Laccaic Acid A Is a Direct, DNA-competitive Inhibitor of DNA Methyltransferase 1*[S]

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Background: Approved DNA demethylators do not directly inhibit Dnmt1, an oncogenic methyltransferase.

Results: Laccaic acid A (LCA) is a direct, DNA-competitive Dnmt1 inhibitor that reactivates genes silenced by DNA methylation in breast cancer cells synergistically with 5-azadC.

Conclusion: LCA is a natural product in a new class of Dnmt1-targeting small molecules.

Significance: By directly inhibiting Dnmt1, we may reveal and block specific carcinogenesis pathways.

Methylation of cytosines in CpG dinucleotides is the predominant epigenetic mark on vertebrate DNA. DNA methylation is associated with transcriptional repression. The pattern of DNA methylation changes during development and with disease. Human DNA methyltransferase 1 (Dnmt1), a 1616-amino acid multidomain enzyme, is essential for maintenance of DNA methylation in proliferating cells and is considered an important cancer drug target. Using a fluorogenic, endonuclease-coupled DNA methylation assay with an activated form of Dnmt1 engineered to lack the replication foci targeting sequence domain, we discovered that laccaic acid A (LCA), a highly substituted anthraquinone natural product, is a direct inhibitor with a 310 nM K_i. LCA is competitive with the DNA substrate in in vitro methylation assays and alters the expression of methylated genes in MCF-7 breast cancer cells synergistically with 5-aza-2′-deoxycytidine. LCA represents a novel class of Dnmt-targeted molecular probes, with biochemical properties that allow it to distinguish between non DNA-bound and DNA-bound Dnmt1.

Methylation of cytosine, which occurs predominantly in CpG[2] dinucleotides, is an important epigenetic mark associated with gene repression. DNA methylation is crucial for development and differentiation, genome stability, genomic imprinting, X-chromosome inactivation, and silencing of retrotransposons (1). Three catalytically active DNA methyltransferases (Dnmts) are responsible for establishing and maintaining DNA

methylation patterns in vertebrates. The Dnmt3 isoforms (Dnmt3a and Dnmt3b), termed de novo methyltransferases, are responsible for establishing DNA methylation marks during germ cell and embryonic development and thus act preferentially on nonmethylated DNA sequences (2). Dnmt1, the most abundant Dnmt, is responsible for maintaining DNA methylation patterns. Thus, hemimethylated DNA sequences are the preferred Dnmt1 substrates (3).

Disruption of normal DNA methylation patterns can lead to a variety of diseases including cancer. Indeed, cancer is the class of illness for which DNA methylation was first proposed as a therapeutic target (4). Increases in Dnmt1 activity and genomic changes in methylation patterns are observed in a variety of malignancies. Multiple common oncogenic pathways lead to the overexpression of Dnmt1 through transcriptional and post-translational mechanisms (5–8). This tends to produce global DNA hypomethylation with concomitant hypermethylation of promoter regions of tumor suppressor genes, enabling tumor-specific gene silencing (9, 10). In preclinical models, targeting of the Dnmt1 isozyme for cancer prevention has been validated genetically. Reducing Dnmt1 copy number and gene activity protects against tumor formation in the Apc^{Min} mouse (11). In addition, targeting Dnmt1 with antisense oligonucleotides inhibits neoplasia in cell culture and in mouse tumor models (12, 13).

Gene-targeted experiments that result in the reduction or elimination of a protein can be used to distinguish among the functions of Dnmt1, Dnmt3a, and Dnmt3b. This is particularly important because Dnmt3a is inactivated in a high proportion of human tumors, such as acute myeloid leukemia (14). In contrast to the isozyme specificity of genetic experiments, most small molecule inhibitors work by mechanisms that are quite distinct from genetic knockouts and knockdowns. Indeed, small molecules that block enzyme activity or inhibit a specific protein-protein interaction would be expected to perform differently than genetic reagents that prevent expression of a protein. This is relevant for Dnmt1, which may be essential for the assembly of multiple protein complexes with different functions in different cell types.

The most commonly used small molecules for cellular and therapeutic DNA demethylation are neither isozyme-specific
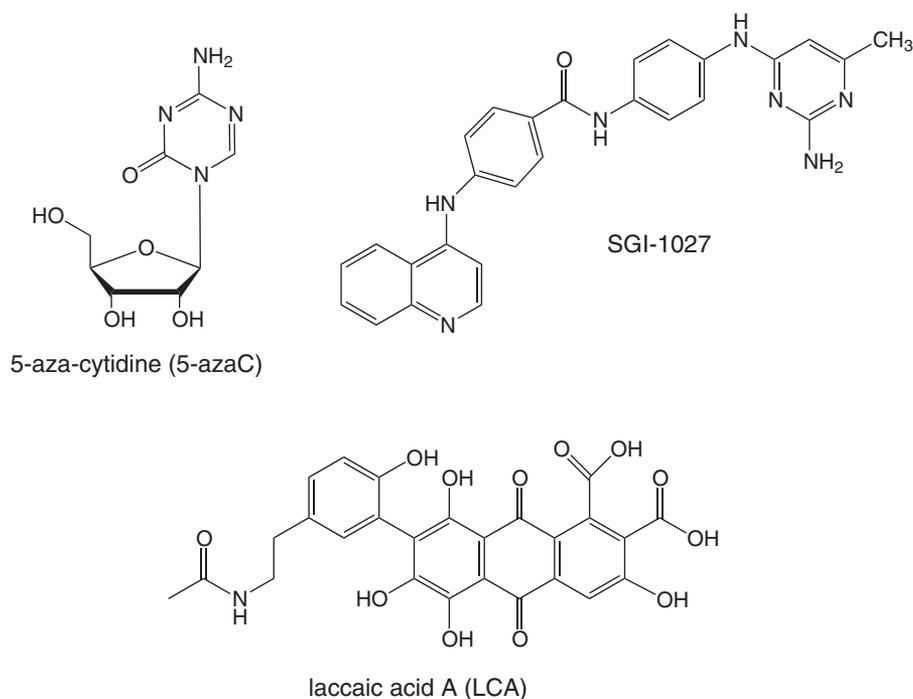
FIGURE 1. **Structures of inhibitory compounds.** 5-AzaC is a known demethylator *in vivo* and an approved drug for treatment of myelodysplastic syndrome (15). SGI-1027 is a previously reported Dnmt inhibitor (31). LCA is an insect-derived natural product and the focus of this work.

nor direct inhibitors of Dnmt1. Agents such as 5-azacytidine (5-azaC; see Fig. 1) and 5-aza-2′-deoxycytidine (5-azadC) effectively cause DNA demethylation and have been approved for use in myelodysplastic syndrome (15). 5-Aza nucleosides have complicated mechanisms of action. After cellular uptake, these compounds are converted to their triphosphate counterparts, which requires an additional ribonucleotide reductase-dependent step for 5-azaC. The resulting 5-azadCTP metabolite is then incorporated into DNA, albeit not specifically at CpG dinucleotides. Incorporated 5-azadCMP nucleotides induce a DNA damage response and covalent trapping of Dnmt isozymes, which is followed by proteolysis of Dnmt proteins, DNA demethylation, and reactivation of hypermethylated genes (15–17). The cytotoxicity of 5-aza nucleosides, lack of direct inhibition of Dnmts, and lack of Dnmt isozyme specificity suggests that there is room for improvement in molecular probes targeted against Dnmt1 enzyme activity. Such compounds might allow further characterization of the role of Dnmt1 and other Dnmt isozymes in carcinogenesis and could also serve as leads for new drug development.

Human Dnmt1 is a 1616-amino acid multidomain protein with a conserved C-terminal catalytic domain and ~1100 amino acids of N-terminal regulatory sequences (supplemental Fig. S1). Multiple globular conserved domains are found in the N-terminal regulatory region including the DMAP1 (DNA methyltransferase-associated protein 1) binding domain (18), the proliferating cell nuclear antigen binding domain (19), the replication focus targeting sequence (RFTS) domain (20), the CXXC domain (21), and two bromo-adjacent homology domains (22).

Recently several structures of Dnmt1 were reported (23–26). The first structures to be solved consisted of nonmethylated duplex DNA bound to a fragment of Dnmt1 that started with

the CXXC domain (23). In these structures the linker between the CXXC domain and the first bromo-adjacent homology domain was positioned in the active site and proposed to serve an autoinhibitory role and occlude nonmethylated DNA binding. However, using a fluorescence-based DNA methylation assay, we showed that the RFTS domain of Dnmt1 (not present in the original crystal structure) is an endogenous DNA-competitive inhibitor of Dnmt1 activity, such that a truncation mutant lacking RFTS is ~640-fold more active than a protein containing RFTS (24). Enzymatic assays and gel shift assays clearly showed that the RFTS domain inhibits association with DNA whether the DNA is hemimethylated or not. The kinetics of RFTS inhibition and the acidic nature of this domain prompted us to conclude that RFTS is the key autoinhibitory domain of Dnmt1 that must be removed from the DNA active site for catalysis to occur in the full-length enzyme (24). Consistent with our biochemical data and prediction, the crystal structure of a mouse Dnmt1 construct containing the RFTS domain confirmed that the RFTS domain binds in the substrate DNA binding pocket (25).

This activated form of Dnmt1 (residues 621–1600; supplemental Fig. S1) and the fluorogenic assay proved to be valuable tools to identify small molecule inhibitors of Dnmt1 activity *in vitro*. Here we show that laccaic acid A (LCA; Fig. 1), a highly substituted anthraquinone natural product, is a direct, DNA-competitive inhibitor of Dnmt1 with a submicromolar K_i. In addition, treatment of MCF-7 cells with LCA results in re-expression of genes known to be silenced by methylation, indicating that LCA is a useful lead compound for Dnmt1 inhibition. Significantly, LCA and 5-azadC synergized in activating expression of silenced tumor suppressor genes, thereby demonstrating the value of obtaining a molecular probe with a direct and
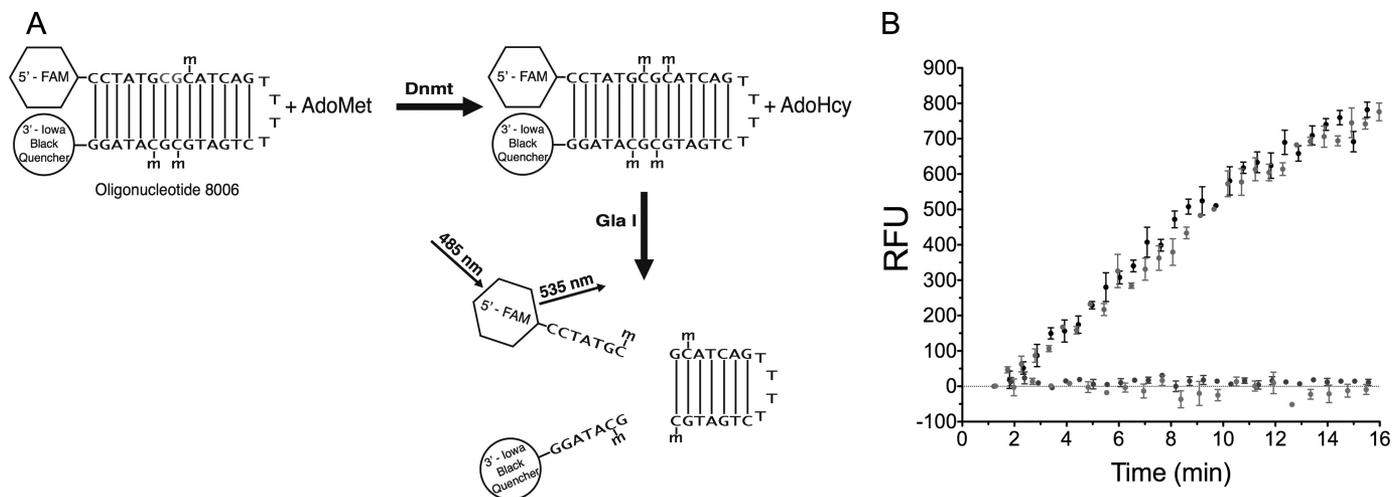
FIGURE 2. **LCA inhibits Dnmt1 activity.** *A*, shown is a schematic of the Gla I-coupled Dnmt assay. Oligonucleotide 8006 contains a single nonmethylated CpG dinucleotide (shown in *red*). The fully methylated product oligonucleotide is a substrate for the restriction endonuclease Gla I. Cleavage of the product oligonucleotide releases the 5′ fluorophore from the 3′ quencher and generates fluorescence. *AdoHcy*, *S*-adenosyl homocysteine. *B*, shown is time dependence of assays containing 10 nM oligonucleotide 8006, 10 μM AdoMet, and 1 nM RFTS-lacking Dnmt1 (amino acids 621–1600) in the presence of DMSO (*black*), 10 μM 5-azaC (*green*), 10 μM SGI-1027 (*blue*), and 10 μM LCA (*red*). The addition of SGI-1027 and LCA fully inhibit Dnmt1 activity, whereas 5-azaC has no effect on fluorescence generation. *RFU*, relative fluorescence units.

complementary mechanism of action to that of 5-aza nucleosides.

EXPERIMENTAL PROCEDURES

Expression and Purification of Dnmts—Truncated versions of human Dnmt1 (amino acids 621–1600 and 351–1600; supplemental Fig. S1) were expressed and purified as previously described (24). The catalytic domain of human Dnmt3a (amino acids 590–912) was expressed as an N-terminal His-tagged maltose-binding protein fusion protein in Rosetta 2(DE3)pLysS competent cells (Novagen). Protein expression was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and cultures were grown at 30 °C for 4 h. After cell lysis, protein was purified via metal affinity using nickel-nitrilotriacetic acid resin (GE Healthcare). The fusion protein was cleaved with tobacco etch virus protease, and a second metal affinity column was used to remove the His-tagged protease and any uncleaved fusion protein. The C-terminal catalytic domain was then further purified using a Heparin HP Hi-Trap column (GE Healthcare). Bound protein was eluted using a linear gradient from 0.2 to 1.5 M NaCl in 20 mM NaP$_i$, pH 7.5, 2 mM DTT buffer. Purified proteins were concentrated and stored at −80 °C in 50% glycerol. All proteins were quantified using A_{280} and calculated extinction coefficients.

DNA Methylation Assay—Dnmt activity was measured using a fluorescence-based DNA methylation assay (24). In short, hemimethylated hairpin oligonucleotide, termed 8006, with a 5′ fluorophore and a 3′ quencher was incubated with Dnmt and AdoMet in the presence of restriction enzyme Gla I, which cleaves the fully methylated product oligonucleotide and releases the fluorophore from the quencher, generating fluorescence in real-time (Fig. 2*A*). A control containing Gla I in the absence of methyltransferase was subtracted from each assay condition to account for the sum of background fluorescence of the internally quenched substrate and slow Gla I cleavage of the hemimethylated substrate oligonucleotide. Assays (0.1 ml)

were conducted in 96-well black half-area plates in either a Wallac VICTOR[2] or Biotek Synergy Neo plate reader at 37 °C in 10 mM Tris, pH 7.5, 100 mM potassium glutamate, 1 mM MgCl$_2$, 1 mM DTT, 0.1 mg/ml BSA, and 5% glycerol. Assays contained varying amounts of oligonucleotide 8006 (5′-FAM-CCTATGC-GmCATCAGTTTTCTGATGmCGmCATAGG-3′-Iowa Black, in which mC denotes 5-methyldeoxycytidylate residues (Integrated DNA Technologies, Coralville, IA), AdoMet (HPLC-purified, Sigma), and small molecule inhibitors (5-azaC, Sigma; SGI-1027, generous gift of Dr. Jian Jin, University of North Carolina; LCA, TCI America). Other anthraquinone compounds examined were purchased from ChemBridge Corp, San Diego, CA (UI1055 is *N,N*-diethyl-1-nitro-9,10-dioxo-9,10-dihydroanthracene-2-carboxamide; UI1060 is ethyl *N*-{[1-(butylthio)-9,10-dioxo-9,10-dihydroanthra-cen-2-yl] carbonyl}glycinate; UI1061 is 1-(butylsulfonyl)-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid) and Sigma (anthraquinone and anthraquinone 2-carboxylic acid). All assays were conducted in triplicate and contained either human Dnmt1 (621–1600), human Dnmt1 (351–1600), human Dnmt3a (590–912), or M.SssI methyltransferase (New England Biolabs) and 0.8 units of Gla I (Sibenzyme, West Roxbury, MA), except for the Gla I control assay, which did not contain a methyltransferase. Data were fitted using Prism (GraphPad Software, Inc). Dose-response data were fit allowing the Hill slope to vary. In all cases, Hill slopes of −0.9 to −1.2 were obtained. Thus, all dose-response data were refitted using a constant Hill slope of −1.

Gla I Activity Assay—Gla I activity was measured using a fluorescence-based assay. Assays (0.1 ml) were conducted in 96-well black half-area plates at 37 °C in a Wallac VICTOR[2] using excitation and emission wavelengths of 485 and 535 nm, respectively. Each assay contained 10 mM Tris, pH 7.5, 100 mM potassium glutamate, 5 mM MgCl$_2$, 1 mM DTT, 0.1 mg/ml BSA, 5% glycerol, 8 nM oligonucleotide 8007 (5′-FAM-CCTA-TGmCGmCATCAGTTTTCTGATGmCGmCATAGG-3′-Iowa

Black, where mC denotes 5-methyldeoxycytidylate residues; Integrated DNA Technologies), 0.4 units Gla I, and 1% DMSO. Assays were conducted in triplicate in the presence and absence of 10 μM LCA. Cleavage of the oligonucleotide by Gla I releases the 5′-FAM fluorophore from the 3′-Iowa Black quencher and generates fluorescence. Data were fitted using Prism.

Differential Scanning Fluorometry (DSF)—DSF (28) was used to assess the ability of small molecules to bind directly to Dnmts in the absence of substrates and alter the observed melting temperature (T_m). Assays (25 μl) were conducted in triplicate in a Bio-Rad C1000 Thermal Cycler-CFX real-time system using the FRET channel and contained 100 mM Hepes, pH 7.5, 100 mM potassium glutamate, 5 mM MgCl$_2$, 1–4 μM Dnmt, 5× Sypro Orange (Invitrogen), 50 or 100 μM concentrations of the compounds of interest, and 1% DMSO. The temperature was increased from 25 to 95 °C in 0.5 °C increments every minute. Fluorescence traces were exported and analyzed by fitting to the Boltzmann equation in Prism to determine the T_m.

Cell Culture—Human breast cancer MCF-7 cells (ATCC) were seeded at 6×10^4 per well in DMEM (Invitrogen) supplemented with 10% fetalplex (Gemini Bio-products) and grown overnight in 5% CO$_2$ at 37 °C. The following day the cells were treated with 200 μM LCA or anthraquinone-2-carboxylic acid. In some experiments 0.5 μM 5-azadC (Sigma) was added. All compounds were dissolved in media. Each day for 5 days the media were replaced with fresh media plus the compounds, and then RNA and/or DNA were isolated from cells.

Microarray Analysis—Total RNA was isolated from 2×10^5 cells and used for microarray analysis (University of Iowa DNA Core Facility) in hybridization to Human Gene ST1.0 Array GeneChips (Affymetrix). Data were analyzed using Partek Genomics Suite Version 6.5. Pathway analysis was performed on the genes that changed expression in response to LCA using the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) (29, 30).

Quantitative PCR—After drug treatment, total RNA was extracted from 2×10^5 cells using TRIzol (Invitrogen). RNA was treated with DNase I (Invitrogen), and cDNA was generated using the First strand cDNA synthesis kit (Invitrogen). Primers (supplemental Table 1) were designed using Primer3web software version 4.0.0, and qRT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems). Reactions were performed in triplicate on three biologically independent samples using the Step One Plus Real-time PCR system (Applied Biosystems). Normalization was performed using cycle threshold (Ct) values for *HPRT*.

RESULTS

LCA Is a Direct, Potent Inhibitor of Dnmt1—Reversing epigenetic changes, such as aberrant DNA methylation, is a promising cancer therapy strategy (9). Because the link between Dnmt1 hyperactivity and cancer is well established (9, 10, 16), we sought to discover novel small molecule modulators of Dnmt1 function. We developed a high throughput assay to screen for compounds that inhibit activity of an activated form of Dnmt1 (621–1600). Deletion of sequences N-terminal of and including the RFTS domain results in an enzyme that has a k_{cat}/K_m of 1.9×10^6 M^{-1}s^{-1} (DNA substrate), which is a 640-

fold improvement over forms of the enzyme that include the RFTS domain (24). This catalytic power and the sensitivity of the fluorogenic assay were exploited to search for and characterize enzyme inhibitors. Using a miniaturized version of our Gla I-coupled DNA methylation assay (24), we screened the 2320 compound Spectrum library (Microsource, Gaylordsville, CT) for agents that inhibit Dnmt1 activity.[3] In this screen, LCA was identified as a promising potential inhibitor with an unknown mechanism of action.

We employed multiple approaches to test whether LCA is an authentic and direct inhibitor of Dnmt1 activity. In the presence of 10 μM LCA, no fluorescence generating activity was observed in the coupled methylation assay using oligonucleotide 8006 (Fig. 2). As a positive control, we used SGI-1027 (Fig. 1), a quinoline compound reported to directly inhibit Dnmt isozymes (31). The addition of 10 μM SGI-1027 resulted in complete inhibition of the activated form of Dnmt1 in our assay (Fig. 2). However, 10 μM 5-azaC had no effect on Dnmt1 activity. This was anticipated because it is known that cytosine nucleoside inhibitors, such as 5-azaC, must be incorporated into DNA to cause cellular DNA demethylation (16). These data indicated that 5-azaC is not a direct inhibitor of Dnmt1 and suggested that LCA and SGI-1027 are either Dnmt1 inhibitors or cause interference with some aspect of the methylation assay.

The possibilities that LCA is an inhibitor of the restriction enzyme Gla I or a quencher of the 5′-FAM product were excluded by examining the effect of LCA on Gla I activity. The addition of 10 μM LCA to an assay containing oligonucleotide 8007, the fully methylated product oligonucleotide, had no effect on Gla I activity (supplemental Fig. S2). We then aimed to ensure that the inhibitory effects observed by the addition of LCA were structurally specific and could not be attributed to the anthracene or anthraquinone core. We, therefore, examined the effect of various anthraquinone compounds on Dnmt1 activity. No other anthraquinone compound examined inhibited Dnmt1 in the *in vitro* assay (supplemental Fig. S3 and Table 2). Thus, Dnmt1 inhibition cannot be attributed to nonspecific redox cycling or another generic characteristic of anthraquinone compounds.

Although LCA is not an inhibitor of Gla I, we aimed to exclude the possibility that the inhibitory activity of LCA stems from an interaction with the DNA substrate. To test if LCA interacts with RFTS-lacking Dnmt1 in the absence of DNA and AdoMet, we used DSF to determine the melting temperature (T_m) of Dnmt1 in the presence and absence of each candidate inhibitor. The addition of SGI-1027 resulted in a 2.0 ± 0.1 °C increase in the T_m (Table 1), indicating that the small molecule binds directly to Dnmt1 in the absence of substrates and stabilizes the enzyme against thermal denaturation. Because 5-azaC is a prodrug that does not directly inhibit Dnmt1, this compound did not alter the T_m. In contrast, the addition of LCA to the Dnmt1 DSF assay resulted in a 1.9 ± 0.1 °C increase in T_m from 43.8 °C in the DMSO control to 45.7 °C in the presence of saturating LCA. These data indicate that LCA binds directly to Dnmt1.

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[3] R. L. Fagan, M. Wu, and C. Brenner, submitted for publication.

TABLE 1

Changes in the observed melting temperature of Dnmt1 (621–1600) as determined by DSF

Compound	ΔT_m
	°C
5-AzaC	0
SGI-1027	2.0 ± 0.1
LCA	1.9 ± 0.1

An authentic inhibitor should show concentration dependence. To calculate an IC_{50} value for each inhibitor, the concentrations of LCA and SGI-1027 were varied from 10 nM to 10 μM under standard assay conditions (10 nM oligonucleotide 8006 and 10 μM AdoMet). The percent activity observed was determined with respect to an uninhibited control. Both compounds showed concentration-dependent inhibition of Dnmt1 (621–1600). However, with an IC_{50} value of 650 ± 40 nM, LCA exhibited ~2.5-fold higher potency at inhibiting Dnmt1 than SGI-1027 (Fig. 3).

We then investigated if LCA acts as a covalent inactivator. LCA was incubated with either Dnmt1 or Dnmt1 plus AdoMet for 30 min. Incubated enzymes were then buffer-exchanged into assay buffer that lacked LCA and used to initiate DNA methylation assays. In both cases, incubation with LCA had no effect on the observed activity of Dnmt1 (supplemental Fig. S4), indicating that LCA does not covalently inactivate the enzyme.

LCA Is a DNA-competitive Inhibitor—The cytosine nucleoside DNA demethylating agents, such as 5-azaC, must be incorporated into DNA to exert their effects (15, 16). Several non-nucleoside inhibitors of DNA methylation have been reported in the literature (32). However, experimental information on the kinetics and mode of inhibition is lacking for many of these compounds. We set out to determine the mode of inhibition of LCA and SGI-1027 using the fluorescence-based DNA methylation assay. We titrated SGI-1027 in assays containing high concentrations of DNA (~13 times $K_{m,\text{DNA}}$) and varied concentrations of AdoMet to determine kinetic parameters. As suggested by previous results (31), SGI-1027 acts as an AdoMet competitor (Fig. 4A). The double reciprocal plot of the data clearly shows competitive inhibition with all lines intersecting on the *y* axis. Fitting the data to an AdoMet competitive model yields a K_i of 1.5 ± 0.3 μM. Surprisingly, LCA was not competitive with AdoMet. Instead, this compound exhibited mixed inhibition with respect to AdoMet (Fig. 4B and supplemental Fig. S5) and was competitive with the oligonucleotide substrate (Fig. 4C and supplemental Fig. S5). Titrating LCA into assays containing AdoMet at ~15 times $K_{m,\text{AdoMet}}$ and varying concentrations of oligonucleotide 8006 yielded a double reciprocal plot in which all lines intersect on the *y* axis, indicating DNA competition. As the concentration of LCA was increased, the apparent $K_{m,\text{DNA}}$ was increased with no effect on the observed V_{max}. Fitting these data by nonlinear regression, a K_i of 310 ± 80 nM was obtained (supplemental Fig. S5). Thus, in our *in vitro* DNA methylation assay, LCA is an ~5-fold more potent inhibitor of Dnmt1 activity than SGI-1027 with a novel mechanism of action.

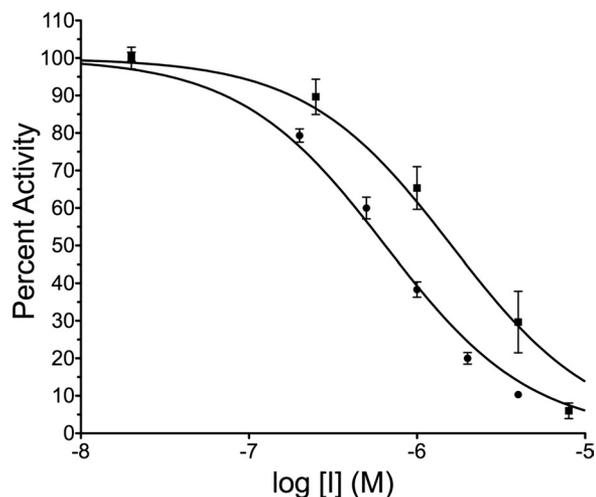The activated form of Dnmt1 (621–1600) was used for probe discovery because it is ~640-fold more active than forms of

FIGURE 3. **LCA is a more potent inhibitor of Dnmt1 than SGI-1027.** The concentration dependence of inhibition by SGI-1027 (●) and LCA (■) was investigated at 10 nM oligonucleotide 8006 and 10 μM AdoMet using 1 nM RFTS-lacking Dnmt1 (amino acids 621–1600). The percent activity of each condition was determined by comparing to an uninhibited DMSO-containing control. Fitting the data using a Hill slope of −1 gives an IC_{50} of 1.6 ± 0.2 μM for SGI-1027 and 650 ± 40 nM for LCA.

Dnmt1 with the RFTS domain. We have previously shown that RFTS functions as a DNA-competitive inhibitor of Dnmt1 (24). To test whether LCA would inhibit Dnmt1-containing RFTS, we first used DSF to test direct binding to Dnmt1 (351–1600). As observed for Dnmt1 (621–1600), the addition of 50 μM LCA shifts the melting temperature of Dnmt1 (351–1600) 2 °C to the right (Fig. 5A). The ability of LCA to stabilize against thermal denaturation of Dnmt1 (351–1600) indicates that the small molecule is capable of binding Dnmt1 even in the presence of the RFTS domain. We then investigated the ability of LCA to inhibit the methyltransferase activity of Dnmt1 (351–1600) under assay conditions suitable for this enzyme (100 nM oligonucleotide 8006 and 100 μM AdoMet). LCA concentration was varied from 2 to 100 μM. Under these conditions, LCA exhibited concentration-dependent inhibition of Dnmt1 with an IC_{50} value of 19.1 ± 1.7 μM (Fig. 5B). The higher IC_{50} for LCA inhibition of Dnmt1 (351–1600) than for Dnmt1 (621–1600) can be explained in two ways. First, the RFTS domain-containing enzyme must be assayed at a 10-fold higher concentration of DNA substrate. Second, because the RFTS domain is also DNA-competitive, a higher concentration of LCA would be required to displace the endogenous inhibitory protein domain. These findings suggest that LCA would be capable of inhibiting Dnmt1 in a cellular context, although at a higher concentration than used against Dnmt1 (621–1600).

LCA Specificity—We next investigated the specificity of LCA inhibition of cytosine DNA methyltransferases. Three catalytically active isoforms of Dnmt exist in vertebrates: Dnmt1, Dnmt3a, and Dnmt3b (1). To determine if LCA can interact directly with other Dnmts, we used DSF to detect changes in the observed melting temperature of the catalytic domain of Dnmt3a. In the presence of 100 μM LCA, the observed melting temperature is shifted more than 2 °C to the right from 43.0 ± 0.1 °C in the absence of LCA to 45.3 ± 0.1 °C, indicating a direct interaction between LCA and Dnmt3a. To further investigate specificity, IC_{50} values were determined for LCA inhibition of
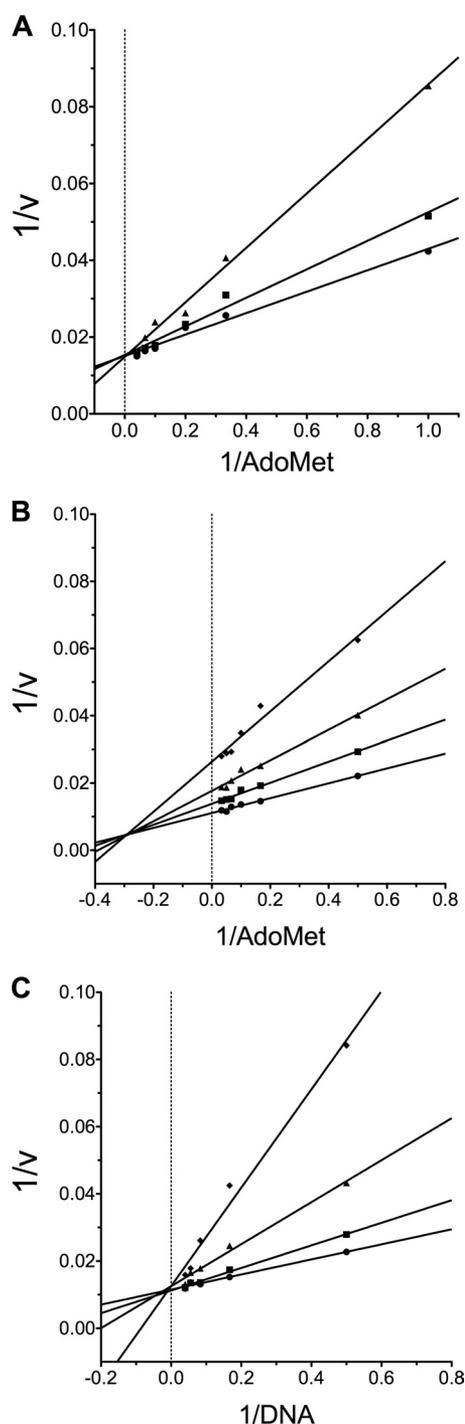
FIGURE 4. LCA is a DNA-competitive inhibitor. *A*, shown are AdoMet-dependent SGI-1027 inhibition kinetics. SGI-1027 was used as an inhibitor in reactions containing 20 nM DNA ($\sim$13$\times$ $K_{m,\mathrm{DNA}}$), and varying concentrations of AdoMet (1–25 μM) and initial velocities (relative fluorescence units/min) were determined. The double reciprocal plot of the data without SGI-1027 (●), 0.5 μM SGI-1027 (■), and 2 μM SGI-1027 (▲) shows that SGI-1027 is competitive with respect to AdoMet. Using nonlinear regression to fit the alteration in $K_{m\,\mathrm{apparent}}$, a K_i of 1.5 $\pm$ 0.3 μM was determined. *B*, shown are AdoMet-dependent LCA inhibition kinetics. LCA was used as an inhibitor in reactions containing 20 nM DNA and varying concentrations of AdoMet (2–30 μM). The double reciprocal plot of the data without LCA (●), 0.4 μM LCA (■), 0.8 μM LCA (▲), and 1.5 μM LCA (◆) shows mixed inhibition, indicating that LCA binds both the free enzyme and the enzyme-AdoMet complex. Using nonlinear regression to fit the data (supplemental Fig. S5), a $K_{i,a}$ of 640 $\pm$ 200 nM and a $K_{I,b}$ of 1.4 $\pm$ 0.4 μM was determined. *C*, DNA-dependent LCA inhibition kinetics are shown. Here, LCA was used as an inhibitor in reactions containing 30 μM AdoMet ($\sim$15$\times$ $K_{m,\mathrm{AdoMet}}$) and varying concentrations of DNA (2–25 nM).
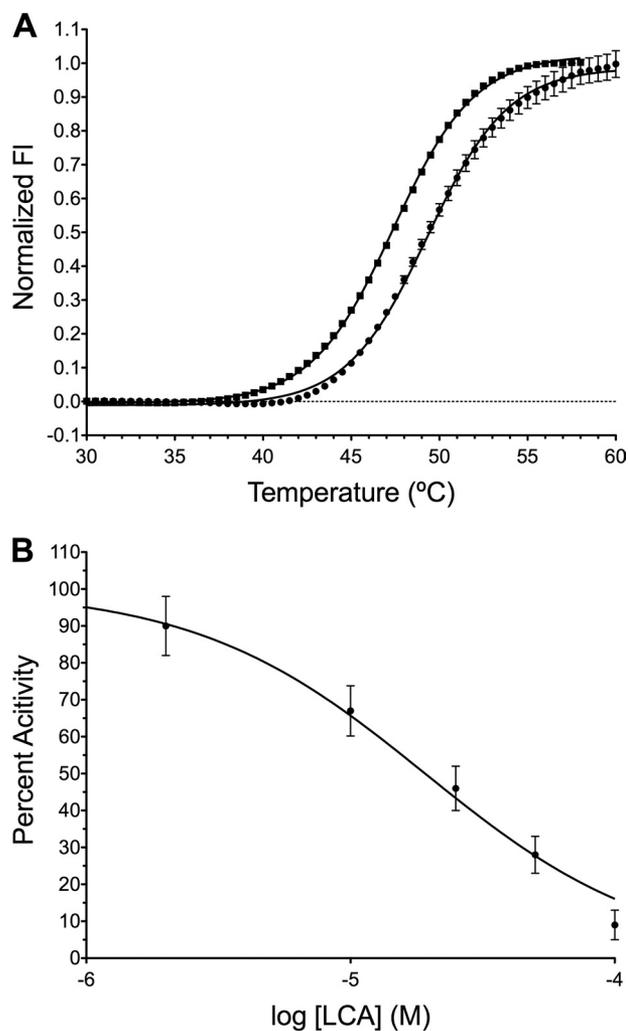

FIGURE 5. LCA is a direct inhibitor of RFTS-containing Dnmt1. *A*, DSF was used to determine the T_m of RFTS-containing Dnmt1 (351–1600) in the presence (●) and absence (■) of 50 μM LCA. Fitting the observed melting traces to the Boltzmann equation gives T_m values of 47.4 $\pm$ 0.1 °C and 49.3 $\pm$ 0.1 °C in the absence and presence of LCA, respectively. The nearly 2 °C shift in the observed T_m is indicative of direct binding. *FI*, fluorescence. *B*, concentration-dependent inhibition by LCA was investigated at 100 nM oligonucleotide 8006 and 100 μM AdoMet using 10 nM RFTS-containing Dnmt1 (amino acids 351–1600). The percent activity of each condition was determined by comparison to an uninhibited DMSO-containing control. Fitting the data using a Hill slope of -1 gives an IC$_{50}$ of 19.1 $\pm$ 1.7 μM.

RFTS-containing Dnmt1 (351–1600), Dnmt3a (590–912), and the M.SssI methyltransferase from *Spiroplasma* sp. strain MQ1 under identical conditions (200 nM oligonucleotide 8006 and 0.25 mM AdoMet in standard assay buffer). LCA was varied from 5 to 100 μM, and the percent activity observed under each condition was determined with respect to an uninhibited control (Fig. 6). The IC$_{50}$ values obtained under these conditions are 27.8 $\pm$ 1.8, 50.5 $\pm$ 4.3, and 53.6 $\pm$ 4.1 μM for RFTS-lacking Dnmt1, the catalytic domain of Dnmt3a, and M.SssI methyltransferase, respectively. Thus, LCA is capable of inhibiting all cytosine methyltransferases investigated. To directly compare potency, one must take into account the assay condition. Here,

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The double reciprocal plot of the data without LCA (●), 0.4 μM LCA (■), 0.8 μM LCA (▲), and 1.5 μM LCA (◆) is indicative of competitive inhibition. Using nonlinear regression to fit the data (supplemental Fig. S5) gives a K_i of 310 $\pm$ 80 nM. In all cases, 1 nM RFTS-lacking Dnmt1 (621–1600) was used.
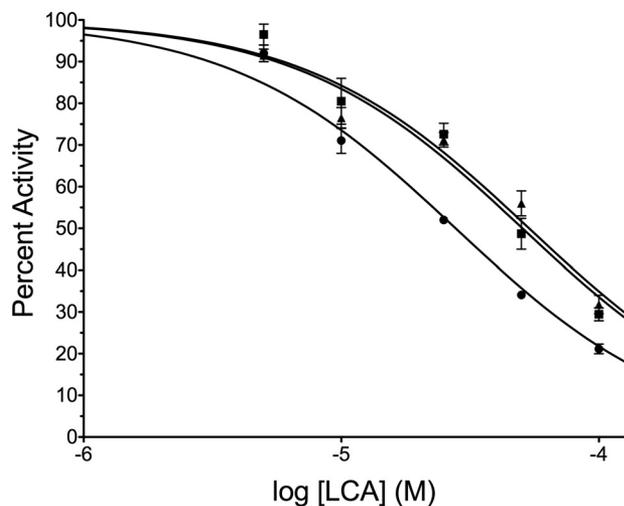
FIGURE 6. **Specificity of LCA inhibition.** LCA inhibition of 10 nM RFTS-containing Dnmt1 (●), catalytic domain of Dnmt3a (■), and M.SssI methyltransferase (▲) was investigated at 200 nM oligonucleotide 8006 and 0.25 mM AdoMet using the Gla I-coupled DNA methylation assay. The percent activity of each condition (5–100 μM LCA) was determined by comparison to an uninhibited DMSO containing control. Fitting the data using a Hill slope of -1 gives IC$_{50}$ values of 27.8 $\pm$ 1.8 μM for RFTS-containing Dnmt1, 50.5 $\pm$ 4.3 μM for Dnmt3a, and 53.6 $\pm$ 4.1 μM for M.SssI methyltransferase.

TABLE 2

qPCR validation of LCA microarray analysis and changes in gene expression caused by treatment with control compound, anthraquinone-2-carboxylic acid

Gene	Microarray	qPCR	Anthraquinone 2-carboxylic acid
ARRB1	+2.29	+2.37 $\pm$ 0.30	+1.05 $\pm$ 0.33
CEACAM5	+3.24	+4.97 $\pm$ 0.23	+1.48 $\pm$ 0.51
CYP1A1	+13.47	+48.77 $\pm$ 15.80	+5.71 $\pm$ 2.31
CYP1B1	+2.26	+2.79 $\pm$ 0.69	+1.65 $\pm$ 0.30
DHRS3	+2.42	+3.00 $\pm$ 0.20	+1.55 $\pm$ 0.27
GCNT1	+2.33	+2.96 $\pm$ 0.18	+0.85 $\pm$ 0.12
PPARG	+2.28	+2.51 $\pm$ 0.13	+1.21 $\pm$ 0.30
RERG	+2.30	+3.32 $\pm$ 0.59	+0.85 $\pm$ 0.08
RGS16	+4.86	+6.72 $\pm$ 0.81	+1.17 $\pm$ 0.58
SNAI2	−3.27	−8.26 $\pm$ 1.99	+0.94 $\pm$ 0.13
TGBF2	−6.10	−4.47 $\pm$ 0.59	+1.40 $\pm$ 0.16
CBX5	+1.02	1.02 $\pm$ 0.10	+0.90 $\pm$ 0.22

200 nM DNA substrate was used. This is ~13 times the reported $K_{m,\mathrm{DNA}}$ for Dnmt1 (351–1600) (24). However, this DNA concentration is roughly equal to the $K_{m,\mathrm{DNA}}$ for M.SssI methyltransferase (33) on a similar hairpin DNA substrate. The $K_{m,\mathrm{DNA}}$ values reported for full-length Dnmt3a are 2.5 μM for 30-mer duplex non-methylated and hemi-methylated DNAs (34), 2.7 μM for poly(dIdC), and 3.5 μM for poly(dGdC) (35). All of these reported values are more than 10-fold larger than the DNA concentration used in the IC$_{50}$ experiment. Yet, as can been seen in Fig. 6, LCA exhibited an ~2-fold specificity for Dnmt1 under this condition. Because DNA substrates were presumably well below saturation for M.SssI methyltransferase and Dnmt3a, LCA appears to have substantial specificity for Dnmt1 *vis à vis* other methyltransferases.

LCA Treatment Alters Gene Expression in MCF-7 Breast Cancer Cells—To determine whether LCA is cellularly available and capable of altering gene expression, we examined the gene expression consequences of LCA on MCF-7 breast cancer cells. Cells were treated with 5–1000 μM LCA in a five-day protocol, and the cells were harvested for RNA purification. qRT-PCR was performed with primers specific for genes known to be methylated in MCF-7 cells and that show increased expression upon 5-azaC treatment (36–41). Of the 10 genes tested, eight (*CLDN6*, *DNMT1*, *FKBP4*, *GSTP1*, *KIF1A*, *PHD3*, *P16*, and *TFPI*-2) showed no change in expression even at the highest concentrations of LCA. In contrast, *VGF* and *MAL* showed 3–5-fold up-regulation at 200 μM and higher concentrations (data not shown). Thus, specific methylated genes showed sensitivity to LCA treatment, indicating that LCA treatment is capable of alteration of gene expression in cell culture.

The selective activation of methylated genes prompted a genome-wide investigation of the effects of LCA. MCF-7 cells were treated for 5 days with 200 μM LCA or media alone as a control. Total RNA was isolated and used for microarray anal-

ysis (Affymetrix, Human Gene ST1.0 gene chip). Of the 21,014 human Refseq genes, 64 showed a 2-fold or greater change in expression with a *p* value of 0.05 or lower (supplemental Table 3 and accession number GSE45804, NCBI GEO database). Of these mRNAs, 35 were significantly up-regulated, and 29 were down-regulated. The majority of these genes (42/64) possess CpG islands within 1000 bp of their transcription start sites and are frequently methylated in cancer.

To determine whether the effects on gene expression are specific for LCA, cells were treated in parallel with a structurally related compound, anthraquinone-2-carboxylic acid, that had no effect on Dnmt1 activity *in vitro* (supplemental Fig. S3). Probes for 12 mRNAs were designed; 9 were up-regulated by LCA, 2 were down-regulated by LCA, and 1 was unmethylated in MCF-7 cells and was unaffected by LCA. qRT-PCR revealed that of the genes tested, only *CYP1A* showed greater than a 2-fold up-regulation in response to the control anthraquinone (Table 2). This finding is consistent with the reported expression of *CYP1A* in response to xenobiotic compounds (42, 43). *CBX5*, a gene that is unmethylated in MCF-7 cells (44), showed no change in expression with either anthraquinone (Table 2). The eight remaining up-regulated genes and two down-regulated genes examined were altered by LCA but not the control anthraquinone (Table 2), indicating that the observed gene expression changes are specific for LCA.

Given that LCA acts as a DNA-competitive inhibitor of Dnmt1, we reasoned that LCA would act synergistically with 5-azadC to re-activate methylated genes. In this treatment combination, incorporated 5-aza nucleotides would deplete Dnmt isozymes that are engaged with DNA, whereas LCA would directly inhibit newly synthesized Dnmt isozymes or Dnmt isozymes that are not DNA-associated, thereby preventing renewed DNA methylation. We were particularly interested in how mRNAs such as *CEACAM5*, *DHRS3*, and *RGS16*, which were strongly and specifically induced by LCA, would accumulate when treated with two agents of complementary mechanisms of action. To test this hypothesis, MCF-7 cells were treated for 5 days with 0.5 μM 5-azadC, 200 μM LCA, or both compounds. qRT-PCR of total RNA showed that each of the genes, *CEACAM5*, *DHRS3*, and *RGS16*, showed a synergistic response to the two compounds. The negative control, *CBX5*, showed no change in expression upon either treatment (Fig. 7A). These findings demonstrate the potential use of LCA to
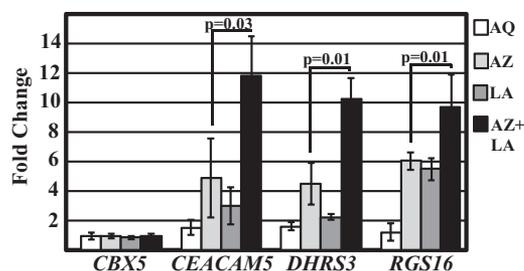
FIGURE 7. **LCA and 5-azadC show synergistic effects on the activation of methylated genes.** MCF-7 breast cancer cells were treated with the indicated compound, total RNA was isolated, and qRT-PCR was performed to examine the expression of 4 mRNAs. *CEACAM5, DHRS3,* and *RGS16* possess CpG islands that are methylated in breast and other types of cancer. *CBX5* possesses a CpG island that is hypomethylated in MCF-7 cells. Assays were performed on three biological replicates. *Error bars* represent the S.D. *p* values were calculated using Student's *t* test. *AQ,* 200 μM anthraquinone-2-carboxylic acid; *AZ,* 0.5 μM 5-azadC; *LA,* 200 μM LCA; *AZ+LA,* 0.5 μM 5-azadC plus 200 μM LCA.

amplify the effects of 5-azadC on the reactivation of genes in cancer cells.

DISCUSSION

Genetic lesions in cancer cells are not reversible and frequently result in the misregulation of gene expression. The plasticity of the epigenome provides an opportunity to correct gene expression. Epigenetic reprogramming could be expected to result in restoration of a more differentiated and less proliferative state and regression to a lower degree of drug resistance (45). Nucleoside demethylating agents, such as 5-azadC, are used in the treatment of myelodysplastic syndrome and acute myeloid leukemia (15, 46). Particularly at high doses, these compounds act non-specifically on the genome, causing the activation of many genes and potentially transposons, which can lead to genomic instability (47, 48). Furthermore, 5-azaC is incorporated into RNA and induces off-target effects (16). Thus, there is a need for the discovery of new compounds that would reduce side effects in patients. In addition, there is a need for research tools that will distinguish genes that require Dnmt1 enzymatic activity from those that require Dnmt1 physical interactions for gene silencing.

Here we show that LCA is a direct inhibitor of Dnmt1. LCA is the first of a new class of Dnmt inhibitors and suggests that specifically substituted anthraquinones may be a useful scaffold for Dnmt inhibitors. LCA, a component of lac dye, is a natural product from the red scales of the insect, *Kerria lacca*, and has been used as a red dye for millennia in food, cosmetics, paint, and fabrics. LCA has mild toxicity (49), unlike DNA demethylators such as 5-azaC, which are cytotoxic (15–17).

LCA is demonstrated to have a DNA-competitive mechanism of action and to inhibit Dnmt3a and M.SssI methyltransferase with moderate selectivity for Dnmt1. Recently, 3-nitroflavanones were identified by screening against Dnmt3a and suggested by molecular docking to compete for DNA binding (50). We suggest that specifically substituted flavones, anthraquinones, and related compounds may produce multiple hits against Dnmts. Potentially by maximizing interactions with the target recognition sequences, which are unique to each isozyme (51), inhibitors of this class may be identified with strong isozyme selectivity.

TABLE 3

Pathways regulated by LCA in MCF-7 breast cancer cells

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; EGFR, EGF receptor.

Pathway	# Genes	*p* value
$\alpha9\beta1$ integrin signaling	13	3.78e-07
Integrin-linked kinase signaling	10	3.78e-07
IL3-mediated signaling	13	3.78e-07
AP-1 transcription factor network	10	3.78e-07
Nectin adhesion pathway	13	3.78e-07
Integrin family cell surface interactions	14	3.78e-07
TRAIL signaling pathway	13	3.89e-07
$\beta1$ integrin cell surface interactions	13	4.16e-07
Regulation of CDC42 activity	10	4.96e-07
EGFR-dependent endothelin signaling	12	4.96e-07

Because LCA is DNA-competitive and Dnmt1 is reported to be highly processive (52, 53), the population of Dnmt1 that is tightly DNA associated should by definition be resistant to inhibition. In contrast, non-DNA-bound enzyme should be sensitive to this class of compound. The set of genes activated by LCA, which appear to be a subset of those silenced by DNA methylation, may serve to be highly revealing of cellular mechanisms of gene silencing. We suggest that the genes that are resistant to LCA but sensitive to 5-azadC are less actively demethylated or more tightly associated with Dnmt proteins than those that are sensitive to LCA. According to this view, LCA-sensitive genes would be those for which Dnmt enzyme activity rather than Dnmt protein complex occupancy is determinative for gene silencing. This can be tested by next generation methods including methylation sequencing and Dnmt-targeted chIPseq.

Interestingly, the genes that changed expression in response to LCA treatment were enriched in 10 common biological pathways, many of which are involved in cell adhesion and signaling (Table 3). These pathways contain shared genes that play roles in breast cancer progression. *LAMA3* (encoding a subunit of laminin 322/laminin 5), up-regulated by LCA treatment, has tumor suppressor functions and aberrant promoter DNA methylation in breast tumors (29, 30). *PPARG* (peroxisome proliferator-activated receptor γ), up-regulated by LCA treatment, induces terminal differentiation of breast cancer cells, providing a natural therapeutic block of proliferation (54). Up-regulation of *PPARG* may provide additional benefits because pioglitazone, a PPARγ agonist, inhibits the expression of aromatase, which mediates estrogen synthesis in adipocytes (55). Thus, knowledge of which genes change expression upon LCA treatment, coupled with genomic profiling of patients, could help to personalize treatment.

We predicted that LCA would potentiate the gene activation effects of 5-azadC at LCA-sensitive genes in MCF-7 cells. The target genes examined, *CEACAM5, DHRS3, RGS16,* are silenced by methylation in breast and other malignancies (46, 56–58), and in all cases, combined treatment resulted in enhanced gene expression as compared with either LCA or 5-azadC alone. This is potentially clinically important because the current goal of expanding the utility of 5-aza nucleosides to solid tumors depends on using low doses that are not cytotoxic (45).

Further development of LCA as a molecular probe or lead compound is expected to depend on structural characterization

and cellular comparisons between LCA and control anthraquinones, such as UI1055 and anthraquinone-2-carboxylic acid, which are inert with respect to Dnmt1. We have been particularly eager to test LCA in cancer models that are driven by Dnmt1. Because Rgs6 functions as a negative regulator of Dnmt1 (27), we tested whether LCA can inhibit Ras-induced proliferative and apoptotic phenotypes in a *rgs6* knock-out model. In these studies, Dnmt1 was strongly overexpressed and drove cellular phenotypes that were effectively reversed by LCA but not by the control anthraquinone (59). These data indicate that LCA is a novel DNA-competitive inhibitor of Dnmt1 with the potential to improve the understanding of biology of DNA methylation and the enzymology of Dnmt isozymes and serve as a lead compound for combination therapy agents.

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