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Effects of siRNA-mediated silencing of DGKA and DGKZ on HL-60 and K562 cell lines

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ABSTRACT

Diacylglycerol kinases (DGKs) are a family of lipid signaling enzymes that decrease diacylglycerol (DAG) signaling while concurrently generating phosphatidic acid (PA), a bioactive lipid involved in various cellular processes as modulating key oncogenic pathways such as Akt/mTOR and MAPK/ERK. Literature studies confirm that some DGKs isoforms are overexpressed and have a role in tumorigenesis. This thesis aims to understand the role of DGKα and DGKζ in AML and CML, represented respectively by HL-60 and K562 cells, as new therapeutic target for those disease. To achieve it siRNA-mediated knockdown of DGKA and DGKZ was performed in different cell lines (HL-60, HEL, THP1 and K562), in this thesis we will discuss HL-60 and K562 silencing as model respectively of acute promyelocytic leukemia (APL), a subtype of AML and chronic myeloid leukemia (CML). Target silencing was confirmed by qPCR and Western blot and assessed viability through Trypan Blue essay and apoptosis/necrosis essays where cell death was found. ERK1/2 phosphorylation status was later analyzed by Western blot, and correlation of DGKα and DGKζ selective inhibitors IC50 (ritanserin, DGKZ-IN-4, BAY 2965501) was determined by X-Y chart with mRNA and DGKα and DGKζ protein levels. DGKζ silencing in HL-60 cells reduced viability by ~37% and induced significant late apoptosis, whereas DGKα knockdown had minimal effect. In K562 cells, instead, neither isoform silencing reduced cell viability. ERK1/2 phosphorylation responded differentially to isoform knockdown in a cell-type specific manner. While X-Y chart showed no correlation within the quantity of DGKα or DGKζ both in mRNA and protein with the IC50 of the related specific inhibitor (ritanserin for DGKα and BAY 2965501 and DGKZ-IN-4 for DGKζ). Our data identify DGKs as a non-redundant regulator of AML cell survival and support its candidacy as a therapeutic target. Future work should validate these findings in primary patient samples and explore combination strategies with existing AML therapies, then develop optimized DGKs inhibitors for clinical purposes.

INTRODUCTION

This study is part of a broader research project investigating the roles of diacylglycerol kinases alpha (DGKA) and zeta (DGKZ) in the pathogenesis of acute myeloid leukemia (AML). To achieve this, we employed gene silencing and pharmacological inhibition approaches in various myeloid leukemia cell lines (HL-60, HEL, THP-1, and K562).

1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with an incidence of over 20,000 cases per year in the United States alone. Large chromosomal translocations and mutations in genes involved in hematopoietic proliferation and differentiation lead to the accumulation of poorly differentiated myeloid cells. AML is a highly heterogeneous disease; although cases can be stratified into favorable, intermediate, and adverse risk groups based on their cytogenetic profile, prognosis within these categories varies significantly [1]. The diagnosis of acute myeloid leukemia (AML) is established through the examination of peripheral blood and bone marrow. A definitive diagnosis is made when leukemic blasts account for more than 20% of bone marrow cells, or when specific cytogenetic abnormalities are identified, such as t (8;21), inv (16), or t (15;17). The assessment of chromosomal and molecular alterations plays a crucial role in risk stratification—distinguishing high-, intermediate-, or low-risk categories—and in monitoring the patient's response to therapy [2].

2. Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a unique subtype of AML (5–10 % of AML), with the first description as a distinct entity in 1957. Patients with APL have a median age between 30–40 years[3]. APL is characterized by a 15;17 chromosome translocation with breakpoints within the retinoic acid alpha receptor (RAR alpha) gene on 17 and the PML gene, which encodes a putative transcription factor, on 15. A PML-RAR alpha fusion protein is formed as a consequence of the translocation [4]. Additional chromosomal aberrations (ACAs) associated with t (15;17) are reported in 23–43 % of APL cases. The clinical impact of these ACAs has not yet been clearly elucidated even if seems to worse the prognosis [56]. Risk stratification is imperative in the treatment of APL patients, as those with low-risk disease (white blood cell

count (WBC) ≤10 000/µl) are generally treated with less intensive regimens than those patients presenting with high-risk disease (WBC >10 000/µl). In the past two decades, therapy for newly diagnosed APL has evolved from an all-*trans* retinoic acid (ATRA)+chemotherapy backbone for all patients to the addition of arsenic trioxide (ATO) to ATRA with omission of chemotherapy in low-risk patients as a new standard of care[7]. Unlike other subtypes of AML, the primary cause of treatment failure in patients with APL is early death, defined as death within the first 30 days of diagnosis and is particularly common in older patients [8,9]. The reasons for early deaths in APL are multiple, although death during induction is most frequently related to the hemorrhagic diathesis because of hyperfibrinolysis, proteolysis and disseminated intravascular coagulation, further complicated by thrombocytopenia [10].

Nowadays treatment for APL is ATRA; tretinoin, the acid form of vitamin A, due to the sensibility of this subtype to this substance in association with traditional chemotherapy. Unlike other chemotherapies, ATRA does not directly kill the malignant cells. ATRA induces the terminal differentiation of the leukemic promyelocytes, after which these differentiated malignant cells undergo spontaneous apoptosis on their own. ATRA alone is capable of inducing remission, but it is short-lived in the absence of concurrent "traditional" chemotherapy [11]. Actual treatments (ATRA + chemotherapy) allow patients to complete remission with 5 years Relapse-Free Survival in 83.8% of cases [12]. This means that good therapeutic options are available, but still new therapeutic targets are needed for relapsing or therapy resisting patients.

3. Chronic myeloid leukemia

The first descriptions of chronic myeloid leukemia (CML), or as it was called at the beginning, chronic granulocytic leukemia, can be dated back to 1845. At that time, almost simultaneously, two independent pathologists, John Bennett and Rudolf Virchow, published case reports of patients with splenomegaly, an enlarged liver and leukocytosis [13]. However, only 100 years later new insight into the disease was gained. Only in 1960 did Peter Nowell and David Hungerford, after improving a method of visualizing chromosomes in mitotic cells (karyotyping), report the identification of an abnormal “minute chromosome” in patients with chronic granulocytic leukemia, representing the first discovery of a link between chromosomes and cancer. This chromosome later became known as the “Philadelphia (Ph) chromosome” [14]. In 1973, Janet Rowley, while using chromosomal staining methods such as quinacrine fluorescence and Giemsa banding, observed that the minute chromosome was the result of

the reciprocal translocation between chromosomes 9 and 22 (t (9;22))[15].

CML belongs to the group of myeloproliferative neoplasms (MPN) characterized by the uncontrolled growth of myeloid cells at different stages of maturation. Patients may present in three disease phases: chronic phase (CP), accelerated phase (AP) and blast phase (BP) or blast crisis (BC), which is characterized by an increasing percentage of blasts of the myeloid, lymphoid or mixed/undifferentiated lineage, with myeloid BC occurring at 70%, approximately twice as frequently as lymphoid BC. According to criteria by the World Health Organization, 10–19% blasts in peripheral blood or bone marrow are counted as AP, while > 20% blasts are considered a criterion for BC [16].

Three main signaling pathways have been linked to the of BCR-ABL1: signal transducer and activator of transcription 5 (Stat5), Ras/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT mediated signaling [17], in particular the activation of Ras/MAPK signaling has been implicated in BCR-ABL1-dependent cellular transformation [18].

Treatment for CML are basically tyrosine kinase inhibitors, the most famous is imatinib. In a 10-year follow-up trial of CML patients on imatinib, it was shown that 83% achieved a complete cytogenetic response [19]. While the success of targeted therapy of CML with imatinib was unprecedented, complications in the form of primary and secondary BCR-ABL1-dependent and -independent resistance to imatinib arose. BCR-ABL1-independent mechanisms of resistance refer to factors such as the bioavailability of the drug, e.g., influx or efflux into or out of the cells, distribution and catabolism or alternative signaling pathways. BCR-ABL1-dependent mechanisms comprise increased expression of BCR-ABL1 or mutations in the *BCR-ABL1* kinase domain [20]. Accordingly, new, improved, second and third generation TKIs, such as nilotinib, dasatinib and bosutinib, were developed. Of the newer agents, the third line TKI ponatinib was specifically developed to target the *BCR-ABL1*^{T315I} and compound mutations, while asciminib (ABL001), an allosteric inhibitor of the ABL-kinase, exerts its effect by binding to the myristoyl and not the catalytic pocket of BCR-ABL1 [21 22 23 24 25]. Nowadays with the current treatments, the survivability of CML is 80.8%, showing an increase of 40.4% after TKI treatments started [26]. Showing that even in this case new therapeutic targets are needed for the minority of patients not responding to present one.

4. Diacylglycerol kinases

Diacylglycerol kinases (DGKs) constitute a family of different enzymes. The human genome

encodes ten DGK isozymes, which are often co-expressed within the same cell type, indicating distinct, non-redundant functions. DGKs are categorized into five subfamilies based on their unique regulatory domains, typically located in the N-terminal region preceding a conserved catalytic domain. This domain includes an ATP-binding site and associated accessory subdomain and is generally preceded by at least two C1 domains homologous to the DAG-binding region of protein kinase C (PKC).

- Class I DGKs (DGKA, DGKB, and DGKG) possess two EF-hand and a recoverin homology domain, conferring calcium sensitivity.
- Class II DGKs (DGKD, DGKH, and DGKK) contain a phosphoinositide-binding PH domain and, except for DGKK, a C-terminal sterile alpha motif involved in protein–protein interactions.
- Class III, represented solely by DGKE, features an amphipathic helix that promotes membrane association and confers substrate specificity for polyunsaturated fatty acids at the sn-2 position.
- Class IV DGKs (DGKZ and DGKI) are characterized by ankyrin repeats implicated in protein–protein interactions.
- Class V includes DGKQ, which contains three C1 domains and a Ras-association domain, the function of which remains unclear [27].

5. DGKA and DGKZ in tumors

In this thesis we will focus on DGKA and DGKZ role in tumorigenesis. The activity of several DGK isoforms has been implicated in both cell transformation and immunosurveillance against tumors [28] and there are also strong evidence supporting the involvement of DGKA activity in the development of glioblastoma [2930], melanoma [31] and hepatocellular carcinoma (Takeishi et al., 2012).

Accordingly, DGKA transduces the proliferative effects of growth factors [33], and oncogenes such as NPL–ALK [34]. Furthermore, depending on the cellular model, other DGK isoforms may be involved in tumorigenesis, as reviewed in [35]. Finally, DGKA and DGKZ act as negative regulators of the immune response by depleting diacylglycerol levels, thereby suppressing the anticancer activity of lymphocytes and natural killer cells [36]. Consistently, targeting DGKA restores impaired T cell receptor (TCR) signaling strength in experimental models of X-linked lymphoproliferative disease 1 and Wiskott–Aldrich syndrome [37].

Since the emerging role of diacylglycerol kinases (DGKs) in tumorigenesis, recent studies have investigated their involvement in the pathogenesis of acute myeloid leukemia (AML). In particular, through employing of the GEPIA2 multiple gene analysis tool to examine the expression of all ten known DGK isoforms. By comparing AML samples from the TCGA database with normal bone marrow tissues from both TCGA and GTEx datasets, a distinct overexpression pattern of several DGKs in AML was identified. Specifically, significant upregulation was observed for DGKα and DGKG (type I), DGKD (type II), DGKE (type III), and DGKZ (type IV), suggesting a potential contribution to leukemic transformation. Functional experiments further supported this hypothesis. AML cell lines, including HL-60 and HEL, were treated with different DGKα inhibitors, R59022, R59949 (non-specific), and CU-3, AMB639752 (DGKα-specific). Among these, R59022 and R59949 demonstrated marked cytotoxic activity, whereas CU-3 and AMB639752 showed limited efficacy, indicating that the therapeutic impact may vary depending on the inhibitor's specificity and cellular context [38].

A more detailed investigation into DGKα revealed its clinical and biological relevance. High DGKα expression was demonstrated to positively correlated with CD34 and was associated with significantly poorer patient prognosis. Furthermore, treatment of Kasumi-1 and KG-1α, other AML cell lines, with ritanserin, a selective DGKα inhibitor, resulted in a dose- and time-dependent reduction in cell proliferation, highlighting its potential as a therapeutic target in AML[40]. The involvement of DGKα extends beyond AML to chronic myeloid leukemia (CML) as well. In K562 cells, DGKα knockdown was found to reduce cell proliferation and induce G0/G1 phase arrest, suggesting a role in CML progression, possibly mediated by nuclear functions associated with cell cycle regulation[41].

In parallel, the role of DGKζ has also been explored, though a lentiviral-mediated knockdown of DGKZ in HL-60 cells induced apoptosis, suppressed proliferation, and caused cell cycle arrest at the G2/M phase. These effects were mechanistically linked to the activation of the MAPK/survivin/caspase signaling cascade, with upregulation of MAPK, caspase-3, caspase-8, and cytochrome c, alongside downregulation of p-MAPK and survivin [43].

Altogether, these studies establish the relevance of DGKs, particularly DGKα and DGKζ, as potential therapeutic targets in both AML and CML, laying the foundation for future translational strategies aimed at inhibiting specific DGK isoforms in hematologic malignancies.

6. Ritanserin

Since DGKs play a role in oncogenesis in AML, the identification of DGK inhibitors becomes crucial for research purposes. Among the available inhibitors, ritanserin, a specific DGKα inhibitor, was selected due to its established safety profile, as supported by clinical studies that documented its pharmacological properties in patients, although these studies originally evaluated it as a serotonin receptor antagonist [39]. Ritanserin has also been evaluated in vivo, where NSG mice were administered the inhibitor (5 mg/kg/day) to assess its efficacy. The treatment significantly reduced AML-associated splenomegaly compared to the vehicle control group (0.3% sodium carboxymethylcellulose), without inducing lethal effects [40]. Structural studies support the existence of a contiguous ligand-binding site in DGKα, comprising the C1, DAGKc, and DAGKa domains within the active site, for this class of inhibitors [42].

Ritanserin cytotoxicity experiments were conducted by the group of work on various leukemic cell lines, like: THP-1, HL-60 and HEL as well as K562. Effects on cell viability were evaluated using the alamarBlue assay after exposure to drug concentrations ranging from 12.5 to 100 μM, then based on cell viability, dose-response curves at 24 and 72 h have been made and the IC50 values for each cell line were calculated:

- THP-1 cells were the most sensitive to ritanserin (37.0 ± 1.4 μM after 24 h and 32.0 ± 1.5 μM after 72 h)
- HL-60 IC50 were (37.6 ± 1.5 μM after 24 h and 25.3 ± 0.9 μM after 72 h)
- HEL IC50 were (49.2 ± 2 μM after 24 h and 29.6 ± 1 μM after 72 h)
- K562 cells were the less sensitive (70.4 ± 2.2 μM after 24 h and 42.5 ± 1.8 μM after 72 h)
- PBLs showed a near complete cell death at 50 μM at both 24 and 72h.

[44]

Moreover, Ritanserin is also known to act as a serotonin receptor antagonist. It was initially characterized as a selective antagonist of 5-HT2A (Ki = 0.45 nM) and 5-HT2C (Ki = 0.71 nM) receptors [45] and has undergone clinical trials for the treatment of schizophrenia and other psychiatric disorders, including obsessive-compulsive disorder, acute mania, and substance use disorders, thus it's not the inhibition of 5HTs receptor the mode of action that drive cytotoxicity [46] [44].

7. DGKZ-IN-4

DGKZ-IN-4 in the patent WO2024165470A1, is highlighted for its potent and selective inhibition of DGKζ. The compound operates by preventing the phosphorylation of diacylglycerol (DAG) into phosphatidic acid, thereby sustaining DAG-mediated signaling downstream of T-cell receptors. This leads to enhanced activation of key immune effector functions, including increased cytokine production (e.g., IFN-γ), proliferation, and cytotoxic granule release from $CD8^+$ T cells and NK cells.

In vivo murine studies demonstrated that DGKZ-IN-4 significantly improved tumor clearance and delayed tumor progression when administered alone or in combination with checkpoint inhibitors. Structurally, the compound belongs to a broader family of substituted aminoquinolones characterized by specific interactions with conserved domains in the DGKζ catalytic site, particularly the DAGKc and DAGKα domains [47].

When tested as described for ritanserin, DGKZ-IN-4 IC50 were as follows:

- HL-60 cells (21.2 ± 1.0 μM after 24 h and 19.9 ± 1.3 μM after 72 h)
- THP-1 cells (31.3 ± 2.0 μM after 24 h and 12.4 ± 0.3 μM after 72 h)
- HEL cells (50.7 ± 5.7 μM after 24 h and 20.7 ± 1.6 μM after 72 h)
- K562 cells showed no detectable cytotoxic effects within the tested concentration range after 24 h, and a mild sensitivity after prolonged exposure (32.9 ± 6.9 μM after 72 h)
- PBLs cells (28.7 ± 3.3 μM after 24h and 15.1 ± 1.8 μM after 72h)

[44]

8. BAY 2965501

BAY 2965501 is a highly selective, cross-species inhibitor of diacylglycerol kinase zeta (DGKζ), effective in both human and mouse models, developed by Bayer AG in collaboration with the German Cancer Research Center (DKFZ). It enhances NK and T-cell-mediated cytotoxicity, and reverses immunosuppression induced by TGF-β, PGE_2.

Even if BAY 2965501 does not directly affect tumor cell proliferation, it significantly boosts the anti-tumor function of T cells expressing tumor-reactive TCRs. Single-cell sequencing identified elevated DGKζ in exhausted $CD8^+$ T-cell clonotypes, indicating its role in immune dysfunction within tumors.

In vivo, BAY 2965501 reduces markers such as PD-1 and TIM-3 and improves antiviral T-cell responses in chronic LCMV infection models. It also reduces tumor growth in multiple syngeneic mouse models (MB49, F9, Hepa129), and shows synergistic effects when combined with anti-PD-L1 therapy [48].

BAY 2965501 IC50 were measured by the work group, as before:

- HL-60 cells (40.7 ± 4.4 μM after 24 h and 34.1 ± 3.0 μM after 72 h)
- THP-1 cells (67.0 ± 3.5 μM after 24 h and 38.0 ± 3.0 μM after 72 h)
- HEL cells (92.0 ± 5.0 μM after 24 h and 58.1 ± 5.8 μM after 72 h)
- K562 cells showed no detectable cytotoxic effects within the tested concentration range after 24 h, and a faint sensitivity after prolonged exposure (100 ± 3.2 μM after 72 h)
- PBLs cells (77.1 ± 4.8 μM after 24h and 35.2 ± 0.7 μM after 72h) [44]

THE OBJECTIVE OF THE THESIS

This thesis objective is to understand the role of DGKα and DGKζ in AML and CML cell lines, using as a model of those two disease HL-60 and THP-1 cell lines. Their relevance was studied through siRNA-mediated silencing, viability assay, apoptosis assay and p-ERK1/2 quantification. This thesis is a part of a larger project in which other cell lines where silenced (HEL and THP-1) and these four cell lines were also treated with specifical DGKα and DGKζ chemical inhibitors.

MATERIALS AND METHODS

REAGENT	**CONCENTRATION**	**CAT. NUMBER**	**PROVIDER**
RPMI-GlutaMAX 1640 Medium	-	61870-010	Thermo Fisher
Fetal bovine serum	-	A5256701	Thermo Fisher
Penicillin and Streptomycin	-	A5955	Sigma-Aldrich
rhIL-2	-	-	PeproTech
Trypan blue	0,4%	15250-061	Thermo Fisher
AlamarBlue	1,5 mg/mL in PBS	A50100	Invitrogen

Phosphate buffered saline	-	P4417	SIGMA
Triton X-100	-	04744977001	Roche Diagnostic
Dimethyl sulfoxide	1%	D2650	Sigma-Aldrich
Ritanserin	-	1955	Tocris
BAY 2965501	-	2732902-08-6	MedChemExpress
DGKζ-IN-4	-	HY-156574	MedChemExpress
Annexin V-FITC	-	BMS500FI-300	Invitrogen
7AAD	-	A9400-1MG	Sigma
DGKα Rabbit PolyAb	1 mg/mL	11547-1-AP	Proteintech
Rabbit Anti-DGKζ pAb	1 mg/mL	AB105195	Abcam
β-actin Monoclonal Antibody	1 mg/mL	MA1-140	Invitrogen
p44/42 MAPK (Erk1/2)	1 mg/mL	9102S	Cell signalling technology
Phospho-p44/42 MAPK (Erk1/2)	1 mg/mL	9101S	Cell signalling technology
Rabbit Anti-Mouse IgG	1 mg/mL	A16160	Invitrogen
Goat Anti-Rabbit IgG	1 mg/mL	A16096	Thermo Fisher
PageRuler prestained protein ladder	-	26616	Thermo Fisher
High-Capacity cDNA Reverse Transcription Kit	-	4368814	Thermo Fisher
TaqMan™ Fast Advanced Master Mix	-	4444556	Thermo Fisher

Table 1: used reagents table.

siRNA

NAME	TYPE	PRODUCER	CATALOG	ID	SEQ. FORWARD	SEQ. REVERSE
siRNA DGKZ	Custom siRNA	Ambion	AM16104	ACFARCW	GCCGCUUUCGGAAUAAGAUTT	AUCUUAUUCCGAAAGCGGCTG
siRNA1 DGKA	Custom siRNA	Invitrogen	10620310		AUAUUUAGCCAUCUCGCCAUCCUCG	CGAGGAUGGCGAGAUGGCUAAAUAU
siRNA2 DGKA	Custom siRNA	Invitrogen	10620310	494910C11	CGAGGAUGGCGAGAUGGCUAAAUA	AUAUUUAGCCAUCUCGCCAUCCUCG
siRNA cntrl (negative control)	Silencer select	Invitrogen	465373			

Table 2: used siRNA table.

Cell culture

The cells used for the experiments were four types of different leukemia cell lines, moreover also PBLs were used. The HL-60 cell line was derived in 1976 from the peripheral blood of a 36-year-old woman diagnosed with acute promyelocytic leukemia [49]. The HEL cell line originated in 1980 from the peripheral blood of a 30-year-old man experiencing a relapse of erythroleukemia following treatment for Hodgkin lymphoma [50]. The THP-1 cell line was established from the peripheral blood of a 1-year-old male patient with acute monocytic leukemia [51]. The K562 cell line was derived from the pleural effusion of a 53-year-old woman in the terminal blast crisis phase of chronic myelogenous leukemia [52]. These cell lines were

employed as in vitro models of acute myeloid leukemia, and PBLs as model of healthy patient. The Cell Line Authentication Test (Eurofins Genomics, Ebersberg, Germany and university internal service) confirmed the identity of cell lines, the resulting markers were 100% consistent with those in the ATCC database. The cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin following ATCC handling information.

UPObiobank, the University biobank provided healthy donor blood samples within the framework of ethical committee authorization (codice autorizzazione comitato etico: parere etico interaziendale AOU Maggiore della carità CE206/2022 sul Progetto MTSGB) with informed consent for research. Peripheral blood mononucleated cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation, washed, and resuspended at 2 x 10^6 cells/mL in RPMI-1640 medium containing 10% heat-inactivated FBS. PBLs (T lymphocytes) were selected by the treatment with 1 µg/mL anti-CD3 (clone OKT3) and anti-CD28 (clone CD28.2) human antibodies for 72 h. Activated T cells were then cultured in complete medium supplemented with 100 IU/mL rhIL-2 (PeproTech) at 0.5 x 10^6 cells/mL.

HL-60, THP-1 and K562 cell lines were cultured in RPMI medium, supplemented with 10% FBS and 1% penicillin and streptomycin at different concentration depending on the cell line utilized. PBLs were cultured in the same medium with the exception of a supplement of IL-2.

Trypan Blue assays

The Trypan blue assay was performed on transfected HL-60, THP-1 and K562 cells to assess cell viability. The trypan blue reagent was added to samples (1:1 v/v) and the number of live/dead cells was automatically recorded by the Automatic counter TC20 (Invitrogen). Each experiment was performed in three technical replicates. The results are reported as the number of total live/dead cells of a representative experiment and as percentage of cell viability of three biological replicates. All data were graphed and analyzed using GraphPad Prism 8 through one-way ANOVA and unpaired t-test.

Transfection

In order to silence DGKA and DGKZ, HL-60 and K562 cells were electroporated with 80nM of siRNA (table 2) using Neon™ 100µL Kit (MPK10025, Invitrogen) with Neon™ electroporator and the following conditions (table 3).

	CONDITION	N° OF CELLS	VITALITY (%)	EFFICIENCY (%)
HL-60	1300V, 35ms, 1P	500.000		
HEL	1350V, 20ms, 2P	760.000	38%	100%
THP-1	1550V, 15ms, 2P	750.000	89%	81%
K562	1350V, 10ms, 4P	1.500.000	87%	71%

Table 3: electroporation conditions.

After siRNA transfection, cells were resuspended in RPMI-1640 medium with 10% heat inactivated FBS without streptomycin and penicillin and incubated for 72h at 37°C and 5% CO_2. Following, silencing efficiency was evaluated for each experiment by RT-PCR and Western blotting and cells were used for viability experiments.

Electroporation protocols were established using BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control, to find the best condition in terms of viability and efficiency of transfection. Samples transfected with BLOCK-iT were analyzed using by "BD FACS Symphony A5" using BD FACSDiva software. Data were gated based on FSC-A vs. FSC-H to exclude cell doublets and FSC-A vs. SSC-A to exclude debris.

Electroporation protocol setting on K562 cell line.

Since there were no pre-existing protocol for the K562 cell line electroporation, we used BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control to establish a new one, that could combine both efficiency and viability of the samples.

The electroporated cells were analyzed by flow cytometry (BD FACSymphony™ A3) with the BD FACS Diva software, data were gated based on FSC-A vs. FSC-H to exclude cell doublets and FSC-A vs. SSC-A to exclude debris and population were identified as positive or negative to the BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control.

Different combination of number of pulses (P), width of the pulse (ms), number of cells (N°) and Volt (V) of the pulse. Finally, the conditions that better fits our purpose were 1550V, 15ms, 2 pulse with 1.500.000 cells, which showed a vitality of 89% and an efficiency of 81% (relative to control) (table 3) (figure 1).

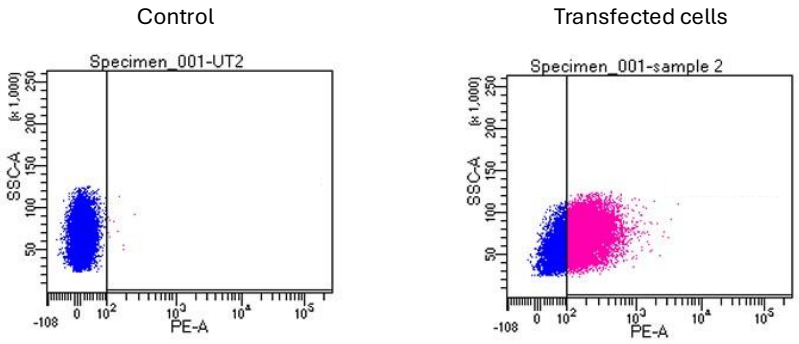

Figure 1: ***efficiency of electroporation protocol on K562 cell line.***

Cells were electroporated with BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control and analyzed by flow cytometry to quantify the percentage of red fluorescent cells at 72h.

Reverse Transcription Polymerase Chain Reaction Assay

Transfected cells were lysed in 200µL of nuclease-free water supplemented with 2% thioglycerol. Total RNA was extracted from lysates by Kingfisher automated nucleic acid extractor (ThermoFisher) with "MVP_2WASH_200_FLEX" protocol and "MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit" (ThermoFisher) and quantified using NanoDrop (ThermoFisher). Then, equal amounts of RNA were retrotranscribed into cDNA using "High-Capacity cDNA Reverse Transcription Kit" (ThermoFisher) following the manufacturer's instructions. The relative expression of DGK isoforms (DGKA Assay ID Hs00176278_m1, DGKZ Assay ID Hs05025727_m1) was assessed by quantitative real-time PCR (qRT-PCR) with TaqMan technology. Glucuronidase beta (GUSB Assay ID Hs00939627_m1) was used as an internal reference gene.

To perform qRT-PCR, a 384-well plate was prepared following the protocol provided by the TaqMan™ Fast Advanced Master Mix kit (Thermo Fisher) with 4µL of mix and 1µL of cDNA and amplified with Bio-Rad CFX384™ real-time system. Amplification protocol: initial denaturation at 95°C for 20s, followed by 50 cycles of denaturation at 95°C for 3s and annealing/extension at 60°C for 30s. The qPCR reactions were performed in technical triplicates.

The data were analyzed with Bio-Rad CFX Maestro software using GUSB as reference and normalizing DGKA and DGKZ expression to the relative control (siRNA control).

Apoptosis and necrosis assessment

Apoptosis was evaluated with the AnnexinV-FITC kit (Invitrogen) according to the manufacturer's instructions except using 7AAD instead of propidium iodide. Analysis was performed by flow cytometry (BD FACSymphony™ A3) with the BD FACS Diva software.

Data were gated based on FSC-A vs. FSC-H to exclude cell doublets and FSC-A vs. SSC-A to exclude debris. Different populations were identified as: live (Annexin V- and 7AAD-), necrotic (Annexin- and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+). Data are shown as the mean ± SD of at least 3 independent experiments analyzed by GraphPad Prism 10.

Western blot

Cells were lysed with lysis buffer (HEPES 25 mM, NaCl 150 mM, EDTA 5 mM EGTA 1 mM NP40 1%, Glycerol 10%) sodium orthovanadate (1mM, ThermoFisher) and protease inhibitor cocktail (0.0089 mg/mL, Sigma Aldrich) mix. The samples were incubated for 15 minutes at 4°C, under gentle, constant shaking, and then centrifuged at 12,000 × g for 15 min at 4°C. The concentrations of proteins in lysates were quantified by Qubit protein br assay kit (A50669, ThermoFisher) following manufacturer's protocol.

Equal amounts of proteins were separated on SDS–PAGE gels and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked by incubating with 3% (w/v) BSA for 1 hour at room temperature and subjected to overnight incubation with respective primary antibodies at 4°C with gentle agitation. The following day, membranes were washed 3 times in TBS-T buffer. They were then incubated with alkaline HRP-conjugated rabbit or mouse secondary antibody (1:5000) for 1 hour at room temperature with gentle agitation. After 3 additional washes with TBS-T, the membranes were developed using the Western Chemiluminescence Substrate (PerkinElmer, USA) using the ChemiDoc™ Imaging System (Bio-Rad). Band volumes were analyzed by densitometry using Bio-Rad Image Lab 6.1 software and normalized on their respective β-actin or total ERK, and subsequently on their respective siRNA cntrls.

Finally, results were analyzed and graphed using GraphPad Prism 8 with one-way ANOVA and unpaired T-test.

RESULTS

1. Role of DGKα and DGKζ on HL-60 viability

DGKζ silencing significantly decreased HL-60 cells' proliferation blocking cell cycle at the G2/M phase and leading to apoptosis (Li et al., 2019). Thus, to investigate the specific relevance of DGKα and ζ isoforms, we knocked down DGKA and DGKZ in HL-60 cells through siRNA. After 72h, we quantify mRNA and protein levels and we observed that DGKA mRNA was reduced by approximately 40% with siRNA1 and 20% with siRNA2, while DGKα protein expression decreased by about 50% with both siRNAs (figure 2A). Concerning DGKZ, we obtained an mRNA reduction of approximately 55% (figure 2C).

To evaluate the biological effects of reducing DGKα and DGKζ protein on HL-60 cells viability, we used a Trypan Blue assay at 72h after transfection. As shown in figure 2B left, reporting the mean live cells percentage from four experiments, the viability is reduced by about 20% in HL-60 cells transfected with DGKA siRNA1 but not affected by siRNA2. On the right in the same panel, the absolute cell count of a representative experiment indicates that both control and DGKA-silenced conditions exhibit a net increase in cell number relative to the initial seeding density (dotted line). However, a modest non-significant reduction in proliferative capacity is observed in cells transfected with siRNA1 DGKA and no differences with siRNA2.

In contrast, upon DGKZ knockdown we observed (Figure 2D, left) a statistically significant reduction in mean live cells percentage of seven experiments by about 37%. The absolute cell number of a representative experiment (Figure 2D, right) shows that only control cells display a net increase relative to the initial seeding density (dotted line), whereas DGKZ-silenced cells remain approximately unchanged in number, indicating a block of proliferation or increased apoptosis (figure 2D, left).

We further investigated the eventual induction of cell death upon DGKZ silencing performing an apoptosis/necrosis assay. As shown in figure 2E, DGKZ silencing elevated total cell death from 2.2% to 3.8%. This increase was primarily attributable to late apoptosis ($P < 0.05$), with minimal contributions from early apoptosis, whereas necrosis is negligible in both siRNA control and DGKZ silenced cells.

Collectively, these results indicate that in HL-60 cells, DGKζ plays a critical role in sustaining cell viability, and its downregulation promotes a strong cytostatic effect and apoptotic cell death. In contrast, DGKα appears to be dispensable for viability under the conditions tested.

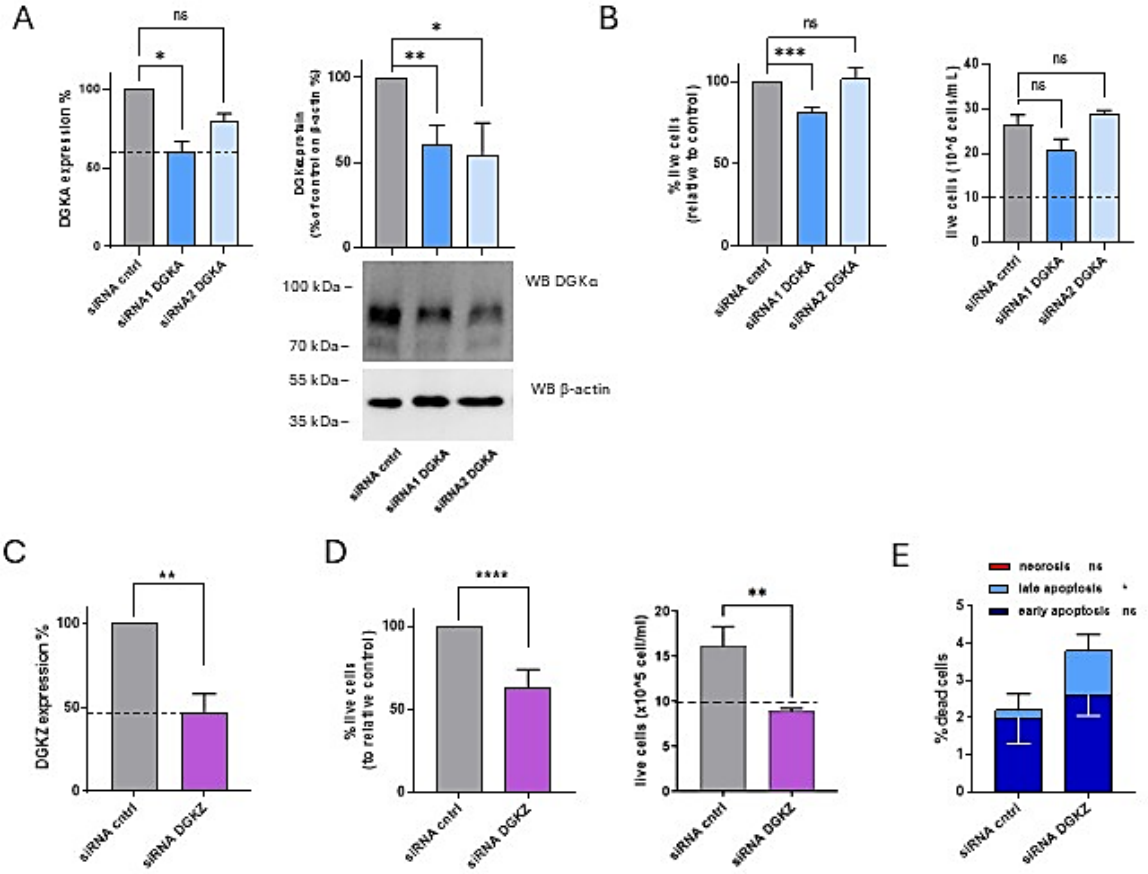

Figure 1: ***Role of DGKA and DGKZ on HL-60 viability***

HL-60 cells were transfected with the indicated siRNA and assessed after 72h.

A. Mean ± SEM of three independent experiments showing the silencing of DGKA on HL-60 cells. Data are presented as percentage of DGKA mRNA expression (left) or DGKA protein (right) in comparison with siRNA cntrl transfected cells. Lower right, a representative western blot for DGKA and β-actin protein.

B. Trypan Blue assay. Mean ± SEM of four independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from the 10*10^5 cells/ml seeded.

C. Mean ± SEM of three independent experiments showing the silencing of DGKZ on HL-60 cells. Data are presented as percentage of DGKZ mRNA expression in comparison with siRNA cntrl transfected cells.

D. Trypan Blue assay. Mean ± SEM of seven independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from the 10*10^5 cells/ml seeded.

E. Annexin V-7AAD assay. Data are the mean ± SD of five independent experiments presenting the percentage of dead cells and the type of cell death.

Two-way ANOVA or unpaired T test versus siRNA cntrl: $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** and $p<0.0001$ ****.

2. Role of DGKα and DGKζ on K562 viability

We later examined the distinct functions of the DGKα and ζ isoforms in K562 cells following the same methodology of HL-60 silencing, but with a different electroporation protocol established by the candidate.

72 hours after the transfection, DGKA mRNA levels were reduced by 20% with siRNA1 and 45% with siRNA2, while DGKα protein expression decreased by 60% and 70%, respectively (Figure 3A). For DGKZ, we observed a reduction of 60% in mRNA expression and a decrease of 70% in protein levels (Figure 3C).

As illustrated in Figure 3B (left panel), the percentage of viable cells remained unchanged across three independent experiments in K562 cells transfected with either siRNA, compared to control cells. The right panel of Figure 3B presents the absolute cell counts, demonstrating that both control and DGKA-silenced cells exhibited an overall increase in cell number relative to the initial seeding density (1*10^6 cells/ml), indicated by the dotted line.

Likewise, after DGKZ knockdown we observed similar viability of the silenced cells as the control one (Figure 3D, left). The right panel of the same figure presents absolute cell counts, showing that both control and DGKZ-silenced cells exhibited a relevant increase in cell number relative to the initial seeding density (showed by the dotted line and the same of DGKA silencing).

Thus, in fast-growing K562 cells, decreasing DGKα and DGKζ protein levels do not result in a reduction of cell viability. This suggests a non-essential or redundant role in cell viability of those two isoforms in this specific cell line.

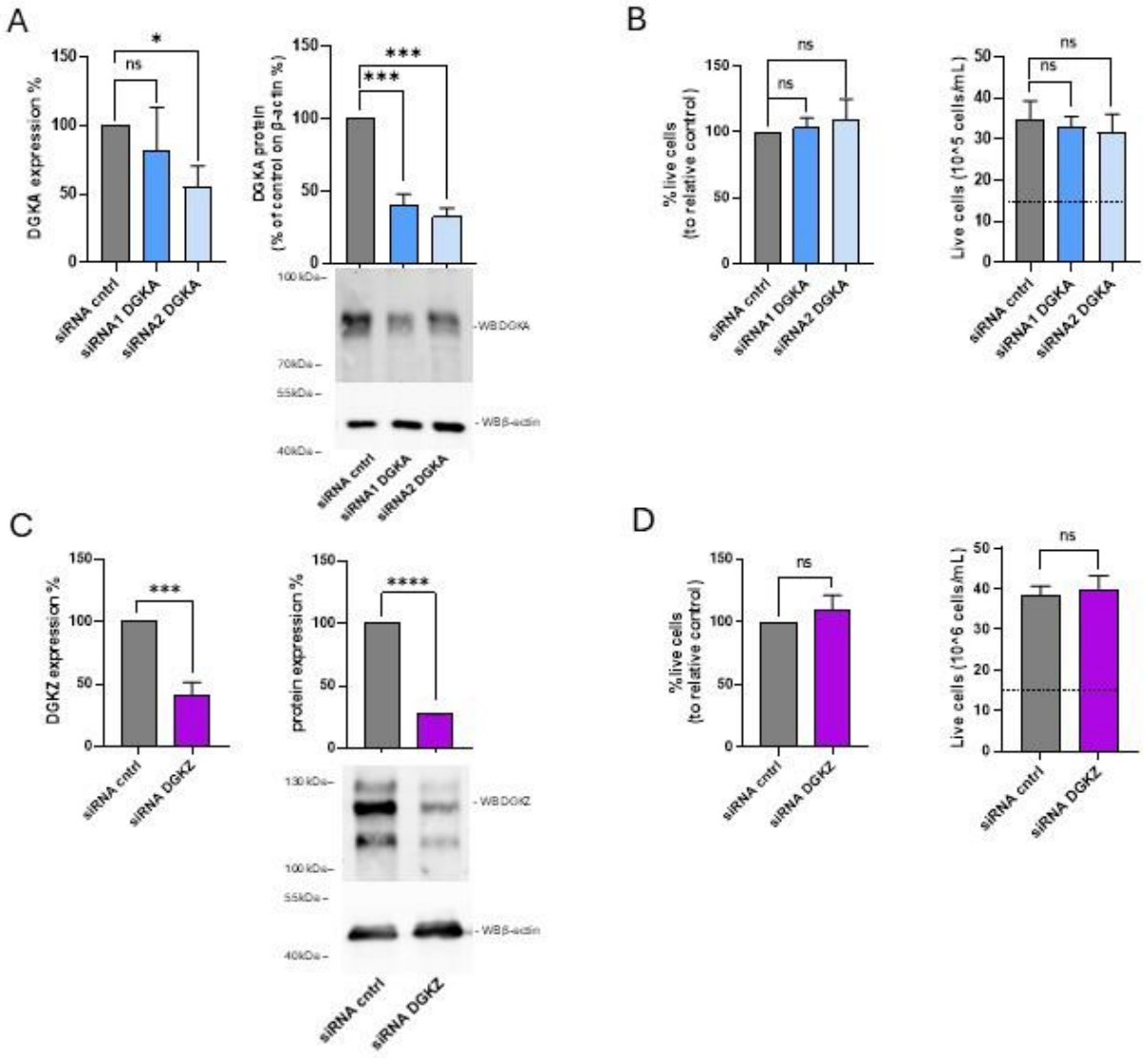

Figure 2: ***Role of DGKA and DGKZ on K562 viability***

K562 cells were transfected with the indicated siRNA and assessed after 72h.

A. Mean ± SEM of three independent experiments showing the silencing of DGKA on K562 cells. Data are presented as percentage of DGKA mRNA expression (left) or DGKA protein (right) in comparison with siRNA cntrl transfected cells. Lower right, a representative wester blot for DGKA and β-actin protein.
B. Trypan Blue assay. Mean ± SEM of three independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from 15*10^5 cells/ml seeded.
C. Mean ± SEM of three independent experiments showing the silencing of DGKZ on K562 cells. Data are presented as percentage of DGKZ mRNA expression (left) or DGKZ protein (right) in comparison with siRNA cntrl transfected cells. Lower right, a representative wester blot for DGKZ and β-actin protein.
D. Trypan Blue assay. Mean ± SEM of three independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from 15*10^5 cells/ml seeded.

Two-way ANOVA or unpaired T test versus siRNA cntrl: p<0.05 *, p<0.01 **, p<0.001 *** and p<0.0001 ****.

3. DGKs silencing drive to a modification of ERK1/2 phosphorylation

We analyzed ERK signaling by Western blotting in DGK-silenced AML cell lines (HL-60, HEL, K562 and THP-1) targeting p-ERK and ERK1/2 as control, finding that DGKs has an important role in the signalling cascade, but each DGK isoform act differently in every cell line, indicating that the effect is not directly dependent on basal expression levels of the target isoform in each cell line.

In detail, in HL-60 the cascade shows an increased level of p-ERK1/2 in DGKA silenced cells with both siRNA anti DGKA, suggesting that other isoform can affect the cascade differently (Figure 4B). In HEL cell line, both siRNA anti-DGKA showed an overall decrease in p-ERK1/2 expression, with respectively 40 and 50% less expression, while the DGKZ siRNA showed an increase of 10% (Figure 4C). K562, meanwhile, showed no statistically significant reduction or increase in p-ERK1/2 but a tendency to an increase of p-ERK in the silenced isoforms (Figure 4D). In THP-1 cell line p-ERK1/2 expression decreased with both DGKA siRNA by respectively 50 and 40%, while with DGKZ siRNA the protein expression remains unchanged (Figure 4A).

Those finding suggest that MAPK signalling cascade may be affected by DGKs silencing, were each DGK isoform may play a crucial role in each different cell line without correlation on the expression of the isoform itself.

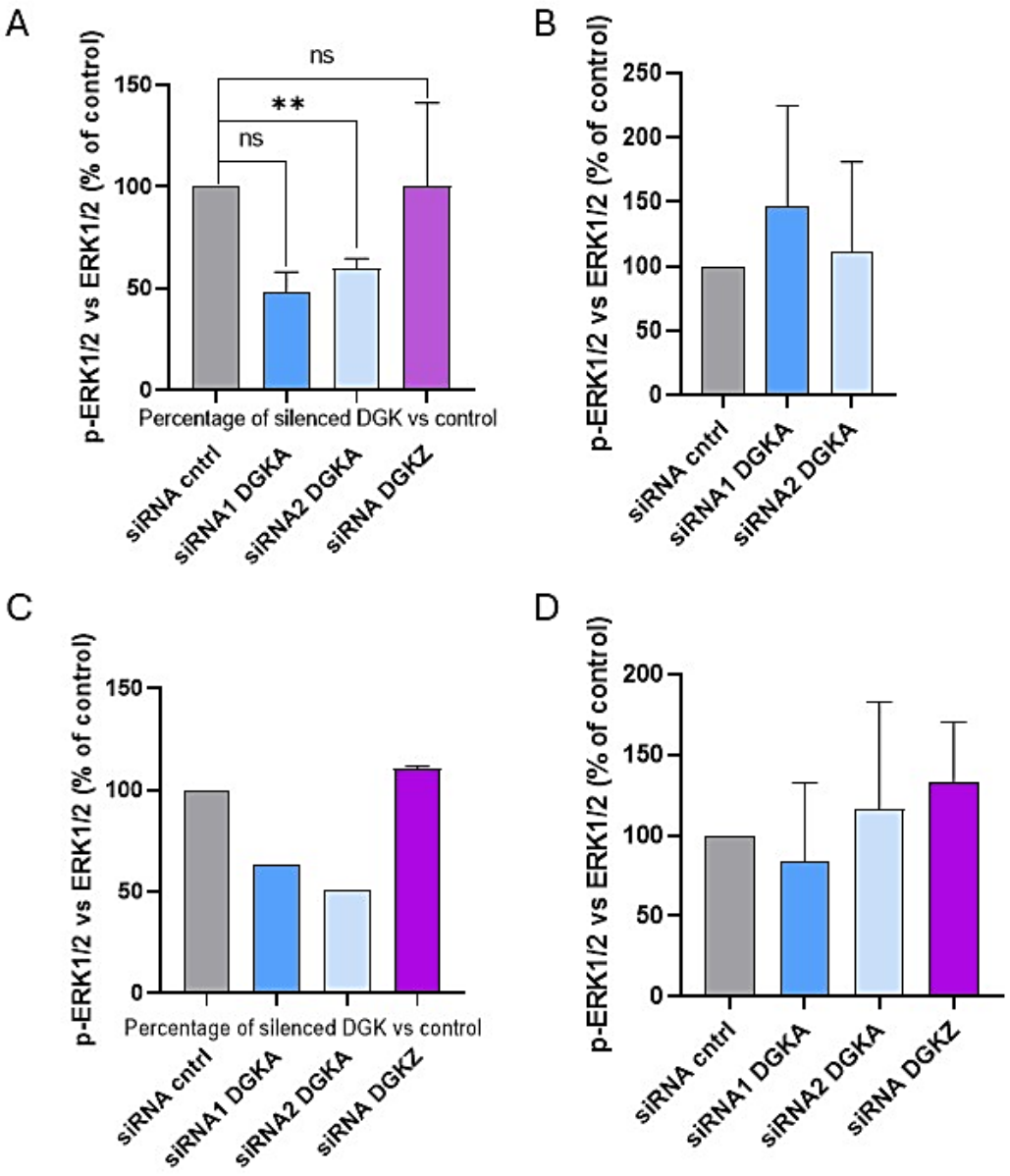

Figure 3: ***DGKA and DGKZ silencing affect ERK1/2 phosphorylation.***

AML cell lines were transfected with the indicated siRNA and assessed after 72h for ERK1/2 phosphorylation by WB with p-ERK1/2 and ERK1/2 antibodies. The percentage of DGKA or DGKZ silencing in the same experimental setting is also indicated. Data are presented as percentage of p-ERK1/2 expression in comparison with siRNA cntrl transfected cells.

A. Mean ± SEM of four independent experiments in THP-1 cells.

B. Mean ± SEM of three independent experiments in HL-60 cells.

C. Mean ± SEM of two independent experiments in HEL cells silenced with siRNA DGKZ and a single experiment with siRNA1 DGKA and siRNA2 DGKA.

D. Mean ± SEM of three independent experiments in K562 cells.

The significance is assessed through paired two-way ANOVA versus siRNA cntrl: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***.

4. Correlation between DGK isoform expression and sensitivity to isoform-specific inhibitors

To understand if target expression was correlated with isoform-specific silencing, I correlated the half-maximal inhibitory concentration (IC50) of drug targeting either DGKA (ritanserin) or DGKZ (DGKZ-IN4 and BAY 2965501) with expression of the target in AML cell lines and of peripheral blood lymphocytes (PBLs).

Panel A shows a weak correlation between DGKA protein expression and ritanserin IC50 at both 24h (R^2 = 0.1557) and 72h (R^2 = 0.0021), suggesting that DGKA protein expression does not predict sensitivity to ritanserin. Panel D demonstrates a similarly weak correlation when DGKA mRNA levels are compared with ritanserin IC50 (24h R^2 = 0.0151; 72h R^2 = 0.0539), reinforcing the notion of limited predictive value of DGKA expression for ritanserin response.

Panels B and E display the correlations between DGKZ expression and sensitivity to DGKZ-IN-4. Protein expression of DGKZ (panel B) shows a moderate inverse correlation with DGKZ-IN-4 IC50 at 24h (R^2 = 0.3064), and no correlation at 72h (R^2 = 0.0149). Moreover, DGKZ mRNA levels (panel E) exhibit a stronger negative correlation at both time points (24h R^2 = 0.04248; 72h R^2 = 0.7963), indicating that sensitivity to DGKZ-IN-4 does not require high expression of DGKZ.

Finally, panels C and F show the correlation of DGKZ expression with sensitivity to BAY 2965501. At the protein level (panel C), DGKZ expression is strongly inversely correlated with BAY 2965501 IC50 at both 24h (R^2 = 0.5884) and 72h (R^2 = 0.09594), in line with what we observed previously. This trend is also observed at the mRNA level (panel F), though slightly less pronounced (24h R^2 = 0.1267; 72h R^2 = 0.7992).

Thus, for both isoform target expression and sensitivity to isoform specific inhibitors are not directly correlated.

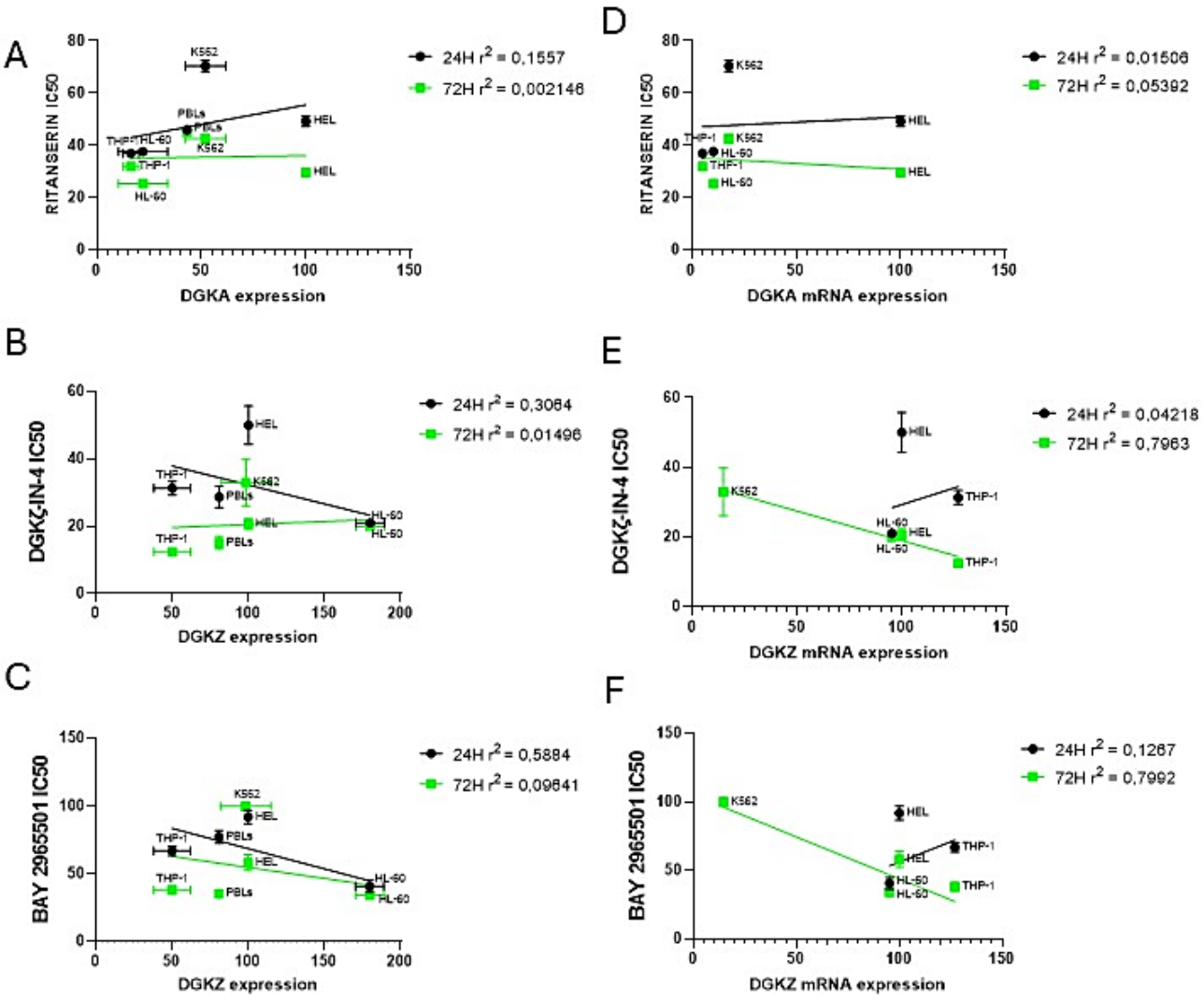

Figure 4: ***Correlation between sensitivity to isoform specific DGKs inhibitors and target espression.***

DGKs expression in PBLs and AML cell lines was measured by western blotting after normalization for protein concentration and rtPCR after normalization for mRNA concentration. Expression is shown as percentage of HEL cells used as a reference.

A. Ritanserin IC50 vs DGKA protein;

B. BAY 2965501 IC50 vs DGKZ protein;

C. DGKζ-IN-4 IC50 vs DGKZ protein;

D. Ritanserin IC50 vs DGKA mRNA;

E. BAY 2965501 IC50 vs DGKZ mRNA;

F. DGKζ-IN-4 IC50 vs DGKZ mRNA.

Data were analyzed using Graphpad prism linear regression. In B, C, E and F data for K562 cell line at 24h were excluded as IC50 is not defined.

DISCUSSION

Diacylglycerol kinases (DGKs) have emerged as key regulators of lipid signaling, primarily through their modulation of the balance between diacylglycerol (DAG) and phosphatidic acid (PA), two crucial second messengers. Several studies have suggested a role for DGK isoforms, especially DGKα and DGKζ, in tumorigenesis, where they modulate cellular proliferation, survival, and anti-tumor immunity by modulating the Akt/mTOR and MAPK/ERK signaling pathways (Olmez et al., 2018; Baldanzi et al., 2016; Mérida et al., 2008).

Since nowadays there are still AML patients who are not responding or relapsing to present therapies in particular for AML and CML, we suppose that DGKs may be new therapeutic targets for those diseases. In particular, the present study focuses on DGKα and DGKζ and examines how their expression correlates with the efficacy of specific inhibitors and the outcomes of siRNA-mediated silencing.

From this study emerges that DGKζ plays a unique and crucial role in AML cell viability, particularly in HL-60 cells, where viability was reduced by 37%, with a silencing of 55% in mRNA expression. Knockdown of DGKζ led to a profound reduction in proliferation and a statistically significant increase in late apoptosis, in line with data from Li et al. (2019), who identified a similar apoptotic phenotype upon DGKZ silencing. These findings suggest that DGKζ may be essential for maintaining the survival of certain AML subtypes and could be exploited as a potential therapeutic target. In the same cells DGKA silencing induced a reduction of viability only with a single siRNA (20%) with mRNA reducing by approximately 40% with siRNA1 and 20% with siRNA2, while DGKα protein expression decreased by about 50% with both siRNAs. This suggests that DGKA silencing is less important in this cell line.

In CML, using as a model K562 cell line, DGKs silencing produced no significant reduction in viability in both DGKA and DGKZ silenced cells, even if DGKA mRNA levels were reduced by 20% with siRNA1 and 45% with siRNA2, while DGKα protein expression decreased by 60% and 70%, respectively and DGKZ showed a reduction of 60% in mRNA expression and a decrease of 70% in protein levels. These findings suggest that in this specific cell line decreasing DGKA and DGKZ alone is not sufficient to reduce vitality of K562 cells, nor induce a block in the proliferation. This suggest that in this cell line multiple isoforms may be involved in cell proliferation and survival, or other pathways compensate for DGKs absence.

Those findings are in contrast with what achieved by Poli et al. of 2019, probably due to a different subtype of K562 cells used, indeed experiments with the original cell line used by Poli are actually

ongoing.

Interestingly, the ERK1/2 pathway, one of the key MAPK signaling cascades involved in proliferation and survival, was differentially modulated upon DGKα and DGKζ silencing across the AML cell lines tested. In K562 cell line there's no significant modification of p-ERK1/2 compared with total ERK1/2, as p-ERK1/2 quantification shows no significant variation in HL-60 cell line. However, in other cell lines (HEL and THP-1), there's a decrease in p-ERK1/2. This might mean that in those cell lines DGKs have a more important role in MAPK signaling cascade. These changes were not directly proportional to the expression level of each isoform, implying a complex, cell-type-specific interaction between DGKs and MAPK signaling. These findings may be due to the BCR/ABL chromosomal modification in K562 cell line in which Ras/MAPK and PI3K/AKT pathways result constitutive activated [17]. Previous work proved also that DGKs can interface with multiple signaling nodes, including Ras/ERK and PI3K/AKT pathways, either through PA production or DAG depletion [53]. Combined DGKs isoform silencing may have a stronger effect on cell viability, in particular in those cell lines where a single isoform didn't achieve significant reduction in cell viability, such as K562.

In all five cell lines, including controls, the sensitivity data for DGKα and DGKζ specific inhibitors (ritanserin, DGKZ-IN-4, and BAY 2965501) obtained from Marco Cristiano Cartella thesis, were correlated with both protein and mRNA expression levels of DGKA and DGKZ. Results indicate the absence of correlations overall, with cells treated with DGKZ-IN-4 or BAY 2965501 exhibited a significant inverse correlation between DGKZ expression and drug sensitivity, especially at 72h. Therefore, baseline DGKA/DGKZ expression does not predict inhibitor efficacy in AML or CML cell lines and cannot be used for patient-tailored therapy.

Moreover, as emerges from Ginevra Lovati thesis, in HEL cell line, which is another AML model, precisely, acute erythroleukemia DGKα silencing drive to a reduction in cell vitality, enhancing apoptosis [54]. This highlights how different AML cell lines are susceptible to different AML silencing and that not only targeting DGKζ could be suited as a future therapeutic target.

This study presents one main limitation, the lack of patient's primary cells affected by AML or CML. As previously shown in the work of Tan et al. regarding the effects of ritanserin, we might verify that our results in cell lines are VALID also in CELLS FROM actual patients, even if is clear that it will be difficult to silence an elevated number of patient's cells rather than only treat them with inhibitors, mainly due to elevated costs and time required. However, a cheaper and less time-consuming future research, still following Tan et al. methodology may also be the developing new inhibitors that specifically target different DGKs isoforms at the same time, to investigate how different DGKs

simultaneous inhibition act on AML cell viability. Even if, the most promising isoform are α and ζ, in the future may discover that combined inhibition with other isoform may achieve relevant results. The findings of this thesis not only clarify the isoform-specific functions of DGKs in AML and CML but also provide a rational basis for further investigation into DGKs inhibitors as targeted therapies. It demonstrates that DGKα and DGKζ could be good therapeutic target in AML cells, but only some cell lines are sensible to DGKα and DGKζ inhibition. This let us suppose that only some patients will be responsive to DGKα and DGKζ inhibitors. Since heterogeneous nature of AML, future research should focus on validating these findings in primary patient samples, exploring combination strategies with existing chemotherapeutics and TKI for CML and ATRA for APL, and performing in vivo studies to assess the therapeutic window and systemic effects of DGKα and DGKζ inhibition. Might be also useful to study how different DGKs simultaneous silencing or inhibition can affect cell viability and since DGKs expression are not corelated with the effect of inhibitors or silencing, other DGKs rather than DGKα and DGKζ should be tested.

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