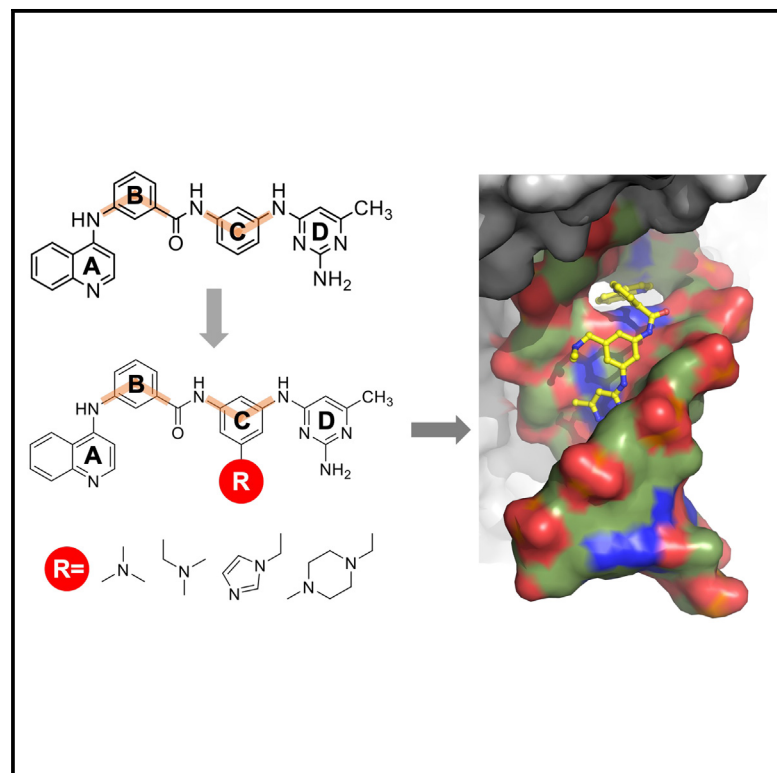


Cell Chemical Biology

Quinoline-based compounds can inhibit diverse enzymes that act on DNA

Graphical abstract



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In brief

Developing non-nucleoside inhibitors for DNA hypomethylation is vital for treating various conditions without the toxicities of current agents. Zhou et al. show that quinoline analogs inhibit multiple DNA-interacting enzymes, including DNMT1, BER glycosylases, DNA/RNA polymerases. Compound 11 intercalates into enzyme-bound DNA, triggering a p53-mediated DNA damage response in cancer cells.

Highlights

- Six quinoline compounds inhibit human and bacterial DNA methyltransferases
- Compounds 9 and 11 intercalate into methyltransferase bound DNA but not into free DNA
- These quinoline derivatives also inhibit DNA glycosylases and DNA/RNA polymerases
- Compound 11 provokes a DNA damage response via p53 activation in cancer cells

Article

Quinoline-based compounds can inhibit diverse enzymes that act on DNA

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SUMMARY

DNA methylation, as exemplified by cytosine-C5 methylation in mammals and adenine-N6 methylation in bacteria, is a key epigenetic process. Developing non-nucleoside inhibitors to cause DNA hypomethylation is crucial for treating various conditions without the toxicities associated with existing cytidine-based hypomethylating agents. This study characterized fifteen quinoline-based analogs, particularly compounds with additions like a methylamine (9) or methylpiperazine (11), which demonstrate similar low micromolar inhibitory potency against human DNMT1 and *Clostridioides difficile* CamA. These compounds (9 and 11) intercalate into CamA-bound DNA via the minor groove, causing a conformational shift that moves the catalytic domain away from the DNA. This study adds to the limited examples of DNA methyltransferases being inhibited by non-nucleotide compounds through DNA intercalation. Additionally, some quinoline-based analogs inhibit other DNA-interacting enzymes, such as polymerases and base excision repair glycosylases. Finally, compound 11 elicits DNA damage response via p53 activation in cancer cells.

INTRODUCTION

DNA methylation, mostly generating 5-methylcytosine (5mC) at CpG dinucleotides, profoundly impacts chromatin structure and gene expression, affecting inheritance, evolution, and human development and health.^{1,2} The earliest observations suggesting that epigenetic silencing involved DNA methylation included studies of promoter CpG hypermethylation transcriptionally repressing β -globin genes³ and tumor-suppressor genes including retinoblastoma (RB),⁴ von Hippel-Lindau (VHL),⁵ and p16/CDKN2.^{6–8} It has become particularly clear that promoter CpG island hypermethylation of tumor-suppressor genes is a common hallmark of human cancers.^{9–11} However, DNA hypomethylation is associated with genomic instability, which might be associated with increased tumor heterogeneity.¹² Modern whole-genome methylation-based approaches, using cell-free DNA circulating in blood, provide a promising biomarker for early cancer detection.^{13–15}

The two FDA-approved DNA hypomethylating agents, Azacitidine (Vidaza)(<https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-azacitidine-newly-diagnosed-juvenile-myelomonocytic-leukemia>) and Decitabine (Dacogen)(<https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-decitabine-and-cedazuridine-myelodysplastic-syndromes>), have been in standard clinical use for some time, particularly in older, medically challenged patients, for the treatment of certain hematological disorders including myelodysplastic syndromes.^{16–23} However, the dose-limiting toxicity of, and limited patient tolerance for, cytidine analogs, the poor median overall survival for elderly patients with acute myeloid leukemia (<1 year), as well as cytidine analogs' ineffectiveness in treating solid tumors,^{24–26} have led to a persistent search for non-nucleoside DNA methyltransferase (DNMT) inhibitors. One such exploration led to the quinoline-based compound SGI-1027 (1), which inhibits the activities of CpG-specific 5mC methyltransferases (MTases), including mammalian DNMT1, 3A and 3B, and bacterial M.SssI.²⁷

Compound 1 contains four ring fragments (A–D in Figure 1A) linked in sequence in a para/para orientation: a 4-aminoquinoline (ring A), a 4-aminobenzoate (ring B), a 1,4-phenylenediamine (ring C), and a 2,4-diamino-6-methylpyrimidine (ring D). Rotating each ring fragment's linkage from the para to the meta or ortho position, or duplicating either the quinoline or pyrimidine moiety, yielded a library of new derivatives (regioisomers and analogs).²⁸

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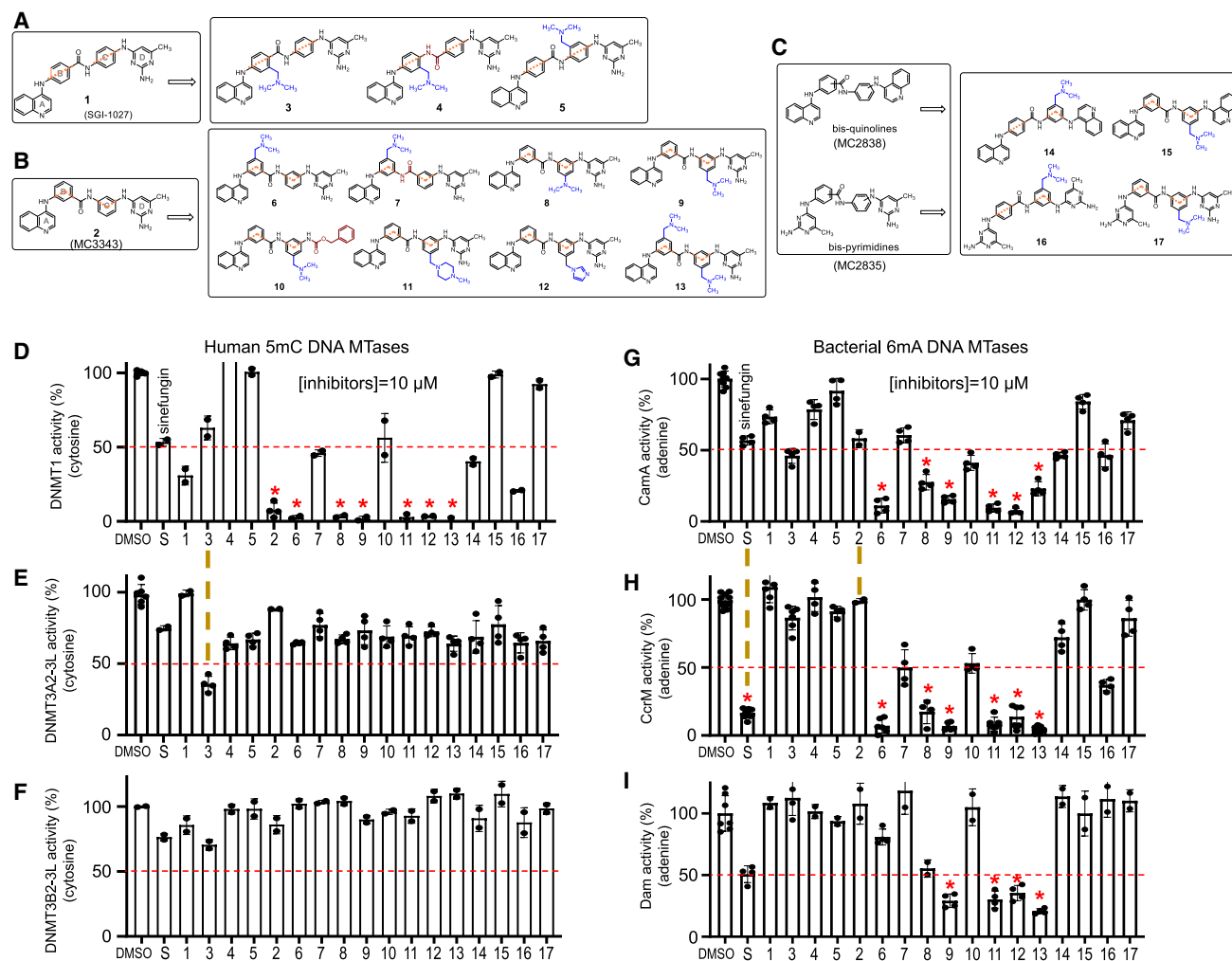


Figure 1. Compounds, used in the inhibition study of six MTase activities, at a single inhibitor concentration of 10 μM

(A) Derivatives (3–5) of compound 1 (SGI-1027).

(B) Derivatives (6–13) of Compound 2 (MC3343).

(C) Derivatives (14–17) of bis-quinolines (MC2838) and bis-pyrimidines (MC2835). [The parent compounds MC3343, MC2838, and MC2835 were reported previously as compounds 5, 10, and 11 by Valente S et al.²⁸] Link orientations are indicated with dashed orange lines and amino-related groups are in blue.

(D–F) Relative inhibition by compounds against three human 5mC DNA MTases.

(G–I) Relative inhibition by compounds against three bacterial 6mA DNA MTases. Compounds indicated by a red asterisk were chosen for further study. DMSO is the control, while S is the known pan-MTase inhibitor sinefungin. The vertical brown dashed lines indicate different inhibition potencies of compound 3 between DNMT1 and DNMT3A/3B, compound 2 between CamA and CcrM/Dam, or sinefungin (S) between CcrM and rest of the five enzymes. Data represent the mean ± SD of N independent determinations (N = 2–4).

One such analog, MC3343 (2), has been further examined *in vitro* and in cancer cells.^{28–31} Interestingly, the mechanism of action of compounds 1 and 2 exhibited “a DNA competitive behavior”, “interacted with DNMT only when the DNA duplex was present” or “inhibited DNMTs by interacting with the DNA substrate”.³² These biochemical observations led us to re-examine the effects of quinoline-based compounds 1–2 and their derivatives (3–17) on the activities of three human DNMTs yielding 5mC, three bacterial DNA adenine MTases yielding 6-methyladenine (6mA), and a few nucleic acid glycosylases and polymerases.

We report here that compounds 9 and 11 exhibit similar low micromolar inhibition potencies for both DNMT1 (a 5mC MTase) and CamA (a 6mA MTase). Structurally, compounds 9 and 11 inter-

calate into CamA-bound DNA, adjacent to the target adenine, via the minor groove. This intercalation leads to a significant conformational shift, moving the enzyme’s catalytic domain away from the DNA. Our findings represent only the second known instance of DNA MTases being inhibited by non-nucleotide compounds through the intercalation of enzyme-bound DNA, following the discovery of dicyanopyridine-based inhibitors against DNMT1.^{33,34}

RESULTS

Quinoline-based compounds

In addition to the two parent compounds, 1 and 2, we prepared fifteen quinoline-based compounds (3–17), all bearing an amino

side chain inserted onto one of the phenyl rings of their structures. Compounds **3–5** follow the **1** structure (*para/para* orientation of the rings B and C) (Figure 1A). Compounds **6–13** were built on the structure of **2** (*meta/meta* orientation between the rings B and C) (Figure 1B). Compounds **6** and **7** contain an *N,N*-dimethylaminomethyl group attached onto ring B, with either the amide (**6**) or the retroamide (**7**) moiety between the B/C rings. Compounds **8–12** have one of the four moieties at ring C: an *N,N*-dimethylamino (**8**), *N,N*-dimethylaminomethyl group (**9**, **10**), (*N*-methylpiperazin-1-yl)methyl (**11**), or a (1*H*-imidazol-1-yl)methyl (**12**) moiety. Differing from **9**, compound **10** replaces the pyrimidine ring (ring D) with a benzyloxycarbonyl moiety. Compound **13** exhibits two *N,N*-dimethylaminomethyl groups inserted respectively at rings B and C. In addition, compounds **14–15** and **16–17** are derivative of bis-quinolines and bis-pyrimidines, respectively (Figure 1C).

Inhibition of DNA MTases generating 5-methylcytosine or 6-methyladenine

Because the two prototypes **1** and **2** inhibit DNA methylation in a DNA-dependent manner, we tested the new quinoline analogs on three human DNMTs, including the maintenance enzyme DNMT1 acting on a hemi-methylated CpG substrate, and two *de novo* enzymes DNMT3A and DNMT3B acting on unmethylated CpG substrates. Instead of the isolated catalytic MTase domain, we used a construct of DNMT1 expressing residues 351–1,600,³⁵ which includes the replication foci-targeting sequence (RFTS) domain, unmethylated CpG-binding CXXC domain, two copies of the bromo-adjacent homology (BAH) domain and a lysine-glycine (KG)-repeat sequence in addition to the C-terminal catalytic domain (Figure S1A). For DNMT3A, we used full-length DNMT3A2, an isoform that lacks the N-terminal region of DNMT3A1³⁶ (Figure S1B), in complex with the effector protein DNMT3L³⁷ (for a review of the different isoforms of human DNMTs, see³⁸). Both DNMT3A2 and DNMT3L are normally expressed at high levels in developing germ cells.³⁹ For DNMT3B, we generated an N-terminally truncated DNMT3B2 (Figure S1C), equivalent to DNMT3A2, and complexed it with DNMT3L.⁴⁰ Initially intended as negative (specificity) controls, we also included three bacterial DNA adenine MTases: *Clostridioides difficile* CamA, required for normal sporulation and persistence of infection,^{41,42} *Caulobacter crescentus* CcrM, a cell cycle-regulated DNA MTase essential for viability,^{43,44} and *Escherichia coli* DNA adenine MTase (Dam), having roles in coordinating DNA replication initiation, DNA mismatch repair, and the regulation of gene transcription.^{45–47} These various DNA MTases have very little amino acid sequence similarity beyond their catalytic motifs, though their three-dimensional catalytic domain structures are related, and all utilize a base-flipping mechanism to access the target nucleotide (Figure S1). Base flipping is often coupled with substantial DNA distortions including bending and unwinding.^{48–51}

We conducted an initial inhibition screen, using a compound concentration of 10 μ M and employing the MTase-Glo biochemical assay,^{52,53} which detects conversion of the methyl donor S-adenosyl-L-methionine (SAM) to S-adenosyl-L-homocysteine (SAH). We included sinefungin, a known pan inhibitor of SAM-dependent MTases, as a positive control (Figures 1D–1I). In the case of DNMT1 ([E]/[DNA] = 1:10), 10 μ M sinefungin and com-

pound **1** demonstrated ~50% and ~70% inhibition, respectively (Figure 1D). Of the fifteen new compounds examined, six of them (compounds **6**, **8**, **9**, **11**, **12**, and **13**)—all being derivatives of compound **2**—were highly effective on DNMT1 at 10 μ M, completely eliminating its activity (Figure 1D). In contrast, the majority of compounds **3–17** showed only about 20% inhibition of the DNMT3A2-3L complex, though compound **3** exhibited over 50% inhibition (Figure 1E). Similarly, minimal or no inhibition was observed for DNMT3B2-3L, with the best compound (**3**) demonstrating just ~20% inhibition (Figure 1F). However, both DNMT3A2-3L and DNMT3B2-3L exhibit low turnover rates under the conditions tested (Figure S2A), complicating the detection of changes in enzymatic activity. Because we observed that quinoline derivatives operate in a DNA substrate-dependent inhibition mode (see in the following text), we repeated the DNMT3A2-3L inhibition assay with an [E]/[DNA] ratio of 1:2 ([DNA] = 1 μ M), reduced from 1:10 ([DNA] = 5 μ M). Under that condition, all 15 new compounds showed >50% inhibition at [I] = 10 μ M (Figure S2F). However, as expected, the inhibition by sinefungin did not change as a function of DNA substrate concentration.

Compounds **3–17** were tested against three bacterial DNA adenine MTases (CamA, CcrM, and Dam) for their specificity. Unexpectedly, we observed robust inhibition of the activities of the three tested DNA adenine MTases by the same set of six compounds (**6**, **8**, **9**, **11**, **12**, and **13**) that had abolished DNMT1 activity (Figures 1G–1I). For comparison, the two parent compounds **1** and **2** exhibited no inhibitory effect at all for CcrM and Dam, and less than 50% inhibition for CamA (Figure 1G). Notably, among these six compounds, compounds **9** and **13** displayed differential activity against CamA, CcrM, and Dam, with both compounds showing stronger inhibition of CcrM. Similarly, sinefungin exhibited significant inhibition of CcrM, the strongest potency among the six MTases examined. As sinefungin is a SAM analog, its limited inhibition of CamA might reflect that the enzyme's unusually weak binding of SAM.⁴² Preferential inhibition of CcrM by compounds **9** and **13** might reflect the fact that CcrM engages in substantially more DNA strand separation, as part of its DNA recognition process, than the other tested MTases.^{44,54,55}

Interestingly, the six most potent compounds (**6**, **8**, **9**, **11**, **12**, and **13**) share common chemical features in the backbone, which are reminiscent of the *meta/meta* orientation of **2**, maintaining identical relative positions of the ring structures but with modifications in either ring B, ring C, or both (Figure 1B). Changes in the amide fragment from compound **6** to **7** (amide vs. inverted amide between B/C rings) or replacement the pyrimidine moiety of ring D from compound **9** to a benzyl carbamate of **10** resulted in loss of inhibition. Conversely, the same modifications on ring B (compound **3**) or ring C (compound **5**) in the backbone of **1** (*para/para* orientation) did not yield the same effect. The *bis*-quinoline (**15**) or *bis*-pyrimidine structure (**17**) resulted in a loss of inhibition ability, highlighting critical importance of the “left” quinoline and the “right” pyrimidine moieties for the derivatives of compound **2**.

DNMT1 inhibition

We selected the six **2**-mimicries (**6**, **8**, **9**, **11**, **12**, and **13**) for a detailed inhibition study, determining their half-maximal

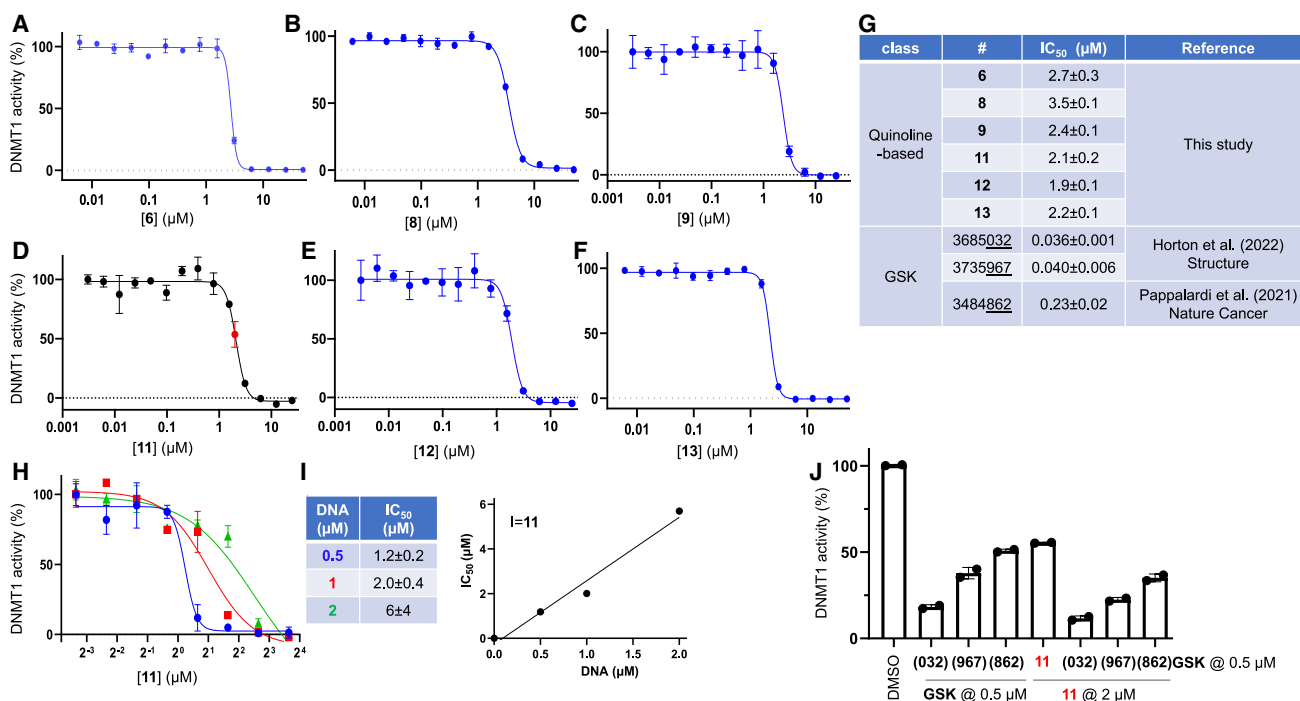


Figure 2. DNMT1 inhibition

(A–F) The IC₅₀ measurements were made by varying inhibitor concentrations in the presence of 1 μM DNA substrate. Data represent the mean ± SD of three independent determinations.

(G) Summary of the IC₅₀ values of inhibition of DNMT1 methylation by quinoline-based compounds and dicyanopyridine-based GSK inhibitors. # indicates compound number.

(H) The IC₅₀ measurements of compound **11** as a function of substrate DNA concentration. *N* = 3 independent measurements.

(I) Plot of the IC₅₀ values of compound **11** versus DNA concentrations.

(J) Additive effect of inhibition of DNMT1 by GSK inhibitor at 0.5 μM together with compound **11** at 2 μM. *N* = 2 independent determinations.

inhibitory concentrations (IC₅₀) in the presence of 0.1 μM DNMT1, 5.5 μM SAM, and 1 μM DNA substrate (Figures 2A–2F). The six compounds exhibited similar IC₅₀ values, ranging from 1.9 to 3.5 μM, with **12** demonstrating 1.8× higher potency than compound **8** (Figure 2G). The six selected quinoline derivatives exhibited undetectable inhibition at low concentrations, transitioning to a steep slope upon reaching the DNA substrate concentration of 1 μM, and plateauing near background levels rapidly thereafter (Figures 2A–2F). This observation suggests that quinoline derivatives operate in a DNA substrate-dependent inhibition mode (Figures 2H and 2I).

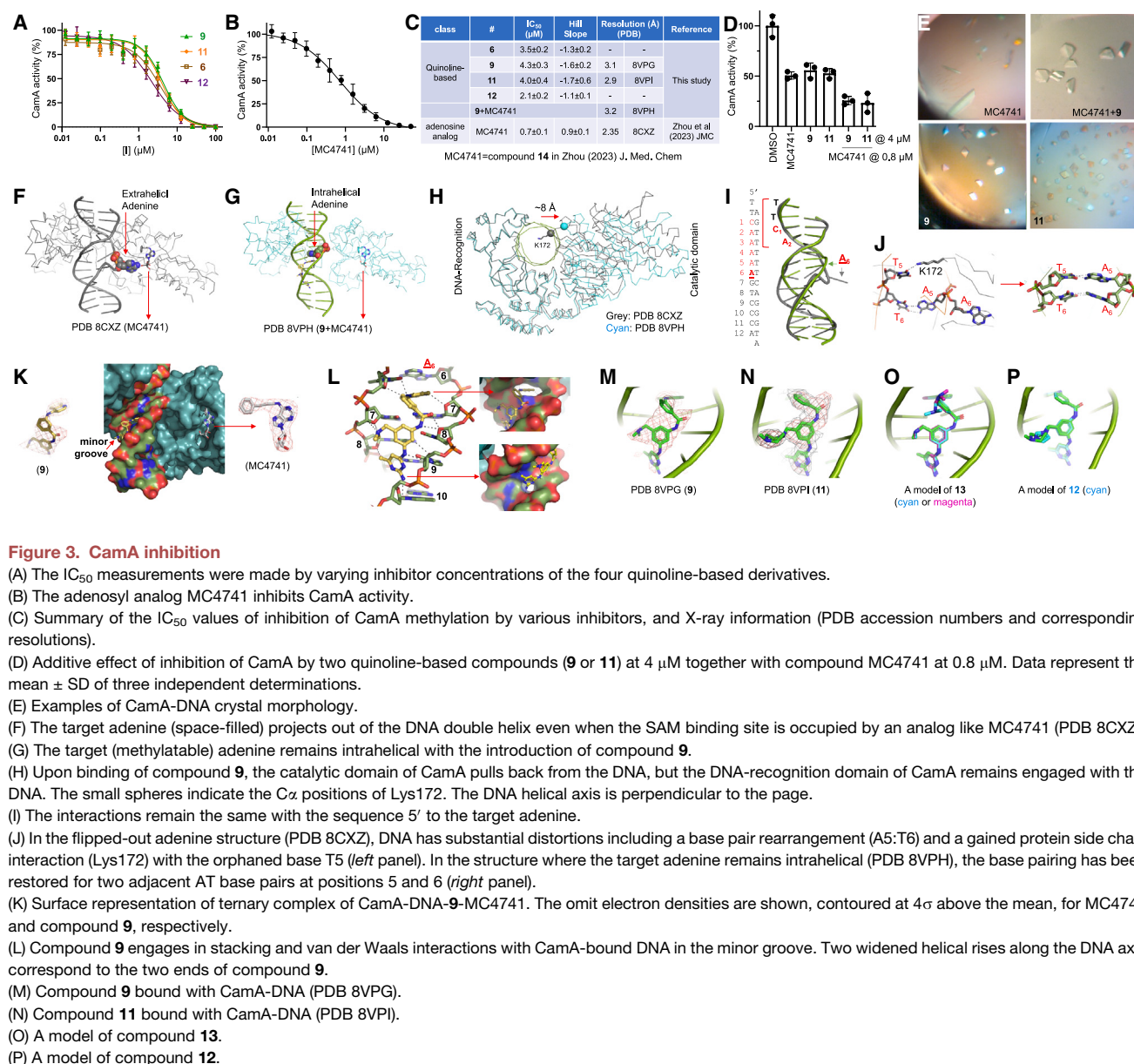
Next, we compared the inhibitory constants with those of three recently developed, dicyanopyridine-containing, non-nucleoside DNMT1-selective GlaxoSmithKline (GSK) inhibitors.^{33,34} Under the same reaction conditions, GSK3685032 and GSK3735967 each displayed an IC₅₀ value of ~0.04 μM, while GSK3484862 showed an IC₅₀ value of 0.23 μM, making them 10–50 times more potent than the quinoline-based compounds (Figure 2G). At or above their respective IC₅₀ values—0.5 μM for GSK inhibitors and 2 μM for compound **11**—the effect of combined treatment is additive, not synergistic (Figure 2J).

We proceeded to examine the impact of the inhibitors in murine embryonic stem cells (mESCs). Unlike other mammalian cells that rely on DNA methylation for viability and proliferation, mESCs can survive without DNA methylation,^{56–58} making

them an ideal cellular system for studying DNA hypomethylation. We used the seven **2**-mimicries (**2**, **6**, **8**, **9**, **11**, **12**, and **13**), in conjunction with GSK3685032 and GSK3484862 (Figure S2G). We assessed global methylation, using methylation of the minor satellite repeats as a proxy, by Southern blot analysis post-digestion with the methylation-sensitive enzyme *HpaII* (which cuts CCGG but not C⁵mCGG). After a two-day treatment, only GSK3685032 and GSK3484862 significantly reduced methylation levels, consistent with severe depletion of Dnmt1⁵⁹ (Figure S2G). In contrast, the **2**-related quinoline compounds did not significantly differ from the DMSO control in their effect on methylation, despite their strong *in vitro* inhibition of DNMT1 (Figure 2). This discrepancy may be partly due to their pharmacokinetic properties, including their capacities to enter the cells and the nuclei, where DNA methylation occurs, their stability in cells, or their interactions with various other enzymes (see in the following text).

CamA inhibition

We determined IC₅₀ values of these compounds against the 6mA MTase CamA. They ranged from 2 to 4 μM, for the four quinoline compounds (**6**, **9**, **11**, and **12**) (Figure 3A) that had exhibited the most potent inhibition against CamA in a single-dose experiment (Figure 1G). These values are in a similar range as we observed for DNMT1 inhibition. Compound **12** inhibited both DNMT1 and



CamA with comparable potency (IC₅₀~2 μM). Subsequently we compared these results to MC4741, a recently developed selective CamA inhibitor that features an adenosine analog carrying a 3-phenylpropyl moiety attached to the adenosyl N6 atom.⁶⁰ MC4741 exhibited stronger inhibition, with an IC₅₀ value of 0.7 μM (Figure 3B), 3× lower than that of compound **12** and 5-6× lower than that of the other three quinoline compounds (Figure 3C). When used in combination, at their respective IC₅₀ values, compounds **9** (or **11**) together with MC4741 exhibited additive effects (Figure 3D).

MC4741 inhibits CamA activity by occupying the binding pocket of methyl donor SAM.⁶⁰ To clarify the inhibition mode of the new quinoline derivatives, we meticulously explored co-crystallization conditions for CamA-DNA complexes in the presence of compound. We determined three structures of CamA-DNA complexes with compounds **9** or **11** individually, or

compound **9** together with MC4741, in a resolution range of 2.9–3.2 Å (Figure 3C). The introduction of the quinoline derivatives into the crystallization mix altered the crystal shapes from elongated rods to diamonds (Figure 3E), though the crystals were formed in the same crystallographic space group and with similar unit cell dimensions (Table S1). Within the crystallographic asymmetric unit, there are three CamA-DNA complexes, and only one of the three complexes showed a bound inhibitor (**9** or **11**). We made the following observations from the quinoline-bound complexes.

First, regarding base flipping, CamA resembles other structurally characterized DNA MTases acting on cytosine and adenine.^{51,61} Specifically, like all other known DNA MTases, CamA flips its target adenine out of the DNA double helix.⁴² This action occurs even when the SAM binding site is occupied by an analog like MC4741 (Figure 3F). However, with the

introduction of compound **9** or **11**, the target adenine either does not flip at all, or it does but returns to its intrahelical position within the double helix (Figure 3G). Superimposition of the complexes reveals that, upon binding of compound **9**, the DNA-recognition domain of CamA remains engaged with DNA, but the catalytic domain retracts by approximately 8 Å (Figure 3H). More specifically, the DNA-recognition domain stays in close contact with the five base pairs of the recognition sequence 5' to the target adenine in the major-groove, such that this portion of the DNA is undisturbed (Figure 3I). However, the catalytic domain retraction disrupts the interaction between the enzyme's catalytic residues and the DNA, especially the Lys172-base T5 interaction, and restores the base pairing of two adjacent AT base pairs at positions 5 and 6 (Figure 3J). The Lys172-containing active-site loop, which normally penetrates the DNA minor groove, undergoes large movement and becomes less ordered when the enzyme transitions in the presence of compound **9**.

Second, compound **9** engages with DNA, in the minor groove (Figure 3K). The aminoquinoline moiety intercalates into the DNA, right after the base pair that includes the target adenine. This insertion occurs chiefly through stacking interactions with the base pairs at positions 6 and 7 (Figure 3L). The subsequent molecular moieties, 3-aminobenzoic acid and 1,3-phenylenediamine, extend across the subsequent three base pairs (7–9). The terminal component, a diamino-6-methylpyrimidine moiety, wedges in between the base pairs at positions 9 and 10 (Figure 3L). Beyond the stacking provided by the aminoquinoline, compound **9**'s primary interactions with the DNA are van der Waals forces with six deoxyribose rings, three on each strand. The *N,N*-dimethylaminomethyl addition to the 1,3-phenylenediamine moiety allows two van der Waals contacts with the deoxyribose of cytosine at position 7. Additionally, the amino and methyl groups on the diamino-6-methylpyrimidine moiety form a hydrogen bond with the cytosine O2 atom at position 10, and a van der Waals interaction with the deoxyribose O4 atom of adenine at position 8, respectively (Figure 3L). The helical rise along the DNA axis is extended at two specific points, corresponding to the two ends of compound **9**: between base pairs 6 and 7 due to the intercalation of the aminoquinoline moiety, and between base pairs 9 and 10 where the diamino-6-methylpyrimidine is positioned (Figures 3L and S3).

Finally, compounds **9** and **11** exhibited similar binding modes when tested independently, without the presence of MC4741 (Figures 4M and 4N). Building upon the structure of compound **9**, we introduced an additional *N,N*-dimethylaminomethyl group onto ring B, creating compound **13** (Figure 3O). In this modeled structure, the second *N,N*-dimethylaminomethyl group of compound **13** is oriented away from the DNA, facing toward the solvent. For compound **11**, we replaced its *N*-methylpiperazin-1-yl moiety with an imidazole ring to form compound **12** (Figure 3P). This substitution of a positively charged imidazole ring allows potential interaction with the nearest DNA backbone phosphate group, potentially contributing to compound **12**'s 2-fold increased potency, as reflected in its IC₅₀ value.

The insertion of planar moieties between adjacent bases is a common mechanism for drug intercalation into DNA or RNA.^{62–64} However, the unique aspect here is the specific insertion of aminoquinoline-based derivatives after the target adenine, within the sequence bound by CamA. While there is

no observed direct interaction between the compound and CamA in the current structure, it seems likely that the inhibitor competes with or displaces the enzyme's active-site loop from the DNA's minor groove. This action results in the reestablishment of Watson-Crick base pairing while the DNA remains bound by the enzyme. In addition, the quinoline-based compounds (**9**, **11**, or **13**) do not interact independently with DNA, with CamA, or with preformed CamA-DNA complexes (Figures S4A–S4H). However compound **9** reduces CamA-DNA interaction when present in the mixture for either wild type (WT) or the catalytically dead mutant N165A (compare Figures S4I and S4J for WT; Figures S4K and S4L for the N165A mutant). This observation suggests that the quinoline-based compounds specifically alter the enzyme's interaction with DNA, providing a structural basis for its inhibitory effect.

Inhibition of base excision repair DNA glycosylases

The finding that compounds **9** and **11** intercalate into CamA-bound DNA, reversing base flipping, prompted us to explore whether our quinoline derivatives can inhibit other DNA base-flipping enzymes. Specifically, DNA base excision repair (BER) enzymes such as uracil-DNA glycosylase (UDG), thymine-DNA glycosylase (TDG), methyl-CpG-binding domain protein 4 (MBD4), and MutY homolog (MYH) all utilize a base-flipping mechanism for excising bases.^{65–67} Initially, we assayed MBD4 and TDG base excision activities on a T:G mismatch substrate (with the mispaired T-strand being FAM-labeled in Figure 4A), using an inhibitor concentration of 2 μM. Similar to their effect on DNMT1 and CamA, the same six compounds (**6**, **8**, **9**, **11**, **12**, and **13**) inhibited MBD4 activity (Figure 4B). However, only compound **13** almost completely inhibited TDG activity, while the other compounds exhibited lesser inhibition (Figure 4C). The variance in inhibition levels between MBD4 and TDG was not observed when the inhibitor dose was increased 10-fold (Figures 4D and 4E). In addition, using compound **13** as an example, we noted that this compound does not independently bind with mismatched DNA (Figure S4E). The inhibition effect on MBD4 is not substrate specific, as compound **13** inhibits MBD4 activity on a U:G mismatch (Figure 4F). Moreover, compound **13** also effectively inhibits the excision activity MYH in the removal of adenine opposite 8-oxo-2'-deoxyguanosine (8-oxoG) (Figure 4G). Notably, the two progenitor compounds, **1** and **2**, do not exhibit effective inhibition at a concentration of 2 μM (Figures 4B, 4C, 4F, and 4G). The lack of effective inhibition by the original compounds at this concentration underscores the importance of molecular modifications in enhancing or altering the inhibitory capabilities of these compounds against base-flipping enzymes.

Continuing our investigation, we explored the excision of uracil by UDG, an enzyme notable for its activity on both double-strand (ds) and single-strand (ss) DNA. We found that compound **13** exhibited less potent inhibition of UDG activity on dsDNA substrates (Figure 4H) compared to the other glycosylases examined in this study (MBD4, TDG, and MYH). However, a critical observation is that compound **13** does not inhibit UDG activity on ssDNA (Figure 4I). This differential effect highlights the substrate specificity of these quinoline-based derivatives using a mechanism of intercalation with dsDNA, as demonstrated by CamA (Figure 3).

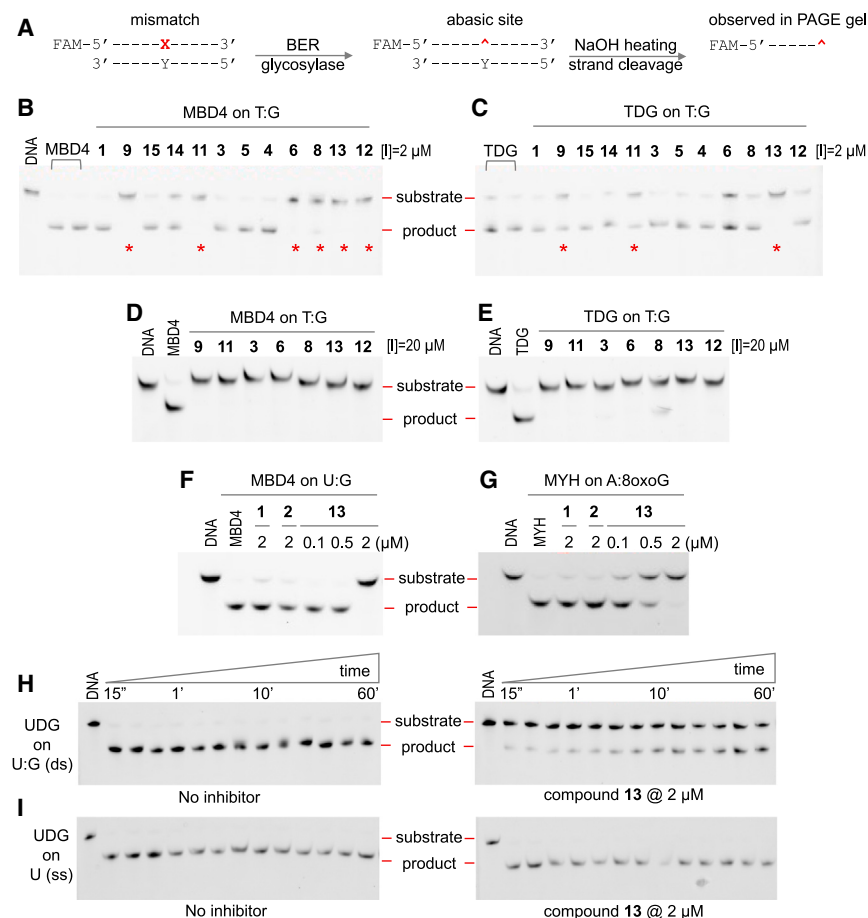


Figure 4. Inhibition of base excision repair (BER) glycosylases on dsDNA containing mismatches

(A) Schematic of strand cleavage at an abasic site in dsDNA arising from treatment with glycosylase and then NaOH.
(B–E) Inhibition of MBD4 and TDG activities on T:G mismatches at inhibitor concentrations of 2 μM (B–C) or 20 μM (D–E).
(F) Inhibition of MBD4 activity on U:G mismatch.
(G) Inhibition of MYH activity on adenine opposite to 8-oxoguanine.
(H) Activity of UDG on uracil of dsDNA without (left panel) and with compound 13 at 2 μM (right panel).
(I) Activities of UDG on uracil of ssDNA without (left panel) and with compound 13 at 2 μM (right panel).

in the presence of 50 μM deoxynucleoside triphosphate substrates (dNTP), compounds 9 and 13 exhibited IC₅₀ values of 7.5 μM and 3.9 μM, respectively, in inhibiting Pol η activity (Figures 5B and 5C).

We then conducted steady-state kinetic assays focusing on the first incoming nucleotide incorporation (dATP) (Figure 5D). Under the laboratory conditions (STAR Methods), Pol η exhibited a catalytic rate constant (k_{cat}) of 60 min^{−1} and a Michaelis constant (K_M of dATP) of 31 μM (Figure 5D). When exposed to a concentration of compound 9 or 13 at twice their respective IC₅₀ values, we observed significant changes in the enzyme kinetics:

Next, we purified two human Nei-like DNA glycosylases, Neil1 and Neil2⁶⁸ (Figures S5A and S5B), and tested the inhibition of their abasic (AP) lyase activity.⁶⁹ While the quinoline derivatives had little effect on Neil1 activity, they significantly inhibited Neil2 at a concentration of 10 μM (Figures S5C and S5D). We focused on compound 13, which showed concentration-dependent inhibition with an estimated IC₅₀ value of ~0.3–0.6 μM (Figure S5E).

Inhibition of polymerases

Next, we considered DNA polymerases, such as Y-family translesion DNA polymerase η (Pol η) and A-family repair DNA polymerase θ (Pol θ), which—like other DNA polymerases—are considered potential targets for cancer therapy.^{70,71} We chose Pol η as it has an enlarged active site for accommodating the bulky lesions (such as UV-induced cyclopurine dimers),⁷² and thus we believe Pol η is an ideal candidate for testing against the quinoline-based derivatives. We chose Pol θ as it mediates the end-joining repair pathway, has an important role in resolving chromosomal double-strand breaks,⁷³ and as drug development against Pol θ is among the most advanced, with over 20 patented inhibitors and several proceeding to clinical trials.⁷⁴

Looking first at the Pol η results, using the known pan MTase inhibitor sinefungin as a control, as expected we observed no impact on polymerase activity (Figures 5A and S6). In contrast, we found that compounds 9 and 13 inhibited >50% of Pol η's activity at a compound concentration of 10 μM (Figure 5A). Further,

a 12-fold or 25-fold reduced k_{cat} values for compounds 9 (5.0 min^{−1}) and 13 (2.4 min^{−1}) respectively, and a 2- to 4-fold increase in K_M^{dATP} values (Figures 5E and 5F). This translates to a 30-fold or 95-fold reduction in catalytic efficiency for compounds 9 and 13, respectively, as evidenced by comparing the k_{cat}/K_M^{dATP} value of 1.9 min^{−1}μM^{−1} (with no inhibition) to 0.06 min^{−1}μM^{−1} (compound 9) or 0.02 min^{−1}μM^{−1} (compound 13).

We next examined Pol θ using the same conditions. We observed that at a concentration of 10 μM, none of the compounds inhibited Pol θ activity by more than 20% (Figure S6A). However, at a 5-fold higher concentration (50 μM), compounds 1 and 9 achieved ~50% inhibition, while compound 13 reached approximately 75% inhibition. Notably, compounds 9 and 13 demonstrated higher IC₅₀ values of 68 μM and 14 μM, respectively (Figures S6B and S6C), making them 9 times and 3.6 times less effective as inhibitors of Pol θ compared to Pol η. The basis for differential inhibition of these two repair polymerases, belonging to different families with distinct difference sequences and structures, remains elusive, but the important point here is that these DNA-intercalating inhibitors of DNA MTases also inhibit some DNA polymerases.

We also evaluated the quinoline derivatives against HIV reverse transcriptase (RT), an enzyme that synthesizes DNA on primed RNA templates. Remarkably, nearly all of the compounds showed approximately 50% inhibition at a concentration of 10 μM (Figure S6D). When tested at the higher concentration

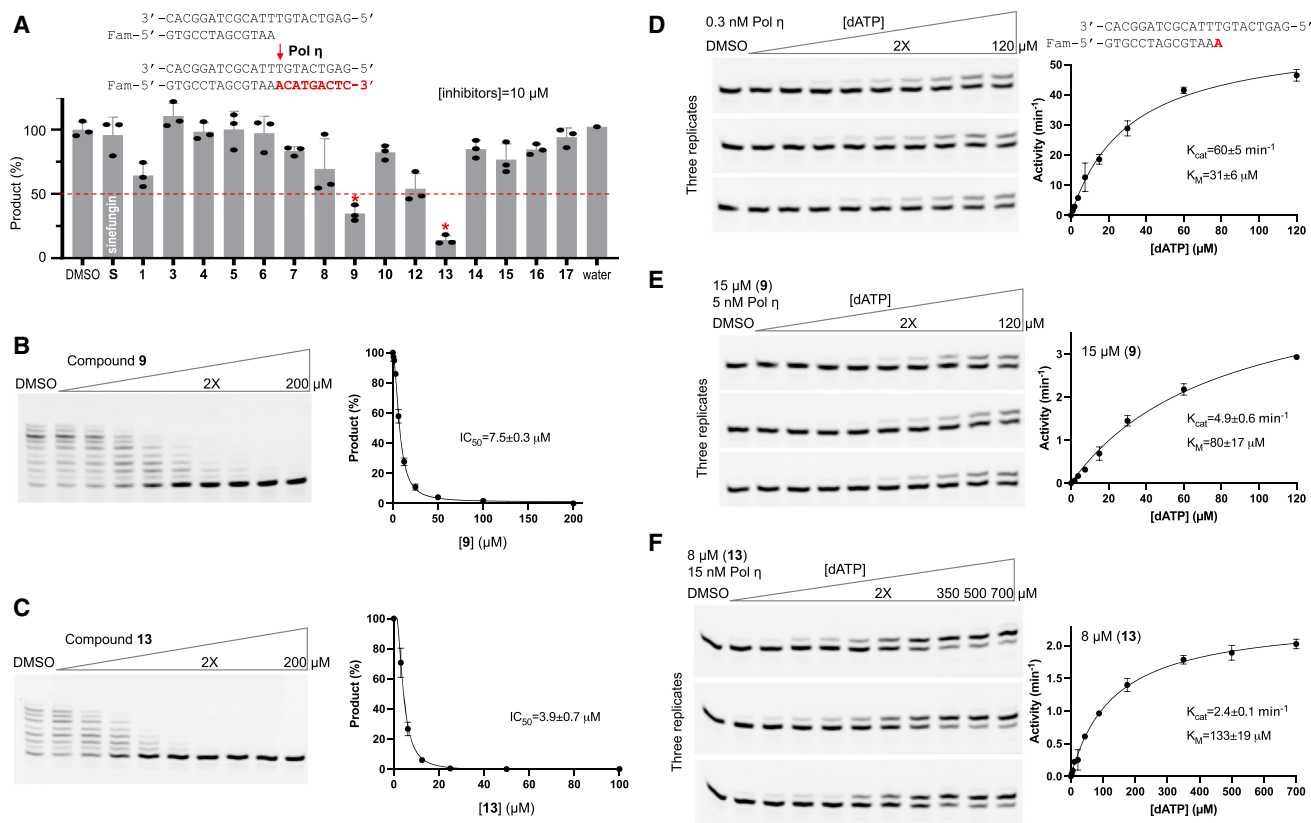


Figure 5. Inhibition of DNA Pol η

(A) The DNA synthesis reaction uses DNA template (top strand) and 5'-fluorescein-labeled primer (bottom strand). Relative inhibition of pol η at a single inhibitor concentration of 10 μM [S, sinefungin; compounds 2 and 11 were unavailable at the time of these assays]. (B and C) Inhibition of Pol η activity with increased concentrations of compounds 9 (B) or 13 (C). (D–F) Kinetics of Pol η on the first adenine incorporation in the absence of inhibitor (D), presence of compound 9 (E), or of compound 13 (F). Data represent the mean $\pm$ SD of three independent determinations.

of 50 μM , four of these compounds (3, 7, 9, and 12) reduced the activity of HIV RT to undetectable levels (Figure S6D).

While our focus here is on enzymes that act on DNA, in our final set of experiments, we tested the quinoline derivatives against a poliovirus RNA-dependent RNA polymerase (RdRp), which synthesizes RNA on primed RNA templates. Intriguingly, the parent compound 1 exhibited the most significant inhibition, with around 75% effectiveness at a concentration of 10 μM (Figure S6E). Notably, the RdRp enzyme demonstrated a unique response compared to the other tested polymerases and MTases, particularly in its sensitivity to compound 1. This finding suggests that quinoline derivatives might differentiate between RNA and DNA duplexes in their mechanism of action. This would not be surprising if, as with CamA, inhibition results from interaction with the enzyme-bound nucleic acid substrate (see above). However, again, the key point for the purposes of this study is that these target-specific intercalating drugs inhibit a wide range of enzymes that act on nucleic acids.

Compound 11 elicits DNA damage response via p53 activation

Given the *in vitro* inhibitory activity of quinoline-based derivatives on various enzymes acting on DNA substrates, including some

that are active in repair, we investigated the effects of compound 11 on the viability of A549 human non-small cell lung carcinoma (NSCLC) cells. Through luminescence-based cell viability assays, we observed that treatment with 11 at a concentration of 4 μM for three days resulted in a reduction of A549 cell survival to $\sim 50\%$ compared to DMSO-treated control cells (Figure 6A). Under the same conditions, compound 3 displayed the greatest cellular toxicity (Figure 6A). In contrast, cells exposed to GSK3484862 exhibited viabilities similar to control cells, as we had observed previously,⁵⁹ whereas GSK3685032 displayed higher toxicity than GSK3484862 but was less effective in reducing cell viability than compound 11 (Figure 6A). To minimize the impact of cell death on subsequent analyses, A549 cells were treated for one day in the following experiments.

Recent studies have revealed the efficacy of dicyanopyridine-based GSK inhibitors in promoting degradation of DNMT1.⁵⁹ We reproduced this observation, demonstrating a complete depletion of DNMT1 in A549 cells treated with GSK inhibitors (Figure 6B). Interestingly, similar treatment conditions with compound 11—a 4 μM concentration for one-day—also resulted in decreased DNMT1 protein levels, albeit to a lesser extent (Figure 6B). We observed no significant changes in DNMT3A protein levels.

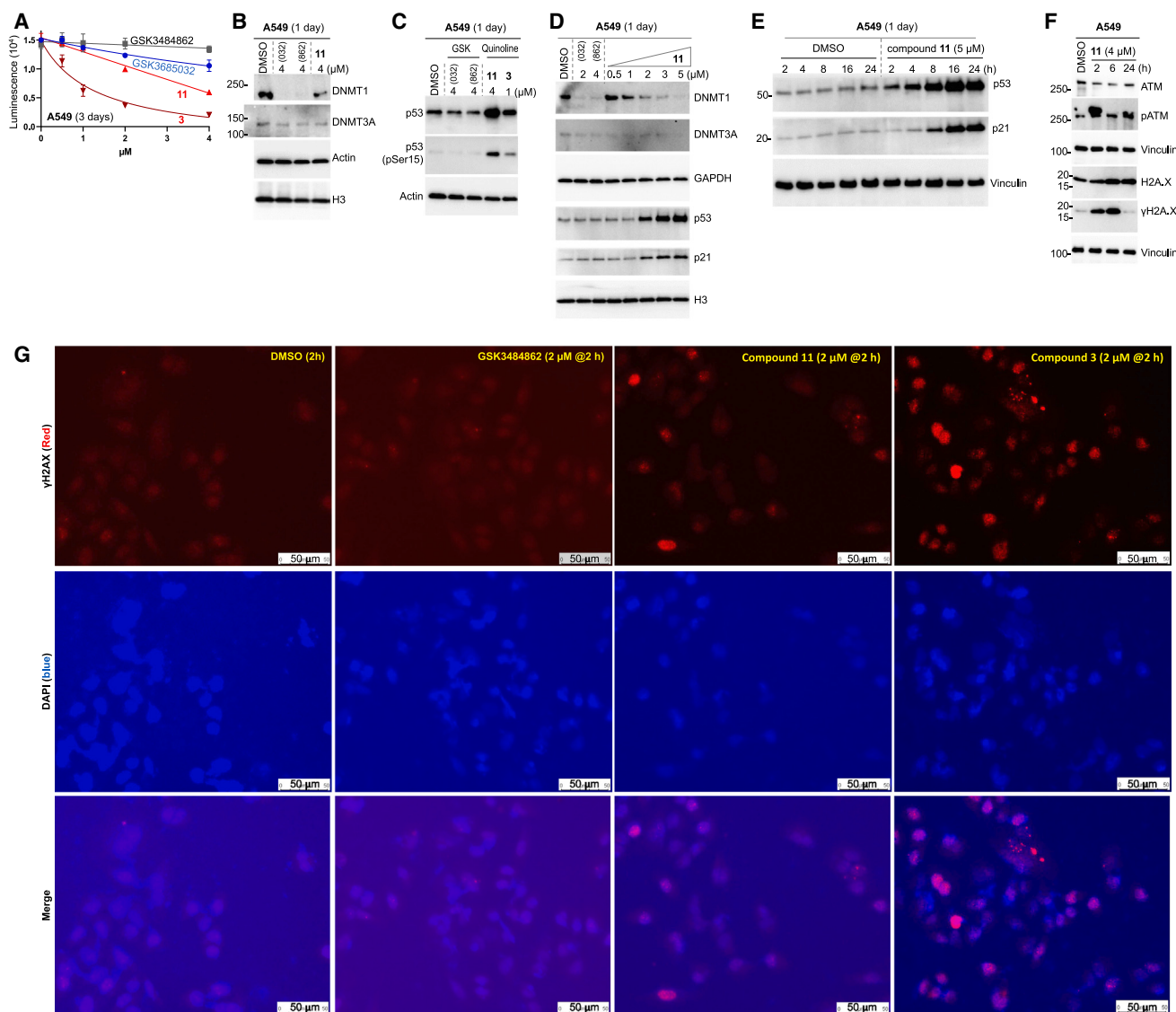


Figure 6. The effect of compound 11 in A549 cells

(A) Cell viability (relative to DMSO treatment). *N* = 3 independent experiments, each with triplicates (mean ± SD).
 (B and C) Western blots showing endogenous levels of DNMT1, DNMT3A, p53 and pSer15 of p53 following treatment with 4 μM of GSK3484862, GSK3685032 and compound 11 for 24 h or compound 3 at 1 μM.
 (D) Dose-dependent decrease of DNMT1 and increase of p53 and p21 by compound 11 treatment.
 (E) Time-dependent increase of p53 and p21 by compound 11 treatment.
 (F) Western blots showing dynamic changes of endogenous levels of ATM (Ataxia Telangiectasia Mutated) phosphorylation, and of γH2AX (phosphorylated H2A histone family member X), following compound 11 treatment.
 (G) γH2AX forms foci in A549 following compound treatment at 2 h, DMSO, GSK3484862, quinoline-based compounds 11 and 3.

The most notable distinction between these two classes of inhibitors lies in their impact on p53—a protein crucial for the cellular DNA damage response^{75,76}—and the phosphorylation of p53 at serine 15 (Ser15) (Figure 6C). This post-translational modification is key for the activation and stabilization of p53 following DNA damage,⁷⁷ highlighting a potential differential mechanism of action between the two chemotypes. Treatment of A549 cells with compound 11 led to dose- and time-dependent increases in levels of p53 and its downstream target p21 (Figures 6D and 6E). Furthermore, upon compound 11 treatment,

we observed ATM autophosphorylation (peaking at 2 h) and of γH2AX (peaking at 2–6 h) (Figure 6F). Both pATM and γH2AX are crucial components of the DNA damage response pathway.⁷⁸ Obvious γH2AX foci were observed in A549 cells at 2-h treatment with compounds 11 (at 2 μM) or 3 (at 2 μM) (Figure 6G). This observation supports quinoline-based derivatives (e.g., compounds 11 and 3) having stronger cellular toxicity (via a DNA-damage effect) than the GSK compounds (Figure 6A). Following an established protocol,⁷⁹ we used siRNA to knock down *TP53* expression, resulting in reduced p53 protein level

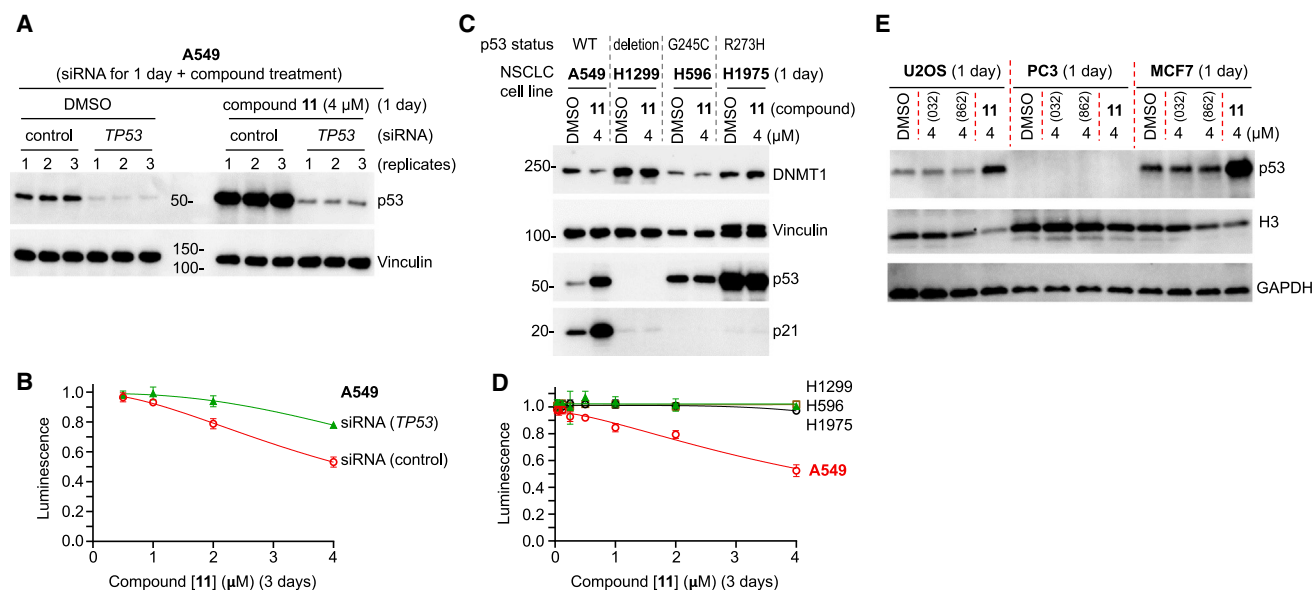


Figure 7. The effects of compound 11 in cancer cells with wild-type p53

(A) Knockdown of *TP53* expression by siRNA. $N = 3$ independent experiments (labeled as 1, 2, and 3).
(B) Anti-*TP53* siRNA decreased cellular toxicity from compound **11** treatment. $N = 3$, each with triplicates (mean $\pm$ SD).
(C) Treatment with compound **11** in a set of non-small cell lung cancer (NSCLC) cell lines, encompassing a variety of p53 genetic backgrounds as indicated.
(D) Cell viability (relative to DMSO treatment) of compound **11** among the four NSCLC cell lines. $N = 3$, each with triplicates (mean $\pm$ SD).
(E) Western blot confirms increased p53 levels in osteosarcoma U2OS (left) and breast-cancer derived MCF7 cells (right) but not in prostate cancer-derived PC3 (middle).

in A549 cells (Figure 7A), which resulted in decreased cellular toxicity from compound **11** treatment (Figure 7B).

Next, we extended our investigation of compound **11**'s effects to a broader array of NSCLC cell lines, encompassing a variety of p53 genetic backgrounds: wild-type p53 (A549), p53 deletion (NCI-H1299), and p53 hotspot mutations Gly245-to-Cys (NCI-H596) and Arg273-to-His (NCI-H1975) (Figure 7C). Treatment of the p53 wild-type (A549) cells led to a partial reduction in DNMT1 levels and the activation of p53 and p21, an indication of DNA damage/cellular stress caused by the compound treatment. However, we observed no significant impact on p53/p21 levels, and little or no cellular toxicity (Figure 7D) in cell lines with p53 deletion or harboring p53 mutations. Additionally, we confirmed that compound **11** treatment elevated p53 protein levels in osteosarcoma-derived U2OS cells and in estrogen receptor-positive, breast cancer-derived MCF7 cells (Figure 7E). As a control, p53 protein was undetectable in prostate cancer-derived, androgen-insensitive PC3 cells, due to deletion of the *TP53* gene.^{80–82} Significantly, these data suggest that the presence of the wild-type p53 in human cancer cells correlates well with successful treatment using quinoline-based compound **11** (Figure S7A).

DISCUSSION

DNA intercalating agents as DNMT inhibitors

Recently, we investigated the structural and biochemical interactions of human DNMT1 with dicyanopyridine-based inhibitors.^{33,34} These inhibitors are unique in their chemotype, featuring a planar dicyanopyridine core that specifically interca-

lates into DNMT1-bound DNA substrates (Figure S7B). Our structural insights into these dicyanopyridine-based compounds, combined with biochemical data showing DNA-dependent inhibition by quinoline derivatives,³² prompted us to re-examine the mechanism of the quinoline derivatives in inhibiting two distinct classes of DNA MTases—those generating 5mC and those generating 6mA.

We identified three key similarities between dicyanopyridine-based and quinoline-based inhibitors, applicable to both compound types. First, intercalation into DNA—both dicyanopyridine and aminoquinoline moieties intercalate into the DNA, specifically at sites where it is bound by MTases (Figures S7B and S7C), disrupting the normal DNA helical rise. Second, conformational shifts in the MTase catalytic domain—the intercalation induces a significant conformational shift, moving the catalytic domain away from the specific DNA sequence. This shift does not depend on catalytic competence, at least in the case of DNMT1: an active-site cysteine-to-serine substitution does not affect the dicyanopyridine compound interaction with DNA in the presence of the mutant DNMT1.⁵⁹ Third, restoration of base pairing—the interaction between the enzyme's active-site residues and the flipped (extrahelical) target cytosine or adenine is disrupted, allowing these bases to return to their normal intrahelical positions (or preventing them from flipping in the first place). While the initial mechanism directing these inhibitors to their specific binding sites remains unclear, given that they bind to neither the DNA alone nor the enzyme alone, we hypothesize that the DNA deformation caused by the base-flipping action of MTases creates an entry point for these intercalating agents, though a single mismatch is insufficient deformation to

allow such intercalation (Figure S4E). Nevertheless, supporting this hypothesis, we also observed that these compounds inhibit DNA BER glycosylases such as MBD4, TDG, MYH, UDG, and NEIL2 (Figures 4 and S5), which employ a base-flipping mechanism for excising bases.^{65–67} This, in turn, led us to speculate that quinoline-based analogs may have a broad spectrum of activity against various DNA-acting enzymes, such as polymerases, that contain a junction of dsDNA and ssDNA during catalysis, which indeed we found to be correct (Figures 5 and S6).

Compound selectivity vs. potency

A significant distinction between dicyanopyridine-based and the quinoline-based inhibitors studied here is their selectivity. Dicyanopyridine-based inhibitors tested to date exhibit exclusive specificity toward DNMT1, whereas quinoline-based inhibitors can inhibit both DNMT1 as well as the three adenine DNA MTases, and six BER glycosylases in our tests. This difference in selectivity is thought to arise from the interaction of dicyanopyridine-based inhibitors with the active-site loop of DNMT1 (Figure S7B),^{33,34} which is likely crucial for their DNMT1-specific action. In contrast, our structural analyses, representing only a snapshot along the inhibition pathway, did not reveal a direct interaction between the quinoline-based compounds **9** and **11** and CamA (Figure S7C). This absence of direct interaction in the observed CamA structures suggests that quinoline-based compounds might have a wider range of targets, potentially inhibiting various other MTases or enzymes that interact with nucleic acids. Aligned with this concept, the quinoline-based compounds demonstrate more pronounced cytotoxic effects than dicyanopyridine-based DNMT1 inhibitors (Figure 6).

However, the quinoline-based inhibitors did not exhibit uniform behavior. For instance, the parent compound **2** displays a distinct selectivity pattern, with 10 μ M eliminating nearly all DNMT1 activity, inhibiting CamA by about 50%, and showing no inhibitory effects on CcrM or Dam (Figure 1). However, derivatives of **2**, such as compounds **9** and **11**, have higher inhibitory potency across all four MTases and lack obvious selectivity among the four.

Regarding BER glycosylases, compound **13** exhibited the most effective inhibition. For polymerases, compounds **1** and **13** demonstrated contrasting inhibitory effects on DNA repair polymerase Pol η and the RdRp. Compound **13** is most effective against Pol η , while compound **1** shows more than 50% inhibition at 10 μ M. Conversely, compound **1** is the most potent against RdRp, whereas compound **13** does not detectably inhibit it, even at a concentration of 50 μ M (Figure S6E). These observations highlight the potential for fine-tuning the potency and selectivity of quinoline-based inhibitors to target specific enzymes more effectively.

Future work includes testing chemical modifications of the selected quinoline compounds that exhibit single-digit micromolar potencies against the three classes of DNA-acting enzymes examined. Guided by the crystal structures of compounds **9** or **11** bound with CamA-DNA, several strategies will be employed to improve potency and selectivity (Figure S7D). Among other modifications, first we will introduce more nitrogen atoms into ring A by replacing the quinoline with a naphthyridine, indole, indazole, or azaindole rings, which should enhance interactions with DNA bases when intercalated between two base

pairs. Second, we will extend the side chain at ring C from methyl- to ethyl- and propyl-amino, allowing deeper insertion between DNA strands. Third, we will replace the amine linker between rings C and D with a carboxamide group to increase molecule length and flexibility. Fourth, we will increase the polarity of the methyl substituent at ring D by changing it to hydroxymethyl, carboxyl, carboxamide, or an amino group. Finally, we will design chimeras by combining our quinolines with GSK DNMT1 inhibitors, substituting either ring A or D with the 2-amino-3,5-dicyano-4-ethyl-6-pyridinyl moiety typical of GSK compounds.

Comparison with other quinoline-based compounds

Quinoline is a versatile nitrogen heterocycle found in many biologically active compounds, including DNA intercalators, G-quadruplex (G4) stabilizers, topoisomerase inhibitors, and inhibitors of histone deacetylases (HDACs) and histone lysine methyltransferases (reviewed by Lauria et al.⁸³). Quinoline-containing DNA intercalators typically feature polycondensed aromatic rings, like the three-ring structure in amsacrine (Figure S7E).^{84,85} This flat molecular moiety allows the compound to insert itself non-specifically between the planar base pairs of DNA.⁸⁶ Similarly, G4 stabilizers often possess large, planar, heteroaromatic, and electron-deficient chromophore that facilitate stacking with G4 conformation, as seen in the four-ring structure of SYUIQ-5 (Figure S7F).^{87,88}

One of the earliest quinoline derivatives used as a topoisomerase inhibitor is camptothecin,⁸⁹ which consists of five polycondensed aromatic rings (Figure S7G). Camptothecin and its derivatives stabilize the topoisomerase cleavage complex by inserting the planar quinoline moiety between two consecutive base pairs, preventing the elongation of the topoisomerase-mediated nick in the DNA.⁹⁰

More recently, quinoline-containing compounds have been developed as inhibitors of HDACs and histone lysine methyltransferases. For example, CHR-3996⁹¹ features a quinoline ring that replaces the benzenic group found in vorinostat (also known as SAHA) (Figure S7H). BIX-01294 (Figure S7I) has served as a lead compound in the development of more active quinazolines against histone H3 lysine 9 (H3K9) methyltransferases.^{92–94}

In contrast to the polycondensed aromatic ring structures, SGI-1027 (compound **1** in this study) belongs to the class of 4-anilinoquinolines (Figure 1A). It comprises four fundamental structural fragments with rotatable linkages: the quinoline, two six-membered aromatic rings linked by a central amide, and the aminopyrimidine group. Although SGI-1027 (compound **1**) was initially modeled to bind in the active site and the SAM binding pocket of DNMTs,^{28,95} our study demonstrates that these quinoline-based MC3343 (compound **2**) derivatives (Figure S7J) exhibited inhibitory activity against various DNA-acting enzymes using double helix substrates. Specifically, compounds **9** and **11** were found to intercalate into a CamA-bound DNA substrate.

Limitations of the study

This study acknowledges several limitations, particularly in the exploration of DNA and RNA interactions with proteins and enzymes acting on double-stranded substrates. Such interactions often result in significant structural changes to the nucleic acids, including kinking, bending, unwinding, base flipping, and

separation of strands, which may facilitate the binding of small molecule intercalators. Our investigation was limited to a select few of these enzymes, leaving a vast array of potential targets (or pathways) yet to be explored. Moreover, the demonstrated specificity of compound **13** in inhibiting UDG activity on dsDNA over ssDNA highlights the possibility of developing inhibitors that are substrate selective for enzymes acting on both forms of substrates, though it also underscores the need for extensive research to fully understand and exploit these mechanisms. Finally, a CRISPR screen in the presence of the compound treatment should identify potential targets of the compound or pathways affected by the quinoline-based compounds, providing insights into their mode of action.

SIGNIFICANCE

For the second time following the discovery of dicyanopyridine-based inhibitors against DNMT1, we have identified quinoline-based compounds as inhibitors of both mammalian and bacterial DNA MTases, acting on either cytosine or adenine through the intercalation of enzyme-bound DNA. Furthermore, the enzymatic inhibition by these compounds extends beyond DNA MTases to include several base excision repair DNA glycosylases and DNA polymerases. Inhibition of DNA pol θ is particularly noteworthy because it would lead to the accumulation of chromosomal double-strand breaks, potentially enhancing the effectiveness of cancer treatments that rely on inducing DNA damage. This broad spectrum of enzymatic inhibition suggests that quinoline-based compounds could serve as versatile agents in cancer therapy by targeting multiple pathways involved in DNA repair. Our research revealed that treating various cancer cells with compound **11**, particularly those with wild-type p53, significantly elevates the protein levels of the tumor suppressor p53 and its downstream effector p21. Once activated, p53 can trigger cell-cycle arrest, apoptosis (programmed cell death), and senescence.⁹⁶ However, in NSCLC cell lines lacking p53 or containing mutated forms of p53, compound **11** showed no substantial effects. This suggests that the quinoline-based compounds examined in our study hold potential for further development into advanced p53-targeting therapeutics for cancers harboring wild-type p53. Conducting comparative studies on different p53 mutational subtypes, treated with a p53-reactivating compound,⁹⁷ could enable us to tailor therapeutic strategies to each specific subtype effectively.

RESOURCE AVAILABILITY

Lead contact

Requests for further information, resources, and reagents should be directed to and will be fulfilled by the Lead Contact, Xiaodong Cheng (xcheng5@mdanderson.org).

Materials availability

All reagents generated in this study are available from the [lead contact](#) with a completed Materials Transfer Agreement. Chemical structures and associated characterization data (NMR and mass spectral data) have been provided for all described compounds.

Data and code availability

- The X-ray structures (coordinates and structure factor files) of the compound-bound CamA-DNA complexes have been deposited to PDB and

are publicly available as of the date of publications. PDB accession numbers 8VPG (compound **9**), 8VPI (compound **11**), and 8VPH (compounds **9** and MC4741) and their corresponding DOI are listed in the [key resources table](#).

- The paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

J.Z. performed CamA and CcrM protein purification, inhibition assays, and crystallization. Q.C. performed cellular inhibition in cancer cell lines. J.Z. and J.R.H. performed X-ray crystallography experiments. R.R. performed protein purifications of DNMTs and their inhibition assays. J.R.H. provided Dam enzyme used in the study. J.Y. performed inhibition assays of DNA glycosylases and ITC experiments. B.L. performed experiments in mESCs. C.C. performed inhibition assays of Pol η and HIV RT. C.L. and L.M. performed inhibition assays of Pol θ . Y.Y. performed inhibition assays of RNA-dependent RNA polymerase. D.R. provided compound MC4741. A.K.J. provided advice on p53 biology and siRNA pools. R.M.B. participated in discussions and in preparing the manuscript. Y.G. guided inhibition of polymerases. S.V. and A.M. designed and provided the compounds **3–17**. X.Z., T.C., and X.C. organized and designed the scope of the study.

DECLARATION OF INTERESTS

The authors declare no competing interest.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
DNMT1	Cell Signaling Technology	Cat#5032; RRID: AB_10548197
DNMT3A	CST	Cat#3598; RRID: AB_2277449
Histone H3	CST	Cat#14269; RRID: AB_2756816
GAPDH	CST	Cat#2118; RRID: AB_561053
H2AX	CST	Cat#2595; RRID: AB_10694556
γH2AX	CST	Cat#9718; RRID: AB_2143587
HRP-conjugated anti-rabbit-IgG	CST	Cat#7074; RRID: AB_2099233
p53 (human)	Santa Cruz Biotechnology	Cat#sc-126 (DO-1); RRID: AB_628082
p53 (mouse)	Leica Biosystems	Cat# NCL-L-p53-CM5p; RRID: AB_2895247
phosphor p53 (Ser15)	Abcam	Cat#ab1431; RRID: AB_301090
ATM	Abcam	Cat#ab17995; RRID: AB_444169
pSer1981-ATM	Abcam	Cat#ab81292; RRID: AB_1640207
HRP-conjugated anti-mouse-IgG	Abcam	Cat#ab6820; RRID: AB_955438
p21	BD Biosciences	Cat#556431; RRID: AB_396415
Vinculin	Sigma-Aldrich	Cat#SAB4200729; RRID: AB_2877646
Actin	Sigma-Aldrich	Cat#A2228; RRID: AB_476697
Chemicals, peptides, and recombinant proteins		
SAM (S-adenosyl-L-methionine)	Promega	Cat#V7601
Sinefungin	Sigma	Cat#S8559
Compound 1 (SGI-1027)	MedChemExpress	Cat#HY-13962
Compound 2 (MC3343 or 475)	Manara et al. ²⁹	MC3343
Compound 3 (MC3671 or 463)	This study	N/A
Compound 4 (MC3682 or 465)	This study	N/A
Compound 5 (MC3672 or 464)	This study	N/A
Compound 6 (MC3716 or 466)	This study	N/A
Compound 7 (MC3653 or 461)	This study	N/A
Compound 8 (MC3807 or 467)	This study	N/A
Compound 9 (MC3563 or 455)	This study	N/A
Compound 10 (MC3652 or 460)	This study	N/A
Compound 11 (MC3669 or 462)	This study	N/A
Compound 12 (MC3821 or 469)	This study	N/A
Compound 13 (MC3817 or 468)	This study	N/A
Compound 14 (MC3646 or 457)	This study	N/A
Compound 15 (MC3639 or 456)	This study	N/A
Compound 16 (MC3647 or 458)	This study	N/A
Compound 17 (MC3649 or 459)	This study	N/A
GSK3685032	MedChemExpress (MCE)	Cat#HY-139664
GSK3735967	MCE	Cat#HY-150249
GSK3484862	MCE	Cat#HY-135146
MC4741	Zhou et al. ⁶⁰	compound 14
E. coli UDG	New England Biolabs	Cat#M0280S
Lipofectamine 3000	Invitrogen	Cat#L3000015
Critical commercial assays		
MTase-Glo™	Promega	Cat#V7601

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
CamA-DNA-compound 9 complex structure	This study	PDB 8VPG; https://doi.org/10.2210/pdb8VPG/pdb
CamA-DNA-compound 11 complex structure	This study	PDB 8VPI; https://doi.org/10.2210/pdb8VPI/pdb
CamA-DNA-compounds 11 and MC4741	This study	PDB 8VPH; https://doi.org/10.2210/pdb8VPH/pdb
Experimental models: Cell lines		
A549-Luc2	American Type Culture Collection (ATCC)	Cat#CCL-185-LUC2
A549	ATCC	Cat#CCL-185
NCI-H1299	ATCC	Cat#CRL-5803
NCI-H596	ATCC	Cat#HTB-178
NCI-H1975	ATCC	Cat#CRL-5908
U2OS	ATCC	Cat#HTB-96
PC3	ATCC	Cat#CRL-1435
MCF7	ATCC	Cat#HTB-22
mESC J1	ATCC	Cat#SCRC-1010
Oligonucleotides		
Table S2	N/A	N/A
Recombinant DNA		
Human DNMT1 expressed in E. coli BL21	Hashimoto et al. ³⁵	pXC915
Human DNMT3A2-DNMT3L expressed in BL21	Hashimoto et al. ³⁵	pXC465, pXC391
Human DNMT3B2-DNMT3L expressed in BL21	Hashimoto et al. ³⁵	pXC273, pXC391
<i>Clostridioides difficile</i> CamA expressed in BL21	Zhou et al. ⁴²	pXC2184
<i>Caulobacter crescentus</i> CcrM expressed in BL21	Horton et al. ⁴⁴	pXC2121
<i>Escherichia coli</i> Dam expressed in BL21	Horton et al. ⁴⁷	pXC1612
Human TDG expressed in BL21	Hashimoto et al. ⁶⁶	pXC1056
Mouse MBD4 expressed in BL21	Hashimoto et al. ⁶⁷	pXC1064
Mouse MYH expressed in BL21	Hong et al. ¹⁰⁰	pXC1321
Human NEIL1 expressed in BL21	Hazra and Mitra ⁶⁸	pXC2395
Human NEIL2 expressed in BL21	Hazra and Mitra ⁶⁸	pXC2396
Human Pol η (pET28pPolH)	Biertümpfel et al. ⁷²	N/A
Human Pol θ (pETsumo2Polq)	Zahn et al. ¹⁰³	N/A
HIV reverse transcriptase (pET21a(+)-HIV-RT-p66-p51)	Tian et al. ¹⁰⁴	N/A
poliovirus RNA-dependent RNA polymerase (pET-SUMO2-PV-3D-L446D)	Gong et al. ¹⁰⁶	N/A
Software and algorithms		
HKL2000	Otwinowski et al. ¹⁰⁸	https://hkl-xray.com/
PHENIX.Refine	Afonine et al. ¹¹⁰	https://phenix-online.org/
COOT	Emsley and Cowtan ¹¹²	https://strucbio.biologie.uni-konstanz.de/ccp4wiki/index.php/Coot
PyMol	DeLano Scientific LLC	https://www.schrodinger.com/products/pymol
Graph-pad prim (version 8.0)	GraphPad Software	https://www.graphpad.com/
MicroCal PEAQ-ITC	Malvern Panalytical	https://www.malvernpanalytical.com/en/products/product-range/microcal-range/microcal-itc-range/microcal-peaq-itc
SMILES string	Grade web server	http://grade.globalphasing.org

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines

Four lung cancer cell lines (A549-Luc2, NCI-H1299, NCI-H596 and NCI-H1975), osteosarcoma U2OS, breast cancer MCF7 and prostate cancer PC3 cell lines were purchased from the American Type Culture Collection (ATCC) and validated at The University of Texas MD Anderson Cancer Center (Houston, TX). A549-Luc2, U2OS and MCF7 contain the wild-type p53 gene; H1299 and PC3 are p53-null; H596 and H1975 contain a hotspot p53 mutation (p.G245C for H596, p.R273H for H1975). All cells were incubated at 37°C with 5% CO₂. A549-Luc2 cells were cultured in ATCC-formulated F-12K Medium (Catalog No. 30-2004) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin. MCF7, U2OS and PC3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 4.5g glucose/L but without sodium pyruvate (Mediatech) supplemented with 10% FBS and 1% penicillin/streptomycin. NCI-H1299, NCI-H596 and NCI-H1975 cells were cultured in ATCC-formulated RPMI-1640 Medium (ATCC 30-2001) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were seeded onto 6-well plates at a cell density of $\sim 5 \times 10^5$ /well. The next day, cells were treated with 0.1% DMSO (control) or compounds at the indicated concentrations and duration. Cells were collected for assays described below.

According to ATCC, A549 was isolated from a male with carcinoma, NCI-H1299 from a male with carcinoma, NCI-H596 from a male with adenocarcinoma, NCI-H1975 from a female with non-small cell lung cancer, and U2OS from a female osteosarcoma patient.

METHOD DETAILS

Chemical synthesis

The detailed synthetic procedures used to prepare compounds **3–17** followed the general procedure established previously,²⁸ and the synthesis schemes are provided in [Data S1](#). Associated characterization data (NMR and mass spectrometry) have been provided for all described compounds ([Data S2](#)), including raw NMR spectra and element analysis ([Data S3](#)). Other inhibitors used in this study include GSK3484862 (MedChemExpress, HY-135146), GSK3685032 (MedChemExpress, HY-139664), and GSK3735967 (MedChemExpress, HY-150249), SGI-1027 (**1**) (MedChemExpress, HY-13962), MC3343 (**2**) (ProbeChem, PC-35290). MC4741 [(2R,3S,4R,5R)-2-(Hydroxymethyl)-5-(6-((3-phenylpropyl)-amino)-9H-purin-9-yl)tetrahydrofuran-3,4-diol] is compound **14** as recently described.⁶⁰

Inhibition assays of DNA methylation

The methyltransferases used in the current study were characterized previously and prepared in our laboratories: human DNMT1 residues 350–1600 (pXC915),³⁵ DNMT3A2-3L (pXC465 and pXC391) and DNMT3B2-3L (pXC273 and pXC391),⁴⁰ *Clostridioides difficile* CamA (pXC2184),⁴² *Caulobacter crescentus* [now called *C. vibrioides*] CcrM (pXC2121),⁴⁴ and *Escherichia coli* Dam (pXC1612).^{46,47,98} The enzymes were expressed in *Escherichia coli* BL21(DE3) as either 6xHis-SUMO-tagged or non-cleavable 6xHis-tagged fusion proteins. They were purified using nickel-charged affinity chromatography, followed by SUMO tag cleavage with ULP1 protease, and further purified by ion exchange and size exclusion chromatography.

The inhibition of DNA methylation was quantified using the Promega bioluminescence assays (MTase-Glo).⁵² This assay utilizes a coupled reaction mechanism, where SAH produced during the methylation process is transformed into ATP in two distinct steps. The resultant ATP is then detected via a luciferase reaction. Luminescence signals were measured using a Synergy 4 multimode microplate reader (BioTek). Concentrations of SAH were determined based on a standard SAH curve, employing a linear regression analysis of the luminescence data. The specific reaction conditions applied for each MTase in the presence of a 10 μ M concentration of the inhibitor are detailed in [Table S2](#).

Inhibition assays of BER glycosylases

Generally, double-stranded DNA molecules (40 nM) featuring a single mismatch and FAM-labeled on the strand containing the mismatched nucleotide were synthesized by Integrated DNA Technologies (IDT). These were incubated with various inhibitor compounds at specified concentrations at room temperature for 10 min in a reaction buffer composed of 20 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Tris (2-carboxyethyl) phosphine (TCEP), and 0.1 mg/mL bovine serum albumin (BSA). The reaction was initiated by adding DNA glycosylases (MBD4, TDG, or MYH) at a concentration of 400 nM. The mixtures were then incubated at room temperature for 60 min, and the reactions were halted by the addition of 0.1 M NaOH and subsequent heating at 95°C for 10 min.^{99,100} Following this, samples were mixed with 2 $\times$ loading buffer (containing 98% formamide, 1 mM EDTA, and trace amounts of bromophenol blue and xylene cyanole), heated again at 95°C for 10 min, and then cooled on ice. A 5- μ L aliquot of each sample was loaded onto a 10 cm $\times$ 10 cm denaturing polyacrylamide gel electrophoresis (PAGE) gel, which contained 15% acrylamide, 7 M urea, and 24% formamide in 1 $\times$ TBE. Electrophoresis was performed using 1X TBE buffer at 200 V for 35 min. Gels were scanned for visualization using a BIO-RAD ChemiDoc MP Imaging system.

For assessing UDG activity on uracil-containing DNA, both double-stranded and single-stranded, 40 nM FAM-labeled DNA was incubated at room temperature for 10 min, with or without 2 μ M of compound **13**. The reaction was commenced by the addition of 20 nM UDG. These mixtures were then incubated at room temperature for the duration specified (from 15 s to 60 min) and were terminated by the addition of 0.1 M NaOH and heating at 95°C for 10 min. The remaining steps were the same.

NEIL1 and NEIL2 purification and inhibition

Plasmid express human NEIL1 (pXC2395) and human NEIL2 (pXC2396) protein with C-terminal 6xHis tag were a gift from Dr. Tapas Hazra's laboratory. Human NEIL1 and NEIL2 proteins were expressed and purified as previously described with modification.⁶⁸ The plasmids were transformed into *Escherichia coli* BL21-codon-plus (DE3)-RIL, and the bacteria were grown in LB broth at 37°C until the OD₆₀₀ reached 0.4, after which the temperature was lowered to 16°C. When the OD₆₀₀ reached 0.8 to 1.0, 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) was added, and the culture was grown for an additional 20 h at 16°C to induce protein expression. Cells were then harvested and lysed in a buffer containing 500 mM NaCl, 20 mM HEPES (pH 8.0), 5% glycerol, 0.5 mM TCEP, and 20 mM imidazole. The lysate was clarified by centrifugation, and the supernatant was applied to a 5 mL HisTrap HP column (GE Healthcare), with NEIL1 or NEIL2 proteins eluted using a linear gradient of 20–500 mM imidazole. Protein-containing fractions were pooled, diluted 3-fold with lysis buffer lacking NaCl and imidazole, and loaded onto a HiTrap SP HP (5 mL) column. The proteins were eluted with a NaCl gradient ranging from 0.1 to 1 M, and the fractions containing the target proteins were aliquoted, flash-frozen, and stored at –80°C.

Oligo containing G:AP was generated in a 1 mL reaction volume by incubating 40 nM G:U oligo with 5 U of *E. coli* uracil DNA glycosylase (catalog #M0280; New England Biolabs) for 30 min in 37°C. To test compound inhibition on NEIL1 and NEIL2 activity, 10 μL 40 nM G:AP DNA was incubated with indicated inhibitor in buffer 20 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Tris (2-carboxyethyl) phosphine (TCEP), and 0.1 mg/mL bovine serum albumin (BSA). An equal volume of either 800 nM NEIL2 or 100 nM NEIL1 protein in the same buffer was added, and the reaction was conducted at room temperature for 30 min. The reaction was terminated by adding 2× loading buffer (containing 98% formamide, 1 mM EDTA, and trace amounts of bromophenol blue and xylene cyanol) without heating. The remaining steps followed the protocol used for other base excision repair (BER) enzymes.

Inhibition assays of polymerases pol η and pol θ

Pol η and Pol θ biochemical assays, for nucleotide incorporation activity, were performed as previously described.^{101–103} The assays were executed using the DNA template and 5'-fluorescein-labeled DNA primer (Table S2). Reactions were conducted at 37°C for 5 min and were stopped by adding formamide quench buffer to the final concentrations of 40% formamide, 50 mM EDTA (pH 8.0), 0.1 mg/mL xylene cyanol, and 0.1 mg/mL bromophenol. After heating to 97°C for 5 min and immediately placing on ice, reaction products were resolved on 22.5% polyacrylamide urea gels. The gels were visualized by a Sapphire Biomolecular Imager and quantified using the built-in software. Visual representation of the acquired data was rendered in Graph Prism.

The initial inhibitor screens (Figure 5A) were performed with 1 nM Pol η or Pol θ and 10 or 50 μM inhibitor, in the reaction buffer of 0.2 μM DNA, 50 μM dNTP, 150 mM KCl, 45 mM Tris (pH 7.5), 5 mM MgCl₂, 10 mM DTT, 0.1 mg/mL bovine serum albumin, 5% glycerol, and 10% DMSO. For IC₅₀ measurement (Figures 5B and 5C), compounds **9** and **13** were serially diluted with DMSO and added to a reaction mixture to a final concentration of 0.01–20 mM, in the reaction buffer contained 0.3–15 nM Pol η or Pol θ. For steady-state kinetics (Figures 5D–5F), the reaction mixture contained 0.2–1 nM Pol η, 120–700 μM dATP, with or without inhibitor (15 μM compound **9** or 8 μM compound **13**).

Inhibition of HIV reverse transcriptase (RT)

HIV RT biochemical assays testing nucleotide incorporation activity were performed as previously described.¹⁰⁴ The inhibition mixture contained 10 or 50 μM inhibitor, 10 nM HIV RT, 0.5 μM DNA, 50 μM dNTP, 50 mM KCl, 45 mM Tris (pH 7.5), 5 mM MgCl₂, 1.3 mM DTT, 0.1 mg/mL bovine serum albumin, 4% glycerol, and 10% DMSO. The synthesis assays were executed using the RNA template and 5'-fluorescein-labeled DNA primer (Table S2). Reactions were conducted at room temperature for 5 min and were stopped by adding formamide quench buffer to the final concentrations of 40% formamide, 50 mM EDTA (pH 8.0), 0.1 mg/mL xylene cyanol, and 0.1 mg/mL bromophenol. The gel products were analyzed similar as for Pol η.

Inhibition of RNA-dependent RNA polymerase (RdRp)

Biochemical assays of RdRp from poliovirus testing nucleotide incorporation activity were performed as previously described.^{105,106} Each compound was assayed at two different concentrations, 10 μM and 50 μM, with 5 μM of RdRp and 1 μM of a 5'-fluorescein-labeled symmetrical self-annealing RNA template (Table S2). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 μM ZnCl₂, 250 μM ribonucleoside triphosphates (rNTPs), 1.3 mM DTT, 0.1 mg/mL bovine serum albumin, 4% glycerol, and 10% DMSO. Reactions were conducted at 30°C for 5 min and were stopped by adding formamide quench buffer to the final concentrations of 40% formamide, 50 mM EDTA (pH 8.0), 0.1 mg/mL xylene cyanol, and 0.1 mg/mL bromophenol. The gel products were analyzed similarly as for Pol η.

Isothermal titration calorimetry

The ITC experiment was performed at a constant temperature of 25°C using the MicroCal PEAQ-ITC automated system (Malvern Instrument Ltd). Data analysis was conducted using the ITC data analysis module supplied by the manufacturer.

Double-stranded T:G mismatch oligonucleotides and compound **13** were prepared in a uniform buffer solution consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 5% glycerol, and 1% DMSO. In the sample cell, DNA was maintained at a concentration of 20 μM, and compound **13**, at a concentration of 200 μM, was injected via syringe. The process entailed thirteen injections, each delivering 3 μL of the compound into the cell. This was done with continuous stirring at a rate of 750 rpm, and the reference power was set to 8 μcal/s. Each injection lasted for 4 s, and there was a 200-s interval between injections to allow the system to reach equilibrium.

For interactions with a 19-bp dsDNA or CamA protein, 200 μ M of compound **9**, **11**, or **13** was titrated against 20 μ M of DNA or CamA. To assess the impact of compound **9** on the CamA-DNA interaction, 200 μ M of compound **9** was pre-incubated separately with 200 μ M of DNA or 20 μ M of CamA in a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.5 mM TCEP, and 1% DMSO.

Southern blot DNA methylation assays

Analysis of DNA methylation at the minor satellite repeats in mouse embryonic stem cells was carried out as described previously.¹⁰⁷ Cells were cultured in gelatin-coated petri dishes in DMEM supplemented with 15% FBS, 0.1 mM nonessential amino acids, 0.1 mM β -mercaptoethanol, 1% penicillin/streptomycin, and 10^3 U/mL leukemia inhibitory factor (LIF). Cells were seeded onto 6-well plates at a cell density of $\sim 5 \times 10^5$ /well, and cells were treated with 0.1% DMSO (control) or at 0.1 μ M for GSK3685032 and GSK3484862, and 2 μ M for quinoline-based compounds for 48 h. Genomic DNA (1 μ g) was digested with the methylation-sensitive restriction enzyme HpaII (New England Biolabs) and analyzed by Southern hybridization with a specific biotin-labeled DNA probe (300 ng) (Table S2). Detection was performed using the North2South Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific).

For analysis of whole cell extracts by western blot, mESCs were lysed in cold RIPA buffer [50 mM Tris-HCl (pH 8.8), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 3 mM MgCl₂, and 1 $\times$ protease inhibitor cocktail (Thermo Fisher Scientific)].

X-Ray crystallography

For crystallization, the CamA-DNA-inhibitor complexes were prepared following previously established methods.^{42,60} In brief, crystal growth was achieved under similar conditions: 21–24% (w/v) polyethylene glycol 3350, 0.1 M Tris-HCl with a pH range of 7.0–7.5, and 0.28 M potassium citrate, all at room temperature ($\sim 19^\circ\text{C}$). The crystals typically appeared after 3–4 days, using the sitting drop vapor diffusion technique. An Art Robbins Gryphon Crystallization Robot was employed for setting up 0.4 μ L drops. Notably, crystals formed with compound **9** or **11** at a concentration of 200 μ M exhibited a distinct shape compared to those without inhibitors. For preservation, these crystals were rapidly frozen in liquid nitrogen following a brief immersion in the reservoir solution, which had been fortified with 20% (v/v) ethylene glycol.

Diffraction data for the crystals were collected at the SER-CAT beamline 22ID of the Advanced Photon Source at Argonne National Laboratory. The crystals were maintained in a cryostream at 100 K, and data collection typically involved rotating each crystal by 0.5° for each of the 800 frames. The crystallographic datasets were processed using HKL2000¹⁰⁸ (Table S1).

Structures of the CamA-DNA-inhibitor ternary complex were resolved using the difference Fourier method.¹⁰⁹ For crystals incorporating only compound **9** or **11**, our previously determined binary CamA-DNA structure (PDB ID: 7LNJ) was used as a search model. For the crystal containing both compounds **9** and MC4741, the CamA-DNA-MC4741 structure (PDB ID: 8CXZ) served as the starting model. Given that the unit cell parameters of all crystals were essentially isomorphous to those of previously obtained crystals, rigid body refinement was employed in the initial refinement cycle to position the new structures within the unit cell. Difference electron density maps (2Fo-Fc and Fo-Fc) were utilized to locate the bound compounds **9** or **11**. For each compound, an SMILES string was submitted to the Grade web server (<http://grade.globalphasing.org>) to generate geometrical restraints, which were provided in a CIF file. This file was used in subsequent refinement cycles and was facilitated the provision of the compound's structure in PDB format.

Initially, the extra densities in the crystal structures suggested the possibility of several conformations for the compounds. However, only the most distinct and likely predominant conformation was modeled and refined within the structure. All refinements were carried out using PHENIX REFINER,¹¹⁰ which included 5% of reflections randomly selected for validation, as indicated by the R-free value¹¹¹ (Table S1). The quality of the structures was continually assessed during the PHENIX refinement process and supplemented with manual inspection using COOT.¹¹² The final structure models underwent validation by the PDB validation server.¹¹³ Images of the structures were generated using PyMol (Schrödinger, LLC).

Compound treatments in cancer cell lines

For Western blotting assay, cells were lysed with sodium dodecyl sulfate (SDS) sample buffer. The lysates were separated by Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% precast polyacrylamide gel (BioRad, #4561096). The proteins were transferred to low fluorescence polyvinylidene difluoride (PVDF) membranes (BioRad, #1620261), which were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h and then probed with primary followed by secondary antibodies. The signals were detected with Clarity Western ECL substrate (Bio-Rad Laboratories, #1705061) and imaged using a ChemiDoc imaging system (Bio-Rad Laboratories).

For immunostaining assay, A549-luc cells were seeded on coverslips. Next day, cells were treated with 0.1% DMSO or 2–4 μ M compounds and fixed with 4% paraformaldehyde at indicated time-points. After washing, the fixed cells were blocked in blocking buffer (1 $\times$ PBS, 0.25% Triton X-100, 10% normal goat serum) for 1 h at room temperature (RT), and then incubated with primary antibody (γ H2AX) for overnight at 4°C . Next day, cells were rinsed and incubated with secondary antibody (anti-Rabbit IgG-conjugated with 555, 1:1000; Molecular Probes/Invitrogen) for 1 h at RT. After washing cells were mounted with antifade-Gold with 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes) and imaged with the fluorescence Leica DMI8 Microscope.

For cell viability assay, A549-Luc2, NCI-H1299, NCI-H596 and NCI-H1975 cells were seeded onto 96-well plates at a cell density of $\sim 1 \times 10^4$ /well. The following day, 0.1% DMSO or compounds at the indicated concentrations were added and incubated for 3 days. The viability was measured by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7572).

For siRNA knockdown *TP53* in A549 cell line (ATCC CCL-185), cells were seeded in 96-well plate at 5000 per well and transfected with control or human *TP53* siRNA using lipofectamine 3000 (Invitrogen, L3000015). The siRNAs were purchased from Dharmacon (now Horizon): siGENOME SMARTpool for Non-Target siRNA pool #1 (Cat# D001206-13-05) and human *TP53* (cat# M-003329-03-0005). After overnight incubation, the culture media with siRNA were removed and replaced with fresh media supplemented with 0.1% DMSO or different dose of compound **11** (4, 2, 1, 0.5 μ M, 2-fold serial dilution). Cells were collected for Western blotting 24 h post-treatment or incubated for 3 days for viability assay (CellTiter-Glo assay).

QUANTIFICATION AND STATISTICAL ANALYSIS

X-ray crystallographic data were measured quantitatively and processed with HKL2000. Structure refinements were performed by PHENIX Refine, with 5% randomly chosen reflections for validation by R-free values. The data collection and refinement statistics are shown in [Table S1](#). Structure quality was analyzed during rounds of PHENIX refinements and validated by the PDB server. Statistics details on inhibition experiments can be found in legends of [Figures 1, 2, 3A–3D, 5, 6A, 7B, 7D, S2, and S6](#). The IC_{50} and Hill slope were calculated using GraphPad Prism 10 through nonlinear regression based on the equation: $Y = \text{Min} + (\text{Max} - \text{Min}) / (1 + (IC_{50}/[I])^n)$, where $[I]$ represents the inhibitor concentration, n is the Hill coefficient (or Hill slope), Y represents the enzyme activity (%), and Max and Min represent the maximum and minimum enzyme activities (%), respectively. The standard deviation (SD) on graphs was calculated using the difference between each data point X_i and the mean X_{avg} ($X_i - X_{\text{avg}}$), squared ($(X_i - X_{\text{avg}})^2$), summed $\sum (X_i - X_{\text{avg}})^2$, and then divided by $N-1$, where N is the number of data points. The square root of this values gives the SD.