CHARLES UNIVERSITY

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Department of Biochemical Sciences

Title:

ISOCITRATE DEHYDROGENASE 2 INHIBITOR ENASIDENIB SYNERGIZES DAUNORUBICIN CYTOTOXICITY BY TARGETING ALDO-KETO REDUCTASE 1C3

Master's Thesis

Thesis Supervisor: Prof. Ing. Vladimír Wsól, Ph.D.

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Hradec Králové 2023

Andro 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 08. 05. 2023

…….

Andro Haddad

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to the following people who have supported and encouraged me throughout my master's thesis:
First, I would like to thank my thesis supervisor prof. Ing. Vladimír Wsól, Ph.D. for professional guidance and assistance in the preparation of the diploma thesis.
I am also deeply grateful to Anselm Morell García, Ph.D. for his exceptional support and guidance. Moreover, Mgr. Lenka Laštovičková, Ph.D., for her insightful comments and suggestions.
My sincere appreciation goes to my family and friends, for their support and encouragement throughout my academic journey.

ABSTRACT

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Treatment with anthracyclines is crucial in treating several oncologic disorders. However, several molecular mechanisms hinder the effectivity of anthracyclines, which is a significant obstacle in cancer therapy. Carbonyl reducing enzymes (CREs), a type of NAD(P)H-dependent oxidoreductase, contribute to anthracycline resistance by reducing these drugs to fewer active alcohols. These enzymes also play a role in the proliferation and differentiation of cancer cells, leading to increased tumour aggressiveness. Therefore, targeting these enzymes is essential for effective anticancer therapy.

This study aimed to uncover the potential off-targets of the isocitrate dehydrogenase (IDH) inhibitor enasidenib (ENA) that could counteract the resistance to anthracycline, specifically in relation to the detoxification role of CREs. For this, we screened the ability of ENA to inhibit different recombinant CREs that can reduce daunorubicin (Daun) to daunorubicinol (Daun-ol). Furthermore, we evaluated how ENA counteracts the metabolism of Daun in different human cell lines that express the Aldo-keto reductase 1C3 (AKR1C3) either endogenously (A549) or exogenously (HCT116). Moreover, the potential synergistic effect of ENA on Daun cytotoxicity was quantified in the same cell models. ENA was found to selectively inhibit AKR1C3-mediated inactivation of Daun both over the recombinant enzyme and in cell lines expressing AKR1C3, which contributed to synergizing Daun cytotoxicity to overcome resistance. This study provides in vitro evidence of ENA targeting the anthracycline resistance actor AKR1C3 at clinically achievable concentrations, thus, suggesting that ENA could be combined with anthracyclines to improve their therapeutic efficacy.

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1 Introduction

Multidrug resistance is a significant obstacle in cancer treatment, as it can lead to the failure of chemotherapy and cancer recurrence. Resistance can be primary, meaning it is present from the start of treatment and is genetically determined; or acquired, which develops during treatment and is often caused by changes in cancer cells. The development of resistance to chemotherapies can involve a range of mechanisms, such as alterations in drug metabolism, changes in drug targets, activation of DNA repair pathways, and increased efflux of drugs from cancer cells. The study of resistance mechanisms and the development of strategies to overcome resistance are essential for improving chemotherapy's efficacy and patient outcomes.

Human aldo-keto reductase family 1 member C3 (AKR1C3) is overexpressed in various metabolic diseases, hormone-dependent and -independent carcinomas, and has been correlated with clinical drug resistance. In recent years, the development of selective AKR1C3 inhibitors has gained attention as a potential therapeutic strategy to overcome resistance to therapeutic drugs, primarily anthracyclines (Liu et al., 2020).

This thesis aims to discern how the isocitrate dehydrogenase (IDH) inhibitor enasidenib (ENA) could influence the activity of AKR1C3 in the context of anthracyclines resistance in vitro.

2 THEORETICAL BACKGROUND

2.1 ALDO-KETOREDUCTASES

Aldo-keto reductases play a crucial role in the biotransformation and elimination of xenobiotics, encompassing phase I and phase II, as well as the efflux transport outside the cells. Lipophilic compounds are usually initially processed by the phase I enzymes, which create or expose an essential functional group for following conjugation reactions. Phase II enzymes are responsible for conjugating the compounds with a polar endogenous compound and often interact with the intermediates the phase I enzymes create. These more polar compounds are then eliminated through active and passive transport mechanisms. The clearance of most xenobiotics happens through multiple enzymes and pathways; the chemical concentration can determine the specific metabolism of a xenobiotic in an individual, the amount of and binding preferences of the enzymes, and the availability of cofactors (Croom, 2012).

The aldo-keto reductases (AKR) superfamily is a group of monomeric NAD(P)(H)-dependent enzymes that perform redox transformations related to biosynthesis, intermediary metabolism, and detoxification. Their target molecules include glucose, steroids, products of sugar modification, by-products of lipid breakdown, and environmental toxins (Barski and Tipparaju, 2008). AKRs are present in nearly all living organisms, from prokaryotes to eukaryotes, including fungi, plants, and humans. In addition, AKRs share a specific structural pattern, composed of an $(\alpha/\beta)_8$-barrel motif with three flexible loops defining the shape of the substrate-binding site (**Figure 1**) (Ahmed Laskar & Younus, 2019).

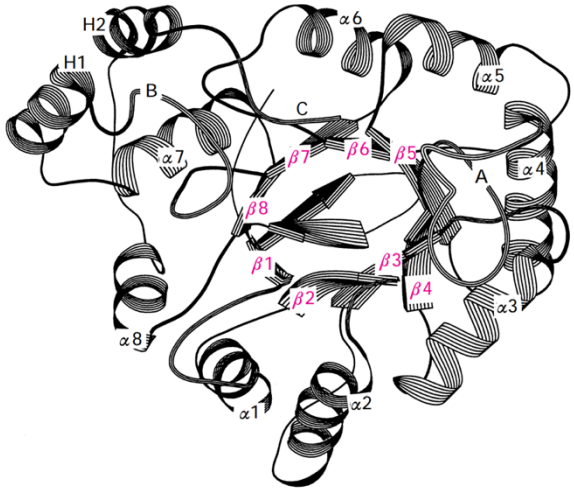

Figure 1. Three-dimensional fold of the common $(\alpha/\beta)_8$-barrel motif of the AKRs with the C-terminus on the top. The α-helices and β-sheets (in pink) of the barrel are indicated. The three loops forming the substrate binding site are indicated as A, B and C. (Jez et al., 1997)

AKRs are mainly located in the cytoplasm and perform oxidation-reduction reactions as monomeric enzymes. Examples of AKR enzyme systems include aldehyde reductase (ALR), aldose reductase (AR), xylose reductase, prostaglandin F synthase, etc. These enzymes metabolize molecules containing a carbonyl group and have a wide range of substrate specificity. So far, 190 AKRs have been identified and are grouped into 16 families labeled AKR1-AKR16.

2.1.1 AKRs Functions

Physiological Functions: - Detoxification and Synthesis:

- Lipoperoxidation Products: The detoxification of lipid aldehydes by AKRs is an essential physiological function of these enzymes. Under conditions of oxidative stress, reactive oxygen species (ROS) can attack polyunsaturated fatty acids (PUFAs) and form lipid peroxides that break down into reactive bifunctional electrophiles, such as 4-hydroxy-2-nonenal (4-HNE). These electrophiles can form protein crosslinks and are implicated in developing diseases such as atherosclerosis and neurodegenerative diseases. Multiple enzyme systems can detoxify 4-HNE, such as AKRs, to limit lipid aldehydes' potential toxicity. AKR1B1 reduces 4-HNE to 1,4dihydroxy-2-nonene (DHE), effectively removing the reactive aldehyde and α,β-unsaturated carbonyl in a single reaction, while AKR1C1-1C4 and AKR1A1 also can detoxify 4-HNE. These AKR1C isoforms appear unique among AKRs because they detoxify bifunctional electrophiles in a single step (Penning & Drury, 2007).

- Glycosylated end products (AGEs): AGEs are created by modifying proteins with carbohydrates or carbohydrate metabolism products containing reactive carbonyl groups. The process begins with forming Schiff bases with amino groups of proteins, followed by forming Amadori products and further oxidative and non-oxidative reactions, which ultimately lead to the formation of advanced glycation end products (AGEs). They accumulate with normal aging and in diseases such as diabetes, atherosclerosis, renal failure, Haemodialysis-associated amyloidosis, and Alzheimer's disease (Barski et al., 2008).

The primary reactive carbonyl compounds from carbohydrates are glyoxal, methylglyoxal, D-arabinose, and 3-deoxyglucosone. In addition to carbohydrates, AGEs can also be derived from polyunsaturated fatty acids, amino acids, and ascorbate. Detoxification of reactive carbonyl compounds proceeds through reduction, conjugation, or oxidation. For example, methylglyoxal is detoxified by the glutathione-dependent glyoxalase system or through reduction by AKR1B1 and AKR1A1 (Barski et al., 2008).

- Prostaglandins: Prostaglandins synthesis involves several stages, with PGH2 being further converted to PGD2, PGE2, PGI2, and PGF2alpha. An enzyme called PGF synthase, or AKR1C3, catalyzes the reaction of PGF2alpha. This enzyme also catalyzes the conversion of PGD2 to 9alpha, 11Beta-PGF2, the active form of PGF2. AKR1C3 reduces PGh2 endoperoxide and the aldehyde group PGD2 but cannot reduce PGE2. AKR1C3 is mainly found in the lungs and spleen but is also in smaller amounts in the kidneys, skeletal muscles, leukocytes, uterus, and liver. Other enzymes, such as AKR1C1, 1C2, and enzymes from other superfamilies, can also participate in these reactions. Defects in the synthesis of AKR1C3 can lead to disorders of vasoconstriction, temperature regulation, smooth muscle constriction, and the sleep/wake cycle in the brain (Barski et al., 2008).

- Steroids: The AKR1C family of enzymes plays a role in the synthesis and metabolism of steroid hormones. These enzymes are 3-alpha-hydroxysteroid dehydrogenases, also known as dihydrodiol dehydrogenase. These enzymes have different substrate specificities and tissue-distribution characteristics, which reflects the diverse roles they play in the formation and inactivation of sex hormones. In the liver, the 3alpha-HSD work with other enzymes to convert dihydro steroids into tetrahydro steroids, thereby inactivating circulating steroid hormones and limiting their biological activity. The liver-specific AKR1C4 enzyme is well-suited to perform this function. In steroid target tissues, the differential distribution of AKR1C isoforms contributes to maintaining a pro-oestrogenic or anti-androgenic state. In the prostate, AKR1C2 and AKR1C3 reduce the androgen dihydrotestosterone to a weakly androgenic metabolite, thus regulating the occupancy of the androgen receptor. In the mammary gland, AKR1C3 converts delta-androstane-3,17-dione to testosterone, which can then be aromatized to estrogenic and progestogenic precursors, contributing to a pro-oestrogenic state. AKR1C1 reduces

progesterone initiating parturition and termination of pregnancy. Downregulated expression of AKR1C1 and C2 have been found in ovarian cancer tissue and are associated with decreased ability to catabolize progesterone. AKR1A and 1B family members also contribute to the catabolism of steroid hormones by reducing corticosteroids, such as iso-corticosterone and iso-cortisol, which have an aldehyde group at the position C-21 and a hydroxyl group linked to C-20. The AKR1A1 displays a marked preference for these iso-corticosteroids (Barski et al., 2008).

- Succinic Semialdehyde: The neurotransmitter GABA is metabolized by an enzyme called monoamine oxidase (MAO, GABA-T), which forms an intermediate compound called succinic semialdehyde. This intermediate can be further metabolized in two ways. One way is through oxidation by an enzyme called succinic semialdehyde dehydrogenase (SSADH), which converts it to succinic acid. The other way is through conversion to a compound called gamma-hydroxybutyrate (GHB) by enzymes called aflatoxin and aldehyde reductases. Both are primarily found in the brain and nerve cells. The enzyme SSADH deficiency has been linked to seizures and mental retardation (Barski et al, 2008).

- Glucouronate-Xylulose pathway and vitamin C biosynthesis: AKR1A is an enzyme crucial in synthesizing vitamin C. It converts D-Glucuronate to L-gluconate, a necessary step in the biosynthesis of vitamin C. Inhibition of AKR1A leads to a significant decrease in urine vitamin C levels. L-gluconate can also be used in the pentose phosphate pathway, mainly active in the renal cortex and proximal tubules. That is the fate of L-gluconate in humans and guinea pigs unable to produce their own vitamin C.

- Glucose: AKR1B1 converts excessive glucose to sorbitol in cells, especially in Schwann cells and cataractous lens, which causes complications associated with diabetes. Studies have attempted to alleviate symptoms by examining AKR1B1 (inhibitors); however, one of the problems is the crucial role in detoxifying lipoperoxides. Thus, AKR1B1 inhibition has a setback; on the one hand, it functions as a detoxifying enzyme that removes toxic lipid peroxidation products. However, on the other hand, mediating hyperglycaemic injury by converting excessive glucose to sorbitol (Barski et al., 2008).

- Nuclear Receptor Signalling: The human AKR enzymes regulate gene transcription by reducing lipophilic substances, such as ketosteroids, prostaglandins, and retinals, which can affect the access of these substances to nuclear receptors and hence affect gene transcription. The principal receptors are:

  - PPAR-gamma: This protein acts as a receptor mainly found in fat cells called adipocytes. It plays a role in these cells' development and differentiation and regulates glucose and fatty acid metabolism. AKR1C3 has two ways of influencing the activity of PPAR. Firstly, it can produce a compound called 15-deoxy-Δ12,14-PGJ2α which binds to PPAR and alter its activity. Secondly, AKR1C3 can also modify PPAR by phosphorylating it, leading to an increase in cell growth or proliferation.

  - RXR: AKR1B10 and AKR1C3 regulate retinoic acid signalling. AKR1B10 is an enzyme found in the small intestine that reduces retinal to retinol, which prevents the formation of 9-cis-retinoic acid, an essential ligand for RXR. RXR heterodimerizes with another receptor called PPARc and affects gene transcription, leading to an anti-proliferative response. The combined action of AKR1C3 and AKR1B10 may regulate this response by targeting different parts of the heterodimeric complex. However, more research is needed to determine whether selective inhibitors of these enzymes could help treat certain medical conditions (Penning & Drury, 2007).

  - CAR, PXR, and FXR: AKR1D1 is an enzyme found in the liver that converts specific steroid molecules into different compounds that can bind to certain receptors known as CAR, PXR, and FXR. These receptors can then increase the activity of specific genes, including those involved in detoxifying drugs (CYPs) (Penning & Drury, 2007).

  - Aryl-hydrocarbon receptor (AhR): is activated by planar polycyclic aromatic hydrocarbon o-quinones, produced by the activity of certain enzymes called AKR1A1 and AKR1C1-AKR1C4 (Dihydrodiol dehydrogenases). This activation leads to increased expression of specific genes, including those detoxifying foreign substances, by binding to the AhR with high affinity. Studies have shown that this process is essential for the induction of the CYP1A1 gene in human cells, and it is also observed that this process is preceded by the movement of AhR in the cell's nucleus (Penning & Drury, 2007).

The function of AKRs towards xenobiotics:

- Aflatoxin: Aflatoxin is a harmful chemical produced by a fungus called Aspergillus flavus, which can cause cancer in the liver. It is activated by an enzyme called P450 3A4, which transforms it into a substance called aflatoxin B1-9,0-epoxide, a potent carcinogen. This substance can be transformed by another enzyme called epoxide hydrolase into aflatoxin-8,9-dihydrodiol. That can cause damage to cells. Then, it is transformed by the AKR7A family members, specifically AKR7A2 and AKR7A3, into less harmful substances, mono- and di-alcohols which can prevent the toxicity caused by aflatoxin.

- Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants that must be metabolically activated to cause cancer. The most well-known PAH is benzo[alpha]pyrene (B[a]P). B[α]P is activated through monooxygenases by the enzymes P4501A1/1B1 on the terminal benzo-ring, which forms the 7R,8S-arene oxide. After a series of reactions that yield (+)-anti-B[a]-diol-epoxide (anti-B[α]PDE), which is highly mutagenic and carcinogenic. Another way B[a]P is activated is through the dihydrodiol dehydrogenase activity of the human AKRs (AKR1A1, 1C1-1C4), which oxidize the B[a]P-7,8-dihydrodiol to yield the corresponding ketol, which rearranges to form a catechol. This catechol can then undergo a 1 electron oxidation step to yield the fully oxidized o-quinone, which can react with DNA, RNA, protein, GSH, and amino acids. This o-quinone is redox-active and can deplete cellular NADPH and amplify ROS production. In the presence of NADPH, the o-quinone can be reduced back to the catechol, allowing for further autooxidation, leading to NADPH depletion and the production of ROS (Penning & Drury, 2007).

- Nicotine-Derived nitro aminoketones (NNK): are components of tobacco smoke that are lung carcinogens. NNK can be activated through alpha-methylene hydroxylation by P450 2A6 and P450 3A4 or alpha-methyl hydroxylation by P450 2A6 and P450 3A4. NNK can be reduced to form a nitrosamine-alcohol (R/S-NNAL). The S-NNAL isomer has similar carcinogenic properties to NNK and is formed by the AKR1C1, 1C2, and 1C4 enzymes. In contrast, the R-NNAL isomer is formed by the 11beta-HSD type 1 and does not have carcinogenic properties. The reduction of NNK to NNAL is a form of

detoxification, as the alcohol allows NNAL to be eliminated through glucuronidation (Penning & Drury, 2007).

Therapeutic implications:

Many drugs have a carbonyl (mainly ketone) group in their structure, making them potential substrates for AKRs. These drugs include a wide range of pharmaceuticals with various effects, such as anticancer, antipsychotic, antidepressants, opiate antagonists, antiasthmatic, antidiabetic, and antiemetic drugs. For some drugs, such as the anticancer Daun, reduction by AKRs decreases its effectiveness. For others, such as the antiemetic dolasetron, modification by AKRs leads to forming the active compound. AKR levels and activities vary among individuals, making it essential to understand their role in drug metabolism to effectively prescribe and monitor drugs for efficacy and toxicity (Barski et al., 2008).

2.1.2 Nomenclature of AKRs

The naming convention introduced and accepted by the Hugo Genome Nomenclature Committee (HGNC) in 1997. And is created based in that of the cytochrome P450 isoenzymes. For example, AKR1C3:

- AKR – Stands for the superfamily of aldo-keto reductases
- 1 – represents the family (Ranging from 1 to 16)
- C – denotes the subfamily
- 3 – represents the unique protein sequence within the family

The family classification is based on 40% similarity in amino acids, while subfamily members have up to 60% similarity. And the amino acid sequence refers to the unique sequence of each of the proteins (Mindnich & Penning, 2009).

2.1.3 The Structure of AKRs

AKRs contain around 320 amino acids and have a molecular weight of 34-37 kDa. These proteins have a structure composed of eight beta-sheet layers that form the core and eight alpha-helices that encase the core giving it a barrel-like shape. On the back side of the protein, three loops named as A, B and C play a role in binding a cofactor and the substrate. The binding site

for the cofactor, which typically prefers NADPH over NADH, is highly conserved and has a nicotinamide head in an anti-conformation to the ribose. Whereas the binding site for the substrate is highly flexible and contains four catalytic residues highly conserved: tyrosine, lysine, aspartic acid, and histidine (**Figure 2**) (Penning & Drury, 2007).

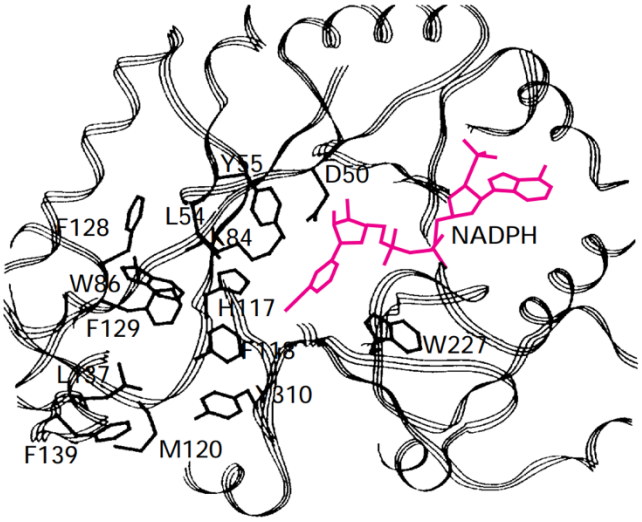

Figure 2. This stereo-view displays the residues of the substrate binding site in relation to the active site residues (Asp-50, Tyr-55, Lys-84 and His-117) and bound NADPH (depicted in pink). (Jez et al., 1997).

2.2 AKR1C3

The AKR family 1 member C (AKR1C) enzymes have been found to have varying expression levels and functions throughout the human body. One of these isoforms, AKR1C3, also known as 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5), plays a role in the production of androgens (such as testosterone and 4α-dihydrotestosterone) and estrogens (such as 17β-estradiol), which can bind to the androgen receptor (AR) and estrogen receptor (ER) in different target tissues. AKR1C3 can also convert prostaglandin D2 (PGD2) into 9alpha,11beta-PGF2alpha, promoting tumour cell proliferation by preventing peroxisome proliferator-activated receptor gamma (PPARγ) activation. Overall, AKR1C3 is crucial in regulating cell growth and differentiation in both hormone-dependent and hormone-independent manners (Penning et al., 2021).

AKRs are enzymes at high levels in a broad range of human cancers, including prostate, breast, glioma, neuroblastoma, non-small and small cell lung cancer, colon cancer, the small intestine,

and acute myeloid leukemia (AML). For example, it has been observed that AKR1C3 is expressed in certain types of cancer, such as non-small cell lung cancer, breast cancer, prostate cancer, and acute myeloid leukemia. The high abundance of AKRs in cancer has led to the suggestion that they can serve as biomarkers for oncological disorders:

- Prostate Cancer: Androgens, hormones that promote the growth of prostate cancer cells, are the primary driver of prostate cancer growth via the androgen receptor (AR). Despite the androgen deprivation therapy (ADT), which is the standard treatment for advanced prostate cancer patients, many patients develop a disease relapse—known as castration-resistant prostate cancer (CRPC). AKR1C3 is an enzyme that plays a crucial role in the biosynthesis of testosterone and estradiol. Compared to prostate cancer, elevated levels of AKR1C3 expression have been reported in CRPC. Recent studies have found an orally available inhibitor of AKR1C3 called ASP952, which has anti-tumour activity in preclinical models (Zeng et al., 2017).

- Acute Myeloid Leukaemia (AML): The enzyme AKR1C3 catalyzes the synthesis of prostaglandins, which help sustain the growth of myeloid precursor cells in the bone marrow. However, the enzyme is overexpressed in specific subtypes of AML, and this overexpression leads to resistance to certain chemotherapy drugs, such as etoposide, anthracyclines, and cytarabine. A study examined the potential of using selective inhibitors of AKR1C3 (KV-49a and KV-49g) in combination with the chemotherapy drug Daun in AML cell lines with varying levels of AKR1C3 expression. The results indicated that the combination did not produce a substantial enhancement effect in cells with low, moderate, and high AKR1C3 expression; however, when the cells were pre-treated with either inhibitor for 24 h, a significant enhancement in the effectiveness of Daun was observed (Penning et al., 2021)

2.3 Anthracyclines

Anthracyclines are among the most effective anti-cancer drugs that have been developed. They were first discovered in the 1960s from a type of bacteria called *Streptomyces peucetius* and were named doxorubicin (Dox) and Daun. Both drugs have similar chemical structures (**Figure 3**), with a tetracyclic ring and adjacent quinone-hydroquinone groups in the C-B rings, a

methoxy substituent a at C-4 in the D ring, and a short chain at C-9 with a carbonyl at C-13. The main difference between the two drugs is that DOX has primary alcohol in its side chain, while DNR has methyl. This slight difference leads to differences in their activity. DOX is recommended for treating breast cancer, childhood solid tumours, soft tissue sarcomas, and aggressive lymphomas. In contrast, DNR is mainly employed in acute lymphoblastic or myeloblastic leukemias (Minotti et al., 2004).

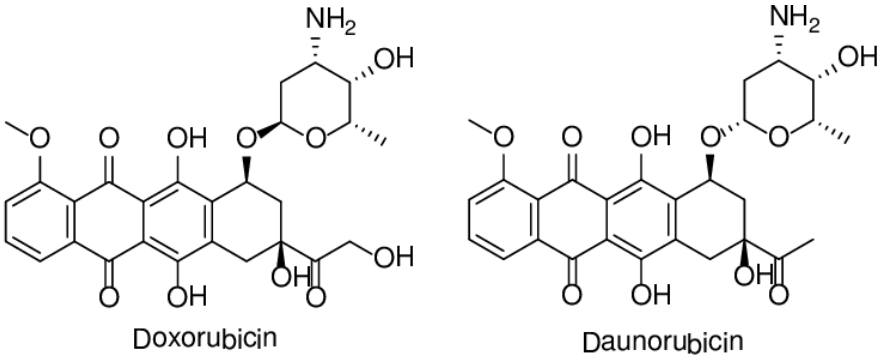

Figure 3. Chemical structures of DOX and Daun.

2.3.1 Mechanism of Action

- Anthracyclines as Topoisomerase II inhibitors: Topoisomerases are enzymes responsible for modifying the DNA structure in cells, and they can cause transient breaks in the DNA strands. The TOP II breaks and reconnects the phosphodiester bond in both DNA strands, allowing replication and transcription to occur. Anthracyclines bind to TOP II and stabilize a reaction intermediate in which the DNA strands are cut and covalently linked to the enzyme. Anthracyclines form a complex with a broken strand of DNA, preventing it from reconnecting. That leads to irreversible DNA breaks and induces apoptosis (Minotti et al., 2004).

- Intercalation into DNA: It is the process of the drugs inserting themselves between the DNA base pairs. The drug inserts itself between the base pairs of DNA and holds on to them through hydrogen bonds and van der Waals interactions. This interaction blocks RNA synthesis and stops DNA replication, leading to cell death (Minotti et al., 2004).

2.3.2 Anthracyclines Cytotoxicity

Anthracyclines are a class of chemotherapy drugs found to have a toxic effect on the heart. The exact mechanism of this toxicity is not fully understood, but the drugs are believed to create free radicals that damage heart cells. Anthracyclines toxicity has been classified into acute, early-onset chronic, and late-onset chronic. Acute cardiotoxicity occurs shortly after treatment, is usually reversible, and symptoms appear within 14 days of treatment. Early-onset chronic cardiotoxicity occurs within a year, is the most common form, and presents as dilated and weak heart muscle with a progressive evolution towards heart failure. Late-onset chronic cardiotoxicity develops years, or even decades, after the end of anthracyclines therapy and is irreversible, with poor prognosis and limited treatment options. However, recent research suggests that cardiotoxicity caused by anthracyclines is a continuous process that starts at the cellular and can lead to heart failure (**Figure 4**). By using biomarkers like troponin, it is possible to identify cardiotoxicity early (Cardinale et al., 2020).

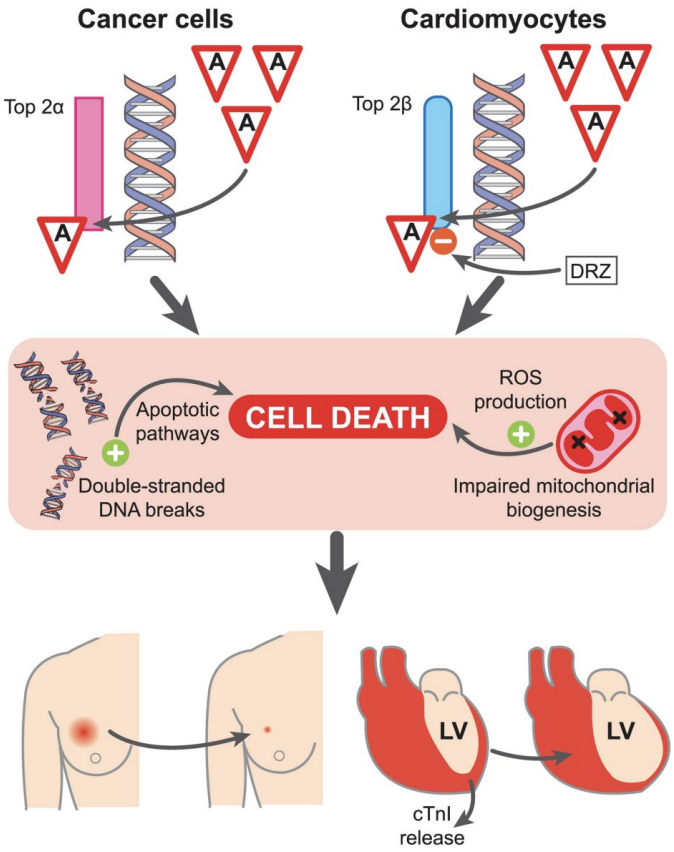

Figure 4. Mechanism of action of anthracyclines and cardiotoxicity ensuing from the drug inhibiting Topoisomerase II. Anthracyclines inhibit Top Iα, while in heart cells they inhibit Top IIβ. Top II inhibition in both cells causes accumulation of double-stranded DNA breaks and

mitochondrial dysfunction leads to the build up of DNA breaks and mitochondrial dysfunction leading to activation of cell death pathways and accumulation of ROS. Taken from (Henriksen, 2018)

Regarding the risk factors, the most factor is the total cumulative dose; for example, in the case of Daun, it is a dose higher than 400-500 mg/m2. Other risk factors include young age (>5 years) or advanced age (>65 years), being female, and the timing of treatment initiation. In general, the later the start of the treatment, the greater the risk. Genetic factors, such as Down syndrome and African-American ancestry, can also play a role. Already established risk factors, such as heart disease, hypertension, diabetes, hyperlipidemia, kidney and liver disease, and other therapy such as radiation therapy or other anticancer drugs (cyclophosphamide, vincristine, bleomycin, mitoxantrone, trastuzumab) also increase the risk. Additionally, lifestyle choices such as smoking, alcohol consumption, and obesity can influence them, too (Cardinale et al., 2020).

2.3.3 Anthracyclines Metabolism

It is estimated that about half of the Dox is eliminated from the body without undergoing any structural changes in humans. At the same time, the rest of the drug is metabolized through three major pathways. The metabolism of anthracycline occurs through hydroxylation (Two electron reduction), semiquinone formation (One electron reduction), or deoxy-aglycone formation, which can lead to the formation of metabolites that either enhance or reduce the anticancer properties of anthracyclines (Edwardson et al., 2015).

- Two electron reduction: The process of adding a hydroxyl group to the C-13 carbonyl group of anthracyclines, also known as two-electron reduction, leads to the formation of secondary alcohol metabolites that have been linked to anthracycline-induced heart damage. This major metabolic pathway of anthracycline is mediated by a family of enzymes called NADPH-dependent carbonyl reductases (CBR) and AKRs. For example, they catalyze the formation of Daun-ol and Dox-ol from their parent drug. For instance, AKR1C3 and CRB1 can convert Daun to Daun-ol (Edwardson et al., 2015).
- One electron reduction: The one-electron reduction of anthracycline is catalyzed by cytochrome P-450 reductase (CRP), NADH dehydrogenase, nitric oxide synthase, and

xanthine oxidase, leading to the conversion of the quinone moiety of anthracycline drugs into a semiquinone radical. This radical is stable in an oxygen-free environment, but when exposed to oxygen, the semiquinone radical is re-oxidized to regenerate the parent quinone. This process produces a superoxide anion and hydrogen peroxide, increasing the formation of reactive oxygen species. The resulting free radicals can cause peroxidation of lipids, protein aggregation, and cell death. The redox cycling of drugs like Dox and Daun has been observed in various cellular compartments such as cytoplasm, mitochondria, and cytoplasmic reticulum. It is believed to contribute to producing toxic aldehydes, which can escape the cell and contribute to anthracycline toxicity (Edwardson et al., 2015).

- Deglycosylation: The final way the anthracyclines are metabolized is through deglycosylation, which entails 1-2% of the drug's metabolism. Anthracyclines are metabolized by breaking down the sugar and side chain carbonyl groups, forming 7-deoxyaglycones and hydroxyaglycones. Enzymes such as NADPH quinone oxidoreductase (NQO1), NADPH-cytochrome P450 reductase (CRP), and xanthine dehydrogenase (XDH) are responsible for this process. Aglycones formed are more lipophilic and can insert themselves into mitochondrial membranes. Studies suggest they may cause heart damage due to their strong oxidizing properties. The two-electron reduction (hydroxylated) product is the most common metabolite with lower percentages than the other products (Edwardson et al., 2015).

2.3.4 Daunorubicin Hydrochloride

Daun is a hygroscopic crystalline, orange-red powder. Its solubility characteristics are easily soluble in water and methanol, slightly soluble in alcohol, and insoluble in acetone. The chemical structure is (8S,10S)-8-Acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride. The molecular weight of Daun is 564 g/mol (European Pharmacopoeia, 2019). It is an antineoplastic drug used to treat and another cytotoxic agent in acute non-lymphocytic leukaemia, including acute myelogenous, promyelocytic, and erythroid leukaemia in adults and acute leukaemia in both children and adults. In addition, as an off-label, Daun is also used as a first-line to treat Kaposi sarcoma in patients with advanced human immunodeficiency virus (HIV) syndrome. Daun can only be administered intravenously. Some of Daun's most common

side effects are bone marrow suppression and cardiotoxicity. Bone marrow suppression can occur in all patients given the therapeutic dose of Daun. Thus, in patients with pre-existing bone marrow suppression, Daun should be used cautiously. The cardiotoxic effect is usually seen after a cumulative dose exceeding 400 to 550 mg/m2. However, it may also occur at 200 mg/m2. Other side effects include alopecia, mucositis, nausea, vomiting, and skin necrosis (Internet 1).

2.3.5 Anthracycline resistance

Despite ongoing efforts to develop new cancer drugs, anthracyclines are still considered the best option for treating AML. AML is a heterogeneous group of blood cancers that occurs in adults and children. Despite advances in understanding the disease and developing new treatments, the standard induction therapy using anthracycline and cytarabine remains the primary treatment for AML. Daun and idarubicin (Ida) are commonly used anthracyclines. However, some leukemia cells may not respond to this treatment due to the mechanisms such as enhanced drug efflux or enzymatic inactivation. NADPH-dependent CBR, specifically within the AKR superfamily, are known to metabolize anthracyclines into less active and cardiotoxic forms, like Daun to Daun-ol (**Figure 5**). Among these enzymes, AKR1A1, 1B10, 1C3, and 7A2 are the most efficient in reducing Daun (Novotná et al., 2020).

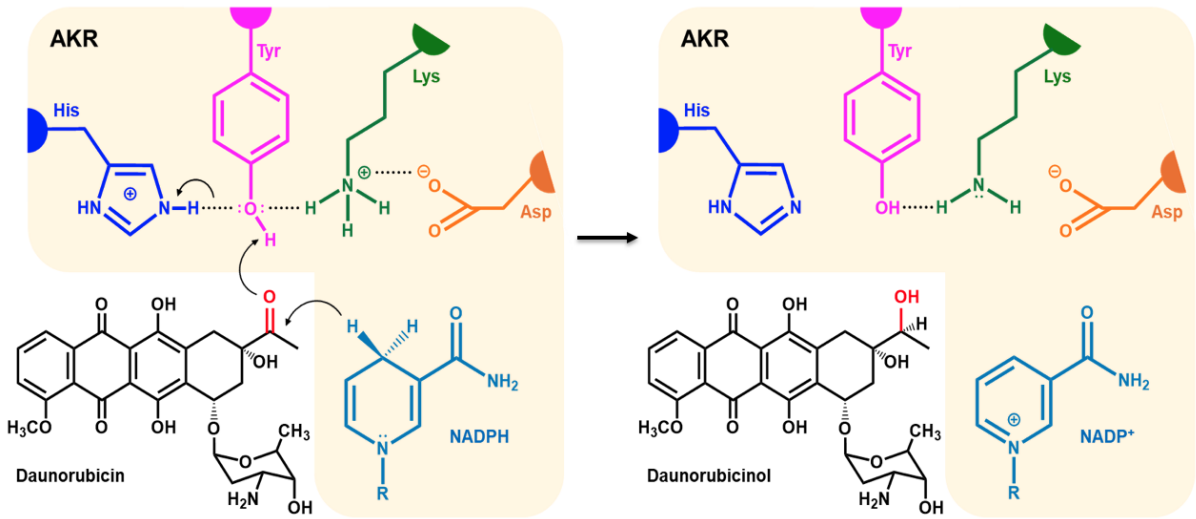

Figure 5. Proposed mechanism involving the catalytic tetrad and cofactor NADPH for the AKR-mediated reduction of Daun to Daun-ol.

Chemotherapy drugs are often used in combination regimens to reduce the likelihood of drug resistance. This approach allows for lower drug doses and increases treatment safety by reducing toxic side effects (Novotná et al., 2020). In recent years, there is an increasing effort to develop new combination regimens that can target those mechanisms involved in anthracyclines resistance and improve the efficiency and safety of these drugs on cancer treatment.

2.4 Targeted Therapy

Cancer treatment typically involves a combination of methods, such as drug therapy, surgery, radiotherapy, and biotherapy. Historically, chemotherapy was the primary form of drug therapy, but it has a significant side effect because it affects both cancer and healthy cells (Zhong et al., 2021). In the late 1800s, Paul Ehrlich proposed the concept "magic bullet," It was initially used to depict the ability of a chemical that targets microorganisms. However, it has since been expanded to include cancer treatment, "Molecular Targeted Therapy." It is a form of cancer treatment that uses drugs or other substances to target specific molecules to stop the growth and spread of cancer cells. Identifying the correct targets is crucial for developing effective molecular-targeted therapies. Cancer occurs when there are changes or mutations in the genetic profile of cells, which can lead to changes in proteins and receptors that promote cell survival and proliferation. These changes can be used as molecular targets for developing drugs to inhibit tumour growth and progression. In addition, through cancer markers and sequencing technology, researchers can study the genetic heterogeneity between malignant and normal cells within an individual (Lee et al., 2018). Recently, there has been a shift towards targeted drugs, which specifically target cancer cells while sparing normal cells, resulting in higher effectiveness and lower toxicity (Zhong et al., 2021). The two main types of targeted therapy are small-molecule drugs and monoclonal antibodies (Internet 2). Small-molecules are compounds with a low molecular weight that can enter cells and target specific proteins. Many small molecule inhibitors, like the recently developed Isocitrate dehydrogenase (IDH) inhibitors, focus on inactivating kinases and disrupting signalling pathways altered in cancer (Lee et al., 2018).

2.4.1 Isocitrate dehydrogenase inhibitors

Isocitrate dehydrogenases (IDH) are enzymes that catalyze the oxidative breakdown of isocitrate into α-ketoglutarate (α-KG) in the tricarboxylic acid (TCA) cycle. However, when there is a mutation in IDH genes, it can lead to the creation of a neomorphic enzyme. It means a new form of the enzyme that catalyzes the conversion of α-ketoglutarate to the oncometabolite called 2-hydroxyglutarate (2-HG). This 2-HG then inhibits the activity enzymes called TET (Ten-Eleven translocation enzyme) and lysine demethylases, which are responsible for removing methyl groups from DNA and histones, respectively. That can cause changes in gene expression and contribute to cancer development (**Figure 6**) (Zhong et al., 2021). These mutations have been found in 20 % of AML patients. Recently, the FDA approved two IDH inhibitors, enasidenib, and ivosidenib, based on clinical trials (Liu & Gong, 2019).

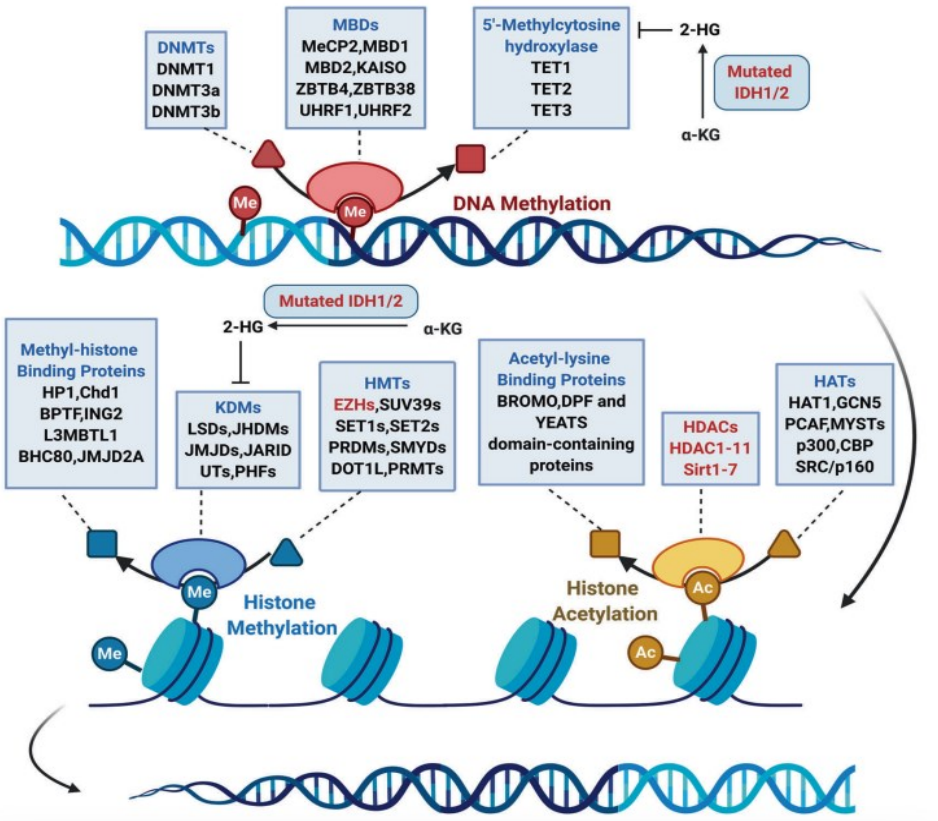

Figure 6. Mutated IDH1/2 causes a conversion of α-KG to 2-HG. This conversion then inhibits the functioning of TET and lysine demethylases, leading to higher levels of DNA methylation altering gene expression and contribute to cancer development.

2.4.2 Enasidenib

Enasidenib (ENA, **Figure 7**) is an oral medication designed to target the IDH2 enzyme present in several cases of AML. Celgene Corporation developed it and has been exclusively licensed by Agios Pharmaceuticals. The FDA has approved it in the United States for treating adults with relapsed or refractory AML with IDH2 mutations. ENA is the first medication of its kind, and it is taken daily at a recommended dosage of 100 mg for a minimum of 6 months. The medication will continue to be taken until the disease progresses, which means the cancer worsens or spreads, or if the patient experiences unacceptable side effects (Kim, 2017). Thus, the patient should stop taking ENA if either of these events occurs before the 6-month minimum period is completed.

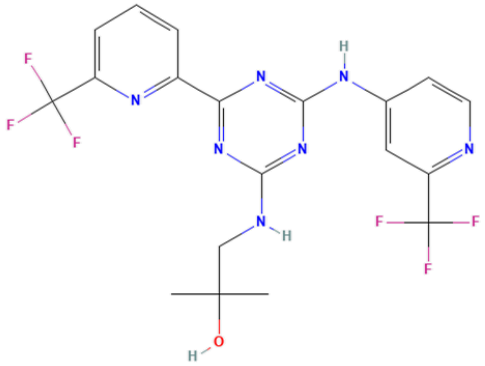

Figure 7. The chemical structure of enasidenib. Obtained from pubchem.ncbi.nlm.nih.gov/

- Pharmacodynamics: ENA is a drug that targets a specific mutation in the IDH2 enzyme in the mitochondria and plays a critical role in the Krebs cycle. The drug works by binding to the active site of the mutant enzyme, preventing it from forming an oncometabolite called R-2-hydroxyglutarate (Kim, 2017).

- Pharmacokinetics: ENA is taken orally and reaches peak concentration in the blood within 4 h of dosing. It has an absolute bioavailability of 57 %, and its exposure increases proportionally with higher doses. The medication is highly protein-bound in the blood and has a volume of distribution of 55.8 L. ENA has a long terminal half-life of 137 h and a mean total body clearance of 0.74 L/h. According to in vitro studies, ENA metabolism is mediated by multiple CYP enzymes (e.g. CYP 1A2, 2C9, 2C19, 2D6, 3A4) and UGTs (e.g. UGT1A1, 2B7, 2B15). The majority of the drug is eliminated in feces (89%), with a small portion eliminated in the urine (11%) (Kim, 2017)

3 AIM OF THE THESIS

The current study aims to investigate the potential of ENA, a clinically available drug, as a novel strategy to prevent anthracyclines resistance mediated by AKRs. The specific objectives of this thesis are:

1. To assess the inhibitory potential of ENA on the metabolism of Daun mediated by recombinant AKRs.

2. To analyze the scope of Daun metabolism inhibition by ENA on cell lines expressing AKR1C3 either endogenously or exogenously.

3. To evaluate how ENA influencing AKR1C3-mediated Daun metabolism in cells improve their sensitivity to cytotoxicity and thus indicates a novel synergistic drug combination.

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)

$MgCl_2$ (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. CR)

Acetonitrile (Sigma-Aldrich, Germany)

PBS (Sigma-Aldrich, Germany)

MTT (Sigma-Aldrich, Germany)

DMEM (Lonza, USA)

4.1.2 Recombinant enzymes

The standard techniques described in Sambrook & MacCallum's laboratory manual (2001) were used to prepare the stocks of recombinant forms of carbonyl reducing enzymes at the Department of Biochemical Sciences of the Faculty of Pharmacy in Hradec Králové. Briefly, the coding sequence of each enzyme was amplified from a human liver cDNA library using PCR with 18-21 nucleotide primers containing restriction sites. The resulting PCR fragments were cloned into pET28b and pET15b vectors. E.coli strain HB101 was used to amplify the vectors, and the correct sequences were confirmed by sequencing. Overexpression of the enzymes was perform in the E.coli strain BL21 (DE3) using IPTG. His-tagged enzymes were purified using Ni-affinity chromatography columns on an Äkta purifier system, and their activity was confirmed by SDS-PAGE. The purified recombinant enzymes were stored in a 20 mM Na-phosphate buffer (pH 7.4) with 20-40% glycerol, and their protein concentration was determined using a BCA Protein Assay Kit.

AKR1A1 (1.7 mg/ml)

AKR1B1 (1.63 mg/ml)
AKR1B10 (1.61 mg/ml)
AKR1C3 (1.3 mg/ml)
CBR1 (2.11 mg/ml)

4.1.3 Enasidenib

Enasidenib was obtained from MCE (MedChemExpress, USA) and reconstituted with DMSO at a stock concentration of 10 mM and stored at -80 ℃. It was prepared at the Department of Biochemical Sciences of the Faculty of Pharmacy in Hradec Králové.

4.2 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 x 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)

4.3 Equipment

Ice cube maker (Scotsman, UK)
Analytical scales (T-Scale, 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 x 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)

4.4 Methods

4.4.1 Assessing Daun Metabolism by recombinant CREs

This method allows to measure the metabolic activity of recombinant forms of CREs over Daun *in vitro*. CREs reduce Daun's carbonyl group by transferring a hydrogen from NADPH cofactor, thus producing NADP+ and consuming the cofactor, which could limit the scope of the reaction. To prevent this, recombinant CREs are incubated in an enzyme substrate combination, called NADPH regeneration system, that simultaneously recovers NADP+ to NADPH by the transformation of the glucose-6-phosphate to 6-phosphoglucono-δ-lactone by the enzyme glucose-6-phosphate dehydrogenase (Figure 8).

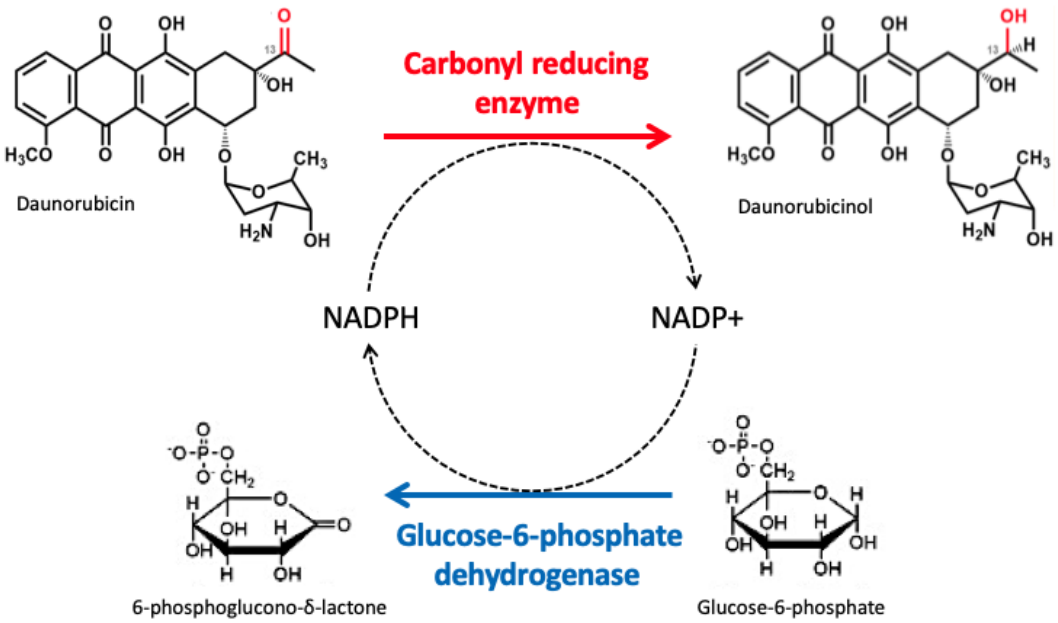

Figure 8. Diagram of the *in vitro* metabolism of Daun by recombinant CREs and the NADPH regeneration system.

Therefore, to asses Daun metabolism by recombinant CREs *in vitro*, reaction mixtures of 100 µl were prepared by mixing pure recombinant enzymes at different amounts depending on the CRE -CBR1 (1 µg), AKR1A1 (1 µg), AKR1B1 (5 µg), AKR1B10 (5 µg) or AKR1C3 (1.5 µg) -, with the substrate Daun at a final concentration of 500 µM, the NADPH regeneration system composed of (final concentration: 2,6 mM NADP+, 19.2 mM glucose-6-phosphate, 0.34 U glucose-6-phosphate dehydrogenase, 9.8 mM $MgCl_2$, 0.1 M phosphate buffer, pH 7.4) and the compound to test, in this case ENA, reaching a specific final concentration. All the experiments always include control reaction mixtures with the vehicle (DMSO) instead of ENA as well as control reaction mixtures of Daun without enzyme. Reaction mixtures were incubated for 30 min (60 min in case of AKR1B1) at 37 ℃; this incubation interval was within the linear phase of the reaction. Subsequently, the reaction was stopped by the addition of 40 µl of 25% ammonia and by cooling on ice. Samples were extracted twice with 1 ml of ethyl acetate by shaking on an automatic shaker for 15 min, which was followed by centrifugation for 2 min at 13,000 rpm. Organic phases were evaporated under vacuum, and residues were then dissolved in a mobile phase and subjected to UHPLC analysis.

4.4.2 Cell Cultures

For this work, two different cell lines (**Figure 9**) were employed:
-Human colorectal carcinoma cell line HCT116 from the European Collection of Authenticated Cell Cultures (ECACC, UK).
-Human lung adenocarcinoma cell line A549 from the American Type Culture Collection (Manassas, USA).

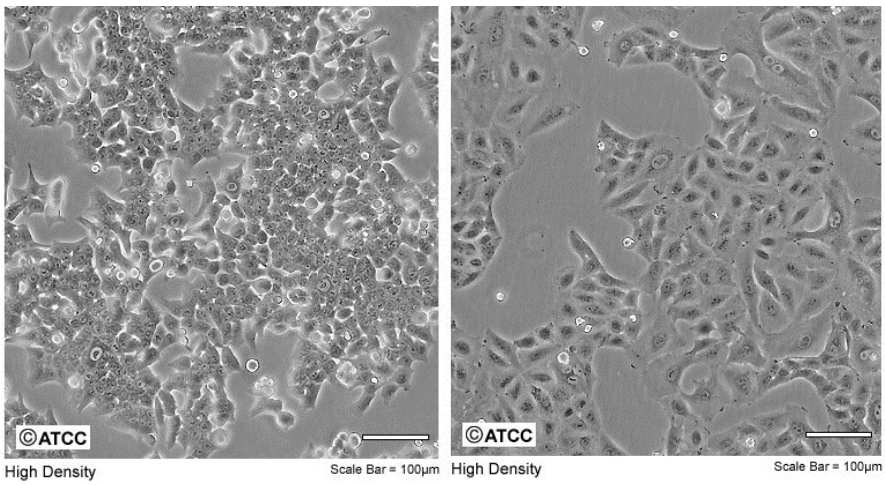

Figure 9. Pictures of the cell morphology of HCT116 (left) and A549 (right) cell lines. (Internet 3 and 4)

Both cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM), a type of cell culture media, supplemented with 10% Fetal Bovine Serum (FBS), which is a supplement added to cell culture media to provide nutrients and growth factors. Cell culture and experimentation was always performed under the same standard conditions: 37 ℃ in a 5% CO_2 atmosphere with humidified air.

4.4.3 Transient transfection of HCT116 cells with a vector coding for human AKR1C3

The pCI Mammalian Expression Vector (**Figure 10**) allows for the constitutive expression of cloned DNA inserts within mammalian cells. This vector incorporates the human cytomegalovirus (CMV) major immediate-early gene enhancer/promoter region, which induces the expression of the inserted gene. In this particular study, a previously cloned pCI vector was utilized, which had been modified to contain the open reading frame (ORF) sequence coding for the human AKR1C3 protein (**Figure 11**).

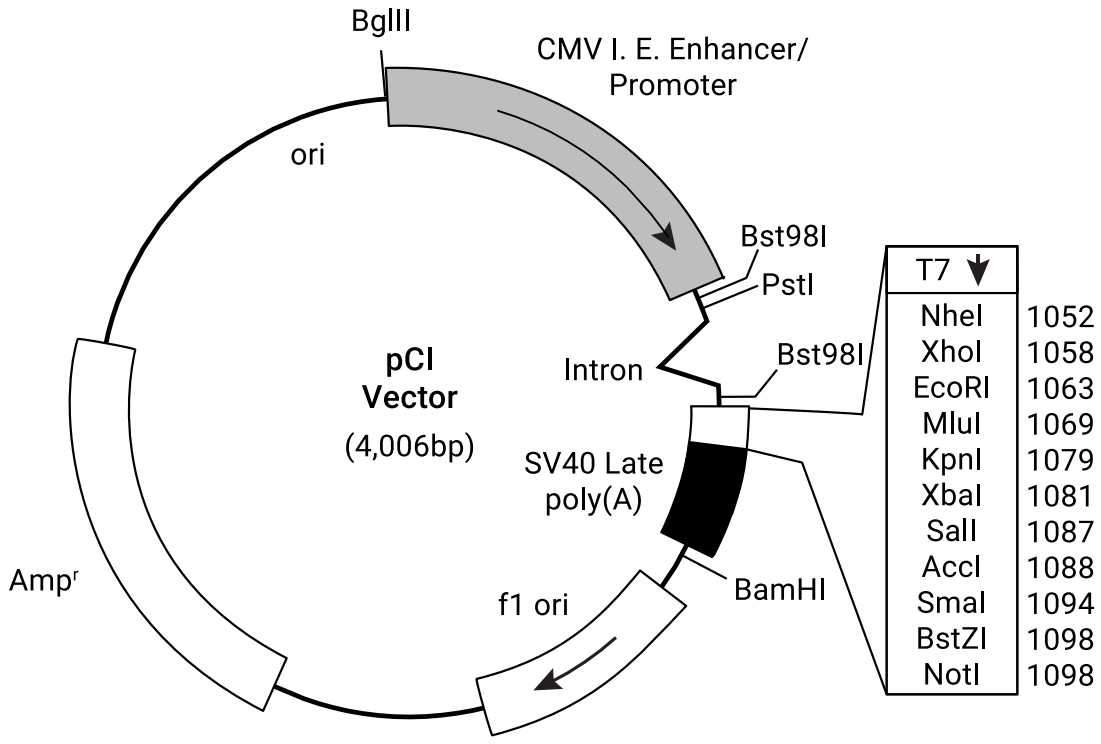

Figure 10. pCI Mammalian Expression Vector GenBank®. This vector was employed in this work as control pCI empty vector (pCI-EV). (Internet 5)

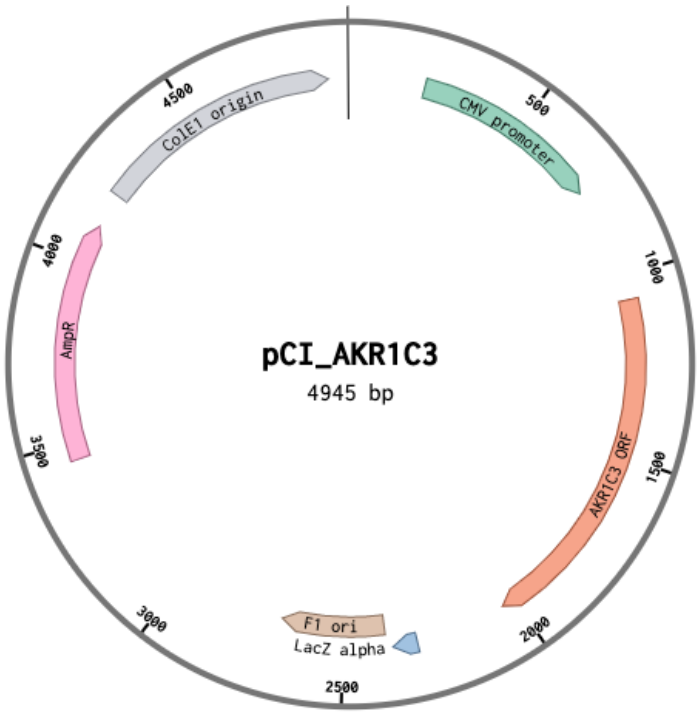

Figure 11. pCI Mammalian Expression Vector containing the ORF coding for the human AKR1C3 protein (pCI_AKR1C3) employed in this study. Scheme generated by (Internet 6)

To conduct a transient expression of the pCI expression vectors (pCI-EV or pCI-AKR1C3) in HCT116 cells, 1.25×10^5 cells were seeded per well in 24-well plates and cultured for 24 h at 37 ℃ in a 5% CO_2 atmosphere in humidified air. The cells media was replaced by fresh DMEM 10% FBS, and then transfected with either pCI_AKR1C3 or empty pCI plasmid using jetPRIME® transfection reagent according to the manufacturer's protocol (jetPrime® Transfection Reagent; Polypus Transfection®, Illkirch, France) (**Figure 12**). For this, 50 µL jetPrime® buffer, 1 µL of transfection reagent jetPrime® and 0.5 µg of plasmid were mixed and incubated for 10 min at room temperature for polyplexes formation. Polyplexes were added carefully to the cells and incubated for 24 h at 37 ℃ and 5% CO_2. Then the expression of recombinant AKR1C3 was verified after 24 h using western blotting (WB) (**Figure 13**).

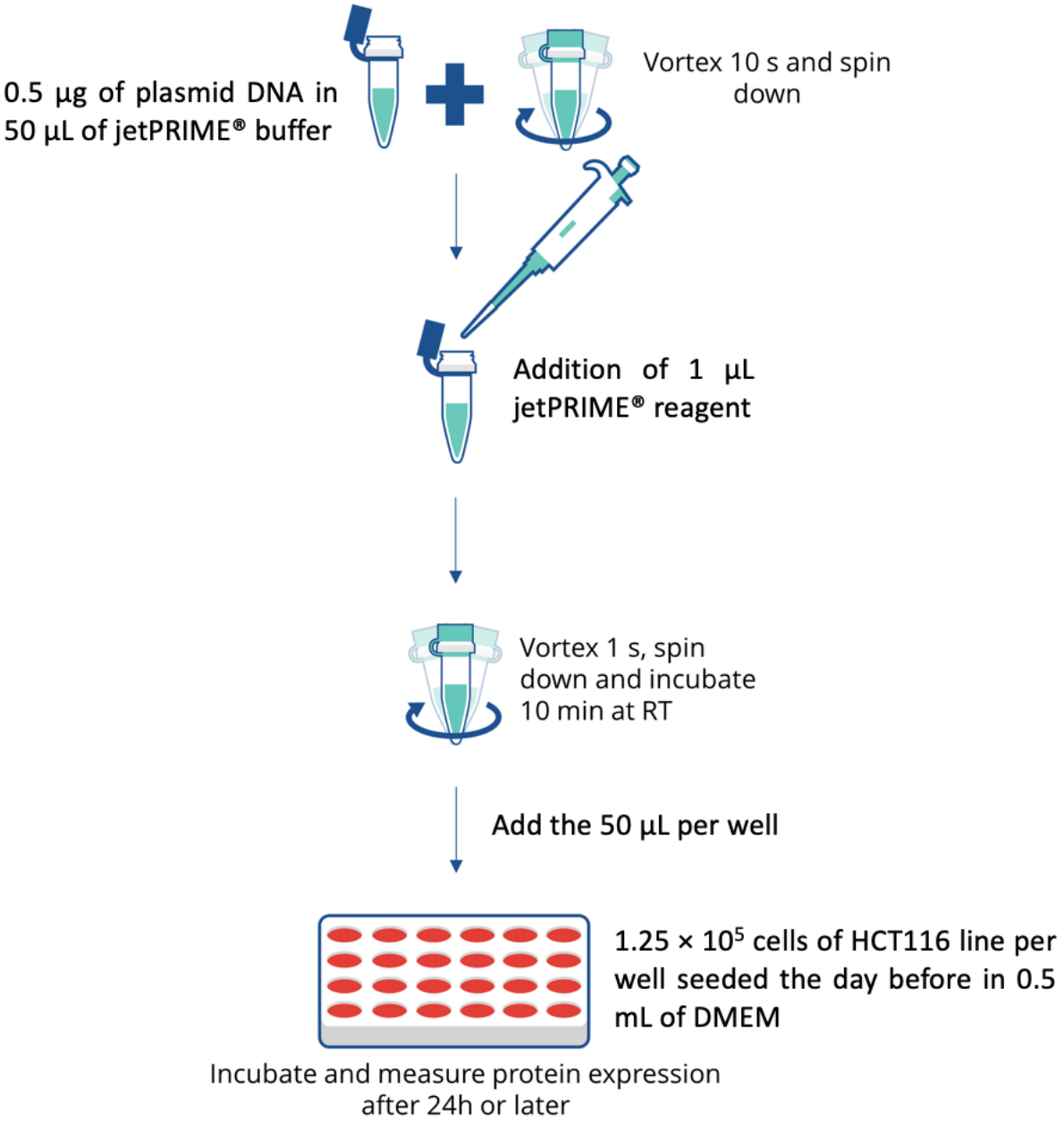

Figure 12. Protocol for transient transfection of pCI vectors using jetPRIME® reagent. Modified from a picture available (Internet 7)

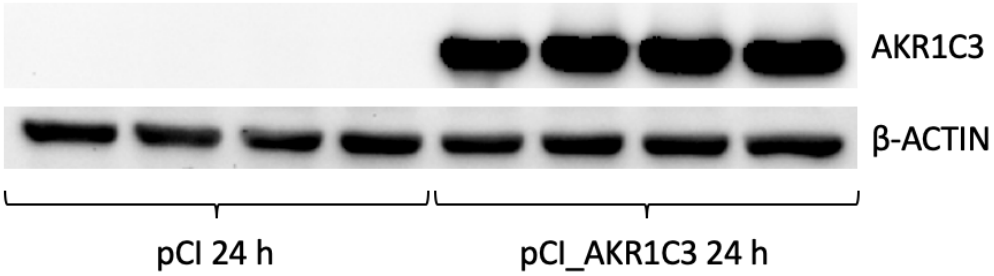

Figure 13. Verification by WB of AKR1C3 protein expression in HCT116 cells 24 h after plasmid transfection. Each of the four samples on the left side correspond to a 24-well containing HCT116 cells transfected with pCI empty vector, whereas the four samples on the right side correspond to HCT116 cells transfected with pCI_AKR1C3. β-actin protein expression was used as loading control.

4.4.4 Determination of Daun Metabolism by Cells

To determine the influence of a specific drug like ENA on the metabolism of Daun by cell lines expressing AKR1C3, we experimented with different cell lines growing in 24-well plate format. On one side, transiently transfected HCT116 were prepared as described above, whereas A549 cells were seeded at a density of 25×10^3 cells per well 24 h before the experiment. Then, cells were treated with 1 μM Daun and varying concentrations (1, 5, and 10 μM) of ENA in DMEM supplemented with 10% FBS. The cells were then incubated at 37 ℃ with 5% CO_2 for 5 h before the medium was collected and the cells were lysed using lysis buffer (25 mM Tris, 150 mM NaCl, and 1% Triton X-100, pH 7.8) at room temperature for 15 min. The harvested medium containing the cell lysate was extracted twice with 1 ml of ethyl acetate by shaking on an automatic shaker for 15 min and centrifuged for 2 min at 13,000 rpm. Furthermore, the organic phases were evaporated under vacuum, and the residues were dissolved in a mobile phase before being subjected to UHPLC analysis.

4.4.5 Daun and Daun-ol quantification by Ultra High Performance Liquid Chromatography (UHPLC)

Analytical characterization is a useful tool for identifying components in various fields such as pharmaceuticals, food, and cosmetics by understanding their chemical composition. High performance liquid chromatography is a separation technique that can be used to analyze compounds with different properties (**Figure 14**). HPLC involves passing a mixture dissolved

in a mobile phase through a stationary phase, and both phases are immiscible. Depending on the analyte's nature, chemical structure, and molecular weight, different types of liquid chromatography can be selected. HPLC was developed in the mid-20th century and has since undergone numerous improvements, including the use of high pressure, computerization, and automation, resulting in ultra-performance liquid chromatography. Many detectors are coupled with the HPLC apparatus such as ultra-visible (UV-Vis) spectroscopy, mass spectroscopy (MS), nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform Raman (FTR) (Lozano-Sánchez et al., 2018). Ultra-high performance liquid chromatography (UHPLC) is a type of liquid chromatography that utilizes columns containing particles smaller than those typically used in HPLC, specifically particles smaller than 2.5-5 µm in size. The underlying principle of UHPLC is like that of HPLC, in that decreasing the size of the column packing particles leads to an increase in efficiency per unit time. Moreover, the use of smaller particles enables faster separations with high peak resolution, ultimately resulting in a significant increase in resolution, sensitivity, and efficiency, while also reducing solvent usage and cost, making the technology more environmentally friendly (Rathod et al., 2019).

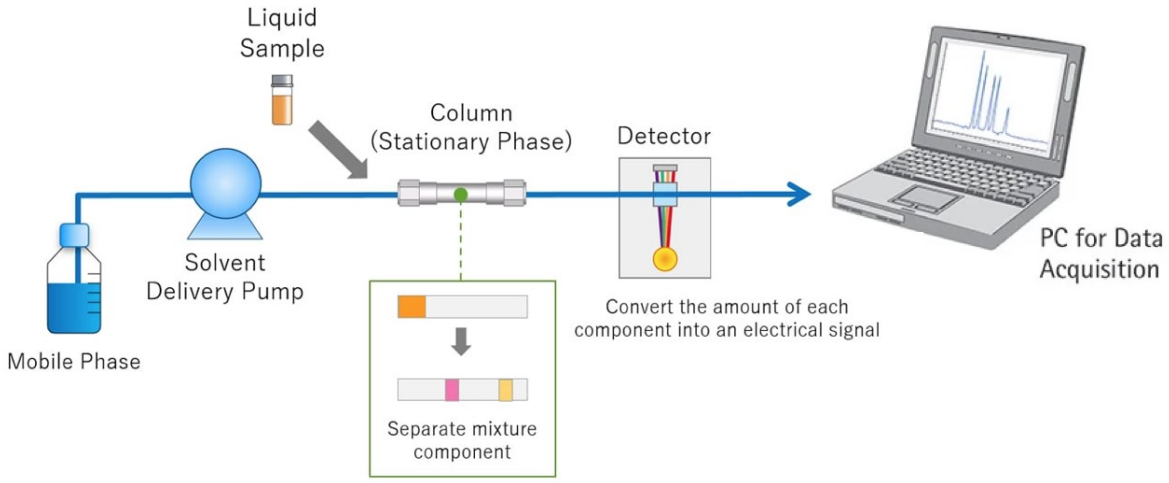

Figure 14. Diagram of the components of HPLC method. Modified picture from (Internet 8)

UHPLC was utilized to quantify the Daun-ol formed after enzymatic conversion of Daun by recombinant CREs *in vitro* and from different cell extracts. Agilent 1290 Series chromatographic system equipped with Zobrax C18 Eclipse Plus (2.1 × 50 mm, 1.8 µm) column and a 1290 infinity inline filter (Agilent, Santa Clara, CA, USA), under the following conditions: an isocratic elution of 1.0 ml/min with 0.1% formic acid a water and acetonitrile

mixture at a ratio of 74:26 (v/v), with the column compartment maintained at 40 ℃. Fluorescence detection was employed, with excitation and emission wavelengths set at 480 and 560 nm, respectively.

4.4.6 Combination assays and cell viability calculation by MTT assay

Combination assays were performed to evaluate the potential improvement of Daun cytotoxicity by the combination with ENA. For this, transfected HCT116 cells (8×10^3 cells/well) or A549 cells (5×10^3 cells/well) were seeded in 96-well plates 24 h prior to experiment. Following this, the medium was replaced by fresh medium containing varying concentrations of Daun along with DMSO or ENA. After 72 h of incubation under standard conditions (37 ℃, 5% CO2), cell viability was measured using the MTT assay (supplied by Sigma-Aldrich, Prague, Czech Republic). For this, 50 µl of MTT solution (3 mg/ml) was added to each well and incubated for 30 min. Then the supernatant was harvested and the cell monolayers were dissolved in 100 µl of DMSO. The absorbance was measured at 570 nm and 690 nm using a microplate reader Infinite M200 (Tecan, Salzburg, Austria); the background values at 690 nm were subtracted from the absorbance obtained at 570 nm. The values of cell survival were calculated as relative to control cells in % and integrated by GraphPad Prism 8.1.2 to calculate the half-maximal inhibitory concentrations (IC_{50}).

4.4.7 Analysis of synergism by Chou-Talalay method.

The Chou-Talalay method is a mathematical model used to analyze drug interactions and synergism. It was developed by Dr. Ting-Chao Chou and Dr. Paul Talalay in the 1980s. The method is based on the median-effect equation (MEE) derived from the physiochemical principles of mass action law. The MEE provides a common link between single entity and multiple entities, and first order and higher-order dynamics. In addition, the combination index theorem and dose reduction index equation are extensions of the MEE. Chou-Talalay method is widely used to evaluate the interaction between two or more drugs, such as whether the drugs have a synergistic effect, an additive effect, or an antagonistic effect. By quantitatively characterizing the interaction between drugs, the Chou-Talalay method can help optimize drug dosing and minimize side effects. It has been applied to the study of a wide range of diseases, including cancer, and has been used to evaluate the effectiveness of various combinations of

drugs (Chou, 2010; Elwakeel et al., 2019). In this work, the Chou-Talalay method was employed to analyzed the interaction between ENA and Daun. For this, the values of cell survival obtained as described in the combination assays were analyzed by the CompuSyn software, version 3.0.1 (ComboSyn Inc., Paramus, NJ, USA).

4.4.8 SDS-PAGE gel electrophoresis and Western blotting

SDS-PAGE gel electrophoresis and Western blotting are used to investigate the presence of a specific protein within cells or tissues. The proteins are denatured and reduced on treatment with reducing buffer and then separated, by virtue of their molecular weight, using SDS-PAGE gel electrophoresis (**Figure 15-1**). The separated proteins are transferred onto a PVDF or nitrocellulose membrane (**Figure 15-2**) and, after blocking, probed with the specific primary and secondary antibody (**Figure 15-3**). Enhanced chemiluminescence (ECL), followed by standard photographic film developing techniques are used to detect the expression of the protein (**Figure 15-4**). Finally, the specific signal from each protein band is analysed by densitometry (**Figure 15-5**).

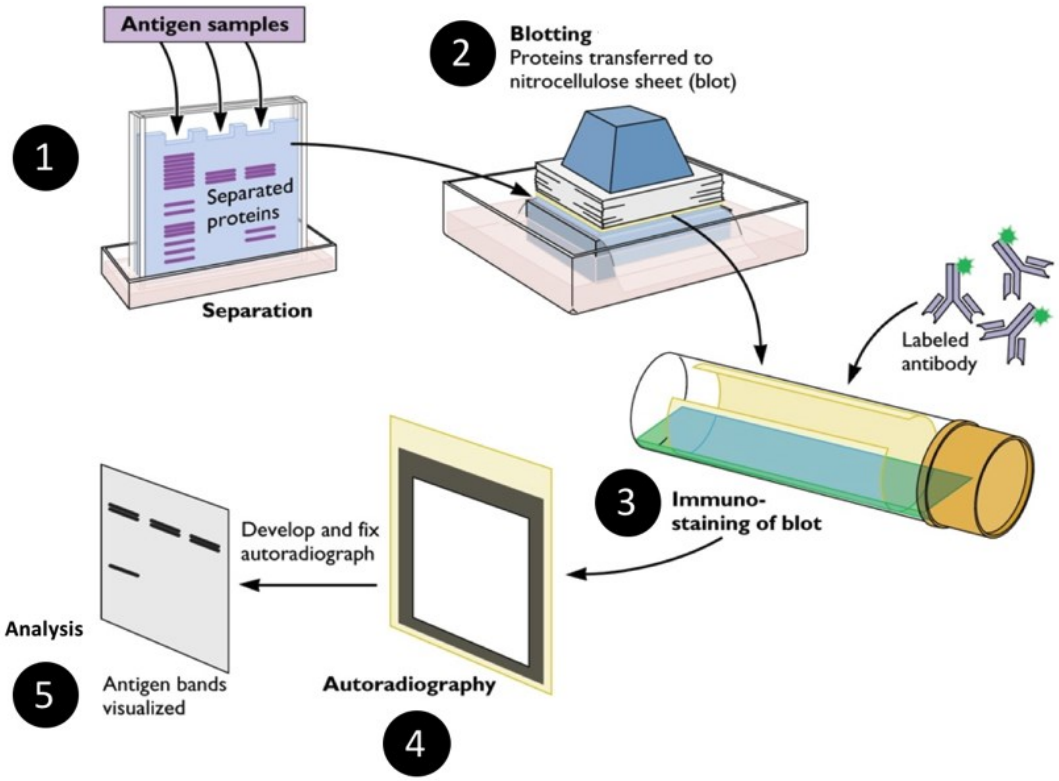

Figure 15. Steps of protein quantification by SDS-PAGE gel electrophoresis and Western blotting.

This work used western blotting analysis to detect the protein expression of AKR1C3 in transfected HCT116 and A549 cells. The cells were lysed on ice with a lysis buffer comprising a 1x protease inhibitor cocktail plus 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, and 1 mM sodium orthovanadate. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used to quantify the protein level in each sample and ensure equal protein loading. SDS-polyacrylamide gel electrophoresis separated the proteins according to their molecular weight. In brief, 15 μg of proteins (denatured) along with a molecular weight protein marker (Precision Plus Protein All Blue Standards; Bio-Rad Laboratories) were loaded onto an acrylamide gel consisting of a 4% acrylamide stacking gel on top of a 12.7% acrylamide resolving gel and run using an application of 100 V (20 min) followed by 150 V (75 min). Using a semi-wet-blotting method, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Then membrane was blocked with 5% non-fat milk in TBS containing 0.1% Tween 20 (TBS-T) and probed with 3% BSA-TBS-T containing rabbit monoclonal anti-AKR1C3 antibody (Abcam (ab209899)), and normalized to a total mouse monoclonal anti-β-actin antibody (Abcam (ab8228)). The enhanced chemiluminescence reagents (ECL Plus; Amersham GE Healthcare, Buckinghamshire, UK) were employed to detect the labelled proteins. Membrane densitometry was performed using ImageJ 1.50i software (Internet 9). The results of the target protein expression are expressed as the % of the densitometry of the endogenous control β-actin.

4.4.9 Statistical Significance

To assess significant changes in Dau metabolism by different recombinant CREs and cell lines, one-way ANOVA was employed. This method was used to compare changes in Dau metabolism among different recombinant CREs and cell lines. In other words, this statistical test determines whether there are any statistically significant differences between the means of the groups. Furthermore, another was test was conducted which is Dunnett's post hoc test to identify significant differences. In addition, Unpaired Student's t-test was used to assess the statistical significance of changes in the Dau-IC_{50} shifts, changes in AKR1C3 protein levels. Moreover, the statistical analysis was performed using GraphPad Prism 8.1.2 software. A p-value less than 0.05 was considered statistically significant, meaning that there was less than a 5% chance that the observed results were dure to rand.

5 RESULTS

5.1 ENA specifically inhibits AKR1C3-mediated carbonyl reduction of Daun

The first step of this study was to evaluate the potential effect of ENA on the carbonyl reducing activity of various CREs towards Daun. For this, two doses of ENA (10 and 50 µM) were tested over the recombinant forms of AKR1A1, 1B1, 1B10, 1C3, and CBR1. Among them, only AKR1C3 was affected, where 10 and 50 µM ENA reduced its carbonyl reducing activity over Daun by 94.52% ± 1.28% and 99.02% ± 0.39%, respectively (Figure A-E). In addition, the inhibitory strength of ENA against AKR1C3 was determined by calculating the half-maximal inhibitory concentration (IC_{50}), which indicates the concentration of ENA required to decrease the activity of the enzyme by 50%. The IC_{50} of ENA towards AKR1C3 mediated carbonyl reduction of Daun was 1.084 µM (**Figure 16**).

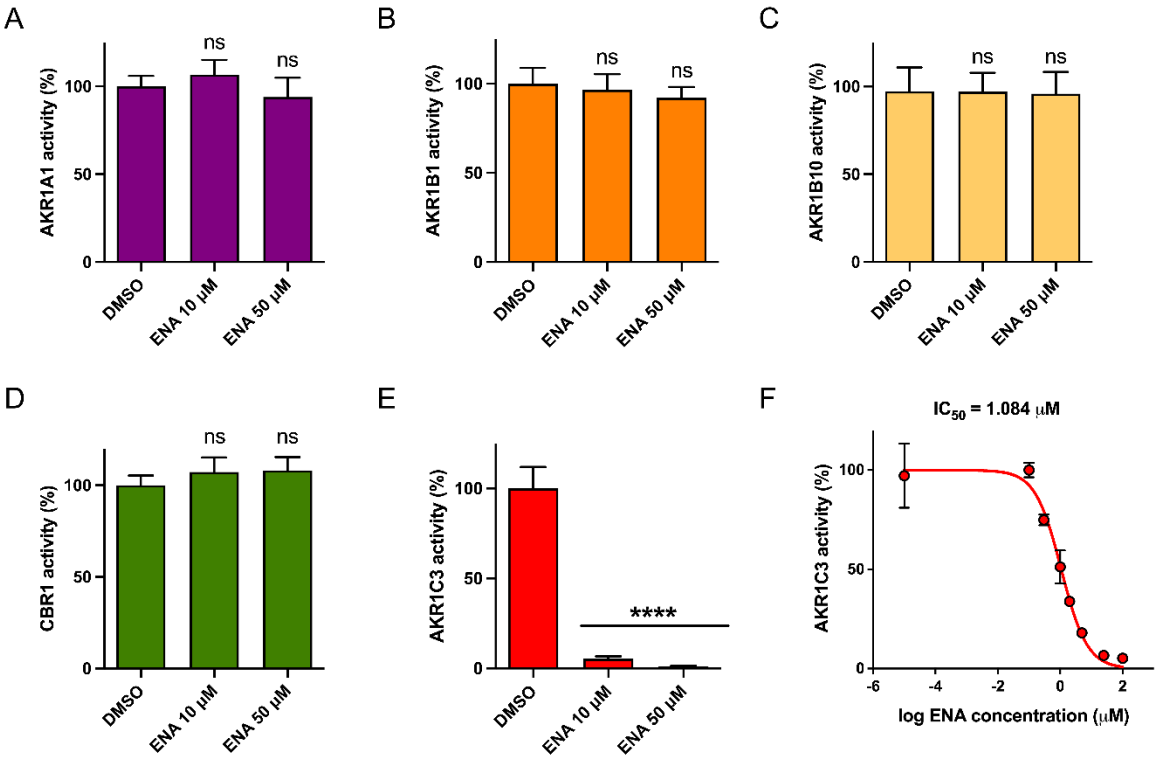

Figure 16. ENA specifically inhibits AKR1C3-mediated carbonyl reduction of Daun. Bars represent the activity of recombinant AKR1A1 (**A**), AKR1B1 (**B**), AKR1B10 (**C**), CBR1 (**D**), and AKR1C3 (**E**) towards Daun when incubated with DMSO or two doses of ENA. The IC_{50} value of ENA's inhibition on AKR1C3-mediated carbonyl reduction of Daun was determined and represented by the **F**. All the enzymatic activities were calculated as a percentage relative to control incubations with DMSO. Bars or data points indicate the mean ± SD of independent experiments performed in triplicate. The P values were calculated using Student's t-test, with ****P < 0.0001 and ns indicating no significant (relative to DMSO).

5.2 ENA dose-dependently inhibits the metabolism of Daun in HCT116 cells expressing AKR1C3

Furthermore, we assessed the inhibitory effect of ENA against AKR1C3 at the cellular level. To accomplish this, we transformed the HCT116 cell line to transiently express a vector coding for the AKR1C3 enzyme (HCT116-C3), along with control cells that were transformed with an empty vector (HCT116-EV). Then we compared the ability of those transformed HCT116 cell lines to transform Daun (1 µM) to Daun-ol during a period of five h and evaluated the ability of ENA to revert it. ENA (0.1, 0.5, 1, 5, and 10 µM) exhibited a dose-dependent inhibition of the HCT116-C3 mediated transformation of Daun to Daun-ol, with reductions of 17.93% ± 10.18%, 23.10% ± 2.45%, 40.70% ± 2.86%, 66.51% ± 6.26%, and 81.04% ± 5.55%, respectively (**Figure 17**). Interestingly, this data indicates that the inhibition of AKR1C3 activity by ENA 10 µM was so effective that it fully prevented the metabolism of Daun in the AKR1C3-overexpressing cells (HCT116-C3), as the levels of Daun-ol were almost comparable to those found in the control cells (HCT116-EV), which do not express the AKR1C3 enzyme.

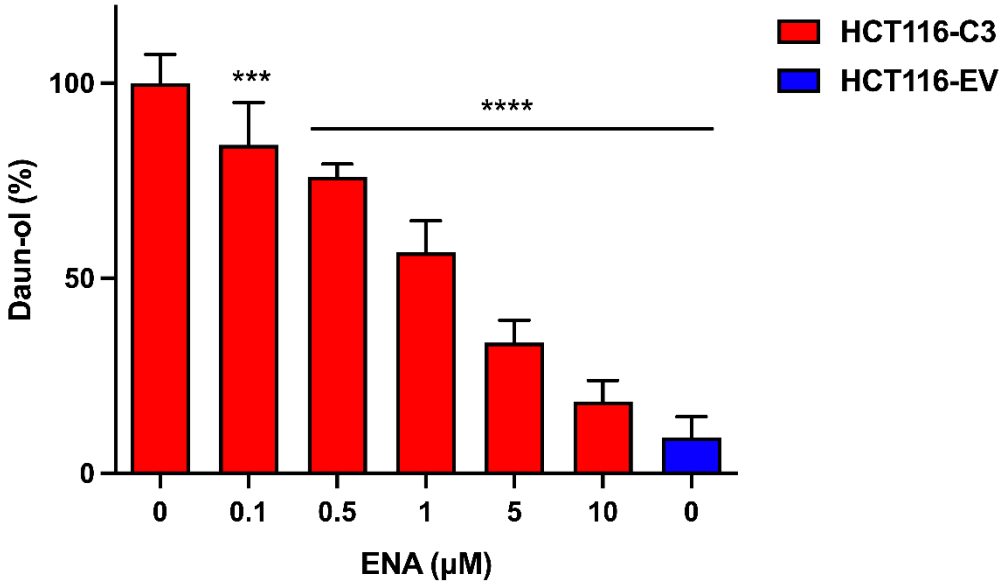

Figure 17. ENA dose-dependently inhibits the metabolism of Daun in HCT116 cells expressing AKR1C3. AKR1C3 coding vector (C3) or empty vector (EV) were transfected into HCT116 cells, followed by incubation with Daun (1 µM) and different concentrations of ENA or DMSO for 5 h. UHPLC was used to analyze the cell extracts. The bars show the mean ± SD (n=6) of detected Daun-ol as a % relative to the amount detected in control HCT116-C3 cells (3.27 ± 0.86 µg/mL). The data was statistically analyzed using one-way ANOVA followed by Dunnett's post hoc test, with ***P<0.001 and ****P<0.0001 as relative to control.

5.3 AKR1C3 overexpression does not influence ENA's toxicity over transformed HCT116 cells

Based on the data from HCT116-C3 cells, we were interested in examining how the decline in Daun metabolism by ENA could upsurge Daun cytotoxicity. However, the experiments to compare the cytotoxic effect of a specific drug over different cell lines imply long incubation periods (72 h), where ENA could present a cytotoxic effect itself. Then, we analyzed how the expression of AKR1C3 in HCT116 cells may have an impact on the ENA's cytotoxicity, which could mislead Daun's related cytotoxicity. To determine that, the IC_{50} values of ENA's effect on cell viability were compared between HCT116-C3 and HCT116-EV cells as the relative resistance (RR). The RR value is the proportion between the IC_{50} in overexpressing cells (HCT116-C3) and the control cells (HCT116-EV). Results indicated that both cell lines were equally sensitive to ENA (RR= 1.00; **Figure 18**), suggesting that the presence of AKR1C3 does not alter the potential cytotoxic effect of ENA itself.

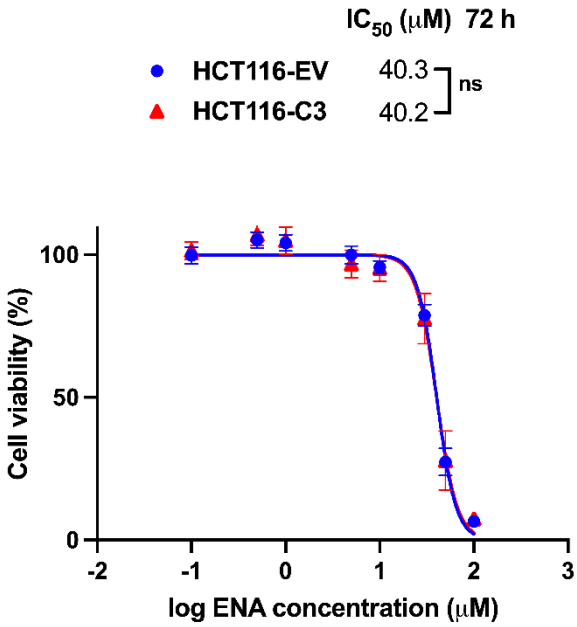

Figure 18. AKR1C3 overexpression does not influence ENA's toxicity over HCT116 cells. HCT116-EV and HCT116-C3 cells were exposed to increasing amounts of ENA for 72 h, and their survival was evaluated using MTT assay. The charts show a comparison of the dose-response curves for normalized cell viability (%) in each cell line, with data points indicating the mean ± SD (n=9). Student's t-test was used to calculate P values, with "ns" indicating not significance.

5.4 ENA synergizes Daun's cytotoxicity in HCT116 cells expressing AKR1C3

The expression of AKR1C3 in HCT116-C3 cells resulted in Daun resistance, with a significantly higher Daun-IC_{50} value (0.61 ± 0.06 µM**) compared to HCT116-EV cells (0.35 ± 0.07 µM). The combination with ENA 1, 5, and 10 µM, which previously displayed potent inhibitory effects of Daun metabolism, effectively reversed AKR1C3-mediated resistance to Daun in a dose-dependent manner, as demonstrated by significant decreases in IC_{50} values in HCT116-C3 cells (**Figure 19-A**). In contrast, the same combinations had no effect on Daun cytotoxicity in HCT116-EV cells (**Figure 19-B**).

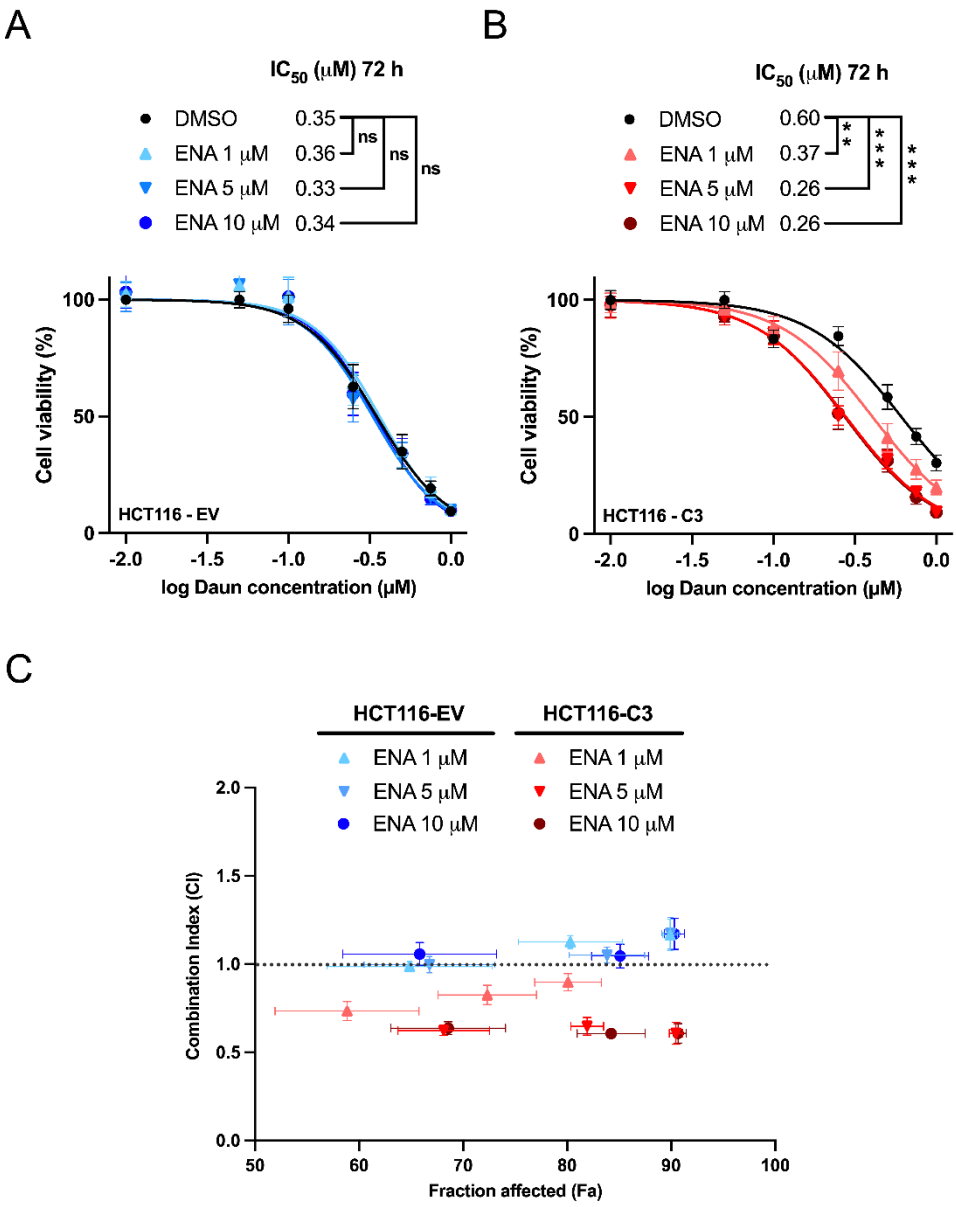

Figure 19. ENA synergizes Daun's cytotoxicity in HCT116 cells expressing AKR1C3. HCT116-EV (**A**) and HCT116-C3 (**B**) cells were incubated with DMSO or different doses of

ENA along with increasing concentrations of Daun for 72 h. Graphs compare the dose-response curves for the viability (in %) of each cell line under different combinations, assessed by MTT assay. P values were calculated using Student's test. **P < 0.01, ***P < 0.001, and ns for not significant. **C**, plots comparing combination index (CI) vs. fraction-affected (Fa) resulting from the Chou-Talalay analysis of the combined treatment of Daun + ENA 1, 5 and 10 μM, based on data from **A-B**. The dashed line separates the CI ranges for synergism (<1) and antagonism (>1). All data are presented as the mean ± SD of three independent experiments performed in triplicate.

In addition, the pharmacodynamic aspects of combining Daun and ENA were analyzed by the Chou-Talalay method (**Figure 19-C** and **Table 1**). This method allows to calculate whether the combination of different drugs results in a synergistic effect (i.e., the combined effect is greater than the sum of the individual effects) or an antagonistic effect (i.e., the combined effect is less than the sum of the individual effects). The combination of ENA and Daun showed index values indicating synergism (CL < 1) in HCT116-C3 cells. However, for HCT116-EV cells, the same combinations showed higher CI values mostly within the range of additivity (CI=1) or antagonism (CI > 1). The results suggest that ENA effectively synergizes with Daun to enhance its cytotoxicity in AKR1C3-expressing cells, overcoming resistance by selectively inhibiting AKR1C3 activity.

Table 1. Synergism between ENA and Daun is related to AKR1C3 expression in HCT116 cells. Values are expressed as the means ± SD from three independent experiments performed in triplicate.

Enasidenib (μM)	**Daunorubicin (μM)**	**Fraction affected (Fa %)**	**Combination index (CI)**
		HCT116 - EV	
1	0.50	64.87 ± 7.94	0.99 ± 0.03
	0.75	80.31 ± 5.02	1.12 ± 0.04
	1.00	89.90 ± 0.70	1.17 ± 0.09

Enasidenib (µM)	Daunorubicin (µM)	Fraction affected (Fa %)	Combination index (CI)
5	0.50	66.78 ± 6.29	1.00 ± 0.05
	0.75	83.83 ± 3.62	1.05 ± 0.05
	1.00	89.95 ± 0.85	1.17 ± 0.09
10	0.50	65.83 ± 7.39	1.06 ± 0.07
	0.75	85.09 ± 2.74	1.05 ± 0.07
	1.00	90.31 ± 0.97	1.17 ± 0.09
		HCT116 - C3	
1	0.50	58.85 ± 6.92	0.73 ± 0.05
	0.75	72.33 ± 4.72	0.83 ± 0.06
	1.00	80.09 ± 3.20	0.90 ± 0.05
5	0.50	68.14 ± 4.39	0.62 ± 0.03
	0.75	81.93 ± 1.57	0.65 ± 0.05
	1.00	90.47 ± 0.65	0.61 ± 0.06
10	0.50	68.57 ± 5.51	0.64 ± 0.04
	0.75	84.23 ± 3.25	0.61 ± 0.00
	1.00	90.66 ± 0.80	0.61 ± 0.05

5.5 ENA dose-dependently reduces intracellular Daun metabolism in A549 cells

Following the assessment of ENA's ability to synergize with Daun's cytotoxicity by selectively inhibiting AKR1C3 in transfected cells, we conducted similar experiments on the A549 lung epithelial cancer cell line. This cell line expresses a considerable amount of AKR1C3, making

it suitable to study the effects of ENA on Daun's metabolism through the enzyme's endogenous form (Hofman et al., 2014). Initially, we found that ENA at various concentrations (0.1, 0.5, 1, 5, and 10 μM) dose-dependently reduced intracellular Daun's metabolism in A549 cells by 11.77% ± 5.13%, 26,46% ± 1.04%, 32.10% ± 3.93%, 38.13% ± 3.75% and 43.88% ± 7.83%, respectively (**Figure 20**).

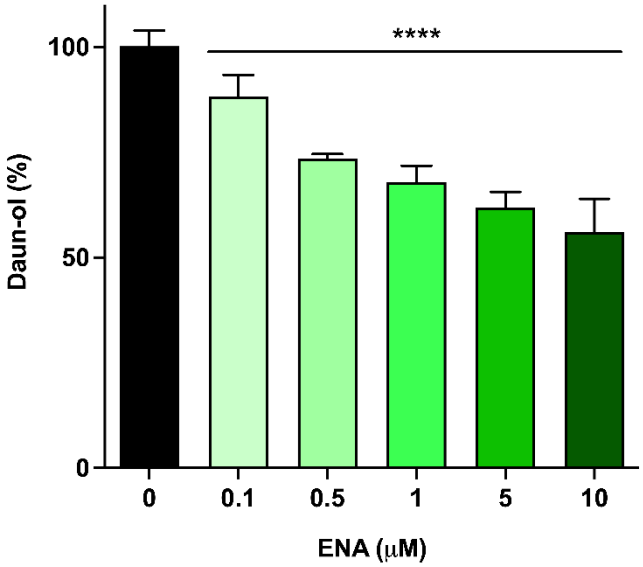

Figure 20. ENA dose-dependently reduces intracellular Daun reduction in A549 cells. A549 cells were treated with a combination of 1 μM Daun and increasing concentrations of ENA or DMSO for 5 h, and the cell extracts were analyzed by UHPLC. The amount of Daun-ol detected is expressed as a percentage relative to the amount detected in the control (2.46 ± 0.31 μg/mL), where bars represent the mean ± SD (n=9). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (****P<0.0001).

5.6 ENA synergises with Daun's cytotoxicity in A549 cells

Moreover, when we incubated A549 cells with Daun and the most effective ENA concentrations (1, 5, and 10 μM) from the previous experiment, Daun-IC_{50} values were dose-dependently enhanced as observed in (**Figure 21-A**). That outcome is consistent with the observed inhibitory effect of ENA on Daun metabolism. We also performed a Chou-Talalay analysis to discern the potential synergism between ENA and Daun on A549 cells. This analysis reported that the majority of the dose combinations exhibited CI values in the range of

synergism (CI < 1), indicating significant improvement in Daun's cytotoxicity in A549 cells (**Figure 21-B** and **Table 2**).

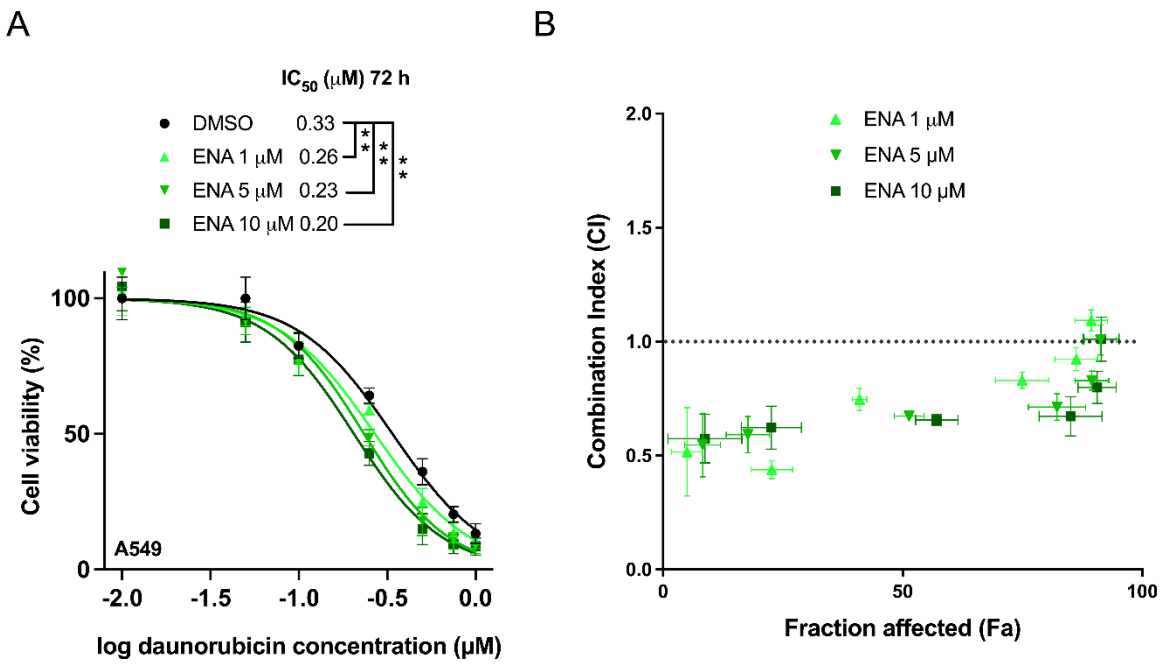

Figure 21. ENA synergises with Daun's cytotoxicity in A549 cells. A, A549 cells were treated with different concentrations of ENA in combination with increasing concentrations of Daun for 72 h. The viability of cells was determined using the MTT assay, and the normalized cell viability (%) was plotted for each combination. **B**, Chou-Talalay analysis of the combined treatment of Daun and ENA based on data in **A**. The CI ranges for synergism (<1) and antagonism (>1) were delineated by a dashed line. Data are presented as the mean ± SD of three independent experiments performed in triplicate. P values were calculated using the Student's test, and ns indicates not significant while **P < 0.01.

Table 2. Synergism between ENA and Daun on A549 cells. Values are expressed as the means ± SD from nine independent experiments.

Enasidenib (μM)	Daunorubicin (μM)	Fraction affected (Fa %)	Combination index (CI)
1	0.05	5.03 ± 3.29	0.52 ± 0.19
	0.10	22.68 ± 4.34	0.44 ± 0.04
	0.25	41.01 ± 1.52	0.75 ± 0.05
	0.50	74.93 ± 5.56	0.83 ± 0.04
	0.75	86.20 ± 4.45	0.92 ± 0.05
	1.00	89.35 ± 3.40	1.09 ± 0.05
5	0.05	8.26 ± 3.73	0.55 ± 0.14
	0.10	17.69 ± 4.51	0.59 ± 0.08
	0.25	51.33 ± 3.10	0.67 ± 0.01
	0.50	82.21 ± 5.98	0.71 ± 0.06
	0.75	89.48 ± 3.51	0.83 ± 0.04
	1.00	91.30 ± 3.30	1.01 ± 0.07
10	0.05	8.74 ± 7.72	1.29 ± 1.25
	0.10	22.61 ± 6.27	0.62 ± 0.09
	0.25	57.11 ± 4.41	0.66 ± 0.03
	0.50	85.04 ± 6.56	0.67 ± 0.09
	0.75	90.60 ± 3.98	0.80 ± 0.07
	1.00	91.44 ± 3.74	1.01 ± 0.10

5.7 ENA does not induce the expression of AKR1C3 in A549 cells

Nevertheless, the decreased activity of AKR1C3 might not result from its selective inhibition but rather from a decrease in its expression. This is important consideration because it suggests that the effects of ENA on AKR1C3 activity may be indirect and potentially involve other cellular pathways. Thus, an additional experiment was performed to investigate whether the reduction in AKR1C3 activity observed in the previous experiments was due to a selective inhibition of the enzyme by ENA, or whether it was related to a reduction in the expression of the enzyme. After treatment of A549 cells with the same concentrations of ENA (1, 5, and 10 µM) for 72 h, any concentration caused significant changes in the expression of AKR1C3 protein (**Figure 22**). This indicates that the increase in Daun sensitivity observed in this cell line might be attributed to the specific inhibition of AKR1C3.

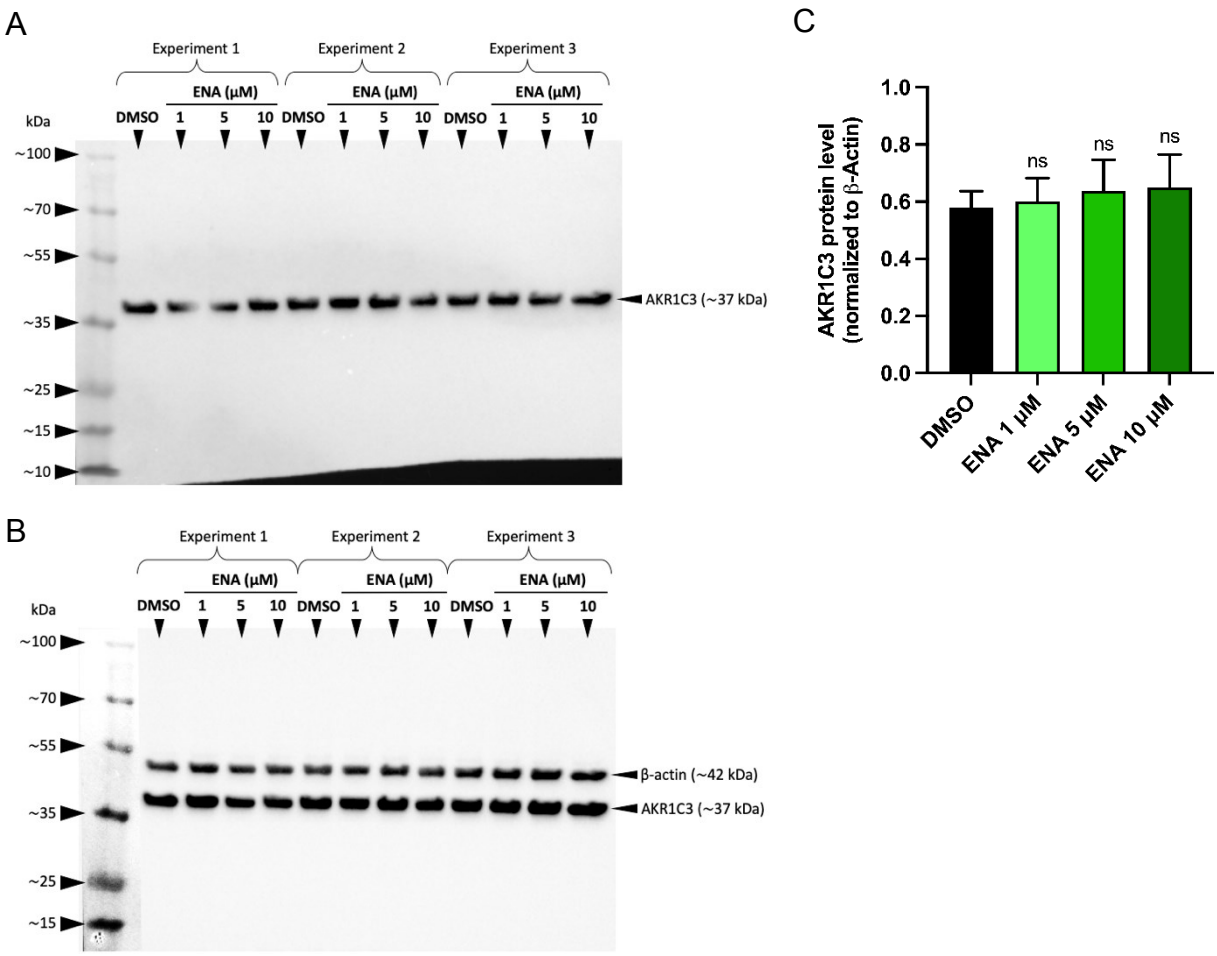

Figure 22. ENA does not induce the expression of AKR1C3 in A549 cells. A549 cells were incubated with DMSO or increasing concentrations of ENA (1, 5, and 10 µM) for 72 h, and then total protein was extracted for analysis of protein expression by WB. **A**, picture of the WB membrane containing samples from the A549 incubations performed in triplicate and probed

for AKR1C3 protein expression. **B**, picture of the same WB membrane as **A**, but blocked and re-probed for β-actin as the housekeeping protein used as loading control. **C**, bars represent AKR1C3 protein levels as determined by densitometry relative to β-actin (n=3, mean ± SD). P values were calculated using Student's t test; ns as not significant.

6 DISCUSSION

Anthracyclines are prone to drug resistance and have some side effect that limit their clinical usefulness. However, identifying the molecular targets responsible for that is challenging due to the numerous mechanisms that confer anthracycline resistance in cancer cells (Patel et al., 2013). Compelling evidence has demonstrated that the presence of CREs in cancer cells makes anthracyclines susceptible to transformation into secondary alcohol metabolites. This metabolic process makes the metabolites more water-soluble, thereby facilitating their elimination from the body through phase II conjugation reaction or directly. Thus, anthracyclines transform into secondary alcohol metabolites exhibit much less cytotoxicity than the parent drugs (Ax et al., 2000; Kuffel et al., 1992; Piska et al., 2017). The research indicates that Doxorubicin and its reduced form, doxorubicinol, have different intracellular distributions in MCF-7 breast cancer cells (Heibein et al., 2012). Doxorubicin primarily accumulates in the nucleus, while doxorubicinol is mainly retained in lysosomes and has a lower affinity for DNA molecules. Even though the reduced form can still block topoisomerase II and produce significant DNA damage, it lacks cytotoxicity due to its reduced intracellular availability and susceptibility to transmembrane extrusion by ABC transporters. This, in combination with the drug's cytosolic metabolism by carbonyl reductases (CREs), creates a defense mechanism that prevents anthracyclines from reaching their targets in the nucleus and thereby increases cancer cell resistance to this chemotherapy (Ferrazzi et al., 1991). Moreover, the reduced form has a higher tendency to accumulate in cardiac tissue, leading to increased cardiotoxicity during anthracycline treatment (Minotti et al., 2001).

Over the past few years, there has been extensive research on AKR1C3 inhibitors because of their strong connection to anthracycline resistance. For instance, it is the most efficient and relevant CRE in AML chemo-resistance. Moreover, this enzyme can contribute to both inherent and acquired resistance. Thus, developing new AKR1C3 inhibitors is very crucial (Liu et al., 2020). Several studies have demonstrated the potential of specific AKR1C3 inhibitors, such as midostaurin, dinaciclib, buparlisib, ibrutinib, and acalabrutinib, to improve the cytotoxicity of anthracyclines in different cell lines expressing AKR1C3 (Bukum et al., 2019; Morell et al., 2021; Novotná et al., 2018).

In this study, ENA was identified as a specific inhibitor of AKR1C3, with an IC50 of 1.08 µM. The inhibitory effect of ENA on AKR1C3 was found to prevent the metabolic inactivation of Daun by exogenously expressed AKR1C3 in the HCT116 cell line, leading to a potential way to overcome Daun resistance (**Figure 23**). This effect was observed not only in HCT116 cells but also in the cell line A549, that naturally expresses AKR1C3. The effective concentrations of ENA used in the study were much lower that the C_{max} achieved in clinical trials with the 100 mg dose, indicating that ENA may cause similar pharmacokinetic interactions when co-administered with Daun *in vivo*. The effectiveness and tolerability of ENA in combination with cytarabine and either Daun or idarubicin were evaluated in a phase 1 multicentre clinical trial (NCT02632708), and ENA-treated patients showed well tolerated and safe induction and consolidation chemotherapy. The best overall complete remission rate was 47% with 12-month survival probabilities of 76%. However, a randomized controlled trial is needed to adequately compare response rates between the combinations and the 7 + 3 schedule alone (Cihalova et al., 2015; Hsiao et al., 2019).

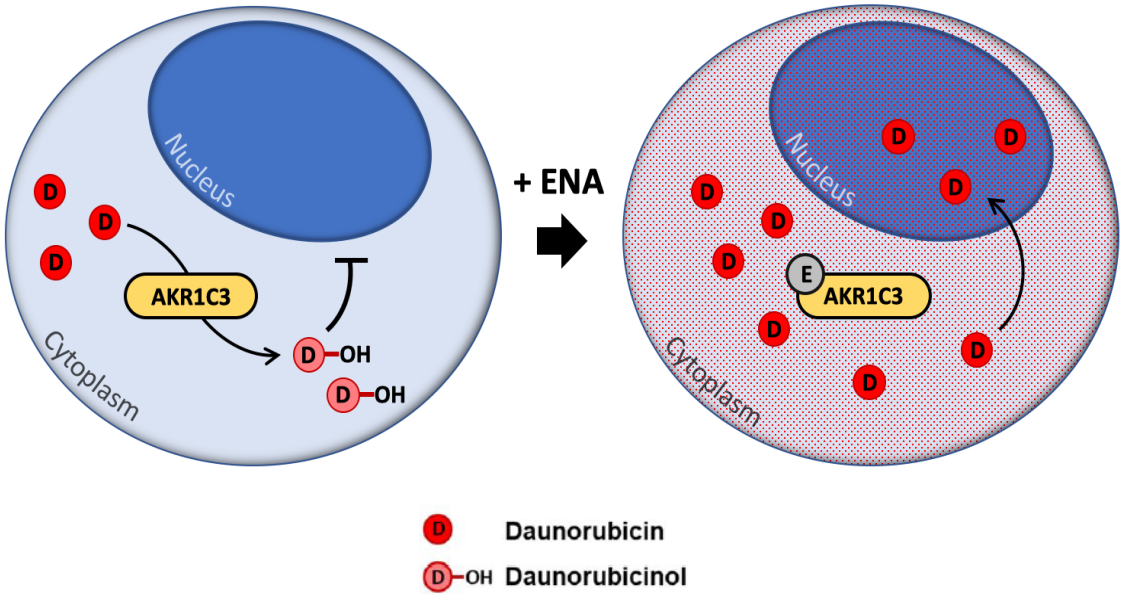

Figure 23. Graphic illustration showing the mechanistic mode of action proposed for ENA on Daun metabolism by AKR1C3 expressing cells. In these cells, AKR1C3 mediates the reduction of Daun to Daun-ol, which has less affinity to the nuclear DNA. However, due to the ability of ENA (E) to inhibit AKR1C3, intracellular accumulation of Daun increases, thus becoming more cytotoxic. Illustration modified from Morell et al. 2021.

Interestingly, AKR1C3 has been found to play a role in the proliferation of leukaemic blasts. Specifically, it transforms prostaglandin D2 (PGD2) to 9α and 11β-prostaglandin F2α (11-PGF2 α), which can drive blast proliferation (Birtwistle et al., 2009). However, blocking AKR1C3 activity can cause PGD2 to dehydrate to 15-deoxyΔ12,14PGJ2 (15dPGJ2), which induces myeloid cell differentiation and apoptosis (Hayden et al., 2009). AKR1C3 also has retinaldehyde reductase activity over all-trans retinoic acid (ATRA), which drives myeloid differentiation and has been successful in differentiation therapies against AML (Brown & Hughes, 2012). Overexpression of AKR1C3 in AML cells has been linked to resistance to PGD2- and ATRA-mediated differentiation. However, the combination of ENA and ATRA was found to significantly enhance the differentiation of IDH2-mutant AML cells by an unknown mechanism (Kim et al., 2020). This suggest that the inhibition of AKR1C3 by ENA may be a potential mechanism against AML leukemogenesis, particularly for patients with concurrent IDH2 mutations and AKR1C3 overexpression. This different pathway involving AKR1C3 is of interest for future research on ENA targeting this enzyme in the context of AML chemotherapy.

7 Conclusions

-Among different recombinant CREs with carbonyl reduction activity over Daun, the isocitrate dehydrogenase inhibitor ENA selectively inhibited AKR1C3.

-ENA dose-dependently prevented Daun inactivation mediated by AKR1C3 during its overexpression in HCT116 cells as well as on the A549 cell line, which naturally expresses AKR1C3.

-The inhibition of Daun metabolism correlated with a synergistic effect of ENA on Daun cytotoxicity in the same cell lines, thus reverting AKR1C3-mediated anthracycline resistance *in vitro*.

-These results suggest that ENA has a novel off-target action with key relevance in the context of anthracycline insensitivity.

-This work provides *in vitro* evidence supporting the potential of combining ENA with standard chemotherapeutics like anthracyclines in the clinical context.

8 List of abbreviations

2-HG: 2-hydroxyglutarate

4-HNE: 4-hydroxy-2-nonenal

A431: Human epithelial carcinoma cell line

A549: Lung adenocarcinoma cells

ABC: ATP binding cassette

ADT: Androgen deprivation therapy

AGEs: Advanced glycation end products

AhR: Aryl -hydrocarbon receptor

AKRs: Aldo-keto reductases

ALR: Aldehyde reductase

AML: Acute myeloid leukaemia

ANT: Anthracyclines

AR: Aldose reductase

AR: Androgen receptor

ATRA: All-trans retinoic acid

BCL-2: B-cell lymphoma 2

BTK: Bruton tyrosine kinase

CBR: Carbonyl reductase

CD33: Cluster of differentiation 33

CDK: Cyclin-dependent kinase

CI: Combination index

Cmax: Maximum concentration

CMV: Cytomegalovirus

CO2: Carbone dioxide

CR: Complete remission

CREs: Carbonyl reducing enzymes

CRP: Cytochrome P-450 reductase

CRPC: Castration-resistant prostate cancer

CYP: Cytochrome

Daun: Daunorubicin

Daun-ol: Daunorubicinol

DHE: 1,4dihydroxy-2-nonene

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNR: Daunorubicin

Dox: Doxorubicin

Dox-ol: Doxorubicinol

ECACC: European Collection of Authenticated Cell Cultures

ENA: Enasidenib

ER: Estrogen Receptor

EV: Empty vector

Fa: Fraction-affected

FBS: Fetal bovine serum

FDA: Food and drug administration

FLT3: FMS-like tyrosine kinase 3

FTR: Fourier transform Raman

GHB: gamma-hydroxybutyrate

GLI: Glioma-associated oncogene homologue

GO: Gemtuzumab ozogamicin

GSH: Glutathione

HCT116: Human colorectal carcinoma cell line

HH: Hedgehog

HPLC: High performance liquid chromatography

HSD: Hydroxy steroid dehydrogenase

IC50: Half-maximal inhibitory concentration

IDH: Isocitrate dehydrogenase

kDa: Kilo Dalton

KG1a: Human leukaemic cell line

MCF-7: Human breast cancer cell line

MDR: Multidrug Resistance

MEE: Median-effect equation

MS: Mass spectroscopy

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH: Nicotine-Derived nitrosaminoketones

NMR: Nuclear magnetic resonance

NNAL: Nitrosamine-alcohol

NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NQO1: NADPH quinone oxidoreductase

ns: Not significant

ORF: Open reading frame

PAHs: Polycyclic aromatic hydrocarbons

pCI: Mammalian Expression Vector

PG: Prostaglandin

PI3K: Phosphoinositide 3-kinase

PPAR-γ: Peroxisome proliferator-activated receptor gamma

ROS: Reactive oxygen species

Rpm: Rounds per minute

RR: Relative resistance

RXR: Retinoid X receptor

SD: Standard Deviation

SSADH: Succinic semialdehyde dehydrogenase

TCA: Tricarboxylic acid

TET: Ten-Eleven translocation enzyme

TOP: Topoisomerase

UGTs: UDP-glucuronosyltransferase

UHPLC: Ultra high-performance liquid chromatography

UV-Vis: Ultraviolet-Visible

WB: Western blot

XDH: Xanthine dehydrogenase

α-KG: α-ketoglutarate

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