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Effects of DGKα and DGKζ inhibitors on AML cell viability

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SUMMARY

1. ABSTRACT

Acute Myeloid Leukaemia (AML) represents a heterogeneous group of clonal haematopoietic neoplasms characterized by the accumulation of myeloid blasts in the bone marrow and peripheral blood. With a 5-year relative survival of 32.9% and with an incidence rate of 4.3 new cases per 100,000 individuals per year, AML represents a clinical urgency and challenge. Diacylglycerol kinases (DGKs) are a family of cytosolic or nuclear enzymes that catalyze DAG phosphorylation generating PA. They both act as dynamic second messengers involved in numerous cellular processes, including proliferation, survival, motility, and metabolic regulation. In AML DGKs are overexpressed and are linked to tumorigenesis. In particular, DGKα and DGKζ are widely studied given their proved role in several oncogenic pathways. Their inhibition or knockdown retrieved interesting results *in vitro* and *in vivo*, demonstrating that it can reduce tumor cell viability and progression while enhancing the immune system antitumor response.

To investigate the role of the two enzymes we selected a DGKα inhibitor (ritanserin) and two DGKζ inhibitors (DGKζ-IN-4 and BAY 2965501). For this purpose, we performed cell viability assays, apoptosis/necrosis analysis by flowcytometry, and proteomic profiling. In this work we demonstrated that these inhibitors affect AML cells viability, with marked heterogeneity, across the cell lines, confirming a role for DGKs but also suggesting that they may be beneficial only in some patients. Even untransformed control cells were affected suggesting potential general toxicity. Additionally, ritanserin, induced apoptosis in all the cell lines, while DGKζ inhibitors induced also necrosis in most of the tested cells. Proteomic analysis on AML cell line treated with ritanserin revealed that many proteins were downregulated and only few were upregulated. Ritanserin downregulated many pathways critical for MHC I-mediated antigen processing and presentation, cell metabolism, cell cycle progression, DNA synthesis and nucleotide excision repair. These pathway alterations are in line with what expected in a growth-arrested and apoptotic cell.

It's important to take in consideration that *in vitro* studies do not replicate the complexity of the tumor microenvironment. Nevertheless, previous *in vivo* studies have shown that these inhibitors can significantly reduce tumor progression and enhance antitumor immune responses in various cancer models. These findings support the hypothesis that even in the context of AML, DGK inhibitors may be effective at lower concentrations, affecting both malignant cells while simultaneously enhancing the immune response within the tumor microenvironment.

2. INTRODUCTION

Acute Myeloid Leukaemia (AML) represents a heterogeneous group of clonal haematopoietic neoplasms characterized by the accumulation of myeloid blasts in the bone marrow and peripheral blood. This accumulation results from a blockade of differentiation and gain of proliferative capacity of myeloid precursor cells, ultimately impairing normal haematopoiesis. AML is classified into several major categories: AML with recurrent genetic abnormalities; AML with myelodysplasia-related changes; therapy-related myeloid neoplasms; AML with germline predisposition; and AML not otherwise specified (NOS).[1,2,3]

Acute myeloid leukaemia with maturation (M2) is a subtype of AML NOS.[1] It is characterized by the presence of ≥ 20% blasts in the bone marrow or peripheral blood, along with evidence of maturation (≥ 10% of maturing cells of granulocytic lineage) in the bone marrow. This subtype accounts for approximately 10% of AML cases. Patients often present with symptoms related to anaemia, thrombocytopenia, and neutropenia. The immunophenotype includes one or more of the myeloid associated antigens: CD13, CD33, CD65, CD11b, and CD15. Flow cytometric analysis reveals patterns associated with granulocytic differentiation. Expression of HLA-DR, CD34, and/or KIT (CD117) is frequently observed, although it may be present only in some blasts. Monocytic markers such as CD14, CD36, and CD64 are usually absent. There is no demonstrated association between AML with maturation and specific recurrent chromosomal abnormalities.[1] For the purposes of our study, we used the HL-60 cell line as a model of AML with maturation. Despite being isolated from a patient initially diagnosed with acute promyelocytic leukaemia (APL), a subtype of AML with recurrent genetic abnormalities, morphological studies indicated that HL-60 is more representative of AML with maturation.[4] The patient, from whom the cell line was established, lacked several morphological features characteristic of APL and exhibited atypical clinical features not described in APL. Additionally, HL-60 cells lack the distinctive t(15;17) translocation typically observed in APL. Nevertheless, they respond to treatment with all-trans retinoic acid (ATRA) by differentiating into more mature stages, such as neutrophils.[5,6,7] This indicates that the t(15;17) translocation is not required for *in vitro* maturation in response to ATRA, as evidenced by the induction of a granulocytic phenotype in HL-60 cells that do not harbor this cytogenetic abnormality.[8] These findings suggest that insights into the mechanism of action of ATRA may support its application to neoplasms beyond APL. Moreover, HL-60 cells exhibit multipotentiality and can differentiate along various myeloid lineages. Upon treatment with phorbol esters such as TPA, they differentiate into macrophage-like

cells[9], while exposure to vitamin D_3 induces monocytic differentiation.[10,11] Furthermore, HL-60 cells are characterized by CD34 and HLA-DR, markers typically absent in APL.[4,12,13,14,15]

Among the AML subtypes with recurrent genetic abnormalities is AML with the t(9;11)(p21.3;q23.3) translocation. This subtype is usually associated with monocytic features and is characterized by the presence in peripheral blood or bone marrow of blasts (≥20%), of which at least 80% of them are of monocytic lineage.[16] These leukaemias typically present monoblasts and promonocytes with frequent extramedullary involvement, and strong non-specific esterase positivity. The immunophenotype includes CD33, CD65, HLA-DR and CD4, while expression of CD34 is usually low. Most cases also express markers of monocytic differentiation, such as CD64, CD11b, CD11c, lysozyme and CD4.[1] This AML subtype is primarily associated with the characteristic chromosomal translocation t(9;11), which generates the AF9-MLL (KMT2A) fusion protein that leads to expression of genes involved in epithelial-mesenchymal transition (EMT), as well as chemoresistant and invasive phenotypes.[17] AML with t(9;11)(p21.3;q23.3) is associated with intermediate survival, which is more favorable than that observed in AML cases with other 11q23.3 translocations. Additionally, MECOM is overexpressed in 40% of cases in this AML subtype. MECOM-positive KMT2A-rearranged AML exhibits genetic, molecular, morphological and immunophenotypic alterations that appear to be associated with a poor prognosis.[1] Standard treatment involves induction therapy with cytarabine and daunorubicin, followed in most cases by a cytarabine-based intensification treatment.[18] Despite current protocols, novel targeted therapies are still needed for this neoplasm. For the purposes of our study, we used THP-1 cell line as a model of this AML subtype, as it harbors the typical t(9;11) chromosomal abnormality. Upon stimulation with phorbol 12-myristate 13-acetate (PMA), THP-1 cells cease proliferation, become adherent, and differentiate into macrophage-like cells. These differentiated cells resemble native monocyte-derived macrophages in several key criteria.[19,20]

Pure erythroid leukaemia (PEL) is a rare and aggressive subtype of AML NOS characterized by a predominance (>80%) of immature erythroid precursors. Immunophenotypically, these cells express CD71, glycophorin and haemoglobin A. E–cadherin, which stains early erythroid forms, is positive in the vast majority of cases and is specific for erythroid differentiation. Blasts are often positive for KIT (CD117) and usually negative for HLA–DR and CD34. Antigens associated with megakaryocytic lineage (i.e. CD41 and CD61) are typically negative. PEL often carries complex karyotypes and TP53 mutations associated with a dismal prognosis (median survival of 3 months).[1]

The response to both conventional chemotherapy and targeted therapies is generally poor, highlighting the urgent need for novel and more effective therapeutic strategies.[21] For the purposes of our study, we employed the HEL cell line as a model of PEL. HEL cells are positive for KIT, have TP53 mutations, and express high level of glycophorin A, making them well suited as a model for this AML subtype.[22]

In addition to the proposed AML cell line models, we also used the K562 cell line. Although K562 cells were derived from a patient with chronic myeloid leukaemia (CML) in blast crisis, they are widely utilized in AML research due to their myeloid characteristics and their ability to differentiate toward erythroid or megakaryocytic lineages under specific treatments.[23,24,25] CML is a myeloproliferative neoplasm (MPN) in which granulocytes are the major proliferative component. It originates from a haematopoietic stem cell and is defined by the chromosomal translocation t(9;22)(q34.1;q11.2), which results in the formation of the Philadelphia (Ph) chromosome containing the BCR-ABL1 fusion gene. The transforming activity of BCR-ABL1 arises from its constitutive tyrosine kinase (TK) activity, which drives the activation of multiple downstream pathways, including JAK/STAT, PI3K/AKT and RAS. This activity leads to cell proliferation, inhibition of differentiation, and resistance to cell death.[1] K562 cells have been widely used for investigating the BCR/ABL1 oncogene and the effects of tyrosine kinase inhibitor, imatinib-mesylate, often used as CML treatment. Further, K562 cells overexpress transferrin receptors (TfR) and have been used as a model for evaluating cytotoxic therapies that exploit receptor-mediated endocytosis for targeted delivery.[26,27]

Looking at AML therapeutic options, despite recent advances in the treatment of hematologic malignancies, including targeted and immune therapies and improved supportive care, outcomes for AML patients remain poor (5-year relative survival of 32.9% with an incidence rate of 4.3 new cases per 100,000 individuals per year)[16]. Several factors contribute to the poor prognosis of AML, including its higher incidence in older adults, poor chemotherapy response, high relapse rates, and limited options for relapsed cases. AML also imposes a significant clinical and financial burden due to its heterogeneity such as varying ages and genetic profiles, prolonged hospitalizations, infections, and the need for stem cell transplants. Standard first-line treatment consists of intensive chemotherapy, with the aim of inducing remission. Patients may then go on to receive additional chemotherapy, radiation therapy, or a stem cell transplant.[16] However, significant unmet clinical needs remain, such as the urgency for more effective and less toxic treatments, strategies to prevent

relapse, and better support for the reduced quality of life.[28] Given the urgent need to identify new therapeutic strategies, we decided to search additional potential molecular targets in AML. One potential candidate for new drugs could be diacylglycerol kinases (DGKs). In this study, we explored the the role of two specific DGK isoforms, DGKα and DGKζ, across the previously described AML models using pharmacological inhibitors targeting specifically those isoforms.

DGKs are a family of cytosolic or nuclear enzymes that catalyze the phosphorylation of the lipid second messenger diacylglycerol (DAG), generating phosphatidic acid (PA). This reaction is mediated by the transfer of the γ-phosphate group from ATP to the hydroxyl group of DAG.[29] Both DAG and PA are primarily localized to cellular membranes where these lipids facilitate the activation of several proteins, such as protein kinase C (PKC), RAS activator proteins, mammalian target of rapamycin (mTOR), and tyrosine kinases.[30,31,32] Through these interactions, DAG an PA act as dynamic second messengers involved in numerous cellular processes, including proliferation, survival, motility, and metabolic regulation.[33] All DGK isoforms share a conserved catalytic domain and two cysteine-rich C1 domains. The catalytic domain is regulated by phospholipids, while the C1 domains mediate membrane targeting and protein interactions.[34,35] DGKα, in particular, contain EF-hand calcium-binding motifs and a recoverin homology (RVH) domain.[36] In T lymphocytes, upon T cell activation, it has been observed that DGKα can be activated by rises in intracellular calcium levels[37,38] and by the lipid products of phosphatidylinositol-3-kinase (PI3K).[39] Once stimulated, DGKα translocates to the plasma membrane, where it catalyzes the conversion of DAG into PA. This not only downregulate T cell activation by attenuating DAG-mediated signalling but also triggers the dissociation of DGKα from the membrane and its return to the cytosol.[40] The role of DGKα in regulating T cell responses was first suggested by its activation in response to interleukin-2 (IL-2), the main T cell growth factor. This enzymatic activity has been shown to be essential for the coordinated transition of T cells from late G1 to the S phase of the cell cycle.[41,42] Beyond the immune system, DGKα also promotes endothelial cell proliferation and migration via vascular endothelial growth factor (VEGF) signalling in PAE-KDR and HUVEC cells. Specific inhibition of this isoform, obtained via chemical inhibition with R59949 or by expression of a DGKα dominant-negative mutant, impairs VEGF-dependent chemotaxis, proliferation and angiogenesis.[43] DGKζ is another DGK isoform well known for its role in the control of the immune system and in tumorigenesis.[44,45] It possesses a MARCKS homology domain and a PDZ-binding motif. Similar to DGKα, DGKζ exerts regulatory effects on the cell cycle and cell proliferation. Knockdown of DGKZ via lentiviral transfection has been shown to suppress proliferation, induce apoptosis, and cause G0/G1 cell cycle

arrest in SiHa and HeLa cells. In addition, it resulted in restrained tumor growth in xenograft mouse models.[46] Alongside DGKα, DGKζ also participates in T cell negative regulation[45], providing a control downstream of the TCR by limiting DAG availability[44]. Tumor-associated overexpression of both DGKα and DGKζ, drives T lymphocytes into a hyporesponsive, anergic state as a result of unbalanced Ca^{2+} and Ras/ERK inactivation. Notably, this immunosuppressive effect can be reversed by chemical inhibition of DGKα and DGKζ using compounds such as R59949 and R59022.[47] Additionally, combined deletion and/or inhibition of these isoforms enhances T-cell-mediated anti-tumor responses, such as priming and antigen-specific effector functions, both *in vitro* and *in vivo* and improves immunotherapy efficacy with a synergic effect.[48,49,50]

In the context of AML specifically, DGKα and DGKζ have been investigated for their potential as therapeutic targets. Analysis of public databases such as The Cancer Genome Atlas (TCGA) has revealed DGKs overexpression across various tumors, including AML[51], suggesting a possible oncogenic role.[52,57,58,61,62] Among the DGK isoforms, α and especially ζ are frequently altered in leukemic cell lines. A recent study has shown that Kasumi-1 and KG-1α cell lines (AML) treated with DGKα specific inhibitor, ritanserin (see below) had a significant decrease in DGKα protein levels, along with downregulation of sphingosine kinase 1 (SphK1) and its active phosphorylated form, indicating disruption of the PLD–PA–SphK1 axis. Additionally, western blot analyses revealed marked suppression of the JAK–STAT and MAPK pathways. Importantly, there was a noticeable downregulation of PARP, caspase-3, MCL1, and Bcl-xL. Ritanserin exerted cytotoxic effects on both Kasumi-1 and KG-1α cell lines, with half maximal inhibitory concentration (IC_{50}) of 51.1 µM and 37.7 µM respectively after 24 hours, and lower IC_{50} values (29.8 µM and 25.9 µM) after 72 hours, indicating a dose- and time-dependent cytotoxic effect. Additionally, bone marrow mononuclear cells (BMMCs) from primary AML samples were treated with ritanserin, inducing primary cell apoptosis up to 70% at a drug concentration of 40 µM. Notably, in normal hematopoietic stem cell samples, ritanserin toxicity was lower (<30% of apoptosis in cells treated with 40 µM ritanserin). These findings suggest that ritanserin affects several AML cell types, reducing viability through a multi-pathway mechanism involving DGKα inhibition, signaling pathway downregulation, and caspase-dependent apoptosis.[57] The *in vitro* results were further validated by *in vivo* evidence: in xenograft mouse models injected with Kasumi-1, ritanserin treatment led to a significantly reduced tumor burden and improved survival. Additional studies have investigated the response of other AML cell lines to DGK inhibitors. In HEL and HL-60 cells, DGKα inhibitors CU-3 and AMB639752 did not significantly affect viability, whereas R59022 and R59949 induced marked cytotoxic effects after

24 hours, with IC_{50} values of 32 µM and 72 µM, respectively, in HL-60 cells, and 49 µM and 80 µM, respectively, in HEL cells.[52] Moreover, R59022 and R59949, at concentrations up to 30 µM, led to an acceleration of differentiation and disruption of cell cycle in HL-60 cells.[58] In THP-1 cells and primary human monocytes, chemical inhibition of DGKs downregulates calcium signalling induced by CCL2. Under normal conditions, this chemokine triggers intracellular calcium responses and promotes DAG generation.[59] Treatment with R59949, suppressed CCL2-induced Ca^{2+} signaling with a half-maximal concentration of 8.6 µM.[60] In K562 cells, DGKα translocates into the nucleus during both the G1 and G2 phases of the cell cycle and its presence appears to be required for efficient cell cycle progression. Inhibition of DGKα in these cells led to a reduction in cell proliferation and altered nuclear morphology, supporting a direct role for this isoform in mitotic regulation.[61] Similarly, knockdown of DGKZ via RNA interference in HL-60 cells induced significant apoptosis and G2/M phase cell cycle arrest. Mechanistically, this effect was associated with downregulation of the MAPK pathway and suppression of the anti-apoptotic protein survivin, alongside activation of caspase-3 and caspase-9.[62]

Among the DGKs inhibitors described in the literature, R59022, R59949 and ritanserin share a similar structure and exhibit anti-tumoral effects in common.[63] Based on these observations, we aimed to further investigate the role of DGKs in AML by expanding current data on ritanserin obtained by Tan et al., in Kasumi-1 and KG-1α cells to additional AML cell models.

Ritanserin is a synthetic compound initially developed as a selective antagonist of serotonin 5-HT_2A and 5-HT_2C receptors with an inhibition constant (Ki) of 0.45 nM and 0.71 nM respectively.[64,65,66] Although it was never approved for clinical use due to safety concerns, it was tested in a range between 1 to 30 mg[67] and its favorable oral bioavailability and tolerability in early trials have maintained its relevance in research settings.[68] Beyond its serotonergic activity, ritanserin has been identified as a potent inhibitor of DGKα.[63] Recent studies have explored ritanserin's potential in treating AML. *In vitro* experiments demonstrated that ritanserin inhibits AML progression in a dose- and time-dependent manner by inducing apoptosis and reducing proliferation in AML cell lines. Moreover, ritanserin negatively regulates sphingosine kinase 1 (SphK1) expression through phospholipase D (PLD) signaling and inhibits the Jak-Stat and MAPK pathways via DGKα inhibition. *In vivo* studies using xenograft mouse models showed that ritanserin effectively reduces leukaemia burden and prolongs survival without significant toxicity to normal hematopoietic stem cells.[57] Other than its established roles as a serotonin receptor antagonist and DGKα inhibitor, ritanserin

has been identified as a modulator of voltage-gated calcium channels, specifically the $Ca_V1.2$ channels in vascular smooth muscle cells. Electrophysiological studies have demonstrated that ritanserin inhibits $Ca_V1.2$ channel currents ($I_{Ca1.2}$) in a concentration-dependent manner, with an apparent dissociation constant (K_r) of approximately 3.61 μM.[69]

Along with ritanserin, we tested various serotonin and dopamine receptors antagonists to exclude potential ritanserin effects that did not involve DGKs.

Risperidone is a second-generation antipsychotic with high affinity for 5-HT_2A (Ki = 0.16 nM) and D_2 (Ki = 3.13 nM)[70] receptors and is widely prescribed for schizophrenia and bipolar disorder with an optimized dosage ranging between 6 to 10 mg/day.[71] Notably, studies in human monocyte-derived THP-1 cells and peripheral blood mononuclear cells (PBMCs) have shown that risperidone modulates immune-related pathways. Moreover, risperidone enhances expression of JAK–STAT1 genes in THP-1 and PBMCs, suggesting a shift toward proinflammatory M1 phenotypes.[72] Canfrán-Duque et al., further demonstrated that risperidone alters sphingolipid and ceramide profiles in THP-1 cells and disrupts lipid raft integrity.[73] Although these findings indicate significant immunomodulatory effects in hematopoietic contexts, no studies have evaluated risperidone's direct cytotoxic or anti-proliferative effects in AML cell lines.

Metoclopramide is a dopamine D_2 receptor antagonist with nanomolar affinity (Ki = 28.8 nM)[74] and additional serotonergic activity, primarily used as an antiemetic. Metoclopramide is generally administered orally in a tablet or solution form. The dosage may vary depending on its purpose from 2 to more than 100 mg.[75] Beyond its clinical application, it has been shown to exert minimal cytotoxic effects *in vitro*. In HL-60 and K562 AML cell lines, metoclopramide directly induced apoptosis by arround 4% at 100 μM without significantly increasing necrosis.[76,77] When combined with ionizing radiation, both acidic and neutral forms of metoclopramide produced additive apoptotic effects, confirming distinct mechanisms from radiation alone and suggesting potential as a radiosensitizing agent. Further, metoclopramide and its derivatives have been implicated in modulating NF-κB signaling. Metoclopramide inhibited NF-κB activation, as demonstrated by reduced surface Ig expression in 70Z/3 pre-B cells and suppression of TNF-α production after LPS stimulation *in vivo*. This benzamide act upstream by blocking IκBβ degradation, a critical step in NF-κB activation.[78]

Altanserin is a highly selective antagonist of the serotonin 5-HT_2A receptor (Ki = 0.13 nM)[79], that can be radiolabeled and employed as a radiotracer in PET neuroimaging studies. It has been tested on

humans as radiotracer in a concentration of 0.81 ± 0.06 mg/kg.[80] Its selectivity is notable, with negligible affinity for other serotonin receptor subtypes or dopaminergic targets. No studies about altanserin effects on AML cells have been made currently.

Previous structural analyses of several DGKζ inhibitors, performed by Luisa Racca, Ph.D., and Prof. A. Massarotti, guided the selection of two compounds that could be potentially effective in AML.

DGKζ-IN-4 is a heteroaryl carboxamide compound that has been reported as a potent and selective inhibitor of DGKζ that can be used as therapeutic agent for cancer associated with the activation of an immunocyte or cancer resistant to anti-PD-1 antibody/anti-PD-L1 antibody therapy. It has been reported to inhibit DGKζ with an IC_{50} of 0.4 nM while also increasing IL-2 by 10-fold in the nanomolar range. In murine models compounds structurally related give a relative tumor growth inhibition of at least 30% and are suitable for oral, intravenous or transmucosal administration.[81]

BAY 2965501 is a highly potent and selective, orally available inhibitor of DGKζ, developed by Bayer AG in collaboration with the German Cancer Research Center. This compound enhances human and mouse T cell reactivity both in the priming and the effector phases. Notably, it overcomes immunosuppressive factors such as adenosine and prostaglandin E2. *In vitro*, BAY 2965501 increased natural killer cell- and T-cell-mediated tumor cell killing and enhanced IL-2-induced natural killer cell activation. In syngeneic murine tumor models, oral administration of BAY 2965501 resulted in improved T cell efficacy, reactivation of exhausted T cell and reduced tumor growth.[82,83] Moreover, pharmacological DGK inhibition with BAY 2965501 overcomes the immunosuppressive effects caused by TGFβ and PGE2, immune modulators commonly found in tumor microenvironment. T cell enhancement is even more remarkable upon combined DGK inhibition, especially in the context of weaker antigenic TCR-stimulation. In syngeneic mouse tumor models, DGKζ inhibition alone was sufficient to elicit maximal anti-tumor responses in certain tumors (F9 and MB49), whereas combined DGKα/ζ inhibition provided additional therapeutic benefits in others, such as MC38.[84] Currently, BAY 2965501 is undergoing a first-in-human Phase 1 clinical trial to evaluate its safety, tolerability, pharmacokinetics, pharmacodynamics, best dosage and preliminary efficacy in patients with advanced solid tumors.[85]

3. OBJECTIVE OF THE THESIS

AML remains an urgent clinical challenge. As stated in literature, DGKs play a significant role in AML cell viability, which can be demonstrated through the use of DGK inhibitors. The aim of this work is to further investigate the effects of selected DGKα and DGKζ inhibitors in AML, expanding existing data on ritanserin and, for the first time, testing two novel DGKζ inhibitors in four selected AML cell line models. For this purpose, we performed cell viability assays, apoptosis/necrosis analysis by flowcytometry, and proteomic profiling.

4. 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
Risperidone	-	R3030	Sigma-Aldrich
Altanserin	-	A8106	Sigma-Aldrich
Metoclopramide	-	M2218	TCI
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

4.1 Cell culture

The HL-60 cell line was derived in 1976 from the peripheral blood of a 36-year-old woman diagnosed with acute promyelocytic leukemia.[86] The HEL cell line originated in 1980 from the peripheral blood of a 30-year-old man who experienced a relapse of erythroleukemia following treatment for Hodgkin lymphoma.[87] The THP-1 cell line was obtained from the peripheral blood of a 1-year-old male patient with acute monocytic leukemia.[88] 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.[89] These four cell lines were employed in this study as *in vitro* models of acute myeloid leukemia. The identity of the cell lines was verified through a Cell Line Authentication Test conducted by Eurofins Genomics (Ebersberg, Germany) and an internal university service. The resulting markers matched 100% with those reported in the ATCC database. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, following the handling protocols provided by ATCC. Additionally, healthy donor blood samples were provided by UPObiobank, the University biobank, under approved ethical committee authorization (PARERE COMITATO ETICO INTERAZIENDALE AOU Maggiore della carità CE206/2022 sul progetto MTSGB) and with informed consent for research use. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE

Healthcare), washed, and resuspended at a concentration of 2 x 10^6 cells/mL in RPMI-1640 medium containing 10% heat-inactivated FBS. Peripheral blood lymphocytes (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 hours. Activated T cells were then cultured in complete medium supplemented with 100 IU/mL recombinant human IL-2 (rhIL-2, PeproTech) at a density of 0.5 x 10^6 cells/mL.

4.2 AlamarBlue assay

The alamarBlue assay was performed to assess cell viability in presence of chemical inhibitors. 50,000 cells per well were seeded in a 96-wells plate in a volume of 100 µL of complete medium together with 10 µL of 10x concentrated inhibitor solution and then incubated for either 24 or 72 hours. Each experimental condition was performed in technical quadruplicates, using cells treated with equal amounts of DMSO (vehicle) as control and medium as background. After incubation, AlamarBlue was added to each well at a final concentration of 0.15 mg/mL. The fluorescence of the samples was measured 24 hours later using a Spark 10M Multimode Plate Reader (Tecan) with an excitation wavelength of 535 nm and an emission wavelength of 590 nm. Thus, for the dose–response curve fitting, fluorescence values were normalized as:

$$Viability\ (\%) = \frac{(sample\ mean\ fluorescence)}{(DMSO\ control\ mean\ fluorescence)} * 100$$

Data are the results of at least 5 independent experiments analyzed with GraphPad Prism 10 as [inhibitor] vs. normalized response curve (variable slope, after manual outlier removal).

4.3 Apoptosis and necrosis assessment

In order to define the type of cell death, we used flow cytometry to detect apoptotic and necrotic cells using “Apoptosis detection Kit Annexin V-FITC” (Invitrogen). For each sample, 500,000 cells, pre-treated for 24 hours, were stained according to manufacturer’s instructions except using 7AAD instead of propidium iodide. Samples were analyzed on a BD FACS Symphony A5 flow cytometer 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. 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 as control vs [inhibitor].

5. RESULTS

5.1 Ritanserin has cytotoxic effects and induces apoptosis in AML cell lines

Studies have shown that ritanserin, a DGKα inhibitor, can suppress AML progression in a dose- and time-dependent manner on Kasumi-1 and KG-1α cell lines with a half maximal inhibitory concentration (IC_{50}) of 51.1 and 37.7 μM, respectively after 24 hours.[57]

The aim of our experiments was to extend this study to other AML cell models. For this purpose, cytotoxicity assays were conducted on: 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. On the basis of cell viability, dose-response curves at 24 and 72 hours have been made (Figure 1A) and the IC_{50} values for each cell line were computed.

Across all the tested cell lines, the IC_{50} values decreased, as expected, with longer treatment time: 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), followed by HL-60 (37.6 ± 1.5 μM after 24 h and 25.3 ± 0.9 μM after 72 h) and HEL (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 from healthy donor, used as control, showed an IC_{50} of 42.9 ± 1.3 μM after 24 h and 32.5 ± 1.9 μM after 72 h (Figure 2).

Apoptosis/necrosis assays were conducted at 24 hours to evaluate the modality of cell death induced by ritanserin at concentrations close to IC_{50}. Ritanserin treatment induced a statistically significant increase in apoptosis among the tested cell lines (Figure 1B,3). In particular, THP-1, HEL and K562 cells underwent early apoptosis while HL-60 cells underwent late apoptosis.

These findings, demonstrating the dose- and time-dependent efficacy of ritanserin in the micromolar range, support previous ones obtained in other AML cell lines[57], while revealing marked heterogeneity and toxic effects on untransformed cells starting from 50 μM.

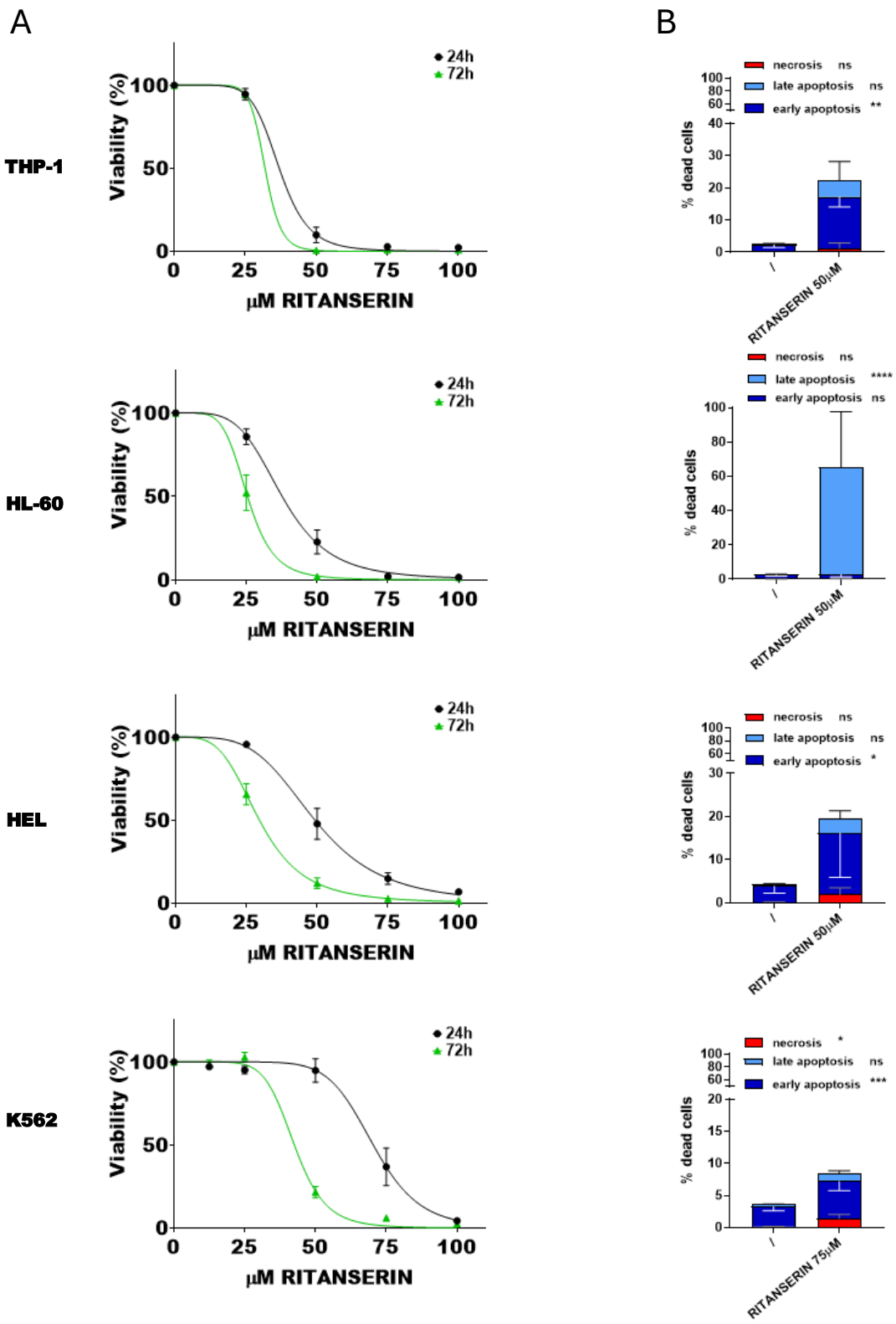

Figure 1: ***effects of ritanserin on AML cell lines viability.***

A: AML cells were treated with increasing concentration of ritanserin for 24 or 72 hours followed by alamarBlue viability assay for additional 24 hours. Experiments were run in quadruplicates. Data are the mean ± SEM of 6 or more experiments interpolated as [inhibitor] vs % viability.

B: AML cells were treated with concentrations of ritanserin near to the IC50s obtained previously. They were analyzed by flow cytometry to detect live (Annexin V- and 7AAD-), necrotic (Annexin- and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations. Data are the mean ± SD of 3 or more independent experiments presenting the percentage of dead cells and the type of cell death. Two-way ANOVA test, p<0.05, p<0.01**, p<0.001*** and p<0.001****.*

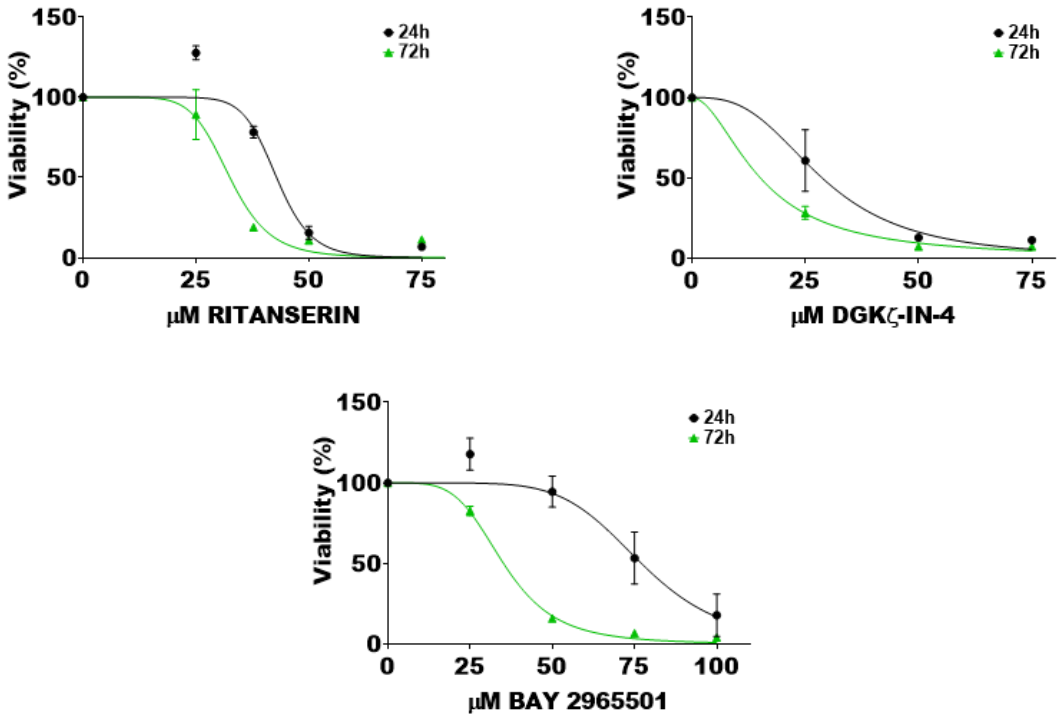

Figure 2: ***effects of DGK inhibitors on primary cells viability.***

PBLs were treated with increasing concentration of ritanserin, DGKζ-IN-4 and BAY 2965501 for 24 or 72 hours followed by alamarBlue viability assay for additional 24 hours. Experiments were run in quadruplicates. Data are the mean ± SEM of 5 or more experiments interpolated as [inhibitor] vs % viability.

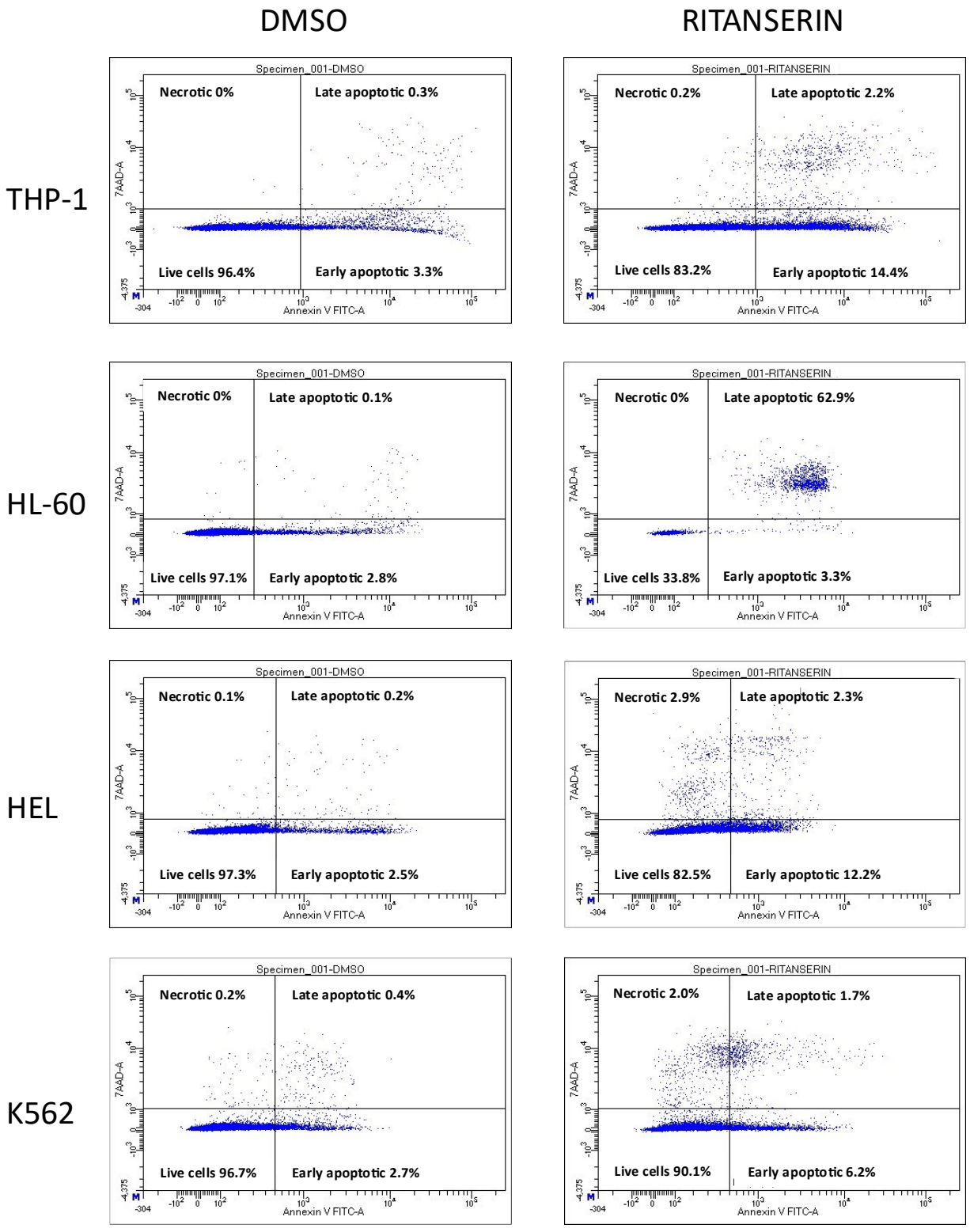

Figure 3: ***ritanserin effects on apoptosis/necrosis in AML cells****.*

Dot plots of a representative experiment of cells treated with ritanserin, analyzed by flow cytometry and divided in the 4 subpopulations.

Cells were treated with ritanserin concentrations close to IC50. After 24 h, cells were stained with Annexin V-7AAD and analyzed by flowcytometry to detect live (Annexin V-and 7AAD-), necrotic (Annexin-and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations.

5.2 Serotonin and dopamine receptors antagonists have no impactful effects on AML cell viability

In order to exclude potential off-target effects of ritanserin, related to its known activity as serotonin 5-HT_2A (Ki = 0.45 nM) and 5-HT_2C (Ki = 0.71 nM)[64] receptors antagonist, as a control we treated AML cells with three chemical compounds: risperidone, metoclopramide and altanserin which are known antagonists of 5-HT_2A and D_2 receptors.[70,74,80]

These compounds were tested with the previously described method for 24 hours.

The resulting dose-response curves (Figure 4) indicate that serotonin receptor antagonists exert negligible effects on AML cell viability, with the highest observed reduction being only 11% following treatment with metoclopramide 100 µM.

These findings support the conclusion that the cytotoxic effects of ritanserin in AML cell lines are unlikely to be attributed to interference with serotonergic signaling.

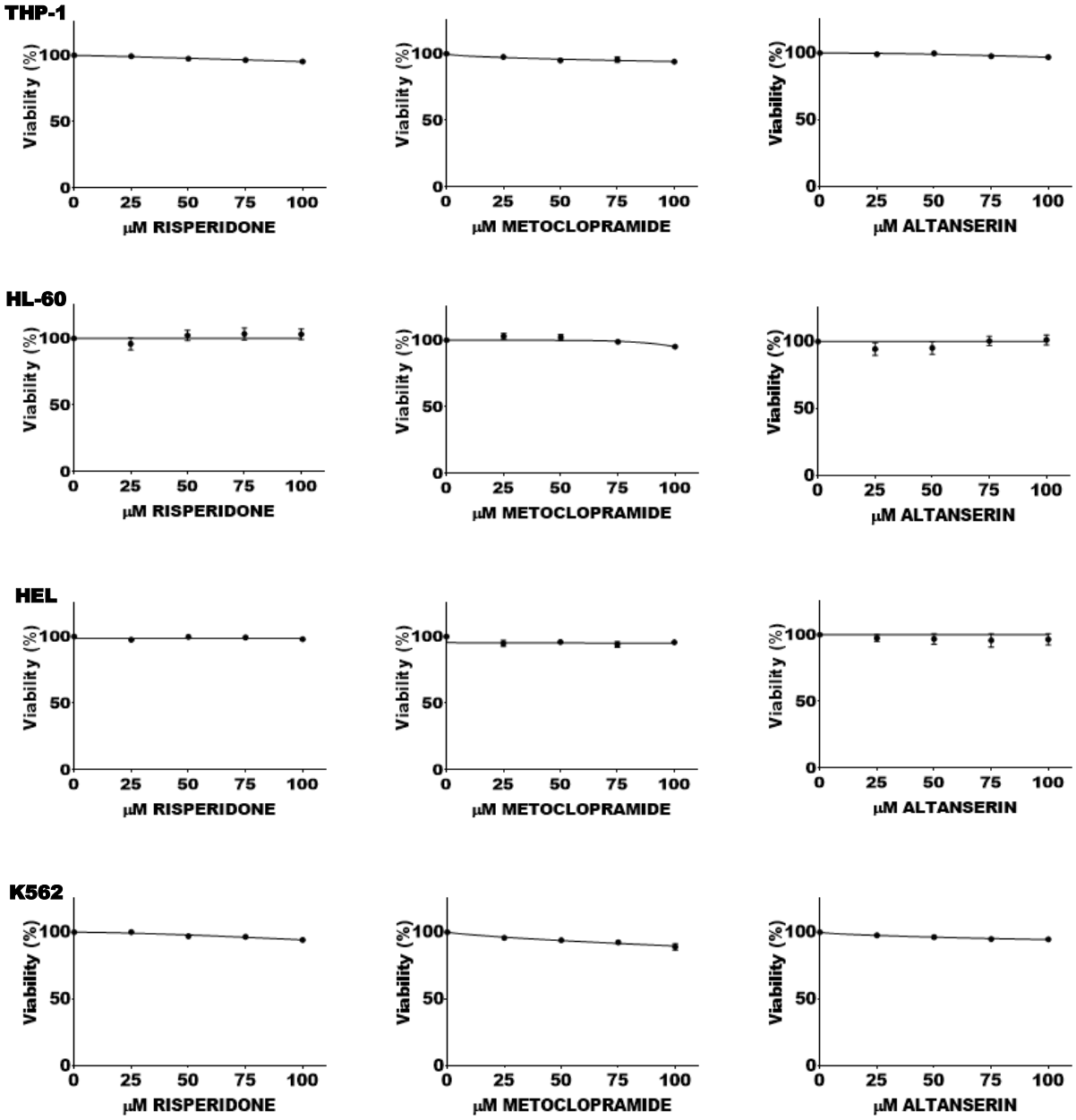

Figure 4: ***effects of serotonin receptor inhibitors on AML cell lines viability.***

AML cells were treated with increasing concentration of the indicated inhibitor for 24 hours followed by alamarBlue viability assay for additional 24 hours. Experiments were run in quadruplicates. Data are the mean ± SEM of 4 or more experiments interpolated as [inhibitor] vs. % viability.

5.3 DGKζ-IN-4 has heterogenic cytotoxic effects and induces both apoptosis and necrosis in AML cell lines

DGKζ has been proven to be overexpressed and involved in key metabolic pathways in RAS-mutated Ba/F3 AML cells.[90] Additionally, in HL-60 cells, DGKζ knockdown induced apoptosis and G2/M phase arrest through the MAPK/surviving/caspase pathway.[62]

Thus, we aimed to assess the impact of inhibiting DGKζ isoform on the viability of our AML panel. For this purpose, DGKζ-IN-4, a patented DGKζ chemical inhibitor[81], was examined with the previously described method (Figure 5A).

As observed with ritanserin, IC_{50} values decreased with extended treatment time: HL-60 cells were the most sensitive to DGKζ-IN-4 after a short exposure (21.2 ± 1.0 µM after 24 h and 19.9 ± 1.3 µM after 72 h), while THP-1 cells were the most responsive after a longer exposure (31.3 ± 2.0 µM after 24 h and 12.4 ± 0.3 µM after 72 h). HEL cells were less sensitive (50.7 ± 5.7 µM after 24 h and 20.7 ± 1.6 µM after 72 h), whereas 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 from healthy donor were sensitive to this drug with an IC_{50} of 28.7 ± 3.3 µM after 24h and 15.1 ± 1.8 µM after 72h (Figure 2).

Apoptosis/necrosis assays were conducted at 24 hours using inhibitor concentrations close to the calculated IC_{50} values. K562 cells were not tested due to the lack of a relevant cytotoxicity. DGKζ-IN-4 treatment induced a statistically significant increase in both apoptosis and, unexpectedly, necrosis across the tested cell lines (Figure 5B,6). In particular, THP-1 and HL-60 cells underwent both late apoptosis and necrosis, while HEL cells underwent both early apoptosis and necrosis.

These findings demonstrate a dose- and time-dependent effect also for DGKζ-IN-4 in the micromolar range, although with substantial variability among different AML cell lines and the existence of insensitive lines. Moreover, untransformed PBLs resulted highly sensitive to this drug. In line with the previously reported pro-apoptotic effects of DGKZ knockdown in HL-60[62], our results reveal that chemical inhibition of DGKζ activity induces not only apoptosis but also necrosis.

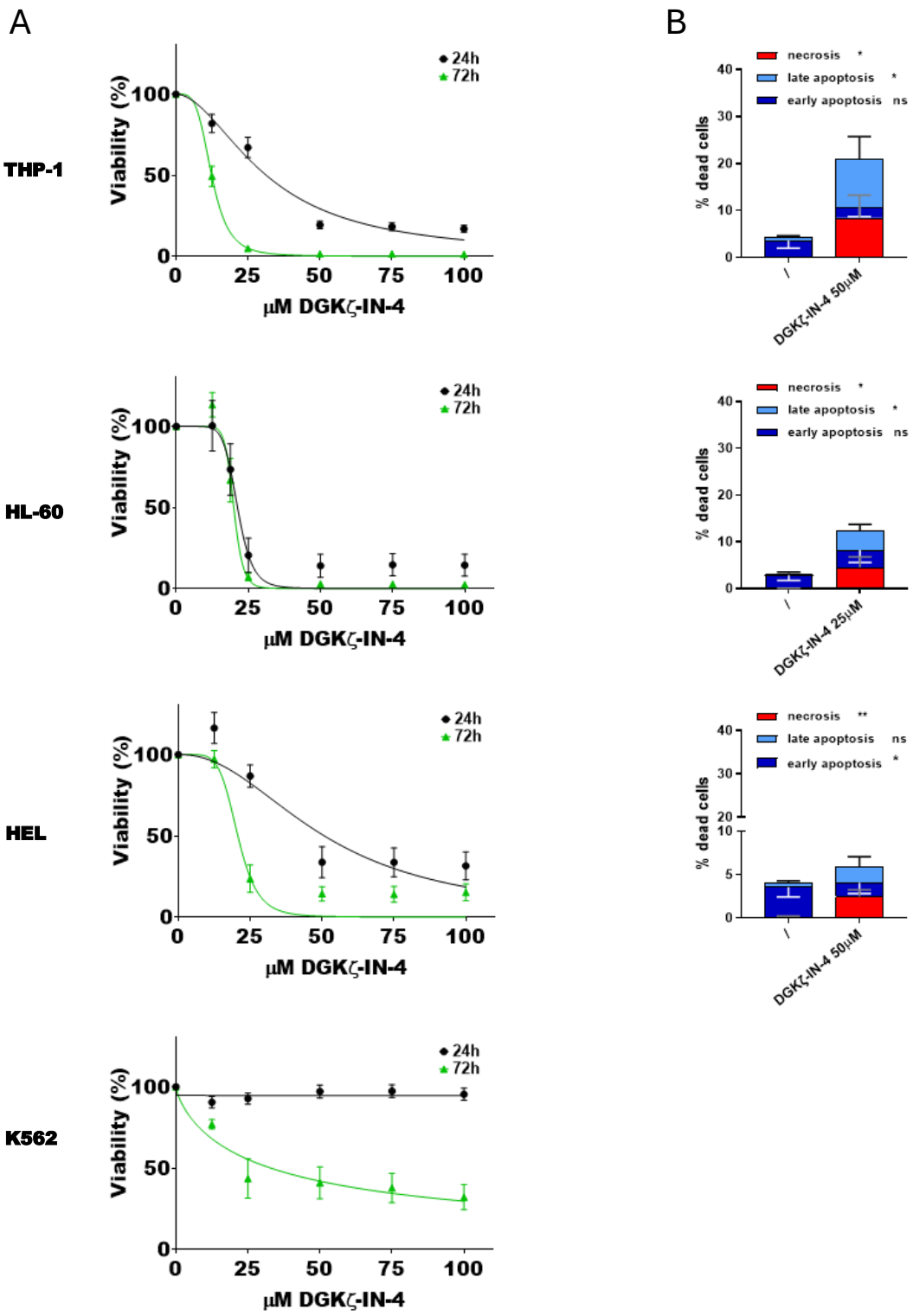

Figure 5: ***effects of DGKζ-IN-4 on AML cell lines viability.***

A: AML cells were treated with increasing concentration of DGKζ-IN-4 for 24 or 72 hours followed by alamarBlue viability assay for additional 24 hours. Experiments were run in quadruplicates. Data are the mean ± SEM of 5 or more experiments interpolated as [inhibitor] vs % viability.

B: AML cells were treated with concentrations of DGKζ-IN-4 near to the IC50s obtained previously. They were analyzed by flow cytometry to detect live (Annexin V- and 7AAD-), necrotic (Annexin- and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations. Data are the mean ± SD of 3 or more independent experiments presenting the percentage of dead cells and the type of cell death. Two-way ANOVA test, p<0.05 and p<0.01**.*

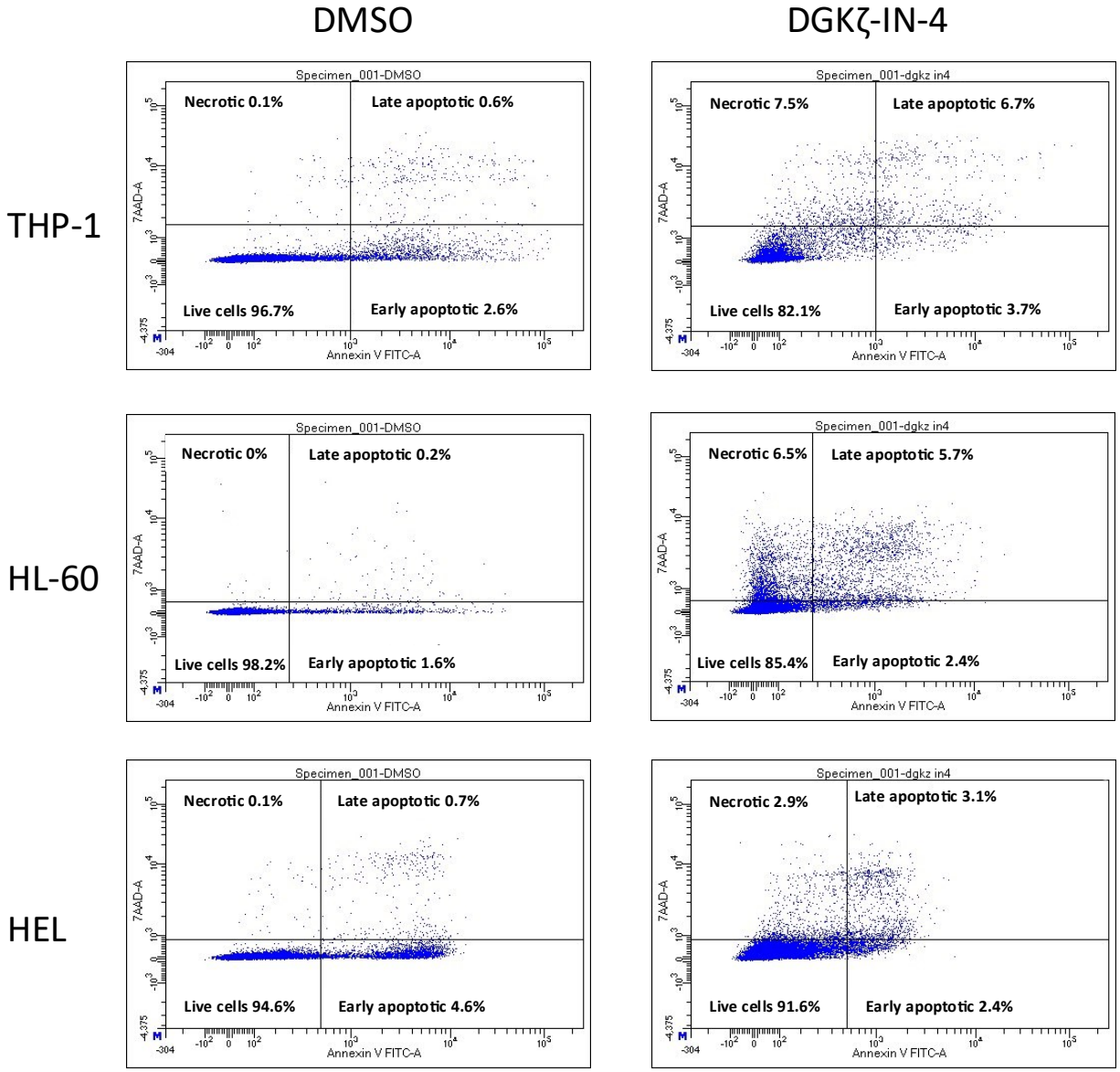

Figure 6: ***DGKζ-IN-4 effects on apoptosis/necrosis in AML cells****.*

Dot plots of a representative experiment of cells treated with DGKζ-IN-4, analyzed by flow cytometry and divided in the 4 subpopulations.

Cells were treated with DGKζ-IN-4 concentrations close to IC50. After 24 h, cells were stained with Annexin V-7AAD and analyzed by flowcytometry to detect live (Annexin V-and 7AAD-), necrotic (Annexin-and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations.

5.4 BAY 2965501 has highly heterogenic cytotoxic effects and induces both apoptosis and necrosis in AML cell lines

To further investigate the effects of DGKζ chemical inhibition in AML cells, we used BAY 2965501, a highly selective DGKζ inhibitor currently involved in a first-in-human study.[85] *In vitro*, BAY 2965501 has been shown to enhance natural killer cell- and T-cell-mediated tumor cell killing, increase interleukin 2-induced natural killer cell activation[82,83], and induce pERK activation in CD8+ cells.[91]

Cytotoxicity assays were conducted following the previously described protocol (Figure 7A).

As expected, IC_{50} values decreased with longer exposure times: HL-60 cells were the most sensitive to BAY 2965501 (40.7 ± 4.4 µM after 24 h and 34.1 ± 3.0 µM after 72 h), followed by THP-1 cells (67.0 ± 3.5 µM after 24 h and 38.0 ± 3.0 µM after 72 h). HEL cells were less sensitive (92.0 ± 5.0 µM after 24 h and 58.1 ± 5.8 µM after 72 h), whereas 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 from healthy donor showed an IC_{50} of 77.1 ± 4.8 µM after 24h and 35.2 ± 0.7 µM after 72h (Figure 2).

Apoptosis/necrosis assays were performed at 24 hours using inhibitor concentrations close to the calculated IC_{50} values. K562 and HEL cells were not tested due to the lack of a relevant cytotoxicity. BAY 2965501 treatment induced a statistically significant increase in both apoptosis and, unexpectedly, necrosis across the tested cell lines (Figure 7B,8). In particular, THP-1 cells underwent early apoptosis, while HL-60 cells underwent both late apoptosis and necrosis.

These findings confirm that BAY 2965501 exerts a dose- and time-dependent cytotoxic effect in the micromolar range, although with substantial variability among different AML cell lines and the presence of insensitive cell lines. Modest sensitivity was observed even in PBLs. Consistent with previously reported effects of DGKζ-IN-4 also BAY 2965501 induces not only apoptosis but also necrosis.

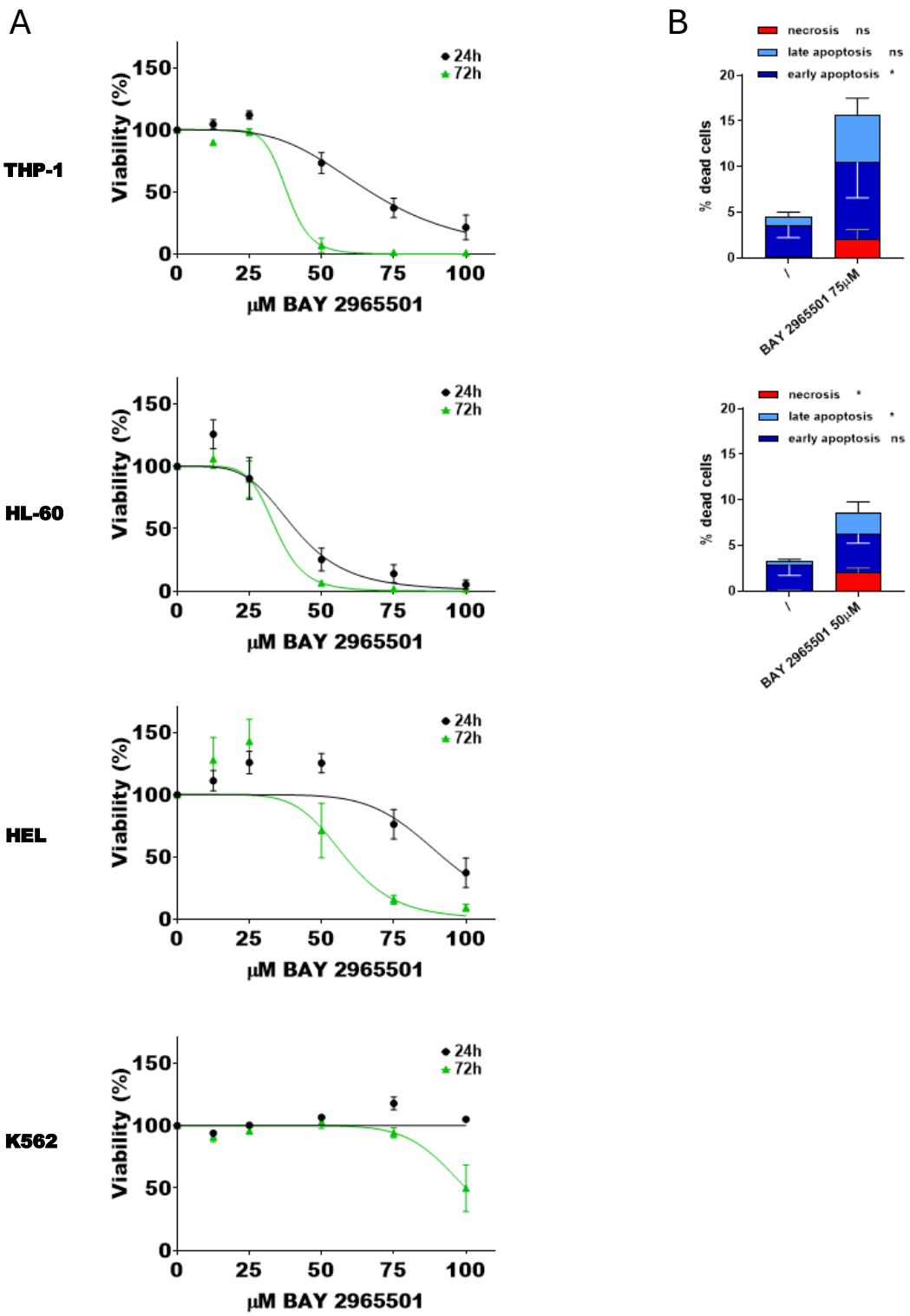

Figure 7: ***effects of BAY 2965501 on AML cell lines viability.***

A: AML cells were treated with increasing concentration of BAY 2965501 for 24 or 72 hours followed by alamarBlue viability assay for additional 24 hours. Experiments were run in quadruplicates. Data are the mean ± SEM of 5 or more experiments interpolated as [inhibitor] vs % viability.

B: AML cells were treated with concentrations of BAY 2965501 near to the IC50s obtained previously. They were analyzed by flow cytometry to detect live (Annexin V- and 7AAD-), necrotic (Annexin- and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations. Data are the mean ± SD of three or more independent experiments presenting the percentage of dead cells and the type of cell death. Two-way ANOVA test, p<0.05.*

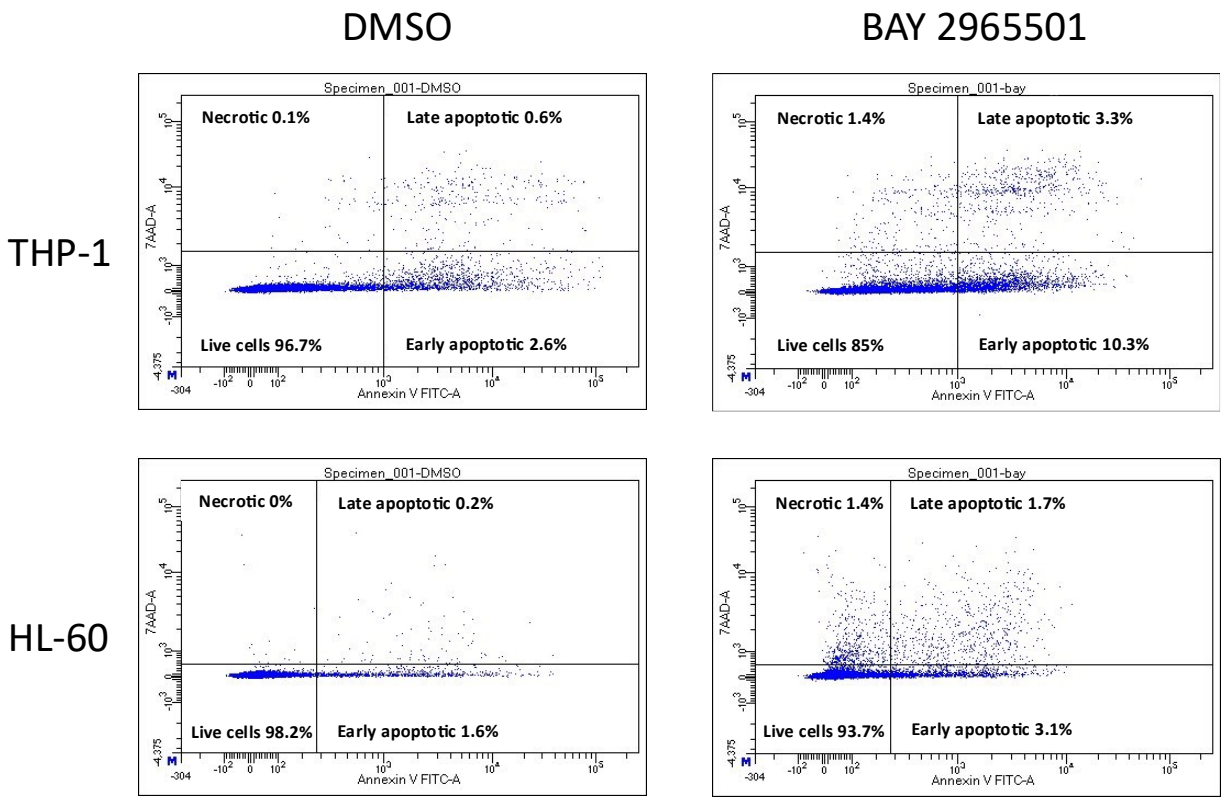

Figure 8: ***BAY 2965501 effects on apoptosis/necrosis in AML cells****.*

Dot plots of a representative experiment of cells treated with BAY 2965501, analyzed by flow cytometry and divided in the 4 subpopulations.

Cells were treated with BAY 2965501 concentrations close to IC50. After 24 h, cells were stained with Annexin V-7AAD and analyzed by flowcytometry to detect live (Annexin V-and 7AAD-), necrotic (Annexin-and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations.

5.5 Ritanserin significantly alters THP-1 proteome

The aim of our experiments was to investigate the effects of ritanserin on the proteome of THP-1 cells as these cells are sensitive to this drug. For this purpose, THP-1 cells were treated for 24 hours with ritanserin concentrations near the IC_{50} value determined in previous experiments and cells treated with equal amounts of DMSO (vehicle) were used as control. Protein extraction and quantification by mass spectrometry were done by the CAAD proteomic and metabolomic facility leaded by Professor M. Manfredi.

Analyses with the support of the CAAD bioinformatic unit, leaded by Professor D. Corà, reveals that out of the 5772 proteins detected, almost 1200 proteins were significantly downregulated while 14 were significantly upregulated (increased by at least two fold or at least halved with an adjusted P-value < 0.05). The majority of proteins are downmodulated in line with growth arrested cells undergoing apoptosis (Figure 9A). Among those, accordingly to what reported by Tan et al., we noticed a significant downregulation of PARP, Caspase3, MAPK, JAK and STAT proteins.[57] The most upregulated proteins (Figure 9A) suggest are involved in: oxidative stress, extracellular matrix remodeling, nuclear integrity/homeostasis, innate immune activation, adhesion changes, lipid metabolism shifts, and possible Notch pathway modulation.

These results revealed that more than 500 pathways were affected by ritanserin with the pathway analysis indicating that ritanserin suppresses pathways essential for MHC I mediated antigen processing and presentation, TCR signaling, cell metabolism, cell cycle progression, DNA/RNA synthesis, protein homeostasis, oxidative stress response and nucleotide excision repair (Figure 9B).

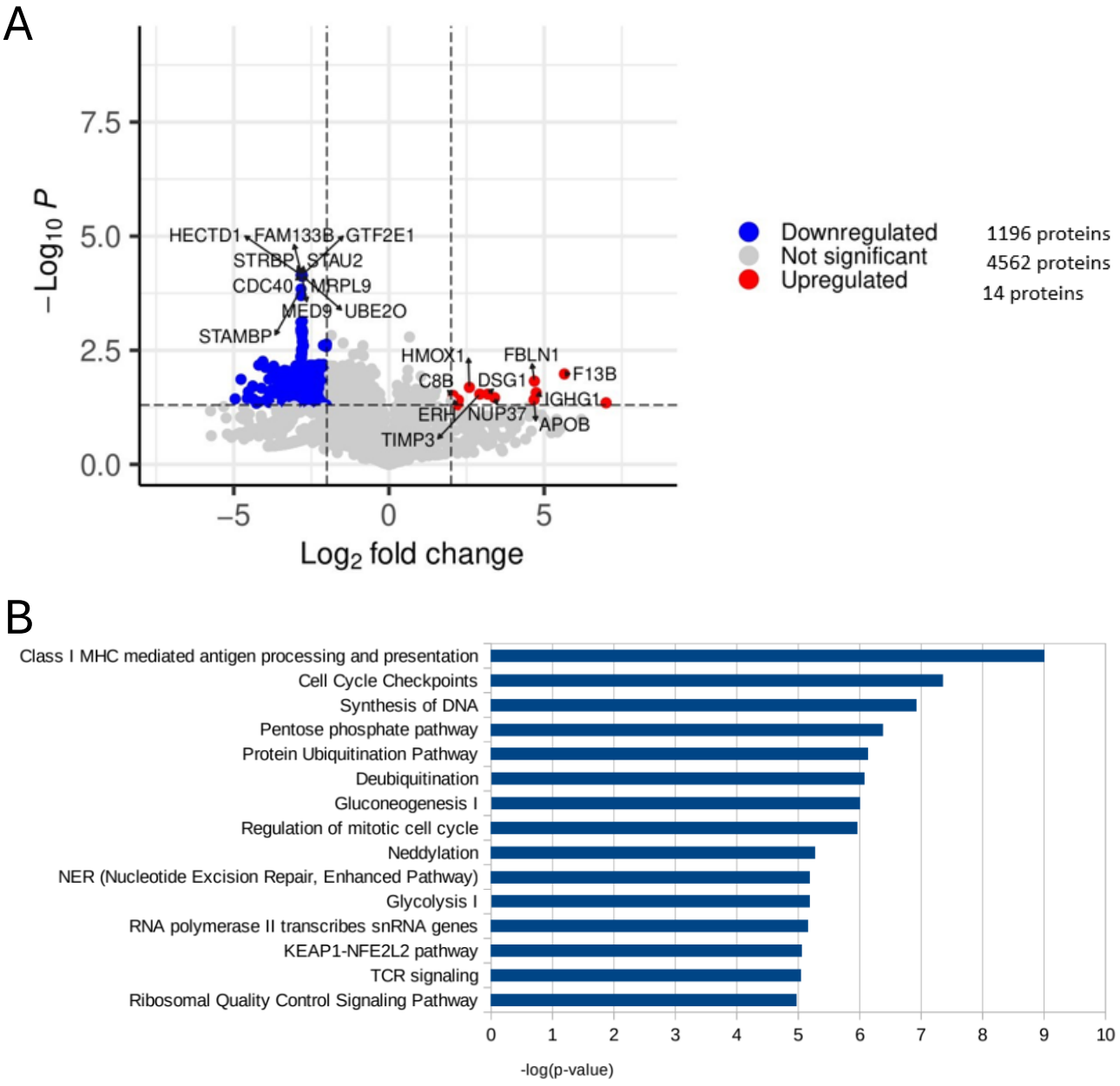

Figure 9: ***ritanserin significantly alters THP-1 proteome****.*

THP-1 cells were treated with ritanserin 50 µM for 24 hours, then washed three times with PBS and analyzed for protein content via mass spectrometry.

A: Volcano plot of the data obtained. The top 10 significantly up and downregulated proteins (log2FC > 2 or < -2, adj. p-value <0.05) are labelled.

B: Ingenuity pathway analysis of the significatively modulated genes. The top 15 downregulated pathways |Z-score|>1 are displayed.

6. DISCUSSION

AML remains a major therapeutic challenge, highlighting the need for novel, effective treatments and the identification of new molecular targets. DGKα and DGKζ play critical roles in regulating many tumorigenic features as well as in the control of immune response to tumors.[52,57,58,61,62,81,82] Inhibition of both DGKα and DGKζ has been shown to induce antitumor effects in AML cell models, including reduced cell viability, cell cycle arrest, induction of apoptosis and differentiation.[52,57,58,61,62] In this present study, we explored the effects of DGKα and DGKζ chemical inhibitors in AML cell viability across multiple AML cellular models.

Ritanserin, a DGKα inhibitor, has been shown to downregulate key signaling molecules including SphK1, JAK–STAT, MAPK, PARP, caspase-3, MCL1, and Bcl-xL in Kasumi-1 and KG-1α (AML) cell lines. Ritanserin exerted cytotoxic effects with IC_{50} values of 51 μM and 38 μM, respectively, after 24 hours of treatment. Additionally, ritanserin induced apoptosis in BMMCs derived from primary AML samples. Importantly, it also reduced tumor burden, and improved survival in mouse models.[57] In HL-60 and HEL cells, the DGKα inhibitors R59022 and R59949, structurally related to ritanserin, exerted significant cytotoxic effects in the micromolar range (IC_{50} between 30 μM and 80 μM) after 24 hours, with HEL cells displaying slightly higher resistance to both inhibitors.[52] In our experiments, we confirmed and extended these findings to additional AML cell models through cytotoxicity assays, apoptosis/necrosis analysis by flowcytometry, and proteomic profiling. Consistent with previously cited literature, ritanserin reduced cell viability across all selected AML models, with THP-1 being the most sensitive (IC_{50} ≈ 37 μM), followed by HL-60 (IC_{50} ≈ 38 μM) and HEL (IC_{50} ≈ 49 μM), while K562 cells were more resistant (IC_{50} ≈ 70 μM). Additionally, the effects of ritanserin increased with longer treatment times, demonstrating dose- and time-dependent cytotoxicity. Notably, untransformed PBLs exhibited complete cell death at a ritanserin concentration of 50 μM. This indicates that ritanserin cytotoxic effects are not restricted to AML cells and suggests that, at the concentrations required to induce AML cell death, ritanserin may exert general toxic effects on the organism.

Since ritanserin is also known as a potent serotonin receptors antagonist, we treated cells with increasing amounts of altanserin, risperidone and metoclopramide, three selective and strong serotonin/dopamine receptors antagonists. These compounds caused negligible effects on AML cell viability, with the highest observed reduction being only 11% after treatment with a

metoclopramide concentration of 100 µM. Thus, ritanserin cytotoxic effects are unlikely to be due to interference with serotonergic signaling, suggesting a specific effect on DGKα.

In line with what reported by Tan et al., apoptosis/necrosis assays revealed that cells treated for 24 hours with a concentration of ritanserin near the calculated IC_{50} led to a significant increase of cell death by apoptosis on all our tested AML models. Moreover, thanks to the CAAD proteomic and metabolomic facility, led by Professor M. Manfredi, and the CAAD bioinformatics unit, led by Professor D. Corà, we were able to study ritanserin effects on THP-1 cells proteome. The majority of proteins detected in cells treated for 24 hours with a ritanserin concentration near the IC_{50} values, were downregulated. Notably, we also noticed a downregulation of PARP, Caspase3, MAPK, JAK and STAT proteins, consistent with findings reported by Tan et al., in Kasumi-1 and KG-1α cells. The most upregulated proteins are involved in: oxidative stress, extracellular matrix remodeling, nuclear integrity and homeostasis, innate immune activation, adhesion changes, lipid metabolism shifts, and Notch pathway modulation. Ritanserin downregulated many pathways critical for MHC I-mediated antigen processing and presentation, cell metabolism, cell cycle progression, DNA synthesis and nucleotide excision repair. These pathway alterations are in line with what expected in a growth-arrested and apoptotic cell.

DGKζ-IN-4 is a selective inhibitor of DGKζ that can be used as therapeutic agent for treating cancer associated with immune cell activation, or cancer resistant to anti-PD-1 antibody/anti-PD-L1 antibody therapy. It has been reported to inhibit DGKζ with an IC_{50} of 0.4 nM while also increasing IL-2 by 10-fold in the nanomolar range. In murine models compounds structurally related give a relative tumor growth inhibition of at least 30% and are suitable for oral, intravenous or transmucosal administration.[81] In our experiments, we treated the tested AML cells with the same methods applied to ritanserin. Similarly to what observed in ritanserin, DGKζ-IN-4 reduced cell viability in some of the selected AML models with HL-60 being the most sensitive (IC_{50} of ~ 21 µM), followed by THP-1 (IC_{50} of ~ 31 µM) and HEL (IC_{50} of ~ 51 µM), while K562 showed no detectable cytotoxic effects within the tested concentration range after 24 hours and mild sensitivity after prolonged exposure (IC_{50} of ~ 33 µM after 72 hours). These are the first data available with a DGζ inhibitor in AML cell lines and suggest a potential application in this tumor type. Unfortunately, PBLs from healthy donor were sensitive to this drug with an IC_{50} of 28.7 ± 3.3 µM after 24 hours, also in this case indicating a potential general toxicity. Apoptosis/necrosis assays revealed that DGKζ-IN-4 treatment induced a statistically significant increase in both apoptosis and, unexpectedly, necrosis

across the tested cell lines apart from K562 cells that were not tested because they responded poorly to this drug. DGKζ may be a valid molecular target in AML cells although we noted heterogenic results, so a better understanding of the AML subtype sensitive to this drug is necessary.

Bayer AG in collaboration with the German Cancer Research Center (DKFZ) developed BAY 2965501, a highly selective inhibitor for DGKζ that enhances human and mouse T cell activity and overcomes T cell tolerance, negative regulation by exogenous factors (i.e., adenosine and prostaglandin E2) and T cell exhaustion.[82,83] A first in human phase I clinical trial with BAY2965501, alone or in combination with pembrolizumab, is currently under recruitment phase[85] to evaluate safety, pharmacokinetics, pharmacodynamics, and tumor response in patients with advanced non-small cell lung cancer (NSCLC), gastric and esophagogastric junction adenocarcinoma (gastric/GEJ AdCa), clear cell renal cell carcinoma (ccRCC) and melanoma.[83] Despite being under clinical trial test, no cytotoxicity studies of this compound are currently publicly available. For this matter we decided to test this compound on our AML cell models. BAY 2965501 reduced cell viability in few of the selected AML models with HL-60 being the most sensitive (IC_{50} of ~ 41 μM), followed by THP-1 (IC_{50} of ~ 67 μM). HEL and K562 cells were resistant to this drug, with minimal cytotoxic effects. Similarly to the previously described drugs, BAY 2965501 had increased effects with longer treatment time. PBLs from healthy donor showed an IC_{50} of 77.1 ± 4.8 μM after 24h and 35.2 ± 0.7 μM after 72h (Figure 2). Also in this case this drug may does affect also untransformed cells ad the concentrations utilized to kill AML cells. Apoptosis/necrosis assays revealed that treatment with BAY 2965501 induced a statistically significant increase in both apoptosis and necrosis across the sensitive cell lines. Among the tested drugs, BAY 2965501 seems to be the one which can better affect HL-60 cells with less cytotoxic effects on healthy cells. Despite that, the effective concentration to treat AML cells appears to be high, making difficult to reach effective doses in AML patients.

Nevertheless, to get a wider view on the effects of DGKζ inhibitors on AML cells, proteomic analyses should be conducted. This would allow a comparison with the previously obtained proteomic data from THP-1 cells treated with ritanserin, in order to determine whether similar pathways are affected.

Despite our data suggest that those molecules are not optimal for treating AML patients, our studies with inhibitors revealed a potential role of DGKα and DGKζ in AML that is also indicated by the literature. This role can be exploited with new compounds. As an example, a recently discovered,

dual activity DGKα/ζ inhibitor, BMS-502, is showing exciting and promising results.[50,92] While ritanserin, R59949 and R59022 have a DGKα IC_{50} > 5 μM and DGKζ IC_{50} > 10 μM[93,94], BMS-502 has a DGKα IC_{50} of 4.6 nM, DGKζ IC_{50} of 2.1 nM. BMS-502 significantly increased IFNγ production and pERK levels in human whole blood, with an EC_{50} of 280 nM, as expected with DGK inhibition.[95] In contrast, ritanserin and R59949 showed no activity at 20 μM. BMS-502 also exhibited similar results in OT-1 murine models. In proliferation assays, on human effector CD8+ T cells, BMS-502 exhibited an EC_{50} of 65 nM while ritanserin and R59949 had negligible activity at 10 μM. In mice, the compound exhibited favorable oral bioavailability, low clearance, and a long half-life while triggering an immune response only in presence of a tested peptide antigen. This indicates that BMS-502 does not directly stimulate T cells; instead, it amplifies inadequate stimulation provided by suboptimal antigen concentrations. Furthermore, DAG levels orchestrate T cell function and perform a key role in tumor immunosurveillance.[96,97]

Additionally, by blocking PD-1/PD-L1 interaction, T cells get reinvigorated. By doing this, T cells eventually become exhausted after some time and lose function if the antigen stimulation is persistent.[98,99] A similar process is suspected to be present in tumors, that will lead to exhaustion of T cells and resistance to PD-1 blockade.[100] Therefore, DGK inhibitors, by potentiating immune response, have the potential to significantly impact immune checkpoint modulation and adoptive T-cell-based immunotherapy, beside direct inhibiting tumor growth. The molecules that we tested *in vitro*, ritanserin, DGKζ-IN-4, and BAY2965501, are already known to be effective in increasing the immune response against tumors and reducing tumor burden.[57,81,83] DGKα inhibition via ritanserin synergizes with anti-PD-1 therapy in SW480 cells and in OT-I xenograft mice, affecting both T cells and tumor cells. This combination resulted in an enhanced efficacy of the therapy, delayed resistance onset to PD-1 blockade, reduced T cells exhaustion and prevented tumor cell growth.[100] DGKζ is involved in T cell exhaustion as well[101] and increased expression of this enzyme was observed in exhausted OT-I T cells from tumors resistant to PD-1 treatment.[100] DGKζ-IN-4 resulted efficient in treating PD-1 blockade resistant tumors and reduced tumor burden in mice.[81] Therefore, simultaneous inhibition of DGKα and DGKζ in T cells may represent a powerful strategy to combine with PD-1 blockade therapies. Considering the complexity and immunological activity of the tumor microenvironment, the compounds that we examined in AML *in vitro* could retain therapeutic effectiveness *in vivo*, even at lower concentrations, since additional actors are involved in the overall tumor response. Moreover, these molecules may augment the efficacy and persistence of CAR-T cells therapies, already approved for certain leukaemias[102] and currently under clinical investigation

for AML[103], especially within immunosuppressive tumor environments. Altogether, this evidence supports the further preclinical evaluation of DGKα/ζ inhibitors in AML, not merely as cytotoxic compounds but as immunologic adjuvants capable of amplifying both innate and engineered anti-leukemic immunity.

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