Cytidine Deaminase Deficiency Reveals New Therapeutic Opportunities against Cancer

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Abstract

Purpose: One of the main challenges in cancer therapy is the identification of molecular mechanisms mediating resistance or sensitivity to treatment. Cytidine deaminase (CDA) was reported to be downregulated in cells derived from patients with Bloom syndrome, a genetic disease associated with a strong predisposition to a wide range of cancers. The purpose of this study was to determine whether CDA deficiency could be associated with tumors from the general population and could constitute a predictive marker of susceptibility to antitumor drugs.

Experimental Design: We analyzed CDA expression in silico, in large datasets for cancer cell lines and tumors and in various cancer cell lines and primary tumor tissues using IHC, PDXs, qRT-PCR, and Western blotting. We also studied the mechanism underlying CDA silencing and searched for molecules that might target specifically CDA-deficient tumor cells using in silico analysis coupled to classical cellular experimental approaches.

Results: We found that CDA expression is downregulated in about 60% of cancer cells and tissues. We demonstrate that DNA methylation is a prevalent mechanism of CDA silencing in tumors. Finally, we show that CDA-deficient tumor cells can be specifically targeted with epigenetic treatments and with the anticancer drug aminoflavone.

Conclusions: CDA expression status identifies new subgroups of cancers, and CDA deficiency appears to be a novel and relevant predictive marker of susceptibility to antitumor drugs, opening up new possibilities for treating cancer.

Introduction

Despite major advances in the development of chemotherapy, many cancers continue to have a poor prognosis, due to the resistance of cancer cells to antineoplastic drugs through intrinsic or acquired mechanisms (1). It is, thus, highly important to identify markers predicting the response to anticancer treatment and new molecular targets for novel anticancer treatments.

Two families of cytidine deaminases exhibit different biological functions, deaminating either the free cytidines, as performed by cytidine deaminase (CDA or CDD; EC 3.5.4.5), or deaminating the cytidines incorporated within the DNA or RNA polymers, as performed by the AID/APOBECs (activation-induced deaminase/apolipoprotein B mRNA editing catalytic polypeptide like) proteins (2, 3). We previously reported that CDA deficiency leads to DNA damage and to genetic instability (4, 5), known to be associated with cancer development, suggesting a possible relationship between CDA underexpression and cancer.

CDA is an enzyme of the pyrimidine salvage pathway catalyzing the hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively (6). In addition to native nucleotides, CDA also deaminates and inactivates nucleoside analogues, such as gemcitabine and cytosine arabinoside (Ara-C), agents widely used to treat cancer (7). CDA thus plays an important role in the sensitivity/resistance of cancer cells to treatment with cytidine analogues (8, 9). Indeed, early severe toxicity has been reported in cancer patients, with low levels of CDA activity treated with gemcitabine (10, 11). CDA overexpression might thus be a marker for resistance to chemotherapy based on cytidine analogues.

CDA gene polymorphism has been widely explored (11–17), but little is known about its transcriptional and posttranscriptional regulation. One study recently reported that miR-484 downregulates CDA gene expression by targeting its 3’-UTR, sensitizing breast cancer cells overexpressing CDA to gemcitabine (18). CDA overexpression is thus a good marker for resistance to chemotherapy based on cytidine analogues.

CDA overexpression has recently been identified as a potential target for anticancer treatment. Indeed, CDA has been shown to be involved in the deamination of oxidized and epigenetically modified cytidine nucleosides (19, 20). These cytidine analogues are...
CDA Deficiency in Cancer

Translational Relevance
This study revealed for the first time that CDA expression is lost in a large proportion of tumors, mostly due to DNA methylation and that tumors from the same classically defined groups may display differences in CDA expression status, resulting in contrasting cellular properties, such as levels of sister chromatids exchanges. Thus, the use of CDA expression status in tumor cells defines two new subgroups: CDA-deficient tumors and CDA-proficient tumors. Our results indicate that IHC assessments of CDA levels could be used to determine the CDA status of tumors, with potential implications for treatment. In particular, we identified aminoavone, which reached phase II clinical trials, as a proof-of-principle candidate for the targeting of CDA-deficient tumor cells, with no effect on CDA-proficient cells. Thus, CDA expression status could be used as a new marker to guide anticancer therapy.

Materials and Methods
Cell culture and treatments
We used 33 cancer cell lines in this study (Supplementary Material S1): 19 breast cancer cell lines from the Translational Research Department of the Curie Institute (Paris, France; ZR75-1, T47D, HCC-1428, BT-474, MCF-7, MDA-MB-361, MDA-MB-468, MDA-MB-231, MDA-MB-436, HCC-38, HCC-70, HCC-1187, HCC-1937, HCC-1143, BT-20, BT-549, HCC-1954, SKBR-3, and HS578T) and two nonmalignant breast cell lines (IGROV-1, SKOV-3, and OVCAR-8) from the NCI (Rockville, MD; refs. 21, 22), one melanoma cell line (A2058) from Dr. Gemble and colleagues (5). In brief, total RNA was extracted from patient-derived xenograft (PDX) tissues and from cell lines with the RNeasy Mini Kit (Qiagen). Reverse transcription was performed on 1 μg of RNA with the GoScript enzyme (Promega). The cDNA obtained was used at a dilution of 1/10 to base-pair resolution (Eurofins Genomics) are presented in Supplementary Material S2.

The procedure for real-time PCR (qRT-PCR) was as described before by Gemble and colleagues (5). In brief, total RNA was extracted from patient-derived xenograft (PDX) tissues and from cell lines with the RNeasy Mini Kit (Qiagen). Reverse transcription was performed on 1 μg of RNA with the GoScript enzyme (Promega). The cDNA obtained was used at a dilution of 1/10 to real-time PCR with the SYBR Green polymerase (Promega). The cDNA obtained was used at a dilution of 1/10 to base-pair resolution (Eurofins Genomics) are presented in Supplementary Material S2.

For Western blotting, cells were harvested by centrifugation and lysed in 8 mol/L urea, 50 mmol/L Tris-HCl, pH 7.5, and 150 mmol/L β-mercaptoethanol buffer supplemented with protease inhibitor (Thermo Scientific). They were then sonicated and heated. Protein concentration was estimated with the BCA Kit (Pierce), and the equivalent of 20 μg or protein per cell lysate was run on a 4% to 12% Bis-Tris precast gel (Life Technologies). The proteins were then transferred to PVDF membranes, which were probed with the appropriate antibody. Protein bands were visualized with a CCD camera (Bio-Rad). Details of the primary and secondary antibodies used are provided in Supplementary Material S2.
IHC
IHC was carried out as described by Baldeyron and colleagues (23). Briefly, paraffin-embedded tissue blocks obtained at the initial diagnosis were retrieved from the archives of the Biopathology Department of Curie Institute Hospital (Paris, France). Sections (3-μm thick) were cut with a microtome from the paraffin-embedded tissue blocks. Tissue sections were dewaxed and rehydrated through a series of xylene and ethanol washes. A primary anti-CDA Ab was used (Supplementary Material S2). The sections were processed with a Dako machine for immunostaining. The specificity of the CDA Ab was confirmed by applying the same protocol to paraffin-embedded human tissue sections and cell block sections. The sections were rehydrated by incubation in PBS for 5 minutes and then incubated with anti-CDA antibody for 1 hour. Antibody binding was detected by incubation with a secondary antibody coupled to a peroxidase-conjugated polymer (Dako Envision+) after treatment with DAB solution (Dako K3468) for 5 minutes and Mayer hematoxylin for 1 minute. The sections were then mounted in resin. We evaluated CDA immunostaining on histologic sections from 19 normal human tissues (20 samples per tissue) and from 6 primary tumor tissues (50 samples per cancer type). For each section, we evaluated two immunohistologic scores: intensity score: score 0, no staining; score 1+, weak staining; score 2+, moderate staining; score 3+, intense staining; and frequency score: score 0, no staining; score 1+, 1% to 33% stained cells; score 2+, 34% to 67% stained cells; score 3+, 68% to 100% stained cells. Then, we defined a final score (H-score = frequency score × intensity score). This H-score was equal to 1 in normal colon tissue, and 1.5 in lung, breast, melanoma, ovary, and endometrium normal tissues. It means that the expression of CDA in normal tissues is between ≥1 and <2.

Thus, the cut-off value of CDA expression in tumor tissues was defined as CDA underepression by H-score between 0 and 1 (CDA low), and CDA overexpression by H-score between 2 and 3 (CDA high). Thus, the data are presented as a combination of the percentage of CDA-positive cells and intensity scores. The analysis was carried out by two independent pathologists.

Breast cancer PDXs
The PDX models used here were established as described by Marangoni and colleagues (24). Briefly, breast cancer fragments were obtained from patients at the time of surgery, with prior written informed consent of the patients. Fragments (30–60 mm³) were grafted subcutaneously into the interscapular fat pad of 8- to 12-week-old female Swiss nude mice, under Avertin anesthesia. Mice were maintained in specific pathogen-free animal housing (Curie Institute) and received estrogen (17 mg/mL) in their drinking water. Xenografts appeared at the graft site 2 to 8 months after grafting. They were subsequently transplanted from mouse to mouse and stored frozen in DMSO–FCS solution or dry frozen in liquid nitrogen for RNA isolation. The experimental protocol was performed in accordance with French regulations.

Sister chromatid exchange assay
This assay was performed as described by Gebble and colleagues (5). In brief, cells were plated on glass slides in the presence of 10 μmol/L 5-bromodeoxyuridine (Sigma Aldrich). After two divisions, colchicine (Sigma Aldrich) was added (0.1 μg/mL), and the cells were incubated for 1 hour. Cells were then incubated in a hypotonic solution [1:5 (v/v) FCS distilled water] and fixed with a 3:1 (v/v) mixture of methanol and acetic acid. They were then stained by incubation with 10 μg/mL Hoechst 33258 (Sigma Aldrich) in distilled water for 20 minutes. The slides were rinsed with 2× SSC (Euromedex) and exposed to UV light at a wavelength of 365 nm and a distance of 10 cm for 105 minutes. The slides were then rinsed in water, stained with 2% Giemsa (VWR) for 16 minutes, rinsed in water, dried, and mounted in EUKITT (Sigma Aldrich). Metaphases were captured and chromosomes were visualized under a Leica DMRB microscope at a magnification of ×100. We determined the number of sister chromatid exchanges (SCE) per chromosome.

DNA methylation data
We analyzed 482,422 CpGs in the NCI-60 cell lines with Illumina Infinium Human Methylation 450 BeadChips. The DNA methylation datasets are available under accession number GSE66872. The methylation values are presented from 0 to 1. The data were normalized and analyzed as described by Nagales and colleagues (25).

The negative correlations between CDA promoter methylation and CDA expression on The Cancer Genome Atlas (TCGA) samples (26) were generated through the Broad Institute FireBrowse portal (27, 28) and the cBioPortal for Cancer Genomics database (29–31), all the cBioPortal data (expression, mutation, copy number, significance analyses) being loaded directly from FireBrowse. The only promoter CpG site presenting a high significant negative correlation with CDA expression in both NCI-60 cell lines and TCGA samples was selected.

Figure 1.
CDA expression levels in cancer cell lines and tissues. A, Transcriptomic datasets in log 2 values for Curie Institute breast cancer cell lines (n = 34) publicly available (see Materials and Methods section; left) and from NCI 60 cancer cell lines data miner (right; ref. 45). Mean and median values are shown as dashed and solid lines, respectively, B, Real-time qRT-PCR and Western blot analyses of CDA expression in a set of 26 cell lines for three different cancers (breast, non-small lung, and ovary) representative of the Curie Institute (left) and NCI60 (right) panels. Hsp90 and β-actin were used as loading controls for Western blotting; TBP and GAPDH were used for qRT-PCR data normalization. Western blotting and qRT-PCR data were reproduced at least twice. C, Real-time qRT-PCR quantification of human (Hs in dark) and mouse (Mm in gray) CDA transcripts relative to human and mouse TBP housekeeping gene transcripts in 66 breast-derived xenografts. D, Immunohistochemical analysis of CDA expression in six different tumor tissues (n = 50/issue). TN, triple negative. Representative images for each tissue (top) and quantitative representations of CDA protein levels in each tissue (bottom) are shown. The results are presented as percentages of tumors expressing low (black) and high (gray) levels of CDA on the basis of the scores obtained (low, scores 0–1; high, scores 2–3). Scale bar (for all images), 50 μm. Black bars, percentage of low-CDA cancer tissues. E, Scatter dot plot with mean ± SD for transcriptomic data for CDA transcripts, comparing unmatched normal and xenograft tissues for the liver (GSE41520), esophagus (GSE38998), cervix (GSE9750), and colon (GSE9348). The data were retrieved from the NextBio (58) and Oncomine (59) data sources and downloaded from GEO and presented as log 2 intensities. F, CDA transcript levels relative to TBP, as quantified by qRT-PCR in a mini cohort of cancerous and noncancerous colon tissues. Error bars, SD. The P values calculated in unpaired two-tailed t tests are considered statistically significant at <0.05.
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Transcriptomic data
A collection of 40 human breast tumor cell lines (mostly from ATCC) was established in the Translational Research Department of the Curie Institute. Gene expression profiles were generated with the Affymetrix Exon Array and GenoSplice algorithms to summarize multiprobe measurements as single mRNA levels.

CDA expression levels were extracted from various transcriptomic datasets: breast tumor cell lines of the Curie Institute collection (32), NCI-60 (CellMiner tools; ref. 33), Cancer Cell Line Encyclopedia (CCLE; ref. 34), Gene Expression Across Normal and Tumor Tissue database (35, 36), the TCGA portal, and the Gene Expression Omnibus database (GEO; ref. 37). All these data are publicly accessible.

Statistical analysis
All data analyses and processing were performed with GraphPad Prism 6 software. Pearson correlation analysis was used to assess the association between two variables. P values for sister SCEs were calculated by Mann–Whitney tests. CDA mRNA levels in normal and cancerous tissues were compared in two-tailed unpaired t tests. Differences in the induction of CDA expression by 5-Aza-dC, as assessed by qRT-PCR, were evaluated in two-tailed paired t tests. Survival curves were compared in paired t tests for HeLa-shCDA versus HeLa-Ctrl cells treated with aminoflavone and HCC-1954 and IGROV-1 cells with and without 5-Aza-dC pretreatment. Unpaired t tests were used for the other cell lines. Differences were considered statistically significant if P < 0.05.

Results
CDA expression is downregulated in a large panel of cancer cell lines and tissues
We first analyzed CDA expression in cancer cell lines by combining in silico analyses on cancer cell lines from the Curie Institute, the NCI, and the CCLE (Broad-Novartis Cancer Cell Line Encyclopedia), and experimental approaches using qRT-PCR and Western blotting on a set of 26 representative cancer cell lines from the Curie Institute and the NCI. We found that CDA was expressed weakly or not at all in 25 of 34 (73%) breast cancer cell lines from the Curie Institute and 44 of 60 (73%) cancer cell lines derived from nine different organs and tissues from the NCI (Fig. 1A, left and right). Similarly, about 60% (700) of the 1,036 cancer cell lines from 26 different cancer tissues from the CCLE database did not express CDA (Supplementary Fig. S1A). We validated these results, by qRT-PCR and Western blotting on a set of 26 representative cancer cell lines from the Curie Institute and the NCI cancer cell line collections (19 breast cancer cell lines, 4 lung cancer cell lines, and 3 ovarian cancer cell lines; Fig. 1B).

We investigated whether the absence of detectable CDA expression observed in the majority of cancer cell lines also applied to primary tumor tissues, by performing qRT-PCR to analyze CDA mRNA levels in human primary breast tumors xenografted into nude mice (PDXs). As control, we analyzed Cda mouse analogue in parallel, to ensure that mouse stroma was not interfering with human tumor data. This approach made it possible to avoid the contamination of primary tumor tissues with normal cells from the stroma (usually up to 30%). We found that 56 of the 66 (~84%) human primary breast tumors studied had no significant CDA expression and did not find any correlation between the levels of mouse Cda expression and human CDA expression, indicating that mouse stroma did not contribute to the xenograft data (Fig. 1C). We also analyzed CDA protein levels in six types of primary cancer tissues (50 per type) and in 19 normal human tissues (20 per tissue) by IHC with an anti-CDA antibody validated by IHC on isogenic Bloom syndrome–derived cells not expressing CDA (BS-Ctrl) or expressing exogenous CDA (BS-CDA; see the Materials and Methods section and Supplementary Fig. S1B, ref. 5). According to CDA staining intensity in normal tissues (Supplementary Fig. S1C, top), we stratified CDA expression into two groups on the basis of staining intensity scores: CDA low (scores of 0 and 1) and CDA high (scores of 2 and 3; Fig. 1D). About 50% (endometrium) to 88% (breast triple-negative, ovary, and colon) of cancer tissues displayed very low levels of CDA expression.

We then compared CDA mRNA levels between healthy and cancerous tissues of different origins, by replotted the CDA mRNA data downloaded from GEO found in different genomic data sources (Nextbio, Oncomine). Tumor tissues are often contaminated with normal tissues that might express CDA, leading to inappropriate interpretations of CDA expression in some tumor tissues. Nevertheless, CDA expression levels were significantly lower in several tumors than in healthy tissues (Fig. 1E and Supplementary Fig. S1D and S1E). These results (summarized in Supplementary Table S1) reveal that CDA is overexpressed in some tumor tissues, such as those of pancreas, stomach, thyroid, and bladder cancers, as previously reported (19, 35), but underexpressed in other tumor tissues, such as those of liver, cervix, colon, and esophagus cancers. We confirmed these results by qRT-PCR on a small in-house cohort of colon tissues. We found that...
CDA expression levels were significantly lower (*P* = 0.0128) in tumor tissues (*n* = 10) than in healthy tissues (*n* = 5, Fig. 1F).

Finally, analysis of a recently published gene expression dataset used to determine the molecular mechanisms of cervical cancer progression (38) revealed that CDA expression decreased considerably with cervical cancer progression (Supplementary Fig. S1F). This result is consistent with the data presented in Fig. 1E, showing lower levels of CDA expression in cervical cancer tissues than in noncancerous tissues. Overall, these results suggest that CDA expression tends to be lost during carcinogenesis, at least in some tissues, such as the cervix.

CDA is downregulated by DNA methylation

We investigated the mechanism underlying the downregulation of the CDA gene in tumor cells by first analyzing CDA copy number in the DNA of the CCLE and NCI60 cell lines. No significant correlation was found between CDA mRNA levels and CDA gene copy number (Supplementary Fig. S2A). The downregulation of CDA levels cannot therefore be attributed to genetic deletions in tumor cells.

We then carried out sequencing analysis to determine whether CDA (promoter and exons) was mutated in 11 breast cancer cell lines that did not express CDA, through comparison with two breast cancer cell lines expressing high levels of CDA (HCC-1143 and MDA-MB-231, see Fig. 1B) and breast cell lines derived from healthy tissues with strong or weak CDA expression (MCF-12A and 184B5, respectively; Supplementary Fig. S2B). No genetic mutation likely to lead to CDA inactivation was identified (Supplementary Table S2A). However, we found several SNPs that had previously been identified and listed in the Single Nucleotide Polymorphism Database (11, 15, 39, 40). These results are consistent with the CDA gene sequencing results for the NCI-60 cell lines (Supplementary Table S2B) and demonstrate that CDA is not inactivated through genetic alterations in cancer cells.

We then explored the possible role of epigenetic regulation of CDA gene expression. We mapped the CpG methylation sites in the CDA gene. Using the NCI-60 methylation datasets, we mapped the CpG methylation sites in the CDA gene and identified 8 CpG methylation sites, three in the body of the gene, two within 1,500 bp of the transcription start site (TSS), one in TSS200, one in 5′-UTR, and one is 3′-UTR (Supplementary Fig. S2C). We then analyzed the levels of methylation of the CDA promoter using the dataset for the methylation of the NCI60 cell lines (25). We calculated Pearson correlation coefficients for the relationships between methylation at the various CpG methylation sites and CDA expression. We found a highly significant negative correlation between CDA transcript and methylation levels at CpG two sites, cg04087271 (TSS200) and cg00784581 (5′-UTR; Pearson *r* = −0.4184, *P* = 0.009; Fig. 2A), the six other exhibiting no significant negative correlation with CDA expression. High levels of CDA mRNA were found in 42% of the CDA-deficient cell lines (19/45), such as MCF-7 and IGROV-1, and no methylation was detected in cell lines overexpressing CDA, such as MDA-MB-231 and HOP-92, except for the LOXIMVI melanoma cell line.

For the validation of these methylation data, we treated a set of cancer cell lines derived from breast, lung, ovarian, and melanoma tumors not expressing CDA (Fig. 1B and Supplementary Fig. S2D) with the DNA methyltransferase activity inhibitor (5-Aza-dC), resulting in DNA demethylation (42). We found that 5-Aza-dC induced a strong increase (up to 1,000-fold induction) in CDA mRNA levels (Fig. 2B) without major toxicity (Supplementary Fig. S2E) in the 7 CDA-deficient cell lines analyzed. By contrast, it had little or no effect on CDA transcription in CDA-deficient melanoma cells (HCC-1143, and HCC-1937 control cell lines, which have constitutively high levels of CDA (~2-fold induction).

It has been reported that the selection of CDA overexpression in response to prolonged drug exposure is responsible for resistance to gemcitabine (18, 43) and that the ectopic expression of CDA in CDA-deficient cancer cells leads to a significant increase in resistance to gemcitabine (18, 44). We thus evaluated the functionality of the CDA protein produced after 5-Aza-dC treatment by breast and ovarian cancer cells, HCC-1954 and IGROV-1, respectively. The cells were left untreated or were subjected to pretreatment with 5-Aza-dC for 96 hours and then to treatment with various concentrations of gemcitabine over a period of 72 hours. The induction of CDA protein production by 5-Aza-dC led to a significant increase in gemcitabine resistance (Fig. 2C). Our data are consistent with 5-Aza-dC inducing the expression of functional CDA protein in CDA-deficient cancer cells, leading to resistance to gemcitabine.

We then analyzed in silico CDA promoter methylation levels (Supplementary Fig. S2C) on TCGA samples for 16 different tissue cancers, using the CBioPortal for cancer genomics (29, 30) and the Broad Institute FireBrowse portal (27). We found a highly significant correlation between CDA transcripts levels and CDA promoter methylation on two CpG sites (cg04087271 and cg24502330). Methylation of the cg04087271 site was the only one correlating with CDA deficiency in both tumor tissues and NCI-60 cell lines (Fig. 2D). In both cancer cell lines and tumor tissues, CDA promoter methylation levels were significantly higher in samples with low CDA transcript levels (Fig. 2D) than in those with high CDA transcript levels. This correlation was not significant in some other cancer types, but we nevertheless identified a subpopulation of these cancers with low CDA expression and high CDA promoter methylation levels (Supplementary Fig. S2F). Thus, the association of methylation status to gene expression is consistent with the hypothesis that promoter methylation is a principal driver of CDA gene expression in cancer.

Loss of CDA expression in tumor cells defines a new tumor subgroup that could be specifically targeted by chemotherapy

We previously reported that CDA deficiency in BS cells or CDA depletion in HeLa cells leads to an increase in SCE frequency, a hallmark of genomic instability (4, 5).

We therefore investigated whether constitutive CDA deficiency in tumor cells was also associated with an increase in SCE frequency by analyzing basal SCE levels in several cancer cell lines derived from breast, lung, and ovary tumors. SCE frequency was significantly higher in the cancer cell lines not expressing CDA than in those expressing CDA (Fig. 3A).

Thus, tumors from the same classically defined groups may display differences in CDA expression status, resulting in contrasting cellular properties, such as SCE levels (e.g., CDA-proficient HCC-1143 cells and CDA-deficient BT-20 cells are both classified as triple-negative breast cancer cells). We thus propose the use of CDA expression status in tumor cells to define two new subgroups: CDA-deficient tumors and CDA-proficient tumors. These new subgroups may differ in their sensitivity to antitumor therapies. The targeting of CDA-deficient tumor cells might therefore open up new possibilities for cancer therapy.
Figure 3.
Drug sensitivity of CDA-deficient cells. **A**, SCE frequency in CDA-deficient and CDA-proficient cells (left) and representation of SCE frequency in cells classified on the basis of their CDA expression status, low or high (right). ns, not significant. P values were calculated by Mann-Whitney tests for at least three independent experiments. P < 0.05 was considered statistically significant. **B**, Scatterplot showing a significant negative correlation between aminoflavone cytotoxicity and CDA expression (Pearson correlation) in the NCI60 panel of cell lines. Colors, origin of the cancer tissue. **C**, Isogenic HeLa cell lines (red, HeLa control cells; blue, CDA-depleted HeLa cells) were treated for 72 hours with the indicated concentrations of aminoflavone, and the percentage of cells surviving is shown. **D**, Breast (MCF-7, MDA-MB-468, and MDA-MB-231) and ovarian (SKOV-3, OVCAR-8, and IGROV-1) cancer cell lines were treated for 72 hours with the indicated concentrations of aminoflavone (AF). Blue, survival curves of cell lines with low levels of CDA expression; red, survival curves of cell lines with high levels of CDA expression. For **C** and **D**, cell viability was assessed in MTT assays. The error bars represent means ± SD for three independent experiments. P < 0.05 was considered statistically significant.
The CellMiner web tool (45) can be used to assess the correlation between gene expression and drug sensitivity/resistance. We searched for drugs with antiproliferative activity significantly correlated with CDA expression levels. We identified 277 such drugs, 94 of which were more toxic to CDA-deficient cells and 183 of which were more active against CDA-proficient cells (Supplementary Table S3). We tested our hypothesis that drugs that do not affect CDA-proficient cells can specifically target CDA-deficient cells by focusing on an aminoflavone derivative (AFP464; NSC 710464), for which we found a highly significant negative correlation (Pearson r = -0.379, P = 0.0031) with CDA deficiency (Supplementary Table S3). Twenty CDA-deficient cell lines of the 43 tested (46.5%), including MCF-7 and IGROV-1, were sensitive to aminoflavone, whereas 13 of the 16 (81.25%) CDA-proficient cell lines, including MDA-MB-231, were resistant to this drug (Fig. 3B).

Among the selected molecules presenting a significant negative correlation with CDA expression, we choose aminoflavone because it has reached phase II clinical trials in the United States for treating solid tumors, including breast cancer (NCT01015521, NCT01233947, NCT00356920, NCT00348699; ref. 46), and because the anititumor efficacy of aminoflavone had been validated in vivo by three independent groups on MCF-7 and MDA-MB-468 xenograft breast cancer models (47–49). No effect of aminoflavone was observed on MDA-MB-231 xenograft breast cancer cells (49). Together with our results showing that MCF-7 and MDA-MB-468 breast cancer cell lines are CDA deficient, and MDA-MB-231 cells are CDA proficient (Fig. 1A and B), these data are consistent with our hypothesis that aminoflavone specifically targets CDA-deficient cancer cells both in vitro and in vivo.

We thus evaluated the causality of the relationship between CDA downregulation and the antiproliferative activity of aminoflavone by shRNA-mediated CDA depletion in HeLa cells (Supplementary Fig. S3). We found that CDA depletion significantly increased sensitivity to aminoflavone treatment (Fig. 3C). We then confirmed the cytotoxicity of aminoflavone in six breast and ovary cancer cell lines, three of which were CDA deficient (MCF-7, MDA-MB-468, and IGROV-1), and the other three being CDA proficient (MDA-MB-231, OVCA-8, and SKOV-3). The CDA-deficient cell lines were highly sensitive to aminoflavone, whereas the CDA-proficient cell lines were resistant (Fig. 3D, left and right).

Our results show that CDA expression status can be used as a predictor of sensitivity to aminoflavone; CDA deficiency is thus a potential new sensitive biomarker or target for anticancer therapies.

**Discussion**

We found that CDA expression was lost in a large proportion of cancer cells and tumor tissues, and our findings identify CDA-deficient tumors as a new subgroup of cancers. SCE frequency was significantly higher in cancer cell lines not expressing CDA than in those expressing CDA. This increase in SCE frequency might reflect a nucleotide pool imbalance due to CDA deficiency that slows down replication fork progression, leading to an increase in replication-associated DNA breaks that could account, at least in part, for SCE formation (4).

The loss of CDA expression is mostly due to DNA methylation, and the treatment of CDA-deficient cells with 5-Aza-dC was sufficient to restore the expression of a functional CDA. This is the first study, to our knowledge, to reveal the extent of CDA inactivation and its epigenetic control in cancer.

DNA methylation may be the predominant mechanism of CDA silencing, but it is clearly not the only one, as some CDA-deficient cell lines present no CDA gene methylation. The other mechanisms involved in regulating CDA gene expression merit further investigation but are beyond the scope of this study.

CDA has already been shown to play a crucial role in the response of cancer cells to widely used nucleoside analogues, such as cytosine arabinoside and gemcitabine, and the dose-limiting toxicity of these drugs (8, 50–53). Our results suggest that IHC assessments of CDA levels could be used to determine the CDA status of tumors, with potential implications for treatment.

Oxidized and epigenetically modified cytidine nucleosides specifically target tumors overexpressing CDA (19, 20). We found that 5-Aza-dC treatment strongly induced the expression of a functional CDA in CDA-deficient tumor cells, with little or no effect on CDA expression in CDA-proficient cells. These findings suggest that DNA-demethylating agents could be assessed as a possible treatment for CDA-deficient tumors to induce CDA overexpression and then sensitize these tumors to treatment with oxidized and epigenetically modified cytidine nucleosides.

Finally, our results suggest that the targeting of CDA deficiency might offer new possibilities for treatment. In silico screening with the NCI CellMiner analysis tool identified aminoflavone as a proof-of-principle candidate for the targeting of CDA-deficient tumor cells. Aminoflavone acts mainly by activating aryl hydrocarbon receptor, which in turn induces the expression of cytochrome P450 CYP1A1/1A2/1B1 that metabolizes aminoflavone to toxic products, inducing replication stress, DNA damage, and apoptosis via a p53/p21-mediated pathway (48, 54–57).

We found that aminoflavone was specifically effective in CDA-deficient tumor cells and that this drug had no effect on CDA-proficient cells, in agreement with the literature (47–49).

Thus, the subgroup of tumors not expressing CDA could be specifically targeted by such treatment, and CDA expression status could be used as a new marker to guide anticancer therapy. Molecules not yet shown to be active against this tumor subgroup will probably be discovered through the systematic screening of CDA-proficient and -deficient cells.

In conclusion, although additional human trials are needed, our results constitute a proof of concept that CDA deficiency may turn out to be a new predictive marker of susceptibility to antitumor drugs that could be used as a new target for anticancer therapies, thus opening up new possibilities for the treatment of cancers.

**Disclosure of Potential Conflicts of Interest**

S. Varma is an employee of HiThru Analytics. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: H. Mameri, Y. Pommier, M. Amor-Guérét

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References


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