Targeting Calcium Signaling Induces Epigenetic Reactivation of Tumor Suppressor Genes in Cancer

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Abstract

Targeting epigenetic pathways is a promising approach for cancer therapy. Here, we report on the unexpected finding that targeting calcium signaling can reverse epigenetic silencing of tumor suppressor genes (TSG). In a screen for drugs that reactivate silenced gene expression in colon cancer cells, we found three classical epigenetic targeted drugs (DNA methylation and histone deacetylase inhibitors) and 11 other drugs that induced methylated and silenced Cpg island promoters driving a reporter gene (GFP) as well as endogenous TSGs in multiple cancer cell lines. These newly identified drugs, most prominently cardiac glycosides, did not change DNA methylation locally or histone modifications globally. Instead, all 11 drugs altered calcium signaling and triggered calcium-calmodulin kinase (CamK) activity, leading to MeCP2 nuclear exclusion. Blocking CamK activity abolished gene reactivation and cancer cell killing by these drugs, showing that triggering calcium fluxes is an essential component of their epigenetic mechanism of action. Our data identify calcium signaling as a new pathway that can be targeted to reactivate TSGs in cancer.

Introduction

In cancer, the epigenome is aberrantly reprogrammed, leading to a wide range of heritable changes in gene expression such as silencing of tumor suppressor genes (TSG; ref. 1). The most studied epigenetic aberrations in cancer involve DNA methylation and histone posttranslational modifications. Acquisition of de novo methylation in cytosine of CpG dinucleotide at the promoter region of TSG results in stable gene silencing through direct inhibition of transcription factor binding or by recruitment of methyl-binding domain (MBD) proteins such as MeCP2 (1, 2). These MBDs are associated with other repressor complexes including histone deacetylases (HDAC) that are responsible for global loss of histone acetylation, resulting in gene silencing and heterochromatin formation (2).

As these epigenetic modifications are reversible, one goal of epigenetic therapy of cancer is to reverse these alterations and induce TSG reactivation, leading to cancer cell differentiation and cancer cell death (3). Clinical efficacy of epigenetic drugs led to their approval for the treatment of hematologic malignancies and occasional proof-of-principle responses can be seen in solid tumors (2, 4). However, treatment options are limited to a small number of epigenetic drugs approved in the clinic with two DNA methylation inhibitors (decitabine and azacitidine) and two HDAC inhibitors (vorinostat and depsipeptide). There is a need to discover new candidate epigenetic drugs, including some that work through other mechanisms of action. Drug discovery initiatives are underway in rare and specific cancer types with well-defined mutations in epigenetic effectors. However, these efforts may take years before approval and may have limited effects outside of a restricted patient population (5).

To discover new epigenetic drugs that can be rapidly tested in the clinic, we performed an unbiased epigenetic drug screen using FDA-approved drug libraries. The rationale is that positive hits can be rapidly repositioned for cancer treatment because these drugs have known safety, pharmacodynamics, and pharmacokinetics (6). As a platform for epigenetic drug
screening, we used the well-characterized YB5 cell-based system, which is derived from the human colon cancer cell line, SW48 (7, 8). YB5 cells contain a single insertion of cytomegalovirus (CMV) promoter driving GFP gene. GFP expression is silenced in >99.9% of YB5 cells by epigenetic mechanisms because its CMV promoter has DNA hypermethylation and is embedded in repressive chromatin with histone deacetylation and histone methylation marks (Fig. 1A; ref. 7). In YB5 cells, GFP behaves similarly to endogenous TSGs silenced by epigenetic mechanisms and it can be reactivated by treatment with DNA methylation inhibitors and/or HDAC inhibitors (7, 8). We previously demonstrated that GFP reactivation induced by decitabine is characterized by both DNA demethylation and chromatin resetting at the CMV promoter region (7). Moreover, we also showed that HDAC inhibitors induced GFP reactivation through chromatin resetting at the CMV promoter with an increase in histone acetylation without any changes in DNA methylation (8). As the goal of the epigenetic therapy is to reactivate silenced TSGs, we used the YB5 system as a cell-based assay for epigenetic drug screening.

Here, our screen revealed a new mechanism where targeting calcium signaling can reactivate TSGs silenced by epigenetic mechanism in cancer cells. The alteration in calcium signaling results in activated calcium-calmodulin kinase (CamK), which played a central role in TSG reactivation and cancer cell killing.

Materials and Methods

Cell culture
Human colon cancer cell line YB5 and its parental cell line SW48 were cultured in L-15 medium. Human colon cancer cell line HCT116 was cultured in McCoy’s medium. In HCT116 human colon cancer cells, GFP sequence was inserted in exon 2 of the TSG secreted frizzled-related protein 1 (SFRP1) making the HCT116 SFRP1-GFP cell line. This locus is silenced by promoter DNA hypermethylation in HCT116 (9). GFP expression detected
by flow cytometry corresponded to the activity of SFRP1 promoter and was measured in less than 10% of the cells. Normal colon cells, CRL-1831, were grown in DMEM F-12 medium supplemented with 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, and 100 mM/L HEPES. Leukemia cell lines, K562 and HL-60, were cultured in RPMI1640. HEK293T were grown in DMEM. All cell culture media were supplemented with 10% FBS. Colon cancer and leukemic cells were grown in log phase in 1% and 5% CO2 atmosphere, respectively. SW48, K562, and HL-60 cell lines were obtained from ATCC (2005–2007) and were authenticated at MD Anderson Cancer Center genomic core facility by DNA fingerprinting in 2011. CRL-1831 cell line was obtained from ATCC in 2012 and used within 2 months from the purchase. HCT116 and HEK293T were obtained from the laboratories of S.B. Baylin and D.L. Gill and were genetically engineered cell lines that were not further authenticated.

**Drugs and treatments**

Drug libraries of FDA-approved drugs were obtained from NCI-Developmental Therapeutics Program (Combo Plate 3948/99 containing 77 drugs, NCI Oncology Drug set 4640/34 containing 52 drugs, NCI Oncology Drug set 4641/34 with 37 drugs) and from commercially available U.S. Drug collection library with 1,040 drugs (MS Discovery). A total of 1,118 unique FDA-approved drugs were screened. Log-phase growing YBS cells were treated with drug libraries (n ≥ 1) using two different schedules. The first schedule consisted of a 72-hour treatment at 10 μmol/L, where drugs and media were replaced every day. Cells were incubated for an additional 24 hours without drugs before analysis. This schedule was designed to discover putative new hypomethylating agents as the induction of DNA demethylation may require several cell divisions to become detectable (2). The second schedule consisted of a 24-hour treatment at 50 μmol/L. Cells were immediately analyzed after the treatment. This schedule was designed to discover drugs that would reactivate GFP by chromatin remodeling because, in this cell line, HDAC inhibitors can reactivate GFP in 24 hours without any changes in DNA methylation at the CMV promoter. For validation purposes and pharmacologic (leptomycin L2913) modulation, drugs were purchased at Sigma-Aldrich and dissolved either in DMSO, ethanol, or sterile PBS according to the manufacturer’s recommendations and stocks were kept frozen at −80°C. CamK inhibitors were purchased from EMD Millipore (Kn-93, 422712 and Kn-92, 422709).

**Flow cytometry**

After treatment, cells were trypsinized and resuspended in cell culture media with propidium iodide (PI) to stain dead cells. For epigenetic drug screening with YB5 cells, GFP and PI fluorescence were measured using BD LSR II flow cytometer. Validations were performed using Millipore Guava flow cytometer with YB5 or SFRP1-GFP HCT116 cells. Flow cytometry analysis was also used prior to any type of extractions (RNA, DNA, and proteins) to ensure a similar level of GFP reactivation following drug treatments for each biologic replicate. Flow cytometry results were plotted as GFP fluorescence on the x-axis and PI on the y-axis (Supplementary Fig. S1A–S1D). Positive hits were designated using two selection criteria. First, we excluded all autofluorescent drugs (like antimarialds) because autofluorescence creates a false positive signal that bleaches into GFP channel. Drugs that showed more than 8% of the cells in the double positive quadrant were discarded. This threshold value was calculated as the sum of the average plus one SD of the percentage of cells detected in the double positive quadrant in the entire screen dataset. Second, the lowest detection threshold for GFP reactivation was set at 2.2% of the cells present in the GFP+ quadrant. This value was established experimentally and corresponded to the lowest GFP value obtained in the screen that was confirmed during our validation step with drugs purchased from independent suppliers.

**RNA extraction, cDNA synthesis, qPCR**

Total RNA (2 μg) was extracted using TRIzol (Invitrogen) and cDNA was synthesized using High-Capacity cDNA Kit (Applied Biosystems). Quantification of cDNA was done by qPCR with the Universal PCR Master Mix (Bio-Rad) using ABI Prism 7900HT system. Results were obtained from at least three independent experiments where each sample was analyzed in triplicate. 18S was used as a reference gene. All primers have been described previously (7, 8).

**DNA extraction and DNA methylation analysis**

DNA extraction, bisulfite conversion, and pyrosequencing were carried out as previously described (7, 8).

**Histone extraction and Western blots**

For acid histone extraction, cells were harvested and washed twice with ice-cold PBS supplemented with 10 mM/L sodium butyrate to retain levels of histone acetylation. Cell pellets were resuspended in Triton Extraction Buffer [TEB; PBS containing 0.5% Triton X100 (v/v), 2 mM/L phenylmethylsulfonylfluoride, 0.02% (w/v) NaN3, complete protease inhibitor cocktail, 10 mM/L sodium butyrate]. Cell lysis was induced after 10 minutes incubation on ice with gentle stirring. Lysates were centrifuged at 6,500 × g for 10 minutes at 4°C. Supernatant was discarded. Cells were washed in half the volume of TEB and centrifuged as before. Pellets were resuspended in 0.2 N HCl. Histones were acid extracted overnight at 4°C. Samples were centrifuged at 6,500 × g for 10 minutes at 4°C. Histone proteins contained in the supernatant were saved and protein concentration was determined using the Bradford assay. Extracted histones were stored at −80°C. Histone extracts were run in 12% SDS-PAGE precast gels (Bio-Rad, 345-0118). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes in 10 mM/L CAPS (pH 11) containing 10% methanol and detected by using specific primary antibodies and horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) and Enhanced Chemiluminescence reagent (Pierce). PVDF membranes were incubated with specific primary antibodies. Commercially available antibodies were purchased for H3K4-3me (Active motif, 39159), H3K9-3me (Abcam, AB 8898), H3K14-Ac (Active motif, 39697), H3K9-Ac (Active motif, 39917), H3K27-3me (Active motif, 39155), H3K27-Ac (Upstate Biotechnology, 07360), H3 (Active motif, 39763), and McEp2 (Abcam, AB2828).

**Statistical analysis**

Differences between groups were assessed using a one-way ANOVA. The P-value was evaluated by the Tukey–Kramer multiple comparison test. Statistics and graphical representations were performed using GraphPad Prism software.
Results

Epigenetic drug screening and positive hits validation

We screened over 1,100 FDA-approved drugs obtained from the NCI and commercial sources using two dose schedules (Fig. 1B). The first schedule (10 μmol/L for 72 hours followed by 24 hours without drug) was designed to discover epigenetic drugs that require several cell cycles to induce robust gene reactivation, as observed with DNA methylation inhibitors. This schedule induced significant DNA demethylation and was associated with gene reactivation in YB5 cells (7). The second schedule (50 μmol/L for 24 hours) was selected to discover epigenetic drugs that produce rapid gene reactivation, as seen with HDAC inhibitors. On the basis of the very low background in the YB5 system (less than 0.1% of the cells express detectable levels of GFP; Supplementary Fig. S1A) and activity seen in negative controls, positive hits in the screen were defined as those non-autofluorescent drugs inducing more than 2.2% of GFP+ cells (Supplementary Fig. S1B–S1E).

The drug screen revealed 23 (2%) and 77 (7%) positive hits at 10 μmol/L (for 72 hours) and at 50 μmol/L (for 24 hours) respectively, whereas 12 drugs (1%) were identified in both schedules (Fig. 1C–E). The highest inducers of GFP expression were the DNA methylation inhibitors decitabine (at 10 μmol/L, for 72 hours) and azacitidine (at 50 μmol/L, for 24 hours) producing, respectively, 30% and 16% GFP+ cells (Supplementary Tables S1 and S2). Azacitidine induced GFP reactivation after a short treatment of 24 hours, which can be explained by the number of YB5 cells in S-phase during the treatment (2). The HDAC inhibitor vorinostat was also identified in this screen producing 2.6% GFP+ cells (Supplementary Table S2). Thus, all three known epigenetic drugs represented in the libraries were rediscovered in this blinded screen, validating the approach.

Because of the unexpected large number of positive hits, we focused on the 12 drugs identified in both dose schedules (Fig. 1E). For validation, compounds were purchased from separate suppliers and tested for GFP reactivation in YB5 cells. Of these 12 hits, all except tilmicosin (which was low positive) were validated. These 11 validated drugs fell into three groups (Fig. 1F). The first one included the known epigenetic drugs decitabine, azacitidine, and vorinostat. The second group encompassed cardiac glycosides such as ouabain, lanatoside C, digoxin, digitoxin, and proscillaridin A. All cardiac glycosides represented in the libraries reactivated GFP, suggesting a drug class effect. The last group was labeled as antibiotics/others and contained oxquinoline (antibiotic), dapsilim (a drug used for alcoholism treatment), and arsenic trioxide (anti-cancer drug; Fig. 1F). In the validation studies, GFP reactivation was tested at multiple doses (Fig. 2A–F). All cardiac glycosides induced dose-dependent GFP reactivation in the nanomolar range at clinically relevant concentrations, with proscillaridin A being the most potent (Supplementary Fig. S2A and S2B). We also validated three additional antibiotics (pyrithion zinc, cycloheximide, and thiram) in the antibiotic/others subgroup that were identified in the screen at 50 μmol/L only but were positive during the validation using both schedules. Therefore, we validated a total of 14 FDA-approved drugs encompassing three well-known epigenetic drugs and 11 candidate epigenetic drugs.

No changes in classical epigenetic pathways

We confirmed GFP mRNA reactivation after 24 hours treatment by qPCR (Fig. 3A). We also detected gene reactivation in three endogenous TSGs silenced by epigenetic mechanism in YB5 cells (CDH13, RIL, TIMP-3) as well as the stress response gene P21 (CDKN1A; Supplementary Fig. S3A–S3D). In addition, we measured SFRP1 reactivation, another DNA hypermethylated TSG in HCT116 colon cancer cells (9, 10). In this cell line, GFP sequence was inserted in exon 2 of SFRP1 making its activation detectable by flow cytometry (9). Similar to YB5 cells, all drugs reactivated SFRP1 gene in the HCT116 SFRP1-GFP system (Fig. 3B). To identify a mechanism of action, we asked whether candidate epigenetic drugs induced gene reactivation through known epigenetic mechanism such as DNA demethylation or histone acetylation. First, we measured DNA methylation locally in the promoter regions of reactivated genes (CMV and endogenous TSGs) and at LINE-1 repeats to assess global DNA methylation levels. Using bisulfite pyrosequencing assays designed in the promoter regions close to the transcription start site (8), we found that none of the drugs reduced DNA methylation at the CMV (Fig. 3C) or TIMP-3 (Supplementary Fig. S4A) promoters or globally at LINE-1 repeats (Fig. 3D), except for decitabine (a well-known hypomethylating drug). We then measured quantitatively histone acetylation levels by mass spectrometry on 9 residues in histone 3 (H3) and histone 4 (H4). No significant changes were observed, except with vorinostat, a well-known HDAC inhibitor that increased histone acetylation levels on lysine 9, 14, 18, and 23 of histone 3 and on lysine 8, 12, and 16 of histone H4 (Fig. 3E and Supplementary Fig. S4B). A similar lack of global effects was observed by Western blots for histone-activating marks (H3K4-me3, H3K9-ac, and H3K27-ac) or histone-inactivating marks (H3K9-me3 and H3K27-me3; Supplementary Fig. S4C).

Intracellular Ca2+ fluxes induced by the newly identified drugs

As neither DNA demethylation nor global chromatin modifications explained TSG reactivation by the newly identified drugs, we searched for alternative mechanisms. Surprisingly, when searching for a mechanism of action of the newly identified drugs, we noted that most of the (9/11) drugs were known to increase intracellular calcium (Ca2+) levels (11–14). No data were found regarding the three known epigenetic drugs and calcium signaling. Interestingly, it was reported in neurons that cytosolic increase in Ca2+ levels activate CamK, which releases the methyl-binding protein MeCP2 from silenced promoters, leading to gene reactivation (15, 16).

To test whether this mechanism could explain TSGs reactivation in cancer cells by the newly identified drugs, we first characterized Ca2+ channels in YB5 cells. Using Ca2+ sensitive dye (fura-2), we determined that YB5 cells do not express functional L-type voltage-operated Ca2+ channels (Supplementary Fig. S5A and SSB). Instead, YB5 cells have functional plasma membrane Ca2+ pump blocker, thapsigargin (Supplementary Fig. SSC; refs. 17, 18).

Then, we characterized the effects of drug treatments in YB5 cells on cytosolic Ca2+ levels at rest, ER Ca2+ levels and SOCE activity in response to Ca2+ ionophore, ionomycin or thapsigargin followed by the addition of 1 mmol/L Ca2+ in the media (a cation-safe solution). Interestingly, known epigenetic drugs did not alter Ca2+ homeostasis (Fig. 4A). However, cardiac glycosides...
and arsenic trioxide reduced ER $\text{Ca}^{2+}$ levels (first peak) while strongly inhibiting SOCe-mediated $\text{Ca}^{2+}$ entry (second peak; Fig. 4B and Supplementary Fig. S5D). Even though ER $\text{Ca}^{2+}$ levels were significantly reduced, we could not measure an increase in cytosolic $\text{Ca}^{2+}$, suggesting a transient spark in cytosolic $\text{Ca}^{2+}$ after treatment with digitalis compounds (19). In another model of HEK293 cells expressing the genetically encoded calcium sensors (D1ER cameleon system) specifically expressed in the ER, we confirmed digitoxin-induced reduction in ER $\text{Ca}^{2+}$ (Supplementary Fig. S5E; 20). Finally, we could detect an increased cytosolic $\text{Ca}^{2+}$ level (higher baseline $\text{Ca}^{2+}$ signal) after only pyrithion zinc treatment whereas both pyrithion zinc and disulfiram increased $\text{Ca}^{2+}$ levels in the ER (first peak; Fig. 4C). Thus, most of the candidate epigenetic drugs induce measurable intracellular $\text{Ca}^{2+}$ changes in YB5 cells by different mechanisms of action, which result in cytosolic $\text{Ca}^{2+}$ sparks or sustained increased levels. It is noteworthy that cardiac glycosides are well-known Na$^+$/K$^+$-ATPase pump blockers used in heart failure treatment (11). To assess the impact of Na$^+$/K$^+$-ATPase pump inhibition on GFP reactivation in YB5 cells, we treated YB5 cells with orthovanadate, an inorganic Na$^+$/K$^+$-ATPase inhibitor and did not detect GFP reactivation. These data suggest that the epigenetic activity of cardiac glycosides might be independent of Na$^+$/K$^+$-ATPase (data not shown).

Figure 2.
Validation of positive hits with known epigenetic drugs, cardiac glycosides, and antibiotics/others subgroups. Dose–response curves were generated measuring GFP expression in a validation step in YB5 cells treated for 24 hours (left) or 72 hours (right) with known epigenetic drugs (A and B), cardiac glycosides (C and D), and antibiotics/others ($n \geq 3$; E and F).

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We further explored the mechanism of SOCe inhibition induced by cardiac glycosides. SOCe is mediated by the highly Ca\(^{2+}\)-selective Orai1 channels, which are activated by STIM proteins in response to a decrease in ER Ca\(^{2+}\) levels. STIM translocates into ER-plasma membrane junctions to tether and activate Orai1 channels (21, 22). For these experiments, we used a well-established model of HEK293 cells overexpressing Orai1-CFP and STIM1-YFP (23). We found that digitoxin did not affect STIM1-Orai1 coupling as maximal FRET signal between STIM1 and Orai1, obtained after ionomycin treatment, was unaltered by digitoxin (Supplementary Fig. S5F). Wild-type and overexpressing STIM1-Orai1 HEK293 cells showed similar inhibition of SOCe and reduction in ER Ca\(^{2+}\) size by digitoxin (Fig. 4D and Supplementary Fig. S5G). Importantly, these effects on Ca\(^{2+}\) levels were not caused by changes in cytosolic pH (Supplementary Fig. S5H; refs. 20, 24, 25). Finally, digitoxin specifically inhibited Ca\(^{2+}\) influx through Orai1 in HEK293 cells expressing either constitutively active STIM1 mutant (STIM1D76A) or constitutively active Orai1 mutant (O1-V102C; Fig. 4E and F; 26). These experiments, we used the antihistamine fexofenadine as a control for specificity of action as this drug treatment does not induce GFP reactivation. Together, the data demonstrate that cardiac glycosides altered Ca\(^{2+}\) levels by causing potent SOCe inhibition.

Ca\(^{2+}\) signaling through CamK is essential for gene reactivation. CamK, a multifunctional serine/threonine kinase, is a central molecule that is activated by an increase in cytosolic Ca\(^{2+}\), leading
to gene expression changes in part caused by the unbinding of chromatin repressors such as the methyl-binding protein MeCP2 from their silenced promoters (15, 27–29). Except for known epigenetic drugs, all candidate epigenetic drugs induce intracellular Ca$^{2+}$ changes. Thus, we tested the role of CamK in YB5 cells treated with known and candidate epigenetic drugs. Drug-induced reactivation of GFP and endogenous TSGs (TIMP-3 and WIF-1) was significantly reduced by pharmacologic inhibition of CamKII using Kn-93 whereas Kn-92, its weaker analogue, had a much smaller effect (Fig. 5A and Supplementary Fig. S6A and S6B; ref. 30). As expected, GFP expression induced by azacitidine was not sensitive to Kn-93 inhibition as this hypomethylating drug did not alter Ca$^{2+}$ fluxes (Fig. 5A).

We next hypothesized that the epigenetic effects of CamK activation by Ca$^{2+}$ signaling relate to unbinding of MeCP2 from repressed promoters, as previously reported in normal neuronal cells (15, 16, 28, 29). Using ChIP-qPCR following cardiac glycoside treatment (Lanatoside C), we found that MeCP2 occupancy was reduced at several DNA hypermethylated promoters (Supplementary Fig. S7), suggesting that drug-induced dissociation of MeCP2 binding is associated with gene reactivation. Most interestingly, using confocal microscopy, we observed nuclear versus cytoplasmic redistribution of MeCP2 after proscillaridin treatment (Fig. 5B). Following 24-hour and 48-hour treatment with proscillaridin at 500 nmol/L, MeCP2 staining was enriched in the cytoplasm in most of the cells (Fig. 5B). In untreated cells, MeCP2

Figure 4.

Newly identified drugs induce changes in intracellular Ca$^{2+}$ levels. Intracellular changes in Ca$^{2+}$ levels in YB5 cells pretreated for 8 hours with known epigenetic drugs (A), cardiac glycosides (B), pyrithion zinc and disulfiram (C) in response to ionomycin, extracellular Ca$^{2+}$, and thapsigargin. D, time-lapse intracellular Ca$^{2+}$ changes in HEK293 cells overexpressing STIM1-Orai proteins pretreated for 8 hours with digoxin in response to ionomycin and extracellular Ca$^{2+}$. Time-lapse intracellular Ca$^{2+}$ changes in HEK293 cells transfected with constitutively active STIM1-mutant protein (D67A; E) or with constitutively active Orai-mutant channel (V102C; F) in response to extracellular Ca$^{2+}$ exposure and SOCe inhibitor (2-APB). The arrows indicate the addition of Ca$^{2+}$ into the extracellular media while a line indicates the time of Ca$^{2+}$ exposure prior to its removal by washes of Ca$^{2+}$-free solution. Fexofenadine was used as a control as this drug does not induce GFP reactivation.


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staining was mainly concentrated in the nucleus and a weak signal was detected in the cytoplasm as already observed by others (31). MeCP2 antibody specificity was verified by using siRNA (in Western blot experiments and confocal microscopy on YB5 untreated and treated with proscillaridin A) and HEK293T cells overexpressing MeCP2 (Supplementary Figs. S8 and S9). Finally, Figure 5.

Drug-induced gene reactivation is dependent on CamK activity. A, GFP reactivation by flow cytometry in YB5 cells pretreated (4 hours) with CamK inhibitor Kn-93 and its weaker analogue Kn-92 and treated for 24 hours. B, fluorescent immunochemistry in SW48 cells (YB5 parental cells) treated for 24 hours and 48 hours with proscillaridin at 500 nmol/L stained with DAPI, MeCP2 (RFP) antibody. White arrows, MeCP2 signal in the cytoplasm post-proscillaridin A treatment. C, percentage of apoptotic cells in YB5 cells pretreated (4 hours) with CamK inhibition and treated for 48 hours.
we showed that these effects of the positive hits identified in the screen may relate to export of regulatory proteins from the nucleus to the cytoplasm. Blocking the exportin pathway of protein transport from the nucleus to the cytoplasm by leptomycin significantly reduced drugs-induced GFP reactivation in YB5 cells (Supplementary Fig. S10).

As CamKII appeared to mediate gene reactivation, we next tested whether the selective cancer cell killing was also CamKII-dependent. Percentages of apoptotic cells, viable cells, and dead cells were measured in colon cancer (YB5 and its parent cell line SW48) and leukemia (K562 and HL-60) cell lines following 48-hour drug treatment alone or in combination with CamKII inhibitor, Kn-93 and its less active derivative, Kn-92. For all cell lines, drugs significantly induced cytotoxicity whereas, as expected for this brief treatment period, hypomethylating drugs did not. Kn-93 rescued almost completely the drug-induced toxic effects (Fig. 5C and Supplementary Figs. S11–S13). These data suggest that CamK activation is a key effector of the anticancer activity of these FDA-approved drugs.

Anticancer activity and cancer selectivity

We next assessed the anticancer activity of the newly identified candidate epigenetic drugs in YB5 cells. Anchorage-dependent colony formation assays were performed for long-term cytotoxicity evaluation. All drugs reduced cancer cell survival in a dose and time-dependent manner (Fig. 6A–C). Anticancer activity was stronger for the newly identified drugs compared with known epigenetic drugs. Anchorage-independent colony assays were then performed by gauging colony formation in soft agar. All drugs produced a significant dose-dependent reduction in the number of colonies; with proscillaridin A being the most potent (Fig. 6D). To address the question of cancer cell selectivity, we compared the viability of normal colon cells (CRL-1831) versus colon cancer cells (SW48). We used YB5’s parental cell line SW48 for this because GFP expression could affect viability measurements performed by flow cytometry. After 48-hour treatment at doses producing the strongest GFP reactivation in YB5 cells, cell viability was reduced in cancer cells but not in normal cells (Fig. 6E) demonstrating the cancer selectivity of these drugs.

Discussion

There is a need to discover new epigenetic targets and drugs to induce TSG reactivation in cancer cells. The arsenal of epigenetic drugs approved in the clinic is currently limited to only four drugs targeting only two separate mechanisms. On the basis of the high number of epigenetic targets in cancer, there is a great interest in discovering new targets that can be used to reactivate TSGs and reprogram cancer cells to eradicate their clonogenic potential and induce their differentiation. We used our live cell-based assay where we measured GFP reactivation as surrogate for TSG reactivation. Overall, we identified 14 hits including 3 well-known epigenetic drugs and 11 other FDA-approved drugs encompassing cardiac glycosides and some antibiotics.

To identify the mechanism responsible for GFP and endogenous TSG reactivation, we first analyzed promoter DNA methylation and histone modifications. No changes were detected after treatment with the cardiac glycosides or the antibiotics while TSG reactivation was detected. Surprisingly, we noticed that most of the newly identified drugs are known to induce alterations in Ca2+ signaling in normal cells. We demonstrated that these changes in Ca2+ signaling also occurred in our cancer cell screening system (YB5 cells) and only after treatment with the newly epigenetic drugs but not with well-known epigenetic drugs such as DNA methylation and HDAC inhibitors. This difference suggested a separate mechanism of action. Our data showed that reactivation of endogenous epigenetically silenced TSGs was linked to Ca2+ signaling and CamK activation. Mechanistically, our data suggest that gene reactivation may involve gene derepression by inducing MeCP2 eviction from repressed promoters to the cytoplasm through the exportin pathway (Supplementary Fig. S14). Interestingly, the anticancer activity could also be modulated by CamK pharmacologic inhibition.

Consistent with our work, other studies have demonstrated a CamK-dependent mechanism producing MeCP2 release from silenced promoters, leading to gene reactivation (15, 28, 29). Interestingly, our data further demonstrate redistribution of MeCP2 to the cytoplasm after drug-induced CamK activation. The relationship between MeCP2 binding to methylated-CpGs and Ca2+ has been described in neurons where MeCP2 phosphorylation is involved in intracellular localization during neuronal differentiation (31, 32). Phosphorylation sites of MeCP2 are known to modulate its binding to other epigenetic cofactors but more studies are needed to clearly understand the role of CamK and MeCP2 phosphorylation sites. Nevertheless, our study shows that the CamK–MeCP2 interaction previously linked only to differentiation can be exploited against cancer cells to reactivate silenced TSG. These data also demonstrate a key role for calcium fluxes in epigenetic silencing in neoplasia.

Interestingly, we identified a class effect for the cardiac glycoside subgroup where all these heart failure drugs present in the libraries scored positive in the screen and also separately validated. These drugs are relevant for cancer treatment because epidemiologic studies have shown that patients treated with cardiac glycosides for heart failure exhibit a reduced cancer rate and less aggressive cancers (11, 33, 34). Moreover, in vitro studies have previously shown selective cancer killing for some of these cardiac glycosides, which we now relate to Ca2+ signaling and TSG reactivation (35). Our data suggest that their chemopreventive or chemotherapeutic activity may be in part due to their ability to target Ca2+ fluxes and reactivate epigenetically silenced TSGs. Another drug we identified as having effects on gene silencing is arsenic trioxide, which has marked activity in acute promyelocytic leukemia (36), a disease characterized by a block in cell differentiation (37). Our data suggest that epigenetic modulation may be part of its antineoplastic mechanism of action. Interestingly, cytotoxic drugs or targeted drugs (e.g., tyrosine kinase inhibitors) used in cancer chemotherapy did not reactivate GFP, pointing to the specificity of the identified hits. This was verified separately from the screen for many cytotoxic drugs (38). Thus, the mechanism of action of the positive hits is likely to be specific disruption of epigenetic pathways involved in gene silencing rather than nonspecific effects seen with many antiproliferatives.

Finally, it is worth noting that Ca2+ signaling is essential to initiating epigenetic reprogramming in early embryogenesis (39) and our data extend these findings to cancer therapy. Also, the mechanism of epigenetic action of these drugs is unlikely to be traced to a single chromatin regulator; rather, Ca2+ signaling through CamK activation may have effects on multiple epigenetic acting proteins simultaneously. It will be interesting to determine what targets, other than MeCP2, might be phosphorylated by CamK activation.
Figure 6. Anticancer efficacy and selectivity of candidate epigenetic drugs. Anchorage-dependent toxicity assays were performed in YB5 cells treated for 72 hours (top) or 24 hours (bottom) with known epigenetic drugs (A), cardiac glycosides (B), and antibiotics/others (n = 3). C, anchorage-independent clonogenic assays in soft agar were performed after a 24-hour treatment at the doses indicated on the graph (n = 3). D, cell viability assays using normal colon cells (CRL-1831) and SW48 (YB5’s parental cell line) after a 48-hour treatment, measured by flow cytometry. Drugs and doses are indicated on the graph (n = 3). Cyclohex, cycloheximide; Oxiquin, oxiquinoline; Pyrition, Pyrition zinc.
In summary, our data identify Ca\(^{2+}\) signaling as a new pathway that can be targeted to reactivate TSGs in cancer. This study suggest that cardiac glycosides and other FDA-approved drugs such as some antibiotics represent interesting drugs for cancer epigenetic chemotherapy aiming to reactivate epigenetically silenced TSGs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: N.J.-M. Raynal, Y. Wang, S.B. Baylin, D.L. Gill, J.-P.J. Issa


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.J.-M. Raynal, J.T. Lee, Y. Wang, A. Beaudry, G. Malouf, S. Dumont, E.J. Dettman, R.A. Henry, S.B. Baylin


**References**


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Other (assisted with selection of compounds tested in this study): W.E. Childers

Other (collaboration by applying HTS using the Center's screening capabilities and compound library): M. Abou Gharbia

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Targeting Calcium Signaling Induces Epigenetic Reactivation of Tumor Suppressor Genes in Cancer

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