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Upregulation of cytidine deaminase in *NAT1* knockout breast cancer cells

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Abstract

Purpose—Arylamine *N*-acetyltransferase 1 (NAT1), a phase II metabolic enzyme, is frequently upregulated in breast cancer. Inhibition or depletion of NAT1 leads to growth retardation in breast cancer cells *in vitro* and *in vivo*. A previous metabolomics study of MDA-MB-231 breast cancer cells suggest that *NAT1* deletion leads to a defect in *de novo* pyrimidine biosynthesis. In the present study, we observed that *NAT1* deletion results in upregulation of cytidine deaminase (CDA), which is involved in the pyrimidine salvage pathway, in multiple breast cancer cell lines (MDA-MB-231, MCF-7 and ZR-75–1). We hypothesized that *NAT1* KO MDA-MB-231 cells show differential sensitivity to drugs that either inhibit cellular pyrimidine homeostasis or are metabolized by CDA.

Methods—The cells were treated with 1) inhibitors of dihydroorotate dehydrogenase or CDA (e.g., teriflunomide and tetrahydrouridine); 2) pyrimidine/nucleoside analogs (e.g., gemcitabine and 5-azacytidine); and 3) naturally occurring, modified cytidines (e.g., 5-formyl-2'-deoxycytidine; 5fdC).

Results—Although *NAT1* KO cells failed to show differential sensitivity to nucleoside analogs that are metabolized by CDA, they were markedly more sensitive to 5fdC which induces DNA damage in the presence of high CDA activity. Co-treatment with 5fdC and a CDA inhibitor, tetrahydrouridine, abrogated the increase in 5fdC cytotoxicity in *NAT1* KO cells, suggesting that the increased sensitivity of *NAT1* KO cells to 5fdC is dependent on their increased CDA activity.

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Author Contributions

The study conception and design were done by Kyung U. Hong and David W. Hein. Material preparation, acquisition, analysis, or interpretation of data was performed by Kyung U. Hong, Afi H. Tagnedji, Mark A. Doll, and Kennedy M. Walls. The first draft of the manuscript was written by Kyung U. Hong and Afi H. Tagnedji, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Conclusions—The present findings suggest a novel therapeutic strategy to treat breast cancer with elevated *NAT1* expression. For instance, NAT1 inhibition may be combined with cytotoxic nucleosides (e.g., 5fdC) for breast cancer treatment.

Keywords

Breast cancer; arylamine N-acetyltransferase; cytidine deaminase; chemotherapeutic; pyrimidine biosynthesis; cytidine analogs

Introduction

Arylamine *N*-acetyltransferase 1 (NAT1) is a phase II metabolic enzyme that metabolizes a variety of drugs (e.g., isoniazid) and carcinogens (e.g., aromatic amines) by transferring an acetyl group from acetyl-CoA to the primary amine of its substrates (Hein et al. 2000; Hein 2002). NAT1 can also hydrolyze acetyl-CoA to CoA in the presence of folate (Laurieri et al. 2014; Stepp et al. 2015, 2017), and accordingly, depletion of NAT1 leads to increased cellular acetyl-CoA level in MDA-MB-231 breast cancer cells (Stepp et al. 2019). More recently, NAT1 has been implicated in breast cancer biology. Studies have reported that NAT1 is frequently upregulated in breast cancer, and its expression is associated with estrogen receptor expression in breast cancer (Zhang et al. 2018; Minchin and Butcher 2018; Carlisle and Hein 2018).

We and others have investigated the role of NAT1 in breast cancer using *in vitro* and *in vivo* model systems. Small molecule inhibitors, gene-specific siRNAs/shRNAs, and CRISPR/Cas9 knockout (KO) approaches have been used to inhibit or deplete NAT1 in breast and other cancer cell types (Tiang et al. 2010, 2011; Stepp et al. 2018, 2019; Leggett et al. 2022). One of the reproducible findings from the previous studies is that inhibition or depletion of NAT1 result in a significant decrease in both anchorage-dependent and -independent cell growth in breast cancer cell lines (Tiang et al. 2010, 2011; Stepp et al. 2019), suggesting that NAT1 contributes to the growth and metastasis of breast cancer cells. In a previous study, the parental and *NAT1* KO MDA-MB-231, triple-negative breast cancer cells, were xenografted into the flank of immunocompromised mice. The primary and secondary tumors originating from *NAT1* KO cells were significantly smaller than those formed by the parental cells (Doll et al. 2022), consistent with the findings *in vitro*. The frequency of lung metastasis, however, was not altered in *NAT1* KO cells (Doll et al. 2022), suggesting that NAT1 is not essential for the process of metastasis. So far, mitochondrial dysfunction has been proposed as a mechanism that leads to the growth retardation observed in *NAT1* KO cancer cells (Wang et al. 2019a; Hong et al. 2022). However, the role of NAT1 in breast cancer pathobiology still remains largely unknown.

To generate hypotheses as to the mechanism(s) by which NAT1 contributes to breast cancer cell growth, our research group has performed omics analyses (i.e., transcriptomics, metabolomics, and proteomics) of the parental versus *NAT1* KO MDA-MB-231 cells. One of the interesting findings from the metabolomics study is that *NAT1* KO cells apparently exhibit a defect in *de novo* pyrimidine biosynthesis (Carlisle et al. 2020). This was indicated by a significant and concomitant decrease in the level of intermediates that are involved

in the *de novo* pyrimidine biosynthetic pathway, including *N*-carbamoyl aspartate, orotate, UTP, and CDP, in two different *NAT1* KO MDA-MB-231 cell lines (Carlisle et al. 2020). In addition, cytidine, a ribosyl pyrimidine, was among the few metabolites whose levels were positively correlated with NAT1 activity (Carlisle et al. 2020). In other words, low or no NAT1 activity was associated with decreased levels of cellular cytidine. Pyrimidines (i.e., cytosine, uracil, and thymine) play a critical role in cellular metabolism (Jones 1980; Traut 1994) and serve as precursors of RNA and DNA. As such, *de novo* pyrimidine biosynthesis is indispensable during cell growth to meet the demand for nucleic acid precursors and other cellular components. Accordingly, the *de novo* pyrimidine biosynthetic pathway is invariably upregulated in rapidly growing cancer cells (Weber 2001). The apparent defect in *de novo* pyrimidine biosynthesis observed in *NAT1* KO cells is also supported by our proteomics study (Hong et al. 2022). According to the study, one of the most highly upregulated proteins in two separate *NAT1 KO* MDA-MB-231 cell lines is cytidine deaminase (CDA). Moreover, our transcriptomics data shows that CDA is also upregulated at the mRNA level in *NAT1* KO cells (Carlisle et al. 2021). CDA catalyzes the conversion of (deoxy)cytidine to (deoxy)uridine, an important step in pyrimidine salvage pathway (Lane and Fan 2015) which recycles nucleosides and free bases generated during DNA or RNA degradation (or present in their environment) and convert them back to nucleotide monophosphates (Lane and Fan 2015). Taken together, it is plausible that the deficiency in the *de novo* pyrimidine synthesis contributes to 1) the growth retardation observed in *NAT1* KO breast cancer cells and 2) upregulation of compensatory, pyrimidine salvage pathway (indicated by upregulation of CDA).

Although the mechanism by which *NAT1* deletion leads to an alteration of the pyrimidine biosynthetic pathway is currently unclear, this finding has an implication in breast cancer therapy. Targeting the pathway has been shown to be a successful strategy in treating different types of tumors (Rabinovich et al. 2015; Mohamad Fairus et al. 2017; Madak et al. 2019; Wang et al. 2019b). Brown and colleagues have reported that resistance to chemotherapy in triple-negative breast cancer cells can be overcome by inhibiting the *de novo* pyrimidine biosynthesis pathway (Brown et al. 2017). In addition, upregulation of CDA also has an implication in cancer therapy. Nucleoside analogs (also known as antimetabolites), such as pyrimidine and purine derivatives, are mainstay in chemotherapy (Galmarini et al. 2003). They inhibit the synthesis of the nucleosides or the nucleotides necessary for proliferation and progression of cancer cells. Among these, the efficacy (and adverse effects) of a group of widely prescribed pyrimidine analogs, namely gemcitabine, capecitabine, cytarabine, and azacytidine, are largely dependent on CDA activity (Serdjebi et al. 2015). CDA catalyzes their deamination, which leads to either deactivation (gemcitabine, cytarabine and azacytidine) or activation (capecitabine). For this reason, genetic and epigenetic polymorphisms in the *CDA* gene, that result in differential CDA activity, have been shown to significantly impact the pharmacokinetics and pharmacodynamics of the aforementioned pyrimidine analogs in patients (Li et al. 2008; Ciccolini et al. 2011; Serdjebi et al. 2015).

In the present study, we verified that *NAT1* deletion leads to a significant upregulation of CDA in multiple breast cancer cell lines. In order to assess the functional importance of this finding, we treated the parental and two *NAT1* KO MDA-MB-231 cell lines with 1)

inhibitors of pyrimidine biosynthesis and 2) nucleoside analogs that are metabolized by CDA; and 3) naturally occurring and epigenetically modified cytidines, and measured the sensitivity of the cell lines to each agent.

Materials and Methods

Cell Culture

The *NAT1* KO cell lines were generated previously using CRISPR/Cas9 technology in breast cancer cell lines, MDA-MB-231, MCF-7, and ZR-75–1 (Stepp et al. 2018, 2019). Two different guide RNAs were used to generate two separate *NAT1* KO cell lines (KO2 and KO5) in MDA-MB-231 and MCF-7 cells, and one *NAT1* KO cell line (KO2) was generated in ZR-75–1 cells. The generation and characterization of *NAT1* KO cell lines have been described elsewhere in detail (Stepp et al. 2018, 2019). All cell lines were cultured in high glucose (4 g/L) DMEM, supplemented with 10% fetal bovine serum and penicillin-streptomycin solution (penicillin 100 U/mL; streptomycin 100 μg/mL). Cells were cultured at 37°C in a humidified incubator with 5% CO_2. Media was changed every three days. At approximately 85% confluence, cells were subcultured using 0.25% trypsin/1 mM EDTA solution. All cell lines described above were authenticated via the ATCC (American Type Culture Collection) cell authentication service.

RT-qPCR Analysis

For isolation of total RNA, E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek) was used according to the manufacturer's instructions with an added, in-column DNase digestion step. For cDNA synthesis, 500 ng total RNA was processed in a 10-μl reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For quantitative PCR, 1 μl of cDNA, the gene-specific primer set, and iTaq Universal SYBR Green Supermix (Bio-Rad) were used. For PCR amplification and quantification, StepOne™ Real-Time PCR System (Applied Biosystems) was used. Samples underwent 40 cycles of 2-step PCR (denaturation at 96 °C [15 sec] followed by annealing and extension at 60 °C [1 min]). For calculation of relative levels of the gene of interest, delta-delta Ct method (a.k.a., the $2^{-\Delta\Delta Ct}$ method) was used using *GAPDH* as an internal control. The following primer sequences were used: *CDA* (human) forward, 5'-AAGGCCGTCTCAGAAGGGTA-3' and reverse, 5'-CCATCCGGCTTGGTCATGTA-3'; *GAPDH* (human) forward, 5'-GGTGAAGCAGGCGTCGGAGG-3' and reverse 5'-GAGGGCAATGCCAGCCCCAG-3'. Statistical significance was assessed by one-way ANOVA with Bonferroni's posttest using GraphPad Prism v8.2.1 (GraphPad Software). The results are expressed as the mean ± the standard error of the mean (SEM). The p values indicate the statistical significance between the parental and each of the two *NAT1* KO cell lines. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Western Blot

The cells were lysed in Laemmli buffer (50 mM Tris-Cl [pH 6.8], 2% sodium dodecyl sulfate [SDS; w/v], 0.1% bromophenol blue, 10% [v/v] glycerol) and boiled for 10 minutes. Protein concentrations were determined using Pierce BCA Assay kit (Thermo Scientific) per manufacturer's instructions. β-mercaptoethanol was then added to each sample and boiled

for additional 5 minutes. Fifty μg of protein per sample was loaded and separated on a 4–12% gradient Bis-Tris Plus polyacrylamide gel (Invitrogen). The gel was transferred to a PVDF membrane and blocked in 5% (w/v) skim milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 30 minutes. Membranes were incubated with primary antibodies at room temperature for 1 hr. Membranes were washed with TBST. Membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies (1:5,000) (Bio-Rad). Membranes were washed, and the protein-antibody complex was detected using chemiluminescent substrate (Thermo Scientific). Polyclonal rabbit anti-CDA (center) antibody (Cat. No. SAB1300717) (Zauri et al. 2015) and mouse monoclonal anti-α-tubulin antibody (clone B-5-1-2) were obtained from Sigma-Aldrich. They were diluted in 5% skim milk in TBST 1:250 for CDA antibody and 1:5,000 for α-tubulin antibody.

CDA Activity Assay

Parental and *NAT1* KO cells were lysed in a lysis buffer containing 20 mM sodium phosphate pH 7.4, 1 mM EDTA, 0.2% Triton X-100, 1 mM DTT, 100 μM PMSF, 1 μg/ml aprotinin, and 2 μM pepstatin A. Cell lysate was centrifuged at 15,000 × g for 20 min in the cold room, and the resulting supernatant was used for the assay. CDA enzymatic reactions were performed using 100 μg of cell lysate and 300 μM cytidine. Reactions were terminated by the addition of 1/10 volume of 1 M acetic acid. Proteins were precipitated by centrifugation (15,000 × g for 10 min) and supernatant was injected onto a C-18 (250 mm × 4 mm; 5 μm) reverse phase column using an Agilent 1260 Infinity II system (Agilent Technologies). The following gradient was used to separate cytidine and uridine: 100% 55 mM sodium phosphate pH 4.0/0% acetonitrile to 0% 55 mM sodium phosphate pH 4.0/100% acetonitrile over 10 mins. The products were detected at 250 nm. Cytidine and uridine (Sigma-Aldrich) served as standards.

Drug Treatment

Drugs tested in this study (Fig. 3) were obtained as follows: teriflunomide (Tocris Bioscience); leflunomide (Selleckchem); zebularine (Cayman Chemical); tetrahydrouridine (THU); gemcitabine, 5-azacytidine, Ara-C, and capecitabine (Sigma-Aldrich); 5-formyl-2'-deoxycytidine (5fdC; Berry & Associates); and 5-hydroxymethyl-2'-deoxycytidine (5hmdC; Cayman Chemical). THU was solubilized in distilled water, and the rest were solubilized in DMSO. The parental and two *NAT1* KO MDA-MB-231 cell lines (KO2 and KO5) were plated the day before the treatment on 96-well plates at density of 9,000 cells per well. For treatment with teriflunomide, leflunomide, THU, or zebularine, the cells were treated with the indicated concentrations for 3 days. For treatment with gemcitabine, 5-azacytidine, Ara-C, capecitabine, 5fdC, or 5hmdC, the cells were treated with the indicated concentrations for 7 days. For the co-treatment experiment with 5fdC and THU, the cells were treated with the indicated concentrations for 3 days.

Cell Viability Measurement

For analysis of cell viability, alamarBlue assay was performed according to the manufacturer's instructions (Thermo Fisher). Briefly, the cell media was replaced with media containing 10% alamarBlue and incubated for 3 hours. The fluorescence was measured on a fluorometric plate reader at 530/590 nm. The background was subtracted

from the resulting values. The relative cell viability was calculated using the vehicle control as the reference and expressed as a percentage of the control value. Statistical significance was assessed by two-way ANOVA with Bonferroni's posttest using GraphPad Prism v8.2.1 (GraphPad Software). The results are expressed as the mean ± the standard error of the mean (SEM). The p values indicate the statistical significance between the parental and each of the two *NAT1* KO cell lines. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

Upregulation of CDA in *NAT1* KO breast cancer cells

In our previous proteomic analysis of the parental vs. *NAT1* KO MDA-MB-231 breast cancer cells, CDA is one of the most upregulated proteins in two different *NAT1* KO cell lines (KO2 and KO5), compared to the parental cells (4.7-fold increase in KO2 and 4.0-fold increase in KO5) (Hong et al. 2022). In accordance, the *CDA* transcripts also were significantly elevated in *NAT1* KO cells in our RNAseq study (1.9-fold increase in KO2 and 3.2-fold increase in KO5) (Carlisle et al. 2021).

We performed RT-qPCR analysis for *CDA* mRNA to verify this data and investigate if the loss of *NAT1* resulted in upregulation of *CDA* mRNA expression in different breast cancer cell lines (MDA-MB-231, MCF-7, and ZR-75–1). The *CDA* mRNA level was invariably and significantly elevated in *NAT1* KO cells, compared to their parental cell counterparts in all three breast cancer cell lines (Fig. 1A). Among three cell lines, the parental MDA-MB-231 cells expressed the highest level of *CDA* transcript, while its level in MCF-7 and ZR-75–1 cells was markedly low (~0.1% of that in MDA-MB-231) (Fig. 1B). Consistent with our findings, a previous study reported that *CDA* mRNA level was the highest in MDA-MB-231 cells among 34 different, publicly available breast cancer cell lines, and that MCF-7 cells are deficient of CDA due to epigenetic silencing of the *CDA* gene (Mameri et al. 2017). Western blot analysis was conducted to compare the CDA protein level in three cell lines and their *NAT1* KO counterparts. The CDA protein was not detected in MCF-7 and ZR-75–1 but was readily detectable in MDA-MB-231 cells (Fig. 1C). Both *NAT1* KO MDA-MB-231 cell lines (i.e., KO2 and KO5) expressed higher levels of the CDA protein, compared to the parental cells (Fig. 1C). Next, we measured CDA enzymatic activity in parental vs. *NAT1* KO cells to investigate if the increased CDA mRNA and protein expression ultimately results in an increase in the enzyme activity. The parental and *NAT1* KO cell lysates were incubated with cytidine, and its conversion to uridine was quantified using HPLC. As expected, *NAT1* KO MDA-MB-231 cells showed a marked increase in the CDA enzyme activity (64.7 ± 38.0 nmoles/min/mg protein in KO2 and 65.7 ± 34.7 nmoles/min/mg protein in KO5; mean ± SD), compared to the parental cells (7.2 ± 1.6 nmoles/min/mg protein) (Fig. 1D). The CDA activity, however, was considerably and relatively low in MCF-7 and ZR-75–1 cells (0.051 ± 0.012 in parental MCF-7 and 0.117 ± 0.056 in parental ZR-75–1), and no increases in the CDA activity was detected in their corresponding *NAT1* KO cell lines. Taken together, these findings indicate that depletion of NAT1 results in upregulation of CDA in breast cancer cell lines.

We analyzed the RNAseq data from our previous transcriptomics study on parental and *NAT1* KO MDA-MB-231 cells (Carlisle et al. 2021) to investigate if additional genes

involved in the pyrimidine salvage pathway were also significantly upregulated in *NAT1* KO cells (Table 1). Along with CDA, *CMPK1* (cytidine monophosphate [UMP-CMP] kinase 1), *ENTPD8* (ectonucleoside triphosphate diphosphohydrolase 8), and *UPRT* (uracil phosphoribosyltransferase; *FUR1* homolog) were also significantly upregulated ("Up") in both *NAT1* KO cell lines, compared to the parental cells (Table 1). Other genes in the pathway, such as *CTPS1* (CTP synthase 1), *CTPS2* (CTP synthase 2), *ENTPD1*, *ENTPD3*, and *UPP1* (uridine phosphorylase 1), were found upregulated in only one of *NAT1* KO cell lines. The only gene that was significantly downregulated ("Down") was *UCK1* (uridine-cytidine kinase 1), which was in *NAT1* KO2 cell line only. Taken together, the current findings suggest that deletion of *NAT1* in breast cancer cells leads to upregulation of CDA and the pyrimidine salvage pathway.

Correlation between *NAT1* and *CDA* expression levels in human tumors and normal tissues

Since there appeared to be an inverse relationship between *NAT1* and *CDA* mRNA levels in the breast cancer cell lines we tested, we analyzed gene expression databases (The Cancer Genome Atlas [TCGA] and Genotype-Tissue Expression [GTEx]) to investigate if an inverse relationship exists in human breast cancer or in normal human tissues. Using graphing and statistical tools available at GEPIA (Tang et al. 2017), we compared the transcript levels of *NAT1* and *CDA* in 1) breast tumors only (TCGA), 2) all tumors (TCGA), and 3) normal tissues (GTEx) (Fig. 2). As previously reported, *NAT1* expression was variable in breast cancer (Fig. 2a). *CDA* expression, however, did not correlate with *NAT1* expression (p value = 0.54; Pearson correlation coefficient, R = −0.019) (Fig. 2a). When expression of *NAT1* and *CDA* were analyzed using all TCGA tumors, a slight, yet negligible, negative correlation (R = −0.057) was found (Fig. 2b). In contrast, in normal tissues from GTEx database, a slight positive correlation (R = 0.055) between *NAT1* and *CDA* expression was observed (Fig. 2c). Based on these data, we found no consistent correlation between expression of two genes.

De novo pyrimidine biosynthesis and CDA inhibitors

Our previous omics analyses (Carlisle et al. 2020; Hong et al. 2022) and the current data suggest that *NAT1* KO MDA-MB-231 cells exhibit a defect in the *de novo* pyrimidine synthesis pathway and as a result, rely on the pyrimidine salvage pathway for their growth, although no direct evidence is available. To test this further, we subjected the parental and *NAT1* KO MDA-MB-231 cells to inhibitors of the *de novo* pyrimidine biosynthesis or CDA. Leflunomide and its active metabolite, teriflunomide, (Fig. 3) selectively and reversibly inhibit dihydro-orotate dehydrogenase, a key enzyme in the *de novo* pyrimidine synthesis pathway (Bar-Or et al. 2014). Tetrahydrouridine (Cohen and Wolfenden 1971) and zebularine (1-(β-d-ribofuranosyl)-1,2-dihydropyrimidin-2-one) (Marquez et al. 2005) (Fig. 3) are known inhibitors of CDA. We reasoned that *NAT1* KO cells would be more resistant to teriflunomide and leflunomide due to activation of the pyrimidine salvage pathway, while being more sensitive to CDA inhibitors (i.e., tetrahydrouridine and zebularine) if they required the pyrimidine salvage pathway for their growth and survival. The parental and two *NAT1* KO cell lines of MDA-MB-231 (KO2 and KO5) were treated with varying concentrations of each inhibitor for 3 days and assessed for cell viability. Compared to the

parental cells, both *NAT1* KO cell lines were moderately, yet concomitantly, resistant to both teriflunomide and leflunomide at selective concentrations (Fig. 4). With teriflunomide, the *NAT1* KO cells were relatively more resistant at concentrations between 6 and 60 μM (Fig. 4). However, the difference in the cell viability was not consistently observed at the highest concentration tested (i.e., 200 μM). Similarly, the relative resistance to leflunomide observed in *NAT1* KO cells was moderate, yet significant, at most concentrations tested (Fig. 4). Interestingly, *NAT1* KO cells were also relatively resistant to two different CDA inhibitors we tested (i.e., tetrahydrouridine and zebularine), and this was contrary to our expectation. At concentrations up to 20 μM of tetrahydrouridine and zebularine, the difference in cell viability was not apparent, but at 60 or 200 μM, *NAT1* KO cells showed a significantly higher cell viability compared to the parental cells, in the presence of either CDA inhibitor (Fig. 4). This data suggested that CDA activity is not required for the normal growth of *NAT1* KO cells.

Pyrimidine nucleoside analogs

Nucleoside analogs, such as pyrimidine and purine derivatives, are commonly employed in chemotherapy. They are known to inhibit proliferation of cancer cells by inhibiting the synthesis of the nucleosides or nucleotides necessary for DNA synthesis (Galmarini et al. 2003). Among these, pyrimidine nucleoside analogs, including gemcitabine, capecitabine, cytarabine, and 5-azacytidine, are widely prescribed in treatment of cancer (Galmarini et al. 2003). CDA is known to metabolize these drugs and influence their efficacy as well as their side effects (Serdjebi et al. 2015). Specifically, deamination of the drugs by CDA leads to either 1) deactivation in case of gemcitabine, cytarabine and 5-azacytidine, or 2) activation in case of capecitabine (Serdjebi et al. 2015). Studies have reported that the patients with reduced CDA activity are prone to early and severe toxicities following administration of gemcitabine, due to an increase in plasma drug concentration (Ciccolini et al. 2010, 2011). Conversely, an elevated CDA enzyme activity in tumors has been associated with resistance to cytarabine and poor clinical outcome (Kirch et al. 1998; Yue et al. 2003).

Based on our observation that *NAT1* KO cells express a higher level of CDA, we hypothesized that *NAT1* KO cells exhibit differential sensitivity to pyrimidine nucleoside analogs that are used as chemotherapeutic agents. To test this, we treated the parental and *NAT1* KO cells of MDA-MB-231 (KO2 and KO5) to varying concentrations of gemcitabine, 5-azacytidine, cytosine-β-D-arabinofuranoside (Ara-C), or capecitabine (see Fig. 3) for 7 days and assessed the cell viability. We expected *NAT1* KO cells, which exhibit higher CDA activity, to be more resistant to gemcitabine, 5-azacytidine, and Ara-C, while being more sensitive to capecitabine. However, the sensitivity to the pyrimidine analogs was not significantly different between parental and *NAT1* KO cells (Fig. 5). There were significant differences in cell viability within a selective range of drug concentrations, but the difference was not concomitantly found in both *NAT1* KO cell lines (Fig. 5), and thus, the effect was not attributed to the *NAT1* status.

Naturally Occurring, Modified Cytidines

Zauri and colleagues reported that naturally occurring and epigenetically modified nucleosides (e.g., 5-methyl-2’-deoxycytidine) are not usually recycled via the nucleotide

salvage pathways, for incorporation of pre-modified bases during replication could adversely affect the fidelity of epigenome and cellular phenotypes (Zauri et al. 2015). The authors showed that enzymes of the nucleotide salvage pathway (e.g., CDA) display substrate selectivity, effectively preventing the incorporation of epigenetically modified forms of cytidine (e.g., 5-hydroxymethyl-2'-deoxycytidine [5hmdC], 5-formyl-2'-deoxycytidine [5fdC] and 5-carboxyl-2'-deoxycytidine [5cadC]) into the newly synthesized DNA. However, a subset of cancer cell lines that express high levels of CDA exhibit a high lethality when exposed to these modified cytidines. The authors reported that CDA converts the modified cytidines into uridine variants which are then incorporated into DNA, resulting in DNA damage and cell death (Zauri et al. 2015). Their finding suggests a new therapeutic option for cancer treatment and implies that cancer cells expressing high levels of CDA can be effectively targeted using pre-modified cytidines (Zauri et al. 2015).

Based on this report, we hypothesized that *NAT1* KO breast cancer cells are more sensitive to the modified cytidines, for they express higher levels of CDA compared to the parental cells. To test this, we treated the parental and two *NAT1* KO cell lines of MDA-MB-231 (KO2 and KO5) to varying concentrations of 5fdC or 5hmdC (see Fig. 3) for 7 days and assessed the cell viability. Cell viability of both *NAT1* KO cell lines declined sharply in response to 5fdC treatment, in contrast to the parental cells (Fig. 6a). At 25 μM of 5fdC, the cell viability of *NAT1* KO cells was less than 10%, whereas the majority of parental cells (>70%) were viable at the same concentration (Fig. 6a). We repeated the experiment using a range of lower concentrations of 5fdC (0 to 6 μM). The increased sensitivity of *NAT1* KO cells to 5fdC was evident even at the lowest concentration tested (i.e., 1 μM), and the cell viability of *NAT1* KO cells continued to decline in a concentration-dependent manner (Fig. 6b). Another modified cytidine, 5hmdC, caused concentration-dependent decline in cell viability in all cell lines tested. However, with the 5hmdC treatment, *NAT1* KO cells did not show significant differences in cell viability when compared to the parental cells (Fig. 6c). Next, we investigated if the increased sensitivity to 5fdC in *NAT1* KO MDA-MB-231 cell lines was reproducible in other *NAT1* KO breast cancer cell lines. The parental and *NAT1* KO cell lines of MCF-7 and ZR-75–1 were treated with 5fdC (0 to 6 μM), and cell viability was assessed after 7 days of treatment. In contrast to *NAT1* KO MDA-MB-231 cells, the viability of neither *NAT1* KO MCF-7 nor *NAT1* KO ZR-75–1 cells was affected by the 5fdC treatment even at the highest concentration tested (Fig. 6d and e). This finding indicated that the increased sensitivity to 5fdC observed in *NAT1* KO MDA-MB-231 cells may not be dependent on the *NAT1* status *per se*, but on the CDA activity.

To investigate if the increased sensitivity to 5fdC by *NAT1* KO MDA-MB-231 cells was indeed dependent on the CDA activity, we co-incubated the parental and *NAT1* KO MDA-MB-231 cells with varying concentrations of 5fdC and a CDA inhibitor, tetrahydrouridine (THU; 60 μM) for 3 days and compared the cell viability. THU at 60 μM alone had a moderate, yet significant, effect on cell viability in all cell lines tested (Fig. 7). With a 3-day treatment, 5fdC at 4 μM did not significantly affect viability in any of the cell lines tested (Fig. 7). At 40 μM of 5fdC, however, both *NAT1* KO cell lines showed significantly lower viability, compared to the parental cells (77.3 ± 2.8% in parental; 38.0 ± 1.5% in KO2; and 46.3 ± 0.9% in KO5. Mean ± SEM) (Fig. 7). Co-treatment of cells with THU abrogated this difference and rescued both *NAT1* KO cell lines from 5fdC cytotoxicity (Fig. 7), suggesting

that the increased sensitivity to 5fdC in *NAT1* KO cells is mediated by the increased CDA activity.

Discussion

The present study showed that *NAT1* deletion resulted in upregulation of CDA in multiple breast cancer cell lines. Along with CDA, several enzymes involved in the pyrimidine salvage pathway were collectively upregulated in *NAT1* KO cells at the transcript level (see Table 1). These findings suggest that *NAT1* KO cells actively utilize the salvage pathway. Although the underlying mechanism for this phenomenon is unknown, it seems plausible that *de novo* synthesis of pyrimidines may be inhibited in *NAT1* KO cells. This hypothesis is supported by our previous metabolomic study (Carlisle et al. 2020). The study shows that multiple intermediates in the *de novo* pyrimidine biosynthetic pathway are decreased concomitantly in both *NAT1* KO cell lines (i.e., KO2 and KO5), compared to the parental MDA-MB-231 cells, suggesting that the *de novo* pyrimidine biosynthesis is compromised in *NAT1* KO cells (Carlisle et al. 2020). In particular, among the differentially abundant metabolites, the cellular level of cytidine (a ribosyl pyrimidine) positively correlated with NAT1 enzymatic activity (i.e., lower in *NAT1* KOs) (Carlisle et al. 2020), suggesting that the cellular cytidine level may be directly or indirectly regulated by NAT1 activity. Additionally, the upregulation of CDA in *NAT1* KO MDA-MB-231 cells, observed in our present study, is also consistent with the decrease in their cellular cytidine level, as cytidine is metabolized to uridine by CDA (Carlisle et al. 2020). The current study, however, does not directly document the involvement of NAT1 in *de novo* pyrimidine biosynthetic pathway or demonstrate the requirement of CDA in the compensatory pyrimidine salvage pathway. That would require measurement of cellular pyrimidine levels in parental vs. *NAT1* KO cells and also examination of changes in the pyrimidine level in the presence or absence of a CDA inhibitor or siRNA-mediated CDA knockdown. Additionally, other differentially regulated genes of the pyrimidine salvage pathway (e.g., UPRT) in *NAT1* KO cells would need to be investigated for their involvement.

Studies by independent research groups have shown that inhibition or depletion of NAT1 in various cancer cell lines results in growth retardation *in vitro* and *in vivo* (Tiang et al. 2010, 2011; Stepp et al. 2018; Doll et al. 2022). A defect in the *de novo* pyrimidine biosynthetic pathway, in part, may contribute to the decrement in cell growth observed in *NAT1* KO breast cancer cells. The *de novo* pathway is indispensable during cell growth to meet the demand for nucleic acid precursors and other cellular components. Cellular pyrimidines (i.e., cytosine, uracil, and thymine) serve as building blocks of DNA and RNA (Jones 1980; Traut 1994). In addition, CDP-diacylglycerol is a key intermediate in the synthesis of phospholipids, and UDP-sugars serve as sugar donors in protein glycosylation (Jones 1980; Traut 1994). For this reason, the pathway is invariably upregulated in rapidly growing cancer cells (Weber 2001). Hence, it is possible that *NAT1* KO cells have activated the salvage pathway to compensate for the defect in the *de novo* pathway. This is supported by our current finding that *CDA* mRNA was invariably upregulated in *NAT1* KO cells of three different breast cancer cell lines (i.e., MDA-MB-231, MCF-7, and ZR-75–1). However, it should be noted that *NAT1* KO cells did not exhibit increased sensitivity to CDA inhibitors (see Fig. 4), which does not support this hypothesis. Additional investigations are required

to determine whether *NAT1* deletion invariably results in a defect in the *de novo* pyrimidine biosynthetic pathway in breast cancer cell lines.

Based on these observations, we sought to investigate if breast cancer cells, in which NAT1 is either inhibited or depleted, become more susceptible to certain classes of conventional as well as potential chemotherapeutic agents. In other words, can inhibition (or depletion) of NAT1 in breast cancer cells be a potential strategy to increase their sensitivity to chemotherapeutic agents? To test this, we treated the parental vs. *NAT1* KO MDA-MB-231 cells with 1) inhibitors of pyrimidine biosynthesis or salvage; 2) pyrimidine/nucleoside analogs; or 3) naturally occurring, modified cytidines. Compared to the parental cells, both NAT1 *KO* MDA-MB-231 cell lines were relatively resistant to two different inhibitors of dihydroorotase (i.e., teriflunomide and leflunomide), an essential enzyme in *de novo* pyrimidine synthesis. Upregulation of CDA and activation of the salvage pathway in *NAT1* KO MDA-MB-231 cells can explain the increased resistance to the *de novo* pyrimidine synthesis inhibitors and suggest that *NAT1* KO cells can better withstand inhibition of *de novo* synthesis. In regard to CDA inhibitors (i.e., tetrahydrouridine and zebularine), however, we expected *NAT1* KO cells to be more sensitive to CDA inhibitors, based on the hypothesis that CDA activity is required to maintain proper cellular level of pyrimidines. However, unexpectedly, *NAT1* KO cells were more resistant to two the CDA inhibitors tested, which does not support our hypothesis. Funamizu and colleagues reported that tetrahydrouridine can suppress E2F1, thereby inhibiting the G1-S cell cycle progression in multiple cancer cell lines (Funamizu et al. 2012). Interestingly, its inhibitory effect on cell cycle progression was independent of CDA expression (Funamizu et al. 2012). The other CDA inhibitor, zebularine, is also known to inhibit DNA methyltransferases. Billam *et al.* have shown that zebularine treatment of two human breast cancer cell lines, MDA-MB-231 and MCF-7, inhibits their cell growth in a concentration-dependent manner by inducing S-phase cell cycle arrest (Billam et al. 2010). In addition, the authors showed that zebularine produces effects that are similar to DNA methyltransferase (DNMT) inhibitors and is able to re-express epigenetically silenced genes (e.g., estrogen and progesterone receptor) in triple-negative MDA-MB-231 cells (Billam et al. 2010). These studies suggest that the inhibitory effects of tetrahydrouridine and zebularine on cancer cell growth can be mediated through CDA-independent mechanisms and may explain the results of the present study.

In adult and pediatric oncology, nucleoside analogs (e.g., gemcitabine, capecitabine, cytarabine, and 5-azacytidine) are widely utilized as anti-metabolites which interfere with the synthesis of the nucleosides or nucleotides necessary for growth in cancer cells. These drugs share a common metabolic pathway driven by a hepatic enzyme, CDA (Serdjebi et al. 2015). The gene coding CDA displays genetic and epigenetic polymorphisms, which leads to a wide variability in CDA activity in patients, ranging from deficient to ultra-rapid (Serdjebi et al. 2015). Patient-to-patient variability in CDA activity has been shown to impact pharmacokinetics and pharmacodynamics of these drugs. For instance, most of gemcitabine is metabolized by CDA into its inactive metabolite, and as a result, variations in CDA activity greatly influence its pharmacokinetics. Many laboratory and case studies in humans report a correlation between deficiency in CDA and an increase in severe hematological toxicities (i.e., adverse effects) from gemcitabine (Yue et al. 2003; Giovannetti et al. 2008; Ciccolini et al. 2010). Capecitabine derives from a purine base

and undergoes a series of transformations in the liver before reaching its active form of 5-fluorouracil (5-FU). CDA is the enzyme responsible for its activation via catalyzing the conversion of 5'-deoxy-5-fluorocytidine into 5'-deoxy-5-fluorouridine (Miwa et al. 1998). Accordingly, Morita and colleagues reported that ectopic overexpression of CDA in a human bladder cancer cell line results in increased sensitivity to capecitabine (Morita et al., 2003). Moreover, relationship between ultra-rapid CDA status and severe toxicities of capecitabine also has been confirmed in patients (Caronia et al. 2011). These studies suggest that the CDA expression or activity is an important determinant of the efficacy and toxicity of the aforementioned nucleoside analogs. This prompted us to investigate the differential sensitivity to nucleoside analogs between the parental vs. *NAT1* KO MDA-MB-231 cells in the present study.

Among the nucleoside analogs tested, *NAT1* KO MDA-MB-231 cells were marginally more sensitive to 5-azacytidine (see Fig. 5), which is inactivated via CDA (Micozzi et al. 2014; Serdjebi et al. 2015). However, this was in contrast to the expected result, for higher CDA activity would normally lead to a greater *resistance* to 5-azacytidine. The rest of the nucleoside analogs we tested, which are also subject to CDA-mediated activation (e.g., capecitabine) or inactivation (e.g., gemcitabine and Ara-C) (Micozzi et al. 2014; Serdjebi et al. 2015), did not produce differential cytotoxicity in *NAT1* KO cells compared to the parental counterparts. This finding suggests that the increased level of CDA alone (in *NAT1* KO cells) is not sufficient to cause differential sensitivity to the nucleoside analogs tested in the study. In addition to CDA, the metabolism and cytotoxicity of these pyrimidine analogs are dependent on additional factors that mediate drug transport, metabolism, activation, deactivation, drug targets and downstream responses. Clinical response to gemcitabine, which is commonly used for treatment of solid tumors (e.g., pancreatic cancer) (Mini et al. 2006; de Sousa Cavalcante and Monteiro 2014), varies widely (Mini et al. 2006). Gemcitabine is a prodrug that needs to be transported into the cell through nucleoside transporters (e.g., SLC28 and 29 family of transporters) and activated to phosphorylated metabolites by kinases (e.g., deoxycytidine kinase, UMP-CMP kinase, and nucleoside diphosphate kinase) before its tri-phosphate metabolite is incorporated into nascent DNA, terminating DNA synthesis (Mini et al. 2006; de Sousa Cavalcante and Monteiro 2014). During its metabolism, gemcitabine (and its metabolites) can be also inactivated by enzymes that catalyze deamination (i.e., CDA) and dephosphorylation (e.g., 5'-nucleotidases; 5'-NTs) (Mini et al. 2006; de Sousa Cavalcante and Monteiro 2014). Li and colleagues identified novel factors that influence the cytotoxicity of gemcitabine and Ara-C in multiple human lymphoblastoid cell lines, and found that downregulation of *NT5C3A* and *FKBP5* altered tumor cell sensitivity to both drugs (Li et al. 2008). *NT5C3A* (5'-nucleotidase, cytosolic IIIA; pyrimidine 5'-nucleotidase 1) encodes an enzyme that catalyzes the dephosphorylation of nucleoside 5'-monophosphates, and, thus, is likely to be directly involved in metabolism of these two pyrimidine analogs. *FKBP5* (FK506-binding protein 5; FKBP prolyl isomerase 5), however, had never been implicated in cytotoxicity of gemcitabine and Ara-C. This study highlights that, besides CDA, multiple factors contribute to metabolism and cytotoxicity of the nucleoside analogs. Thus, it is possible that in addition to CDA, the factors involved in transport and/or metabolism of the pyrimidine analogs might be altered in *NAT1* KO

MDA-MB-231 cells, thereby altering the cytotoxicity of the nucleoside analogs tested in the present study.

NAT1 KO MDA-MB-231 cells showed a marked increase in sensitivity to one of the 'modified cytidines' tested, 5fdC (see Fig. 6a and b). It represents one of naturally occurring, oxidized forms of 5-methylcytidine (Tahiliani et al. 2009; Ito et al. 2011). Normally, 5fdC is not salvaged (i.e., deaminated) by CDA. With relatively high CDA expression, however, 5fdC (and other oxidized products of 5-methyl-2'-deoxycytidine [5mdC], such as 5hmdC) can be converted to the corresponding, noncanonical uridine analog, which then can be phosphorylated and incorporated back into DNA, causing DNA damage and cell death (Klungland et al. 2001; Zauri et al. 2015). Based on this finding, Zauri and colleagues proposed that oxidized epigenetic bases (e.g., 5fdC and 5hmdC) would serve as alternative chemotherapeutic agents in treatment of high CDA-expressing cancers (Zauri et al. 2015). The marked increase in the sensitivity to 5fdC in *NAT1* KO MDA-MB-231 cells (that exhibit higher CDA activities) supports this idea. However, another oxidized epigenetic base, 5hmdC, failed to produce a similar effect in *NAT1* KO cells. As a result, there was no difference in the sensitivity to 5hmdC between cell lines tested. Currently, the underlying mechanism of the selective sensitivity to 5fdC by *NAT1* KO cells is not clear. Zauri and colleagues reported that among dC (2'-deoxycytidine), 5mdC, 5hmdC, and 5fdC, the second-best substrate (i.e., with the second highest kcat) for CDA, after dC, is 5fdC (Zauri et al. 2015). Molecular modelling suggests that 5fdC docks to the catalytic site and retain the amino group position close to the active site containing Zn^{2+}. In contrast, 5hmdC docks in the active site by displacing the amino group, which can explain its lower catalytic turnover compared to 5fdC (Zauri et al. 2015). However, the difference in kcat between 5fdC and 5hmdC does not explain the apparent lack of differential sensitivity to 5hmdC (at concentrations up to 100 μM) in parental and *NAT1* KO cells. It is possible that the pathways of metabolic activation and inactivation of 5fdC and 5hmdC are deviant and that their cytotoxicity is not solely dictated by cellular CDA activity. Importantly, the increased 5fdC cytotoxicity in *NAT1* KO cells was dependent on CDA activity, as co-treatment with a CDA inhibitor prevented cell death induced by 5fdC in *NAT1* KO MDA-MB-231 cells (see Fig. 7). Notably, neither parental nor *NAT1* KO MCF-7 or ZR-75–1 cells, which do not exhibit robust CDA activity, were differentially sensitive to 5fdC (see Fig. 6d and e), in contrast to *NAT1* KO MDA-MB-231 cells. This suggests that sensitivity to 5fdC is not dependent on the *NAT1* gene status *per se*, but requires CDA expression.

Delineating the direct cause and functional consequences of CDA upregulation (following *NAT1* deletion in breast cancer cells) requires additional investigations. Findings reported in this paper suggest a novel therapeutic strategy to treat breast cancer with elevated *NAT1* expression. For instance, NAT1 inhibition may be combined with cytotoxic nucleosides (e.g., 5fdC) for breast cancer treatment.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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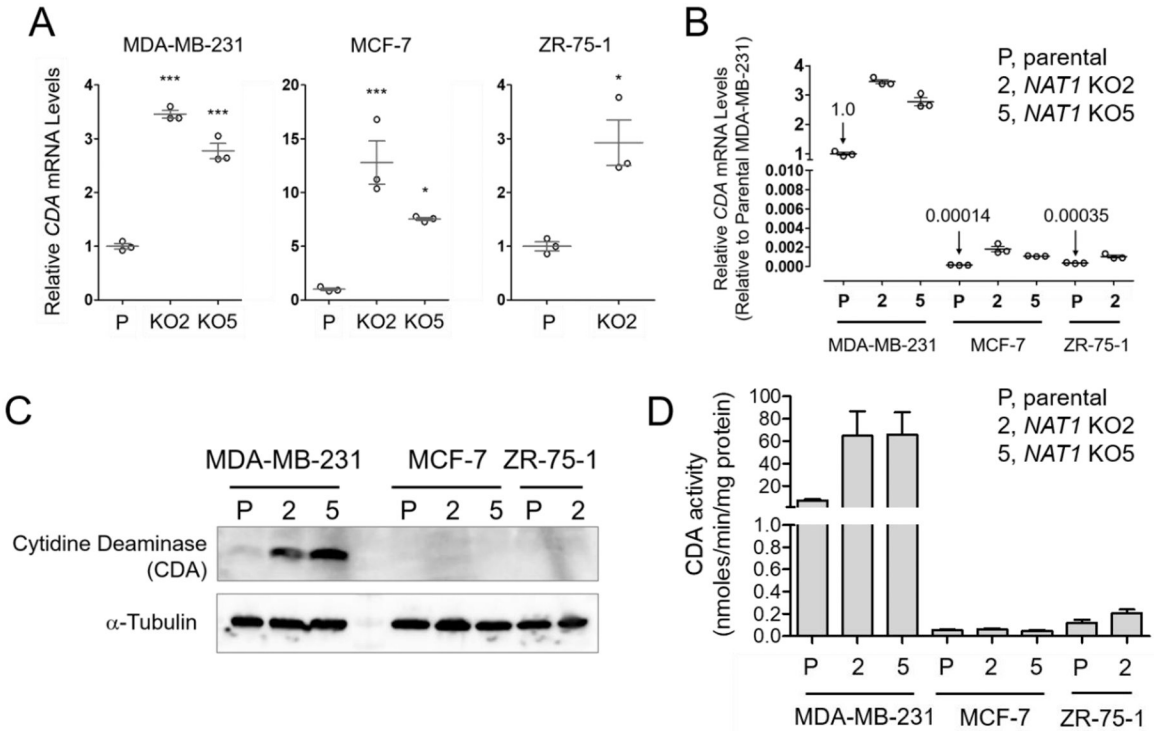

Fig. 1. Deletion of *NAT1* leads to increases in CDA mRNA, protein, and activity levels in breast cancer cell lines

NAT1 KO cell lines, previously generated using three breast cancer cell lines (MDA-MB-231, MCF-7, and ZR-75–1), and their corresponding parental cells were analyzed for CDA (cytidine deaminase) expression. A and B, *CDA* mRNA expression. Total RNA isolated from the indicated cell lines were analyzed for the relative level of *CDA* mRNA using RT-qPCR. N = 3 per group. C, Western blot for CDA protein. Total cell lysates from the indicated cell lines were separated on an SDS-PAGE gel and blotted with a CDA-specific antibody. α-tubulin served as an internal, loading control. D, CDA enzyme activity assay. Lysates from the indicated cell lines were incubated with cytidine (substrate), and the formation of uridine (product) was quantified using HPLC. N = 3 per group. P, parental; 2, *NAT1* KO2; 5, *NAT1* KO5. The graphs show mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The p values are for the difference between the parental and each of the *NAT1* KO cell lines.

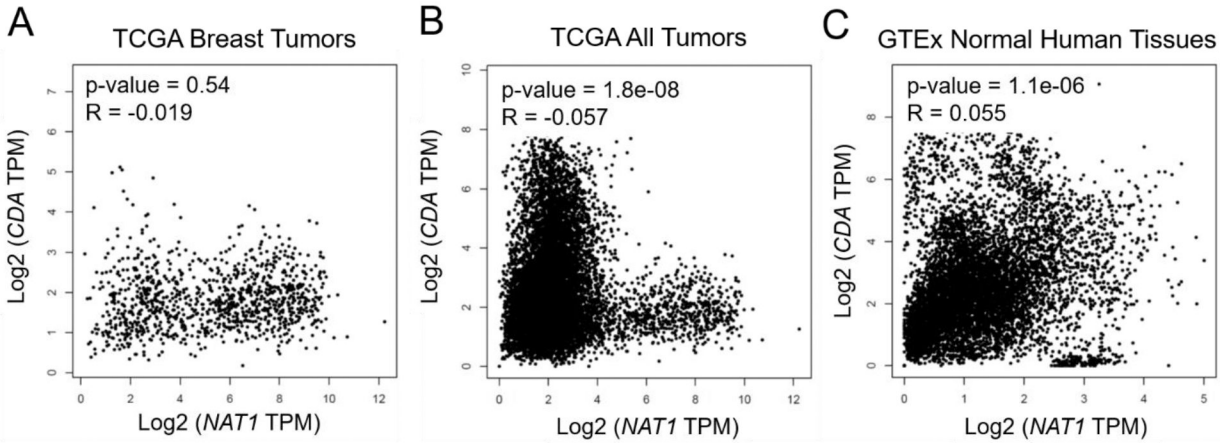

Fig. 2. Correlation between *NAT1* and *CDA* transcript levels in human tumors and normal tissues

The dot plots show the mRNA level (expressed in $\log_2$ of transcripts per kilobase million [TPM]) of *NAT1* (x-axis) and the corresponding mRNA level of *CDA* (y-axis) in the same individual samples. A, *NAT1* vs. *CDA* expression in TCGA breast tumors only. B, *NAT1* vs. *CDA* expression in all tumors in TCGA database. C, *NAT1* vs. *CDA* expression in normal human tissues from GTEx database. R, Pearson correlation coefficient. The analysis was done using tools available at GEPIA (Tang et al. 2017).

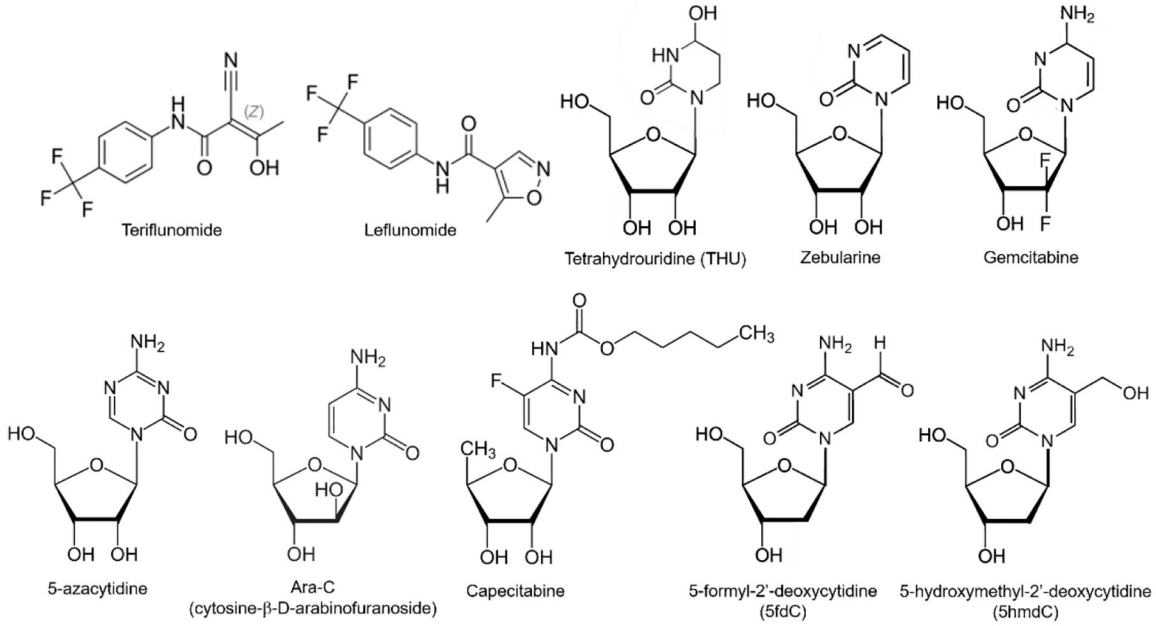

Fig. 3.

Chemical structures of the drugs tested in the present study

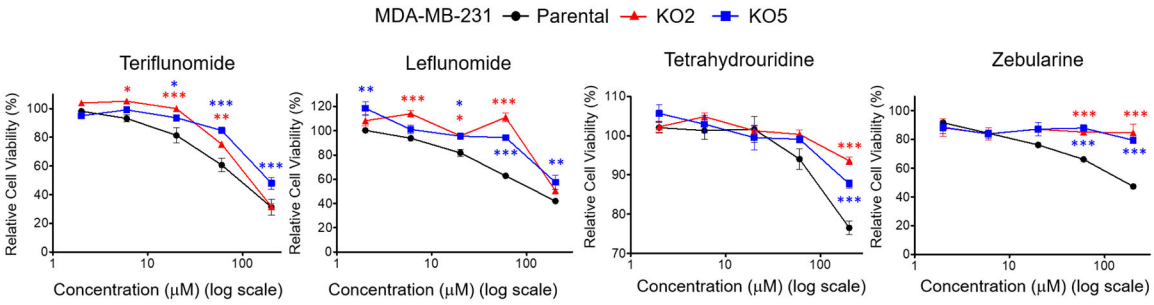

Fig. 4. Sensitivity of parental vs. *NAT1* KO MDA-MB-231 cells to inhibitors of *de novo* pyrimidine synthesis and CDA

To compare the sensitivity of the parental and two *NAT1* KO MDA-MB-231 cells (KO2 and KO5) to inhibitors of pyrimidine biosynthesis or salvage pathway, the cells were treated with the indicated concentrations of teriflunomide and leflunomide (inhibitors of dihydro-orotate dehydrogenase, a key enzyme in the *de novo* pyrimidine synthesis pathway) or tetrahydrouridine and zebularine (inhibitors of CDA) for 3 days. Following the treatment, cell viability was measured using alamarBlue assay and expressed as 'relative cell viability' (relative to the untreated control group). Drug concentrations are shown in a log scale. $N = 4$ per concentration. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The p values are for the difference between the parental and each of the *NAT1* KO cell lines. Red asterisks indicate the significance of the difference between parental and KO2, while blue asterisks indicate the significance of the difference between parental and KO5.

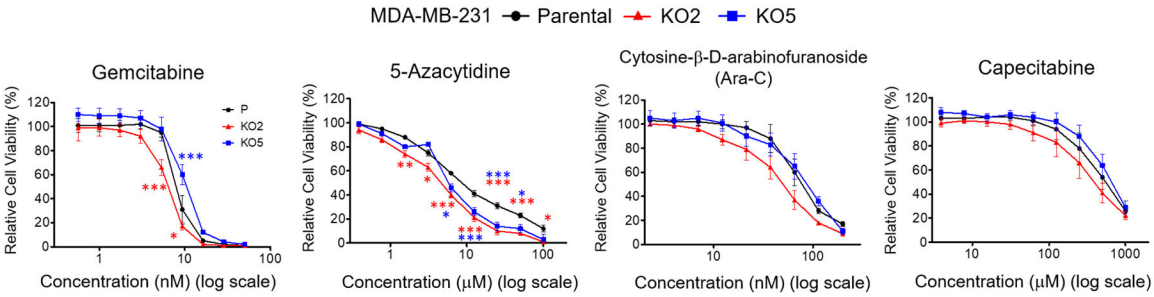

Fig. 5. Sensitivity of the parental vs. *NAT1* KO MDA-MB-231 cells to pyrimidine/nucleoside analogs

To compare the sensitivity of the parental (P) and *NAT1* KO MDA-MB-231 cells (KO2 and KO5) to chemotherapeutic drugs that are subject to metabolism by CDA, the cells were treated with the indicated concentrations of gemcitabine, 5-azacytidine, cytosine-β-D-arabinofuranoside (Ara-C) or capecitabine (see Fig. 3 for their structures) for 7 days. Following the treatment, cell viability was measured using alamarBlue assay and expressed as 'relative cell viability' (relative to the untreated control group). Drug concentrations are shown in a log scale. $N = 4$ per concentration. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The p values are for the difference between the parental and each of the *NAT1* KO cell lines. Red asterisks indicate the significance of the difference between parental and KO2, while blue asterisks indicate the significance of the difference between parental and KO5.

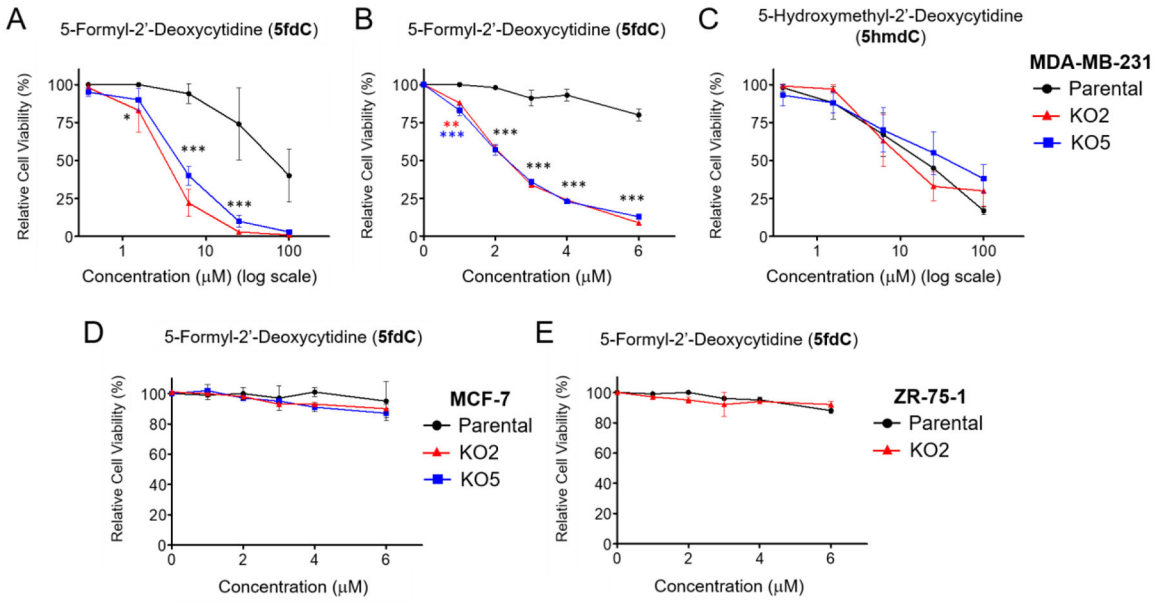

Fig. 6. Sensitivity of the parental vs. *NAT1* KO breast cancer cells to naturally occurring, epigenetically modified cytidines

To compare the sensitivity of the parental and *NAT1* KO breast cancer cell lines to naturally occurring, modified cytidines, the cells were treated with the indicated concentrations of 5fdC (5-formyl-2'-deoxycytidine) (panels A, B, D, and E) or 5hmdC (5-hydroxymethyl-2'-deoxycytidine) (panel C) for 7 days. Following the treatment, cell viability was measured using alamarBlue assay and expressed as 'relative cell viability' (relative to the untreated control group). A-C, Parental vs. *NAT1* KO (KO2 and KO5) MDA-MB-231 cells. D, Parental vs. *NAT1* KO (KO2 and KO5) MCF-7 cells. E. Parental vs. *NAT1* KO (KO2) ZR-75–1 cells. Drug concentrations are shown in a log scale for panels A and C only. $N = 4$ per concentration. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The p values are for the difference between the parental and each of the *NAT1* KO cell lines. Red asterisks indicate the significance of the difference between parental and KO2, while blue asterisks indicate it between parental and KO5.

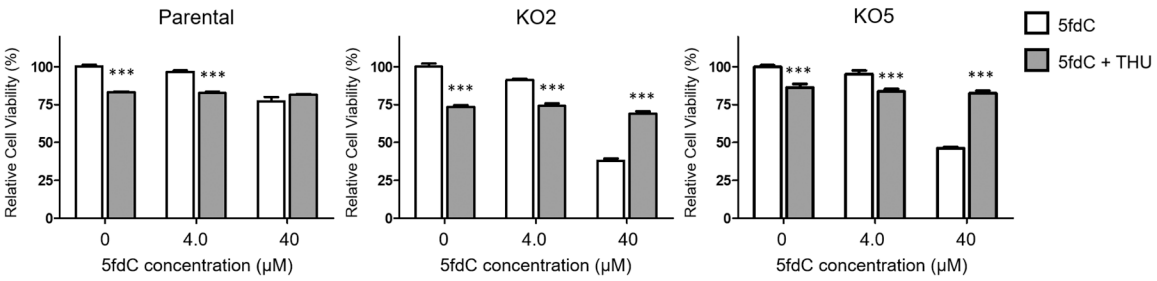

Fig. 7. Increased sensitivity of *NAT1* KO MDA-MB-231 cells to 5fdC is dependent on CDA activity.

Parental and two *NAT1* KO MDA-MB-231 cell lines (KO2 and KO5) were treated with the indicated concentrations of 5fdC (5-formyl-2'-deoxycytidine) alone (white bars) or co-treated with 5fdC and a CDA inhibitor, tetrahydrouridine (THU; 60 μM) (gray bars) for 3 days. Following the treatment, cell viability was measured using alamarBlue assay and expressed as 'relative cell viability' (relative to the control group, i.e., 0 μM 5fdC without THU). $N = 4$ per group. ***, $p < 0.001$. The p values are for the difference between the 5fdC only and 5fdC + THU treatment groups within each cell line.

Table 1.

Relative mRNA Expression of Genes Involved in the Pyrimidine Salvage Pathway in *NAT1* KO MDA-MB-231 Cells (from Carlisle et al. 2021)

Gene Symbol | Gene Name	Average RPKM			P vs. KO2 *p* value	Direction of Change	P vs. KO5 *p* value	Direction of Change
	Parental (P)	*NAT1* KO2	*NAT1* KO5				
CDA | cytidine deaminase	24.3	46.8	76.5	5.00E-05	**Up**	5.00E-05	**Up**
CANT1 | calcium activated nucleotidase 1	30.2	27.3	29.5	0.440	n.s.	0.958	n.s.
CMPK1 | cytidine monophosphate (UMP-CMP) kinase 1, cytosolic	89.8	149.8	126.8	5.00E-05	**Up**	0.001	**Up**
CMPK2 | cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	0.0	0.1	0.0	1.000	n.s.	1.000	n.s.
CTPS1 | CTP synthase 1	82.6	109.6	122.9	0.101	n.s.	0.007	**Up**
CTPS2 | CTP synthase 2	10.2	21.8	10.1	0.002	**Up**	0.954	n.s.
ENTPD1 | ectonucleoside triphosphate diphosphohydrolase 1	0.9	2.0	1.1	1.00E-04	**Up**	0.243	n.s.
ENTPD3 | ectonucleoside triphosphate diphosphohydrolase 3	0.2	16.0	5.2	5.00E-05	**Up**	5.00E-05	**Up**
ENTPD8 | ectonucleoside triphosphate diphosphohydrolase 8	0.0	0.0	0.0	1.000	n.s.	1.000	n.s.
NTPCR | nucleoside-triphosphatase, cancer-related	26.1	28.0	32.5	0.717	n.s.	0.128	n.s.
NTSC | pyrimidine specific 5'-nucleotidase	n.d.	n.d.	n.d.	-	-	-	-
UCK1 | uridine-cytidine kinase 1	12.8	8.6	13.8	0.030	**Down**	0.586	n.s.
UCK2 | uridine-cytidine kinase 2	40.0	46.4	52.9	0.601	n.s.	0.210	n.s.
UPP1 | uridine phosphorylase 1	39.5	36.4	68.0	0.617	n.s.	5.00E-05	**Up**
UPP2 | uridine phosphorylase 2	0.013	0.000	0.000	1.000	n.s.	1.000	n.s.
UPRT | uracil phosphoribosyltransferase (FUR1) homolog	5.4	11.9	8.7	5.00E-05	**Up**	2.00E-04	**Up**
URH1 | uridine nucleosidase	n.d.	n.d.	n.d.	-	-	-	-

P, parental; 2, *NAT1* KO2; 5, *NAT1* KO5. n.s., not statistically significant. n.d., not detected. RPKM, reads per kilobase of transcript per million mapped reads.