-yl]ethan-1-one (15)
Yellow solid; yield 39%; column eluants: EtOAc, EtOAc/MeOH 9:1; m.p.: 216.2–216.8°C; 1H NMR (300 MHz, DMSO-d_6) \( \delta \) 11.70 (br s, NH), 8.65–8.61 (m, 2H), 7.87–7.82 (m, 2H), 7.50–7.45 (m, 1H), 7.15 (s, 1H), 6.95 (br d, 1H), 3.68 (br s, 4H), 3.37 (br s, 2H), 2.67 (s, 3H), 2.56 (br s, 4H), 2.37 (s, 3H); 13C NMR (75 MHz, DMSO-d_6) \( \delta \) 192.6, 167.2, 151.0, 148.1, 144.7, 135.7, 134.3, 132.3, 131.5, 125.1, 124.1, 123.5, 121.2, 116.2, 111.6, 66.7, 53.4, 52.9, 21.7, 15.7; IR (KBr): 3414, 3213, 2828, 1621, 1454, 1267, 1301, 817 \nu_{max}/cm^{-1}; MS (ESI) m/z: 377 [M + H]^+; Anal. Calcd. for C_{31}H_{28}N_{2}O_{2}: C, 70.19; H: 6.43; N: 14.88; found C, 70.21; H: 6.44; N: 14.73.
2.1.12. 1-(2,6-Dimethyl-1H-indol-3-yl)-2-(4-(3-methoxybenzoyl)piperazin-1-yl)ethan-1-one (17)
Yellow solid; yield 32%; column eluants: EtOAc, EtOAc/MeOH 1:1; m.p.: 187–189°C; 1H NMR (300 MHz, DMSO-d6) δ 11.71 (br s, NH), 7.85 (d, J = 7.9 Hz, 1H), 7.34 (t, 1H), 1.75 (br s, 1H), 0.92–6.92 (m, 4H), 3.78 (br s, 3H), 3.68 (br s, 3H), 3.35 (br s, 3H), 2.67 (br s, 7H), 2.01 (br s, 7H), 13C NMR (75 MHz, DMSO-d6) δ 192.7, 169.1, 159.7, 144.7, 137.9, 131.5, 130.2, 125.3, 123.4, 119.4, 115.7, 112.7, 111.6, 66.8, 55.8, 53.3, 53.1, 47.4, 21.7, 15.7; IR (KBr): 3131, 3049, 2944, 1645, 1455, 1292, 910, 402 192.6, 191.7, 172.4, 145.4, 135.5, 132.1, 124.0, 123.6, 120.4, 111.4, 116.2, 66.2, 53.3, 53.1, 45.3, 41.2, 34.5, 31.6, 28.9, 25.0, 22.5, 21.5, 15.6, 14.1; MS (ESI) m/z: 412 [M + H]+; Anal. Calcd. for C23H23N3O2: C, 72.03; H, 8.67; N, 10.96; found C, 72.03; H, 8.73; N, 11.21.

2.1.13. 2-(4-(3,4-Difluorobenzoyl)piperazin-1-yl)-2-(4-(2,6-dimethyl-1H-indol-3-yl)ethan-1-one (18)
Yellow solid; yield 27%; column eluants: EtOAc, EtOAc/MeOH 1:1; m.p.: 233.8–235.0°C; 1H NMR (300 MHz, DMSO-d6) δ 11.71 (br s, NH), 7.85 (d, J = 7.9 Hz, 1H), 7.56–7.47 (m, 2H), 7.28 (br s, 1H), 7.15 (s, 1H), 6.96 (d, J = 7.9 Hz, 1H), 3.68 (br s, 2H), 3.33 (br s, 2H), 2.67 (br s, 7H), 2.01 (br s, 7H), 13C NMR (75 MHz, DMSO-d6) δ 192.7, 169.1, 159.7, 144.7, 137.9, 131.5, 130.2, 125.3, 123.4, 119.4, 115.7, 112.7, 111.6, 66.8, 55.8, 53.3, 53.1, 47.4, 21.7, 15.7; IR (KBr): 3131, 3049, 2944, 1645, 1455, 1292, 910, 402 192.6, 191.7, 172.4, 145.4, 135.5, 132.1, 124.0, 123.6, 120.4, 111.4, 116.2, 66.2, 53.3, 53.1, 45.3, 41.2, 34.5, 31.6, 28.9, 25.0, 22.5, 21.5, 15.6, 14.1; MS (ESI) m/z: 412 [M + H]+; IR (KBr): 3131, 3049, 2944, 1645, 1455, 1292, 910, 402 192.6, 191.7, 172.4, 145.4, 135.5, 132.1, 124.0, 123.6, 120.4, 111.4, 116.2, 66.2, 53.3, 53.1, 45.3, 41.2, 34.5, 31.6, 28.9, 25.0, 22.5, 21.5, 15.6, 14.1; MS (ESI) m/z: 412 [M + H]+; Anal. Calcd. for C23H23N3O2: C, 72.03; H, 8.73; N, 11.21.

2.2. Cell lines
Madin-Darby canine kidney (MDCK) cells stably expressing One Step Tag DGKα (OST-DGKα) were prepared by infecting MDCK cells with a vector expressing an inducible OST tagged DGKα constructs. MDCK cells infected with empty vector were used as controls. MDCK cells were cultured in MEM (minimal essential medium) with 5% FBS (foetal bovine serum) and 1% antibiotic–antimyotic solution. Routinely, cells were splitted every 3–4 days with trypsin–EDTA 0.25% in standard 100 mm dishes.

Human embryonic kidney 293T cells (10 cm² plates) were cultured in RPMI with 10% FBS and 1% penicillin/streptomycin and cultures were maintained by splitting them for every 2–3 days using trypsin–EDTA 0.25%.

Michigan Cancer Foundation 7 (MCF7) cells were cultured in DMEM with 10% FBS + 1% penicillin/streptomycin and cultures were maintained by splitting them for every 2–3 days using trypsin–EDTA 0.25%.

2.3. Primary cells
PBL were isolated from healthy anonymous human donors by Ficoll-Paque PLUS (GE Healthcare, Chicago, IL) density gradient centrifugation, washed, and resuspended at 2 × 10⁶ cell/mL in RPMI-GlutaMAX containing 10% heat inactivated FCS, 2 mM glutamine, and 100 U/mL of penicillin and streptomycin. T cells were activated with 1 μg/mL anti-CD3 (UCHT1) and anti-CD28 (clone CD28.2) antibodies. After three days, activated T cells were washed and cultured in medium additioned of 100 IU/mL rIL-2 (Peprotech, Rocky Hill, NJ) at 1.2 × 10⁶ cells/mL for ≥7 days by changing media for every 2–3 days.

Human monocytes were isolated from healthy anonymous humanuffy coats (provided by the Transfusion Service of...
Ospedale Maggiore della Carità, Novara, Italy) by the standard technique of dextran sedimentation and Histopaque (density = 1.077 g cm\(^{-3}\), Sigma-Aldrich, Milano, Italy) gradient centrifugation (400×g, 30 min, room temperature) and recovered by fine suction at the interface, as described previously.\(^{32}\) Purified monocytes populations were obtained by adhesion (90 min, 37 °C, 5% CO\(_2\)) in serum-free RPMI 1640 medium (Sigma-Aldrich, Milano, Italy) supplemented with 2 mM glutamine and antibiotics. Cell viability (trypan blue dye exclusion) was usually >98%.

### 2.4. Preparation of DGK\(_x\) enriched homogenates

Large cultures of MDCK cells for enzyme preparation were done by plating 5 × 10\(^6\) cells in 245 mm\(^2\) dishes. Once they reached nearly 70% confluence, cells were treated with doxycycline (1 μg/mL, two days). After two days of treatment, each plate was washed in cold PBS and cells homogenised in 5 mL of homogenate buffer (25 mM Hepes (pH 8), 20% glycerol, 135 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(beta-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 mM sodium orthovanadate, and protease inhibitor cocktail) for each dish. Cells were collected with a rubber scraper, homogenised by passing them through a 29-G-needle syringe 20 times and stored in aliquots at −80 °C. Presence of OST-DGK\(_x\) was confirmed by western blotting and enzyme assay, transduced DGK\(_x\) has an activity >100 folds the endogenous DGK.

### 2.5. Preparation of DGK\(_i\), and DGK\(_{ii}\) enriched homogenates

293T cells were transiently transfected with indicated DGK isoform plasmid DNA using Lipofectamine 3000, Invitrogen (Carlsbad, CA). Forty eight hours after transfection, cells were harvested and homogenised with a 29-G-needle using 500 μL of homogenate buffer for each dish and immediately stored in aliquots at −80 °C. Cells transfected with empty vector were used as controls, overexpressed DGK has an activity >50 folds the endogenous.

### 2.6. DGK assay

Essentially the same procedure was followed as reported previously in Velnatì et al.\(^9\) In brief, DGK activity was assayed by measuring initial velocities (5 min at 27 °C) in presence of saturating substrate concentrations. Reaction conditions: 0.9 μg/μL 1,2-dioleoyl-sn-glycerol, 5 mM ATP, 0.01 μCl/μL \(_{[32P]}\)-ATP, 1 mM sodium orthovanadate, 10 mM MgCl\(_2\), 1.2 mM EGTA in 7.5 mM Hepes pH 8.\(^{12}\) Reaction mixture is assembled mixing enzyme (24.5 μL of homogenate), 100 × inhibitor or DMSO (0.5 μL), 5 μL ATP solution (10 μL of 25 mM ATP, 0.05 μCl/μL \(_{[32P]}\)-ATP (Perkin-Elmer, Milan, Italy), 5 mM sodium orthovanadate, 50 mM MgCl\(_2\)), and 3.3 × DAG solution (15 μL of 3 μg/μL 1,2-dioleoyl-sn-glycerol resuspended by sonicication in 4 mM EGTA in 25 mM Hepes pH 8). The reaction was stopped after 5 min by adding 200 μL of freshly prepared 1 M HCl and lipid was extracted by adding 200 μL of CH\(_3\)OH:CHCl\(_3\) 1:1 solution and vortexing for 1 min. The two phases were separated by centrifugation (12,000 RCF for 2 min). Twenty-five microlitres of the lower organic phase was spotted in small drops on silica TLC plates. TLC was run 10 cm and dried before radioactive signals were detected by GS-250 molecular imager and was quantified by quantity one (Bio-Rad, Hercules, CA) software assuring the absence of saturated spots.

Percentage residual activity was calculated as follows: (OST-DGK\(_x\) homogenate with inhibitor – vector homogenate)/(OST-DGK\(_x\) homogenate with DMSO – vector homogenate)×100.

### 2.7. Superoxide anion (O\(_2^{-}\)) production

All the experiments were performed in triplicate using cells isolated from each single donor.

Monocytes (250,000 cells/well) were treated for 1 h with the indicated drugs (10 μM) with or without serotonin (1 μM). Then, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Milano, Italy) 1 μM for 30 min. PMA is a well-known stimulus that induces a strong and significant respiratory burst via PKC activation.\(^{35}\) Superoxide anion production was then evaluated by the superoxide dismutase (SOD)-sensitive cytochrome C (CytC) reduction assay and expressed as nmol Cytc reduced/10\(^6\) cells/30 min, using an extinction coefficient of 21.1 mM. To avoid interference with spectrophotometrical recordings, cells were incubated with RPMI 1640 without phenol red, antibiotics, and FBS.

### 2.8. RICD assay in SAP silenced T cells

Activated human PBLs were transfected with 200 pmol of siRNA oligonucleotides specific for the target protein (Stealth Select siRNA; Life Technologies, Carlsbad, CA) or a non-specific control oligo (Life Technologies, Carlsbad, CA). Transient transfections were performed using Amaxa nucleofector kits for human T cells (Lonza, Basel, Switzerland) and the Amaxa Nucleofector II or 4D systems (programmes T-20 or EI-115). Cells were cultured in IL-2 (100 IU/mL) for four days to allow target gene knockdown. Knockdown efficiency was periodically evaluated by Western blotting.

Non-specific Stealth RNAi Negative Control Duplexes (12935-300, Life Technologies, Carlsbad, CA) were used as a negative control.

siRNA SAP: sense strand UGUACUGCCUAUGUGUGCUGUAUCA, antisense strand UGAUACAGCAUAGGCGAGUACA.

To test restimulation induced cell death, activated T cells (10\(^5\) cells/well) were plated in triplicate in 96-well round-bottom plate and treated with anti-CD3 (clone OKT3) (10 ng/mL) in RPMI-Glutamax supplemented with 100 IU/mL rhIL-2 for 24 h. In these assays, inhibitors (10 μM) were added 30 min before the restimulation with OKT3. 24 h after treatment, cells were stained with 20 ng/mL propidium iodide and collected for a constant time of 30 s per sample on Attune NXT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA). Cell death is expressed as % cell loss and calculated as:

\[
\% \text{ cell loss} = \left(1 - \frac{\text{number of viable cells in sample}}{\text{number of viable cells in control}}\right) \times 100
\]

Results were expressed as mean±standard error of the mean (SEM). We always compared controls and SAP silenced lymphocytes from the same donors as there is a large individual variability in RICD sensitivity.

### 2.9. Migration assays

Cell migration assays were performed using the Culture-Insert 2 well in μ-Dish (ibidi GmbH, Martinsried, Germany).

Briefly, 25,000 MCF7 cells were plated in each well and cultured for 24 h. After the culture insert was removed and the cells were washed with PBS before treating them with respective DGK\(_x\) inhibitors (10 μM) or DMSO for 15 h in complete medium (DMEM 10% FBS + 1% penicillin/streptomycin), while medium without FBS was used as a negative control for migration.
Phase-contrast pictures were taken immediately after treatment (0 h) and after 15 h under 5× magnification. Finally, wound areas were determined using ImageJ software (NIH, Bethesda, MD). Wound reduction was calculated by using the following formula: (wound area at 15 h/wound at 0 h)×100, the values obtained were expressed as the percentage of wound area compared to the initial area.

2.10. Quantification and statistical analysis

Data for the screen on OST-DGKα homogenates are the mean of duplicates. The compounds showing inhibitory activity in this assay were tested >4 times and the mean ± SEM is reported.

To calculate IC₅₀ values of active inhibitors, the inhibitor activity was measured at least three times at 0.1, 1.0, 10.0, and 100.0 μM concentration. Data were analysed using [inhibitor] vs. normalised response parameters with least square [ordinary] curve fitting method in GraphPad PRISM 8.0 software (GraphPad Software, La Jolla, CA) mentioning 95% confidence interval and IC₅₀ values always greater than 0.0. Graph shows the mean ± SEM of inhibitor activity at the indicated concentration. In all the experiments, the data were normalised with the controls.

Evaluation of *in vitro* assays across multiple treatments (RICD), SOD-sensitive CytC reduction assay, migration assays were analysed by using one-way ANOVA with multiple comparisons correction using GraphPad PRISM 8.0 software (GraphPad Software, La Jolla, CA). Error bars are described in figure legends as ± SEM or ± SD where appropriate. A single, double, triple and four asterisk denotes significance of a *p* value ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 respectively in all experiments.

2.11. Pharmacophoric model

A representative 3D structure of each compound was generated using OMEGA2 software. The generated file was used to generate a pharmacophore model with the Pharmagist web server (bioinfo3d.cs.tau.ac.il/PharmaGist)³⁷.

3. Results

3.1. Chemistry

At the beginning, we purchased 14 analogues of Amb639752 by vendors (Figure 3), while one analogue (2), being not commercially available, was synthesised (see Supplementary material). All the compounds were evaluated for their inhibitory activity on DGKα at a concentration of 100 μM (Table 1).

![Figure 3. First set of compounds tested for their inhibitory activity on DGKα.](image-url)
Each inhibitor was tested in duplicate at least once, and DGKα activity was expressed as percentage of residual DGKα activity compared to DMSO control in the same assay. Assay uses OST-DGKα overexpressing cell lysates in presence of saturating exogenous DAG and ATP. We considered R59022 (commercially available) and the lead compound Amb63975230 as our reference molecules. As expected, our reference inhibitors R59022 and Amb639752 featured 73% and 96% inhibition respectively, confirming the quality of data obtained.

This first screening showed us how Amb639752 exhibits a rigid structure activity relationship. Indeed, both the methyl groups on the 2,6 position of indole are mandatory, the NH indole cannot be alkylated as well as ramifications on the alkyl chain are detrimental. We then focused our attention on furan ring knowing its intrinsic toxicity via metabolic activation\textsuperscript{38}. Unfortunately, there were no analogues available by vendors. Our first goal was to replace the furan moiety with the phenyl ring, investigating two different synthetic pathways.

In the first one, the commercially available 2,6-dimethyl-\textit{1H}-indole 3 was acylated with 2-chloroacetyl chloride 4, in the presence of DBU in dichloroethane\textsuperscript{39} to give the derivative 5\textsuperscript{90\%} yield. Then, the acylated compound 5 was reacted with N-Boc piperazine in the presence of potassium carbonate and potassium iodide to afford the piperazinic derivative 6 in 63% yield. Boc deprotection with trifluoroacetic acid, followed by coupling with benzoic acid using the condensing agent EDCI afforded the final compound 8 (Scheme 1).

In the second synthetic strategy, we initially coupled the benzoic acid with N-Boc piperazine in the presence of EDCI to give piperazinic derivative 9 in 49\% yield. Boc deprotection gave in quantitative yield the compound 10. Due to its high aqueous solubility, solvent was evaporated and the crude as trifluoroacetate salt was directly used for the next step, where it was reacted with the acylated indole 5 to give the final compound 8 in 25\% yield (Scheme 2) (see Supplementary material for full synthetic details).

Overall yield calculation was 11\% for both strategies, but with the first route it was possible to use a common synthetic intermediate 7 which can be coupled with different carboxylic acids. Furthermore, the second route requires more purification steps. For this reason, we applied the first route and coupled the advanced intermediate 7 with 12 different carboxylic acids (Figure 4) to afford compounds 11–22 (Figure 5).

All those molecules were dissolved in DMSO and tested at a concentration of 100 \textmu M for the ability to inhibit DGKα using equal amounts of DMSO as control. We identified eight compounds capable of reducing OST-DGKα activity similar or superior to R59022 (Table 2).

### 3.2. Potency and isoform specificity of active molecules

To measure the inhibitor potency, we determined the IC\textsubscript{50} values for the compounds that resulted active when tested at 100 \textmu M by measuring the residual OST-DGKα activity over a dose range of inhibitor concentrations (0.1 \textmu M, 1.0 \textmu M, 10.0 \textmu M, and 100.0 \textmu M).

#### Table 1. Inhibitory activity on DGKα (I).

| Compound       | Residue activity at 100 \textmu M |
|----------------|----------------------------------|
| R59022         | 27                               |
| R59949         | 28                               |
| Amb639752      | 4                                |
| Amb758976      | 67                               |
| Amb37496       | 114                              |
| Amb626577      | 127                              |
| Amb1926062     | 88                               |
| Amb94966       | 196                              |
| Amb730692      | 45                               |
| Amb98433       | 81                               |
| Amb18718       | 135                              |
| Amb726054      | 126                              |
| Amb731111      | 130                              |
| Amb22018852    | 160                              |
| Amb667392      | 115                              |
| Amb758392      | 150                              |
| Amb629700      | 101                              |
| 2              | 135                              |

Scheme 1. The first synthetic route for the compound 8.
For R59022 and Amb639752, we measured IC50 values of 15.2 ± 5.8 μM and 6.9 ± 3.0 μM respectively which were comparable to previous reports using similar assay conditions. Considering those two as reference/template compounds, we measured the IC50 values of 8, 11, 12, 13, 14, 16, 19, and 20 as 3.2 ± 1.0, 1.6 ± 0.4, 3.6 ± 1.2, 6.9 ± 2.3, 3.0 ± 1.0, 32.8 ± 11.5, 49.7 ± 31.7, and 1.8 ± 0.4 μM, respectively, signifying that their activity is equal or superior to the template compounds (Figure 6).

Due to their higher IC50 values, we thus decided to exclude 16 and 19 for further experiments. In summary, we recognised six compounds with equal or superior inhibitory activity compared to commercially available DGKα inhibitors.

To check the isoform specificity of those active molecules, we tested them, along with Amb639752, for their ability to inhibit DGKα, DGKβ (the other major DGK isoform expressed in lymphocytes), and the more distantly related and widely expressed DGKδ. At the highest concentration of 100 μM, all those molecules resulted in highly specific against DGKα as like their parent molecule, Amb639752 by completely inhibit DGKα whereas they do not have significant effects on DGKβ and DGKδ apart from 20 which, at the contrary, acts as an activator of DGKδ (Figure 7).

### 3.4. DGKα inhibitors restore RICD in SAP deficient T cells

Ruffo et al. demonstrated that the defective RICD observed in T cells from XLP-1 patients was rescued by silencing DGKα expression or by pre-treatment with DGKα inhibitors R59949 or R59022. Interestingly, R59022 also showed beneficial effects in an in vivo model of XLP-1, but due to its poor pharmacological properties, its use in human patients results unlikely. We therefore tested the effect all those active molecules along with Amb639752 on RICD sensitivity of SAP-deficient T cells. As additional controls, we also included ritanserin and ketanserin to evaluate the contribution of serotonin antagonism to the effects observed.

To evaluate inhibitor efficacy in physiological context, we modelled XLP-1 by silencing SAP in primary peripheral blood T lymphocytes (PBLs) and restimulating them with anti-CD3 antibody (OKT3 10 ng/mL, 24 h). We pre-treated the cells with the indicated
inhibitors for 30 min at a concentration of 10 μM. In control siRNA-transfected cells, DGKα inhibitors poorly affect RICD, with Amb639752, 11 and 14 slightly reducing it (Figure 9). Conversely, DGKα inhibitors significantly rescued the apoptotic defect of SAP-deficient T cells although not reaching control levels. At 10 μM, all the new molecules showed an efficacy comparable to Amb637952 and ritanserin used as positive reference molecules. Conversely, the serotonin antagonist ketanserin is inactive, excluding the involvement of serotonin receptors in rescuing the RICD in SAP-deficient T cells (Figure 9).

In summary, these data confirm that the newly identified DGKα inhibitors can rescue RICD susceptibility in T cell models of XLP-1 suggesting a putative use for XLP-1 therapy.

**Table 2. Inhibitory activity on DGKα (II).**

| Compound | Residue activity at 100 μM | IC₅₀ (μM) |
|----------|---------------------------|-----------|
| 8        | 5                         | 3.2       |
| 11       | 6                         | 1.6       |
| 12       | 6                         | 3.6       |
| 13       | 14                        | 6.9       |
| 14       | 6                         | 3.0       |
| 15       | 89                        | –         |
| 16       | 27                        | 32.8      |
| 17       | 41                        | –         |
| 18       | 47                        | –         |
| 19       | 26                        | 49.7      |
| 20       | 3                         | 1.8       |
| 21       | 39                        | –         |
| 22       | 48                        | –         |

**Figure 5.** Putative DGKα inhibitors synthesised. In brackets the yield of the coupling reaction with the common intermediate 7.
3.5. DGKα inhibitors reduce migration of the cancer cells (MCF7)

Previous studies conducted in our laboratory demonstrated that the inhibition of DGK activity decreases chemotaxis, proliferation, migration, and invasion of many cancer cell lines\textsuperscript{12-14}. To evaluate if our newly synthesised DGKα inhibitors were effective in impairing cancer cell migration, we measured serum induced wound healing in MCF7 breast cancer cells in presence of 10 μM inhibitor. In presence of serum, none of the inhibitors is toxic for MCF7 cells even after prolonged treatment (data not shown). After 15 h of treatment, all the newly synthesised active molecules

Figure 6. Dose–response curves for novel DGKα inhibitors. Dose–response of the most active compounds along with their IC\textsubscript{50} values. Data from at least three independent experiments performed in triplicate.
equally reduced cell migration when compared to the vehicle (DMSO) delaying wound closure (Figure 10).

Besides being in good agreement with the notion that DGKα is required for cancer cells migration, this observation indicates that our new DGKα inhibitors reduce cancer cell motility, suggesting a potential utility in a metastasis setting.

3.6. Generation of a pharmacophore hypothesis

From the data obtained, it is possible to identify some key pharmacophoric points crucial for the biological activity of the Amb compounds on DGKs namely: (i) a basic nitrogen; (ii) the methyl groups at the 2 and 6 position of the indole nucleus, and (iii) a (hetero)aromatic ring. This information allows us to build a four-point pharmacophoric model represented in Figure 11 superimposed with the minimised structure of compound 11. Although, we are not able to evaluate the importance of the two carbonyl groups, it represents the first attempts in order to identify the minimum structural request to interact with DGK catalytic site considering the molecular structure of the four most active inhibitors discovered to date (Amb639752, ritanserin, R59022, and R59949). We feel that this model might be useful to identify novel
compounds active on DGKα through more targeted virtual screening campaigns, overcoming the current scaffolds.

4. Discussion

As a key component of several signal transduction pathways, DGKα represent an emerging pharmacological target. We have demonstrated the efficacy of DGKα inhibitors for XLP-1 treatment\textsuperscript{16}, while others have proposed them for cancer treatment\textsuperscript{22} and to remove immune-checkpoints promoting immune vigilance against cancer\textsuperscript{40}. Commercially available DGKα inhibitors are limited by poor specificity\textsuperscript{35,41} and pharmacokinetic\textsuperscript{20}. The CU-3 molecule described by other features a noteworthy activity and specificity but its reactive chemical structure make unlikely an in vivo use\textsuperscript{30}. With intent of developing molecules suitable for therapeutic use we selected Amb639752 as a novel inhibitor with remarkable DGKα activity. Amb639752 also features improved selectivity for DGKα as it does not affect serotonin signalling\textsuperscript{9}. Despite numerous efforts a structure of mammalian DGKs is still missing, thus we decided to explore the structure–activity relationship of this molecule to improve its activity and pave the way for further developments. Our efforts allowed us to build a pharmacophoric model for DGKα inhibitors characterised by three required features. We also characterised a set of novel compounds with improved IC\textsubscript{50} in the low μM range and identified the most profitable synthetic route for them. The mode of DGKα inhibition by those molecules is still unknown apart for ritanserin, which binds at the same time the DGKα catalytic accessary domain and the C1 domain putatively promoting a close inactive conformation\textsuperscript{41}.

The second-generation inhibitors we described in this work maintain the specificity of Amb639752 as they do not affect DGKγ, the predominant isofrom of lymphocytes\textsuperscript{42} and the broadly expressed DGKβ\textsuperscript{43}. Those DGKα inhibitors are active in a lymphocyte based XLP-1 assay and in a cancer cell migration assay, holding the promise for a potential therapeutic application. However, their efficacy is still to be determined in in vivo models of disease where some of the parental compounds showed efficacy but poor pharmacokinetic\textsuperscript{8,20}.

Disclosure statement

The authors declare no competing financial interest.

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