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MODULATORY EFFECTS OF DEACETYLATED SIALIC ACIDS ON BREAST CANCER RESISTANCE PROTEIN-MEDIATED MULTIDRUG RESISTANCE AND RECEPTOR TYROSINE KINASE-TARGETED THERAPY

BY

ISAAC TUFFOUR

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Biochemistry

South Dakota State University

2023

DISSERTATION ACCEPTANCE PAGE
Isaac Tuffour

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Rachel Willand-Charnley
Advisor Date

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Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

This dissertation is dedicated to the Almighty God whom I am highly indebted to for giving me the strength and wisdom to accomplish this feat. Secondly, I dedicate this to my parents, Mr. Jacob Tuffour, and Mrs. Grace Tuffour, for their prayers and huge investment in my education and upbringing in spite of all the numerous constraints.

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CONTENTS

ABBREVIATIONS……………………………………………………..……………ix

LIST OF FIGURES………………………………………………………….……...x

LIST OF TABLES…………………………………………………….…..……………xii

Abstract…………………………………………………………………………...xiii

Chapter 1……………………………………………………….…………..............1

1.2 Introduction……………..………..…………………………….…………………………2

1.2.1 Multidrug resistance in Cancer……..…………...………………….....................2

1.2.2 Human ABC Transporters and Multidrug resistance……..…………….…......3

1.2.3 BCRP and Multidrug resistance……..…………...……..…………….…...…6

1.2.4 Glycans and BCRP-mediated Multidrug resistance......…..…...………..…......9

1.2.5 Role of Sialic acid in (Multi)drug resistance………….................................11

1.2.6 Acetylated Sialic acid and (Multi)drug resistance...13

1.2.7 Scope of Study…….……………………….………………………...…..15

1.2.8 Published Study Findings…….………….……………..…………………......16

1.3 Materials and Methods……………………………………………….…....17

1.3.1 Chemicals and reagents…………..…………………………………….........17

1.3.2 Cell lines and cell culture…………………...……………………….……17

1.3.2 Generation of BCRP overexpression HEK 293 Cell line …………………....18

1.3.3 Western Blot Analysis.……………………….…….................................19

1.3.4 Immunofluorescence Assay…………………………………………….20

1.3.5 Hoechst 33342 Accumulation Assay………………………………………..20

1.3.6 Cell viability Assay……………………………………..............................21

1.3.7 Cell Proliferation Assay…………………………………………………...21

1.3.8 mRNA Expression Analysis…………………………………………………22

1.3.9 Expression of CASD1 and SIAE in clinical samples..………………………23

1.3.10 Scientific Rigor………………………………………………………………24

1.4 Results and Discussion ……………….…………….………………..…………24

1.4.1 Generation of BCRP Overexpressing HEK 293 Cells…...............................24

1.4.3 CASD1 knockout upregulates BCRP expression………………………...26

1.4.4 CASD1 knockout decreases intracellular accumulation of Hoechst 33342……………………………..…..……………………………………………28

1.4.4 CASD1 confers resistance to Mitoxantrone……...…..………………………30

1.4.5 CASD1 knockout enhances cell proliferation.……...……………………..34

1.4.6 CASD1 knockout increases expression of cell survival proteins……………………………………………………......…………………...35

1.4.7 CASD1 knockout upregulates BCRP mRNA expression..…………………..39

1.4.8 Lysosomal pathway is involved in decreased BCRP protein stability and levels…………………..……………………………………………………………40

1.4.2 CASD1 expression favors survival in lung adenocarcinoma………………...42

1.4.3 Infographics………..……………………………………………………………44

1.5 Conclusion……….......……………………………….……...……………44

1.6 References………………………………………………………………45

Chapter 2..………………………………………………...…………………..51

2.2 Introduction…………………………………………………………………...52

2.2.1 Conventional Chemotherapy in Cancer Treatment…......................................52

2.2.2 Challenges associated with Conventional Chemotherapy……………………53

2.2.2.1 Adverse side effect due to non-selectivity…………………………………...53

2.2.2.2 Glycan (Sialic acid) mediated drug resistance……………………………….54

2.2.3 Targeted Therapy……..……………………………………..…………..55

2.2.4 Growth Factor Kinase Targeted Chemotherapies..…………………………...56

2.2.5 Natural products as targeted therapeutics……………………………….....60

2.2.6 Cucurbitacin……………………………………………………………..61

2.2.7 Cucurbitacin Inspired Estrone Analogs……………………………...…..63

2.2.8 Published Study Findings…………………………………………….…66

2.3 Materials And Methods……………………………………...……………67

2.3.1 Chemicals And Reagents…...…………………………..……..……...……67

2.3.2 Cell Lines And Cell Culture………………. ...……………………………….67

2.3.3 Cell Viability Assay…….……………………………………….……….....68

2.3.4 Apoptosis Assay……….……….…………………..………………………68

2.3.5 Cell Cycle Analysis……………………………………………. ………69

2.3.6 Immunofluorescence Assay…………………………………………...…...69

2.3.7 In-Cell ELISA…………………………………………………………..70

2.3.8 Scientific Rigor…………………………..……………….……………..70

2.4 Results and Discussion………………………………………………...…..71

2.4.1 Effects of Deacetylated Sia on Inhibitory Activity of CIEAs………………..71

2.4.2 Apoptotic Effects of CIEAs………………...……………………………..73

2.4.3 Effects of CIEAs on the Cell Cycle……………………… …………………76

2.4.4 Effects of Deacetylated Sia on Phosphorylated EGFR……………………..…..79

2.4.5 Effects of CIEAs on ERK and mTOR Phosphorylation……..........................81

2.4.6 Infographics………..……………………..………………………………84

2.5 Conclusion………………..……………………………………………..…..…85

2.6 References…………………………………………………….………..….86

ABBREVIATIONS

ABC	ATP-binding cassette
ABCG2	ATP-binding cassette super-family G member 2
BcL-2	B cell Lymphoma 2
BCRP	Breast Cancer Resistance Protein
CIEAs	Cucurbitacin inspired estrone analogs
DMEM	Dulbecco's Modified Eagle Medium
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated kinase
G_1	Gap 1 phase (cell cycle)
G_2	Gap 2 phase (cell cycle)
IC_{50}	Concentration needed to obtain 50% inhibition
MDR	Multidrug resistance
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PARP	Poly-ADP ribose polymerase
RPMI	Rosewell Park Memorial Institute Medium
SIGLECS	Sialic-acid-binding immunoglobulin-like lectins

LIST OF FIGURES

Chapter 1

Figure 1.1 Mechanisms of drug resistance in cancer…………………….…...…3

Figure 1.2 ABC transporter and subfamily genes identified in the human genome…………………………………………..…………………..…4

Figure 1.3 Structure of P-gp depicting the general structure and organization of Human ABC transporters.....……………………………………………..5

Figure 1.4 Topology and domain structure of BCRP monomer…...……...…..6

Figure 1.5 Transport/Efflux mechanism of ABC Proteins (BCRP) ..………..8

Figure 1.6 Glycan chain composition on BCRP……………....……………10

Figure 1.7 Contribution of Canonical and Non-canonical forms of sialic acid in cancer……..……………………………………………………..12

Figure 1.8 Sia acetylation scheme showing CASD1 and SIAE mediated 9-O and 7,9-O acetylation and deacetylation…………………...…..……....13

Figure 1.9 Screenshot of title page of published article……..…………..16

Figure 1. Generation of BCRP/ABCG2 overexpressing cell line………..…25

Figure 2. BCRP (ABCG2) expression in Wild type and knockout cell line...27

Figure 3. BCRP (ABCG2) expression in Wild type and Knockout cell lines.28

Figure 4. Intracellular accumulation of Hoechst 33342 in HEK293 and A549 cells…..……………………………………………………………….29

Figure 5. Cytotoxic effect of Mitoxantrone on HCT 116 and A549 cells…...32

Figure 6. Comparative cell proliferation/growth assessment………………..34

Figure 7. Bcl-2 Protein Expression in Wild type and Knockout cell lines….36

Figure 8. Cleaved PARP levels in Wild type and Knockout cell lines……...37

Figure 9. BCRP (ABCG2) mRNA expression in Wild type and Knockout cell lines…………………..…………………………………………………………...39

Figure 10. BCRP (ABCG2) expression in Wild type and Knockout cell lines following Bafilomycin A1 treatment…………………… . ……………….....41

Figure 11. Kaplan-Meier survival plots for CASD1 and SIAE mRNA expression and overall survival in clinical Lung (LUAD) and Colon (COAD) adenocarcinoma samples……………………………………………….…43

Figure 12. Infographics……………………………....……..…………..44

Chapter 2

Figure 2.1 Classification of Conventional Chemotherapies based on mechanism of action……………………………………………………..…..53

Figure 2.2 CASD1 enzyme mediates 9-O and 7, 9-O acetylation of Sialic acid (Neu5Ac)…………………………………………………………….....54

Figure 2.3 Mechanisms of actions of Targeted Chemotherapeutics…...……56

Figure 2.4 Schematic diagram of ligand-bound dimeric EGFR showing domains/modules…………………………………..…………………58

Figure 2.5 The MAPK Signaling Pathway………….……………..……….59

Figure 2.6 Skeletal Structure of Cucurbitacin………………….………..…….62

Figure 2.7 Biologically Oriented Organic Synthesis of Cucurbitacin-Inspired Estrone Analogs……………..……………………………………………64

Figure 2.8 Structures of CIEAs used in this study (A) MMA 294 (B) MMA320 (C) MMA 321..……………………………………………..…..65

Figure 2.9 Screenshot of title page of published article……………………..66

Figure 2. Apoptotic effects of CIEAs in A549 cells……….……………………..74

Figure 3. Apoptotic of CIEAs in HCT 116 cells…………….……………75

Figure 4. Effects of CIEAs on A549 cell cycle…………………………..……......77

Figure 5. Effects of CIEAs on A549 cell cycle………...…...…………….......78

Figure 6. Effects of Sia deacetylation on Phosphorylated EGFR (pEGFR) expression………………………...…………………………………...…80

Figure 7. Effects of CIEAs on phosphorylated ERK and mTOR levels…….83

Figure 8. Infographic (Cartoon summary of Findings) …………………….…84

LIST OF TABLES

Chapter 1

Table S1. Comparison between HEK293 Wildtype and BCRP overexpressing cells……………………………………...………..…25

Table 1. Effect of Acetyl Sia on Cellular response to Mitoxantrone...31

Chapter 2

Table 1. Inhibitory effects of CIEAs on HCT116 and A549 cells.…………72

ABSTRACT

MODULATORY EFFECTS OF DEACETYLATED SIALIC ACIDS ON BREAST CANCER RESISTANCE PROTEIN-MEDIATED MULTIDRUG RESISTANCE AND RECEPTOR TYROSINE KINASE-TARGETED THERAPY

ISAAC TUFFOUR

2023

Multidrug resistance (MDR) remains a major challenge in cancer treatment, accounting for over 90% of chemotherapeutic failures. Cancers utilize sugar residues to engage in multidrug resistance. The underlying mechanism of action involving glycans, specifically the glycan sialic acid (Sia) and its various functional group alterations, has not been explored. ATP-binding cassette (ABC) transporter proteins, key proteins utilized by cancers to engage in MDR pathways, contain Sias in their extracellular domains. Modulating the expression of acetylated-Sias on Breast Cancer Resistance Protein (BCRP), a significant ABC transporter implicated in MDR, in lung and colon cancer cells directly impacted the ability of cancer cells to either retain or efflux chemotherapeutics. Acetylation was modulated by the removal of CAS1 Domain-containing protein (CASD1) and Sialate O-Acetyl esterase (SIAE) genes via CRISPR-Cas 9 gene editing. Using a variety of cell and molecular based approaches, we confirmed that deacetylated Sias regulated a MDR pathway in colon and lung cancer in early *in vitro* models. When deacetylated Sias were expressed on BCRP, colon and lung cancer were able to export high levels of BCRP to the cell's surface, resulting in an increased BCRP efflux activity, reduced sensitivity to the anticancer drug Mitoxantrone, and high proliferation relative to control cells. These observations correlated with increased levels of cell survival proteins, BcL-2 and PARP1. Further studies also implicated the lysosomal pathway for the observed variation in BCRP

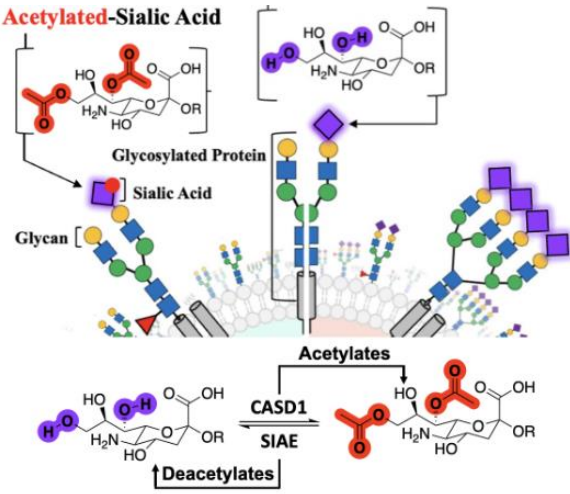
levels among the cell variants. RNAseq data analysis of clinical samples revealed higher *CASD1* expression as a favorable marker of survival in lung adenocarcinoma.

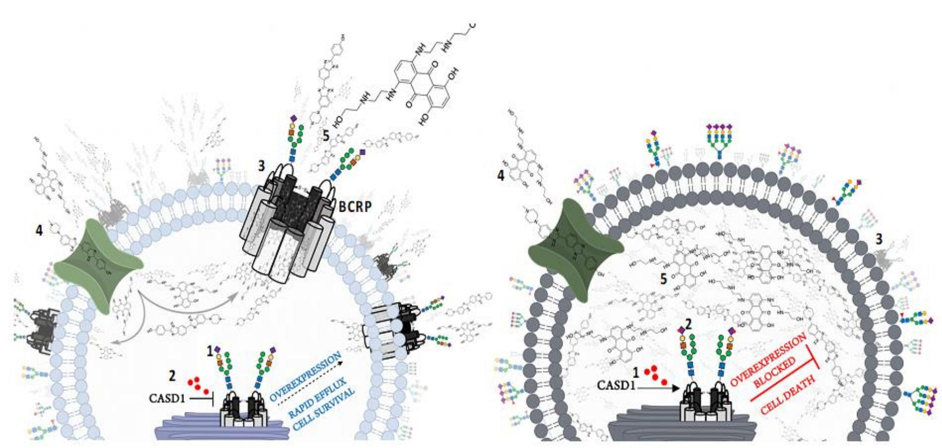
Using same *in vitro* models, we further explored the role deacetylated Sia plays in the EGFR targeted therapy. Specifically, we investigated how deacetylated Sia modulated the activity of three characterized novel Cucurbitacin-Inspired Estrone analogs (CIEAs: MMA 294, MMA 321, and MMA 320) with potent anti-proliferative and dual inhibitory activities, targeting the EGFR and MAPK pathways. Compared to control cells, the CIEAs elicited a ˜2 to 17 -fold sensitivity, induced a G2/M cell cycle arrest and apoptosis in deacetylated Sia-expressing knockout cancer cells. Further studies implicated overexpression of CIEAs' cognate protein target, phosphorylated EGFR, in the chemosensitivity of the deacetylated Sia-expressing knockout cells. This observation correlated with significantly decreased levels of key downstream proteins (phosphorylated ERK and mTOR) of the EGFR pathway in knockout cells compared with controls when treated with CIEAs.

Collectively, our findings indicate that deacetylated Sia is utilized by colon and lung cancers to engage in MDR via overexpression and efflux action of BCRP and renders lung and colon cancer cells susceptible to EGFR therapeutics. This study provides great insights for future therapeutic interventions.

Chapter 1

Deacetylated Sialic Acids Modulate Multi-Drug Resistance in Colon and Lung Cancers Via Breast Cancer Resistance Protein (BCRP/ABCG2)





1.1 Introduction

1.2.1 Multi-drug Resistance in Cancer

Cancer remains a global health menace worldwide and the second leading cause of mortality after heart disease, accounting for nearly 10 million deaths (i.e., 1 out of 6 deaths) according to the World Health Organization. The most common cancers are breast, lung, colon and rectum and prostate cancers [1]. In 2023, it is estimated that the United States will record 1,958,310 new cancer cases and 609,820 cancer deaths with lung cancer predicted to be the leading cause of cancer deaths [2]. Current cancer treatment options include chemotherapy, radiotherapy, immunotherapy, and surgery; however, chemotherapy remains the traditional approach for managing cancer [3]. Despite the substantial progress made over the years in chemotherapy, significantly low survival rates in many cancers still remain mainly due to multidrug resistance (MDR) mechanisms.

Multidrug resistance (MDR) involves the simultaneous resistance of cancer cells to a wide variety of drugs that differ in structure and mechanism of action [4]. A variety of current chemotherapeutic treatments are compromised by multidrug resistance (MDR), resulting in therapeutic failure and extended treatments with second or third-line therapies. MDR occurs in 90% of deaths in cancer patients receiving conventional or novel-targeted therapeutics [5]. It is therefore imperative to intensify research efforts to understand and prevent MDR pathways. Cancers employ MDR pathways, as shown in Figure 1.1, via a multitude of mechanisms including mutations in drug targets, altered drug metabolism, diminished apoptosis (cell death)

signaling, and reduced drug accumulation [6], with reduced drug accumulation accounting for the majority of chemotherapy resistance [7-9]. This particular MDR mechanism occurs through the overexpression of a group of heavily glycosylated transmembrane proteins referred to as ATP-binding cassette (ABC) transporters.

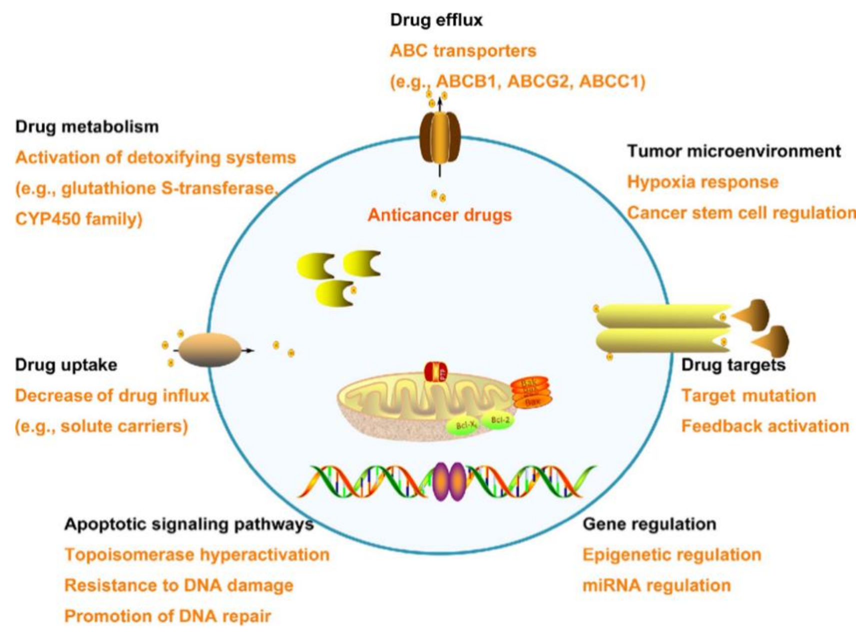


Figure 1.1 Mechanisms of multidrug resistance in cancer [10]

1.2.2 Human ABC Transporters and Multidrug Resistance

ABC transporters, also known as efflux pumps, belong to a large protein superfamily comprising of 48 members that facilitate the energy-dependent extrusion of a broad range of compounds against a concentration gradient [11]. These 48 members have been classified into seven subfamilies (A to G) according to their structural organization and sequence homology (Figure 1.2). The general structure of ABC transporters comprises of nucleotide binding domains (NBD) located on the cytoplasmic side of the

membrane and two sets of transmembrane domains (TMD). Each TMD contains six trans membranes spanning α- helices (TMHs) [12, 13] which form substrate translocation pathway. Both amine and carboxylic end of the transporter are located on the cytoplasmic side of the cell membrane (Figure 1.3). The physiological roles of human ABC transporters include transport of amino acids, peptides, lipids and inorganic ions. Mutations in ABC transporter genes impair their physiological transport function, implicating them in a myriad of debilitating human diseases. Among these diseases include cystic fibrosis (ABCC7), gout (ABCG2), schizophrenia (ABCA13), intrahepatic cholestasis (ABCB4), sitosterolemia (ABCG5 and ABCG8), inflammatory bowel disease (ABCB1), Dubin-Johnson syndrome (ABCC2), Tangier disease (ABCA1) and surfactant metabolism syndrome (ABCA3) [14, 15].

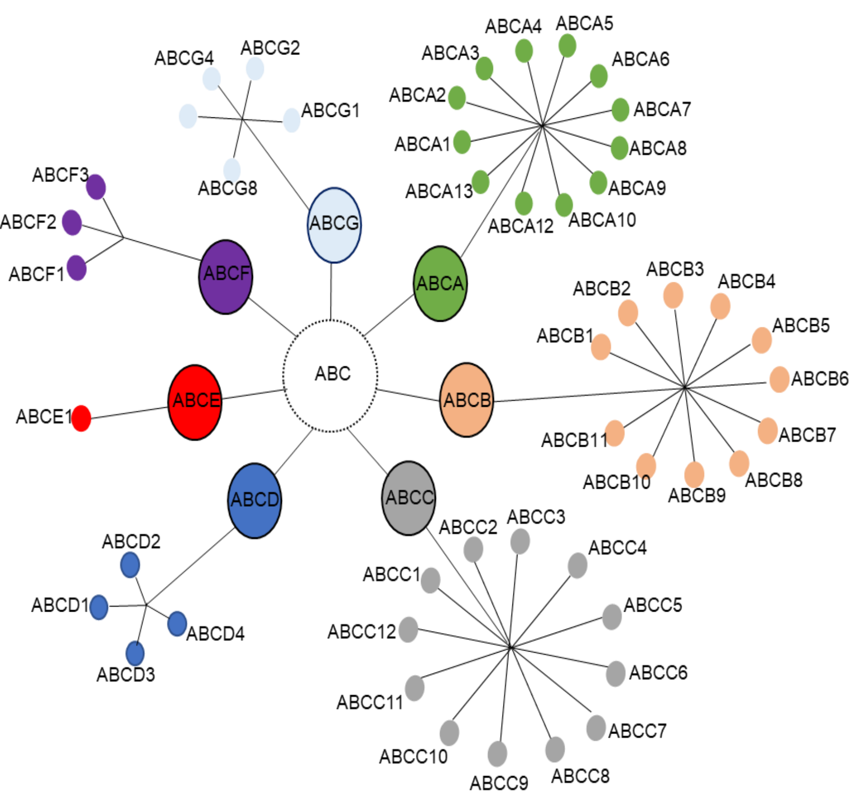


Figure 1.2 ABC transporter and subfamily genes identified in the human genome

Three major ABC transporters are implicated in clinical MDR due to their ability to efflux xenobiotics. These include the permeability-glycoprotein (P-gp, ABCB1), the multidrug resistance protein 1 (MRP1, ABCC1), and the breast cancer resistance protein (BCRP, ABCG2) [16, 17]. These transporters are reported to govern the transit of both endogenous and drugs substrates across major organs and physiological barriers (e.g. liver, kidney, blood-brain barrier, blood-testis barrier and non-polarized cells) and thus influence clinical drug efficacy and drug safety [13, 18].

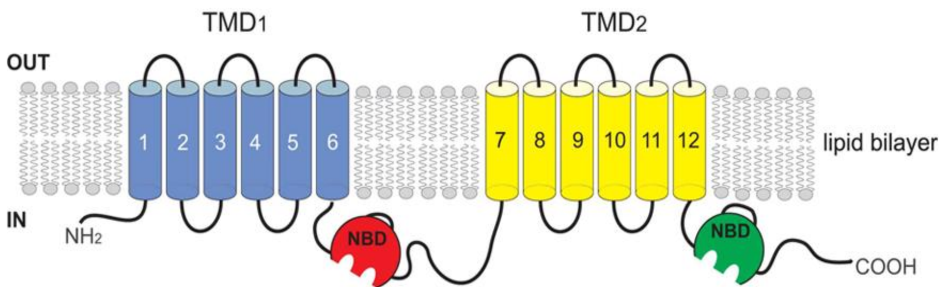


Figure 1.3 Structure of P-gp depicting the general structure and organization of Human ABC transporters.

Physiologically, they are expressed at low levels in tissues however, in disease conditions such as malignancies, their expression levels increase thus enhancing their drug efflux activities and ultimately contributing to drug resistance/therapeutic failure. P-gp has been reported to transport and confer resistance to various anticancer drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxins and clinically important therapeutics like HIV protease inhibitors, cardiac glycosides, immunosuppressive agents, antibiotics and corticoids [19]. MRP1 also transports and confers resistance to a broad range

of antineoplastic agents such as vinca alkaloids, epipodophyllotoxins, methotrexate and camptothecins and other clinically important therapeutics such as antibiotics, antivirals, antidepressants, and statins [20, 21]. This study focused on BCRP due to its broad substrate specificity.

1.2.3 BCRP and Multidrug Resistance

Human BCRP is the second member of the ATP binding cassette sub-family G (ABCG2), encoded by the *ABCG2* gene that is located on chromosome 4q22 [22]. The gene spans approximately 66 kb, has 16 exons and encodes 655 amino acid residues [23]. Unlike P-gp and MRP1, BCRP is considered as a half-transporter because it comprises of six transmembrane helices and one ATP-binding domain. Also, glycosylation of BCRP occurs on Asparagine residue in position 596 (Asn 596) located on the extracellular loop connecting transmembrane helix 5 and 6 [22, 24] (Figure 1.4).

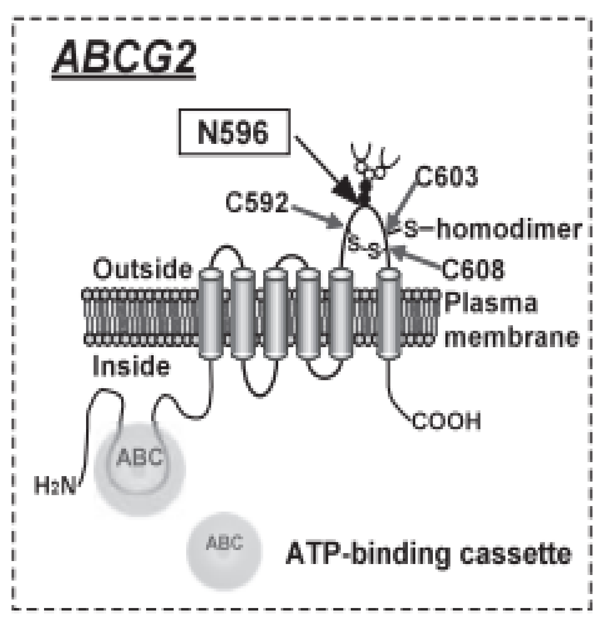


Figure 1.4 Topology and domain structure of BCRP monomer [25]

Physiologically, BCRP exists as a monomer, however, current evidence in a mechanism that is not well understood shows that homodimerization via the formation of a disulfide bridge at cysteine residue in position 603 (Cys 603) located on the extracellular loop is required for BCRP transport activity [26]. Like all ABC transporters, the NBDs have a highly conserved motif known as Walker A and Walker B domains used in ATP binding. The TMDs provide substrate binding sites and contribute to transporter specificity [27].

Several mechanisms of transport have been proposed for BCRP and ABC transporters in general. Among these include the ATP- switch, alternating catalytic site, constant contact, and the reciprocating twin-channel models [28-31]. The ATP- switch model proposed by Higgins and Linton is the most commonly cited mechanistic model for ABC proteins. According to this model as depicted in Figure 1.4, the NBDs alternate between a closed and an open conformation. These movements result in ligand/drug/substrate translocation [32]. The closed conformation has two ATP molecules bound to the NBD dimer interface forming a nucleotide pocket. ATP then binds to the Walker A and Walker B motifs within the nucleotide pocket. Transport is initiated by the binding of substrate to the TMDs in the open NBD conformation which increases affinity to ATP [32, 33]. The binding of ATP triggers changes in the formation of closed NBDs dimer which subsequently induces a major conformational change in the TMDs to begin substrate transport. ATP is subsequently hydrolyzed with release of ADP, and inorganic phosphate and triggering of NBD dimer dissolution [33, 34]. The basal state of transporter is then restored to reinitiate another transport cycle.

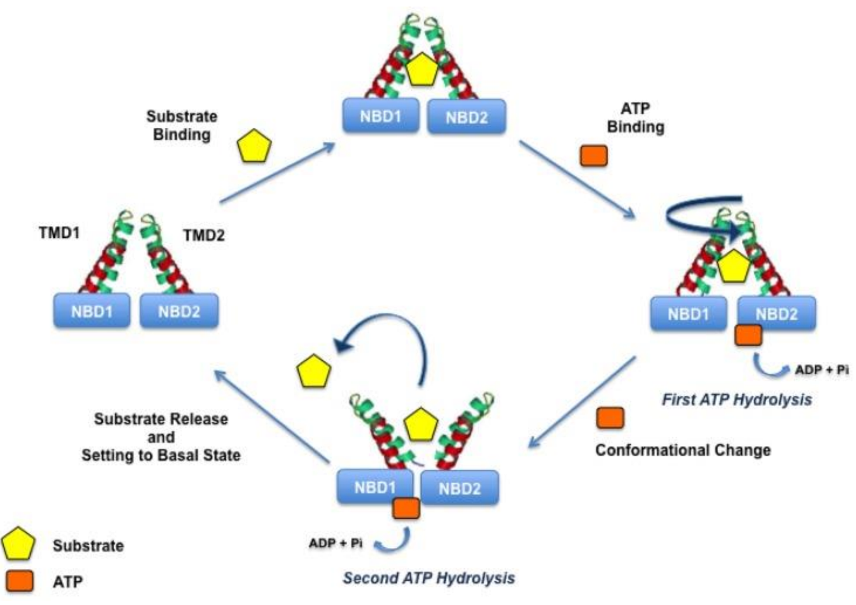


Figure 1.5 Transport/Efflux mechanism of ABC Proteins (BCRP) [28]

BCRP is physiologically expressed in several tissues including placenta, brain micro-vessel endothelium, mammary gland, colon, liver, small intestine, biliary tract, ovary, testis, kidney, and hematopoietic stem cells; contributing to the pharmacokinetics of drugs and endogenous compounds as well as protecting tissues from toxic xenobiotics [35-37]. It actively transports a broad spectrum of conventional anticancer agents, including doxorubicin, mitoxantrone, topotecan, methotrexate, and daunorubicin as well as novel targeted molecules such as the tyrosine kinase inhibitors, imatinib and gefitinib [38, 39]. BCRP is overexpressed in many malignancies and contributes to poor chemotherapeutic response in acute myelogenous leukemia (AML), chronic myeloid leukemia (CML), pancreatic ductal adenocarcinomas, non-small cell lung cancer (NSCLC), and other solid tumors [35]. Existing therapeutic strategies aimed at overcoming BCRP mediated MDR include transcriptional regulation with miRNAs targeting

BCRP/ABCG2 as well as inhibition with various pharmacological classes of inhibitors such as tyrosine kinase inhibitors (TKIs), HCV protease inhibitors, antifungal azoles, and immune suppressants [40-43]. Although these inhibitors have shown MDR reversal activity in pre-clinical studies, they have yielded limited clinical success due to toxicity and off-target drug interactions when co-administered with additional anticancer drugs, prompting the need for alternative clinically relevant therapeutics [44-46].

1.2.4 Glycans and BCRP-mediated Multidrug resistance

A key feature of all ABC transporter proteins is glycosylation (i.e., the presence of glycan chains) on Asparagine residues (N-linked recognition sites) present in specific extracellular loops connecting the transmembrane helices of the transporter proteins [22, 47, 48]. P-gp is glycosylated on three N-linked recognition sites (Asn 91, 94, and 99). Post-translation modification studies have also revealed that MRP1 is glycosylated at Asn 19 and Asn 23 [49]. As mentioned earlier, BCRP is glycosylated on Asn 596 located on the extracellular loop linking transmembrane helix 5 and 6. Lectin binding experiments have revealed these glycans to be branched oligosaccharide chains comprising of mannose, N-acetyl glucosamine and galactose sugars terminally capped with either α-2,6 or α-2,3 linkage of sialic acids (Figure 1.6) [50-53].

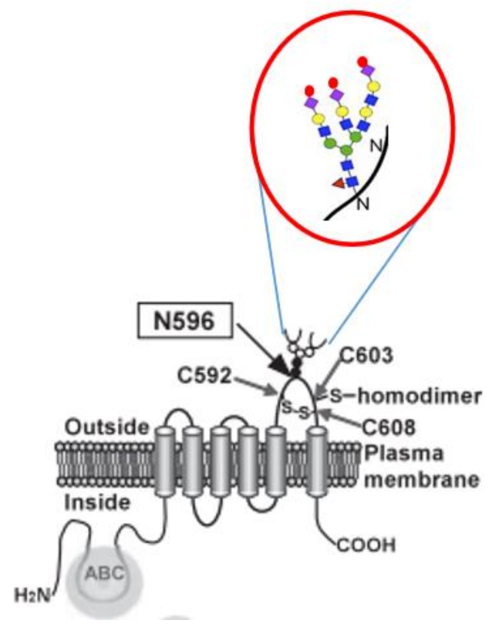


Figure 1.6 Glycan chain composition (zoomed in) on BCRP [25]

Several studies have reported a positive correlation between glycosylation status of BCRP and drug resistance (poor therapeutic outcomes) in cancers. For instance, Wojtowicz and colleagues in 2015 reported significant reversal of MDR phenotypes in ovarian and colorectal cancers following chemical (PNGase and Tunicamycin) inhibition of BCRP glycosylation [54]. Also, a study focused on understanding the functional checkpoints of BCRP revealed that, mutagenic substitution of Asparagine 596, the crucial glycosylation site on BCRP with Glutamine, disrupted N-linked glycosylation, interfered with localization and function of BCRP and sensitized hitherto MDR cancer cells to chemotherapeutics [25]. Probing further, the researchers discovered that absence of the N-glycan chain in mutant BCRP enhanced ubiquitin-mediated proteasomal degradation of the protein, accounting for a two-third reduction in mutant BCRP expression relative to wild type (glycosylated) BCRP. These findings highlight the

important role glycosylation plays in BCRP protein stability and suggest the potential therapeutic value of glycans or sugar residues in the circumvention of MDR, that warrants further exploration.

1.2.5 Role of Sialic acid in (Multi)drug resistance

Sialic acids (Sia e.g., N-acetylneuraminic acid [Neu5Ac]) are a family of 9-carbon sugars that are predominantly found as terminal residues on cell surface glycoconjugates such as glycoproteins (including ABC transporters), glycolipids, and gangliosides [55-57]. Such glycoconjugates are termed as Sialoglycans and their synthesis involves addition of sialic acids to growing glycan chains via specific linkages catalyzed by enzymes known as Sialyltransferases. Among the reported primary cellular roles of Sia include binding to microbes, toxins, and lectins as well as neural transmission [58].

Over 50 kinds of sialic acids are known, all of which can be derived from a molecule of neuraminic acid by substituting its amino group or one of its hydroxyl groups [56]. The most predominant forms found in nature include N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-nonulosonic acid (KDN). Neu5Ac is the most common sialic acid found in humans while Neu5Gc and KDN are some examples of nonhuman sialic acids [59]. Several studies have implicated altered expression of these Sia variants in several disease conditions including cancers (Figure 1.7)

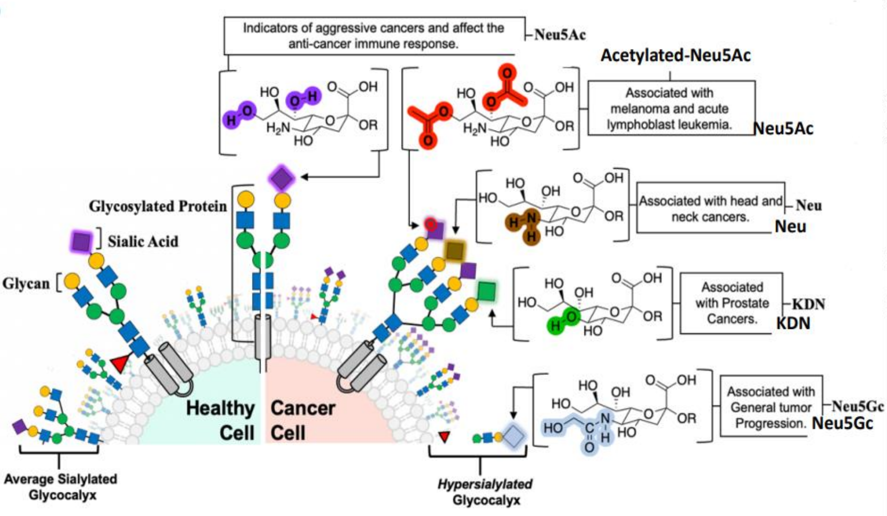


Figure 1.7 Contribution of Canonical and Non-canonical forms of Sialic acid in cancer [60]

One of the ways by which cancer cells develop resistance toward chemotherapeutics is overexpression of Sialyltransferases, key enzymes that add Sia to glycoconjugates. For instance, ST6Gal-I sialyltransferase, an enzyme upregulated in numerous cancers has been reported to promote survival and resistance in ovarian, pancreatic, and colorectal cancers via hypersialylation of tumor necrosis factor receptor 1 (TNFR1) and Fas Receptor (FasR) death receptor [61, 62] . Hypersialylation blocks receptor internalization and the formation of the death-inducing signaling complex (DISC), thereby disabling apoptotic signaling thus leading to cell survival [61, 62]. In a comparative study, Shultz and his colleagues reported an increased expression of ST6Gal-I transferase in cisplatin-resistant ovarian cancer cells relative to their non-resistant counterpart. They further demonstrated that knockout of *ST6Gal I gene* significantly sensitized resistant ovarian cancer cells to cisplatin [63]. Additionally, high levels of ST3GAL5 and ST8SIA4 sialyltransferases were overexpressed in drug resistant human acute myeloid leukemia (AML) cells relative to non-drug resistant cancer cells in both *in*

vitro and *in vivo* experimental models [64]. Further studies revealed that the altered levels of ST3GAL5 and ST8SIA4 correlated with high expression levels of P-gp and MRP1, suggesting a strong association between glycan sialylation and MDR.

1.2.6 O-Acetylated Sialic acid and (Multi) drug resistance

The hydroxyl groups associated with Sia may be further chemically modified with various functional groups including acetyl, lactyl, methyl, sulfate, or phosphate groups [56], contributing to its (Sia) vast structural diversity. Acetylated Sias are the most clinically studied and relevant modification. Of specific interest are the modifications to the C6 hydroxylated tail. Modulation of the acetyl functional group C6 tail are mediated by two enzymes, Sialate-O acetyl transferase (CASD1) and Sialate-O acetyl esterase (SIAE) respectively in the Golgi apparatus during the post-translational modification of Sia-capped glycoconjugates [57].

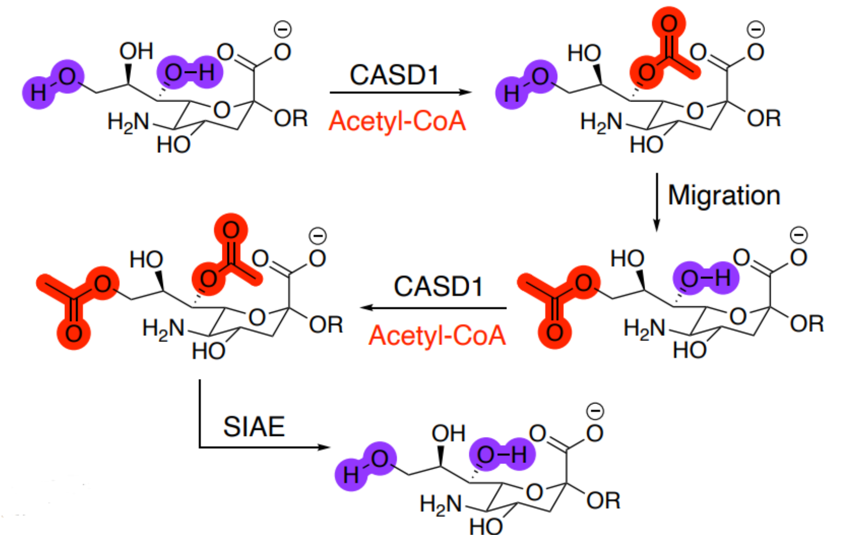


Figure 1.8 Sia acetylation scheme showing CASD1 and SIAE mediated 9-O and 7,9-O acetylation and deacetylation [60]

As shown in Fig 1.8, CASD1 and SIAE enzymes modulate 9-O and 7,9-O acetylation of Sia (Neu 5Ac). CASD1 adds acetyl functional groups, via an Acetyl CoA donor, to the seventh carbon of Sia, from which it migrates to the ninth carbon (Neu 5,9Ac2) under physiological conditions. This facilitates the addition of new acetyl group to the seventh carbon by CASD1 yielding Neu 5,7,9Ac3 [65].

Cancer cells differentially acquire these O-acetyl modifications in the perpetuation of unique survival hallmarks such as immune cell evasion and aggressive phenotype development [66]. For instance, Grabenstein et al. (2021) in an extensive study, reported that Sia acetylation reduces engagement of cancer associated Siglecs and increases NK mediated cytotoxicity in colon and lung cancer cells. Comparative studies on colon-derived mucins have revealed over 50% acetylation of Sia in healthy tissues and predominantly high deacetylated Sia in cancerous tissues. Research has also shown that hypoacetylation of Sia on the tumor associated antigen, Sialyl Lewis X motifs, is the key alteration associated with metastatic colorectal cancers [67]. Furthermore, high levels of 9-O acetyl sialylation of sialoglycoproteins have been shown to be distinctly expressed in human acute lymphoblastic leukemia (ALL) and used as diagnostic biomarker for the detection of pediatric ALL [68]. It has also been touted to contribute to the survival and drug resistance characteristics observed in mouse and *in vitro* models of pre-B ALL [69]. Despite the prognostic and diagnostic value of Sia acetyl functional group alterations, its role in modulating ABC transporter mediated MDR, the clinically major MDR pathway in cancers, remains unexplored.

1.2.7 Scope of Study

Considering the enormous involvement of glycans in several cancer hallmarks and driven by our recent discovery that the overexpression of deacetylated-Sia on colon and lung cancer cell lines results in immune evasion, of Natural Killer (NK) mediated cytotoxicity via the Siglec-Sia pathway [57], we began turning our attention to additional mechanistic (i.e. MDR) pathways undermining the effective prevention of cancers involving glycosylation, specifically deacetyl-Sias. The role acetyl functional group on Sia played in MDR remained unexplored. As such, we investigated how modulating levels of Sia acetyl functional groups affected BCRP-mediated MDR in *in vitro* models of two of the most common and deadliest cancers (i.e., lung and colon cancer). Specifically, lung (A549) and colon cancer (HCT 116) cells that lack acetyl groups and those that express acetyl groups were generated by removal of *CASD1* and *SIAE* gene respectively via CRISPR Cas9 gene editing. We subsequently evaluated BCRP-mediated MDR by assessing BCRP protein and gene expression, efflux function, cell proliferation and survival in knockout cells relative to control (parental) cells. The clinical significance of this study was further evaluated by assessing the correlation between patient survival and Sia acetyl functional group modulating genes expression. Specifically, we used *CASD1* and *SIAE* gene expression data from lung and colon adenocarcinoma patients. Datasets were obtained from The Cancer Genome Atlas (TCGA) and stratified into high and low gene expression groups based on the median. We performed survival analysis to compare the overall survival between high and low expression groups.

1.2.8 Published Study Findings

Interesting and high impact findings from this work have been successfully published as an original article in the Cancer Molecular targets and Therapeutics section of Frontiers in Oncology Journal (Figure 1.9) [70] . Article can be accessed via the link below:

https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2023.1145333/full

frontiers | Frontiers in Oncology

TYPE Original Research
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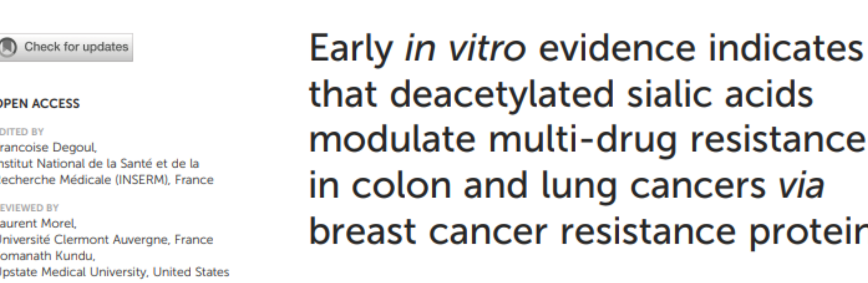
DOI 10.3389/fonc.2023.1145333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Early *in vitro* evidence indicates that deacetylated sialic acids modulate multi-drug resistance in colon and lung cancers *via* breast cancer resistance protein

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Figure 1.9 Screenshot of title page of published article

1.3 Materials and Methods

1.3.1 Chemicals and Reagents

Fetal bovine serum (FBS) and penicillin/streptomycin (P/S) were obtained from Corning Incorporated (Corning, NY). The GAPDH loading control monoclonal antibody (ref AM4300, lot #: 00939504), Alexa Fluor 488 conjugated goat anti-mouse IgG cross-adsorbed secondary antibody (2 mg/mL, Cat #A32723), PowerUp SYBR green PCR master mix (Cat # A25741) and paraformaldehyde were obtained from Thermo Fisher Scientific Inc (Rockford, IL). Anti-ABCG2 antibodies, BCRP/ABCG2 (Cat #EPR20080, lot #: 3026758) and clone BXP-21 (Cat #MAB4146) were obtained from Abcam (Waltham, MA) and Millipore (Billerica, MA) respectively. IRDye Goat anti-mouse and anti-rabbit IgG secondary antibody (lot#: C90130-02) were obtained from LI-COR (Lincoln, NE). Mitoxantrone, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), Ko143, Bafilomycin A1, and Triton X-100 were purchased from Sigma Chemical Co (St. Louis, MO). All other chemicals used were of analytical grade.

1.3.2 Cell Lines and Cell culture

A549 cells were cultured in Dulbecco modified Eagle medium (Corning) supplemented with 10% FBS and 1% P/S (Corning). HCT 116 cells were cultured in RPMI 1640 medium (Corning) supplemented with 10% FBS and 1% P/S. All cell lines were originally purchased from American Type Culture Collection (location). Cell Dissociation Buffer (Gibco, Waltham, MA) was exclusively used for passaging cells.

CASD1 and SIAE knockout A549 cell lines were obtained from Dr. Colin Parrish (Cornell University, Ithaca, NY). CRISPR-Cas9 stably expressing cells and the third-generation lentiviral system were gifted from Dr. Michael Bassik (Stanford University, Stanford, CA). HEK 293/PcDNA 3.1 cells were a generous gift from Dr. Surtaj Iram (American University of Iraq, Sulaimani, Iraq). CRISPR-Cas9 editing of CASD1 and SIAE in HCT 116 and A549 cells was previously published [57, 58]. Briefly, paired Cas9 plasmids targeting adjacent sites in early exons of *CASD1* and *SIAE* were transfected using TransIT-X2 (Mirus Bio LLC, Madison, WI). Transfected cells were selected with puromycin, and single cell clones screened with a specific virolectin recognizing 9-O-acetyl Sia (PToV-P4 HE-Fc) [57, 58, 71]. Full sequencing was used to confirm loss of *CASD1* and *SIAE* function in both alleles. qPCR and western blot analysis were also performed to confirm knockout efficiencies.

1.3.3 Generation of BCRP overexpressing HEK 293 Cell line

A BCRP (ABCG2)-overexpressing cell line was developed and used as a positive control for efflux studies. Briefly, HEK 293 Wildtype cells were plated in 6 well plate (Corning, NY) at a density of $5x10^5$ cells/ml and cultured until 80% confluency was attained. Cells were then transiently transfected with 2 µg PcDNA 3.1 plasmid containing Human ABCG2 cDNA ORF clone (GenScript Biotech, NJ). Transfection was carried out with Lipofectamine 3000 (Invitrogen, MA) in Opti-MEM medium (Gibco, MD), according to manufacturer's protocol [72]. ABCG2-expressing stable cells were selected by treating cells with 0.8mg/ml G418 for up to a week. A G418 (Geneticin) (Gibco, MA) kill curve was performed prior to transfection on

HEK 293 Wildtype cells to select appropriate G418 concentration for stable cell line generation. Western blot analysis was used to confirm stable transfection.

1.3.4 Western Blot Analysis

Cell lysates were prepared in RIPA buffer (Thermo-Fisher Scientific, MA) supplemented with Protease and Phosphatase Inhibitor (ThermoFisher Scientific, MA). Protein concentration was determined using Pierce BCA Protein Assay (ThermoFisher Scientific, MA). Cell lysates (20-30 μg protein) were electrophoresed on 4-12% Criterion TGX Precast gels (BioRad, CA) and transferred onto Trans-Blot turbo nitrocellulose membranes (BioRad, CA). Membranes were blocked with Intercept blocking buffer (LI-COR, NE) for 1 h at room temperature and incubated overnight at 4°C with monoclonal anti-BCRP antibody (Abcam, MA), anti-GAPDH antibody (Thermo Fisher Scientific, IL), anti-Bcl2 antibody (Thermo Fisher Scientific, IL) and anti-PARP1 antibody (Thermo Fisher Scientific, IL) at 1:500, 1:2000, 1:1000 and 1:1000 dilutions in blocking buffer respectively. Secondary antibody incubation (1:1000 dilution in PBS containing 0.1%Tween 20) was performed using goat anti-mouse IRDye secondary antibody (LiCOR, NE) and goat anti-rabbit IRDye secondary (Li-COR, NE) for 1 h at room temperature. Target proteins were detected using the Odyssey CLx Imager (LiCOR, NE). For protein expression comparison, protein band density was analyzed using Image StudioLite (LI-COR, NE) software and corrected for uneven sample loading and transfer using GAPDH as the loading control. For expression levels of Cleaved PARP1 determination, cells were challenged with 0.5μg/ml Mitoxantrone for 48h prior to lysate preparation. This same approach was used

to investigate the effect of the lysosomal enzyme inhibitor, Bafilomycin A1 (BMA), on BCRP protein expression levels. Cells (4×10^5 cell/ml) were challenged with 100nM BMA for 2h prior to lysate preparation using a previously established protocol [25].

1.3.5 Immunofluorescence Assay

Cellular expression of BCRP was determined by immunofluorescence microscopy as previously described [73]. In brief, HCT 116, A549 and HEK/BCRP cells (2×10^4 cells per well) were grown on glass coverslip. Cells were then fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Slides were blocked in a buffer containing 0.01% goat serum,0.01% saponin, and 0.05% glycine in PBS for 1 h. After incubation with 1:20 dilution of human ABCG2 antibody BXP-21 (Millipore, MA) in PBS (containing 0.1% BSA) overnight at 4ºC, cells were incubated with 1:200 dilution Alexa Fluor 488 conjugated goat anti-mouse IgG cross-adsorbed secondary antibody (Thermo Fischer, IL) in PBS (containing 0.1% BSA) in the dark for 1 h. DAPI (Sigma, MO) (1µg/mL final concentration) was used to stain nuclei of cells. A BioTek Cytation Live Cell imager (BioTek, WA) was used to collect immunofluorescence images.

1.3.6 Hoechst 33342 Accumulation Assay

Intracellular fluorescence of Hoechst 33342 was analyzed with a fluorescence microscope (Carl Zeiss, Göttingen, Germany) as described previously [74]. Briefly, cells were cultured in a 6-well plate (Corning, NY) containing poly-L lysine coated glass cover slips overnight at a density of 5×10^5 cells/ml. Cells were exposed to 1µM Hoechst 33342 in the presence or

absence of 1µM Ko143. Cells were then washed twice with ice-cold PBS, covered with HEPES buffer and fluorescence images were measured. Image StudioLite (LI-COR, NE) software was used to quantify fluorescence intensity (Hoechst 33342 accumulation).

1.3.7 Cell viability Assay

Differential sensitivity of cells to Mitoxantrone was measured using the MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) colorimetric assay as described previously [75]. Briefly, cells were plated at a density of $2x10^5$ cells/ml in a 96 well plate (Corning, NY) and challenged with varying concentrations (0-5 µg/ml) of Mitoxantrone for 48 h in the presence or absence of the BCRP inhibitor, Ko143 (2µM). MTT solution (5 mg/ml) was then added to each well and cells further incubated for 4 h at 37°C. The amount of formazan produced was measured at a wavelength of 570 nm with a microplate reader (BioTek Cytation 3, WA). Absorbance values were recorded and used to evaluate the percentage cell viability. GraphPad Prism 8 software was used to evaluate the individual IC_{50} values, following which fold resistance (ratio of IC_{50} value for each cell line to IC_{50} of its corresponding Wildtype cell line) was estimated.

1.3.8 Cell Proliferation Assay

Growth rate of cells was measured and compared via the method described by Barnard et al. (2019). Briefly, cells were plated $2x10^5$ cells/ml in a 96 well plate (Corning, NY) overnight. The cells were subsequently harvested, stained with trypan blue and counted every day for up to 4 days with an automated cell counter (BioRad, CA). Culture medium was replaced

with fresh culture medium after the second day for days 3 and 4 of the experiments.

1.3.9 mRNA Expression Analysis

Total RNA was extracted from cells using Direct-zol RNA mini-prep kit (Zymo Research Corporation, Irvine, CA), according to the manufacturer's instructions. First-strand cDNA was prepared from the extracted total RNA in a reverse transcriptase reaction with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and random hexamers as a primer, according to the manufacturer's protocol. As described by Nakagawa et al (2009), the first-strand cDNA from the mRNA of human ABCG2 and GAPDH were amplified by PCR in an iCycler™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) using the following specific primer sets (Integrated DNA Technologies, Coralville, IA): human:

ABCG2:5'-GATCTCTACCCTGGGGCTTGTGGA-3';

5'-TGTGCAACAGTGTGATGGCAAGGGA-3'

GAPDH:5'-ACTGCCAACGTGTCAGTGGTGGACCTGA-3';

5'-GGCTGGTGGTCCAGGGGTCTTACTCCTT-3′.

The PCR reaction consisted of hot-start incubation at 94°C for 2min, and 30 cycles at 94°C for 30s, 59°C for 30s and 72°C for 30s. After the PCR, the resulting amplicons (123bp for BCRP and 233bp for GAPDH) were separated by 1% (w/v) agarose-gel electrophoresis cDNA concentrations were measured with a Nanodrop spectrophotometer (ThermoFisher, USA) at a wavelength of 260 nm. cDNA quality was evaluated using electrophoresis in 1% agarose gels quality. Using the same primer sets shown above, BCRP relative gene

expression was further investigated by quantitative real-time PCR. Each cDNA sample (1 µl) was amplified with 10 µl of Thermo Scientific PowerUp SYBR Green qPCR Master Mix and 1 µM of each primer. Amplification was performed in a Quant Studio 3 Real Time PCR system (Applied Biosystems, Foster City, USA) with the following parameters: UDG activation at 50ºC, Activation (Dual-Lock DNA polymerase) at 95ºC for 2 min followed by 40 cycles of denaturation at 95 ºC for 15 s, annealing/extension at 60ºC for 1 min. The relative expression levels of ABCG2 in each sample (normalized to that of GAPDH) were determined using $2^{-\Delta\Delta Ct}$ method. RT-qPCR experiments were repeated three times.

1.3.10 Expression of *CASD1* and *SIAE* in clinical samples

To investigate the potential prognostic value of *CASD1* and *SIAE*, we performed survival analysis of *CASD1* and *SIAE* mRNA expression and overall survival in clinical lung adenocarcinoma (LUAD) samples (n=501) and clinical colon adenocarcinoma (COAD) samples (n=374) from the TCGA PanCancer Atlas study [1]. Survival data and gene expression data were downloaded via cBioPortal (https://www.cbioportal.org/) [2, 3]. Gene expression data downloaded for our analysis were *mRNA Expression, RSEM (Batch normalized from Illumina HiSeq_RNASeqV2)*. Survival analysis and visualization were performed with R programming language [4] using *ggsurvfit* [5] and *survival* [6, 7] packages. Kaplan-Meier plots were generated to visually compare survival across gene expression groups and cancer types. Samples were split into two groups, high and low, based on the median expression per gene (high: >=median, low: <median). Overall survival status was coded 1 for deceased and 0 for censored, overall survival

time was expressed in months, and gene (*CASD1* or *SIAE*) expression status was coded 1 for low and 2 for high. Univariable Cox proportional hazard regression models were fit using survival outcome (based on overall survival status and overall survival time) as response variable and gene expression status as explanatory variable. The Cox proportional hazard regression hazard ratio (HR), 95% confidence intervals (CI) and log-rank p value were reported in each Kaplan-Meier plot. Statistical significance was set a log-rank p value < 0.05.

1.3.11 Scientific Rigor

All reagents/antibodies/cell lines have been selected based on published figures and purchased from companies that provide validation. Experiments were always performed in technical and then biological replicates (Specifically 3 times with each done in triplicates). We used t-tests or ANOVA followed by Tukey post-tests for multiple comparisons using GraphPad Prism 8 (San Diego, CA). Data, as seen below, is presented as mean ± standard deviation with P < 0.05 indicating significance.

1.4 Results and Discussion

1.4.1 Generation of BCRP Overexpressing HEK 293 Cells

To develop a stable BCRP-overexpressing cell line to serve as a positive control for functional studies, we first constructed a Geneticin (G418) kill curve to determine appropriate G418 concentration for stable cell line selection. The results revealed that 0.8mg/ml G418 inhibited 99% cell growth (Fig 1a). HEK 293 Wildtype were then transiently transfected with a PcDNA 3.1 vector containing Human ABCG2 cDNA ORF clone and selected for

stably transfected cells by applying 0.8mg/ml G418 selection for a week. Results of transfection revealed high BCRP/ABCG2 protein expression in transfected cells (HEK293/BCRP) compared to the wildtype cell line (HEK 293) and cell transfected with an empty vector (HEK/PcDNA3.1) (Fig. 1b). Also, the generated HEK 293/BCRP cell line was functionally characterized by comparing it to HEK 293 wild-type WT in terms of their response or sensitivity to the chemotherapeutic drug, Mitoxantrone. Our results showed significantly increased cell viability (Fig. 1c) and 5-fold increased resistance (Supplementary Table. 1) in transfected cells compared to wild type cells.

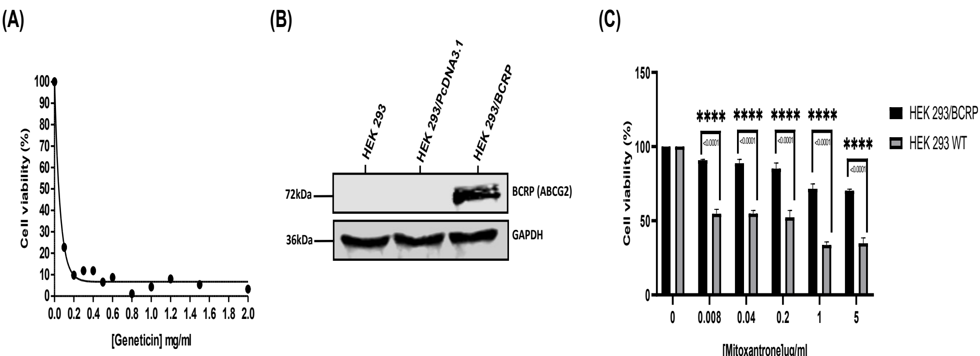


Figure 1. Generation of BCRP/ABCG2 overexpressing cell line. **(A)** G418 Kill curve on HEK 293 Wildtype cell line **(B)** Immunoblot comparison of BCRP expression in Transfected and Untransfected cells **(C)** Effect of mitoxantrone on Transfected and Wildtype HEK 293 cell lines. Data reported as mean $\pm$ sd of three technical replicates (A) or Two-way ANOVA with Tukey's posttest for multiple comparison (C). *P < 0.05

Table S1. Comparison between HEK 293 Wildtype and BCRP Overexpressing cells

Cell line	IC_{50}[a] (µg/ml)	
	Mitoxantrone	Fold resistance[b]
HEK 293 Wildtype	0.190 ± 0.074	1.00
HEK 293/BCRP	1.013 ± 0.012*	5.33

[a]Mean ± SD of three independent experiments performed in triplicate *P < 0.05. [b] Fold resistance determined by dividing the IC_{50} value for HEK 293/BCRP Cell line by the IC_{50} value for HEK 293 Wildtype

1.4.2 CASD1 knockout upregulates BCRP/ABCG2 expression

To determine the effect of O-acetyl modification of Sia on BCRP, western blot analysis and immunofluorescence assay were performed to compare BCRP protein expression in wild type HCT 116 and A549 cell lines to those without O-acetyl Sia (CASD1 knockout) and those that express high levels of O-acetyl Sia (SIAE knockout). The results indicate that knockout cells showed overexpression of BCRP (ABCG2) in both HCT 116 (Fig. 2a, 3a and 3c) and A549 (Fig. 2b, 3b and 3d) cell lines as compared to wild type cells with CASD1 knockout cells recording the most significant overexpression of BCRP followed by SIAE knockout cells. The wild type cell line showed the lowest BCRP (ABCG2) expression. Also, CASD1 knockout A549 cells comparatively hypertrophic (Fig. 3b). The CASD1 knockout cell lines (i.e., cells that lack O-acetyl Sia) in particular, demonstrated significantly higher levels of BCRP expression. With respect to Sia modifications, prior studies have reported a positive correlation between Sialyl-transferases, ST3Gal5 and ST8Sia4 expression and efflux proteins, P-gp and MRP1 expression in human acute myeloid leukemia [76]. Our data suggests that there is a strong correlation between dysregulation of O-acetylation in cells, particularly loss of CASD1, and BCRP expression. Although all cells were imaged under same magnification (scale bar: 100µm), a key observation made during microscopic analysis worth mentioning is the hypertrophy of CASD1 knockout cells relative to the other cell variants. This may be attributed to its comparatively high expression of the BCRP efflux pump. Studies have shown that overexpression of membrane proteins reshape membrane domain and induces

elongation of the membrane, ultimately increasing cell size (Cornet et al., 2022).

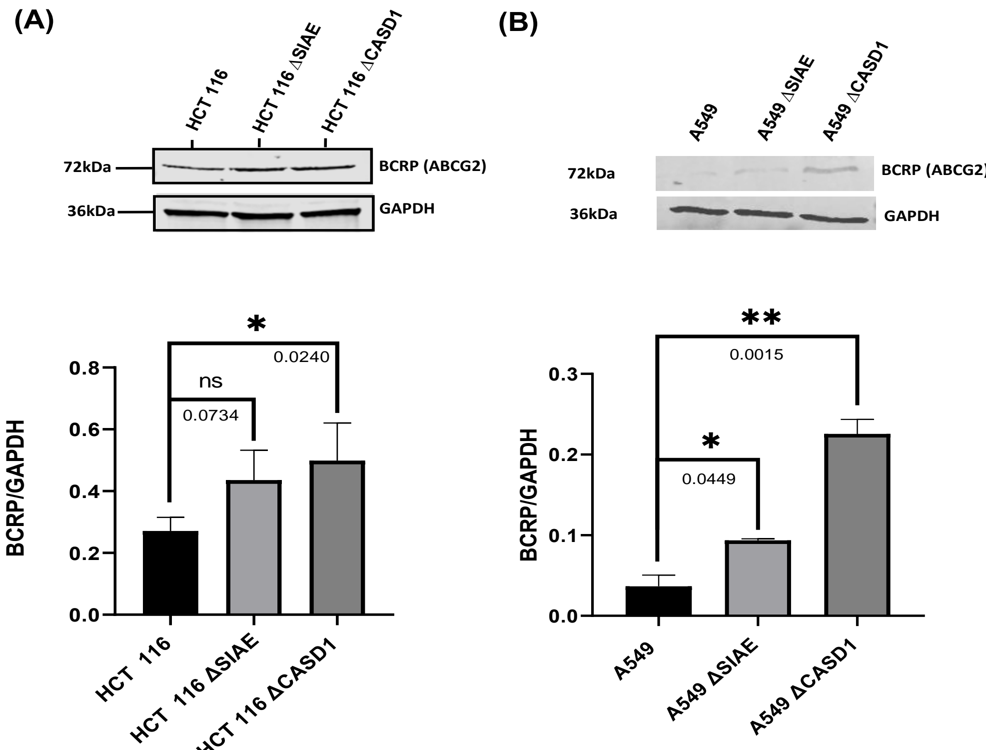


Figure 2. BCRP (ABCG2) expression in Wild type, Knockout (CASD1 and SIAE) cell lines. Western blot analysis was performed on whole cell lysate using BCRP-specific antibodies. GAPDH was used as loading control (A) Immunoblot analysis of whole cell lysates (30ug) prepared from HCT 116 cell lines. Below is normalized Protein band density. (B) Immunoblot analysis of whole cell lysates (20ug) prepared from A549 cell lines. Below is normalized Protein band density. One-way ANOVA with Tukey's posttest for multiple comparison, *$P < 0.05$ and **$P < 0.01$.

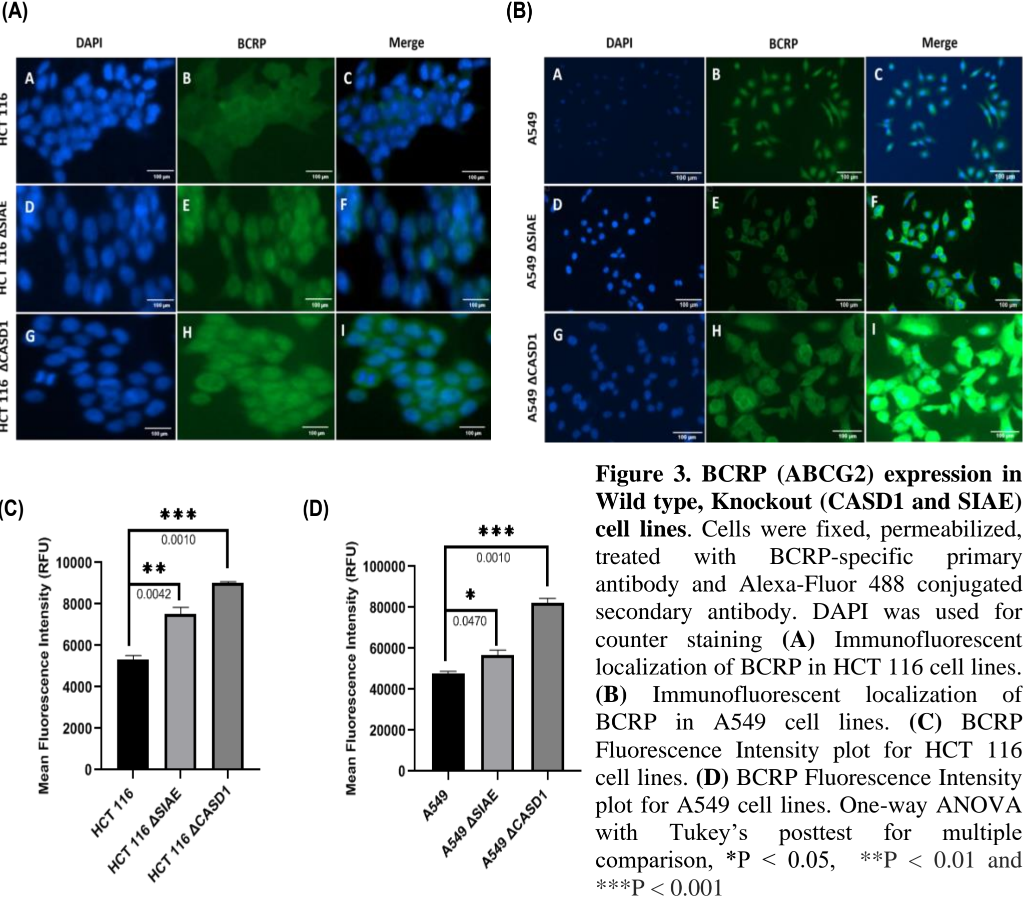


Figure 3. BCRP (ABCG2) expression in Wild type, Knockout (CASD1 and SIAE) cell lines. Cells were fixed, permeabilized, treated with BCRP-specific primary antibody and Alexa-Fluor 488 conjugated secondary antibody. DAPI was used for counter staining **(A)** Immunofluorescent localization of BCRP in HCT 116 cell lines. **(B)** Immunofluorescent localization of BCRP in A549 cell lines. **(C)** BCRP Fluorescence Intensity plot for HCT 116 cell lines. **(D)** BCRP Fluorescence Intensity plot for A549 cell lines. One-way ANOVA with Tukey's posttest for multiple comparison, *P < 0.05, **P < 0.01 and ***P < 0.001

1.4.3 CASD1 knockout decreases intracellular accumulation of Hoechst 33342

To investigate the effect of O-acetylated Sia modifications on BCRP (ABCG2) function, a BCRP fluorescent substrate, Hoechst 33342 was used to assess transport (efflux) competence of wild type and knockout cell lines. Our data shows generally high and significant fluorescent intensities for Ko143-inhibited cell lines compared to control (vehicle-treated) cell lines (Fig. 4). Also, our results show a significantly reduced accumulation of Hoechst 33342 in CASD1 knockout cell lines of both HCT 116 (Panel A of Fig. 4a, and A549 (Fig. 4) cell lines compared to Wild type and SIAE knockout cells. To further test BCRP's involvement in this efflux phenomenon, potent BCRP inhibitor, Ko143 was used to block efflux function. As shown in Panel B of Fig. 4a and

4c as well as Fig 4b and Fig 4d, Hoechst accumulation increases, resulting in higher nuclei fluorescent intensity compared to the vehicle treated.

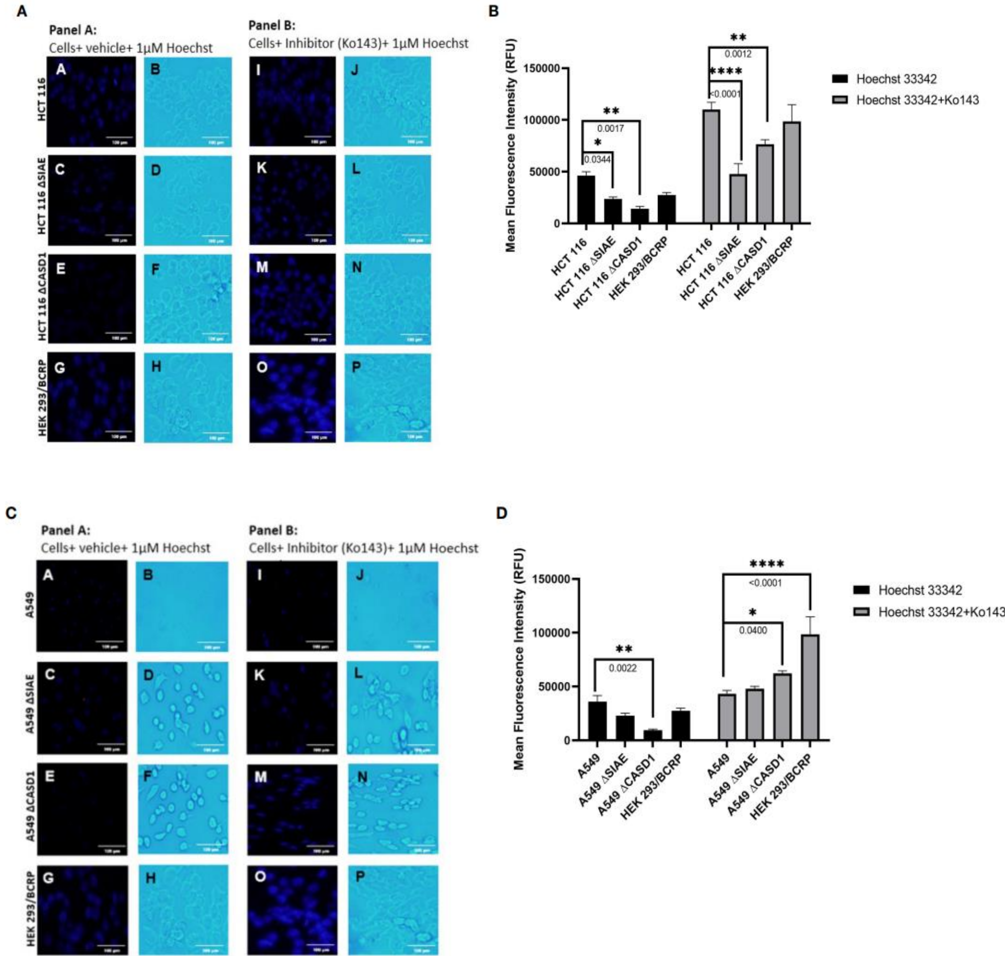


Figure 4. Intracellular accumulation of Hoechst 33342 in HEK293 and A549 Cells. Cells were treated with Hoechst 33342 in the presence or absence of Ko143 and stained with Hoechst 33342. **(A)** Fluorescent and Bright field images of HCT 116 cells. **(B)** Fold change in Hoechst 33342 accumulation in HCT 116 cells. One-way ANOVA with Tukey's posttest for multiple comparison, *P < 0.05. **(C)** Fluorescent and Bright field images of A549 cells. **(D)** Fold change in Hoechst 33342 accumulation in A549 cells. One-way ANOVA with post hoc Tukey's test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001

Hoechst33342(2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H benzimidazole trihydrochloride trihydrate) is a cell permeable dye that emits blue fluorescence when bound to double stranded DNA [77]. Mitoxantrone on the other hand, is a chemotherapeutic agent that is clinically used to treat solid tumors, leukemias, and as an immune system modulator in multiple sclerosis. It functions as an anticancer agent by inhibiting topoisomerase II, an enzyme involved in DNA replication, chromosome

condensation and segregation [78]. This leads to increase in the incidence of double strand breaks and ultimately resulting in cell death. Our results showed significantly high levels of Hoechst 33342 accumulated in the nuclei of wildtype cell lines as compared to the knockout cell lines. The CASD1 knockout cell lines accumulated the least amount in their nuclei, hence recording the lowest fluorescent intensity. This may be attributed to the earlier reported variation in BCRP expression levels across the individual cell types. Generally, there is a positive correlation between efflux activity and levels of efflux proteins (i.e., BCRP) present in the cell. The wildtype cells extrude small amounts of Hoechst 33342 due to its low BCRP expression levels hence more of this fluorescent substrate accumulates in its nuclei. The knockout cells on the other hand, express high levels of BCRP hence they extrude large amounts of Hoechst 33342, ultimately resulting in the observed low intracellular (nuclei) levels, especially in CASD1 knockout cells.

1.4.4 CASD1 knockout confers resistance to Mitoxantrone

To investigate the effect of O-acetyl Sia on cancer cell response to the chemotherapeutic drug, Mitoxantrone, the MTT (tetrazolium-based) cell viability assay was performed. Following exposure to varying concentrations (0-5µg/ml) of Mitoxantrone for 48h in the presence or absence of BCRP inhibitor (Ko143), the result revealed reduced cell viability in all cancer lines in the presence of Ko143 inhibitor as compared to vehicle treated (uninhibited) cells (Fig. 5a-c and 5e-g). The results further showed a reduced sensitivity/response in CASD1 knockout cell lines of both cancer cells as compared to the wildtypes and SIAE knockout cells. This is inferred from the

IC_{50} values of 0.512, 0.658 and 1.009 µg/ml recorded for HCT 116 wildtype, HCT 116 SIAE knockout and HCT 116 CASD1 knockout cell lines respectively (Table 1 and Fig. 5d). A549 wildtype, A549 SIAE knockout and A549 CASD1 knockout cell lines also recorded IC_{50} values of 0.423, 0.710 and 1.226 µg/ml respectively (Table 1 and Fig. 5h). In effect, CASD1 knockouts of both cancer cells exhibited significantly higher resistance towards Mitoxantrone (i.e., ~2-fold resistance and 3-fold resistance for HCT 116 and A549 respectively when compared to their Wildtype cells) (Table 1)

Table 1. Effect of Acetyl Sia on Cellular response to Mitoxantrone

Cell line	IC_{50} (µg/ml)	
	Mitoxantrone	**Fold Resistance**
HCT 116 Wild type	0.512 $\pm$ 0.070	1.00
HCT SIAE Knockout	0.658 $\pm$ 0.012	1.28
HCT CASD1 Knockout	1.009 $\pm$ 0.017*	1.97
HEK 293/BCRP	1.013 $\pm$ 0.012*	1.96
A549 Wild type	0.423 $\pm$ 0.132	1.00
A549 SIAE Knockout	0.710 $\pm$ 0.177	1.68
A549 CASD1 Knockout	1.226 $\pm$ 0.479**	2.90
HEK 293/BCRP	1.013 $\pm$ 0.012**	2.39

Mean $\pm$ SD of three independent experiments performed in triplicate. Fold resistance determined by dividing the IC_{50} value for each cell line by the IC50 value of Wild type. *P < 0.05 and **P < 0.01 significantly different from the wild type cell line

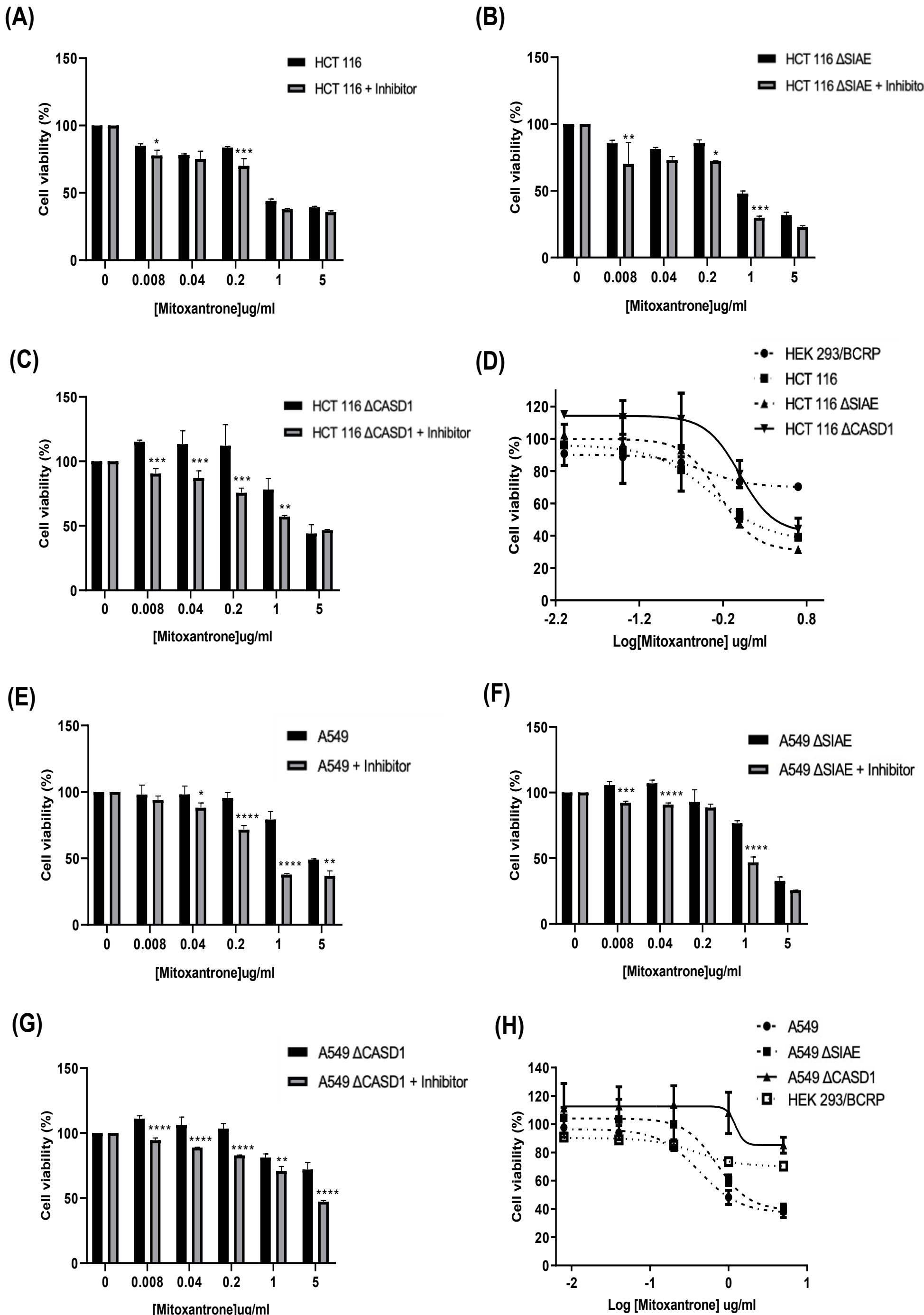


Figure 5. Cytotoxic effect of Mitoxantrone on HCT 116 and A549 cells. Cells were treated with Mitoxantrone (0-5ug/ml) in the presence or absence of 2uM Ko143. Dose-response of the effect of Mitoxantrone on **(A)** HCT 116 Wildtype cells **(B)** HCT 116 SIAE knockout cells **(C)** HCT 116 CASD1 knockout cells **(D)** Comparative cell viability profiles. Data reported as mean $\pm$ sd of three independent experiments. Two-way ANOVA with Tukey's posttest for multiple comparison, *P < 0.05. Cytotoxic effect of Mitoxantrone on A549 cells. Cells were treated with Mitoxantrone (0-5ug/ml) in the presence or absence of 2uM Ko143. Dose-response of the effect of Mitoxantrone on **(E)** A549 Wildtype cells **(F)** A549 SIAE knockout cells **(G)** A549 CASD1 knockout cells **(H)** Comparative cell viability profiles. Data reported as mean $\pm$ sd of three independent experiments. Two-way ANOVA with Tukey's posttest for multiple comparison, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Overexpression of BCRP confers resistance to several novel targeted molecules such as tyrosine kinase inhibitors as well as wide range of chemotherapeutic drugs including mitoxantrone, methotrexate and flavopiridol [79]. Mitoxantrone is a chemotherapeutic agent that is clinically used to treat solid tumors, leukemias, and as an immune system modulator in multiple sclerosis. It functions as an anticancer agent by inhibiting topoisomerase II, an enzyme involved in DNA replication, chromosome condensation and segregation [78]. This leads to increase in the incidence of double strand breaks and ultimately resulting in cell death. Variations in BCRP efflux activity account for the observed responses of the various cell types towards the chemotherapeutic drug, mitoxantrone. Wildtype of both experimented cancer cells were observed to be more sensitive to mitoxantrone. This may be due to their inability to extrude high amounts of mitoxantrone because of their inherently low BCRP levels. Thus, a high amount of this cytotoxic agent is retained intracellularly, leading to a reduction in cell viability. Conversely, the CASD1 knockout cells were observed to be more refractory (2- and 3-fold resistance for colorectal and lung cancer cells respectively) to mitoxantrone. The high efflux activity culminating from high BCRP levels in CASD1 knockout cells may account for this observation. These cells minimize intracellular levels of mitoxantrone and its associated cytotoxic effect by actively pumping out relatively high amounts of mitoxantrone out of the cells. Our data therefore suggests that the absence of O-acetyl Sia (Deacetylated Sia) confers drug resistance characteristics in lung and colon cancer cells.

1.4.5 CASD1 knockout enhances cell proliferation

To examine the association of O-acetyl Sia as well as BCRP expression in phenotypic drug resistant (aggressive) characteristics, we monitored the growth rate/cell proliferation of the wild type and knockout cells over a period of 4 days. Our results revealed significantly increased cell proliferation in the knockout cells compared to the wild type for the A549 cells (Fig. 6b). The CASD1 knockout cell line demonstrated the highest growth rate over the 4-day period of examination followed by the SIAE knockout cell line (Fig. 6b). A similar cell proliferation pattern was observed for HCT 116 cell lines (Fig. 6a), however, there was no significant difference between growth rate of wild type and knockout cells.

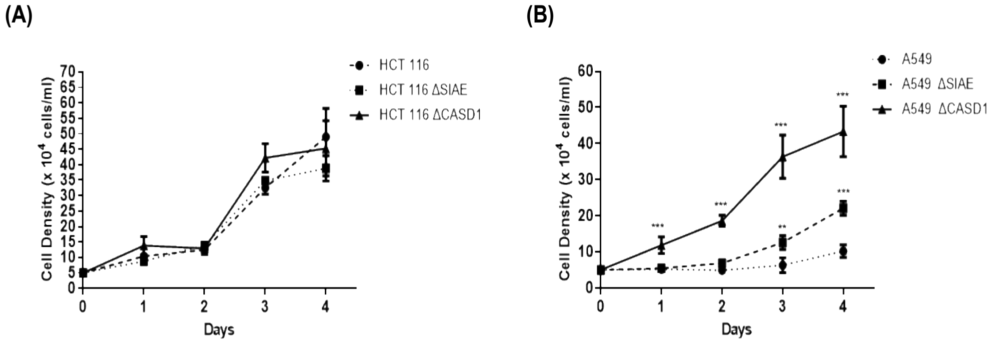


Figure 6. Comparative cell proliferation/growth assessment. $5X10^4$ cells/ml was seeded and cell density was measured every 24h for 4 days (A) Cell growth profile for HCT 116 cell lines (B) Cell growth profile for A549 cell lines. Data reported as mean $\pm$ sd of three independent experiments performed in triplicates. *P < 0.05, **P < 0.01 and ***P < 0.001.

This observation is consistent with earlier reports that implicate Sia hypo/deacetylation as key alteration associated with aggressive and metastatic colorectal cancers [67]. BCRP is also known to be a stem cell marker, whose expression in cancer cells is driven by metabolic and signaling pathways that confer multiple mechanisms of drug resistance, invasiveness (aggressiveness),

and self-renewal [26] hence confirming the observed high proliferative ability of the BCRP-overexpressing CASD1 knockout cells.

1.4.6 CASD1 knockout increases expression of cell survival proteins

To better understand the mechanism of CASD1 knockout-mediated cell proliferation as well as the role O-acetyl Sia plays in cell survival, we investigated the levels of cell survival proteins Bcl-2 and PARP1 in the wild type and knockout cell lines. Our results showed significantly higher levels (~2 fold) of Bcl-2 expression in SIAE and CASD1 knockout HCT 116 cell lines relative to their counterpart wild type cell line (Fig. 7a). In comparison to the wildtype cell line, the CASD1 and SIAE knockouts of A549 cell lines also recorded higher Bcl2 protein expression; however, between the two, CASD1 knockout cell showed a much higher and significant (~2 fold) Bcl-2 protein expression (Fig. 7b). To elucidate the protein expression levels of the 25 kDa PARP1 fragment (cleaved PARP 1) cells were incubated in the presence or absence of the apoptosis inducer, Mitoxantrone. Generally low levels of cleaved PARP1 were observed in the absence of Mitoxantrone, however, levels increased significantly in treated Wild type and SIAE knockout cell lines (Fig 8a, b). On the other hand, the increase in Cleaved PARP 1 levels in Mitoxantrone-treated CASD1 knockouts for both HCT 116 and A549 cell lines were not significant compared its untreated counterpart. Also, CASD1 knockout cell lines expressed the lowest levels (~2 fold less) cleaved PARP1 compared wild type and SIAE knockout cell lines (Fig 8a, b).

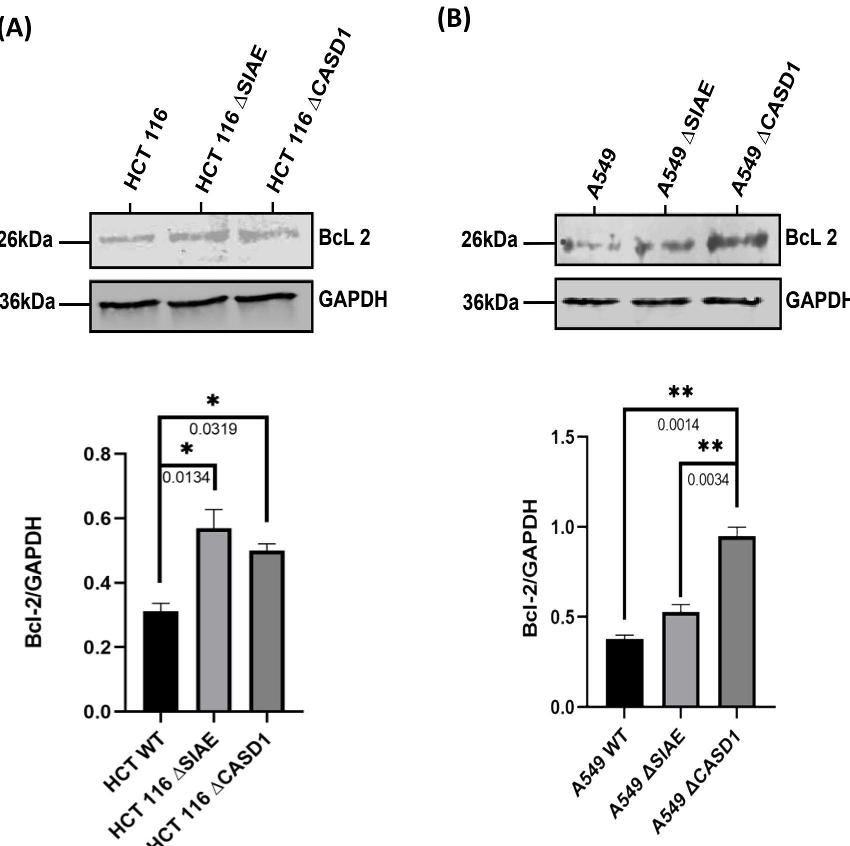


Figure 7. Bcl-2 Protein Expression in Wild type, Knockout (CASD1 and SIAE) cell lines. Western blot analysis was performed on whole cell lysate using BcL2-specific antibodies. GAPDH was used as loading control (A) Immunoblot analysis of whole cell lysates prepared from HCT 116 cell lines Below is the normalized Protein band density (B) Immunoblot analysis of whole cell lysates prepared from A549 cell lines. Below is Protein band density. One-way ANOVA with Tukey's posttest for multiple comparison, *$P < 0.05$ and **$P < 0.01$.

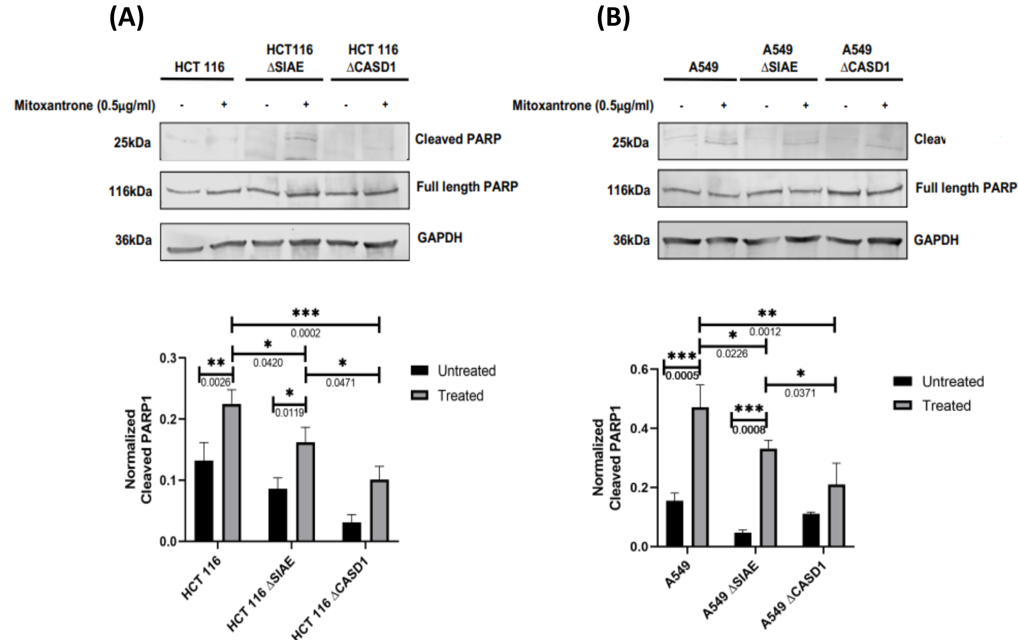


Figure 8. Cleaved PARP levels in wild type, knockout (CASD1 and SIAE) cell lines. Western blot was performed on whole cell lysate using cleaved PARP-specific antibodies. GAPDH was used as loading (A) Immunoblot analysis of whole cell lysates prepared from HCT 116 cell lines Below is the normalized Protein band density (B) Immunoblot analysis of whole cell lysates prepared from A549 cell lines. Below is Protein band density. One-way ANOVA with Tukey's posttest for multiple comparison, *P < 0.05 and **P < 0.01.

BcL-2 and Poly-ADP Ribose Polymerase (PARP) are two proteins that have been shown to be key drivers of cell proliferation and cell survival. BcL-2 family of proteins are key regulator of apoptosis that primarily function by either inhibiting or promoting cell death. The fate of a cell is dependent on the balance between pro-apoptotic and pro-survival members of the Bcl-2 family. Pro-apoptotic members of this family such as Bax and Bak promote cell death by direct binding interaction that disrupts mitochondrial outer membrane potential leading to irreversible release of intermembrane space-bound protein cytochrome C, subsequent caspase activation and apoptosis [80]. Pro-survival BcL-2 family members such as Bcl-2 and Bcl-xL on the other hand, prevent apoptosis by inhibiting mitochondrial outer membrane depolarization and promoting cell survival and proliferation [81]. Our results

revealed consistently significant high levels of the pro-survival protein BcL-2 in CASD1 knockout cells compared to the wild type and SIAE knockout cell lines for the lung cancers and wild type for the colon cancers. This once again corroborates the significantly high proliferation observed earlier in the CASD1 knockout cells. PARP 1, a 116kDa protein, belonging to the PARP superfamily, is a crucial protein involved in ensuring cell survival. Physiologically, full or active PARP1 is known for its involvement in DNA repair processes [82]. Enzymatic cleavage of PARP 1 by active Caspase 3 results in 2 inactive fragments that are unable to facilitate DNA repair leading to genomic instability and ultimately cell death. Evidence from several studies has reported upregulation of PARP activity in some cancer types. For instance, a study conducted on hepatocellular carcinoma patients showed significantly increased levels of PARP in tumor tissues than adjacent non-tumorous tissues [83]. Also, strikingly high PARP 1 mRNA levels have been reported in triple negative breast cancer tumors and tumors of the endometrium, lung, ovary, and skin [84]. In comparison to the wild type and SIAE knockout cells, our results showed significantly low levels of 25kDa cleaved/inactive PARP1 fragments in the CASD1 knockout lung and colon cancer cells when challenged with mitoxantrone. This observation may be attributed to the rapid efflux of mitoxantrone by CASD1 cells resulting in significant reduction of the cytotoxic concentration required to stimulate the protein mediators of the apoptosis cascade which includes Cleaved PARP1 (Uto et al., 2015). This data also confirms and corroborates our earlier findings that sialic acid deacetylation promotes cell survival in lung and colon cancers.

1.4.7 CASD1 Knockout upregulates BCRP/ABCG2 mRNA expression levels

We explored gene expression events as a possible factor linking deacetylated Sia and BCRP protein expression by analyzing the relative expression levels of BCRP mRNA. Our results showed statistically comparable BCRP mRNA levels in Wild type and SIAE Knockout variants of both HCT 116 and A549 cells. However, significantly high expression levels were observed in CASD1 knock out cells (i.e., ˜2.5 fold and ˜ 6-fold increase in HCT 116 and A549 cells lines respectively) (Fig. 9a and 9b).

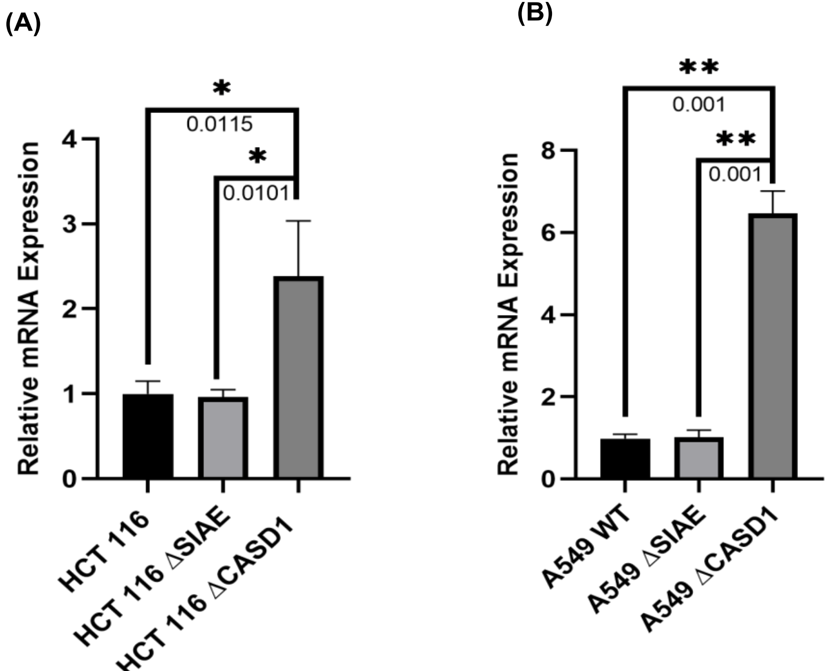


Fig 9. BCRP (ABCG2) mRNA expression in Wild type, Knockout (SIAE and CASD1) cell lines via qPCR. (A) BCRP mRNA expression profile from HCT 116 cell lines. Below is normalized mRNA plot (B) BCRP mRNA expression profile of A549 cell lines. One-way ANOVA with Tukey's post-test for multiple comparison. *P < 0.05 and **P < 0.01

Gene expression modulatory factors and post-translational modification check points at the endoplasmic reticulum (ER) and endosome-lysosome govern the relative expression levels and stability of most proteins [24]. Thus,

to better understand the factor responsible for the varied protein expression levels and function of BCRP, we determined the levels of BCRP mRNA in each of the cell variants. CASD1 knockout cells significantly expressed high levels of BCRP mRNA relative to wild type and SIAE knockouts of all cell types. This finding supports/confirms the BCRP protein expression data, suggesting that deacetylation of sialic acid acids alters (increases) BCRP mRNA transcript levels and consequently upregulates level of expressed proteins. This may also be attributed to alterations in the cell cycle possibly by the increase in DNA content in deacetylated cells. Studies by Anim *et al.*, 2023 have shown that CASD1 knockout affects the cell cycle by increasing cells in the G2/M phase.(Ref)

1.4.8 Lysosomal pathway is involved in decreased BCRP (ABCG2) protein stability and levels

Protein degradation occurs in two major sites, namely lysosomes and proteasomes. To elucidate the involvement of the lysosomal pathway in the observed differences in BCRP protein level across the various cell lines (i.e., Wildtype and knockouts), cells were cultured in the presence or absence of 100 nM of potent lysosomal enzyme inhibitor, Bafilomycin A1 (BMA), and levels of BCRP in cell lysate determined via western blot. Whereas BCRP levels in CASD1 knockout cells remained significantly unchanged following BMA treatment in both cell lines, our results show significant increases in BCRP level for wild type and SIAE knockout cells of HCT 116 cells (p-values= 0.0121 and 0.0119 respectively) (Fig. 10a). A significant increase in BCRP levels was also recorded for wild type of A549 cells (p-value= 0.0010),

however no significant increase was seen in the SIAE knockout cells (p-value= 0.9879) (Fig. 10b).

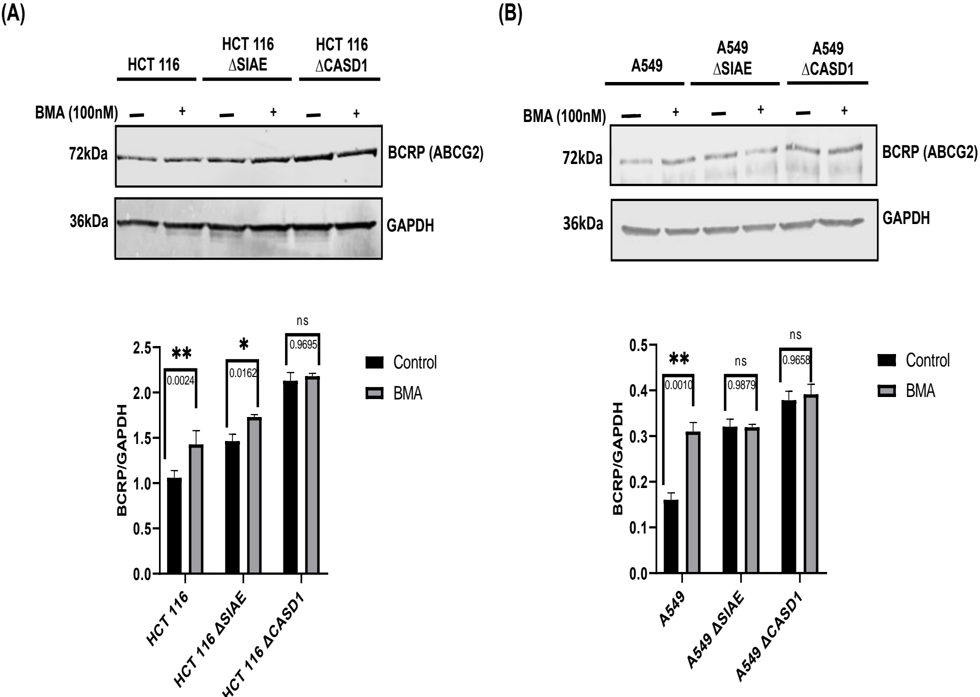


Figure 10. BCRP (ABCG2) expression in Wild type, Knockout (CASD1 and SIAE) cell lines following Bafilomycin A1 treatment (grey bars). Cells were treated with 100nM BMA, lysed and Immunoblot analysis was performed on whole cell lysate using BCRP-specific antibodies. GAPDH was used as loading control (A) Immunoblot analysis of whole cell lysates prepared from HCT 116 cell lines. **(A)** Immunoblot analysis of whole cell lysates prepared from HCT 116 cell lines. Below is normalized Protein band density. **(B)** Immunoblot analysis of whole cell lysates prepared from A549 cell lines. Below is normalized Protein band density. Two-way ANOVA with Tukey's posttest for multiple comparison, $*P < 0.05$.

CASD1 is localized at the trans Golgi membrane where it modifies terminal sialic acids on glycoproteins before they are trafficked to the plasma membrane. An earlier study conducted by Barnard et al. (2019) and Visser et al. (2021) reported that majority of plasma-membrane bound glycoproteins with O-acetyl functional group modifications are retained in the Golgi compartment (i.e., 7,9-O- and 9-O-Ac) whereas only some (9-O-Ac) are transported to the cell surface. After remaining in the plasma membrane domain for a brief period, these unstable modified glycoproteins are recycled and degraded via the endosome-lysosome pathway. We therefore blocked

protein degradation via the lysosome by treating all cell variants with Bafilomycin A1 . BMA is a macrolide antibiotic that prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes [85]. It also serves as a specific inhibitor of vacuolar H^+ ATPase (V-ATPase) causing alkalinization of the lysosome lumen, impairing lysosomal enzyme function by acid hydrolysis inhibition and subsequently leading to cellular accumulation of lysosome-bound proteins [86]. We observed significantly increased levels of BCRP in BMA-treated cells in the wildtype and SIAE knockout cells in both lung and colorectal cancer cell lines. However, no significant increase was recorded in BCRP level for BMA-treated CASD1 knockout cancer cells suggesting that lysosomal degradation pathway may be responsible for modulating levels of acetyl modified glycoproteins including BCRP.

1.4.9 CASD1 expression favors survival in lung adenocarcinoma

After observing that CASD1 knockout promotes cell survival and proliferation in cancer cell lines, we investigated the clinical relevance of *CASD1* and *SIAE* expression in clinical samples by examining the relationship between overall patient survival and *CASD1* and *SIAE* mRNA expression in lung adenocarcinoma (LUAD) and colon adenocarcinoma (COAD) clinical samples from The Cancer Genome Atlas (TCGA). LUAD samples with high expression levels of *CASD1* (red line) were significantly associated with high patient survival rates (HR = 0.73, 95% CI: 0.54 – 0.98, log-rank p value = 0.034) compared to those with low expression (blue line) (Fig. 11A). Median survival in the high and low *CASD1* expression groups are 53 months and 40 months respectively. This finding suggests that higher *CASD1* expression is a

favorable marker of survival in LUAD. No significant association was observed in survival rates for (COAD) samples with high expression levels of *CASD1* compared to those with low expression (HR = 1.15, 95% CI: 0.74 – 1.77, log-rank p value = 0.500) (Fig. 11C). Similarly, no significant associations were observed in *SIAE* expression and survival rates for patients with LUAD and COAD (log-rank p-value = 0.922 and 0.130 respectively) (Figures 11B, 11D). This implies that CASD1-mediated sialic acid acetylation may represent a potential therapeutic target in LUAD requiring further studies to understand how the mechanism and modifiers of this process counteract BCRP-mediated drug resistance.

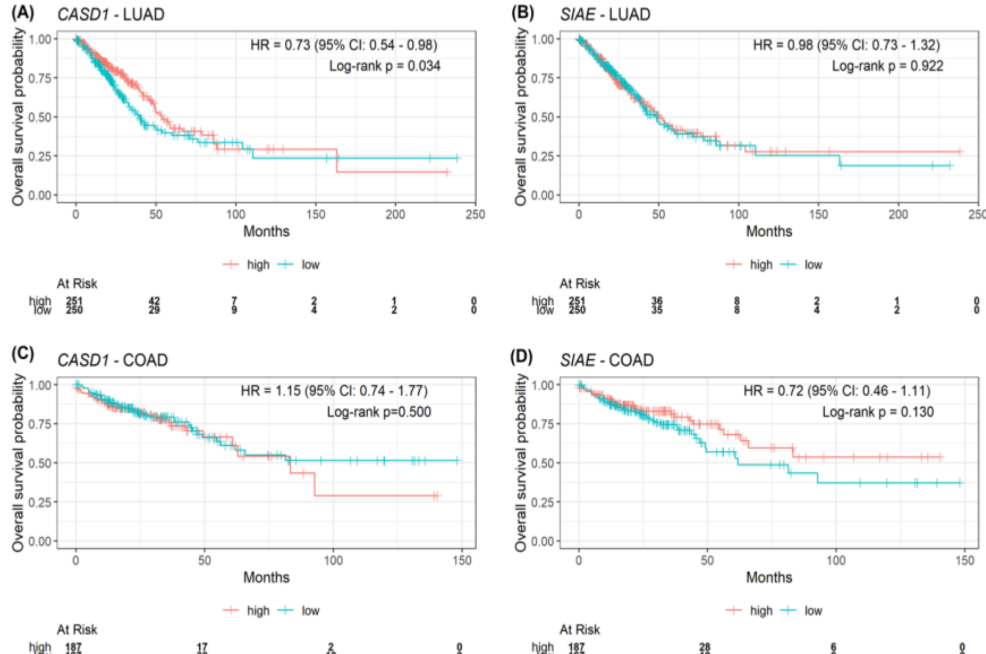


Figure 11. Kaplan-Meier survival plots for CASD1 and SIAE mRNA expression and overall survival in clinical lung adenocarcinoma (LUAD) and colon adenocarcinoma samples (A) CASD1 mRNA expression in LUAD samples **(B)** SIAE mRNA expression in LUAD samples **(C)** CASD1 mRNA expression in COAD samples **(D)** SIAE mRNA expression in COAD samples. Cox proportional hazard regression hazard ration, HR (with 95% confidence interval, CI) and log-rank p value are annotated on each plot. Risk table is shown beneath each Kaplan-Meier plot. Samples were split into high (red) and low (blue) expression groups per gene using the median (high ≥ median, low< median)

1.4.10 Infographics

The cartoon below summarizes the findings of this study (Figure 12).

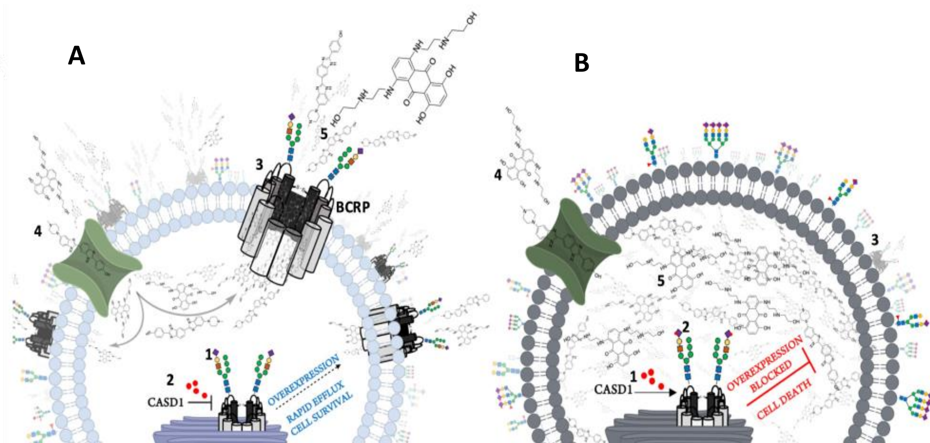


Figure 12. Proposed Mechanism **(A)** Sia (purple diamonds) is added to the glycan chain on BCRP in the golgi by Sialyltransferases **(2)** In the absence of CASD1 (i.e., CASD1 Knockout), no acetyl groups (red circles) are added to the sialylated transporter **(3)** Majority of deacetylated transporters are successfully trafficked to the cell surface **(4)** Chemotherapeutics and BCRP substrates targeted at cells, permeate into the cytoplasm **(5)** The high expression levels of BCRP at the cell surface confers a multidrug resistance phenotype as large amounts of chemotherapeutics are rapidly pumped out enabling the cell to survive. **(D)** CASD1 adds acetyl groups (red circles) to **(2)** sialylated glycan chain on transporter **(3)** The majority of the transporter with acetyl group modification is transported to the lysosome for degradation **(4)** Chemotherapeutics and BCRP substrates targeted at cells, permeate into the cytoplasm **(5)** Due to the low expression levels of BCRP at the cell surface, only a small fraction of chemotherapeutics is pumped out of the cell with high amount accumulated in the cytoplasm exerting prolonged pharmacological effect leading to cell death (apoptosis).

1.5 Conclusion

Taken together, our results highlight the crucial role O-acetyl Sia modification plays in cancer related MDR. Specifically, this study provides empirical evidence that modulation of acetyl Sia (specifically deacetylated Sia) upregulates BCRP expression and promotes survival in lung and colon cancer cell lines. Clinical data also validated that in patients with LUAD, lower levels of CASD1 and thus less O-acetyl Sia expression had lower survival rates than those patients with high CASD1 expression. Findings from this study may have relevance to a broader spectrum of malignancies and provide a promising avenue for future MDR-circumventing therapeutics.

Further studies are however warranted to explore the effect of acetyl Sia modulation on other cancers as well as other relevant MDR efflux proteins such as P-gp and MRP1.

1.6 References

[1] J. Ferlay *et al.*, "Cancer statistics for the year 2020: An overview," (in eng), *Int J Cancer,* Apr 5 2021, doi: 10.1002/ijc.33588.

[2] R. L. Siegel, K. D. Miller, H. E. Fuchs, and A. Jemal, "Cancer statistics, 2022," (in eng), *CA Cancer J Clin,* vol. 72, no. 1, pp. 7-33, Jan 2022, doi: 10.3322/caac.21708.

[3] M. M. Gottesman, T. Fojo, and S. E. Bates, "Multidrug resistance in cancer: role of ATP-dependent transporters," (in eng), *Nat Rev Cancer,* vol. 2, no. 1, pp. 48-58, Jan 2002, doi: 10.1038/nrc706.

[4] B. C. Baguley, "Multiple drug resistance mechanisms in cancer," (in eng), *Mol Biotechnol,* vol. 46, no. 3, pp. 308-16, Nov 2010, doi: 10.1007/s12033-010-9321-2.

[5] K. Bukowski, M. Kciuk, and R. Kontek, "Mechanisms of Multidrug Resistance in Cancer Chemotherapy," (in eng), *Int J Mol Sci,* vol. 21, no. 9, May 2 2020, doi: 10.3390/ijms21093233.

[6] M. M. Gottesman, "Mechanisms of cancer drug resistance," *Annu Rev Med,* vol. 53, pp. 615-27, 2002, doi: 10.1146/annurev.med.53.082901.103929.

[7] K. O. Alfarouk *et al.*, "Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp," (in eng), *Cancer Cell Int,* vol. 15, p. 71, 2015, doi: 10.1186/s12935-015-0221-1.

[8] R. K. Vadlapatla, A. D. Vadlapudi, D. Pal, and A. K. Mitra, "Mechanisms of drug resistance in cancer chemotherapy: coordinated role and regulation of efflux transporters and metabolizing enzymes," *Curr Pharm Des,* vol. 19, no. 40, pp. 7126-40, 2013, doi: 10.2174/13816128113199990493.

[9] C. P. Wu, A. M. Calcagno, and S. V. Ambudkar, "Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies," *Curr Mol Pharmacol,* vol. 1, no. 2, pp. 93-105, Jun 2008, doi: 10.2174/1874467210801020093.

[10] H. L. Martin *et al.*, "High-content, high-throughput screening for the identification of cytotoxic compounds based on cell morphology and cell proliferation markers," (in eng), *PLoS One,* vol. 9, no. 2, p. e88338, 2014, doi: 10.1371/journal.pone.0088338.

[11] M. D. Hall, M. D. Handley, and M. M. Gottesman, "Is resistance useless? Multidrug resistance and collateral sensitivity," *Trends Pharmacol Sci,* vol. 30, no. 10, pp. 546-56, Oct 2009, doi: 10.1016/j.tips.2009.07.003.

[12] F. J. Sharom, "ABC multidrug transporters: structure, function and role in chemoresistance," (in eng), *Pharmacogenomics,* vol. 9, no. 1, pp. 105-27, Jan 2008, doi: 10.2217/14622416.9.1.105.

[13] S. Cheepala *et al.*, "Cyclic nucleotide compartmentalization: contributions of phosphodiesterases and ATP-binding cassette transporters," (in eng), *Annu Rev Pharmacol Toxicol,* vol. 53, pp. 231-53, 2013, doi: 10.1146/annurev-pharmtox-010611-134609.

[14] M. Dean, A. Rzhetsky, and R. Allikmets, "The human ATP-binding cassette (ABC) transporter superfamily," (in eng), *Genome Res,* vol. 11, no. 7, pp. 1156-66, Jul 2001, doi: 10.1101/gr.184901.

[15] K. Moitra and M. Dean, "Evolution of ABC transporters by gene duplication and their role in human disease," (in eng), *Biol Chem,* vol. 392, no. 1-2, pp. 29-37, Jan 2011, doi: 10.1515/bc.2011.006.

[16] U. A. Germann and T. C. Chambers, "Molecular analysis of the multidrug transporter, P-glycoprotein," *Cytotechnology,* vol. 27, no. 1-3, pp. 31-60, Sep 1998, doi: 10.1023/A:1008023629269.

[17] R. G. Deeley and S. P. Cole, "Substrate recognition and transport by multidrug resistance protein 1 (ABCC1)," *FEBS Lett,* vol. 580, no. 4, pp. 1103-11, Feb 13 2006, doi: 10.1016/j.febslet.2005.12.036.

[18] M. K. DeGorter, C. Q. Xia, J. J. Yang, and R. B. Kim, "Drug transporters in drug efficacy and toxicity," (in eng), *Annu Rev Pharmacol Toxicol,* vol. 52, pp. 249-73, 2012, doi: 10.1146/annurev-pharmtox-010611-134529.

[19] A. H. Schinkel and J. W. Jonker, "Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview," (in eng), *Adv Drug Deliv Rev,* vol. 55, no. 1, pp. 3-29, Jan 21 2003, doi: 10.1016/s0169-409x(02)00169-2.

[20] S. P. Cole, "Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future," (in eng), *Annu Rev Pharmacol Toxicol,* vol. 54, pp. 95-117, 2014, doi: 10.1146/annurev-pharmtox-011613-135959.

[21] S. H. Lee *et al.*, "MRP1 polymorphisms associated with citalopram response in patients with major depression," (in eng), *J Clin Psychopharmacol,* vol. 30, no. 2, pp. 116-25, Apr 2010, doi: 10.1097/JCP.0b013e3181d2ef42.

[22] Q. Mao and J. D. Unadkat, "Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update," *AAPS J,* vol. 17, no. 1, pp. 65-82, Jan 2015, doi: 10.1208/s12248-014-9668-6.

[23] K. J. Bailey-Dell, B. Hassel, L. A. Doyle, and D. D. Ross, "Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene," (in eng), *Biochim Biophys Acta,* vol. 1520, no. 3, pp. 234-41, Sep 21 2001, doi: 10.1016/s0167-4781(01)00270-6.

[24] T. Furukawa *et al.*, "Major SNP (Q141K) variant of human ABC transporter ABCG2 undergoes lysosomal and proteasomal degradations," *Pharm Res,* vol. 26, no. 2, pp. 469-79, Feb 2009, doi: 10.1007/s11095-008-9752-7.

[25] H. Nakagawa, K. Wakabayashi-Nakao, A. Tamura, Y. Toyoda, S. Koshiba, and T. Ishikawa, "Disruption of N-linked glycosylation enhances ubiquitin-mediated proteasomal degradation of the human ATP-binding cassette transporter ABCG2," *FEBS J,* vol. 276, no. 24, pp. 7237-52, Dec 2009, doi: 10.1111/j.1742-4658.2009.07423.x.

[26] T. Nakanishi and D. D. Ross, "Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression," *Chin J Cancer,* vol. 31, no. 2, pp. 73-99, Feb 2012, doi: 10.5732/cjc.011.10320.

[27] S. H. Iram and S. P. Cole, "Differential functional rescue of Lys(513) and Lys(516) processing mutants of MRP1 (ABCC1) by chemical chaperones reveals different domain-domain interactions of the transporter," (in eng), *Biochim Biophys Acta,* vol. 1838, no. 3, pp. 756-65, Mar 2014, doi: 10.1016/j.bbamem.2013.11.002.

[28] C. F. Higgins and K. J. Linton, "The ATP switch model for ABC transporters," (in eng), *Nat Struct Mol Biol,* vol. 11, no. 10, pp. 918-26, Oct 2004, doi: 10.1038/nsmb836.

[29] A. E. Senior, M. K. al-Shawi, and I. L. Urbatsch, "The catalytic cycle of P-glycoprotein," (in eng), *FEBS Lett,* vol. 377, no. 3, pp. 285-9, Dec 27 1995, doi: 10.1016/0014-5793(95)01345-8.

[30] B. Verhalen and S. Wilkens, "P-glycoprotein retains drug-stimulated ATPase activity upon covalent linkage of the two nucleotide binding domains at their C-terminal

ends," (in eng), *J Biol Chem,* vol. 286, no. 12, pp. 10476-82, Mar 25 2011, doi: 10.1074/jbc.M110.193151.

[31] P. M. Jones and A. M. George, "A reciprocating twin-channel model for ABC transporters," (in eng), *Q Rev Biophys,* vol. 47, no. 3, pp. 189-220, Aug 2014, doi: 10.1017/s0033583514000031.

[32] Y. K. Zhang, Y. J. Wang, P. Gupta, and Z. S. Chen, "Multidrug Resistance Proteins (MRPs) and Cancer Therapy," (in eng), *Aaps j,* vol. 17, no. 4, pp. 802-12, Jul 2015, doi: 10.1208/s12248-015-9757-1.

[33] K. Hollenstein, D. C. Frei, and K. P. Locher, "Structure of an ABC transporter in complex with its binding protein," (in eng), *Nature,* vol. 446, no. 7132, pp. 213-6, Mar 8 2007, doi: 10.1038/nature05626.

[34] E. M. Leslie, R. G. Deeley, and S. P. Cole, "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense," (in eng), *Toxicol Appl Pharmacol,* vol. 204, no. 3, pp. 216-37, May 1 2005, doi: 10.1016/j.taap.2004.10.012.

[35] M. Maliepaard *et al.*, "Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues," *Cancer Res,* vol. 61, no. 8, pp. 3458-64, Apr 15 2001. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/11309308.

[36] E. Pollex, A. Lubetsky, and G. Koren, "The role of placental breast cancer resistance protein in the efflux of glyburide across the human placenta," *Placenta,* vol. 29, no. 8, pp. 743-7, Aug 2008, doi: 10.1016/j.placenta.2008.05.001.

[37] M. Huls *et al.*, "The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane," *Kidney Int,* vol. 73, no. 2, pp. 220-5, Jan 2008, doi: 10.1038/sj.ki.5002645.

[38] L. Doyle and D. D. Ross, "Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)," *Oncogene,* vol. 22, no. 47, pp. 7340-58, Oct 20 2003, doi: 10.1038/sj.onc.1206938.

[39] M. Maliepaard *et al.*, "Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line," *Cancer Res,* vol. 59, no. 18, pp. 4559-63, Sep 15 1999. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/10493507.

[40] A. Gupta, Y. Zhang, J. D. Unadkat, and Q. Mao, "HIV protease inhibitors are inhibitors but not substrates of the human breast cancer resistance protein (BCRP/ABCG2)," *J Pharmacol Exp Ther,* vol. 310, no. 1, pp. 334-41, Jul 2004, doi: 10.1124/jpet.104.065342.

[41] P. J. Houghton *et al.*, "Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro," *Cancer Res,* vol. 64, no. 7, pp. 2333-7, Apr 1 2004, doi: 10.1158/0008-5472.can-03-3344.

[42] C. Ozvegy-Laczka *et al.*, "High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter," *Mol Pharmacol,* vol. 65, no. 6, pp. 1485-95, Jun 2004, doi: 10.1124/mol.65.6.1485.

[43] Z. Shi *et al.*, "Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance," *Cancer Res,* vol. 67, no. 22, pp. 11012-20, Nov 15 2007, doi: 10.1158/0008-5472.CAN-07-2686.

[44] R. J. Kathawala, P. Gupta, C. R. Ashby, Jr., and Z. S. Chen, "The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade," *Drug Resist Updat,* vol. 18, pp. 1-17, Jan 2015, doi: 10.1016/j.drup.2014.11.002.

[45] Z. Binkhathlan and A. Lavasanifar, "P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future

perspectives," (in eng), *Curr Cancer Drug Targets,* vol. 13, no. 3, pp. 326-46, Mar 2013, doi: 10.2174/1568009611313999007б.

[46] A. Palmeira, E. Sousa, M. H. Vasconcelos, and M. M. Pinto, "Three decades of P-gp inhibitors: skimming through several generations and scaffolds," (in eng), *Curr Med Chem,* vol. 19, no. 13, pp. 1946-2025, 2012, doi: 10.2174/092986712800167392.

[47] S. P. C. Cole, "Targeting Multidrug Resistance Protein 1 (MRP1, ABCC1): Past, Present, and Future," *Annual Review of Pharmacology and Toxicology,* vol. 54, no. 1, pp. 95-117, 2014/01/06 2014, doi: 10.1146/annurev-pharmtox-011613-135959.

[48] S. V. Ambudkar, C. Kimchi-Sarfaty, Z. E. Sauna, and M. M. Gottesman, "P-glycoprotein: from genomics to mechanism," *Oncogene,* vol. 22, no. 47, pp. 7468-7485, 2003/10/01 2003, doi: 10.1038/sj.onc.1206948.

[49] D. R. Hipfner *et al.*, "Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH2 terminus," (in eng), *J Biol Chem,* vol. 272, no. 38, pp. 23623-30, Sep 19 1997, doi: 10.1074/jbc.272.38.23623.

[50] D. A. Greer and S. Ivey, "Distinct N-glycan glycosylation of P-glycoprotein isolated from the human uterine sarcoma cell line MES-SA/Dx5," *Biochim Biophys Acta,* vol. 1770, no. 9, pp. 1275-82, Sep 2007, doi: 10.1016/j.bbagen.2007.07.005.

[51] O. Wesolowska, M. Paprocka, J. Kozlak, N. Motohashi, D. Dus, and K. Michalak, "Human sarcoma cell lines MES-SA and MES-SA/Dx5 as a model for multidrug resistance modulators screening," (in eng), *Anticancer Res,* vol. 25, no. 1a, pp. 383-9, Jan-Feb 2005.

[52] D. A. Greer and S. Ivey, "Distinct N-glycan glycosylation of P-glycoprotein isolated from the human uterine sarcoma cell line MES-SA/Dx5," (in eng), *Biochimica et biophysica acta,* vol. 1770, no. 9, pp. 1275-1282, 2007, doi: 10.1016/j.bbagen.2007.07.005.

[53] H. Nakagawa *et al.*, "Ubiquitin-mediated proteasomal degradation of non-synonymous SNP variants of human ABC transporter ABCG2," (in eng), *Biochem J,* vol. 411, no. 3, pp. 623-31, May 1 2008, doi: 10.1042/bj20071229.

[54] N. M. Varki and A. Varki, "Diversity in cell surface sialic acid presentations: implications for biology and disease," *Lab Invest,* vol. 87, no. 9, pp. 851-7, Sep 2007, doi: 10.1038/labinvest.3700656.

[55] A. Varki and R. Schauer, "Sialic Acids," in *Essentials of Glycobiology*, nd *et al.* Eds. Cold Spring Harbor (NY), 2009.

[56] S. Grabenstein *et al.*, "Deacetylated sialic acids modulate immune mediated cytotoxicity via the sialic acid-Siglec pathway," *Glycobiology,* vol. 31, no. 10, pp. 1279-1294, Nov 18 2021, doi: 10.1093/glycob/cwab068.

[57] K. N. Barnard *et al.*, "Expression of 9-O- and 7,9-O-Acetyl Modified Sialic Acid in Cells and Their Effects on Influenza Viruses," *mBio,* vol. 10, no. 6, Dec 3 2019, doi: 10.1128/mBio.02490-19.

[58] R. Schauer, "Sialic acids: fascinating sugars in higher animals and man," (in eng), *Zoology (Jena),* vol. 107, no. 1, pp. 49-64, 2004, doi: 10.1016/j.zool.2003.10.002.

[59] I. Tuffour, S. Amuzu, H. Bayoumi, I. Surtaj, C. Parrish, and R. Willand-Charnley, "Early in vitro evidence indicates that deacetylated sialic acids modulate multi-drug resistance in colon and lung cancers via breast cancer resistance protein," (in eng), *Front Oncol,* vol. 13, p. 1145333, 2023, doi: 10.3389/fonc.2023.1145333.

[60] A. F. Swindall and S. L. Bellis, "Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells," (in eng), *The Journal of biological chemistry,* vol. 286, no. 26, pp. 22982-22990, 2011, doi: 10.1074/jbc.M110.211375.

[61] A. T. Holdbrooks, C. M. Britain, and S. L. Bellis, "ST6Gal-I sialyltransferase promotes tumor necrosis factor (TNF)-mediated cancer cell survival via sialylation of the TNF receptor 1 (TNFR1) death receptor," *J Biol Chem,* vol. 293, no. 5, pp. 1610-1622, Feb 2 2018, doi: 10.1074/jbc.M117.801480.

[62] M. J. Schultz, A. F. Swindall, J. W. Wright, E. S. Sztul, C. N. Landen, and S. L. Bellis, "ST6Gal-I sialyltransferase confers cisplatin resistance in ovarian tumor cells," (in eng), *J Ovarian Res,* vol. 6, no. 1, p. 25, Apr 11 2013, doi: 10.1186/1757-2215-6-25.

[63] H. Ma, H. Zhou, X. Song, S. Shi, J. Zhang, and L. Jia, "Modification of sialylation is associated with multidrug resistance in human acute myeloid leukemia," *Oncogene,* vol. 34, no. 6, pp. 726-740, 2015/02/01 2015, doi: 10.1038/onc.2014.7.

[64] A. M. Baumann *et al.*, "9-O-Acetylation of sialic acids is catalysed by CASD1 via a covalent acetyl-enzyme intermediate," (in eng), *Nat Commun,* vol. 6, p. 7673, Jul 14 2015, doi: 10.1038/ncomms8673.

[65] O. M. Pearce and H. Laubli, "Sialic acids in cancer biology and immunity," *Glycobiology,* vol. 26, no. 2, pp. 111-28, Feb 2016, doi: 10.1093/glycob/cwv097.

[66] Y. Shen *et al.*, "O-acetylation and de-O-acetylation of sialic acids in human colorectal carcinoma," *Eur J Biochem,* vol. 271, no. 2, pp. 281-90, Jan 2004, doi: 10.1046/j.1432-1033.2003.03927.x.

[67] S. Pal *et al.*, "Purification and characterization of 9-O-acetylated sialoglycoproteins from leukemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia," *Glycobiology,* vol. 14, no. 10, pp. 859-70, Oct 2004, doi: 10.1093/glycob/cwh111.

[68] R. Parameswaran *et al.*, "O-acetylated N-acetylneuraminic acid as a novel target for therapy in human pre-B acute lymphoblastic leukemia," *J Exp Med,* vol. 210, no. 4, pp. 805-19, Apr 8 2013, doi: 10.1084/jem.20121482.

[69] M. A. Langereis, H. H. Rabouw, M. Holwerda, L. J. Visser, and F. J. van Kuppeveld, "Knockout of cGAS and STING Rescues Virus Infection of Plasmid DNA-Transfected Cells," *J Virol,* vol. 89, no. 21, pp. 11169-73, Nov 2015, doi: 10.1128/JVI.01781-15.

[70] T. Wang, L. M. Larcher, L. Ma, and R. N. Veedu, "Systematic Screening of Commonly Used Commercial Transfection Reagents towards Efficient Transfection of Single-Stranded Oligonucleotides," *Molecules,* vol. 23, no. 10, Oct 8 2018, doi: 10.3390/molecules23102564.

[71] J. Q. Wang *et al.*, "Reversal of Cancer Multidrug Resistance (MDR) Mediated by ATP-Binding Cassette Transporter G2 (ABCG2) by AZ-628, a RAF Kinase Inhibitor," *Front Cell Dev Biol,* vol. 8, p. 601400, 2020, doi: 10.3389/fcell.2020.601400.

[72] T. Uto *et al.*, "Antiproliferative and Pro-Apoptotic Activity of Diarylheptanoids Isolated from the Bark of Alnus japonica in Human Leukemia Cell Lines," *Am J Chin Med,* vol. 43, no. 4, pp. 757-67, 2015, doi: 10.1142/S0192415X15500470.

[73] D. Osei-Safo *et al.*, "Constituents of the Roots of Dichapetalum pallidum and Their Anti-Proliferative Activity," *Molecules,* vol. 22, no. 4, Mar 27 2017, doi: 10.3390/molecules22040532.

[74] H. Ma, H. Zhou, X. Song, S. Shi, J. Zhang, and L. Jia, "Modification of sialylation is associated with multidrug resistance in human acute myeloid leukemia," *Oncogene,* vol. 34, no. 6, pp. 726-40, Feb 5 2015, doi: 10.1038/onc.2014.7.

[75] M. Kim *et al.*, "The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells," *Clin Cancer Res,* vol. 8, no. 1, pp. 22-8, Jan 2002. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/11801536.

[76] M. P. Boland, K. A. Fitzgerald, and L. A. O'Neill, "Topoisomerase II is required for mitoxantrone to signal nuclear factor kappa B activation in HL60 cells," *J Biol Chem,* vol. 275, no. 33, pp. 25231-8, Aug 18 2000, doi: 10.1074/jbc.275.33.25231.

[77] A. E. Stacy, P. J. Jansson, and D. R. Richardson, "Molecular pharmacology of ABCG2 and its role in chemoresistance," *Mol Pharmacol,* vol. 84, no. 5, pp. 655-69, Nov 2013, doi: 10.1124/mol.113.088609.

[78] J. Kale, E. J. Osterlund, and D. W. Andrews, "BCL-2 family proteins: changing partners in the dance towards death," *Cell Death Differ,* vol. 25, no. 1, pp. 65-80, Jan 2018, doi: 10.1038/cdd.2017.186.

[79] Y. Tsujimoto, "Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?," *Genes Cells,* vol. 3, no. 11, pp. 697-707, Nov 1998, doi: 10.1046/j.1365-2443.1998.00223.x.

[80] G. V. Chaitanya, A. J. Steven, and P. P. Babu, "PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration," *Cell Commun Signal,* vol. 8, p. 31, Dec 22 2010, doi: 10.1186/1478-811X-8-31.

[81] F. Nomura *et al.*, "Enhancement of poly-adenosine diphosphate-ribosylation in human hepatocellular carcinoma," *J Gastroenterol Hepatol,* vol. 15, no. 5, pp. 529-35, May 2000, doi: 10.1046/j.1440-1746.2000.02193.x.

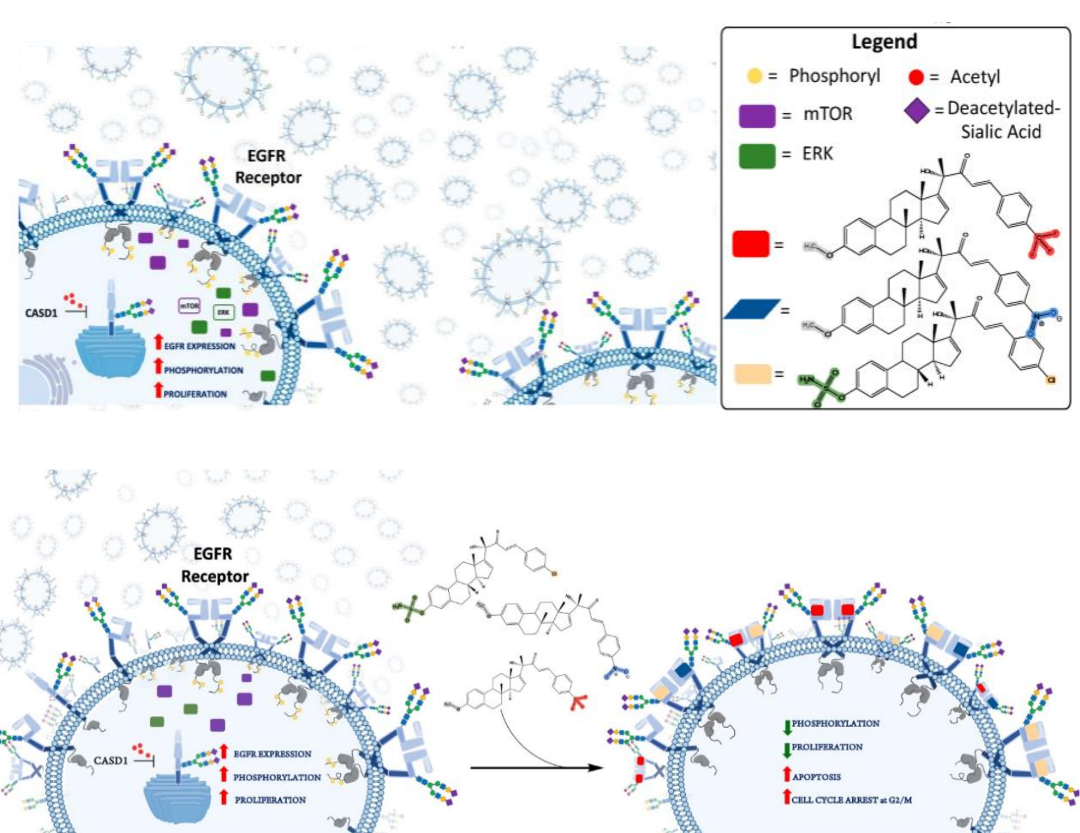
[82] V. Ossovskaya, I. C. Koo, E. P. Kaldjian, C. Alvares, and B. M. Sherman, "Upregulation of Poly (ADP-Ribose) Polymerase-1 (PARP1) in Triple-Negative Breast Cancer and Other Primary Human Tumor Types," *Genes Cancer,* vol. 1, no. 8, pp. 812-21, Aug 2010, doi: 10.1177/1947601910383418.

[83] Y. Yan *et al.*, "Bafilomycin A1 induces caspase-independent cell death in hepatocellular carcinoma cells via targeting of autophagy and MAPK pathways," *Sci Rep,* vol. 6, p. 37052, Nov 15 2016, doi: 10.1038/srep37052.

[84] S. Wang, F. Liu, Z. Zeng, H. Yang, and H. Jiang, "The Protective Effect of Bafilomycin A1 Against Cobalt Nanoparticle-Induced Cytotoxicity and Aseptic Inflammation in Macrophages In Vitro," *Biol Trace Elem Res,* vol. 169, no. 1, pp. 94-105, Jan 2016, doi: 10.1007/s12011-015-0381-9.

Chapter 2

Deacetylated Sialic Acid Sensitizes Lung and Colon Cancers to Novel Cucurbitacin-Inspired Estrone Epidermal Growth Factor Receptor (EGFR) Inhibitor Analogs



2.2 Introduction

2.2.1 Conventional Chemotherapy in Cancer Treatment

Cancer is an abnormal state of cells that is characterized by uncontrolled proliferation arising from a series of genomic alterations/transformations. These alterations cause aggressive malignancies and debilitating hallmarks that accounts for millions of deaths annually [87]. Increasing understanding of the underlying molecular mechanisms over the years, has led to the development of several treatment modalities including chemotherapy, radiotherapy, immunotherapy and surgery to combat cancers [3]. Despite the several aforementioned treatment options, chemotherapy still remains the first-line treatment for many cancers.

Primarily, conventional chemotherapeutics work by effectively targeting processes employed by cancer cells to grow and divide, such as the ability of the cancer cells to replicate their DNA. Thus, based on their mechanisms of action, conventional chemotherapies can be classified into Antimetabolites, Alkylating agents, Mitotic Spindle inhibitors and Topoisomerase inhibitors [5, 88]. Antimetabolites inhibit the synthesis of nucleic acids (i.e., DNA and RNA or their components); Alkylating agents induce inter/ intra strand DNA crosslinks that destabilize DNA and cause DNA breakage. Mitotic spindle inhibitors on the other hand act on tubulin to impede the formation of mitotic spindle and obstruct cell division whereas topoisomerase inhibitors block the action of DNA unwinding enzymes (Figure 2.1)

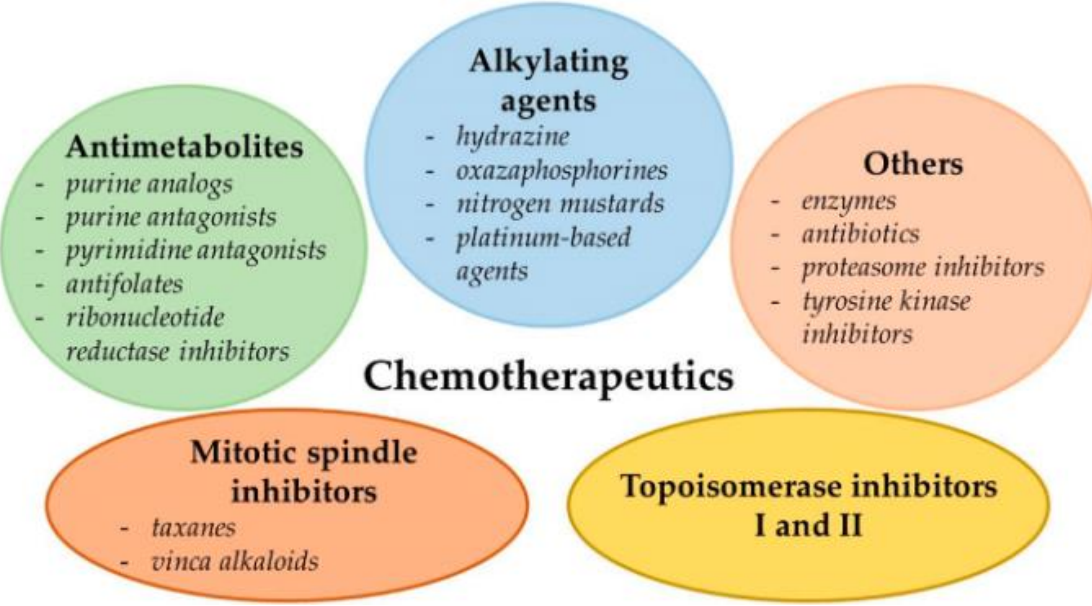


Figure 2.1 Classification of Conventional Chemotherapies based on mechanism of action [5]

2.2.2 Challenges associated with Conventional Chemotherapy

2.2.2.1 Adverse side effect due to non-selectivity

Conventional chemotherapeutics primarily function by effectively targeting processes that cancer cells need to grow and divide, such as the ability of the cancer cells to replicate their DNA [89]. However, since normal healthy cells use the same replication process to grow and divide, they tend to be also damaged by conventional chemotherapeutic drugs. The use of conventional chemotherapy therefore produces many, and often severe, side effects including fatigue, loss of appetite, nausea, bowel issues such as constipation or diarrhea, hair loss, mouth sores, skin, and nail problems among others [90]. Most cancer patients on conventional chemotherapies are at risk of infections. This is due to the non-selective toxicity of chemotherapy towards white blood cells, which are necessary for fighting infections.

2.2.2.2 Glycan (Sialic acid) mediated drug resistance

Hypersialylation of tumor necrosis factor receptor 1 (TNFR1) and Fas Receptor (FasR) death receptor blocks receptor internalization and the formation of the death-inducing signaling complex (DISC), thereby disabling apoptotic signaling thus rendering several conventional chemotherapeutics ineffective [61, 62]. Shultz and his colleagues revealed in a comparative study that hypersialylation facilitated by increased expression of ST6Gal-I transferase conferred cisplatin (an alkylating agent) resistance in ovarian cancer cells. [63]. Additionally, elevated levels of ST3GAL5 and ST8SIA4 sialyltransferases were detected to be overexpressed in drug resistant human acute myeloid leukemia (AML) cells relative to parental cancer cells in both *in vitro* and *in vivo* experimental models [64]. Further studies revealed that the altered levels of ST3GAL5 and ST8SIA4 correlated with high expression levels of P-gp and MRP1, suggesting a strong association between glycan sialylation and MDR.

The acetylation of Sia, a biological phenomenon which is mediated by Sialate-O-acetyl transferase (CASD1) enzyme (Figure 2.2), for instance, has been reported to affect immune cells' activity, and growth of tumors [57, 70, 91].

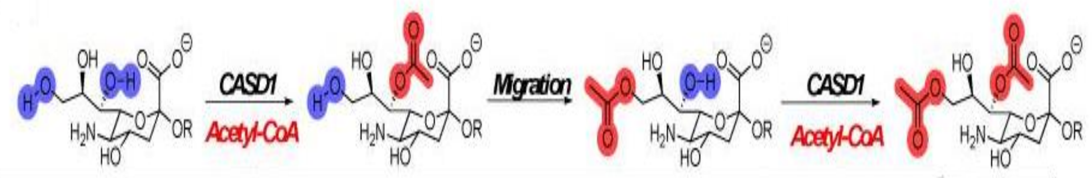


Fig 2.2 CASD1 enzyme mediates 9-O and 7,9-O acetylation of Sia (Neu 5Ac) [92]

Specifically, aberrant expression of CASD1 has been reported to slow down apoptosis and promote rapid growth of tumors [93]. Tumors in such scenarios respond poorly to chemotherapy, thus warranting alternative treatment regimens. Targeted

therapies tend to be a better treatment alternative when these conventional chemotherapies become ineffective [94].

2.2.3 Targeted Therapy

Targeted therapy is a type of cancer treatment that targets proteins that control how cancer cells grow, divide, and spread [95]. Increasing knowledge about the specific differences between normal and cancerous cells over the years has allowed for the development of treatments targeted at cancer-specific activities. One of the most fundamental changes found in cancer cells is the presence of mutations in the genes that are responsible for causing cell growth (oncogenes) [96]. The defective or overexpressed proteins produced by these altered genes are prime candidates for targeted therapies. Thus, unlike conventional chemotherapies, targeted therapies are considered relatively safe and more effective as they do not interfere with the activity of the normal/healthy cells. Cancer targeted therapies employ either monoclonal antibodies or small molecule inhibitors to selectively inhibit growth of cancer cells based on their distinctive phenotypes. Monoclonal antibodies, also known as therapeutic antibodies, are proteins designed to attach to specific targets found on cancer cells. Some monoclonal antibodies mark cancer cells for visibility and destruction by the immune system [95]. Other monoclonal antibodies directly stop cancer cells from growing or cause them to self-destruct while others carry toxins to cancer cells [97]. Among some of the targeted monoclonal antibodies used in cancer treatment include; rituximab (for chronic lymphocytic leukemia (CLL) and some types of non-Hodgkin lymphoma), cetuximab (for advanced bowel cancer and head and neck cancer) trastuzumab (used to treat breast cancer and stomach cancer), Nivolumab and pembrolizumab (used to treat several different types of cancer) [98].

Targeted Small-molecule drugs, also referred to as targeted chemotherapies, are low molecular weight organic compounds and their small size enables them to penetrate into cells to inhibit specific enzymes or molecules that play key roles in cancer-promoting signaling pathways [99]. Cancer targeted chemotherapies are designed to inhibit several cancer-promoting processes (Figure 2.3). Based on mechanisms of action existing targeted chemotherapeutics can be characterized into Growth Factor Kinase inhibitors, Apoptosis inducers, DNA damage response inhibitors, Epigenetic re-programmers, Telomerase inhibitors, Redox modulators, Metabolic re-programmers, Proteasome inhibitors and Immuno-modulators [99].

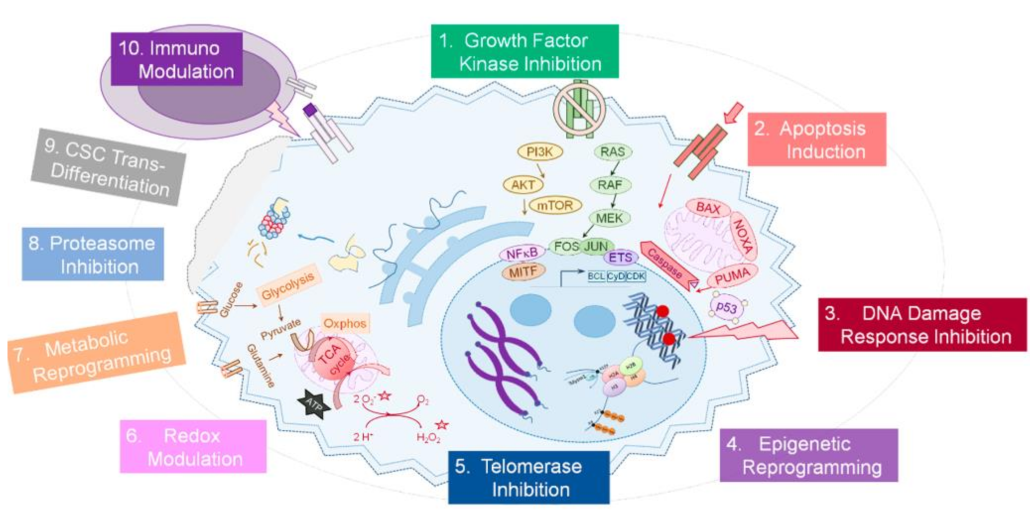


Figure 2.3 Mechanisms of actions of Targeted chemotherapeutics

2.2.4 Growth Factor Kinase Targeted Chemotherapies

The major drawback of most conventional chemotherapeutic treatments is their non-specificity or inability to ascertain and target cancerous cells directly. In the past few decades, one of the major and clinically successful cancer therapeutics is the Growth Factor Kinase targeted chemotherapies. Kinases are enzymes that catalyze the

transfer of phosphate groups from high-energy phosphate-donating molecules (e.g. ATP) to specific substrates in a process known as phosphorylation [100]. Depending on the substrates that receive the phosphate groups, kinases can be classified into several groups. Among these include Serine/Threonine kinases which transfer phosphate groups from ATP to serine or threonine amino acids residues of the receiving protein and Tyrosine kinases which transfer phosphate groups from ATP to tyrosine residues. Other established kinases are lipid kinases, sugar kinases, nucleotide kinases and dual-specificity kinases [101]. One tyrosine kinase of particular interest is the Epidermal growth factor receptor (EGFR) that controls several downstream regulatory pathways. EGFR/EGFR pathway is one of the key pathways that have been successfully targeted to alleviate cancer.

EGFR is a ~170kDa heavily glycosylated and sialylated transmembrane receptor tyrosine kinase. Crystallographic analyses have revealed EGFR to consist of the ectodomain (ECD) (further subdivided into four subdomains, DI–IV), the transmembrane domain (TMD), and the intracellular tyrosine kinase domain (TKD) (Fig 2.4). The extracellular domain has been reported to be post-translationally modified with glycan chains attached to specific Asparagine (Asn) residues [102, 103]. About 11 N-glycosylation sites have been identified on the ectodomain of the EGFR with each site specified by the sequence NXS/T, where X can be any amino acid excluding proline [104]. Further studies have shown glycosylation to be crucial for proper functioning of the receptor. Site directed mutagenic studies on key Asparagine residue (Asn 597) as well as glycan transferase inhibition studies, which resulted in non-glycosylated EGFR were reported to abrogate ligand binding and subsequently led to loss of intracellular kinase activity [103, 105, 106].

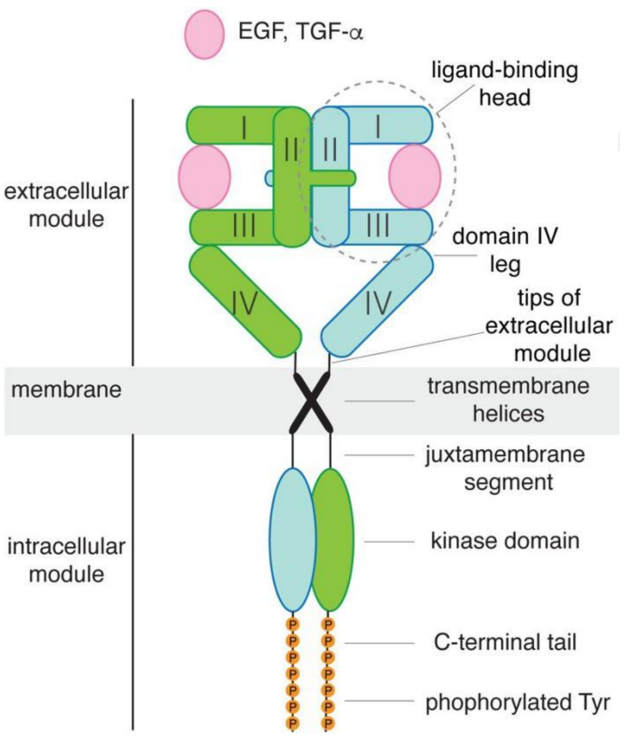


Figure 2.4 Schematic diagram of ligand-bound dimeric EGFR showing domains/modules [107]

Dimerization of two monomers is key for receptor activation. EGFR ligands bind to pre-formed dimerized EGFR receptors. Among the several known ligands of the EGFR receptor includes: epidermal growth factor (EGF), amphiregulin (AR), betacellulin (BTC) and the transforming growth factor α (TGF-α) [108]. Upon EGF binding, EGFR undergoes conformational changes to dimerize, resulting in kinase activation, autophosphorylation and downstream signaling pathways such as the Mitogen activated protein kinase (MAPK), JAK/STAT3 and the PI3K/AKT pathways that are responsible for regulating several cellular processes including cell growth, differentiation, survival, and apoptosis [109, 110]. Specifically, the MAPK pathway (also referred to as the RAS/RAF/MEK/ERK pathway), which is one the well-studied pathways begin with phosphotyrosine residues in the intracellular domain of activated/dimerized EGFR recruiting SH2 domain containing Grb2 protein following extracellular ligand (EGF) binding. Grb2 protein then recruits guanine exchange

factor called Son of sevenless (SOS) bringing it in close proximity to Ras protein which is tethered via its C-terminal hydrophobic tail to the inner leaflet of the plasma membrane. SOS induces activation of Ras by facilitating the release of GDP from Ras, replacing it with binding of GTP. Activated (GTP bound) Ras has increased affinity for the cytosolic-bound Raf, thus recruiting and anchoring it (Raf) to the plasma membrane. This is followed by a series of downstream phosphorylation/activation events in which Raf which is a kinase, proceeds to phosphorylate another protein kinase known as the mitogen-activated protein/extracellular signal-regulated kinase (MEK) [111]. Activated MEK further phosphorylates two other kinases, the extracellular signal-regulated kinase 1 (Erk1) and Erk2. Activated Erk 1/Erk2 translocate into the nucleus and activates transcription factors such as CREB, Fos and Elk-1that regulates transcription of protein synthesis, cell proliferation, differentiation and survival genes [112].

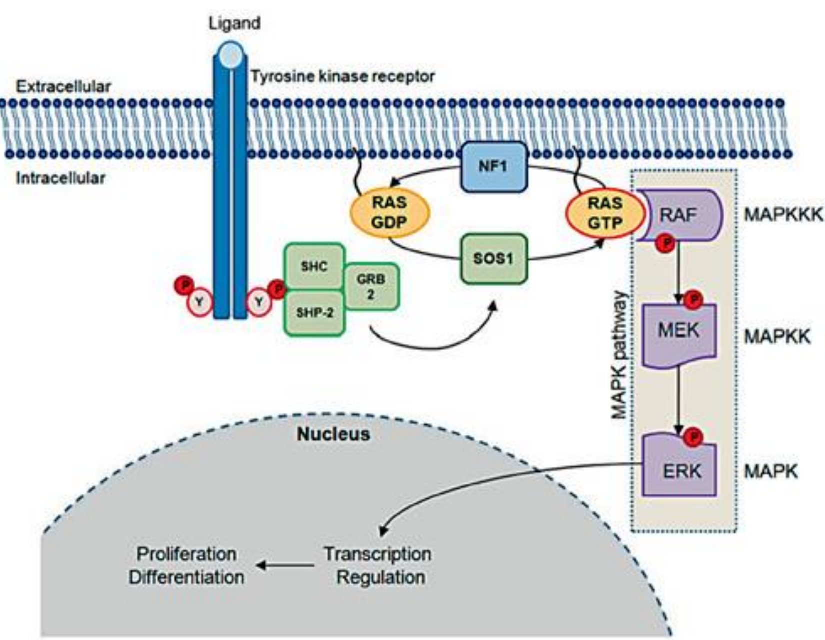


Figure 2.5 The MAPK Signaling Pathway [113]

Defects in the regulation of this pathway can lead to uncontrolled growth in cells, leading to cancer. For instance, aberrant expression (overexpression) of EGFR has been reported in the prognosis of several cancers including lung, breast, prostate, colorectal and bladder cancers. Clinically, 14-91% of all non-small cell lung cancer (NSCLC) 27-77% of colorectal cancer, 30-50% pancreatic cancer and 40-80% lung cancer have increased EGFR expression levels [114]. Also, Ras mutations have been reported in 90% pancreatic cancers, 60% papillary thyroid cancers, 50% colon cancers and 30% of NSCLC. B-Raf mutation have also been reported in 70% melanoma, 50% papillary thyroid cancers and 10% colon cancers [115]. Over the last decade, important advances have been made in developing novel agents that modulate key proteins in this pathway. Existing conventional EGFR/EGFR pathway-targeting therapeutics include monoclonal antibodies such as Cetuximab, panitumumab, zalutumumab, nimotuzumab, and matuzumab as well as small molecule inhibitors such as sorafenib, gefitinib, erlotinib, afatinib, brigatinib and icotinib [116-118] . Barriers to the clinical effectiveness of these aforementioned EGFR-targeting therapeutics, however, abound. Among these include untoward side effects such as high blood pressure, fatigue, loss of hair, skin problems, diarrhea, and the emergence of resistance due to mutations in the drug targets, thus warranting the need for new and effective therapeutics [90].

2.2.5 Natural products as targeted therapeutics

Natural products have been used for the treatment of various diseases and are becoming an important research area for drug discovery. Historically, natural products and their drug derivatives are sources of many therapeutic agents for treatment of approximately 87% classified human diseases [119]. It is estimated that between 1981 and 2019, approximately 25% of all newly approved anti-cancer drugs were related to

natural products [120]. These products, especially those from plant sources (phytochemicals) have been studied extensively and shown to possess potent anti-cancer properties. Most of these phytochemicals function by interfering with the initiation, development, and progression of cancer via the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis [121]. Various phytochemicals obtained from vegetables, fruits, spices, teas, herbs, and medicinal plants, such as flavonoids carotenoids, phenolic compounds and terpenoids, have been extensively investigated for their anti-cancer activities due to their safety, low toxicity and general availability compared to synthetic drugs. Phytochemicals, including curcumin, resveratrol, apigenin, quercetin, genistein, lycopene, isothiocyanates have shown to elicit targeted effected on cancer via plethora of their mechanisms including anti-oxidation, inhibition of cell cycle, induction of apoptosis, regulation of angiogenesis [120]. The remarkable structural and chemical diversity of natural products make them suitable for semi- and total synthetic modifications. They therefore serve as significant sources of new pharmacological leads in the drug discovery and development process.

This study focused on synthetic analogs of Cucurbitacins, a group of naturally occurring triterpenoids isolated from medicinal plants with potent anti-inflammatory and anticancer properties.

2.2.6 Cucurbitacin

Cucurbitacins are a class of diverse compounds made up of highly oxidized tetracyclic triterpenoids isolated from different species of the plant family *Cucurbitaceae* such as cucumber, honeydew melons, cantaloupe, squash, angled gourd, watermelon, pumpkin, Crenshaw melon, and colocynth [122]. Cucurbitacins

have been reported to exhibit wide range of biological activities including cytotoxic, anti-tumor, hepatoprotective, anti-inflammation, antimicrobial, anthelmintic, cardiovascular, and anti-diabetic effects in both *in vitro* and *in vivo* studies [123-125]. The Structure of cucurbitacin is characterized by the tetracyclic cucurbitane nucleus skeleton (triterpenes built from six isoprene units) (Figure 2.7). Cucurbitacins are derivatives of the hypothetical triterpene hydrocarbon cucurbitane named 19-(10→9-β)-abeo-5 alpha- lanostane (also known as 9-β-methyl-19-nor-lanosta-5-ene), with a variety of oxygen substitutions at different positions.[125]

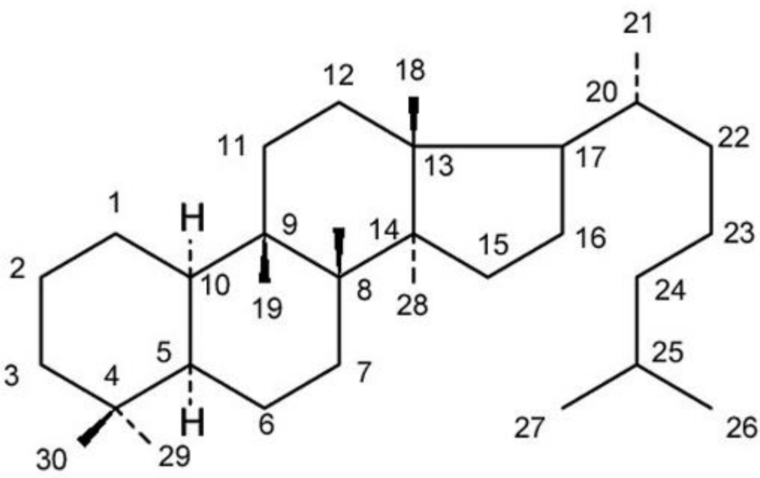


Figure 2.6 Skeletal structure of Cucurbitacin (Gry et al.,2006)

Cucurbitacins are divided into twelve categories. Of these, cucurbitacin E, cucurbitacin B, cucurbitacin D and cucurbitacin I, are the most widely used for *in vitro* and *in vivo* tumor inhibition studies [126-128]. The diversity of the different cucurbitacin classes is based on the positions of substituent molecules (hydroxylated, unsaturated, keto, glycosyl and acyl groups) attached to them. [142]. Several cucurbitacin compounds have been found to exhibit antiproliferative activities on numerous human cancer cell lines and tumor xenografts, including breast, prostate,

lung, uterine cervix, liver, skin, and brain cancers [129]. For instance, Cucurbitacin B, D, E, and I have been reported to show 80% anti-proliferative effect on colon (HCT-116), breast (MCF-7), lung (NCI-H460) and brain (SF-268) cancer cell lines [130, 131]. *In vitro* and nude mice xenograft model studies have also shown Cucurbitacin I to significantly reduce growth in breast (MDA-MB-231, MDA-MB-468) and prostate carcinoma (Panc-1) cell lines [132, 133]. Cucurbitacin B and E have also been reported to elicit growth inhibition in breast cancer cell lines (MCF-7 and MDA-MB-231) via cell cycle arrest and apoptosis [129, 130, 134]. They also modulated the expression of proteins involved in cell-cycle regulation in both of the estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) human breast cancer cell lines. The induction of cell cycle arrest and apoptosis through inhibiting the PI3KAkt signaling pathway in multiple cancer cell lines have been reported for cucurbitacin C [135, 136].

2.2.7 Cucurbitacin Inspired Estrone Analogs

Despite the numerous reported therapeutic benefits of Cucurbitacin including their remarkable anticancer properties, they are still limited as sources of pharmacological candidates. Cucurbitacins are mostly available at small concentrations in their natural (plant) sources making isolating them in large concentrations laborious, cost intensive and usually unsuccessful. Also, due to cucurbitacin structural complexity and functional group diversity, total chemical synthesis is extremely difficult to achieve. Lastly, Cucurbitacins induce toxic adverse effects in mammals even at low doses. Thus, as a way of overcoming these limitations and to develop suitable drug candidates with potential clinical relevance, the Halaweish research group in a biologically oriented organic synthesis (BIOS) approach, generated a panel of novel analogs by coupling biologically active

pharmacophores of cucurbitacin to a steroidal (i.e., estrone) scaffold (Figure 2.8) [137].

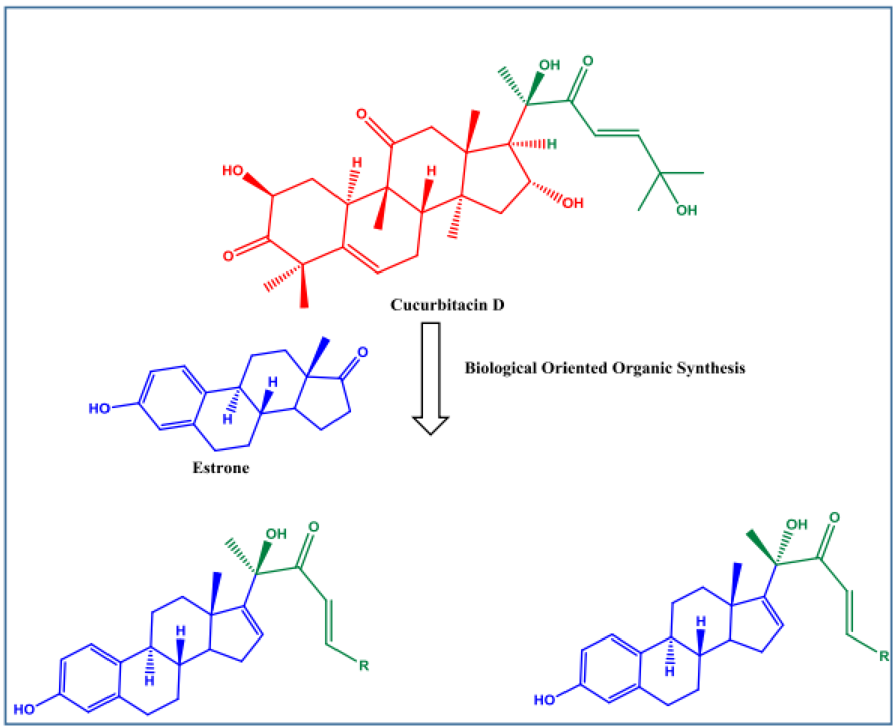


Figure 2.7 Biologically Oriented Organic Synthesis of Cucurbitacin-Inspired Estrone Analogs [137]

These analogs in a comprehensive mechanistic biological investigation were reported possess potent anticancer activity via dual inhibitory effect towards the EGFR and MAPK pathways as well cell cycle arrest induction [105, 137]. Our study focused on three of these EFR-targeting cucurbitacin-inspired estrone analogs namely: MA 320, MMA 321, and MMA 294 (Figure 2.8)

Figure 2.8 Structures of CIEAs used in this study (A) MMA 294 (B) MMA 320 (C) MMA 321

Previously, we have shown that deacetylated Sia is exploited by colon and lung cancers to evade natural killer cell-mediated cytotoxicity via the Sia–Siglec pathway and also to confer multidrug resistance (MDR) phenotype via breast cancer resistance protein [57, 70]. In this study, we focused our attention on how deacetylated Sia affects the effectiveness of EGFR-targeted therapies. We hypothesized that altering levels of acetyl-Sia may affect cancer cell responses to EGFR-targeted therapeutics. Specifically, we investigated how CASD1 gene knockout (i.e., lack of acetyl Sia) affects the activity of the three Cucurbitacin-inspired estrone analogs (CIEAs), MMA 320, MMA 321, and MMA 294, in *in vitro* models of lung and colon cancer cells.

2.2.8 Published Study Findings

Interesting findings from this work have been successfully published as an original article in the “Special issue: Bioorganic Chemistry: Current and Future Perspectives” of Molecules Journal (Figure 2.9) [92]. Article can be accessed via the link below: https://www.mdpi.com/1420-3049/28/17/6257

Article

Deacetylated Sialic Acid Sensitizes Lung and Colon Cancers to Novel Cucurbitacin-Inspired Estrone Epidermal Growth Factor Receptor (EGFR) Inhibitor Analogs

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Figure 2.9 Screenshot of title /abstract page of published article

2.3 Materials and Methods

2.3.1 Chemicals and Reagents

Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Alexa Fluor 488 conjugated goat anti-mouse IgG cross-adsorbed secondary antibody (2 mg/mL, Cat #A32723) and paraformaldehyde were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Phosphorylated EGFR monoclonal IgG1 mouse antibody (15A2:sc-81488) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Annexin V binding buffer 1x, APC Annexin V, 7-Amino-Actinomycin D (7-AAD) and PI/RNase staining buffer were obtained from BD Biosciences (San Jose, CA, USA). The secondary goat anti-mouse IRDye secondary antibody was purchased from LI-COR Biotechnologies (Lincoln, NE, USA), and the Vectashield Antifade Mounting medium with DAPI (H-1200-10) was purchased from Vector Laboratories (Burlingame, CA, USA). CIEAs were provided by Dr. Halaweish (South Dakota Ste University, Brookings, SD). All other chemicals used were of analytical grade.

2.3.2 Cell Lines And Cell Culture

The A549 and HCT 116 cells were grown in Dulbecco modified Eagle medium (DMEM) (Corning) and RPMI 1640 medium, respectively. The media were supplemented with 10% fetal bovine serum (Corning) and 1% pen/strep (Cytiva Hyclone). All the cell lines were originally purchased from the American Type Culture Collection (Rockville, MD, USA). Cell dissociation buffer (Gibco, Waltham, MA, USA) was used exclusively to passage cells. The CASD1 knockout HCT 116 and A549 cell lines were obtained from the group of Colin Parrish (Cornell University). CRISPR-Cas9 editing of CASD1 in HCT116 and A549 cells was

previously published [57, 58]. Briefly, paired Cas9 plasmids targeting adjacent sites in early exons of CASD1 were transfected using TransIT-X2 (Mirus Bio LLC, Madison, WI). Transfected cells were selected with puromycin, and single cell clones screened with PToV-P4 HE-Fc and sequence-verified to confirm loss of CASD1 function in both alleles [138]. qPCR was also performed to confirm deletion of the CASD1 gene.

2.3.3 Cell Viability Assay

The sensitivity of wild type and knockout cells to CIEAs was evaluated using the MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) colorimetric assay as previously described [70, 139]. Briefly, cells were plated at a density of 2×10^5 cells/mL in a 96-well plate (Corning, Corning, NY, USA) and treated with varying concentrations (0–100µM) of CIEAs for 48 h at 37 °C. MTT solution (5 mg/mL) was then added to each well and cells were further incubated for 4 h at 37 °C. When formazan crystals formed, they were then dissolved by adding stopping solution (Acidified 15% SDS). Absorbance was then measured at 570 nm with a microplate reader (BioTek Cytation 3, Charlotte, WA, USA) and used to estimate the percentage cell viability as shown below. GraphPad Prism 8 software was used to evaluate the individual IC_{50} values.

$$\%Cell\ viability = \left(\frac{Absorbance\ of\ treated\ cells - Absorbance\ of\ blank}{Absorbance\ of\ untreated\ cells - Absorbance\ of\ blank}\right) \times 100$$

2.3.4 Apoptosis Assay

The apoptosis-inducing effects of the CIEAs were evaluated using flow cytometry as previously described [140]. Briefly, cells were plated at a density of 1×10^5 cells/well in a 6-well plate (Corning, NY) and challenged with IC_{50} concentrations

of the individual CIEAs for 48 h at 37 °C. The cells were then harvested, washed three times with 1X PBS (Corning, USA) and resuspended in Annexin V binding buffer 1x (BD Biosciences, San Jose, CA). The cells were then stained with APC Annexin V (BD, USA) and 7-AAD (BD Biosciences, San Jose, CA), incubated in the dark for 25 min and analyzed with an Accuri C6 Pus flow cytometer (BD, Biosciences, San Jose, CA).

2.3.5 Cell Cycle Analysis

Cell cycle analysis was performed on wild type and knockout cells as described previously [141]. In brief, cells (1×10^5 cells/dish) were plated in a 6 cm dish. Following treatment with the IC_{50} concentration of CIEAs for 48 h, the cells were centrifuged and washed twice with an assay buffer. The cells were then fixed with 70% ethanol, suspended with staining solution containing propidium iodide (PI) and RNase A, and incubated at room temperature in the dark for 15 min. Analysis was performed within an hour with an Accuri C6 Pus flow cytometer (BD Biosciences, San Jose, CA).

2.3.6 Immunofluorescence Assay

Phosphorylated EGFR expression levels were determined by immunofluorescence microscopy as previously described [70]. Briefly, cells (5×10^5 cells/mL) were cultured on a glass coverslip. The cells were then fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The slides were blocked in a buffer containing 0.01% goat serum, 0.01% saponin, and 0.05% glycine in PBS for 1 h and incubated with human phosphorylated EGFR monoclonal IgG1 mouse antibody (Santa Cruz Biotechnology, TX) overnight at 4ºC. The cells were then incubated with Alexa Fluor 488 conjugated goat anti-mouse IgG cross-adsorbed

secondary antibody (ThermoFisher Scientific, Waltham, IL, USA) in the dark for 1 h. Vectashield Antifade Mounting medium with DAPI (Burlingame, CA, USA) was placed on each coverslip and inverted on a microscope. A BioTek Cytation 3 Live Cell imager (BioTek, WA) was used to collect immunofluorescence images.

2.3.7 In-Cell ELISA

The effect of CIEAs on levels of phosphorylated mTOR and ERK was evaluated using the in-cell ELISA method as described previously [141]. Briefly, cells (2×10^5 cells/mL) were plated in 96-well plate and treated with IC_{50} concentrations of CIEAs for 24 h. The cells were then fixed in 3.7% paraformaldehyde and permeabilized with 0.5% Triton X-100. The plates were then blocked with 1x fish gel PBS for 1 h at room temperature and incubated overnight at 4 °C with individual antibodies for ERK, pERK, mTOR, pmTOR and GAPDH (Santa Cruz Biotechnology, CA) at specific dilutions in blocking buffer. Secondary antibody incubation (1:1000 dilution in PBS containing 0.1%Tween 20) was performed using anti-mouse IRDye secondary antibody (LiCOR, NE) and goat anti-rabbit IRDye secondary antibody (Li-COR, NE) 1 h at room temperature. The target proteins were detected using the Odyssey Fc Imager (LiCOR, NE). Fluorescence quantification was performed with Image StudioLite (LI-COR, NE) software and levels of the target proteins (pERK and pmTOR) were normalized and expressed as a percentage of controls.

2.3.8 Scientific Rigor

All reagents, antibodies and cell lines used in this study were selected based on published figures and purchased from companies that provide validation. The experiments were all performed in technical and then biological replicates. The data were analyzed with one- and two-way ANOVA followed by Tukey post-tests for

multiple comparisons using GraphPad Prism 9 (San Diego, CA, USA). The data are all presented as mean ± standard deviation, with $p < 0.05$ indicating significance.

2.4 Results and Discussion

2.4.1 Effects of Deacetylated Sia on Inhibitory Activity of CIEAs

Modulating the expression levels of acetyl groups on Sias has been reported to influence influenza C and D infection [58]. Also, the role deacetylated sialic acids play in helping cancer cells evade NK-mediated cytotoxicity has been demonstrated [57]. In this study, we investigated the impact of deacetylated Sia on cancer cell response to targeted cancer therapy. Small molecules (MMA 294, MMA 320, and MMA 321) that are structural analogs of Cucurbitacin (i.e., CIEAs) and inhibitors of pEGFR were used as drug candidates. Sorafenib, a known targeted therapy and a multi-kinase inhibitor was used as a positive control drug. The MTT (tetrazolium-based) cell viability assay was performed to screen the CIEAs. Following exposure to varying concentrations (0–100 μM) of CIEAs for 48 h, we observed a general inhibitory effect of all CIEAs and Sorafenib (positive control) on the cancer cells (Table 1). Compared with wild type cells, CASD1 knockout HCT 116 cells, for instance, showed ~two-fold sensitivity to MMA 294 (IC_{50}= 4.29 μM), MMA 321 (IC_{50}= 6.17 μM) and ~three-fold sensitivity to MMA 320 (IC_{50}= 3.14 μM). Knockout variants of A549 cells showed relatively much higher sensitivity towards the CIEAs compared with their wild type counterpart. MMA 294 exhibited ~four-fold inhibitory activity against the CASD1 knockout. MMA 321 on the other hand, interestingly recorded ~17-fold inhibitory activity against the CASD1 knockout cell line.

Table 1. Inhibitory effects of CIEAs on HCT116 and A549 cells.

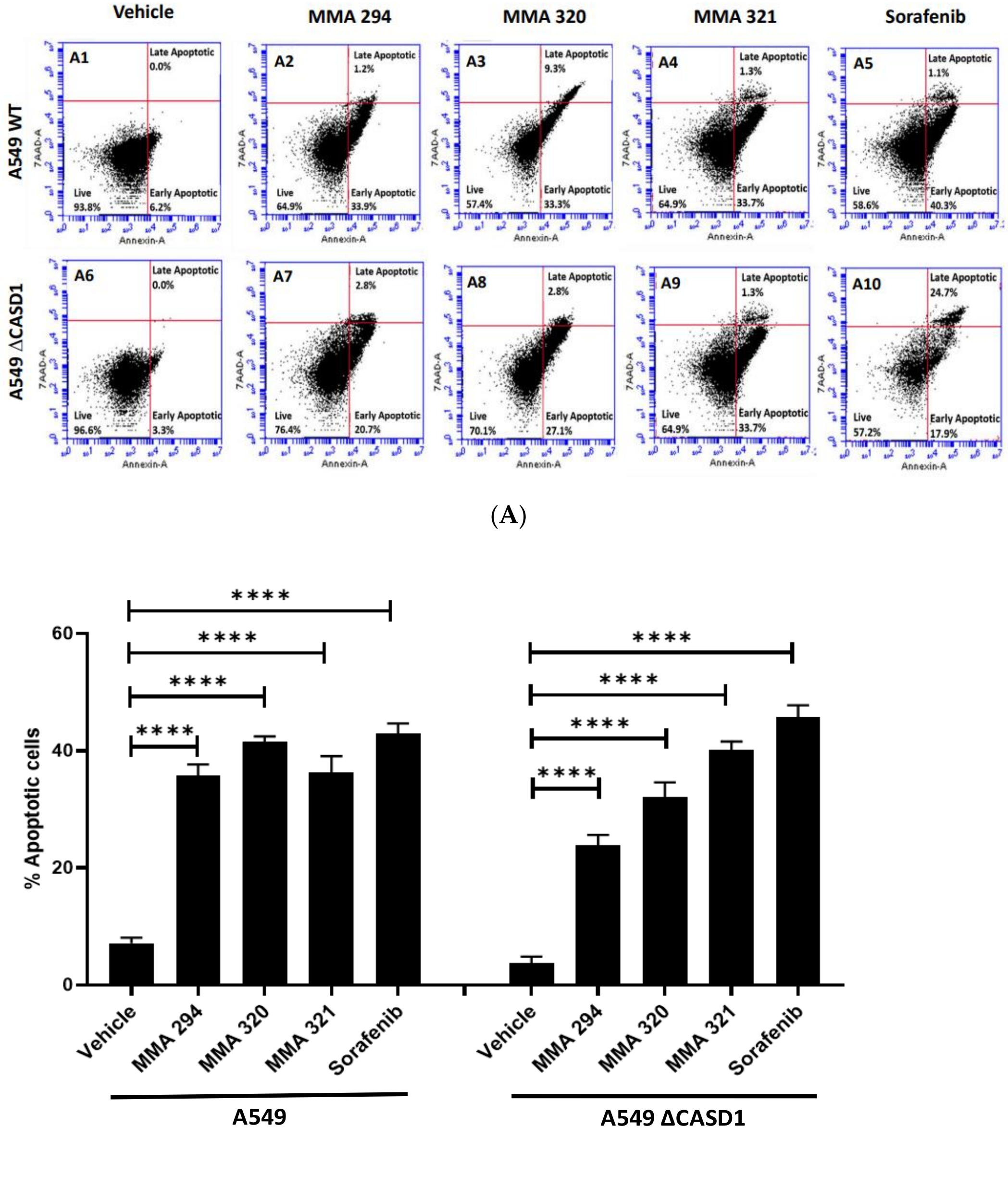
Compound	IC_{50} (µM)	
	A549 WT	**A549 ΔCASD1**
MMA 294	19.07 ± 5.01	4.77 ± 2.60 (4.0)
MMA 320	9.02 ± 2.32	6.49 ± 1.67 (1.4)
MMA 321	20.97 ± 5.19	1.24 ± 0.09 (16.9)
Sorafenib	26.74 ± 4.38	9.96 ± 3.41 (2.7)
	HCT 116 WT	**HCT 116 ΔCASD1**
MMA 294	8.82 ± 0.87	4.29 ± 0.16 (2.1)
MMA 320	8.65 ± 1.14	3.14 ± 0.08 (2.8)
MMA 321	12.72 ± 1.07	6.17 ± 1.74 (2.1)
Sorafenib	9.66 ± 1.58	7.79 ± 0.38 (1.2)

Mean ± SD of three independent experiments performed in triplicate. Fold sensitivity (in brackets) is determined by dividing IC_{50} value of wild type cells by the IC_{50} value of knockout cells.

Our results further suggest that Sia deacetylation stemming from removal of the Sia acetyl group functionalizing enzyme, CASD1, though reported in earlier studies to confer multi-drug resistance (MDR) phenotype via upregulation of breast cancer resistance protein [70] (a drug efflux pump that contributes to multidrug resistance, invasiveness (aggressiveness), and self-renewal in cancers [26]), induces some form of chemo-sensitivity in these tumor cells when challenged with the EGFR-targeted CIEAs. This provides insights into a possible alternative chemotherapeutic approach for combating MDR cancer cells.

2.4.2 Apoptotic Effects of CIEAs

We explored apoptosis as a mechanism underlying the observed remarkable growth inhibitory effects of the CIEAs. Apoptosis is a programmed cell death utilized by cells in controlling tissue or organ development as well as cellular turnover [142]. The characteristic features of chromatin condensation, cell volume reduction, endonuclease cleavage of nuclear DNA, loss of nuclear membrane integrity, loss of plasma membrane symmetry and expulsion of phosphatidylserine onto the outer plasma membrane make cells undergoing apoptosis unique [143, 144]. In this study, we successfully utilized two of these key apoptotic features in sorting and characterizing CIEA-treated cells to evaluate whether their growth inhibitory effects were via apoptosis. CIEA-challenged cells were further treated with APC-Annexin and 7AAD, two well-known reagents that bind to exposed phosphatidylserine (PS) and nuclear DNA, respectively, to detect cells that are undergoing apoptosis [145]. The mode of cytotoxicity or growth inhibition elicited by MMA 294, 320, 321 and Sorafenib was observed to be apoptosis for both HCT 116 and A549 cell lines, which is characterized by the increase in cells found positive for APC-Annexin V only (lower right quadrant: early apoptosis) or in combination with 7-AAD (upper right quadrant: late apoptosis) (Figures 2A(A1–A10), 2B and 3A(A1-A10), 3B).



(B)

Figure 2. Apoptotic effects of CIEAs in A549 cells. Cells were challenged with the CIEAs, harvested, washed, and resuspended in Annexin V binding buffer. Cells were then stained with APC Annexin V and 7-AAD, incubated in the dark for 25 min and analyzed via flow cytometry. (**A**) Flow cytometry histograms of A549 wild type 2A(A1-A5) and CASD1 knockout 2A(A6-A10) cell lines. A1&A6: Vehicle treatment, A2&A7: MMA 294 treatment, A3&A8: MMA 320 treatment, A4&A9: MMA 321 treatment and A5&A10: Sorafenib treatment (**B**) Comparative apoptosis analysis of A549 cell lines. Two-way ANOVA with Tukey's post-test was used for multiple comparisons, **** $p < 0.0001$.

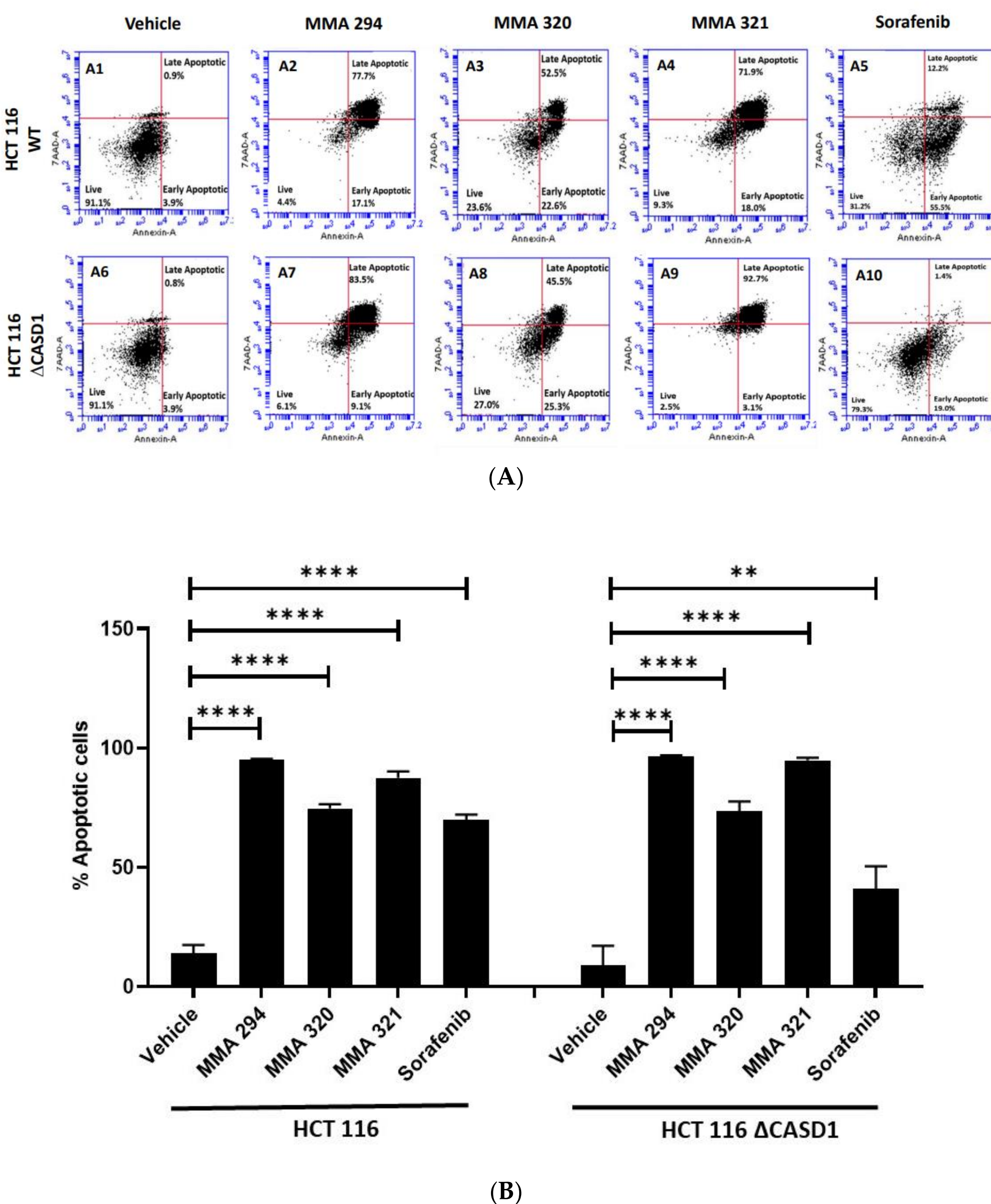


Figure 3. Apoptotic effects of CIEAs in HCT 116 cells. Cells were challenged with the CIEAs, harvested, washed, and resuspended in Annexin V binding buffer. Cells were then stained with APC Annexin V and 7-AAD, incubated in the dark for 25 min and analyzed via flow cytometry. (**A**) Flow cytometry histograms of HCT 116 wild type 3A(A1-A5)) and CASD1 knockout 3A(A6-A10) cell lines. A1&A6: Vehicle treatment, A2&A7: MMA 294 treatment, A3&A8: MMA 320 treatment, A4&A9: MMA 321 treatment and A5&A10: Sorafenib treatment (**B**) Comparative apoptosis analysis of HCT 116 cell lines. Two-way ANOVA with Tukey's post-test was used for multiple comparisons, ** $p < 0.01$ and **** $p < 0.0001$.

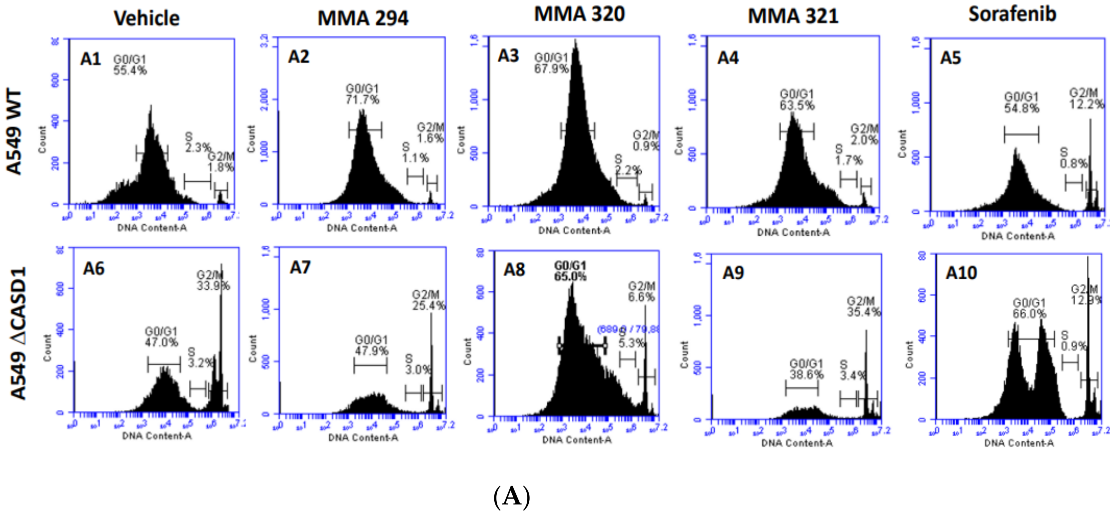
These findings confirm and are consistent with previous studies by Mahnashi and colleagues 2019, who reported increased levels of key mediators of apoptosis (i.e., cleaved Caspase 9, cleaved Caspase 3, and cleaved PARP-1) in hepatocellular carcinoma (HepG2) cells when treated with MMA 132, a structural analog of the

CIEAs used in this study. In addition, these CIEAs have been shown in earlier studies to be potent EFGR inhibitors, and the inhibition of these EGFR pathways is reported to allow the activation of pro-apoptotic proteins, including Bim, Caspase-9, and Bad [146-148], thus promoting apoptosis. MMA 294, MMA 320, and MMA 321 could, therefore, be further explored as potential drug candidates for targeted therapeutics against lung and colon cancers. Comparatively, no significant differences in apoptosis were observed between wild type and CASD1 knockout cells following CIEAs treatment. This suggests that alterations in other cell growth regulatory pathways, including the cell cycle signaling pathway and EGFR signaling pathways, may contribute to the observed sensitivity of knockout cells to the CIEAs compared with wild type cells [149, 150].

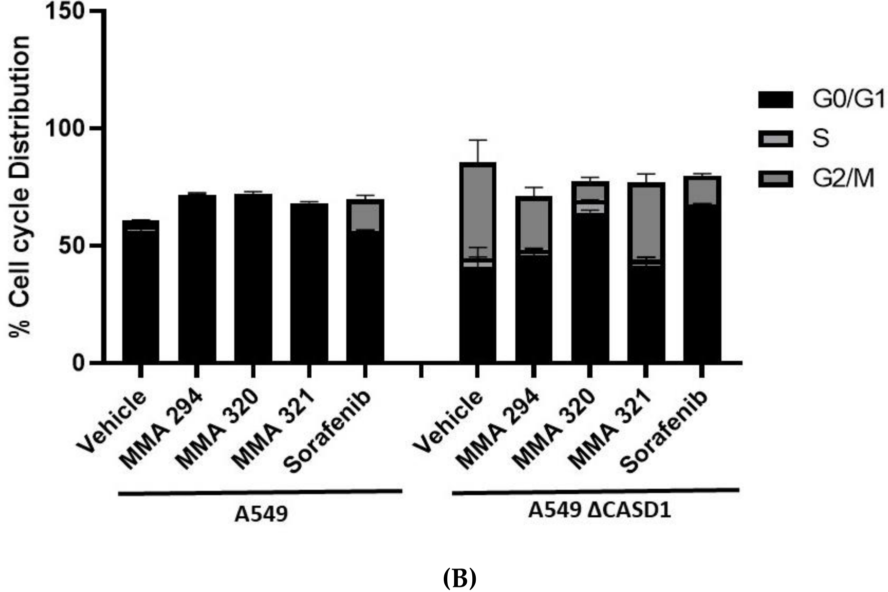
2.4.3 Effects of CIEAs on the Cell Cycle

The cell cycle is primarily involved in replication and plays a crucial role in the coordination between cell proliferation and cell death. Cancer cells rapidly replicate and divide by circumventing the tightly regulated cell cycle [151]. Growth factors are critical for the initiation of signaling events that trigger cell cycle progression. In addition to their targeted functions, growth factor/growth factor receptor inhibitors have been reported to dysregulate normal progression of the cell cycle, often resulting in cytostatic or cycle arrest phenomena [152]. Thus, understanding of the coordination between the cell cycle and EGFR inhibitor is crucial to the development of novel and effective cancer therapies. In this study, we elucidated how Sia deacetylation affects the cell cycle (distribution/phases) in HCT 116 and A549 cells following treatment with the CIEAs. The distribution of cells in various phases of the cell cycle (G0/G1, S, G2/M) was observed to be dependent on the type of CIEA and cell type. All the tested CIEAs and Sorafenib were observed to

elicit a G0/G1 cell cycle arrest in wild types of both A549 and HCT 116 cells (Figures 4A(A1–A5), 4B and 5A(A1-A5), 5B).

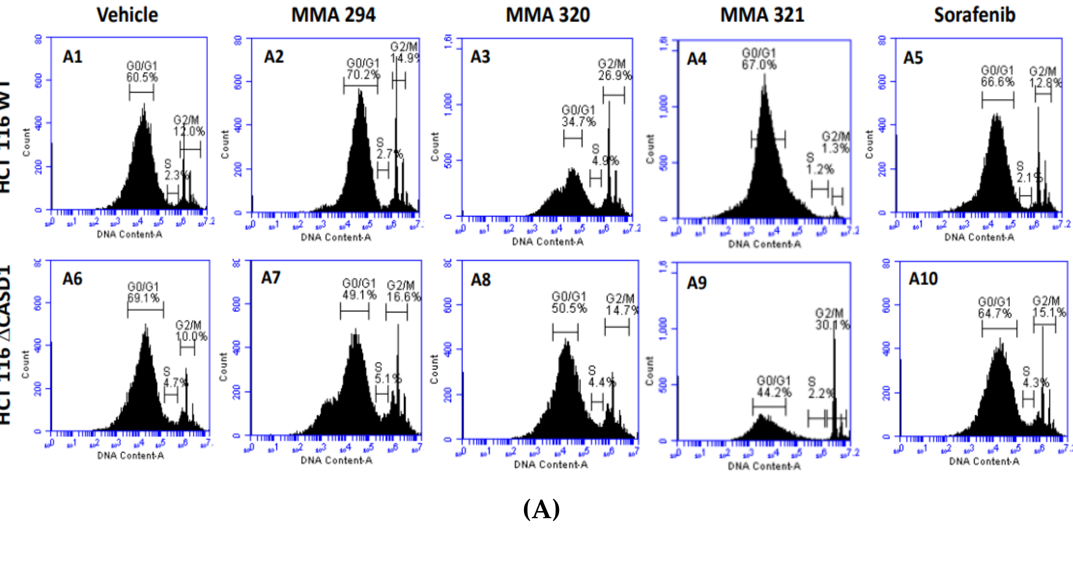


(A)

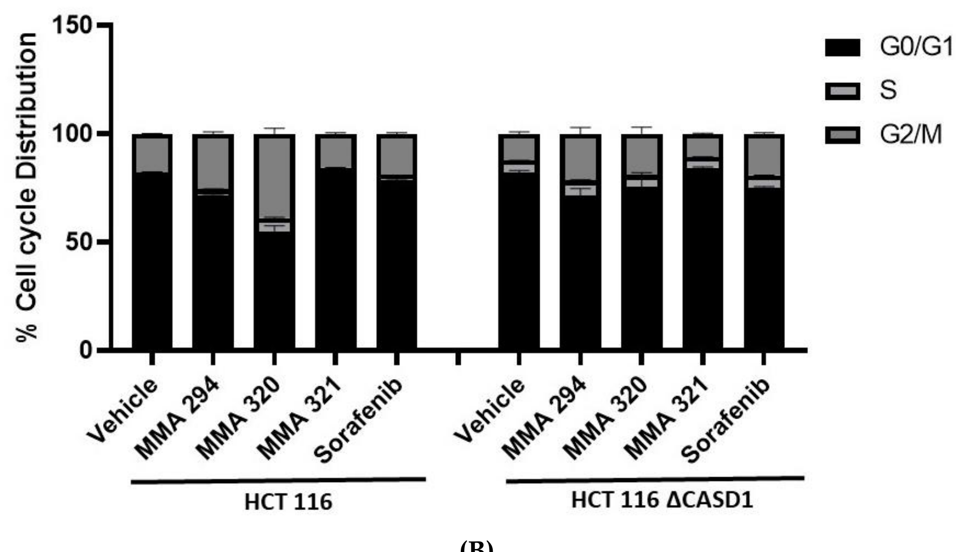


(B)

Figure 4. Effects of CIEAs on A549 Cell Cycle. Cells were challenged with CIEAs, washed, fixed with 70% ethanol, stained with propidium iodide/RNAse A solution, and analyzed via flow cytometry. (**A**) Flow cytometry histograms of A549 wild type 4A(A1-A5) and CASD1 knockout 4A(A6-A10) cell lines. A1&A6: Vehicle treatment, A2&A7: MMA 294 treatment, A3&A8: MMA 320 treatment, A4&A9: MMA 321 treatment and A5&A10: Sorafenib treatment (**B**) Comparative cell cycle phase distribution profile of A549 cell lines.



(A)



(B)

Figure 5. Effects of CIEAs on HCT 116 Cell Cycle. Cells were challenged with CIEAs, washed, fixed with 70% ethanol, stained with propidium iodide/RNAse A solution, and analyzed via flow cytometry. (**A**) Flow cytometry histograms of HCT116 wild type 4A(A1-A5) and CASD1 knockout 4A(A6-A10) cell lines. A1&A6: Vehicle treatment, A2&A7: MMA 294 treatment, A3&A8: MMA 320 treatment, A4&A9: MMA 321 treatment and A5&A10: Sorafenib treatment (**B**) Comparative cell cycle phase distribution profile of A549 cell lines.

Sorafenib in an earlier study was reported to have multiple targets in cells but to arrest HCT 116 and A549 wild type cells at G0/G1 [153, 154]. However, irradiation of these same cells before Sorafenib treatment causes a G2/M arrest cell cycle [153], suggesting that altering the genetic composition of a cell could cause changes in how it responds to drugs. Our data suggests that treating CASD1 knockout cells with the

same CIEAs causes a switch in cell cycle arrest from the observed G0/G1 in wild type cells to a G2/M cell cycle arrest in A549 cells (Figure 4A(A6-A10), 4B), corroborating earlier findings. Again, an earlier study revealed that drugs that elicited G2/M arrest did so at concentrations less than those associated with G0/G1 [155]. This may explain why the recorded IC_{50} values were much lower in the knockout cells compared with the wild type cells of both A549 and HCT 116. Also, the G2/M checkpoint prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damaged cells [156]. This may explain the observed decrease in viable cells and increased sensitivity of CASD1 knockout cells relative to wild type cells when challenged with CIEA.

2.4.4 Effects of Deacetylated Sia on Phosphorylated EGFR Expression

Our data on the viability of the cells following CIEAs treatment revealed increased growth inhibition in the CASD1 knockout HCT 116 and A549 cell lines compared with their wild type counterparts. We, therefore, sought to understand the underlying reason for this observed variation in sensitivities to the CIEAs. CIEAs in earlier in silico and in vitro experimental studies have been reported as potent EGFR inhibitors that reduce levels of pEGFR [105, 137, 157]. We, therefore, explored levels of activated (pEGFR) as a possible contributory factor. Our immunofluorescence data revealed significantly high expression levels of pEGFR in knockouts of the A549 cell line (*p*-value = 0.0019) (Figures 6A(A5-A6), 6B) and HCT 116 cell line (*p*-value = 0.0002) (Figure 6C(C5-C6), 6D) compared with their wild type cell lines (6A(A2-A3), 6B) and 6C(C2-C3), 6D). pEGFR overexpression has been reported in several studies as a major factor in poor tumor prognosis. It is associated with a more aggressive clinical progression in several cancers including lung, breast, ovarian,

bladder, esophageal and cervical, cancers [158-160]. This indicates that CASD1 knockout cells that relatively overexpress pEGFR are a more aggressive cancer type.

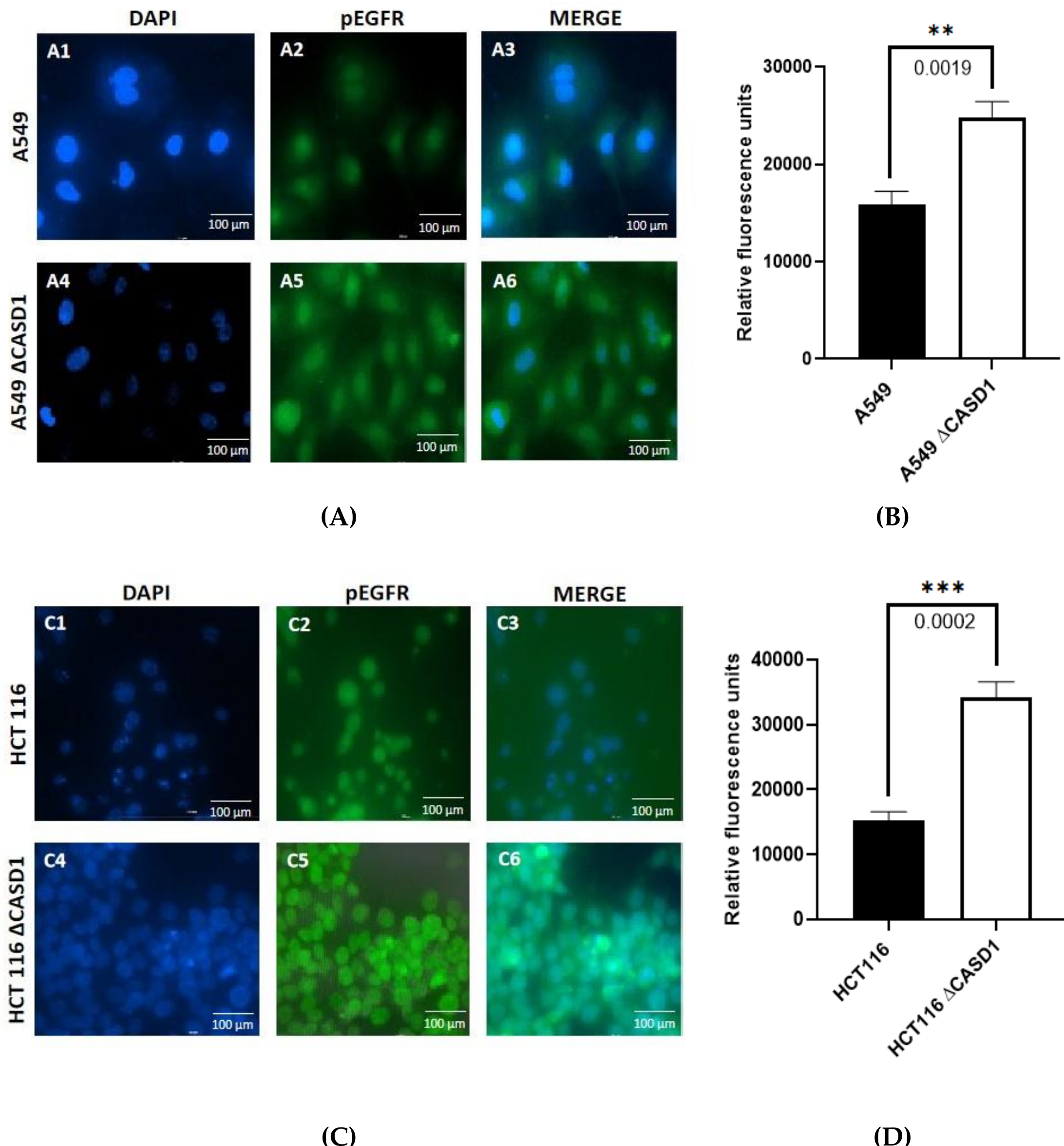


Figure 6. Effects of Sia deacetylation on Phosphorylated EGFR (pEGFR) expression. Cells were fixed, permeabilized, treated with pEGFR-specific primary antibody and Alexa-Fluor 488 conjugated secondary antibody. DAPI was used for counter staining**. (A)** Immunofluorescent localization of pEGFR in A549 wild type 6A(A1-A3) and CASD1 Knockout 6A(A4-A6) cell lines. A1&A4: DAPI stained nuclei, A2&A5: pEGFR fluorescence and A3&A6: Merged DAPI stained nuclei and fluorescent pEGFR **(B)** pEGFR fluorescence intensity for A549 cell lines. **(C)** Immunofluorescent localization of pEGFR in HCT 116 wild type 6C(C1-C3) and CASD1 Knockout 6C(C4-C6) cell lines. A1&A4: DAPI stained nuclei, A2&A5: pEGFR fluorescence and A3&A6: Merged DAPI stained nuclei and fluorescent pEGFR **(D)** pEGFR fluorescence intensity plot for A549 cell lines. One-way ANOVA with Tukey's post-test was used for multiple plot comparisons, ** $p < 0.01$ and *** $p \leq 0.001$.

This speculation is consistent with our earlier studies that reported increased cell proliferation in colon and lung cancer cells with deacetylated Sia [58, 70]. The findings of this present study, however, show that, this acquired enhanced proliferation trait could also render these deacetylated Sia-expressing CASD1 knockout cells very sensitive to cytotoxicity by pEGFR inhibitors. The increased expression of these growth receptors ensures there are relatively more potential targets for the pEGFR inhibitors (Figure 8C), and this could account for the much lower IC_{50} values recorded in the knockout cells relative to the wild type cells. The removal of acetyl functional groups increases Sialylation (i.e., free Sia) relative to levels of modified Sia; thus, our results are consistent with work conducted by Yen et al., 2015 who reported that Sialylation enhances the sensitivity of resistant lung cancer cells to the EGRF-targeted therapeutic, gefitinib. Our data also supports a previous study that reported a decrease in IC_{50} values in cells that overexpress EGFR when a targeted EFGR chemotherapeutic agent is used [161]. We, therefore, speculate that altered (increased) levels of the protein targets (pEGFR) account for the observed increased susceptibility of knockout cells to CIEAs. The mechanism underlying this observed phenomenon is, however, unknown, and thus warrants further investigation.

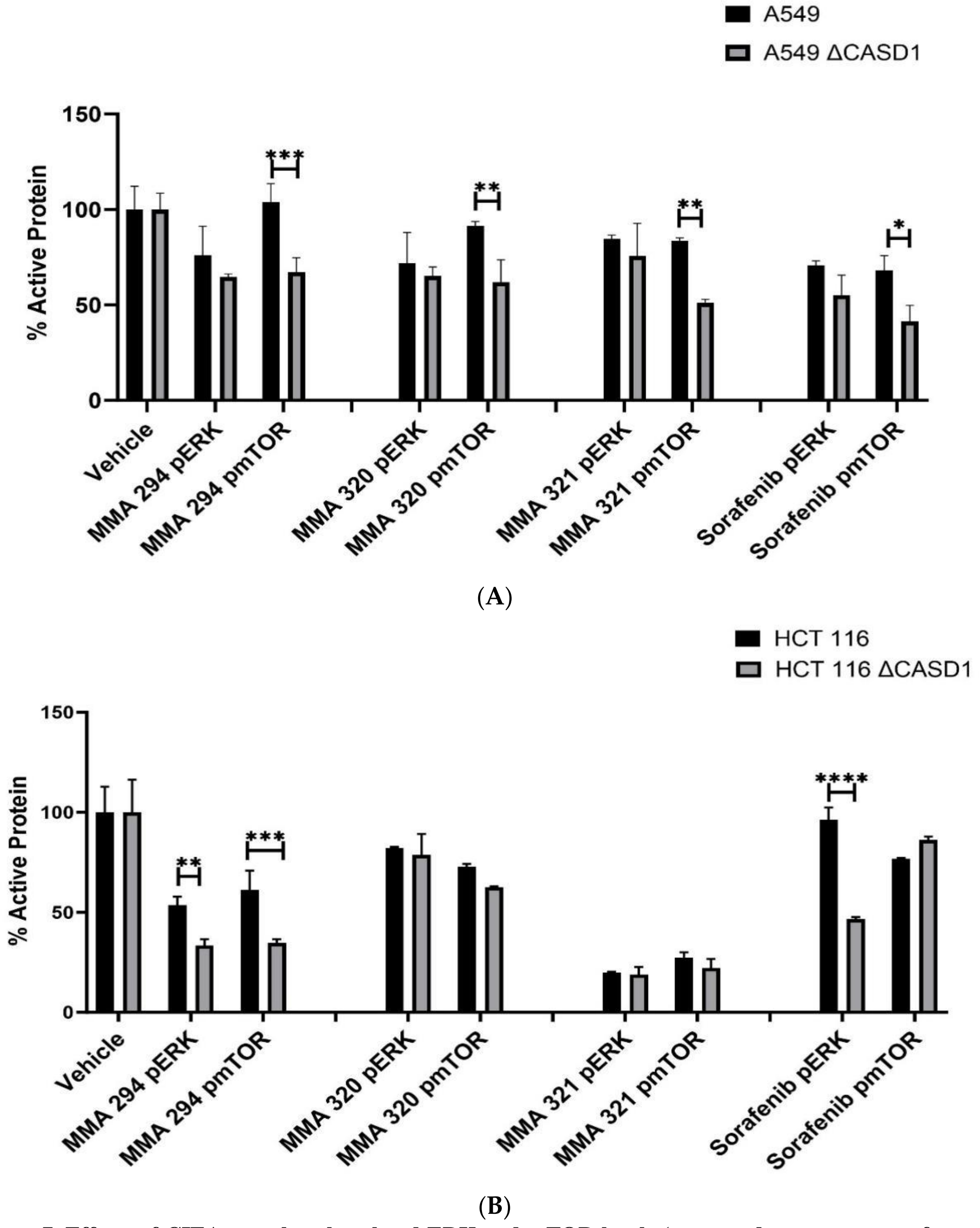
2.4.5 Effects of CIEAs on ERK and mTOR Phosphorylation Levels

To further confirm earlier findings that increased protein target (i.e., pEGFR) renders knockout cells susceptible to the CIEAs relative to wild type cells, we elucidated the effect of CIEAs on the expression of two crucial downstream effectors/proteins of the EGFR signaling pathway: extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR). Our results revealed generally reduced levels of active ERK and more significantly reduced active mTOR in knockout cells relative to wild type cells when treated with CIEAs (Figure 7A, B).

Compared with wild type cells, MMA 294, MMA 320 and MMA 321 elicited significant decreases in the levels of pmTOR in CASD1 knockout A549 cell lines (*p*-values = 0.0010, 0.0056 and 0.0026, respectively). Sorafenib treatment also showed reduced levels of pERK and pmTOR (*p*-value= 0.0108) (Figure 7A). In HCT 116 cells, MMA 294 elicited a significant reduction in pERK and pmTOR levels in CASD1 knockout cell lines (*p*-values = 0.0063 and 0.0007, respectively) (Figure 7B). The sorafenib treatment resulted in significant decreases in pERK levels in CASD1 knockout cells (*p*-value = <0.0001).

The EGFR signaling pathway is one of the most conserved and well-studied pathways in eukaryotes. It basically entails the transmission of activating signals to downstream protein kinases following ligand (EGF)–receptor (EGFR) binding/interactions. Ultimately, these activating signals activate genes in the nucleus that are involved in cell proliferation, differentiation, migration, and survival [109, 148, 152]. ERK and mTOR are two of the crucial downstream proteins activated in this signaling cascade. Upon activation by phosphorylation, ERK translocate to the nucleus where it activates several transcription factors, including CREB, Fos and Elk-1, ultimately leading to effector protein synthesis and causing changes in cell proliferation and survival [162]. ERK activation also promotes the development of tumors by phosphorylating the two pro-apoptotic proteins, Bim and Bid, causing the proteasome degradation of Bim and the sequestration of Bad to the phosphoserine-binding proteins, thereby inhibiting apoptosis [163]. mTOR is also involved in multiple signaling pathways in the body including phosphoinositide-3-kinase (PI3K)/AKT and serves as a key mediator in the regulation of cell proliferation, autophagy, immune cell differentiation, tumor metabolism and apoptosis [164]. ERK

and mTOR are often activated in tumors and have, thus, become hot targets in anti-tumor therapy research.



(**A**)

(**B**)

Figure 7. Effects of CIEAs on phosphorylated ERK and mTOR levels (expressed as percentage of control). Cells were challenged with CIEAs, fixed, permeabilized, treated with either (p)ERK or (p)mTOR specific primary antibody and goat anti-mouse or anti-rabbit IRDye secondary antibody. (**A**) A549 cell lines. (**B**) HCT 116 cell lines. Two-way ANOVA with Tukey's post-test was used for multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Our findings are consistent with earlier studies by Mahnashi and colleagues, 2019 who reported that MMA 132, a structural analog of our CIEAs, exhibited dual

inhibitory mechanisms against the phosphorylating pathways of the EGFR and MAPK (RAS/RAF/MEK/ERK) pathways. Also, the observed significant decrease in levels of activated mTOR and partly activated ERK in CASD1 knockouts of A549 and HCT 116 cells as compared with wild type cells, especially for MMA 249, suggests that there may be more protein targets (pEGFR) in these deacetylated Sia-expressing cells available for inhibition by the CIEAs, hence the corresponding reduced levels of downstream activated proteins (i.e., pERK and pmTOR). Furthermore, the observed reductions in levels of these proteins could explain the comparatively reduced viability/proliferation and increased apoptosis observed in CASD1 knockout cells when challenged with CIEAs (Figure 8D).

2.4.6 Infographics

The cartoon below summarizes the findings of this study (Figure 8).

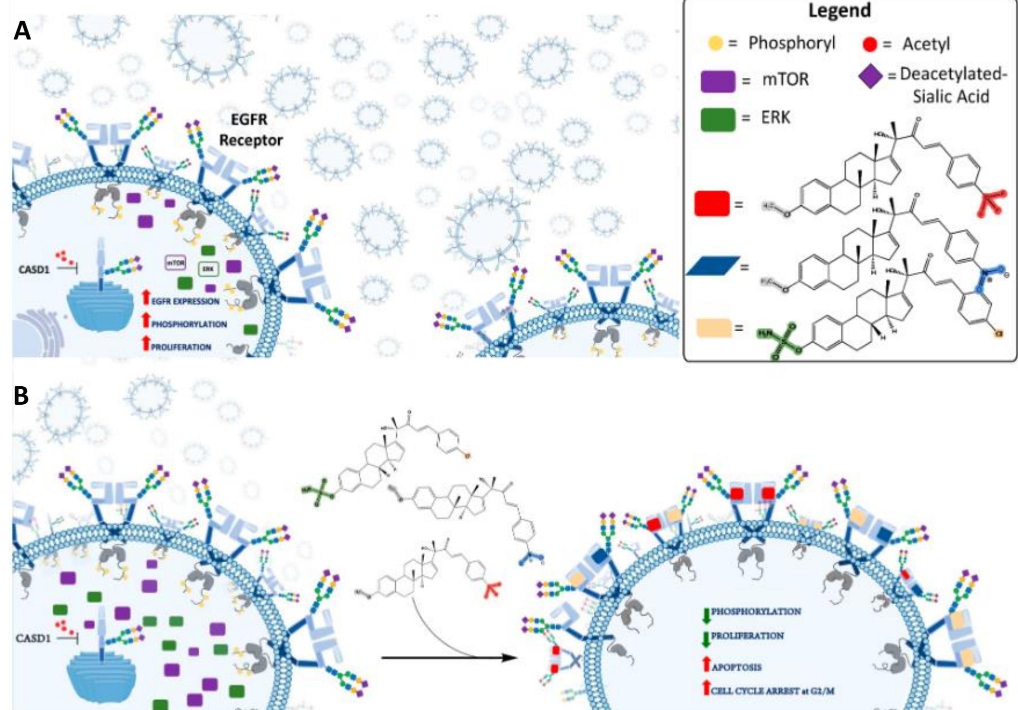


Figure 8. **(A)** Absence of CASD1 abrogates the addition of acetyl groups to Sia residues on the EGFR glycan chain resulting in the overexpression of activated (Phosphorylated) EGFR, increased levels of activated downstream proteins such as ERK and mTOR and increased proliferation of cells **(B)** Treatment of CASD1-deficient/Deacetylated Sia-expressing cells with CIEAs (i.e., EGFR inhibitor) inhibits EGFR activation, decrease levels of phosphorylated(activated) downstream proteins (i.e., mTOR and ERK), decreased cell proliferation and induces apoptosis as well as G2/M cell cycle

2.5 Conclusion

Targeted therapeutics against cancer are increasing in demand due to their ability to target cancer cells specifically and effectively, sparing normal healthy cells. This offers such therapies some advantages over traditional chemotherapeutics. In this study, we have shown that there is a possibility for improving the effectiveness of targeted therapeutics by selectively targeting a second unrelated protein. More specifically, by knocking out Sia acetyl modifying proteins, CASD1 via CRISPR Cas9 genome editing, lung and colon cancer cells tend to upregulate their activated EGFR expression levels to compensate for the genetic aberration. This deacetylated Sia phenotype further makes cancers more vulnerable to EGFR inhibitors (CIEAs and Sorafenib) and selectively provides an improved alternative approach through which they can be targeted and killed. Thus, a multidrug targeted therapy that exploits Sia acetyl group inhibitors (or acetyltransferase inhibitors) and EGFR inhibitors could be explored in cancer treatment. Subsequent studies involving animal models are however required to further confirm the pre-clinical relevance of these findings.

2.6 References

[1] J. Ferlay *et al.*, "Cancer statistics for the year 2020: An overview," (in eng), *Int J Cancer,* Apr 5 2021, doi: 10.1002/ijc.33588.

[2] R. L. Siegel, K. D. Miller, H. E. Fuchs, and A. Jemal, "Cancer statistics, 2022," (in eng), *CA Cancer J Clin,* vol. 72, no. 1, pp. 7-33, Jan 2022, doi: 10.3322/caac.21708.

[3] M. M. Gottesman, T. Fojo, and S. E. Bates, "Multidrug resistance in cancer: role of ATP-dependent transporters," (in eng), *Nat Rev Cancer,* vol. 2, no. 1, pp. 48-58, Jan 2002, doi: 10.1038/nrc706.

[4] B. C. Baguley, "Multiple drug resistance mechanisms in cancer," (in eng), *Mol Biotechnol,* vol. 46, no. 3, pp. 308-16, Nov 2010, doi: 10.1007/s12033-010-9321-2.

[5] K. Bukowski, M. Kciuk, and R. Kontek, "Mechanisms of Multidrug Resistance in Cancer Chemotherapy," (in eng), *Int J Mol Sci,* vol. 21, no. 9, May 2 2020, doi: 10.3390/ijms21093233.

[6] M. M. Gottesman, "Mechanisms of cancer drug resistance," *Annu Rev Med,* vol. 53, pp. 615-27, 2002, doi: 10.1146/annurev.med.53.082901.103929.

[7] K. O. Alfarouk *et al.*, "Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp," (in eng), *Cancer Cell Int,* vol. 15, p. 71, 2015, doi: 10.1186/s12935-015-0221-1.

[8] R. K. Vadlapatla, A. D. Vadlapudi, D. Pal, and A. K. Mitra, "Mechanisms of drug resistance in cancer chemotherapy: coordinated role and regulation of efflux transporters and metabolizing enzymes," *Curr Pharm Des,* vol. 19, no. 40, pp. 7126-40, 2013, doi: 10.2174/13816128113199990493.

[9] C. P. Wu, A. M. Calcagno, and S. V. Ambudkar, "Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies," *Curr Mol Pharmacol,* vol. 1, no. 2, pp. 93-105, Jun 2008, doi: 10.2174/1874467210801020093.

[10] H. L. Martin *et al.*, "High-content, high-throughput screening for the identification of cytotoxic compounds based on cell morphology and cell proliferation markers," (in eng), *PLoS One,* vol. 9, no. 2, p. e88338, 2014, doi: 10.1371/journal.pone.0088338.

[11] M. D. Hall, M. D. Handley, and M. M. Gottesman, "Is resistance useless? Multidrug resistance and collateral sensitivity," *Trends Pharmacol Sci,* vol. 30, no. 10, pp. 546-56, Oct 2009, doi: 10.1016/j.tips.2009.07.003.

[12] F. J. Sharom, "ABC multidrug transporters: structure, function and role in chemoresistance," (in eng), *Pharmacogenomics,* vol. 9, no. 1, pp. 105-27, Jan 2008, doi: 10.2217/14622416.9.1.105.

[13] S. Cheepala *et al.*, "Cyclic nucleotide compartmentalization: contributions of phosphodiesterases and ATP-binding cassette transporters," (in eng), *Annu Rev Pharmacol Toxicol,* vol. 53, pp. 231-53, 2013, doi: 10.1146/annurev-pharmtox-010611-134609.

[14] M. Dean, A. Rzhetsky, and R. Allikmets, "The human ATP-binding cassette (ABC) transporter superfamily," (in eng), *Genome Res,* vol. 11, no. 7, pp. 1156-66, Jul 2001, doi: 10.1101/gr.184901.

[15] K. Moitra and M. Dean, "Evolution of ABC transporters by gene duplication and their role in human disease," (in eng), *Biol Chem,* vol. 392, no. 1-2, pp. 29-37, Jan 2011, doi: 10.1515/bc.2011.006.

[16] U. A. Germann and T. C. Chambers, "Molecular analysis of the multidrug transporter, P-glycoprotein," *Cytotechnology,* vol. 27, no. 1-3, pp. 31-60, Sep 1998, doi: 10.1023/A:1008023629269.

[17] R. G. Deeley and S. P. Cole, "Substrate recognition and transport by multidrug resistance protein 1 (ABCC1)," *FEBS Lett,* vol. 580, no. 4, pp. 1103-11, Feb 13 2006, doi: 10.1016/j.febslet.2005.12.036.

[18] M. K. DeGorter, C. Q. Xia, J. J. Yang, and R. B. Kim, "Drug transporters in drug efficacy and toxicity," (in eng), *Annu Rev Pharmacol Toxicol,* vol. 52, pp. 249-73, 2012, doi: 10.1146/annurev-pharmtox-010611-134529.

[19] A. H. Schinkel and J. W. Jonker, "Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview," (in eng), *Adv Drug Deliv Rev,* vol. 55, no. 1, pp. 3-29, Jan 21 2003, doi: 10.1016/s0169-409x(02)00169-2.

[20] S. P. Cole, "Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future," (in eng), *Annu Rev Pharmacol Toxicol,* vol. 54, pp. 95-117, 2014, doi: 10.1146/annurev-pharmtox-011613-135959.

[21] S. H. Lee *et al.*, "MRP1 polymorphisms associated with citalopram response in patients with major depression," (in eng), *J Clin Psychopharmacol,* vol. 30, no. 2, pp. 116-25, Apr 2010, doi: 10.1097/JCP.0b013e3181d2ef42.

[22] Q. Mao and J. D. Unadkat, "Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update," *AAPS J,* vol. 17, no. 1, pp. 65-82, Jan 2015, doi: 10.1208/s12248-014-9668-6.

[23] K. J. Bailey-Dell, B. Hassel, L. A. Doyle, and D. D. Ross, "Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene," (in eng), *Biochim Biophys Acta,* vol. 1520, no. 3, pp. 234-41, Sep 21 2001, doi: 10.1016/s0167-4781(01)00270-6.

[24] T. Furukawa *et al.*, "Major SNP (Q141K) variant of human ABC transporter ABCG2 undergoes lysosomal and proteasomal degradations," *Pharm Res,* vol. 26, no. 2, pp. 469-79, Feb 2009, doi: 10.1007/s11095-008-9752-7.

[25] H. Nakagawa, K. Wakabayashi-Nakao, A. Tamura, Y. Toyoda, S. Koshiba, and T. Ishikawa, "Disruption of N-linked glycosylation enhances ubiquitin-mediated proteasomal degradation of the human ATP-binding cassette transporter ABCG2," *FEBS J,* vol. 276, no. 24, pp. 7237-52, Dec 2009, doi: 10.1111/j.1742-4658.2009.07423.x.

[26] T. Nakanishi and D. D. Ross, "Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression," *Chin J Cancer,* vol. 31, no. 2, pp. 73-99, Feb 2012, doi: 10.5732/cjc.011.10320.

[27] S. H. Iram and S. P. Cole, "Differential functional rescue of Lys(513) and Lys(516) processing mutants of MRP1 (ABCC1) by chemical chaperones reveals different domain-domain interactions of the transporter," (in eng), *Biochim Biophys Acta,* vol. 1838, no. 3, pp. 756-65, Mar 2014, doi: 10.1016/j.bbamem.2013.11.002.

[28] C. F. Higgins and K. J. Linton, "The ATP switch model for ABC transporters," (in eng), *Nat Struct Mol Biol,* vol. 11, no. 10, pp. 918-26, Oct 2004, doi: 10.1038/nsmb836.

[29] A. E. Senior, M. K. al-Shawi, and I. L. Urbatsch, "The catalytic cycle of P-glycoprotein," (in eng), *FEBS Lett,* vol. 377, no. 3, pp. 285-9, Dec 27 1995, doi: 10.1016/0014-5793(95)01345-8.

[30] B. Verhalen and S. Wilkens, "P-glycoprotein retains drug-stimulated ATPase activity upon covalent linkage of the two nucleotide binding domains at their C-terminal ends," (in eng), *J Biol Chem,* vol. 286, no. 12, pp. 10476-82, Mar 25 2011, doi: 10.1074/jbc.M110.193151.

[31] P. M. Jones and A. M. George, "A reciprocating twin-channel model for ABC transporters," (in eng), *Q Rev Biophys,* vol. 47, no. 3, pp. 189-220, Aug 2014, doi: 10.1017/s0033583514000031.

[32] Y. K. Zhang, Y. J. Wang, P. Gupta, and Z. S. Chen, "Multidrug Resistance Proteins (MRPs) and Cancer Therapy," (in eng), *Aaps j,* vol. 17, no. 4, pp. 802-12, Jul 2015, doi: 10.1208/s12248-015-9757-1.

[33] K. Hollenstein, D. C. Frei, and K. P. Locher, "Structure of an ABC transporter in complex with its binding protein," (in eng), *Nature,* vol. 446, no. 7132, pp. 213-6, Mar 8 2007, doi: 10.1038/nature05626.

[34] E. M. Leslie, R. G. Deeley, and S. P. Cole, "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense," (in eng), *Toxicol Appl Pharmacol,* vol. 204, no. 3, pp. 216-37, May 1 2005, doi: 10.1016/j.taap.2004.10.012.

[35] M. Maliepaard *et al.*, "Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues," *Cancer Res,* vol. 61, no. 8, pp. 3458-64, Apr 15 2001. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/11309308.

[36] E. Pollex, A. Lubetsky, and G. Koren, "The role of placental breast cancer resistance protein in the efflux of glyburide across the human placenta," *Placenta,* vol. 29, no. 8, pp. 743-7, Aug 2008, doi: 10.1016/j.placenta.2008.05.001.

[37] M. Huls *et al.*, "The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane," *Kidney Int,* vol. 73, no. 2, pp. 220-5, Jan 2008, doi: 10.1038/sj.ki.5002645.

[38] L. Doyle and D. D. Ross, "Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)," *Oncogene,* vol. 22, no. 47, pp. 7340-58, Oct 20 2003, doi: 10.1038/sj.onc.1206938.

[39] M. Maliepaard *et al.*, "Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line," *Cancer Res,* vol. 59, no. 18, pp. 4559-63, Sep 15 1999. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/10493507.

[40] A. Gupta, Y. Zhang, J. D. Unadkat, and Q. Mao, "HIV protease inhibitors are inhibitors but not substrates of the human breast cancer resistance protein (BCRP/ABCG2)," *J Pharmacol Exp Ther,* vol. 310, no. 1, pp. 334-41, Jul 2004, doi: 10.1124/jpet.104.065342.

[41] P. J. Houghton *et al.*, "Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro," *Cancer Res,* vol. 64, no. 7, pp. 2333-7, Apr 1 2004, doi: 10.1158/0008-5472.can-03-3344.

[42] C. Ozvegy-Laczka *et al.*, "High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter," *Mol Pharmacol,* vol. 65, no. 6, pp. 1485-95, Jun 2004, doi: 10.1124/mol.65.6.1485.

[43] Z. Shi *et al.*, "Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance," *Cancer Res,* vol. 67, no. 22, pp. 11012-20, Nov 15 2007, doi: 10.1158/0008-5472.CAN-07-2686.

[44] R. J. Kathawala, P. Gupta, C. R. Ashby, Jr., and Z. S. Chen, "The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade," *Drug Resist Updat,* vol. 18, pp. 1-17, Jan 2015, doi: 10.1016/j.drup.2014.11.002.

[45] Z. Binkhathlan and A. Lavasanifar, "P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future perspectives," (in eng), *Curr Cancer Drug Targets,* vol. 13, no. 3, pp. 326-46, Mar 2013, doi: 10.2174/15680096113139990076.

[46] A. Palmeira, E. Sousa, M. H. Vasconcelos, and M. M. Pinto, "Three decades of P-gp inhibitors: skimming through several generations and scaffolds," (in eng), *Curr Med Chem,* vol. 19, no. 13, pp. 1946-2025, 2012, doi: 10.2174/092986712800167392.

[47] S. P. C. Cole, "Targeting Multidrug Resistance Protein 1 (MRP1, ABCC1): Past, Present, and Future," *Annual Review of Pharmacology and Toxicology,* vol. 54, no. 1, pp. 95-117, 2014/01/06 2014, doi: 10.1146/annurev-pharmtox-011613-135959.

[48] S. V. Ambudkar, C. Kimchi-Sarfaty, Z. E. Sauna, and M. M. Gottesman, "P-glycoprotein: from genomics to mechanism," *Oncogene,* vol. 22, no. 47, pp. 7468-7485, 2003/10/01 2003, doi: 10.1038/sj.onc.1206948.

[49] D. R. Hipfner *et al.*, "Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH2 terminus," (in eng), *J Biol Chem,* vol. 272, no. 38, pp. 23623-30, Sep 19 1997, doi: 10.1074/jbc.272.38.23623.

[50] D. A. Greer and S. Ivey, "Distinct N-glycan glycosylation of P-glycoprotein isolated from the human uterine sarcoma cell line MES-SA/Dx5," *Biochim Biophys Acta,* vol. 1770, no. 9, pp. 1275-82, Sep 2007, doi: 10.1016/j.bbagen.2007.07.005.

[51] O. Wesolowska, M. Paprocka, J. Kozlak, N. Motohashi, D. Dus, and K. Michalak, "Human sarcoma cell lines MES-SA and MES-SA/Dx5 as a model for multidrug resistance modulators screening," (in eng), *Anticancer Res,* vol. 25, no. 1a, pp. 383-9, Jan-Feb 2005.

[52] D. A. Greer and S. Ivey, "Distinct N-glycan glycosylation of P-glycoprotein isolated from the human uterine sarcoma cell line MES-SA/Dx5," (in eng), *Biochimica et biophysica acta,* vol. 1770, no. 9, pp. 1275-1282, 2007, doi: 10.1016/j.bbagen.2007.07.005.

[53] H. Nakagawa *et al.*, "Ubiquitin-mediated proteasomal degradation of non-synonymous SNP variants of human ABC transporter ABCG2," (in eng), *Biochem J,* vol. 411, no. 3, pp. 623-31, May 1 2008, doi: 10.1042/bj20071229.

[54] K. Wojtowicz, R. Januchowski, M. Nowicki, and M. Zabel, "Inhibition of protein glycosylation reverses the MDR phenotype of cancer cell lines," *Biomedicine & Pharmacotherapy,* vol. 74, pp. 49-56, 2015/08/01/ 2015, doi: https://doi.org/10.1016/j.biopha.2015.07.001.

[55] N. M. Varki and A. Varki, "Diversity in cell surface sialic acid presentations: implications for biology and disease," *Lab Invest,* vol. 87, no. 9, pp. 851-7, Sep 2007, doi: 10.1038/labinvest.3700656.

[56] A. Varki and R. Schauer, "Sialic Acids," in *Essentials of Glycobiology*, nd *et al.* Eds. Cold Spring Harbor (NY), 2009.

[57] S. Grabenstein *et al.*, "Deacetylated sialic acids modulates immune mediated cytotoxicity via the sialic acid-Siglec pathway," *Glycobiology,* vol. 31, no. 10, pp. 1279-1294, Nov 18 2021, doi: 10.1093/glycob/cwab068.

[58] K. N. Barnard *et al.*, "Expression of 9-O- and 7,9-O-Acetyl Modified Sialic Acid in Cells and Their Effects on Influenza Viruses," *mBio,* vol. 10, no. 6, Dec 3 2019, doi: 10.1128/mBio.02490-19.

[59] R. Schauer, "Sialic acids: fascinating sugars in higher animals and man," (in eng), *Zoology (Jena),* vol. 107, no. 1, pp. 49-64, 2004, doi: 10.1016/j.zool.2003.10.002.

[60] I. Tuffour, S. Amuzu, H. Bayoumi, I. Surtaj, C. Parrish, and R. Willand-Charnley, "Early in vitro evidence indicates that deacetylated sialic acids modulate multi-drug resistance in colon and lung cancers via breast cancer resistance protein," (in eng), *Front Oncol,* vol. 13, p. 1145333, 2023, doi: 10.3389/fonc.2023.1145333.

[61] A. F. Swindall and S. L. Bellis, "Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells," (in eng), *The Journal of biological chemistry,* vol. 286, no. 26, pp. 22982-22990, 2011, doi: 10.1074/jbc.M110.211375.

[62] A. T. Holdbrooks, C. M. Britain, and S. L. Bellis, "ST6Gal-I sialyltransferase promotes tumor necrosis factor (TNF)-mediated cancer cell survival via sialylation of the TNF

receptor 1 (TNFR1) death receptor," *J Biol Chem,* vol. 293, no. 5, pp. 1610-1622, Feb 2 2018, doi: 10.1074/jbc.M117.801480.

[63] M. J. Schultz, A. F. Swindall, J. W. Wright, E. S. Sztul, C. N. Landen, and S. L. Bellis, "ST6Gal-I sialyltransferase confers cisplatin resistance in ovarian tumor cells," (in eng), *J Ovarian Res,* vol. 6, no. 1, p. 25, Apr 11 2013, doi: 10.1186/1757-2215-6-25.

[64] H. Ma, H. Zhou, X. Song, S. Shi, J. Zhang, and L. Jia, "Modification of sialylation is associated with multidrug resistance in human acute myeloid leukemia," *Oncogene,* vol. 34, no. 6, pp. 726-740, 2015/02/01 2015, doi: 10.1038/onc.2014.7.

[65] A. M. Baumann *et al.*, "9-O-Acetylation of sialic acids is catalysed by CASD1 via a covalent acetyl-enzyme intermediate," (in eng), *Nat Commun,* vol. 6, p. 7673, Jul 14 2015, doi: 10.1038/ncomms8673.

[66] O. M. Pearce and H. Laubli, "Sialic acids in cancer biology and immunity," *Glycobiology,* vol. 26, no. 2, pp. 111-28, Feb 2016, doi: 10.1093/glycob/cwv097.

[67] Y. Shen *et al.*, "O-acetylation and de-O-acetylation of sialic acids in human colorectal carcinoma," *Eur J Biochem,* vol. 271, no. 2, pp. 281-90, Jan 2004, doi: 10.1046/j.1432-1033.2003.03927.x.

[68] S. Pal *et al.*, "Purification and characterization of 9-O-acetylated sialoglycoproteins from leukemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia," *Glycobiology,* vol. 14, no. 10, pp. 859-70, Oct 2004, doi: 10.1093/glycob/cwh111.

[69] R. Parameswaran *et al.*, "O-acetylated N-acetylneuraminic acid as a novel target for therapy in human pre-B acute lymphoblastic leukemia," *J Exp Med,* vol. 210, no. 4, pp. 805-19, Apr 8 2013, doi: 10.1084/jem.20121482.

[70] I. Tuffour, S. Amuzu, H. Bayoumi, I. Surtaj, C. Parrish, and R. Willand-Charnley, "Early in vitro evidence indicates that deacetylated sialic acids modulate multi-drug resistance in colon and lung cancers via breast cancer resistance protein," (in English), *Frontiers in Oncology,* Original Research vol. 13, 2023-June-12 2023, doi: 10.3389/fonc.2023.1145333.

[71] M. A. Langereis, H. H. Rabouw, M. Holwerda, L. J. Visser, and F. J. van Kuppeveld, "Knockout of cGAS and STING Rescues Virus Infection of Plasmid DNA-Transfected Cells," *J Virol,* vol. 89, no. 21, pp. 11169-73, Nov 2015, doi: 10.1128/JVI.01781-15.

[72] T. Wang, L. M. Larcher, L. Ma, and R. N. Veedu, "Systematic Screening of Commonly Used Commercial Transfection Reagents towards Efficient Transfection of Single-Stranded Oligonucleotides," *Molecules,* vol. 23, no. 10, Oct 8 2018, doi: 10.3390/molecules23102564.

[73] J. Q. Wang *et al.*, "Reversal of Cancer Multidrug Resistance (MDR) Mediated by ATP-Binding Cassette Transporter G2 (ABCG2) by AZ-628, a RAF Kinase Inhibitor," *Front Cell Dev Biol,* vol. 8, p. 601400, 2020, doi: 10.3389/fcell.2020.601400.

[74] T. Uto *et al.*, "Antiproliferative and Pro-Apoptotic Activity of Diarylheptanoids Isolated from the Bark of Alnus japonica in Human Leukemia Cell Lines," *Am J Chin Med,* vol. 43, no. 4, pp. 757-67, 2015, doi: 10.1142/S0192415X15500470.

[75] D. Osei-Safo *et al.*, "Constituents of the Roots of Dichapetalum pallidum and Their Anti-Proliferative Activity," *Molecules,* vol. 22, no. 4, Mar 27 2017, doi: 10.3390/molecules22040532.

[76] H. Ma, H. Zhou, X. Song, S. Shi, J. Zhang, and L. Jia, "Modification of sialylation is associated with multidrug resistance in human acute myeloid leukemia," *Oncogene,* vol. 34, no. 6, pp. 726-40, Feb 5 2015, doi: 10.1038/onc.2014.7.

[77] M. Kim *et al.*, "The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells," *Clin Cancer Res,* vol. 8, no. 1, pp. 22-8, Jan 2002. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/11801536.

[78] M. P. Boland, K. A. Fitzgerald, and L. A. O'Neill, "Topoisomerase II is required for mitoxantrone to signal nuclear factor kappa B activation in HL60 cells," *J Biol Chem,* vol. 275, no. 33, pp. 25231-8, Aug 18 2000, doi: 10.1074/jbc.275.33.25231.

[79] A. E. Stacy, P. J. Jansson, and D. R. Richardson, "Molecular pharmacology of ABCG2 and its role in chemoresistance," *Mol Pharmacol,* vol. 84, no. 5, pp. 655-69, Nov 2013, doi: 10.1124/mol.113.088609.

[80] J. Kale, E. J. Osterlund, and D. W. Andrews, "BCL-2 family proteins: changing partners in the dance towards death," *Cell Death Differ,* vol. 25, no. 1, pp. 65-80, Jan 2018, doi: 10.1038/cdd.2017.186.

[81] Y. Tsujimoto, "Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?," *Genes Cells,* vol. 3, no. 11, pp. 697-707, Nov 1998, doi: 10.1046/j.1365-2443.1998.00223.x.

[82] G. V. Chaitanya, A. J. Steven, and P. P. Babu, "PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration," *Cell Commun Signal,* vol. 8, p. 31, Dec 22 2010, doi: 10.1186/1478-811X-8-31.

[83] F. Nomura *et al.*, "Enhancement of poly-adenosine diphosphate-ribosylation in human hepatocellular carcinoma," *J Gastroenterol Hepatol,* vol. 15, no. 5, pp. 529-35, May 2000, doi: 10.1046/j.1440-1746.2000.02193.x.

[84] V. Ossovskaya, I. C. Koo, E. P. Kaldjian, C. Alvares, and B. M. Sherman, "Upregulation of Poly (ADP-Ribose) Polymerase-1 (PARP1) in Triple-Negative Breast Cancer and Other Primary Human Tumor Types," *Genes Cancer,* vol. 1, no. 8, pp. 812-21, Aug 2010, doi: 10.1177/1947601910383418.

[85] Y. Yan *et al.*, "Bafilomycin A1 induces caspase-independent cell death in hepatocellular carcinoma cells via targeting of autophagy and MAPK pathways," *Sci Rep,* vol. 6, p. 37052, Nov 15 2016, doi: 10.1038/srep37052.

[86] S. Wang, F. Liu, Z. Zeng, H. Yang, and H. Jiang, "The Protective Effect of Bafilomycin A1 Against Cobalt Nanoparticle-Induced Cytotoxicity and Aseptic Inflammation in Macrophages In Vitro," *Biol Trace Elem Res,* vol. 169, no. 1, pp. 94-105, Jan 2016, doi: 10.1007/s12011-015-0381-9.

[87] U. Anand *et al.*, "Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics," *Genes & Diseases,* vol. 10, no. 4, pp. 1367-1401, 2023/07/01/ 2023, doi: https://doi.org/10.1016/j.gendis.2022.02.007.

[88] C. M. Tilsed, S. A. Fisher, A. K. Nowak, R. A. Lake, and W. J. Lesterhuis, "Cancer chemotherapy: insights into cellular and tumor microenvironmental mechanisms of action," (in eng), *Front Oncol,* vol. 12, p. 960317, 2022, doi: 10.3389/fonc.2022.960317.

[89] J. Minguet, K. H. Smith, C. P. Bramlage, and P. Bramlage, "Targeted therapies for treatment of renal cell carcinoma: recent advances and future perspectives," (in eng), *Cancer Chemother Pharmacol,* vol. 76, no. 2, pp. 219-33, Aug 2015, doi: 10.1007/s00280-015-2770-3.

[90] M. Herrera-Juárez, C. Serrano-Gómez, H. Bote-de-Cabo, and L. Paz-Ares, "Targeted therapy for lung cancer: Beyond EGFR and ALK," (in eng), *Cancer,* vol. 129, no. 12, pp. 1803-1820, Jun 15 2023, doi: 10.1002/cncr.34757.

[91] E. R. Sjoberg, L. D. Powell, A. Klein, and A. Varki, "Natural ligands of the B cell adhesion molecule CD22 beta can be masked by 9-O-acetylation of sialic acids," *Journal of Cell Biology,* vol. 126, no. 2, pp. 549-562, 1994, doi: 10.1083/jcb.126.2.549.

[92] M. T. Anim *et al.*, "Deacetylated Sialic Acid Sensitizes Lung and Colon Cancers to Novel Cucurbitacin-Inspired Estrone Epidermal Growth Factor Receptor (EGFR)

Inhibitor Analogs," *Molecules,* vol. 28, no. 17, p. 6257, 2023. [Online]. Available: https://www.mdpi.com/1420-3049/28/17/6257.

[93] R. L. Mather, K. F. Loveson, and H. L. Fillmore, "Human Sialic acid O-acetyl esterase (SIAE) – mediated changes in sensitivity to etoposide in a medulloblastoma cell line," *Scientific Reports,* vol. 9, no. 1, p. 8609, 2019/06/13 2019, doi: 10.1038/s41598-019-44950-5.

[94] M. Markman, "The Promise and Perils of 'Targeted Therapy' of Advanced Ovarian Cancer," *Oncology,* vol. 74, pp. 1 - 6, 2008.

[95] G. J. Peters, "From 'Targeted Therapy' to Targeted Therapy," (in eng), *Anticancer Res,* vol. 39, no. 7, pp. 3341-3345, Jul 2019, doi: 10.21873/anticanres.13476.

[96] M. Kudo, "Targeted therapy for liver cancer: updated review in 2012," (in eng), *Curr Cancer Drug Targets,* vol. 12, no. 9, pp. 1062-72, Nov 1 2012.

[97] Y. Yang, G. Wang, T. Song, C. B. Lebrilla, and A. J. R. Heck, "Resolving the micro-heterogeneity and structural integrity of monoclonal antibodies by hybrid mass spectrometric approaches," (in eng), *MAbs,* vol. 9, no. 4, pp. 638-645, May/Jun 2017, doi: 10.1080/19420862.2017.1290033.

[98] S. M. Chiavenna, J. P. Jaworski, and A. Vendrell, "State of the art in anti-cancer mAbs," *Journal of Biomedical Science,* vol. 24, no. 1, p. 15, 2017/02/20 2017, doi: 10.1186/s12929-016-0311-y.

[99] M. V. Gatzka, "Targeted Tumor Therapy Remixed—An Update on the Use of Small-Molecule Drugs in Combination Therapies," *Cancers,* vol. 10, no. 6, p. 155, 2018. [Online]. Available: https://www.mdpi.com/2072-6694/10/6/155.

[100] D. P. Byrne, D. M. Foulkes, and P. A. Eyers, "Pseudokinases: update on their functions and evaluation as new drug targets," (in eng), *Future Med Chem,* vol. 9, no. 2, pp. 245-265, Jan 2017, doi: 10.4155/fmc-2016-0207.

[101] D. Fabbro, S. W. Cowan-Jacob, and H. Moebitz, "Ten things you should know about protein kinases: IUPHAR Review 14," (in eng), *Br J Pharmacol,* vol. 172, no. 11, pp. 2675-700, Jun 2015, doi: 10.1111/bph.13096.

[102] A. M. Soderquist and G. Carpenter, "Glycosylation of the epidermal growth factor receptor in A-431 cells. The contribution of carbohydrate to receptor function," (in eng), *J Biol Chem,* vol. 259, no. 20, pp. 12586-94, Oct 25 1984.

[103] K. B. Whitson *et al.*, "Functional effects of glycosylation at Asn-579 of the epidermal growth factor receptor," (in eng), *Biochemistry,* vol. 44, no. 45, pp. 14920-31, Nov 15 2005, doi: 10.1021/bi050751j.

[104] A. Ullrich *et al.*, "Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells," (in eng), *Nature,* vol. 309, no. 5967, pp. 418-25, May 31-Jun 6 1984, doi: 10.1038/309418a0.

[105] F. Acheampong, T. Ostlund, M. Mahnashi, and F. Halaweish, "Antiproliferation and apoptosis studies of estrone pharmacophores in triple-negative breast cancer," (in eng), *Chem Biol Drug Des,* Jul 27 2023, doi: 10.1111/cbdd.14303.

[106] D. Cunningham *et al.*, "Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer," (in eng), *N Engl J Med,* vol. 351, no. 4, pp. 337-45, Jul 22 2004, doi: 10.1056/NEJMoa033025.

[107] Y. Huang *et al.*, "A molecular mechanism for the generation of ligand-dependent differential outputs by the epidermal growth factor receptor," *eLife,* vol. 10, p. e73218, 2021/11/30 2021, doi: 10.7554/eLife.73218.

[108] M. Azimzadeh Irani, S. Kannan, and C. Verma, "Role of N-glycosylation in EGFR ectodomain ligand binding," *Proteins: Structure, Function, and Bioinformatics,* vol. 85, no. 8, pp. 1529-1549, 2017.

[109] Z. Wang, "ErbB Receptors and Cancer," (in eng), *Methods Mol Biol,* vol. 1652, pp. 3-35, 2017, doi: 10.1007/978-1-4939-7219-7_1.

[110] D. L. Cadena, C. L. Chan, and G. N. Gill, "The intracellular tyrosine kinase domain of the epidermal growth factor receptor undergoes a conformational change upon autophosphorylation," (in eng), *J Biol Chem,* vol. 269, no. 1, pp. 260-5, Jan 7 1994.

[111] M. D. Brown and D. B. Sacks, "Protein scaffolds in MAP kinase signalling," (in eng), *Cell Signal,* vol. 21, no. 4, pp. 462-9, Apr 2009, doi: 10.1016/j.cellsig.2008.11.013.

[112] R. A. MacCorkle and T. H. Tan, "Mitogen-activated protein kinases in cell-cycle control," (in eng), *Cell Biochem Biophys,* vol. 43, no. 3, pp. 451-61, 2005, doi: 10.1385/cbb:43:3:451.

[113] A. A. Jorge, A. C. Malaquias, I. J. Arnhold, and B. B. Mendonca, "Noonan syndrome and related disorders: a review of clinical features and mutations in genes of the RAS/MAPK pathway," (in eng), *Horm Res,* vol. 71, no. 4, pp. 185-93, 2009, doi: 10.1159/000201106.

[114] J. Rotow and T. G. Bivona, "Understanding and targeting resistance mechanisms in NSCLC," (in eng), *Nat Rev Cancer,* vol. 17, no. 11, pp. 637-658, Oct 25 2017, doi: 10.1038/nrc.2017.84.

[115] P. J. Roberts and C. J. Der, "Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer," (in eng), *Oncogene,* vol. 26, no. 22, pp. 3291-310, May 14 2007, doi: 10.1038/sj.onc.1210422.

[116] J. G. Paez *et al.*, "EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy," (in eng), *Science,* vol. 304, no. 5676, pp. 1497-500, Jun 4 2004, doi: 10.1126/science.1099314.

[117] W. Liang *et al.*, "Network meta-analysis of erlotinib, gefitinib, afatinib and icotinib in patients with advanced non-small-cell lung cancer harboring EGFR mutations," (in eng), *PLoS One,* vol. 9, no. 2, p. e85245, 2014, doi: 10.1371/journal.pone.0085245.

[118] R. G. P. Gijtenbeek *et al.*, "Randomised controlled trial of first-line tyrosine-kinase inhibitor (TKI) versus intercalated TKI with chemotherapy for EGFR-mutated nonsmall cell lung cancer," (in eng), *ERJ Open Res,* vol. 8, no. 4, Oct 2022, doi: 10.1183/23120541.00239-2022.

[119] D. J. Newman, G. M. Cragg, and K. M. Snader, "Natural products as sources of new drugs over the period 1981-2002," (in eng), *J Nat Prod,* vol. 66, no. 7, pp. 1022-37, Jul 2003, doi: 10.1021/np030096l.

[120] M. Huang, J. J. Lu, and J. Ding, "Natural Products in Cancer Therapy: Past, Present and Future," (in eng), *Nat Prod Bioprospect,* vol. 11, no. 1, pp. 5-13, Feb 2021, doi: 10.1007/s13659-020-00293-7.

[121] E. Rajesh, L. S. Sankari, L. Malathi, and J. R. Krupaa, "Naturally occurring products in cancer therapy," (in eng), *J Pharm Bioallied Sci,* vol. 7, no. Suppl 1, pp. S181-3, Apr 2015, doi: 10.4103/0975-7406.155895.

[122] M. Miró, "Cucurbitacins and their pharmacological effects," *Phytotherapy Research,* vol. 9, no. 3, pp. 159-168, 1995, doi: https://doi.org/10.1002/ptr.2650090302.

[123] M. Clericuzio, M. Mella, P. Vita-Finzi, M. Zema, and G. Vidari, "Cucurbitane triterpenoids from Leucopaxillus gentianeus," (in eng), *J Nat Prod,* vol. 67, no. 11, pp. 1823-8, Nov 2004, doi: 10.1021/np049883o.

[124] M. C. Recio *et al.*, "Anti-inflammatory activity of two cucurbitacins isolated from Cayaponia tayuya roots," (in eng), *Planta Med,* vol. 70, no. 5, pp. 414-20, May 2004, doi: 10.1055/s-2004-818968.

[125] A. A. Alghasham, "Cucurbitacins - a promising target for cancer therapy," (in eng), *Int J Health Sci (Qassim),* vol. 7, no. 1, pp. 77-89, Jan 2013, doi: 10.12816/0006025.

[126] J. C. Chen, M. H. Chiu, R. L. Nie, G. A. Cordell, and S. X. Qiu, "Cucurbitacins and cucurbitane glycosides: structures and biological activities," (in eng), *Nat Prod Rep,* vol. 22, no. 3, pp. 386-99, Jun 2005, doi: 10.1039/b418841c.

[127] K. L. Duncan, M. D. Duncan, M. C. Alley, and E. A. Sausville, "Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells," (in eng), *Biochem Pharmacol,* vol. 52, no. 10, pp. 1553-60, Nov 22 1996, doi: 10.1016/s0006-2952(96)00557-6.

[128] N. Wakimoto *et al.*, "Cucurbitacin B has a potent antiproliferative effect on breast cancer cells in vitro and in vivo," (in eng), *Cancer Sci,* vol. 99, no. 9, pp. 1793-7, Sep 2008, doi: 10.1111/j.1349-7006.2008.00899.x.

[129] X. Chen *et al.*, "Biological activities and potential molecular targets of cucurbitacins: a focus on cancer," (in eng), *Anticancer Drugs,* vol. 23, no. 8, pp. 777-87, Sep 2012, doi: 10.1097/CAD.0b013e3283541384.

[130] S. Garg, S. C. Kaul, and R. Wadhwa, "Cucurbitacin B and cancer intervention: Chemistry, biology and mechanisms (Review)," (in eng), *Int J Oncol,* vol. 52, no. 1, pp. 19-37, Jan 2018, doi: 10.3892/ijo.2017.4203.

[131] B. Jayaprakasam, N. P. Seeram, and M. G. Nair, "Anticancer and antiinflammatory activities of cucurbitacins from Cucurbita andreana," (in eng), *Cancer Lett,* vol. 189, no. 1, pp. 11-6, Jan 10 2003, doi: 10.1016/s0304-3835(02)00497-4.

[132] J. Sun, M. A. Blaskovich, R. Jove, S. K. Livingston, D. Coppola, and S. M. Sebti, "Cucurbitacin Q: a selective STAT3 activation inhibitor with potent antitumor activity," (in eng), *Oncogene,* vol. 24, no. 20, pp. 3236-45, May 5 2005, doi: 10.1038/sj.onc.1208470.

[133] M. A. Blaskovich, J. Sun, A. Cantor, J. Turkson, R. Jove, and S. M. Sebti, "Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice," (in eng), *Cancer Res,* vol. 63, no. 6, pp. 1270-9, Mar 15 2003.

[134] P. Yang, W. Liu, R. Fu, G.-b. Ding, S. Amin, and Z. Li, "Cucurbitacin E Chemosensitizes Colorectal Cancer Cells via Mitigating TFAP4/Wnt/β-Catenin Signaling," *Journal of Agricultural and Food Chemistry,* vol. 68, no. 48, pp. 14148-14160, 2020/12/02 2020, doi: 10.1021/acs.jafc.0c05551.

[135] S. Yasuda, S. Yogosawa, Y. Izutani, Y. Nakamura, H. Watanabe, and T. Sakai, "Cucurbitacin B induces G2 arrest and apoptosis via a reactive oxygen species-dependent mechanism in human colon adenocarcinoma SW480 cells," (in eng), *Mol Nutr Food Res,* vol. 54, no. 4, pp. 559-65, Apr 2010, doi: 10.1002/mnfr.200900165.

[136] A. C. Cheng, Y. C. Hsu, and C. C. Tsai, "The effects of cucurbitacin E on GADD45β-trigger G2/M arrest and JNK-independent pathway in brain cancer cells," (in eng), *J Cell Mol Med,* vol. 23, no. 5, pp. 3512-3519, May 2019, doi: 10.1111/jcmm.14250.

[137] M. Mahnashi, S. M. Elgazwi, M. S. Ahmed, and F. T. Halaweish, "Cucurbitacins inspired organic synthesis: Potential dual inhibitors targeting EGFR – MAPK pathway," *European Journal of Medicinal Chemistry,* vol. 173, pp. 294-304, 2019/07/01/ 2019, doi: https://doi.org/10.1016/j.ejmech.2019.04.018.

[138] J. D. Langereis and M. I. de Jonge, "Invasive Disease Caused by Nontypeable Haemophilus influenzae," (in eng), *Emerg Infect Dis,* vol. 21, no. 10, pp. 1711-8, Oct 2015, doi: 10.3201/eid2110.150004.

[139] D. Osei-Safo *et al.*, "Constituents of the Roots of Dichapetalum pallidum and Their Anti-Proliferative Activity," *Molecules,* vol. 22, no. 4, p. 532, 2017. [Online]. Available: https://www.mdpi.com/1420-3049/22/4/532.

[140] I. Tuffour, I. Ayi, T. M. Gwira, E. Dumashie, Y. Ashong, and R. Appiah-Opong, "Schistosoma Egg Antigen Induces Oncogenic Alterations in Human Prostate Cells,"

(in eng), *Anal Cell Pathol (Amst),* vol. 2018, p. 4675380, 2018, doi: 10.1155/2018/4675380.

[141] T. Ostlund *et al.*, "Triazole-estradiol analogs: A potential cancer therapeutic targeting ovarian and colorectal cancer," *Steroids,* vol. 177, p. 108950, 2022/01/01/ 2022, doi: https://doi.org/10.1016/j.steroids.2021.108950.

[142] G. Koopman, C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals, and M. H. van Oers, "Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis," (in eng), *Blood,* vol. 84, no. 5, pp. 1415-20, Sep 1 1994.

[143] B. Verhoven, R. A. Schlegel, and P. Williamson, "Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes," *Journal of Experimental Medicine,* vol. 182, no. 5, pp. 1597-1601, 1995, doi: 10.1084/jem.182.5.1597.

[144] V. A. Fadok, D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson, "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages," (in eng), *J Immunol,* vol. 148, no. 7, pp. 2207-16, Apr 1 1992.

[145] G. Zhang, V. Gurtu, S. R. Kain, and G. Yan, "Early detection of apoptosis using a fluorescent conjugate of annexin V," (in eng), *Biotechniques,* vol. 23, no. 3, pp. 525-31, Sep 1997, doi: 10.2144/97233pf01.

[146] J. C. Chambard, R. Lefloch, J. Pouysségur, and P. Lenormand, "ERK implication in cell cycle regulation," (in eng), *Biochim Biophys Acta,* vol. 1773, no. 8, pp. 1299-310, Aug 2007, doi: 10.1016/j.bbamcr.2006.11.010.

[147] S. R. Datta *et al.*, "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery," (in eng), *Cell,* vol. 91, no. 2, pp. 231-41, Oct 17 1997, doi: 10.1016/s0092-8674(00)80405-5.

[148] M. H. Cardone *et al.*, "Regulation of cell death protease caspase-9 by phosphorylation," (in eng), *Science,* vol. 282, no. 5392, pp. 1318-21, Nov 13 1998, doi: 10.1126/science.282.5392.1318.

[149] R. J. Duronio and Y. Xiong, "Signaling pathways that control cell proliferation," (in eng), *Cold Spring Harb Perspect Biol,* vol. 5, no. 3, p. a008904, Mar 1 2013, doi: 10.1101/cshperspect.a008904.

[150] P. Wee and Z. Wang, "Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways," (in eng), *Cancers (Basel),* vol. 9, no. 5, May 17 2017, doi: 10.3390/cancers9050052.

[151] J. H. Chung, Y. Zhang, and F. Bunz, "Checkpoint bypass and cell viability," (in eng), *Cell Cycle,* vol. 9, no. 11, pp. 2102-7, Jun 1 2010, doi: 10.4161/cc.9.11.11849.

[152] Z. Wang, "Regulation of Cell Cycle Progression by Growth Factor-Induced Cell Signaling," (in eng), *Cells,* vol. 10, no. 12, Nov 26 2021, doi: 10.3390/cells10123327.

[153] J. P. Plastaras *et al.*, "Cell cycle dependent and schedule-dependent antitumor effects of sorafenib combined with radiation," (in eng), *Cancer Res,* vol. 67, no. 19, pp. 9443-54, Oct 1 2007, doi: 10.1158/0008-5472.Can-07-1473.

[154] H. Zhang, Z. Li, and K. Wang, "Combining sorafenib with celecoxib synergistically inhibits tumor growth of non-small cell lung cancer cells in vitro and in vivo," (in eng), *Oncol Rep,* vol. 31, no. 4, pp. 1954-60, Apr 2014, doi: 10.3892/or.2014.3026.

[155] M. G. Ormerod, R. M. Orr, and J. H. Peacock, "The role of apoptosis in cell killing by cisplatin: a flow cytometric study," (in eng), *Br J Cancer,* vol. 69, no. 1, pp. 93-100, Jan 1994, doi: 10.1038/bjc.1994.14.

[156] G. R. Stark and W. R. Taylor, "Analyzing the G2/M checkpoint," (in eng), *Methods Mol Biol,* vol. 280, pp. 51-82, 2004, doi: 10.1385/1-59259-788-2:051.

[157] M. S. Ahmed, F. El-Senduny, J. Taylor, and F. T. Halaweish, "Biological screening of cucurbitacin inspired estrone analogs targeting mitogen-activated protein kinase (MAPK) pathway," (in eng), *Chem Biol Drug Des,* vol. 90, no. 3, pp. 478-484, Sep 2017, doi: 10.1111/cbdd.12963.

[158] F. J. Hendler and B. W. Ozanne, "Human squamous cell lung cancers express increased epidermal growth factor receptors," (in eng), *J Clin Invest,* vol. 74, no. 2, pp. 647-51, Aug 1984, doi: 10.1172/jci111463.

[159] J. Richard, C. Sainsbury, G. Needham, J. Farndon, A. Malcolm, and A. Harris, "EPIDERMAL-GROWTH-FACTOR RECEPTOR STATUS AS PREDICTOR OF EARLY RECURRENCE OF AND DEATH FROM BREAST CANCER," *The Lancet,* vol. 329, no. 8547, pp. 1398-1402, 1987/06/20/ 1987, doi: https://doi.org/10.1016/S0140-6736(87)90593-9.

[160] D. E. Neal *et al.*, "Epidermal-growth-factor receptors in human bladder cancer: comparison of invasive and superficial tumours," (in eng), *Lancet,* vol. 1, no. 8425, pp. 366-8, Feb 16 1985, doi: 10.1016/s0140-6736(85)91386-8.

[161] C. Deng *et al.*, "Novel recombinant immunotoxin of EGFR specific nanobody fused with cucurmosin, construction and antitumor efficiency in vitro," (in eng), *Oncotarget,* vol. 8, no. 24, pp. 38568-38580, Jun 13 2017, doi: 10.18632/oncotarget.16930.

[162] Y. D. Shaul and R. Seger, "The MEK/ERK cascade: from signaling specificity to diverse functions," (in eng), *Biochim Biophys Acta,* vol. 1773, no. 8, pp. 1213-26, Aug 2007, doi: 10.1016/j.bbamcr.2006.10.005.

[163] J. W. Ramos, "The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells," (in eng), *Int J Biochem Cell Biol,* vol. 40, no. 12, pp. 2707-19, 2008, doi: 10.1016/j.biocel.2008.04.009.

[164] Z. Zou, T. Tao, H. Li, and X. Zhu, "mTOR signaling pathway and mTOR inhibitors in cancer: progress and challenges," *Cell & Bioscience,* vol. 10, no. 1, p. 31, 2020/03/10 2020, doi: 10.1186/s13578-020-00396-1.