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Title:

THE POTENTIAL OF ALDO-KETO REDUCTASE 1C3 INHIBITION AS AN OFF-TARGET EFFECT OF ISOCITRATE DEHYDROGENASE 2 INHIBITOR ENASIDENIB IN LEUKEMIA

Master's Thesis

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Consultant: Anselm Morell García, Ph.D.

Hradec Králové 2024 Melodie Haddad

DECLARATION

"I declare that this thesis is my original work. All literature and other sources from which I have drawn during the processing of this thesis are listed in the bibliography and properly cited in the text. The thesis has not been used to obtain any other or the same degree."

In Hradec Králové 2024

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Melodie Haddad

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ABSTRACT

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Acute myeloid leukemia (AML) involves an excessive proliferation of clonal myeloid blasts. Several drugs targeting AML mutations are under study, with enasidenib (ENA) as the first selective inhibitor of isocitrate dehydrogenases 2 (IDH2) mutants approved by the FDA. A current multicenter clinical trial has shown encouraging results of combining ENA with standard intensive induction therapy, including daunorubicin (Daun). However, expression in AML cells of ATP-binding cassette (ABC) transporters and the aldo-keto reductase family member 1C3 (AKR1C3) may conduct extrusion and hydroxylation of Daun, respectively, that lessens Daun's cytotoxic effects. This study aimed to evaluate the effect of ENA on Daun accumulation and/or inactivation in the leukemic cell line KG1α. ENA produced a synergistic effect on Daun cytotoxicity in KG1α cells related to its higher accumulation and lower metabolization. Furthermore, AKR1C3 also catalyzes the conversion of prostaglandin D2 (PGD_2) to 9α and 11β-prostaglandin F2α (11β-PGF2α), which induces the proliferation of leukemic blasts. This work also provides a preliminary determination of ENA inhibiting AKR1C3 metabolization of PGD_2 as well as KG1α cells proliferative induction by PGD_2. In conclusion, this work deciphered novel IDH2-independent molecular targets for ENA that support its potential effectivity against AML without IDH2 mutations as well as its combination with standard chemotherapeutics like Daun in AML therapy.

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1 Introduction

Multidrug resistance is a challenge in anti-cancer chemotherapy treatment, especially in the context of Acute Myeloid Leukemia. It refers to the ability of cancer cells to develop resistance to multiple drugs with different structures and mechanisms of action. Resistance can be primary, genetically determined, or caused by changes in cancer cells during treatment.

AML is a type of cancer that affects the blood and bone marrow, characterized by the rapid growth of abnormal white cells. Hence, accumulating in the bone marrow affects the production of healthy blood cells, including red blood cells, white blood cells, and platelets. While chemotherapy is the primary treatment for AML, multidrug resistance (MDR) poses a significant challenge to successful therapy. MDR in AML can arise through increased drug efflux transporters such as P-glycoprotein, altered drug metabolism, DNA repair mechanisms, apoptosis resistance, and other ways. These mechanisms collectively reduce the effectiveness of chemotherapy drugs, leading to treatment failure and disease relapse.

Aldo-keto reductase 1C3 (AKR1C3), a member of the AKR superfamily is among the factors influencing MDR in AML. It has been implicated in both anthracycline metabolism and leukemogenesis (cell proliferation); two critical aspects of AML biology and treatment. Anthracyclines, such as Daun and idarubicin, are cornerstone chemotherapeutic agents in AML. AKR1C3, known for its role in anthracycline metabolism, can contribute to drug resistance by catalyzing the reduction of anthracyclines to less cytotoxic metabolites.

Understanding the interplay between AKR1C3, anthracycline metabolism, and leukemogenesis, in addition to targeting AKR1C3-mediated pathways, hold a promising role in improving AML treatment outcomes.

This thesis seeks to investigate the impact of the isocitrate dehydrogenase (IDH) inhibitor enasidenib on the function of AKR1C3 in the context of AML development and treatment.

2 THEORETICAL BACKGROUND

2.1 ALDO-KETO REDUCTASES

Aldo-keto reductases (AKRs) are a superfamily of NAD(P)(H) —dependent enzymes with a pivotal role in mammalian metabolism. Due to their substrate versatility, these enzymes participate in phase I metabolic reactions, yielding the biotransformation of several xenobiotics and drugs (Yaqoob et al., 2022).

Through phase I metabolism, the lipophilic compounds are processed by the involved enzymes to generate a more polar compound, enabling conjugation reactions in phase II. In phase II, conjugating enzymes increase hydrophilicity that compound by the addition of hydrophilic groups, ready to be eliminated from the body through passive and active transport. Numerous enzymes and pathways could participate in this whole process. However, the determining factors that often decide which metabolic reactions dominate in each individual are enzyme affinity, quantity, cofactor availability, and the relationship between chemical concentrations. In this context, AKRs perform oxido-reduction reactions, mostly catalyzing the detoxification of carbonyl groups.

AKRs are mainly active in the cytoplasm and expressed in almost all mammalian tissues, being more active in the kidney and liver. More than 190 members of this superfamily have been discovered to this day and they are grouped into 16 families named from AKR1-AKR16, and some families are furthermore divided into subfamilies (Jez et al., 1997).

2.1.1 Aldo-keto reductases nomenclature

The nomenclature for AKR was introduced and accepted in 1997 by the Hugo Genome Nomenclature Committee.

A given example would be AKR1C3 in which.

- AKR – stands for the superfamily of aldo-keto reductases
- 1 – represents the number of the family ranging from 1-16
- C - represents the subfamily
- 3 - represents the unique protein sequence within the family

Forty percent similarity in amino acids is found in each family, while sixty percent is found in

subfamily members. The amino acid sequence is unique to each of the proteins (Mindnich & Penning, 2009).

2.1.2 AKRs structure

Regarding AKRs' structure, they present eight beta-sheet layers forming the core and eight alpha helices covering it, forming a barrel shape. Between the beta-sheets and alpha helices there are three flexible loops defining the shape and flexibility of the catalytic site, including a binding region for the NAPDH cofactor (Figure 1). These two structural features are shared by more than one hundred structurally described AKRs. It is thought that the structure originates from an already extinct multifunctional ancestor. It is compact and, at the same time, adapts itself to multiple structural variations, allowing the binding of a wide, chemically diverse range of substrates, especially containing carbonyl groups (Amaro et al., 2003).

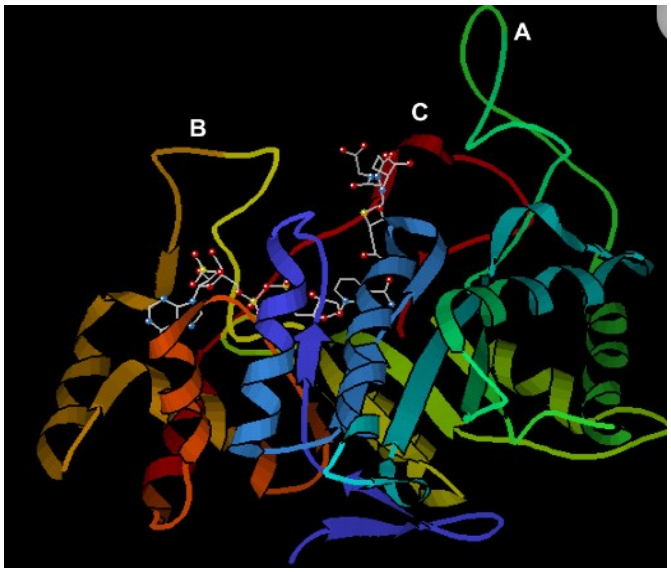


Figure 1. Side-view representation of the protein with NADPH bound to the catalytic site at loop B. The catalytic site loops are marked – A, B, and C.

2.1.3 AKRs functions

Aldo-keto reductases involvement in physiological functions like metabolism, biosynthesis, and detoxification:

Prostaglandins: The synthesis of prostaglandins starts with the conversion of arachidonic acid into PGH2 by cyclo-oxygenase. PGH2 is then converted into PGD_2, PGE2, PGI2, or thromboxane A4. PGF synthase, which belongs to the AKR superfamily and is known as

AKR1C3 in humans, catalyzes the conversion of PGH2 to PGF2α. This enzyme also catalyzes the conversion of PGD_2 to 9α,11β-PGF2, which is the active form of PGF2. AKR1C3 is primarily found in the lungs and spleen and in smaller amounts in kidneys, skeletal muscles, leukocytes, uterus, and liver. It has been shown that defects in AKR1C3 correlate with urinary constriction, contraction of coronary arteries, and increased blood pressure (Urade et al., 1990). Other AKRs, such as AKR1C1 and AKR1C2, can also participate in this process (Figure 2) (Dozier et al., 2008).

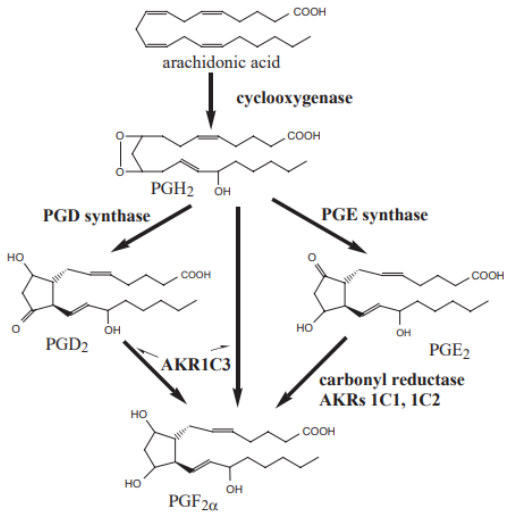


Figure 2. Prostaglandins synthesis. Enzymes belonging to the AKRs participate in the conversion of PGH2, PGD_2, and OGE2 to PGF2.

Lipoperoxidation: Aldo-keto reductases play an essential role in the detoxification of the resulting metabolites of oxidative stress. Under oxidative stress, the production of reactive oxygen species (ROS) and free radicals increases. ROS can attack polyunsaturated fatty acids (PUFAs), leading to the formation of unstable products due to lipid peroxidation. Carbonyls and aldehydes, especially the toxic hydroxy-2-nominal (4-HNE) and the C5 esterified aldehyde [1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine] (POVPC) are notably among the most dangerous products generated, that was proven to increase the incidence of Alzheimer's disease, diabetes mellitus, atherosclerosis, and heart failure. AKR1B1 catalyzes the reduction of HNE and POVPC in addition to a wide spectrum of saturated aldehyde. AKR1C1-4 and AKR1A1 also participate in the detoxification of 4-HNE. What is uniqe about the reduction reaction is that AKRs can perform it in a single-step reaction (Penning & Drury, 2007).

Advanced glycosylated end products (AGEs): AGEs arise from protein modification and carbohydrate introduction or their metabolites having one or multiple reactive carbonyl groups in a process described as “carbonyl stress” by Izuhara in 2002 (Izuhara et al., 2002). The present reactive carbonyl groups form Schiff bases with amino groups, which then progress into Amadori products. Further oxidative and non-oxidative reactions lead to the formation of AGEs. Natural aging and diseases like diabetes mellitus, atherosclerosis, renal failure, hemodialysis-associated amyloidosis, and Alzheimer’s disease result in the accumulation of AGEs. Major reactive carbonyl compounds derived from carbohydrates include glyoxal, methylglyoxal, D-arabinose, and 3-deoxyglucosone (3-DG). Detoxification of these reactive carbonyl compounds will be through reduction, conjugation, or oxidation reactions. Specifically, the reduction of methylglyoxal is performed by AKR1B1 and AKR1A1 (Verzijl et al., 2002).

Steroids: Members of the AKR1C family are actively engaged in both biosynthesis and metabolism of steroid hormones. They are enzymes called 3α-hydroxysteroid dehydrogenases, also known as di-hydro diol dehydrogenases. These enzymes differ in substrate specificity and tissue distribution, which mirror their diverse contribution to the generation and inactivation of sex hormones. Located in the liver, AKR1C4 is well suited for the role of converting dihydro steroids into tetra hydro steroids, thereby inactivating circulating steroid hormones. AKR1C isoforms in steroid-responsive tissues contribute to either a pro-estrogenic or a pro-androgenic state, orchestrating the occupancy of the receptors. In the prostate, AKR1C2 and AKR1C3 reduce dihydrotestosterone to the weaker androgenic metabolite 3α-diol. A decline in AKR1C2 levels showed a significant increase in prostate-related diseases, especially cancer. AKR1C3 on the mammary glands contributes to a pro-estrogenic state by converting delta-androstane-3,17-dione to testosterone, which can be aromatized to progesterone and estrogen precursors. AKR1C1 in the ovaries reduces progesterone to the inactive metabolite 20α-hydroxyprogesterone. Diminished levels of AKR1C1 yield lower progesterone catabolism, therefore increasing the risk of ovarian cancer. Members of AKR1A and 1B family also play a role in the reduction of corticosteroids, like iso-corticosterone and iso-cortisol, which have an aldehyde group at the position C-21 and a hydroxyl group linked to C-20 (Ji et al., 2003).

Glucose: AKR1B1 converts glucose to sorbitol in cells, especially Schwann cells and cataractous lenses, causing complications in patients with diabetes mellitus due to sorbitol

accumulation. Thus, studies have been conducted on AKR1B1 inhibition. However, it also plays a role in removing toxic products resulting from lipid peroxidation. Therefore, inhibiting AKR1B1 is a setback rather than an advantage (Song, 2003).

Glucuronate-Xylose pathway and vitamin C biosynthesis: AKR1A serves as a catalyst for the conversion of D-glucuronate into L-gluconate, a pivotal step in the biosynthesis of vitamin C. Moreover, L-gluconate participates in the pentose phosphate pathway following its transformation into D-xylulose-5 phosphate. This pathway's activity is high in the renal cortex due to the abundance of AKR1A there (Barski et al., 2005).

Succinic semialdehyde: GABA neurotransmitter is metabolized by an enzyme called monoamine oxidase, forming the intermediate called succinic semialdehyde. Succinic semialdehyde is then metabolized in two ways. The first one to succinic acid is by the enzyme succinic semialdehyde dehydrogenase (SSADH). The second way is to gamma-hydroxybutyrate (GHB) by the aflatoxin and aldehyde reductases. Low levels of SSADH are related to seizures and mental retardation (Buzzi et al., 2006).

Nuclear receptor signaling: Aldo-keto reductases possess the capacity to metabolize a wide range of hydrophobic compounds, such as ketosteroids, keto-prostaglandins, and retinals. Consequently, these enzymes play a significant role in modulating the availability of ligands to nuclear receptors, ultimately impacting the process of gene transcription. The main receptors involved:

-PPAR-gamma: This receptor is a master of adipogenesis and can be influenced by AKR1C3 in two ways. The first one is a compound produced by AKR1C3 called 15-deoxy-Δ12,14-PGJ2α, which binds to PPAR-gamma and alters its activity. The second way is AKR1C3 phosphorylating PPAR-gamma, resulting in an increase in cell growth and proliferation (Penning & Drury, 2007).

-RXR: AKR1B10 has been implicated in the signaling of retinoic acid. Aldehyde dehydrogenase (ALDH) catalyzes the transformation of retinal to retinoic acid. Retinals can also be reduced to retinol by AKR1B10. This conversion hinders the generation of 9-cis-retinoic acid, a vital ligand for RXR, therefore affecting RXR heterodimerization with PPAR-gamma. This results in an anti-proliferative response. It is worth noting that AKR1C3 and AKR1B10 might achieve this by individually targeting distinct receptors within the

heterodimeric complex formed by PPAR-gamma and RXR. These processes raise the demand for selective inhibitors to these enzymes as a target for novel anti-proliferative agents (Penning & Drury, 2007).

-CAR, PXR, and FXR: AKR1D1, expressed in the liver, plays an important role in converting steroid molecules into different compounds that bind to receptors like CAR, PXR, and FXR. This process leads to an increase in the activity of specific genes, especially those involved in detoxifying drugs, like cytochromes (CYPs) (Penning & Drury, 2007).

-Aryl-hydrocarbon receptor (AhR): AKR1A1 and AKR1C1-AKR1C4 participate in the di-hydro diol dehydrogenase activity which generates products like o-quinones. These planar polycyclic aromatic hydrocarbons influence the aryl-hydrocarbon receptor leading to its activation. Activation of AhR leads to the induction of the CYP1A1 gene in human hepatoma cells or CYP1A1 and CYP1B1 in bronchoalveolar H358 cells (Penning & Drury, 2007).

AKRs function towards xenobiotics:

AKRs are known to be involved in the metabolic processing of three distinct classes of chemical carcinogens: nicotine-derived into aminoketones (NNK), polycyclic aromatic hydrocarbons (PAHs), and aflatoxin.

-Nicotine-Derived into aminoketones (NNKs): NNKs that arise from nicotine in tobacco are lung carcinogens. They are activated by α-methylene hydroxylation performed by P450 2A6 and 3A4. Or their activity can be neutralized by the reduction into nicotine-derived nitrosamino-alcohol (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol), known as R-NNAL and S-NNAL. The presence of the alcohol group facilitates the elimination process by glucuronidation. What is interesting is that S-NNAL is comparable to NNK's carcinogenic potency. And is formed by the AKR1C1, 1C2, and 1C4 enzymes. On the other hand, R-NNAL isomer does not have carcinogenic properties (Penning & Drury, 2007).

-Polycyclic aromatic hydrocarbons (PAHs): PAHs are widespread environmental pollutants that need to be activated to exert their carcinogenic effects. The activation process is best exemplified by the representative compound benzo[a]pyrene. A key pathway proceeds by P4501A1/b-mediated monooxygenation on the terminal benzo-ring, resulting in the formation of the 7R,8S-arene oxide. Epoxide hydrolase then hydrolyzes oxide to yield (-)7R,8R-

dihydroxy-7,8-dihydro-B[a]P, known as B [a]P-7,8-dihydrodiol. Subsequent monooxygenation of B[a]P7,8-dihydrodiol leads to the formation of (+) anti-B[a]P-diol-epoxide, a highly carcinogenic compound. Another pathway proceeds by the activation of B[a]P through the di-hydro diol dehydrogenase activity of the human AKRs (AKR1A1, 1C1-1C4), which oxidizes the B[a]P-7,8-di-hydrodiol resulting in a ketol which rearranges to form a catechol. This catechol can undergo a 1-step oxidation to yield the fully oxidized o-quinone. O-quinone can react with DNA, RNA, proteins, GSH, and amino acids. It is also redox-active and capable of cellular NADPH depletion and ROS production intensification (Penning & Drury, 2007).

Aflatoxin: aflatoxin is a potent carcinogen. It is activated through P450 3A4 to produce aflatoxin B1-8,9-epoxide, an ultimate carcinogenic form. Epoxide hydrolase hydrates B1-8,9-epoxide to generate aflatoxin-8,9-dihydrodiol, which then undergoes ring opening to form a cytotoxic dialdehyde. This aldehyde has the capability to create protein adducts by reacting with the ε-amino group of lysine residues. Aflatoxin aldehyde reductases, known as the AKR7A family, play a role in the reduction of this dialdehyde to mono- and di-alcohols. Mainly, AKR7A2 and AKR7A3 are responsible for this action. This process helps provide protection against the hepatotoxic effects of aflatoxin (Penning & Drury, 2007).

Therapeutic association:

Many drugs, such as anticancer, antipsychotic, opioid antagonists, antidiabetics, antidepressants, and antiemetics, have a carbonyl group, mainly a ketone. This is a great substrate for AKRs. In this sense, AKRs can activate drugs such as antiemetics, like dolasetron. However, for anticancer drugs, AKRs decrease their effectiveness. Levels of AKRs in the human body vary in each individual. Therefore, understanding precisely their role in drug metabolism helps prescribe the right medication and monitor drug toxicity and efficacy.

2.2 ALDO-KETO REDUCTASE 1C3

2.2.1 AKR family 1

AKR family 1 exerts high control over the levels of active ligands for nuclear receptors. They play a pivotal role in regulating ligand occupancy and the subsequent transactivation of these receptors. Beyond this, AKR1 enzymes have a direct influence on the concentration of neuro-steroids, which in turn modulate the activity of N-methyl-d-aspartic acid (NMDA) and gamma-aminobutyric acid (GABA) receptors. This multifaceted role positions AKR1 enzymes as integral participants in the pre-receptor modulation of both nuclear and membrane-bound receptors. Any deficiency or dysregulation of specific AKR1C genes has been associated with the emergence of prostate, breast, and endometrial cancers (Internet 1).

2.2.2. AKR1C3

AKR1C3 is also known as 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5). It belongs to the family number 1-member C. Its identification and synthesis were initially achieved using a human prostate cDNA library. AKR1C3 operates functionally as an NADP(H)-dependent soluble monomeric oxidoreductase (Figure 3). It also shares different percentages of sequence identity with other representatives like AKR1C1, AKR1C2, and AKR1C4 (Penning, 2019).

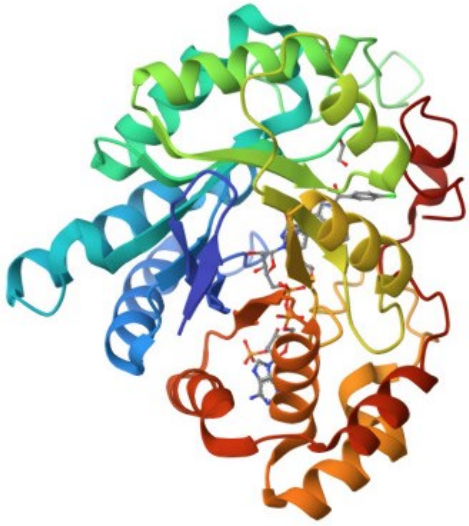


Figure 3. Crystal structure of AKR1C3, complexed with an inhibitor.

2.2.3 AKR1C3 Functions and relation to cancer

AKR1C3 plays important roles in the metabolic and biosynthetic processes of various hormones, such as estrogen, androgen, progesterone, and prostaglandins.

-Prostaglandin D2 (PGD_2) and prostate cancer. AKR1C3 is involved in the conversion of PGD_2 into prostaglandin F2α (PGF2α). Subsequently, enhancing prostate cell proliferation by engaging G-protein coupled receptors specific to PGF2α and activating the phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) signaling pathway. It is evident that elevated levels of AKR1C3 are recognized for the capacity to eliminate reactive oxygen species (ROS) and foster PGF2α accumulation. This, in turn, urges prostate cancer cell (PCa) proliferation, in addition to reinforcing PCa cell resistance against radiation. This process is achieved through the activation of the mitogen-activated protein kinase pathway, which is characterized by the increased expression of p-MEK (phosphorylated mitogen-activated protein/extracellular signal-regulated kinase) and p-ERK (extracellular signal-regulated kinase), coupled with diminished PPAR-gamma expression. Furthermore, what directly regulates AKR1C3 expression in PCa cells is the transcription factor ETS-related gene (ERG). ERG also contributes to cell migration, invasion, epithelial-to-mesenchymal transition (EMT), and androgen receptor signal transduction.

-AKR1C3 in its pivotal role in androgen synthesis, and furthermore its role in PCa receptor activation. It has been shown that the AKR1C3 gene is overexpressed in aggressive PCa lines. Elevated levels of AKR1C3 have the potential to enhance the conversion of PGD_2 to 11b-PGF2α. This process, in turn, facilitates the activation of transcription factors associated with proliferation, such as the nuclear factor kappa-B (NF-κB) complex. Further research has demonstrated that the increased expression of AKR1C3 can amplify the viability and angiogenic properties of PC-3 cells (a specific human prostate cancer cell line). Here, we reach the importance of highlighting androgen and estrogen metabolism in regulating AKR1C3-mediated tumor angiogenesis (Xiao et al., 2021).

-Breast cancer. Breast cancer patients showing increased AKR1C3 expression experience a more unfavorable prognosis compared to those with lower levels of expression. This enzyme, AKR1C3, can elevate the ratio of 17β-estradiol to progesterone within breast tissue. Additionally, the production of PGF2α epimers has been established as a mechanism that activates PGF receptors, thereby reducing the availability of anti-proliferative prostaglandin J2

(PGJ2) ligands for PPAR-gamma. Another investigation unveiled that 11β-PGF2a, generated through the catalytic activity of AKR1C3, phosphorylates ERK and cAMP response element-binding protein (CREB), subsequently prompting the overexpression of slug (a highly conserved zinc finger transcriptional repressor) in RBC cells via the PGF2α.

This process ultimately renders RBC cells less responsive to chemotherapeutic agents. This investigation confirmed the potent influence of 11 β-PGF2α on slugs, which is intricately associated with epithelial-to-mesenchymal transition (EMT). Moreover, there is evidence demonstrating that the upregulation of AKR1C3 led to the loss of the tumor suppressor Phosphatase and tensin homolog (PTEN), resulting in a notable elevation of activated AKT. This cascade of events underscores the multifaceted impact of AKR1C3 on key signaling pathways and cellular processes in the context of breast cancer (Xiao et al., 2021).

-Endometrial cancer. In the context of endometrial cancer, AKR1C3 assumes a pivotal role in influencing estrogen levels. The actions of estrogen and progestin undergo regulation at both receptor and pre-receptor tiers. This entails modulation through the expression of estrogen and progesterone receptors and, on a pre-receptor level, involves the transformation of active hormones into their inactive counterparts. The presence of AKR1C1 and AKR1C3 within endometrial cancer establishes the balance between pregnenolone and estradiol, thereby exerting a discernible impact on the advancement of endometrial carcinoma. Moreover, it has been shown that various endometrial cancer cell lines manifest sensitivity to the growth-retarding influence of 15-deoxy-Δ 12,14-PGJ2, a recognized ligand for PPAR-gamma. Notably, 15d-PGJ2 significantly enhances the expression of AKR1C3 protein across three endometrial cancer cell lines, consequently inducing a G2 phase arrest in the endometrial cancer cell cycle (Xiao et al., 2021).

-Urinary bladder carcinoma. Regarding the interrelation of AKR1C3 and urinary bladder carcinoma (UBC), the studies highlighted a robust correlation between the susceptibility to UBC and genetic variations in genes pertinent to the metabolic processing of polycyclic aromatic hydrocarbons (PAHs) and aromatic amines. Studies have examined 65 SNPs within 15 selected genes recognized for their activation by tobacco-derived carcinogens. These genes play a pivotal role in orchestrating the transcription of metabolic genes or encoding products that facilitate the activation or detoxification of PAHs or aromatic amines. The outcomes of the study unveiled that hereditary diversity implicated in the metabolism of carcinogens,

prominently including AKR1C3, could potentially underline the elevated UBC risk (Xiao et al., 2021).

-AML. AML is a disorder that primarily affects the bone marrow, manifesting as an aberration in hematopoietic stem cells resulting from genetic modifications in precursor blood cells. This leads to the excessive generation of neoplastic clonal myeloid stem cells. While occurrences of extramedullary manifestations can arise, such as myeloid sarcomas or leukemia cutis, the fundamental pathology stems from irregularities in hematological cellular production. Mainly, genetic anomalies play a role in causing the disease. These anomalies manifest as either chromosomal abnormalities or isolated gene mutations, often without apparent causal agents. Diagnosis of AML can be symptomatic; patients may present with symptoms like infections, bleeding tendencies, fever, lethargy/fatigue, pallor, or disseminated intravascular coagulation. It can also be shown in blood tests. Most accurately, to confirm diagnosis is by the examination of the bone marrow.

AML is additionally categorized into three distinct prognostic risk groups: favorable, intermediate, and adverse. These classifications are informed by a combination of cytogenic profiles and the relatively recent identification of molecular disease (Table 1).

Table 1. Classification of risk profiles based on molecular and cytogenic abnormalities (Döhner et al., 2017).

Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1;
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD/low FLT3-ITD
	Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD(high)
	Wild-type NPM1 without FLT3-ITD/ low FLT3-ITD (normal karyotype)
	t(9;11)(p21.3;q23.3)MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t(9;22)(q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	-5 or del(5q); -7; -17/abn(17p)
	Complex karyotype
	Monosomal karyotype
	Wild-type NPM1 and high FLT3-ITD
	Mutated RUNX1
	Mutated ASXL1
	Mutated TP53

Adapted from Blood 2017 129:424-447

AML continues to be a rare yet highly fatal malignancy. Timely diagnosis coupled with swift evaluation of cytogenic and molecular anomalies (such as NPM1 and FLT-3 mutations) assumes paramount importance in tailoring the most effective treatment for patients. While chemotherapy stands as the foundation of treatment, stem cell transplantation remains the most promising avenue for achieving a cure, particularly for patients harboring adverse cytogenic risk profiles (Vasconcelos et al., 2021).

AKR1C3 performs an important role in modulating the differentiation, proliferation, and programmed cell death (apoptosis) of myeloid cells. Investigations into AML have demonstrated that overexpression of AKR1C3 hampers the capacity of AML cells to undergo differentiation when prompted by all-trans retinoic acid (ATRA). Conversely, the downregulation of AKR1C3 in AML cells has been established as a mechanism promoting differentiation. A study revealed that a combined therapy involving AKR1C3 inhibitor

alongside either etoposide (a topoisomerase II inhibitor) or Daun (an anthracycline) produced a potent adjuvant effect in an AML cell line. This synergy led to a remarkable enhancement in the cytotoxicity of Daun (over 10-fold) and etoposide (up to 6.25-fold).

AKR1C3 orchestrates diverse effects on tumor cells through intricate signaling pathways, yielding a complex array of outcomes (Figure 4). AKR1C3's influence spans the promotion of tumor cell proliferation, radio resistance, enhanced survival mechanisms, initiation of epithelial-to-mesenchymal transition (EMT), fostering metastasis, driving angiogenesis, and facilitating invasion. Simultaneously, it inhibits apoptosis and thwarts the differentiation of tumor cells, all achieved through the following key signal pathways and interactions. The intricate interplay of these pathways underscores the complexity of AKR1C3's role in tumor biology, exerting multifaceted effects on a spectrum of cellular behaviors (Xiao et al., 2021).

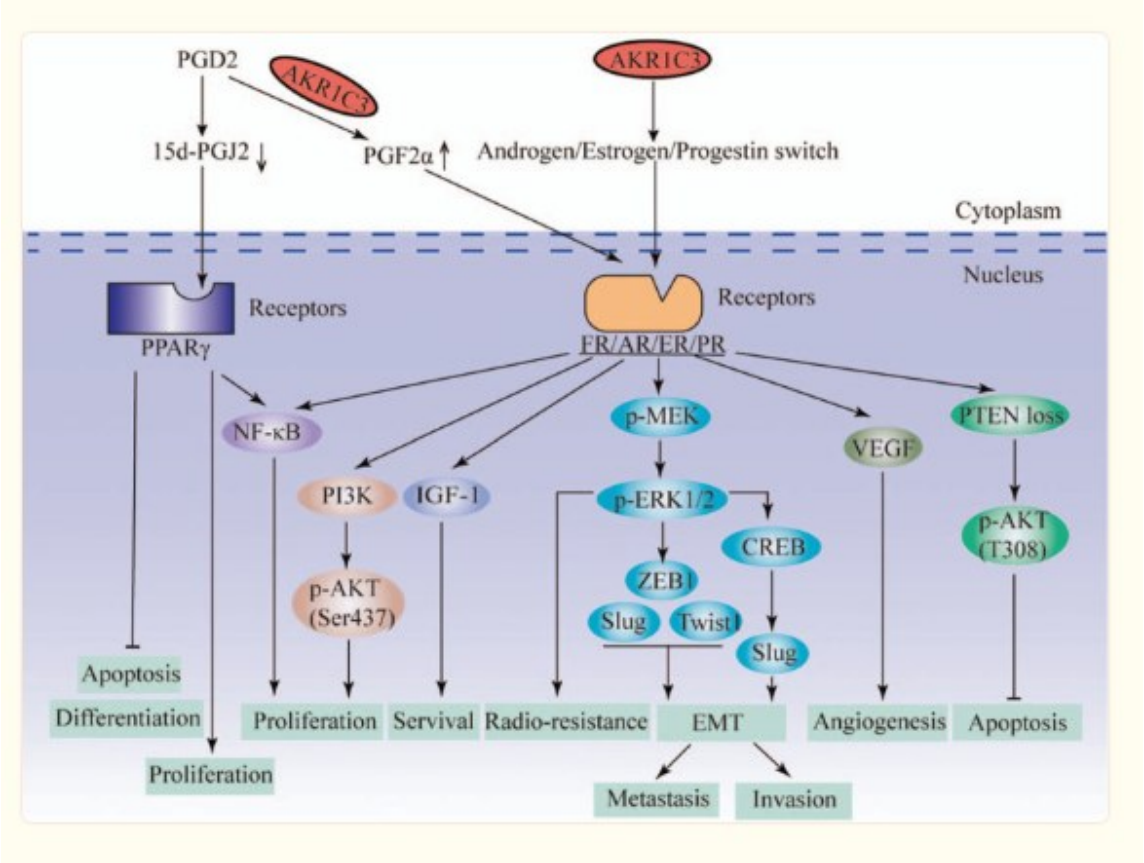


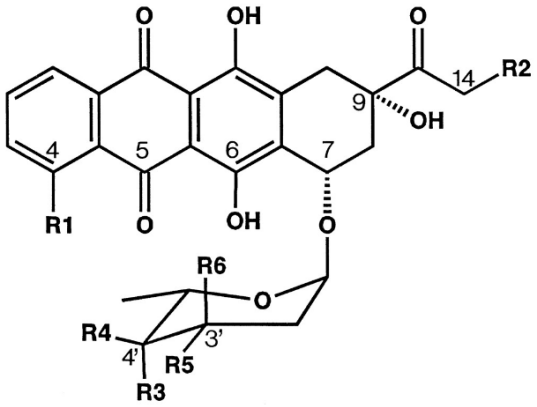
Figure 4. Overview of major pathways governing AKR1C3's impact on tumor cells.

In conclusion, a mounting body of evidence substantiates the pivotal role of AKR1C3 in malignancies. The dysregulation, whether heightened or diminished, of AKR1C3 expression

transcends the boundaries of hormone-dependent and hormone-independent tumors. Hormone-dependent malignancies encompass prostate cancer (PCa), breast cancer, bladder cancer, and endometrial cancer. While the hormone-independent category encompasses AML, esophageal cancer, lung cancer, gastric cancer, and brain tumors. The mechanisms through which AKR1C3 impacts malignant tumors are intrinsically linked to the multifaceted nature of this enzyme. Nonetheless, manipulating AKR1C3 expression via knockdown or overexpression in cancer cells could elucidate its potential impact on diverse facets of tumor behavior (Xiao et al., 2021).

2.3 ANTHRACYCLINES

Anthracyclines constitute a class of pharmaceutical compounds derived from Streptomyces bacterium, and they find application in the treatment of diverse cancers. These pharmaceutical agents stand out as some of the most efficacious and potent anticancer interventions available. The distinct varieties employed for therapeutic interventions encompass Daun, Doxorubicin (Dox), Epirubicin, Valrubicin, Mitoxantrone, and Idarubicin. Administered via injections, intravenously, intraarterially, and intravesical application (Internet 2). They are implemented in treating various malignancies, including leukemia, aggressive lymphomas, lung cancer, breast cancer, non-Hodgkin's lymphoma, and multiple hematologic and solid tumors (Dhingra et al., 2017).



	Compound	R1	R2	R3	R4	R5	R6
I	Doxorubicin	OCH_3	OH	OH	H	NH_2	H
II	Daunorubicin	OCH_3	H	OH	H	NH_2	H
III	Idarubicin	H	H	OH	H	NH_2	H

Figure 5. Anthracycline antibiotics chemical structure (Binaschi et al., 1997).

2.3.1 Anthracyclines mechanism of action

The mechanisms behind the cytostatic and cytotoxic effects of anthracyclines include several distinct pathways:

Enzymatic interaction: The prevailing and widely embraced rationale behind anthracyclines' action resides in their interaction with topoisomerase-II. This interaction yields a ternary complex, effectively hindering the rejoining of double-stranded DNA breaks. Therefore, cellular growth arrest occurs, ultimately culminating in apoptotic cell demise (Internet 2).

-DNA intercalation: Anthracyclines are equipped with a chromophoric unit that possesses an intercalative capacity **(Figure 6)**. When these compounds localize within the cell nucleus, they insert themselves between adjacent base pairs of DNAs. This intercalation impedes the processes of DNA and RNA synthesis, particularly in vigorously replicating cells, thereby obstructing cell division (Internet 2).

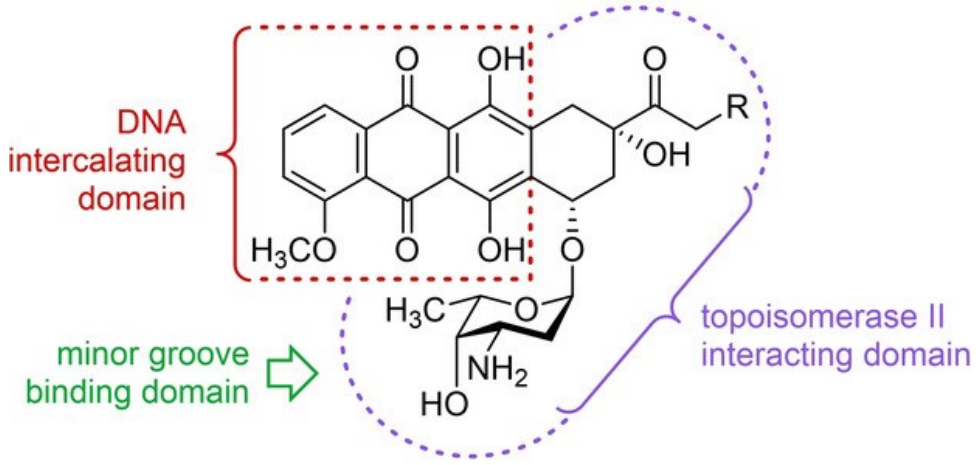


Figure 6. Anthracyclines structural parts. Including the DNA intercalating domain, topoisomerase II interacting domain, and minor groove binding domain (Martins-Teixeira & Carvalho, 2020).

Reactive oxygen species regeneration: Through redox reactions facilitated by enzymes such as cytochrome P450 reductase, NADH dehydrogenase, and xanthine oxidase, reactive oxygen species (ROS) are generated. Excess ROS cannot be effectively neutralized, leading to a state of oxidative stress. This stress results in DNA impairment and lipid peroxidation and ultimately initiates cell apoptosis (Internet 2).

Formation of DNA adducts: These pharmaceutical agents are recognized for their capability to form adducts with DNA, often facilitated by pro-drugs that release formaldehyde. The resultant adducts interfere with specific transcription factors, eventually triggering apoptosis (Internet 2).

2.3.2 Anthracyclines metabolism

Within the human body, anthracycline metabolism ensues via hydroxylation, the formation of semiquinones, or deoxy aglycone. This process can lead to the generation of metabolites that either enhance or attenuate anthracyclines' anticancer attributes.

-Hydroxylation: the process of hydroxylation of anthracyclines at the C-13 carbonyl group, often termed as two-electron reduction, leads to the creation of secondary alcohol metabolites. These metabolites have been implicated in the development of anthracycline-induced cardiotoxicity. This prominent biotransformation pathway of anthracyclines is orchestrated by a diverse superfamilies of cytosolic and microsomal NADPH dependent short chain

dehydrogenases reductases (SDRs) and AKRs, collectively known as carbonyl reducing enzymes (CREs). These enzymes catalyze the conversion of the parent drugs into their respective secondary alcohol forms, such as doxorubicinol (Doxol), daunorubicinol (Daunol), epirubicinol (Epiol), and idarubicinol (Idaol). In the context of dox hydroxylation in the human heart, the AKRs serve as the primary enzymes involved, while the SDRs exhibit a more prominent role in the hydroxylation of Daun (Edwardson et al., 2015).

-One-electron reduction: the process of one-electron reduction in anthracyclines is facilitated by enzymes including CYP450 reductase (CPR), nitric oxide synthase (NOS), NADH dehydrogenase (NDUFS), and xanthine oxidase. This enzymatic action leads to the conversion of the quinone segment of anthracycline drugs into a semiquinone radical state. While this radical remains stable in anaerobic conditions, its presence alongside oxygen triggers rapid re-oxidation of the semi-quinone radical, restoring the original quinone form and concurrently generating hydrogen peroxide, and superoxide anion. This cascade ultimately amplifies the production of ROS. These resultant free radicals have the potential to initiate lipid peroxidation within cellular membranes, instigate cellular demise, and provoke protein aggregation. This cyclic redox process involving Dox, Daun and other analogous anthracyclines occurs within the mitochondria, cytoplasm, and sarcoplasmic reticulum. It has been implicated in the production of toxic aldehydes that can exit the cell, contributing to anthracycline-associated toxicity (Edwardson et al., 2015).

-De-glycosidation: de-glycosidation is the ultimate metabolic pathway; it affects roughly 1-2% of anthracycline antibiotics. This pathway involves the reductive cleavage of the glycosidic bond alongside the carbonyl group in the side chain, yielding the formation of 7-deoxy aglycones and hydroxy aglycones. It is documented that this transformation is catalyzed by enzymatic entities that are not extensively characterized, including NADPH quinone oxidoreductases (NQO1), xanthine dehydrogenase (XDH), and NADPH-cytochrome P450 reductase (CPR). The production of hydroxy aglycones is attributed to NADPH-dependent enzymes present within the cytosol, whereas the genesis of 7-deoxy aglycones could potentially involve microsomal or mitochondrial oxidoreductases. These generated aglycones possess heightened lipophilicity compared to the parent anthracyclines and are suggested to intercalate into mitochondrial membranes. Furthermore, studies suggest that the anthracycline aglycones could potentially induce myocardial damage due to their pronounced oxidizing properties that

direct more electrons towards oxygen within the mitochondria. The product of two-electron reduction (hydroxylated form) generally constitutes the predominant metabolite for anthracyclines (Edwardson et al., 2015).

2.3.3 Anthracyclines cardiotoxicity

Cardiotoxicity stands as a concerning adverse outcome that might curtail the practical application of anthracyclines. It is represented by hypokinetic cardiomyopathy manifested by a decline in left ventricular ejection fraction, gradually advancing to symptomatic heart failure if not diagnosed or treated early.

Cardiomyocytes, cardiac progenitor cells, cardiac fibroblasts, and endothelial cells are the main targets of anthracycline-induced toxicity within the heart (Figure 7). The precise mechanisms responsible for anthracycline-induced cardiotoxicity continue to be unclear. One potential pathway involves the generation of ROS, alterations in iron metabolism, and perturbations in calcium signaling. Moreover, topoisomerase 2β emerged as a pivotal mediator of anthracycline's adverse cardiac effects. As it is involved in unwinding DNA strands during processes such as recombination, transcription, and replication. The inhibition of topoisomerase 2β by anthracyclines disrupts mitochondrial functionality, setting off cascades of cellular death pathways and accumulation of ROS (Cardinale et al., 2020).

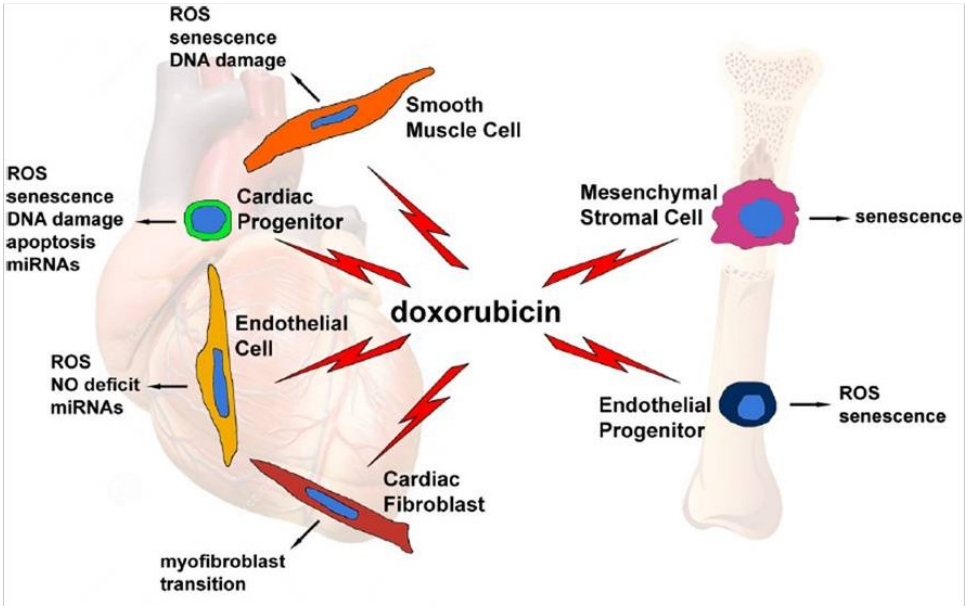


Figure 7. Visual description of diverse cellular entities impacted by Dox, alongside adverse consequences and the cellular and molecular occurrences triggered by the medication (Cardinale et al., 2020).

2.3.4 AKR1C2, AKR1C3, and AKR1B10 specific roles in anthracyclines metabolism and resistance

While various AKRs have been subject to scrutiny concerning their involvement in anthracycline metabolism, only a subset distinctly influences the biochemical and cytotoxic attributes of anthracyclines. While exogenously introduced doxycycline localizes to the nuclei of breast tumor cells, exogenous Doxol accumulates within lysosomes. This observation suggests that the hydroxylation of this anthracycline impedes its nuclear entry, possibly due to its diminished affinity for proteasome binding. Moreover, it has been noted that Doxol exhibited considerably reduced DNA affinity when compared to Dox, potentially explaining the former's lack of nuclear localization. Research also discovered that selecting MCF-7 breast tumor cells for Dox resistance led to heightened transcription of genes like AKR1C2, AKR1C3, and AKR1B10. Intriguingly, the AKR inhibitor β-cholanic acid managed to restore the nuclear localization of Dox and not Doxol in Dox-resistant cells, implying that the inhibitor curbed Dox hydroxylation. This notion was further supported by the fact that the intercellular

concentration of Dox in Dox-resistant cells increased in the presence of β-cholanic acid (Edwardson et al., 2015).

As previously mentioned, anthracyclines have been identified as potent inhibitors of DNA topoisomerase II and as inducers of substantial DNA damage in tumor cells. Interestingly, the hydroxylation of the C-13 carbonyl group in two specific anthracyclines, Dox and IDA, exhibited limited impact on their capacity to inhibit topoisomerase II. Furthermore, both these anthracyclines and their alcohol metabolites displayed robust abilities to induce DNA damage. Remarkably, in isolated nuclei, the hydroxylated forms of these drugs induced double the amount of DNA damage compared to their parent compounds. Given that the hydroxylation of most anthracyclines significantly diminishes their cytotoxic effects, these findings collectively suggest that their cytotoxicity, at least within an in vitro context, is not solely dependent on their ability to inhibit topoisomerase or to induce DNA damage (Edwardson et al., 2015).

2.3.5 Daunorubicin hydrochloride

Daunorubicin hydrochloride (7S,9S)-9-acetyl-7- [(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl] oxy-6,9,11-trihydroxy-4-methoxy-8,10-dihydro-7H-tertacene-5,12-dione; hydrochloride, is a hygroscopic crystalline, orange-red powder, with a molecular weight of 564 g/mol. It represents the hydrochloride variant of an anthracycline antineoplastic antibiotic, exhibiting therapeutic properties the same as those of doxorubicin. Its cytotoxic effects stem from interactions facilitated by topoisomerase with DNA, resulting in the inhibition of processes such as DNA replication, repair, RNA synthesis, and protein synthesis. This highly potent anthracycline aminoglycoside antineoplastic agent is derived from Streptomyces paucities and similar sources. It finds application in treating leukemia and other neoplasms (Internet 3). Daun is exclusively suitable for intravenous administration, specifically through swift infusions. Intramuscular or subcutaneous administration is unsuitable due to the potential risk of inducing serious local tissue necrosis caused by the drug seeping into the adjacent tissue. Patients under the age of 60 are given a dose of 45 mg/m2 intravenously as a single dose on days 1 to 2 for the initial two cycles. For subsequent cycles, in the third one, for example, a 45 mg/m2 single dose is given on days 1 to 3 of each cycle. This regimen should be combined with cytarabine. For patients older than 60 years of age, the same process is followed as in younger patients, but the dose is reduced to 30 mg/m2. Additionally, the dose should be reduced by 50% in patients with renal or hepatic impairment.

Daun exhibits a range of adverse effects, some of which are dose-limiting, while others are not restricted by dosage. Among the prevalent and severe dose-limiting adverse effects are bone marrow suppression and cardiotoxicity. Other adverse effects not limited by dosage are reversible like alopecia, nausea, vomiting, possible skin necrosis, cellulitis, mucositis, and induration of the skin due to extravasation into tissues. Less frequently observed adverse effects encompass secondary leukemias, urticaria, local dermatitis, tumor lysis syndrome, hypersensitivity reactions, and hyperuricemia. Treatment with Daun necessitates vigilant patient monitoring and regular follow-ups. Regular check-ups are recommended, especially blood count, kidney and liver functions, bone marrow suppression, and infections. Any onset of infection requires treatment before the start of another Daun course (Internet 4).

2.3.6 Anthracyclines application in acute myeloid leukemia therapy (the 7+3 induction method)

Despite growing insights into the pathobiology of AML, the prognosis remains unfavorable, particularly for individuals aged 60 or older, a group more likely to have therapy-related AML (tAML) and secondary AML (sAML). For many years, the standard treatment for AML has involved combining cytarabine (a potent inhibitor of DNA polymerase) and Daun or idarubicin. Commonly administered as the “7+3” induction regimen. CPX-351, a liposomal-encapsulated blend of Daun and cytarabine in a fixed molar ratio of 5:1, was approved by the FDA in 2017 for treating newly diagnosed tAML or AML with myelodysplasia-related changes. This balanced dosing might be more efficacious than administering either drug at its maximum tolerated dose. Cytarabine (100 mg/m2) is administered via a continuous infusion over a 7-day period, accompanied by Daun (45 mg/m2) or idarubicin on days 1,2, and 3. In this “7+3” regimen, remission rates ranged from 60-80% among younger adults (below 60 years of age) and 40-60% among older adults (above 60 years of age). Numerous endeavors aimed at enhancing the “7+3” regimen have ultimately fallen short of definitively replacing it. Efforts involving thioguanine with a dual induction strategy or the incorporation of etoposide during induction similarly demonstrated no substantial enhancements in complete remission (CR) rates or disease-free survival rates. In more recent times, positive responses were observed through the application of the histone deacetylase inhibitor “vorinostat”. In combination with the idarubicin and cytarabine (Ara-C) foundation, nevertheless, this endeavor also proved unable to surpass the outcome of the “7+3” approach (Chen et al., 2018).

2.3.7 Anthracycline resistance

Resistance to chemotherapy presents a significant obstacle in cancer treatment. Metabolic adjustments have been identified as contributors to therapy resistance, yet a comprehensive understanding of the extent of these alterations and whether specific treatments induce distinct metabolite changes remains limited (McGuirk et al., 2021).

AML is a prevalent hematological cancer that predominantly affects adult patients. The challenges of AML mainly lie in its limited overall survival rates and the development of resistance to treatment strategies. The primary approach in AML treatment is the induction phase of chemotherapy as discussed above "the 3+7 strategy" comprising Daun and cytarabine. Cytarabine cellular entry occurs through nucleoside transport proteins belonging to the solute carrier (SLC) family, specifically, human equilibrated nucleoside transporter (hENT) 1 and 2, as well as hCNT3 (human concentrative nucleoside transporter 3). Additionally, the transmembrane proteins ABCC10 (MRP7) and ABCC11 (MRP8) also regulate the uptake of cytarabine. Following cellular entry, cytarabine undergoes conversion by the enzyme deoxycytidine kinase (DCK) into cytarabine monophosphate. Subsequently, cytarabine is phosphorylated into cytarabine diphosphate and cytarabine triphosphate through the activities of deoxycytidine monophosphate kinase and nucleoside diphosphate kinase (NDK). Respectively, among these forms, cytarabine triphosphate (Ara-CTP) is the active state that effectively inhibits DNA polymerase activity. Ara-CTP competes with deoxycytidine triphosphate (dCTP) during DNA transcription, acting as a hindrance to DNA elongation. This interference leads to the hindrance of DNA synthesis, DNA repair, and DNA elongation, resulting in the arrest of the cell cycle from the G1 phase to the S phase. Ultimately, this cascade of events culminates in cell death. Whilst Daun can act as a DNA intercalating agent, inhibit topoisomerase II, and form free radicals (Arwanih et al., 2022).

Resistance to Daun in AML can manifest through various mechanisms, including heightened expression of the ATP-binding cassette (ABC) drug efflux transporter, diminished activity of the target enzyme (DNA topoisomerase II), and resistance triggered via the apoptotic pathway. A significant drug efflux transporter scrutinized in these contexts is p-glycoprotein, a member of the ABC family encoded by the MDR1 gene. This transporter is also classified as a multidrug resistance-associated protein (MRP). Overexpression of these transporter proteins can confer resistance of AML cells to anthracycline drugs, such as daunorubicin (Arwanih et al., 2022).

DNA topoisomerase II enzyme activity is a central target of daunorubicin-based therapy. Thus, decreased activity of this enzyme can lead to AML cell resistance against Daun. Research indicated that DNA topoisomerase II enzyme activity is notably diminished in AML cells resistant to anthracyclines, in contrast to their sensitive counterparts. The apoptotic pathway also contributes to AML resistance against Daun, often attributed to mutations in the p53 gene, a pivotal tumor suppressor gene. Notably, p53 gene mutations have been identified in around 15 % of the adult AML patient population (Arwanih et al., 2022).

Currently, chemotherapies, whether employed as standalone treatments or in conjunction with other therapies, continue to be a foundational approach for AML treatment. Nevertheless, AML cells possess diverse mechanisms, including drug efflux transporters, enzyme inactivation, gene mutations, and modulation of signaling pathways, that can confer resistance to chemotherapy. Among the prevalent gene mutations observed in AML are FLT3-ITD and DNMT3A mutations (Arwanih et al., 2022).

2.4 TARGETED THERAPY

Cancer therapy involves many strategies, including drug therapy, radiotherapy, biotherapy, surgery, and chemotherapy. In the past, chemotherapy stood as the primary modality of drug-based treatment; however, its notable drawback lies in the substantial side effects it induces, owing to its impact on both cancerous and normal cells (Zhong et al., 2021). During the 1800s, Paul Ehrlich introduced the notion of the "magic bullet," initially coined to illustrate the potential of a chemical agent targeting microorganisms. Subsequently, this concept was extended to encompass cancer treatment, giving rise to "Molecular Targeted Therapy". This therapeutic approach employs substances or medications to specifically target particular molecules, thereby impeding the expansion and dissemination of cancer cells. The accurate identification of these targets holds pivotal significance in the development of efficacious molecular-targeted treatments. The genesis of cancer is marked by genetic alterations or mutations in cell profiles, leading to modifications in proteins and receptors that bolster cell survival and proliferation. These modifications can be harnessed as molecular targets for crafting drugs aimed at restraining the advancement and progression of tumors. Moreover, advancements in cancer markers and sequencing technology enable researchers to investigate the genetic diversity between malignant and normal cells within an individual (Lee et al., 2018).

Molecular targeted therapies have ushered in a paradigm shift in the field of therapeutics by selectively impeding distinct molecules to impede the advancement, evolution, and spread of cancer. Numerous such therapies, sanctioned by the Food and Drug Administration (FDA), have showcased impressive clinical accomplishments in addressing a diverse array of malignancies, encompassing breast, leukemia, colorectal, lung, and ovarian cancers. This evaluation delivers an up-to-date account of the various categories of molecular targeted therapies harnessed in cancer management. It underscores the foundational aspects of molecular targeted therapy, elucidates its operational mechanism within the realm of cancer treatment, and represents a comprehensive assessment of its strengths and constraints. There are two main types of targeted therapy: small-molecule drugs and monoclonal antibodies (Lee et al., 2018).

Small molecules are characterized as compounds possessing comparably modest molecular weights (< 900Da), enabling their penetration into cells with the capability to pinpoint proteins within these cells. Numerous recognized small molecule inhibitors are designed to incapacitate

kinases and disrupt the signaling pathways that undergo aberration during carcinogenesis. Moreover, small molecules offer the potential to target proteasomes, cyclin-dependent kinases (CDKs), and poly ADP-ribose polymerase. Recently developed isocitrate dehydrogenase inhibitors (IDH) are designed to deactivate kinases and disturb signaling pathways that undergo changes in cancer (Lee et al., 2018).

Monoclonal antibodies (MABs) represent a category of targeted therapeutic drugs. These medications possess the capability to identify and locate distinct proteins situated in cancer cells. A diverse array of monoclonal antibodies is available for cancer treatment, each functioning through distinct mechanisms to either eliminate the cancerous cell or impede its proliferation. Certain MABs aid the immune system in targeting and eliminating cancer cells. These MABs also fall under the umbrella of immunotherapy (Internet 5).

2.4.1 Isocitrate dehydrogenase inhibitors (IDH)

Isocitrate dehydrogenase stands as a pivotal enzyme within the tricarboxylic acid (TCA) cycle, orchestrating the transformation of isocitrate into α-ketoglutarate (α-KG). The mutation in IDH generates a novel enzyme variant, resulting in the aberrant accumulation of R-2-hydroxyglutarate (R-2-HG), thereby fostering the development of leukemia. Additionally, IDH mutations are consistently associated with NPM1 (nucleophosmin 1) mutations while remaining mutually exclusive with TET2 (Ten-Eleven translocation enzyme 2) mutations. IDH mutations are evident in about 20% of patients with AML, with notable instances encompassing IDH1R132, IDH2 R140, and IDH2 R172. The distinct mutant forms carry varying prognostic implications. A promising clinical response has been proven in AML patients with the mutated IDH1 and IDH2 inhibitors, ivosidenib and ENA, respectively. These inhibitors are designated for treating adult cases or relapsed or refractory (R/R) AML harboring IDH2 and IDH1 mutations (Han et al., 2020).

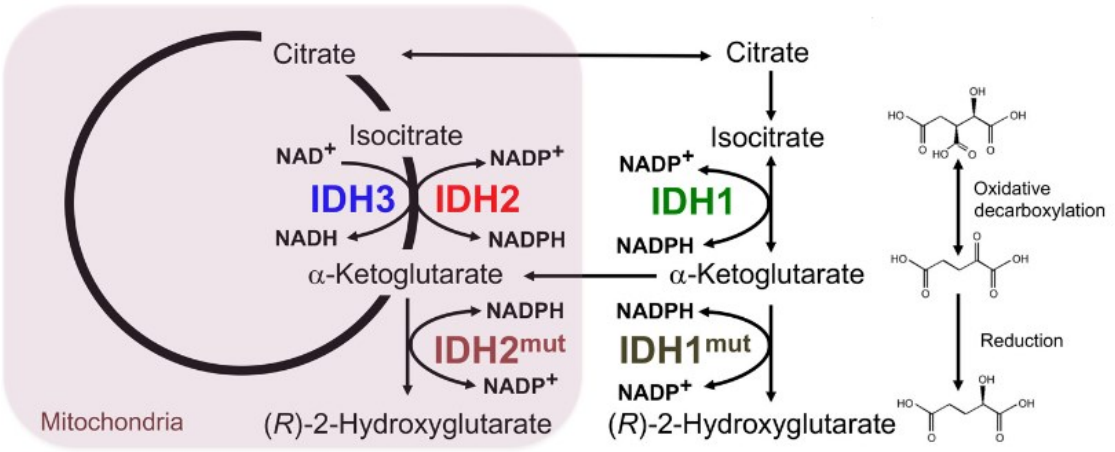


Figure 8. Localization within subcellular compartments and the chemical reactions mediated by the native IDH and IDH mutant enzymes derived from tumors (Tommasini-Ghelfi et al., 2019).

2.4.2 Enasidenib

ENA (ENA) is classified as a 1,3,5-triazine compound featuring substitutions at positions 2,4, and 6. Specifically, it is substituted by groups including (2-hydroxy-2-methylpropyl) nitrilo, 6-(trifluoromethyl) pyridin-2-yl, and [2-(trifluoromethyl) pyridin-4-yl] nitrilo. This compound operates as an isocitrate dehydrogenase-2 inhibitor and has obtained approval for treating adults dealing with relapsed or refractory AML. From a chemical standpoint, it belongs to the categories of aminopyridines, organofluorine compounds, secondary amino compounds, tertiary alcohols, members of 1,3,5-triazines, and aromatic amines (Internet 6).

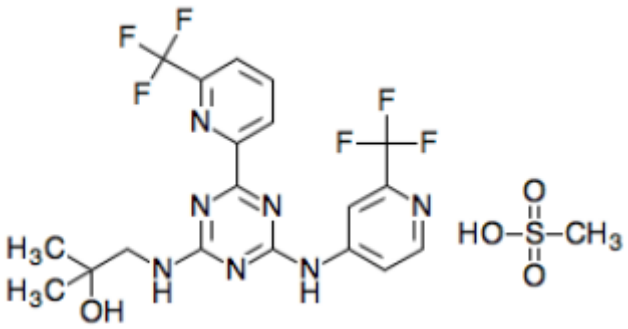


Figure 9. Enasidenib chemical structure (Internet 7).

ENA's pharmacokinetics have been comprehensively studied in both healthy individuals and subjects with relapsed and refractory AML. Following a 100 mg oral dose, the absolute bioavailability of ENA was approximately 57%. After a single oral dose, it took about 4 h for the median time to reach maximum concentration (Cmax, Tmax). Food intake does not significantly affect ENA plasma levels in a clinically meaningful way. The binding of ENA to human plasma proteins was measured at 98.5%. The mean volume of distribution after intravenous administration was 55.8 L; indicating widespread distribution outside the bloodstream. In healthy subjects, the terminal half-life of ENA was approximately 25 h, and its plasma clearance was around 2.5 L/hour. However, in subjects with relapsed and refractory AML, the half-life was considerably longer at approximately 137 h, and the clearance was smaller at 0.74 L/hour. Multiple CYP enzymes are involved in ENA's metabolism, including (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4), as well as multiple UGT enzymes such as (UGT1A1, 1A3, 1A4, 1A9, 2B7, 2B15). The N-dealkylation product AGI-16903 is the most abundant metabolite of ENA. However, it accounted for less than 10% of ENA exposure in patients with AML who received multiple doses. In steady-state conditions, ENA represents 89% of the radioactivity in circulation, while AGI-16903 constitutes 10% of circulating radioactivity. The elimination of ENA took place primarily through feces (89%) and, to a lesser extent, through urine (11%). Notably, unchanged ENA accounted for 34% of the radiolabeled drug excreted in feces and 0.4% in urine (Li et al., 2018).

2.4.3 Enasidenib in AML

AML persists as one of the most lethal hematologic disorders, with refractory cases exhibiting long-term survival rates as low as <10%. This involves the rampant proliferation of clonal myeloid blasts infiltrating various tissues, primarily the bone marrow. The heterogeneity of AML has been linked to multiple genetic irregularities, establishing refined prognostic factors in recent times. Current AML classification incorporates mutations in genes governing signaling intermediates that initiate cell growth and in epigenetic regulators impeding blast differentiation. The latter category of mutations is prevalent in over 50% of AML cases, with mutated forms of isocitrate dehydrogenase 1 and 2 being among the most frequently reported (Morell et al., 2022).

Focusing on mutations that trigger AML has become a refined therapeutic strategy in recent times. ENA, the pioneering selective inhibitor of mutated isocitrate dehydrogenase 2 (IDH2) forms, has gained approval for treating relapsed/refractory AML. Alongside its monotherapy use, ongoing evaluation involves ENA in combination with standard intensive induction therapy (Daun + cytarabine). Studies aimed to elucidate ENA's molecular mechanisms of action against anthracycline resistance, involving processes like reduction by carbonyl-reducing enzymes (CREs) and drug efflux through ATP-binding cassette (ABC) transporters (Morell et al., 2022).

ENA exhibits selective inhibition of AKR1C3-mediated Daun deactivation both in vitro and in cell lines expressing AKR1C3. It also hindered the extrusion of Daun by ABCB1, ABCG2, and ABCC1 transporters, leading to a synergistic enhancement of Daun's cytotoxic effects and effectively overcoming resistance. These findings offer in vitro evidence of ENA's ability to target anthracycline resistance-associated factors, such as AKR1C3 and ABC transporters, under clinically achievable concentrations. Potentially, combining ENA with intensive chemotherapy hints at the possibility that ENA's effectiveness in AML may extend beyond its targeting of mutant IDH2. Beyond IDH inhibitors and specifically, ENA's monotherapy approval by the FDA in 2017. Ongoing clinical trials are exploring the safety and efficacy of combining IDH inhibitors with standard intensive induction therapy (7+3) schedule, where promising results and favorable tolerability for the ENA combination have been observed (Morell et al., 2022).

3 AIMS OF THE THESIS

The present diploma thesis seeks to explore the effectiveness of using ENA, an already clinically utilized medication, to counteract AKR1C3 functions in AML cell proliferation and drug resistance. The precise objectives of this thesis are as follows:

1. To evaluate the potential of ENA counteracting Daun resistance mediated by AKR1C3 in KG1α cells.

2. To assess the inhibitory potential of ENA on the biosynthesis of prostaglandins by recombinant AKR1C3.

3. To study if ENA can inhibit PGD_2-induced proliferation of KG1α cells.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Chemicals

NADP+ (Roche diagnostics, Germany)

Glucose-6-phosphate (Applichem, Germany)

Glucose-6-phosphate dehydrogenase (Roche diagnostics, Germany)

MgCl2 (0.1M, Sigma-Aldrich, Germany)

Na-phosphate buffer (pH 7.4, 0.1M, Sigma-Aldrich, Germany)

Daunorubicin hydrochloride (Toronto Research Chemicals, Canada)

Daunorubicinol hydrochloride (Toronto Research Chemicals, Canada)

Distilled water (Braun, Czech Republic)

Methanol (Penta, Czech Republic)

DMSO (MP Biomedicals, China)

Formic acid, 0.1% (Penta, Czech Republic)

Acetonitrile (Sigma-Aldrich, Germany)

PBS (Sigma-Aldrich, Germany)

MTT (Sigma-Aldrich, Germany)

DMEM (Lonza, USA)

Practice ELISA antiserum (Cayman Chemical, USA)

Practice Ache Tracer (Cayman Chemical, USA)

Practice ELISA standard (Cayman Chemical, USA)

ELISA Buffer Concentrate (Cayman Chemical, USA)

Polysorbate 20 (Cayman Chemical, USA)

Ellman’s Reagent (Cayman Chemical, USA)

ELISA tracer dye (Cayman Chemical, USA)

ELISA Antiserum Dye (Cayman Chemical, USA)

4.1.2 Enasidenib

ENA, sourced from MCE (MedChem Express, USA) and reconstituted with DMSO at a stock concentration of 10 mM and stored at -80 °C. It was prepared at the Department of Biochemical Sciences of the Faculty of Pharmacy in Hradec Králové.

4.1.3 Tools

Automatic pipettes (0.5-1000 µl, Eppendorf, Germany)

Pipetting tips (Sartorius Biohit)

Test tube rack (Eppendorf, Germany)

Test tubes (1.5 mL, 2 mL, 5 mL, Eppendorf, Germany)

Measuring spoons (Thermo Fisher Scientific, USA)

Gloves (Vulcan Medical, Czech Republic)

Timer (Thermo Fisher Scientific, USA)

Plastic syringe (Terumo, Japan)

Injection needle (0.9 × 40 mm, Braun, Czech Republic)

Syringe filter with membrane (0.2 µm PTFE, UK)

Inserts (Agilent, technologies, California, USA)

1 mL glass vials (Agilent, technologies, California, USA)

Screw caps for vials (Agilent, technologies, California, USA)

Septa to vials (Agilent, technologies, California, USA)

Sterile tools for working in a laminar box: tips, automatic pipettes, test tube stands, tweezers, tray (Eppendorf, Germany)

Mouse Anti-rabbit IgG Coated Plate (Cayman Chemical, USA)

96-Well Cover Sheet (Cayman Chemical, USA)

4.1.4 Equipment

Ice cube maker (Scotsman, UK)

Analytical scales (T-scales, Taiwan)

Centrifuge Mini Spin Plus (Eppendorf, Germany)

Thermomixer (Thermomixer compact, Eppendorf)

Shaker (Biotech, Czech Republic)

UHPLC Agilent 1290 Series (Agilent Technologies, USA)

Column (2.1 × 50 mm, 1.8 μm, Zorbax Eclipse Plus C18)

Incubator (Biotech, Czech Republic)

Laminar box (Euroclone , Italy)

Suction pump (P-lab, Czech Republic)

Culture plate counter (Tecan, Switzerland)

Hidex plate reader (FAF, Czech Republic)

4.1.5 Recombinant enzymes

The methodology outlined in Sambrook & MacCallum's laboratory manual (2001) was employed for the preparation of recombinant forms of CREs at the Department of Biochemical Sciences, Faculty of Pharmacy, Hradec Králové. In summary, the coding sequences of each enzyme were amplified from a human liver cDNA library through PCR, utilizing 18-21 nucleotide primers with integrated restriction sites. Subsequently, the resulting PCR fragments were inserted into pET28b and PET15b vectors. Vector amplification was conducted using E. coli strain HB101, and sequence accuracy was validated through sequencing procedures. Enzyme overexpression was induced in E. coli strain BL21 (DE3) utilizing IPTG. Purification of His-tagged enzymes was achieved using Ni-affinity chromatography column via an Äkta purifier system, with enzyme activity confirmation accomplished through SDS-PAGE

analysis. The purified recombinant enzymes were preserved in a 20 mM Na-phosphate buffer (pH 7.4) supplemented with 20-40% glycerol, while their protein concentration was determined utilizing a BA Protein Assay Kit. The concentration of purified recombinant enzymes as follows:

AKR1C3: 1.3 mg/mL

4.2 METHODS

4.2.1 Cell Cultures

KG1α acute myelogenous leukemia cells were procured from ECACC (Salisbury, UK) and cultured in IDMEM supplemented with 20% FBS and 2mM L-glutamine. The cell line was maintained at 37 ℃ in a humidified atmosphere containing 5% CO2 (Morell et al., 2022).

4.2.2 Determination of 11β-PGF2a by ELISA

Enzyme-linked immunosorbent Assays (ELISAs), a type of heterogeneous enzyme immunoassay (EIA), exploit the catalytic properties of enzymes to detect and quantify immunological reactions, particularly in clinical settings. ELISA involves the nonspecific adsorption or covalent binding of one reaction component to a solid phase, such as a microtiter well, magnetic particle, or plastic bead. This attachment facilitates the distinction between bound and free-labeled reactants, enabling the analysis (Internet 8).

For the determination of prostaglandins, Cayman Chemical (Internet 9) (1180 E. Ellsworth Rd. Ann Arbor, MI. USA) ELISA kit was used. Storage conditions -20 ℃. Pre-assay preparation included buffer preparation using ultra-pure water; free from ionic residues and trace organic contaminants (FAF laboratory). ELISA buffer preparation was achieved by diluting the content of one vial of ELISA buffer concentrate (10×) supplied from the ELISA kit with 90 mL of Ultrapure water. In addition, to washing buffer preparation, dilute a 5 mL vials containing the wash buffer to a total volume of 2 L with Ultrapure water, finishing the procedure by adding 1 mL of polysorbate 20. Practice ELISA standard reagent specific for this assay was prepared by equilibrating a pipette tip in ethanol by repeatedly filling and expelling ethanol several times. Using the equilibrated pipette tip, 100 µl of the Practice ELISA Standard was transferred into a clean test tube, which was then diluted with 900 µl with Ultrapure water. The final concentration of this solution was 10 ng/mL. ELISA standard was prepared for use; eight clean test tubes, numbered sequentially from 1 to 8 were prepared. 900 µl of ELISA buffer was pipetted into tube number one and 500 µl into each of tubes two until eight. Subsequent dilutions were prepared according to **(Figure 10)**.

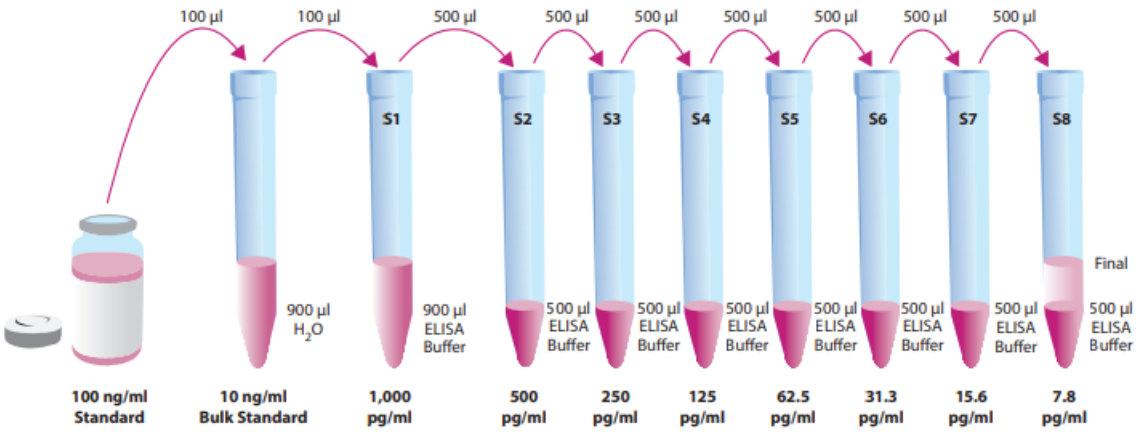


Figure 10. Preparation of ELISA standards (Internet 9)

Following the proper pipetting inside each well, the plate was centrifuged and taken to the Hidex plate reader previously heated to 37 °C. Fluorescence was measured with an excitation of 340 nm and emission 460 nm.

3.2.3 Daun quantification by HPLC

In various disciplines such as pharmaceuticals, food science, and cosmetics, characterization techniques play a vital role in elucidating the chemical composition of materials, thereby aiding in component identification. High-performance-liquid-chromatography (HPLC) stands out as a powerful separation technique capable of resolving compounds based on their physicochemical properties **(Figure 11)**. This method relies on the passage of a dissolved sample known as the mobile phase through a stationary phase, with both phases being immiscible. The selection of a particular HPLC variant depends on the analyte's nature, including its chemical structure and molecular weight. Since its mid-20 century inception, HPLC has undergone significant advancements, incorporating high pressure, computerization, and automation, culminating in the development of ultra-high-performance liquid chromatography (UHPLC). HPLC instrumentation can be coupled with various detectors, including ultraviolet-visible (UV-Vis) spectroscopy, mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared spectroscopy which uses attenuated total reflectance (Sánchez et al., 2018).

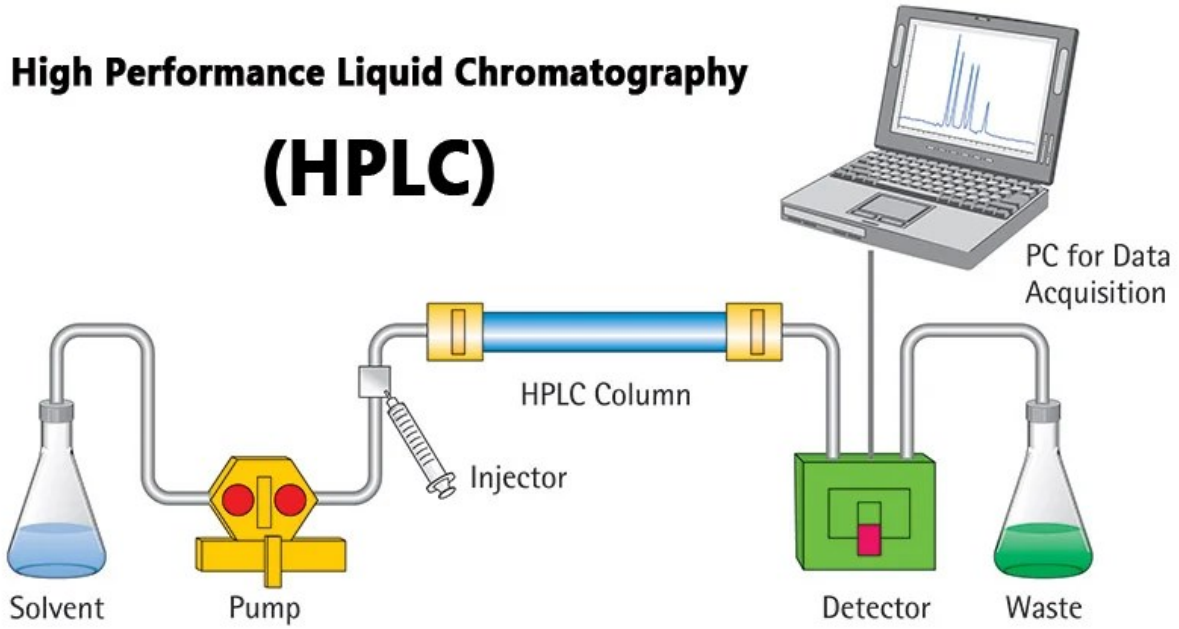


Figure 11. Diagram of HPLC chromatograph (Internet 10)

4.2.4 Determination of Daun accumulation in cells

KG1α cells (1×10^5 cells/well) were cultured in 24-well plates with 0.5 mL of media supplemented with DMSO, LY335979 (1 μM), or ENA (1, 5, 10, 25, and 50 μM) for 15 min. Following this, Daun was introduced at a final concentration of 1 μM for 1 hour. The cells were then harvested, centrifuged, and washed twice with cold PBS for subsequent analysis of Daun fluorescence using a Sony Sa3800 spectral cell analyzer flow cytometer (Sonny Biotechnology, San Jose, CA, USA). The excitation/emission wavelengths for Daun were set at 490/565 nm. Data analysis was performed using Sa3800 software (Sonny Biotechnology, San Jose, CA, USA).

4.2.5 Determination of PGD_2 metabolism by recombinant AKR1C3

The determination of the inhibitory effect of ENA over PGD_2 metabolism by AKR1C3 was performed as follow: reactions were prepared by mixing 5 μg of recombinant AKR1C3, 1 μM PGD_2, and 2.3 mM NADPH in 100 μL total volume of 100 mM potassium phosphate buffer (pH 7.0). The mixture was incubated at 37 °C for 2 h, then the reaction was stopped by adding 400 μl ethyl acetate. The mixtures were extracted for 15 min with the ethyl acetate, twice. The ethyl acetate was isolated and evaporated to dryness under a stream of nitrogen at room temperature. Finally, the residue was resuspended in 500 μl of ELISA buffer and vortexed. Then sample was added to the ELISA plate as described above.

4.2.6 Determination of Daun and PGD_2 metabolism in KG1α cells

To assess Daun metabolism in KG1α cells, a total of 1 x 10^6 cells were cultured in 0.5 mL of media within 24-well plates, and treated with either DMSO, LY335979 (1 μM), or ENA (5 and 10 μM) for a duration of 15 min. Following this initial treatment, Daun was introduced to reach final concentrations of 0.5, 0.75, and 1 μM, and the plates were then incubated for 24 h. Subsequently, the cells were harvested and transferred into Eppendorf tubes. From each sample, 50 μL was isolated and underwent to washes with cold PBS for subsequent examination of Daun accumulation via flow cytometry. Each washed sample was subjected to centrifugation at 1500 rpm for 5 min, separating the supernatant into a new Eppendorf tube, while the cell pellet was resuspended in lysis buffer and incubated for 15 min. The collected medium as combined with the cell lysate for further extraction and subsequent analysis of metabolites (Morell et al., 2022).

To analyze the effect of ENA on PGD_2 metabolization by KG1α cells, 5 x 10^5 cells were seeded in 0.5 mL of media in a 24 well plate. Cells were incubated with PGD_2 1 μM with or without ENA (5 and 10 μM) for 24 h. Then, cells were centrifuged 1500 rpm, 10 min, and the supernatant was removed for further analysis by ELISA.

4.2.7 Determination of synergism by using KG1α cell line

The synergistic impact of ENA on Daun cytotoxicity was assessed across all cell lines through IC_{50} shift analysis and Chou-Talalay evaluation, suspension cell line KG1α (20 × 10^4 cells/well) were seeded onto 96-well plates, with incubation periods of 24 h or 30 min preceding the experiment, respectively. Subsequently, the medium was refreshed for adherent cells or supplemented for KG1α cells with fresh medium containing escalating doses of Daun along with either DMSO or ENA (final concentrations: 1, 5, and/or 10 μM). Following 72 h of incubation (48 h for the transporter aspect) under standard conditions (37 °C, 5% CO_2), cell viability was determined using cell proliferation kit II (XTT) for KG1α cells (both kits sourced from Sigma-Aldrich, Prague, Czech Republic). The half-maximal inhibitory concentrations (IC_{50}) were computed utilizing GraphPad Prism 8.1.2 software. Pharmacodynamic evaluations of drug combinations were conducted employing the Chou-Talalay method, facilitated by CompuSyn version 3.0.1 software (ComboSyn Inc., Paramus, NJ, USA) (Morell et al., 2022).

4.2.8 Analysis of synergism by Chou-Talalay method

The Chou-Talalay method serves as a mathematical framework for analyzing drug interactions and synergy, devised by Dr. Ting-Chao Chou and Dr. Paul Talalay during the 1980s. This method, rooted in the median effect equation (MEE), is derived from the fundamental principles of mass action law in physiochemistry. By offering a common link between single and multiple entities, as well as first-order and higher-order dynamics, the MEE lays the groundwork for extensions such as the combination index theorem and the dose reduction index equation. Widely employed in pharmacology, the Chou-Talalay method enables the valuation of interactions among two or more drugs, determining whether they exhibit synergistic, additive, or antagonistic effects. Through quantitative characterization of drug interactions, this method aids in optimizing drug dosages and mitigating adverse effects. Its utility spans various disease domains prominently cancer research, where it has been instrumental in assessing the efficacy of diverse drug combinations (Chou, 2010), (Elwakeel et al., 2019).In this study, the Chou-Talalay method was employed to analyze the interaction between ENA and Daun. Utilizing the ComuSyn software version 3.0.1 (ComboSyn Inc., Paramus, NJ, USA), cell survival values obtained from combination assays were subjected to rigorous analysis.

4.2.9 SDS-PAGE gel electrophoresis and Western blotting

SDS-PAGE gel electrophoresis and western blotting serve as a complementary technique combination to identify specific proteins within complex cellular or tissue samples. The process begins with protein denaturation and reduction using a specialized buffer **(Figure 12-1)**, ensuring they migrate solely based on their molecular weight during SDS-PAGE **(Figure 12-2)**. Following separation, the proteins are transferred not a PVDF or nitrocellulose membrane **(Figure 12-3)**. To enhance detection specificity, non-specific binding sites on the membrane are blocked **(Figure 12-4)** before incubation with targeted primary and secondary antibodies. Then incubation with specific primary and secondary antibody **(Figure 12-5-6)**. Protein expression is visualized through enhanced chemiluminescence and standard film development techniques. Finally, densitometry analysis quantifies the signal intensity from each protein band **(Figure 12-7)**.

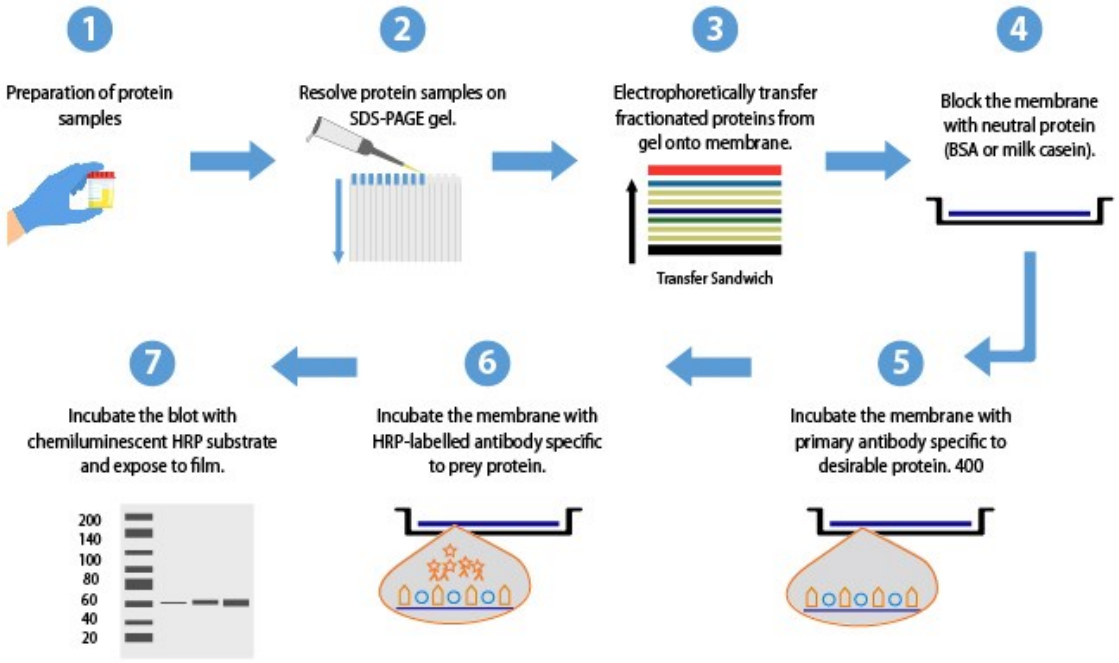


Figure 12. SDS-PAGE gel electrophoresis and Western blotting in protein quantification (Internet 11)

4.2.10 Statistical significance

To evaluate notable alterations in metabolism across recombinant AKR1C3 and KG1α cells, one-way ANOVA was employed. This statistical method facilitated a comparison of changes in the level of metabolites, discerning any statistically significant differences between their means. Furthermore, the Unpaired Student's t-test was utilized to determine the statistical significance of changes observed in Daun- IC_{50} shifts and alterations in AKR1C3 protein levels. The statistical analyses were conducted using GraphPad Prism 8.1.2 software. A p-value below 0.05 was deemed statistically significant, indicating a less than 5% probability that the observed outcomes were due to chance.

4.2.11 Induction studies

ENA-induced protein expression of AKR1C3 was investigated in KG1α cells, with a seeding density of 0.5×10^6 cells/well in 6-well plates, followed by a 72-hour incubation period. Protein extraction and western blot analysis procedures were realized as described above (Morell et al., 2022).

5 RESULTS

5.1 The cooperative effect observed between Daun and ENA in KG1α leukemia cells

To further investigate ENA's potential in treating AML, we sought to expand upon previous findings regarding its ability to counteract resistance in a cell line closely resembling this disease. To achieve this objective, we opted for the AML cell line KG1α, recognized as the standard model for anthracycline resistance (Verma et al., 2016). KG1α cells exhibit consistent expression levels of AKR1C3 (Birtwistle et al., 2009), (Verma et al., 2016)and ABC transporters (J D Bailly, 1995), (Fardel et al., 1998). As previously reported, ENA is a potent inhibitor of AKR1C3-mediated reduction of Daun to Daunol *in vitro* (Morell et al., 2022). Therefore, we assessed the potential impact of ENA on the activity and expression of AKR1C3 in KG1α cells, recognizing that alterations in their expression levels could potentially obscure the true magnitude of ENA's inhibitory effects on Daun efflux and metabolism. No significant alterations in the expression of AKR1C3 protein were detected following a 72-hour incubation period with a concentration of 10 μM of ENA **(Figure 13A)**. Moreover, ENA exhibited a dose-dependent increase in Daun accumulation in KG1α cells **(Figure 13B)**, indicating robust inhibition of drug efflux.

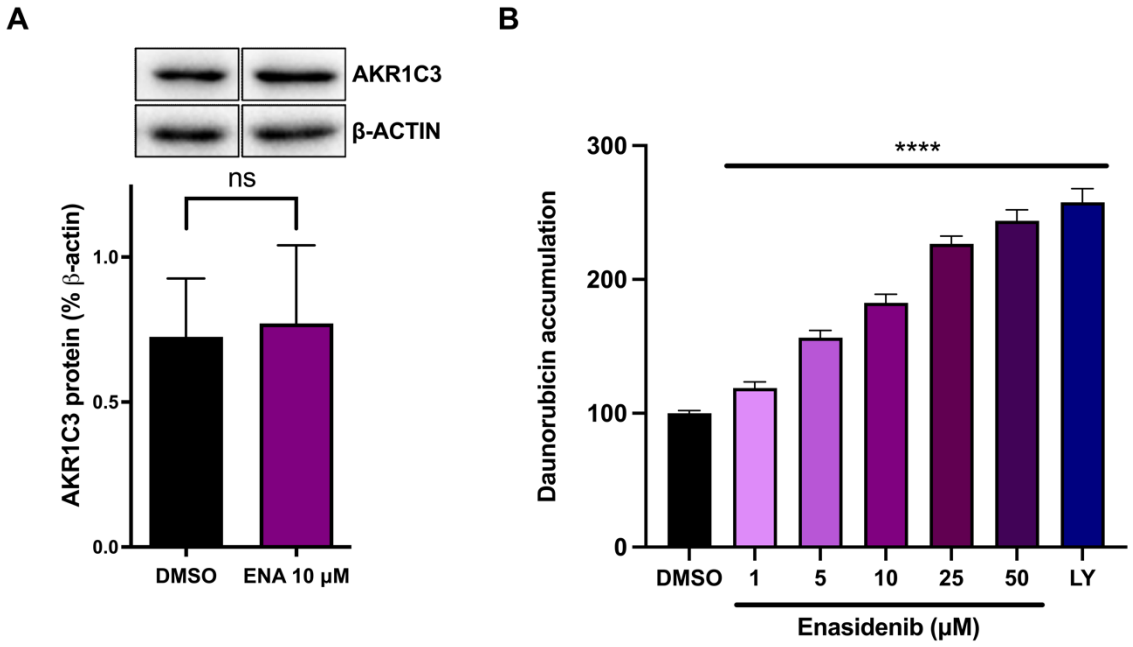


Figure 13. In KG1α cell, ENA does not affect AKR1C3 expression but dose-dependently increases Daun accumulation. A, KG1α cells were treated with either vehicle or 10 μM ENA for 72 h, followed by the analysis of total protein extracts for AKR1C3 expression using Western blotting. Representative Western blot bands are depicted, with bars corresponding to AKR1C3 protein levels determined by densitometry relative to β-actin (n=3, mean±SD). Protein levels were compared by unpaired t-test, where ns is not significant. B, KG1α cells were exposed to DMSO, escalating concentrations of ENA (1-50 μM), or LY335979 (1 μM) for 15 min, then treated with 1 μM Daun for 1 hour. Subsequently, Daun accumulation was quantified as fluorescence within the cells using flow cytometry. Bar graphs represent Daun accumulation as a percentage relative to DMSO. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (****P<0.0001).

We performed drug combination studies on KG1α cells, observing that ENA dose-dependently enhances Daun cytotoxicity, as evidenced by significant reductions in the IC_{50} values (figure 16A and table 2). Moreover, all these dose combinations demonstrated CI values <1, indicating a synergistic effect for all the tested combinations **(Figure 16B)**.

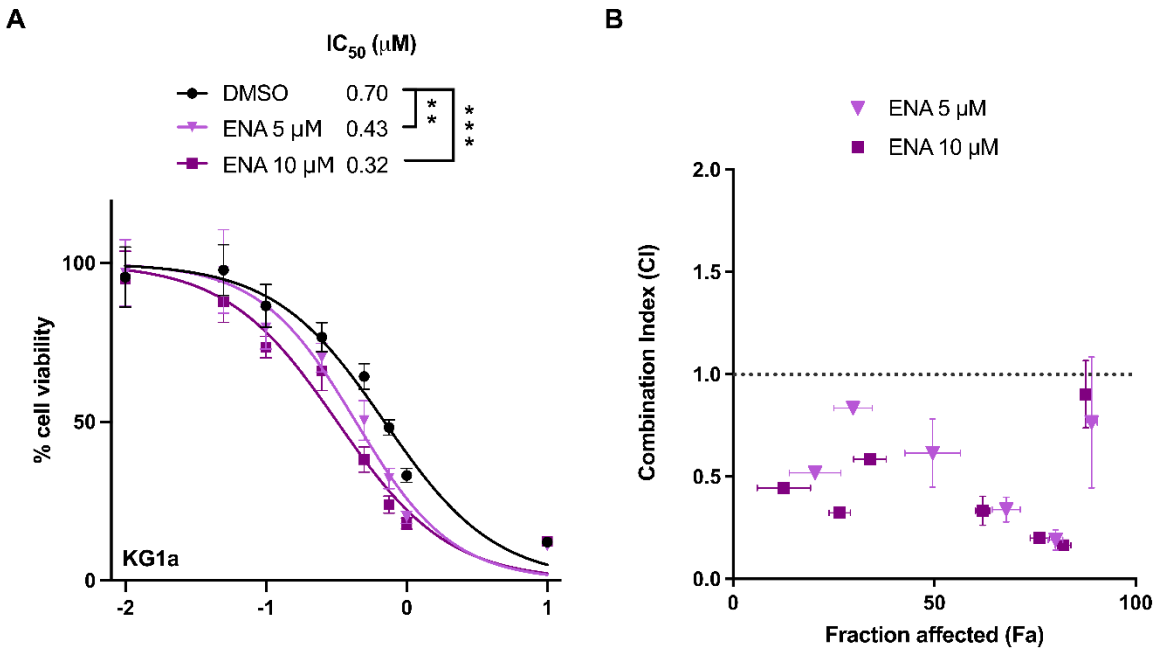


Figure 14. KG1α cells were treated with vehicle or ENA (5 and 10 µM) in combination with increasing concentrations of Daun for 72 h. Cell viability was assessed using XTT assay. A, the graph illustrates a comparison of the dose-response curves of normalized KG1α cell viability under different combinations. IC_{50} values were compared using unpaired t-test (**P<0.01 and ***P<0.001). B, combination index versus fraction-affected (Fa) plots were obtained after Chou-Talalay analysis of the combined treatment involving Daun+ENA, based on data from A. The dashed line delineates the CI ranges for synergy (<1) and antagonism (>1).

Table 2. Synergism between ENA and Daun on KG1α cells.

Enasidenib (µM)	Daunorubicin (µM)	Fraction affected (Fa %)	Combination index (CI)
5	0.10	20.40 ± 6.42	0.52 ± 0.00
	0.25	29.80 ± 4.78	0.83 ± 0.00
	0.50	49.60 ± 6.89	0.62 ± 0.17
	0.75	67.87 ± 3.50	0.34 ± 0.06
	1.00	80.13 ± 1.81	0.19 ± 0.05
	10.00	89.10 ± 1.39	0.76 ± 0.05
10	0.10	26.50 ± 2.66	0.32 ± 0.00
	0.25	34.04 ± 4.04	0.59 ± 0.00
	0.50	62.00 ± 1.73	0.33 ± 0.07
	0.75	76.17 ± 2.39	0.20 ± 0.03
	1.00	82.00 ± 1.93	0.16 ± 0.02
	10.00	87.63 ± 0.51	0.90 ± 0.16

Values are expressed as the means ± SD from nine independent experiments.

Hence, it remains to be determined whether ENA's synergy with Daun in KG1α cells is solely attributed to its enhanced cellular accumulation or to a reduced metabolism. To address this inquiry, KG1α cells were pre-treated with either vehicle, ENA at concentrations of 5 and 10 µM, or the ABCB1 inhibitor LY335979 (1 µM), and subsequently exposed to escalating concentrations of Daun (0.5, 0.75 and 1 µM) for 24 h. The cells were then split for the simultaneous assessment of Daun accumulation within intact cells and the levels of extracted Daunol. Plotting both parameters **(figure 15)** revealed a linear correlation between cellular

accumulation and Daunol amounts, indicating that increasing Daun concentration in KG1α cells may heighten susceptibility to AKR1C3-mediated reduction. However, linear regression of combinations with ENA were notably steeper than those with LY335979. This discrepancy may arise because while LY335979 exclusively blocks ABCB1-mediated efflux (as evidenced by a linear rise between cell accumulation and Daunol production), ENA concurrently inhibits Daun efflux and its AKR1C3-mediated reduction (as indicated by lower Daunol levels despite comparable cell accumulation to LY335979). Thus, it seems that ENA could inhibit simultaneously different mechanisms that contribute to Daun insensitivity. These findings position ENA as a distinctive multi-specific modulator of Daun resistance.

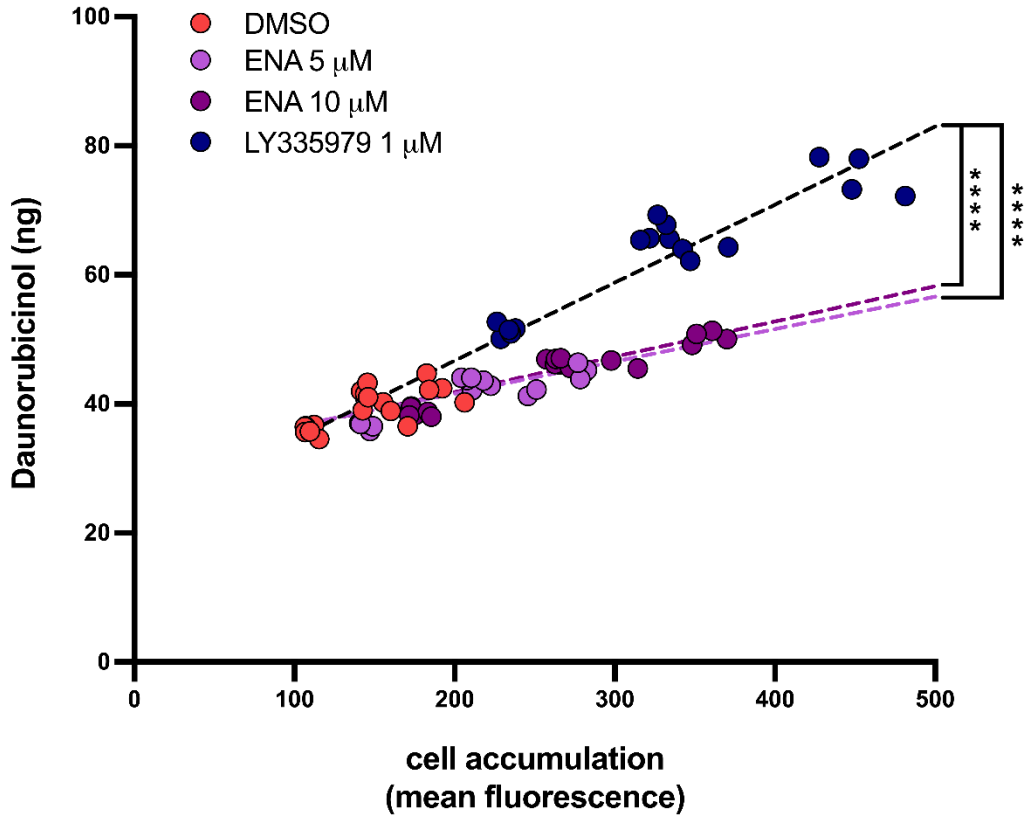


Figure 15. ENA concurrently inhibits Daun efflux and AKR1C3-mediated reduction. KG1α cells were incubated with DMSO, LY335979 (1 μM), and ENA (5 or 10 μM) for 15 min, followed by the addition of Daun to final concentrations of 0.5, 0.75, or 1 μM for 24 h. Subsequently, cells were split

for simultaneous analysis of Daun accumulation by flow-cytometry and Daunol production by UHPLC. In the scatter plot, each dot represents the accumulation of Daun in cells as mean fluorescence versus the corresponding amount of extracted Daunol in ng. Dashed lines represent linear regressions of dots from each combination, performed three times in duplicate (n=6). Linear regressions in were compared using unpaired t-tests (****P<0.0001).

5.2 ENA dose-dependently inhibits AKR1C3 mediated transformation of PDG2 to 11β-PGF2α

AKR1C3 transforms PDG_2 to the 9α and 11β epimers of PGF2α, which promote leukemia cell proliferation. As previously reported, ENA specifically inhibits AKR1C3-mediated metabolism of Daun (Morell et al., 2022). Therefore, we evaluated ENA's potential inhibitory effect on PDG_2 metabolism. Figure 18 shows how ENA was able to significantly reduce the amount of 11β-PGF2α produced by the recombinant AKR1C3 after incubation with PGD_2.

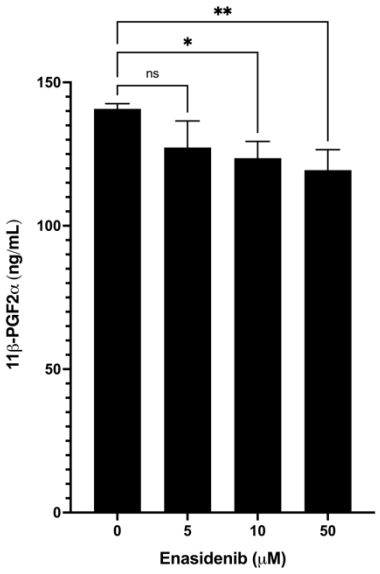


Figure 16. ENA dose-dependently inhibits AKR1C3-mediated transformation of PGD_2 to 11β-PGF2α. Bars represent the total amount of 11β-PGF2α (ng/mL) produced after incubating recombinant AKR1C3 with PGD_2 (1 μM) and DMSO or ENA (n=3, mean±SD). The 11β-PGF2α quantification was performed by ELISA. 11β-PGF2α levels were compared by one-way ANOVA, where ns is not significant, *P<0.05 and **P<0.01.

5.3 ENA reductases KG1α cells' metabolism of PDG2 to 11β-PGF2α and proliferation

Once we determined that ENA can reduce AKR1C3-mediated transformation of PDG_2 in vitro, we further evaluated if this effect could extend to leukemia cells expressing AKR1C3. For this, we first determined that because KG1α cells do not carry any IDH mutation(Steinhäuser et al., 2023), ENA alone does not significantly affect this AML line proliferation **(figure 17A)**. This would help to point out that ENA's potential effects on KG1α cells relate to PDG_2 metabolism and not to a decrease in cell viability. Furthermore, we observed that, similarly to the in vitro incubations with recombinant AKR1C3, ENA dose-dependently inhibited the metabolization of PGD_2 to 11β-PGF2a by KG1α cells **(figure 17B)**. This correlated with a significant increase by ENA 10 µM of the cytotoxicity of increasing concentrations of PGD_2 over KG1α cells **(figure 17C)**. This ENA's effect could relate to the inhibition of AKR1C3, directing the metabolism of PGD_2 to the pro-proliferative PGF2α epimers and, therefore, boosting instead the production of PGJ_2 that induces cell differentiation.

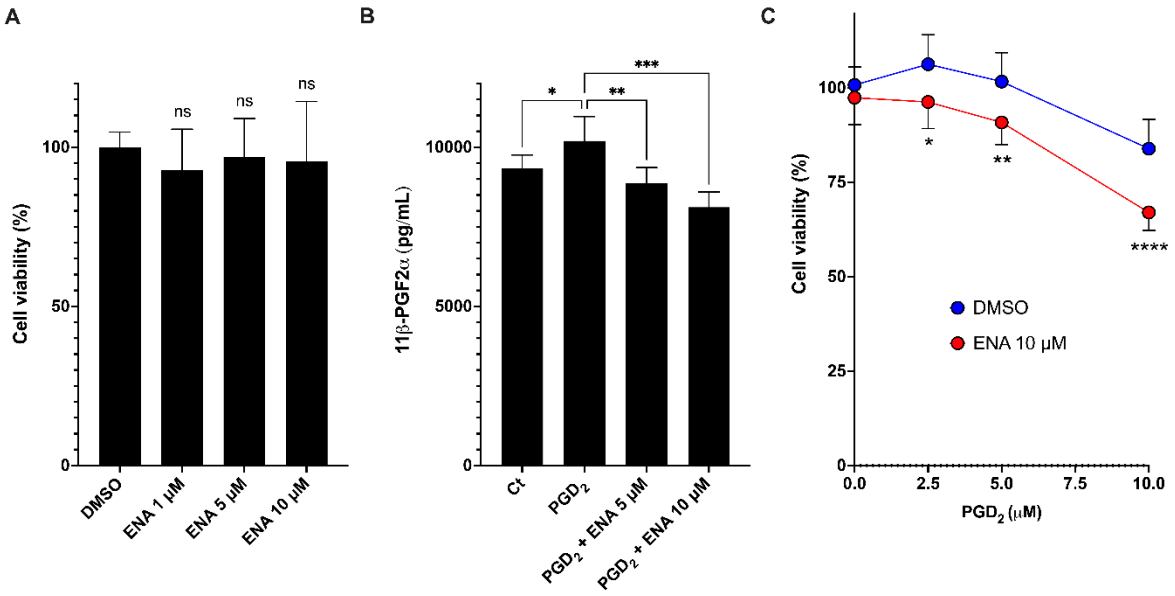


Figure 17. ENA reduces KG1α cells' metabolism of PGD_2 to 11β-PDG2a and proliferation. A, KG1α cells were treated with vehicle or ENA (1, 5, and 10 µM) for 72 h. Cell viability was assessed using an XTT assay. Bar graphs represent cell viability as a percentage relative to DMSO (n=3, mean±SD). Statistical analysis was performed using one-way ANOVA, where ns is not significant. B, bars represent the total amount of 11β-PGF2α (ng/mL) produced after incubating KG1α cells with PGD_2 (1 µM) with or without ENA (5 and 10 µM) for 24 h. The 11β-PGF2α quantification was performed by ELISA

(n=3, mean±SD). 11β-PGF2α levels were compared by one-way ANOVA, where ns is not significant, *P<0.05, **P<0.01, and ***P<0.001. C, KG1α cells were incubated with increasing concentrations of PGD_2 (2.5, 5, and 10 μM) with or without ENA (10 μM) for 72 h. The dots represent cell viability as a percentage relative to control cells, as determined by XTT assay (n=8, mean±SD). Statistical analysis was performed using two-way ANOVA (*P<0.05, **P<0.01, and ***P<0.001).

6 DISCUSSION

The effectiveness of cytotoxic chemotherapies against AML hinges on the rapid proliferation rate of blasts. However, these treatments also damage healthy cells and tissues, causing various side effects. To target AML cells more precisely, researchers have developed novel targeted therapies beyond the standard "7+3" regimen, which has remained unchanged for nearly four decades. The FDA has approved eight such compounds that target specific, abnormal signaling pathways driving AML development (leukemogenesis). These include gemtuzumab ozogamicin (GO), an anti-CD33 antibody; venetoclax, a B-cell lymphoma 2 (BCL-2) inhibitor; midostaurin, gilteritinib, and quizartinib, all FMS-like tyrosine kinase inhibitors; ivosidenib and ENA, IDH1 and 2 inhibitors; and glasdegib, an inhibitor of the hedgehog (HH/glioma-associated oncogene homologue (GLI) signaling pathway (Fiorentini et al., 2020), (M.-K. Song et al., 2021), (Thol & Heuser, 2021). Additionally, promising results are emerging for other targeted agents with different mechanisms of action, such as the cyclin-dependent kinase inhibitor dinaciclib (Gojo et al., 2013), (Boffo et al., 2018); the phosphoinositide 3-kinase inhibitor buparlisib (Ragon et al., 2017), and the Bruton tyrosine kinase inhibitor ibrutinib (Cortes et al., 2019). While these drugs offer greater specificity, recent in vitro studies suggest that they may have unforeseen effects on non-targeted pathways at clinically relevant doses. Understanding these off-target activities is critical for fully comprehending their therapeutic benefits and potential side effects.

Some of the unintended effects of targeted drugs can work together (synergism) with standard chemotherapy drugs like anthracyclines (Novotná et al., 2018), (Bukum et al., 2019), (Morell, Novotná, et al., 2020), (Morell, Čermáková, et al., 2020).However, anthracyclines themselves are often ineffective due to drug resistance and cause severe side effects that limit their usefulness (Patel et al., 2013), (Gurnari et al., 2020). Identifying the exact molecular targets that underline this synergism with anthracyclines is challenging because AML cells can develop resistance through many different mechanisms. For instance, the presence of ATP-binding cassette (ABC) transporter proteins allows AML cells to pump anthracyclines back out of the cell, preventing them from accumulating inside and working effectively. Several studies have shown a link between lower complete remission (CR) rates in AML patients treated with anthracycline and cytarabine and higher levels of specific ABC transporters, such as ABCB1, ABCG2, and ABCC1 as the most active transporters (Marzac et al., 2011), (Chauhan et al.,

2012), (Gupta et al., 2016), (B. Liu et al., 2018), (Vasconcelos et al., 2021). Interestingly, clinical trials that combined these standard anthracycline treatments (Daun and Cytarabine) with drugs that block ABCB1 transporters did not improve patient's outcome (Kolitz et al., 2004), (Cripe et al., 2010). This suggests that blocking just one type of transporter might not be enough, and a more comprehensive approach may be needed. Additionally, other mechanisms beyond drug efflux also contribute to anthracycline resistance in patients, such as the inactivation of the drugs by certain enzymes. Strong evidence suggests that CREs make anthracyclines vulnerable to a process called NADPH-dependent reduction; (Bains et al., 2010), (Bains et al., 2013), (Penning et al., 2021). This process converts anthracyclines into less toxic secondary alcohol metabolites (Kuffel & Ames, 1995), (Ax et al., 2000), (Piska et al., 2017), (Heibein et al., 2012); observed that doxorubicin, an anthracycline drug, accumulated in the nucleus of MCF-7 breast cancer cells, where it can damage DNA, but its reduced form, Doxol, was mainly found in lysosomes, which are cellular waste disposal units, and showed less attraction to DNA. While these reduced forms might still be able to block an enzyme called topoisomerase II and even cause DNA damage, their lack of cytotoxicity (cell-killing ability) seems to be related to their location within the cell (Ferrazzi et al., 1991), (Kuffel et al., 1992). It must be presumed that these metabolites lack cytotoxicity based on the intracellular distribution. In this context, the susceptibility of reduced forms to transmembrane extrusion via ABC transporters has been highlighted (Kuffel & Ames, 1995), thereby diminishing their intracellular availability. Despite the passive diffusion capacity of anthracyclines across plasma membranes, their cytosolic metabolism by CREs and drug efflux may constitute a concerted defense mechanism, hindering drug access to their primary targets, notably DNA in the nucleus, thereby rendering cancer cells more resistant to the cytotoxic effects of this class of chemotherapeutics. Furthermore, reduced forms exhibit a heightened propensity for accumulation in cardiac tissue, implicating CREs' reductive metabolism in the increased cardiotoxicity observed during anthracycline treatment (Mordente et al., 2001).

Of all identified CREs demonstrating reductive activity towards anthracyclines, AKR1C3 emerges as the most efficient and pertinent in conferring chemo-resistance in AML. Multiple studies have elucidated the inherent or acquired anthracycline insensitivity mediated by AKR1C3 expression across various cancer cell types, underscoring the potential of specific inhibitors to enhance the cytotoxic effect of anthracyclines (Y. Liu et al., 2020). (Verma et al.,

2016), demonstrated the efficacy of several synthetic AKR1C3 inhibitors augmenting the cytotoxic effect of Daun by up to tenfold in AML cell lines and primary AML cells expressing AKR1C3. Similarly, prior investigations have reported the potency of midostaurin (Morell, Novotná, et al., 2020a), dinaciclib (Novotná et al., 2018b), buparlisib (Bukum et al., 2019), ibrutinib, and acalabrutinib (Morell, Čermáková, et al., 2020b) in enhancing Daun cytotoxicity through specific inhibition of AKR1C3.

In a previous study, ENA was identified as a specific inhibitor of recombinant AKR1C3, exhibiting an IC_{50} of 1.08 μM. Moreover, ENA demonstrated a dose-dependent ability to hinder the metabolic inactivation of Daun by exogenously expressed AKR1C3 in the HCT116 cell line. Subsequent investigations in HCT116-based assays revealed that this inhibitory interaction could effectively overcome Daun resistance. Consistent results were also observed in cell lines naturally expressing AKR1C3 like A549. Furthermore, in this work, we described how ENA enhanced the intracellular accumulation of Daun in KG1α cells, suggesting potential interference with drug transport mechanisms. This was corroborated by Jakub Hoffman's group by experiments conducted with A431 cell models, wherein ENA dose-dependently inhibited Daun efflux mediated by over-expressed ABC transporters (ABCB1, ABCG2, and ABCC1). Additionally, the inhibition of Daun transport correlated with a synergistic enhancement of its cytotoxicity in all A431 lines over-expressing the same ABC transporters but not in the A431 parent cells.

The effective concentrations of ENA utilized across all cell models (1, 5, and 10 μM) were substantially lower than the clinically achieved Cmax of approximately 27 μM observed in trials with a 100 mg dose (CHMP: Idhifa 2019). Consequently, we anticipate that ENA may induce similar pharmacokinetic interactions when co-administered with Daun in vivo. Particularly noteworthy from our work is that KG1α cells were ENA's dual effect of increasing intracellular Daun levels while preventing its reduction by AKR1C3. These findings align with those of other targeted drugs demonstrating inhibitory effects in vitro against both AKR1C3 and ABC transporters at clinically attainable concentrations, as reported separately for ibrutinib (Zhang et al., 2014), (Morell, Čermáková, et al., 2020b),and dinaciclib (Cihalova et al., 2015), (Novotná et al., 2018), or as previously demonstrated in Morell et al. (Morell, Novotná, et al., 2020a) wherein midostaurin synergistically enhanced Daun effects by concurrently inhibiting both mechanisms.

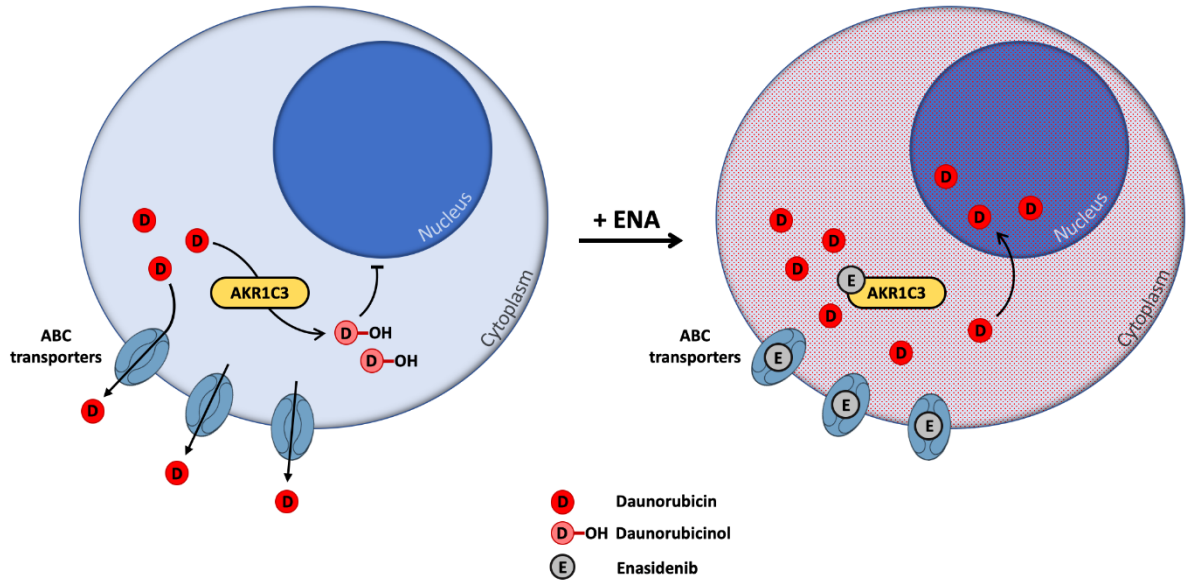


Figure 18. ENA concurrently inhibits Daun efflux and AKR1C3-mediated reduction in AML cells. In this work, we demonstrated that in KG1α cells, ENA has a dual effect by increasing intracellular Daun levels while preventing its reduction to Daunol by AKR1C3. These mechanisms may increase Daun intracellular levels and explain the synergistic effect of ENA over Daun cytotoxicity in KG1α cells that we also described in this work.

Midostaurin demonstrated the ability to counteract drug efflux by ABCB1 but not by ABCG2 or ABCC1 (Hsiao et al., 2019), (N. Ji et al., 2019).In contrast, ENA exhibited an inhibitory effect on all three transporters, thereby enhancing the likelihood of success across a broad spectrum of anthracycline-resistant AML phenotypes. Clinical trials combining Dau, cytarabine, and midostaurin in AML patients demonstrated higher complete remission (CR) rates than previously reported (Deutsch et al., 2019), suggesting potential success for ENA combinations in clinical settings. Both inhibitors improve Dau efficacy through similar mechanisms, irrespective of their primary targets FLT-3 and mIDH2, respectively. Currently, the phase 1 multi-center clinical trial NCT02632708 (Stein et al., 2021)has assessed the effectiveness and tolerability of induction therapy comprising continuous ivosidenib 500 mg/day or ENA 100 mg/day in combination with cytarabine (200 mg/m2/day for 7days) and either Dau (60 mg/m2/day for 3 days) or idarubicin (12 mg/m2/for 3 days). Among ENA-treated patients, the combination therapy was well-tolerated, demonstrating a safety profile consistent with that of induction and consolidation chemotherapy. The best overall CR rate achieved was 47%, with 12-month survival probabilities reaching 76%. Despite these

promising outcomes, the absence of a comparison group receiving intensive chemotherapy alone underscores the necessity for a randomized controlled trial to adequately compare response rates between combination therapies and the 7+3 schedule alone.

Furthermore, the proliferation of leukemic blasts may be driven by the AKR1C3-mediated conversion of prostaglandin D2 (PGD_2) to 9α and 11β-prostaglandin F2α (11β-PGF2α)(Desmond, 2003), (Birtwistle et al., 2009).However, by inhibiting AKR1C3 activity, PGD_2 spontaneously dehydrates to 15-deoxyΔ12,14PGJ2 (15dPGJ2), which binds to PPARγ, prompting myeloid cell differentiation and apoptosis (Shiraki et al., 2005), (Hayden et al., 2008). Additionally, AKR1C3 exhibits retinaldehyde reductase activity toward all-trans-retinoic acid (ATRA) (Ruiz et al., 2011), a compound pivotal for myeloid differentiation and widely used in differentiation therapies against AML (Brown & Hughes, 2012). Consequently, several studies have indicated that AKR1C3 over-expression in AML cells leads to resistance to PGD_2- and ATRA-mediated differentiation, suggesting that AKR1C3 inhibitors could re-sensitize AML cells to these mediators (Mills et al., 1998), (Bunce et al., 1996)as demonstrated by (Desmond, 2003)in the case of ATRA. In this sense, by this work, we preliminary demonstrated how ENA significantly inhibits AKR1C3-mediated transformation of PGD_2 to 11β-PGF2α in vitro and how this inhibition related to a re-sensitization of KG1α cells to PGD_2 **(Figure 19)**.

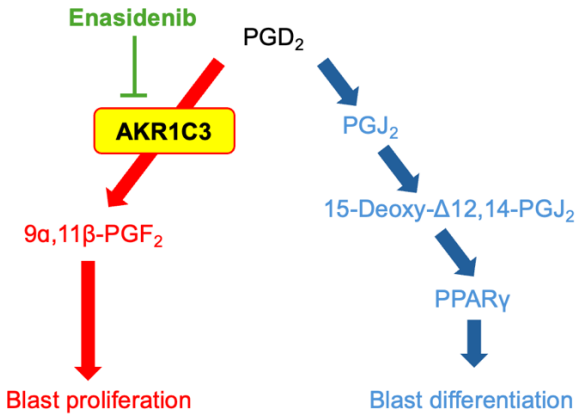


Figure 19. ENA reduces KG1α cells' metabolization of PDG_2 to 11β-PGF2a and proliferation. In this work, we preliminary demonstrated that ENA inhibits AKR1C3 mediated metabolism of PGD_2 in vitro. This mechanism may relate to ENA decreasing KG1α cells' metabolization of PDG_2 to 11β-PGF2a and the induced proliferation.

Therefore, we demonstrated novel molecular targets for ENA in the context of AML treatment. This is relevant because it could extend the use of this drug to those AML patients not carrying IDH2 mutations. It is still a question if the IDH1 inhibitor ivosidenib could also target AKR1C3 and ABC transporters like ENA, further experiments are required for this. On the other hand, more experiments are needed to ensure that ENA specifically inhibits AKR1C3-mediated metabolism of PGD_2 in AML cells and if this mechanism can affect the proliferation/differentiation of leukemic blasts.

7 CONCLUSIONS

In conclusion, the present study delved into the effectiveness of ENA, an isocitrate dehydrogenase inhibitor, in targeting AKR1C3 functions implicated in AML cell proliferation and drug resistance. The objectives outlined were pursued to shed light on ENA's potential therapeutic role in overcoming AKR1C3-mediated resistance mechanisms in AML.

-ENA exhibited a dual effect not only with increasing intracellular Daun levels but also prevented AKR1C3 from converting Daun to its less cytotoxic form, Daunol. This dual action of ENA corresponded with a synergistic enhancement of Daun cytotoxicity in KG1α cell, suggesting a promising strategy for combating drug resistance in AML.

-Preliminary evidence indicating that ENA significantly inhibited AKR1C3-mediated transformation of PGD_2 to its metabolite 11β-PGF2α *in vitro*. This inhibition underscores the therapeutic potential of ENA in disrupting key pathways implicated in AML pathogenesis.

-This study suggests that ENA's inhibition of AKR1C3 may be associated with a re-sensitization of KG1α cells to PGD_2. But further experiments are still needed.

In summary, our study underscores the potential of ENA as a promising therapeutic agent for overcoming AKR1C3-mediated drug resistance and blast proliferation in AML. By targeting key pathways involved in AML pathogenesis, ENA offers a versatile approach to enhance the efficacy of conventional chemotherapeutic agents and mitigate drug resistance.

8 LIST OF ABBREVIATIONS

ABC: ATP-binding cassette

ABCB1: ATB-binding cassette sub-family member 1

ABCC1: ATP-binding cassette sub-family C member 1

ABCG2: ATP-binding cassette sub-family G member 2

AGEs: Advanced glycation end products

AKR: Aldo-keto reductase

AML: Acute myeloid leukemia

Ara-C: Cytarabine

ATRA: All-trans retinoic acid CAR

CAR: Chimeric antigen receptor

CDK: cyclin-dependent kinase

cDNA: Complementary DNA

Cmax: Maximum concentration

CR: Complete Remission

CREB: cAMP response element-binding protein

CREs: Carbonyl reducing enzymes

CYP450: Cytochrome 450

Daun: Daunorubicin

Daunol: Daunorubicinol

DCK: Deoxycytidine Kinase

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNMT3A: DNA methyltransferase 3A

Dox: Doxorubicin

Doxol: Doxorubicinol

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial to mesenchymal transition

ENA: Enasidenib

Epiol: Epirubicinol

ERK: Extracellular signal-regulated kinase

ETS: ETS- related gene

FDA: Food and drug administration

FLT3-ITD: FMS-like tyrosine kinase – internal tandem duplication

GABA: Gamma-aminobutyric acid

GHB: Gamma-hydroxybutyrate

GLI: Glioma-associated oncogene homologue

GO: Gemtuzumab Ozogamicin

hENT1, hENT2, and hCNT3: Human equilibrated nucleoside transporter 1,2 and human concentrative nucleoside transporter 3

HPLC: High-performance liquid chromatography

IC_{50}: Half-maximal inhibitory concentration

Idarubicinol: Idaol

IDH: Isocitrate dehydrogenase

KG1α: cell line

MEK: Mitogen-activated protein/ Extracellular signal-regulated kinase

MRP7 and MRP8: Multidrug resistance-associated protein 7 and 8

MS: Mass spectrometry

NADPH: Nictoinamide Adenine Dinucleotide Phosphate

NDK: Nucleoside diphosphate kinase

NF-κB: Nuclear factor kappa-B

NMDA: N-methyl-D-aspartate

NMR: Nuclear magnetic resonance

NNK: Nicotine-derived nitrosamino-alcohol

NQO1: NADPH quinone oxido reductase

PAH: Polycyclic aromatic hydrocarbons

PBS: Phosphate-buffered saline

PCa: Prostate cancer

PCR: Polymerase chain reaction

PGH2: Prostaglandin H2

PI3K/Akt: Phosphatidylinositol-3 kinase/ Protein kinase B

POVPC: (1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocoline)

PPAR: Peroxisome proliferator activated receptor

PTEN: Phosphatase and tensin homologue deleted on chromosome ten

PUFA: Polyunsaturated fatty acids

PXR: Protein X receptor

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

RBC: Red blood cells

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RXR: Retinoid X receptor

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNPs: Single nucleotide polymorphisms

SSADH: Succinic semialdehyde dehydrogenase

Tmax: Time to reach maximum concentration

UGT: UDP-glucuronosyltransferase

UHPLC: Ultra-high performance liquid chromatography

UV-VIS: Ultra-violet visible

XDH: Xanthine dehydrogenase

μM: Micromolar

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