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Letters

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Identification of Novel Inhibitors of DNA Methylation by Screening of a Chemical Library

Alexandre Ceccaldi,[§,†,‡,○] Arumugam Rajavelu,[∥,○] Sergey Ragozin,[∥] Catherine Sénamaud-Beaufort,[†,‡] Pavel Bashtrykov,[∥] Noé Testa,[†,‡] Hana Dali-Ali,[†,‡] Christine Maulay-Bailly,[⊥] Séverine Amand,[⊥] Dominique Guianvarc'h,[#] Albert Jeltsch,[*,∥] and Paola B. Arimondo*[,†,‡,▽]

[†]CNRS-MNHN UMR 7196, 43 rue Cuvier, 75005 Paris, France

[‡]INSERM UR565, 43 rue Cuvier, 75005 Paris, France

[§]Université Pierre et Marie Curie, Paris 6, place Jussieu, 75005 Paris, France

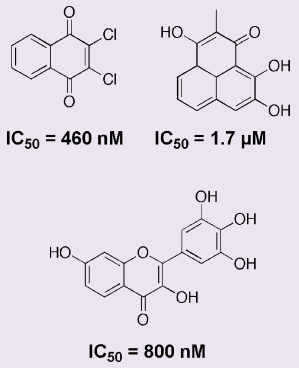
[∥]Institute of Biochemistry, Faculty of Chemistry, University Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany

[⊥]CNRS-MNHN UMR 7245 RDDM, CP 54, 57 rue Cuvier, 75005 Paris, France

[#]UPMC Paris 06−ENS−CNRS, UMR 7203, Laboratoire des Biomolécules and FR2769 Chimie Moléculaire, Université Pierre et Marie Curie, 4 place Jussieu, 75005 Paris, France

Supporting Information

ABSTRACT: In order to discover new inhibitors of the DNA methyltransferase 3A/3L complex, we used a medium-throughput nonradioactive screen on a random collection of 1120 small organic compounds. After a primary hit detection against DNA methylation activity of the murine Dnmt3A/3L catalytic complex, we further evaluated the EC_{50} of the 12 most potent hits as well as their cytotoxicity on DU145 prostate cancer cultured cells. Interestingly, most of the inhibitors showed low micromolar activities and little cytotoxicity. Dichlone, a small halogenated naphthoquinone, classically used as pesticide and fungicide, showed the lowest EC_{50} at 460 nM. We briefly assessed the selectivity of a subset of our new inhibitors against hDNMT1 and bacterial Dnmts, including M. SssI and EcoDam, and the protein lysine methyltransferase PKMT G9a and the mode of inhibition. Globally, the tested molecules showed a clear preference for the DNA methyltransferases, but poor selectivity among them. Two molecules including Dichlone efficiently reactivated YFP gene expression in a stable HEK293 cell line by promoter demethylation. Their efficacy was comparable to the DNMT inhibitor of reference 5-azacytidine.



DNA methylation is a major component of epigenetics along with histone methylation and acetylation, nucleosomes positioning, and small RNAs.[1] These regulations of gene expression not encoded in the genome sequence play a central role in normal and pathological cells.[2] DNA methylation participates in restriction/modification systems of bacteria by protecting their genome from autodigestion by the endonucleases that digest foreign nucleic acids. In eukaryotes, methylation of cytosine residues in the context of CpG dinucleotides grouped in CpG islands is a common way to silence genes and transposons and is involved in many biological processes ranging from embryogenesis, genomic imprinting, and cell differentiation to complex cognitive phenomena. Abnormal DNA methylation of the genome can also lead to pathologies like schizophrenia,[3] Rett Syndrome[4] and cancer.[5] In the latter, a disrupted epigenetic landscape is observed with global hypomethylation of the genome and concomitantly hypermethylation of CpG islands in the promoter regions of some specific genes like tumor suppressor genes, inducing their silencing. On the contrary to genomic modifications such as gene mutations that are irreversible, epigenetic regulations are highly dynamic and pharmacologically reversible, which make them a very attractive therapeutic target.

Epigenetic drugs form a novel class of therapeutics to fight cancer.[6,7] Histone-deacetylases inhibitors (HDACi) like SAHA have benefited from massive research efforts since they can stop the progression of lymphoma, leukemia, and other types of tumors with lower cytotoxicity than standard chemotherapies. Still, HDACi efficacy seems to depend drastically on the type of tumor and to be very prone to drug resistance.[8] We focus on epigenetic drugs that could inhibit DNA methylation by the DNA methyltransferases (DNMT). DNMTs catalyze the transfer of a methyl group form the cofactor *S*-adenosyl-L-methionine (AdoMet) to the position 5 of cytosine, preferentially in a CpG context.[9] DNMT inhibitors (DNMTi) have been known for decades. They come from two distinguished chemical classes. The cytidine analogues share a common mode of action: the modified base forms a covalent complex that traps the DNMT on DNA by mimicking

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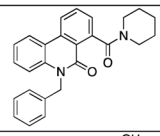
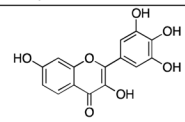
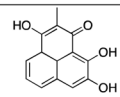
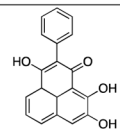
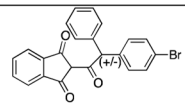
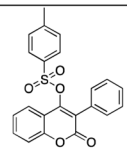
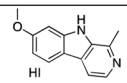
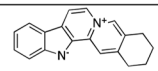
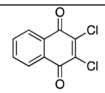
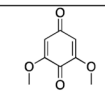
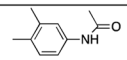
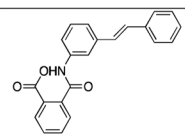
cytosine. FDA has authorized the commercialization of the 5-azacytidine (Vidaza) and the 5-aza-2′-deoxycytidine (Dacogen) for the treatment of the myelodysplastic syndrome.[10] To be active, these suicide substrates need to be integrated into the DNA during replication. However, they integrate everywhere in the genome and the ribose analogue also in the RNA, resulting in toxicity. The 5-aza derivatives are also chemically unstable when exposed to physiological conditions.[11] Other cytidine analogues like zebularine are undergoing preclinical development on several solid tumors.[12] The second class of DNMTi, the noncytidine analogues, shows a much broader chemical diversity. In most cases, these drugs have several cellular targets among which DNMTs have been enlightened incidentally. Only RG108[13] and SGI1027[14] have been described as specific against DNMTs. Globally, this second class of drugs lacks very potent and specific inhibitors. This calls for the discovery of more efficient and less toxic DNMTi. Therefore, we designed a cost-efficient, straightforward, and nonradioactive HTS against the DNMTs, based on fluorescence detection in 96-well microplates.[15] Thanks to it, we have already discovered a new family of DNMTi through the screening of a collection of flavone and flavanone derivatives on the murine Dnmt3A/3L catalytic complex.[15] Here, we describe a larger random screening of 1120 compounds from the chemical library of the Muséum National d'Histoire Naturelle in Paris, France. A total of 61 hits were discovered. We further studied the most active molecules by measuring their apparent EC_{50}, through the same *in vitro* fluorescent assay, and by assessing their mode of action. Selectivity against different methyltransferases was evaluated for the best hits by a complementary kinetics assay. Finally, the two most active compounds were able to reactivate gene expression of an epigenetic reporter system in cells by demethylation of the promoter.

In order to find new inhibitors of the DNMTs, we used a medium throughput screening assay against the murine complex formed by the C-terminal domains of mDnmt3A and its cofactor mDnmt3L that we already described in ref 15. A double modified DNA substrate, bearing a biotin and a FAM at the 5′ end of each strand, respectively, is immobilized on a 96-well microplate through biotin/avidin interactions. This DNA duplex comprises a unique CpG site in a sequence that is recognized by a methylation-sensible restriction enzyme. The principle of the assay is to protect the DNA substrate against cleavage by the restriction enzyme due to methylation by the mDnmt3A/3L. If a molecule is able to inhibit the DNMT, the unmethylated substrate will be cut in the next step, the FAM will be lost during final washing, and the fluorescence signal will decrease. On the contrary, an inactive molecule will not allow cleavage, and thereby, no loss of the fluorophore will be monitored.

The chemical library of the Muséum National d'Histoire Naturelle of Paris containing 1120 small organic compounds (average MW = 280 g/mol) was screened at the concentration of 40 μg/mL (on average 140 μM). This collection has never been tested before for DNMT inhibition. A typical result after the screen of 80 compounds in a single 96-wells plate is shown in Supplementary Figure 1. This primary screen gave a total of 61 hit molecules, 5.1% of total tested compounds, which is consistent with a screening at high concentration. Interestingly, some of the hit molecules like compound **2** (known as Robinetin) are flavone derivatives as the 3-nitroflavones and 3-chloro-nitroflavanones derivatives that we described as a new family of DNMT inhibitors in ref 15. A secondary screen of the hit molecules on Dnmt3A/3L at 1 μM and 0.5 μM (data not shown) selected the 12 best candidates that were further evaluated. Table 1 gives the chemical structure of the 12 hits of the secondary screen and the apparent EC_{50} (concentration at

Table 1. Biochemical and Biological Activity of the 12 Most Active Compounds; Inhibition of Murine Catalytic Complex Dnmt3A/3L Is Reported As EC50 (μM); Inhibition of M. SssI Is Reported at 10 μM of Drug; Cytotoxicity on DU145 and HCT116 Cell Lines Is Reported As TC50 (μM)

Cpds	Chemical Structure	mDnmt3A/3L EC_{50} (μM)[a]	M.Sss I at 10 μM	DU145[b] TC50 (μM)
1		23.7 (19.0 to 29.6)	+/-	N.D.[c]
2 (Robinetin)		0.80 (0.69 to 0.91)	+	39
3		1.7 (1.1 to 2.4)	+/-	40
4		1.5 (0.9 to 2.6)	+	27
5		21.0 (15.6 to 28.2)	+	N.D.
6		20.3 (15.4 to 26.5)	-	155
7 (Harmine)		2.3 (1.5 to 3.5)	+	26
8 (Sempervirin)		12.4 (9.3 to 16.5)	+/-	N.D.
9 (Dichlone)		0.46 (0.37 to 0.57)	+	8
10		2.3 (1,4 to 3,6)	-	9
11		20.3 (13.4 to 30.8)	-	N.D.
12		30.0 (21.4 to 42.1)	+	N.D.

[a]Mean value of 2 to 6 experiments of the concentration at which 50% of inhibition of the enzyme activity is observed (with 95% confidence interval). [b]Mean value of three experiments of the concentration at which 50% of inhibition of cellular proliferation is observed. [c]N.D. = not determined.

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which they inhibit at 50% Dnmt3A/3L methylation activity). Figure 1A exemplifies an experiment of EC_{50} determination for compound **2** through 6 independent experiments, while Figure 1B summarize it for all 12 hits. Data are reported in Table 1. Six molecules showed lower micromolar or high nanomolar average EC_{50}, down to 0.46 μM for compound **9**. For comparison, RG108, a reference inhibitor that has been discovered on a virtual screening against DNMT1[13] previously showed an EC_{50} of 350 μM against mDnmt3A/3L in the same assay.[15]

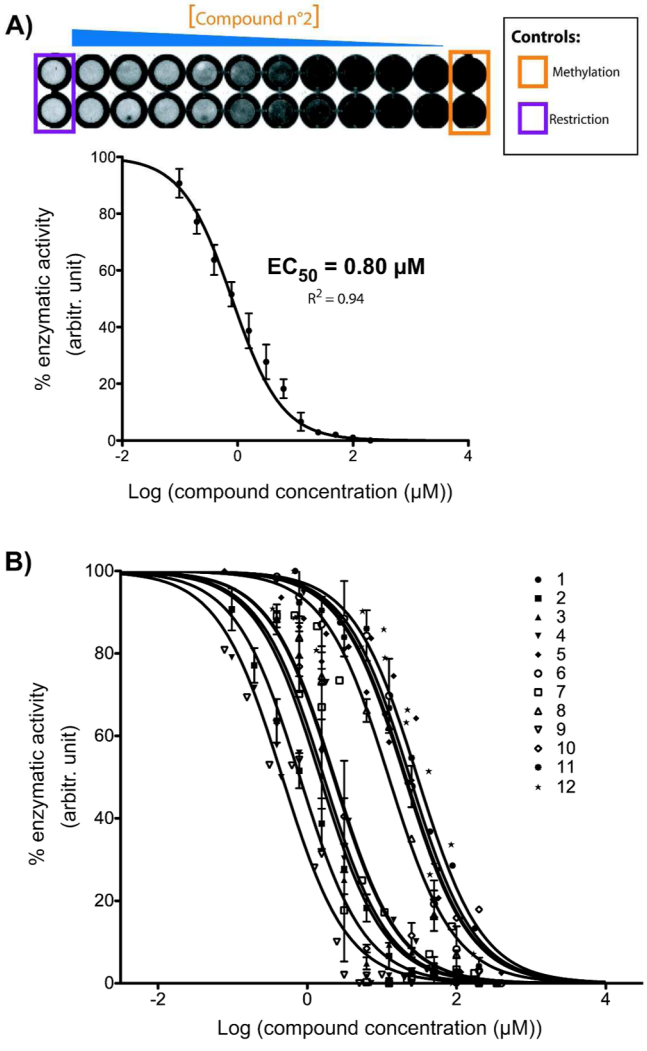

Figure 1. Determination of the concentration of compound (EC_{50}) at which 50% of the methylation activity is inhibited. (A) Example of compound 2. Each line of the microplate corresponds to a dose–response study of the hit molecule. The first and last columns are dedicated to assay control wells. The range of concentration was 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.2 μM, 1.6 nM, 800 nM, 400 nM, 200 nM, and 100 nM. After quantification, results were plotted as normalized methylation activity (which is inversionally proportional to the fluorescence signal intensity) against the log of inhibitor concentration. EC_{50} values were evaluated after fitting the dose–response plots by nonlinear regression through the GraphPad Prism software, with constant slope equations, on 6 independent experiments. Errors bars are represented at each concentration. (B) Plots for the 12 hits. Two to six experiments for each hit molecule were plotted as normalized methylation activity against the log of inhibitor concentration and fitted by nonlinear regression through the GraphPad Prism software, with constant slope equations. Different concentration ranges were used for each compound; errors bars are represented.

The large majority of the hit molecules are small polycyclic organic compounds. Some of them probably act as DNA intercalators like compound **8**, a natural compound called Sempervirin,[16] which is already known to be active against DNA topoisomerase I.[17] Interestingly, compounds **3** and **4** share the same structure in which a methyl group (**3**) is replaced by a phenyl group (**4**). This substitution does not have a significant impact on inhibition activity (EC_{50} 1.7 μM vs 1.5 μM, respectively). Compound **7** is a natural alkaloid named Harmine that is present in medicinal plants such as *Peganum harmala*. Its anticancer activity, through apoptosis induction and neovascularization inhibition, has been described.[18,19]

The most active molecule, compound **9**, Dichlone, has an apparent EC_{50} of 0.46 μM. It is an halogenated naphthoquinone, a five-ringed alkaloid, classically used as a fungicide and pesticide. Indeed, it shares a quinone moiety with other compounds among the 60 hit molecules. It cannot be excluded that it might inhibit the mDnmt3A/3L complex and its catalytic cysteine through redox properties. More generally, the mode of action of phytosanitary chemicals on their targets as well as their undesirable impacts on the environment or human health is a very active field of research in toxicology.[20−22] In particular, the epigenetic (i.e., nonmutagenic) mechanisms of action of carcinogenic organochlorine pesticides, like Dichlone, have been suggested since the early 1980s.[23,24] In addition, flavanol **2** was once used as insecticide and fungicide. Our results propose that the study of short and long terms disruptions of the epigenetic landscape, in particular, global DNA hypomethylation, caused by **9** on cultured cells could give precious insights to assess the mechanisms of toxicity of such pesticides.

To explore the selectivity of the hit compounds between different C5 DNA methyltransferases, the compounds were tested at 10 μM against M.SssI, a bacterial methyltransferase that methylates cytosine residues in a CpG context (Supplementary Figure 2). Globally, the hit molecules showed poor selectivity between Dnmt3A/3L and M. SssI. Compounds **2**, **4**, **5**, **7**, **9**, and **12** were fully active on M.SssI, while compounds **1**, **3** and **8** showed lower activity. Compounds **6**, **10** and **11** were inactive on the M. SssI at 10 μM. Noteworthy, the most active compounds on mDnmt3A/3L were also active on M. SssI, with the exception of compound **10** (EC_{50} = 2.3 μM) that did not inhibit M SssI. Compound **10**, a benzoquinone, has been described throughout the literature for its inhibition activity on various enzymes and cellular tests,[25−28] suggesting that it is not a specific inhibitor, probably because of its redox properties. It will be interesting to further explore the two quinone derivatives (**9** and **10**) in order to increase their specificity and cellular activity.

Compounds **3** and **9** presented particularly interesting features both for their chemical skeleton and their strong biological activity. A complementary kinetics assay,[15] based on the incorporation of radioactivity from tritiated SAM by human methyltransferase hDNMT1 revealed an inhibition with EC_{50} values of 13.3 and 1.55 μM, respectively. These two compounds were further analyzed for their selectivity on the bacterial adenine methyltransferase EcoDam and the human protein lysine methyltransferase PKMT G9a. They both have a clear preference for the DNMTs: compound **3** showed an EC_{50} of 31 ± 5 μM on EcoDam and more than 1 mM on G9a, while **9** showed an EC_{50} of 11 ± 2 μM on EcoDam and more than

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600 μM on G9a. This is a very encouraging result since the C5-DNA methyltransferases, EcoDam, and PKMT G9a all use the same donor of methyl groups, the SAM.

We then studied the mode of action of compounds **3** and **9**. Kinetics done at different concentrations of inhibitor and AdoMet (Supplementary Figure 3) suggested an uncompetitive mode of inhibition. Furthermore, we did not observe any binding to the DNA in ethidium bromide displacement experiments (data not shown). Altogether these data conclude that inhibitors **3** and **9** do not bind to the AdoMet pocket and bind after AdoMet binding.

To investigate the demethylation effect of both compounds in cells, we established a cell-based assay to monitor effects of DNA demethylating compounds like inhibitors of DNMT1 in living cells. We prepared a reporter cell line in which the yellow fluorescent protein (YFP) is stable inserted, and its expression is under control of a methylated Cytomegalovirus (CMV) immediate-early promoter and enhancer. The demethylation of the promoter restores YFP expression allowing to monitor the compounds that induce this demethylation. The cell line was treated for three days with the inhibitors at concentration at which the compounds were not toxic (data not shown), and the YFP fluorescence was determined. As control, cells were treated with 5-aza-deoxycytidine, which is a well characterized inhibitor of DNA methylation.[29] With both compounds a clear onset of expression of YFP was observed (Figure 2A) This was further validated by the reduction of the DNA methylation at the CMV promoter as observed by bisulfite analysis (Figure 2B) with compounds **3** and **9** treated cells compared to the DMSO control (reduction of 17% and 29%, respectively).

In particular, compound **9** showed a strong induction of YFP expression and promoter demethylation that is in agreement with its lower EC_{50} in the inhibition of DNA methyltransferases.

The next step will be the evaluation of these new DNMTi to induce stable demethylation of specific genes in cancers, in particular, the hypermethylated promoters of tumor-suppressor genes to revert the tumoral status. Indeed, the necessity to find new drugs to reset the corrupted patterns of epigenetic marks in cancer cells is a major priority in cancer drug innovation.[6] The cytotoxicity of the inhibitors showing an EC_{50} lower than 5 μM on mDnmt3A/3L was evaluated through an MTS test (Table 1) on human prostate carcinoma DU145 cells. Interestingly, all molecules showed a TC_{50} (concentration of the drug that induces 50% of cell death) that is at least 1 order of magnitude higher than their apparent *in vitro* EC_{50} against mDnmt3A/3L. Interestingly, the most potent compounds show little cytotoxicity, which should help to enlighten their potential epigenetic effect on cancer cells separately from nonspecific cytotoxic effects.

In conclusion, 1120 small organic molecules were screened for their ability to inhibit the catalytic murine complex Dnmt3A/3L. The EC_{50} of 12 hits were evaluated, 6 of them showed a lower micromolar activity, down to 0.46 μM for Dichlone, a molecule that is classically used as fungicide. Two compounds were selective of the DNA methyltransferase and were able to efficiently reactivate the reporter gene by promoter demethylation. This may open new perspectives both in developing new epigenetic therapies and reinforcing the understanding of fungicide impacts on the environment.

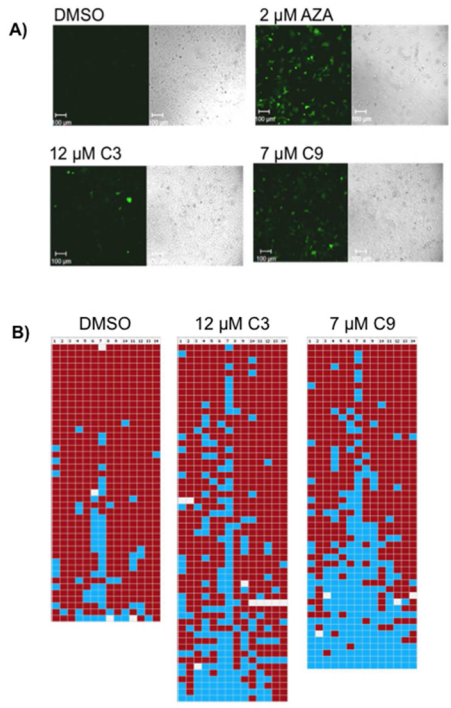

Figure 2. (A) Confocal fluorescence image of the YFP fluorescence in the epigenetic reporter cell line after incubation for three days with compound **3** (12 μM) and compound **9** (7 μM). As control, DMSO-treated cells and cells demethylated by 5-aza-deoxycytosine (AZA) treatment (2 μM) are shown. (B) Methylation analysis of CMV promoter in HEK293 reporter cells for compounds **3** (12 μM, 73.8% total methylation) and **9** (7 μM, 63.1% total methylation). More than 20% reduction of methylation was observed in inhibitor-treated cells compared to the DMSO control (88.8% total methylation). Each row corresponds to one clone of bisulfite-converted PCR products. Each column corresponds to one CpG site in the studied region. The color code indicates different methylation states of the CpG sites (blue, unmethylated; red, methylated; white, methylation state unknown). The image was generated using BISMA.[35]

METHODS

Chemicals and Drugs. All the drugs were dissolved in 100% DMSO and stored at −20 °C. SAM was purchased from Sigma Aldrich; preweighted aliquots were stored at −80 °C. The chemical library containing 1120 synthetic compounds was obtained from the Muséum National d'Histoire Naturelle, Paris, France. The structure and purity of compounds **1** to **12** were verified by ^{1}H nuclear magnetic resonance and mass spectrometry.

Screening Test. The test and data analysis is described in ref 15. The concentration in DMSO was constant in every screening and EC_{50} determination experiments, with final 3.5%.

Complementary Experiments. Kinetics experiments were carried out on the purified bacterial EcoDam N-6 DNA methyltransferase, as described in ref 30, and the catalytic domain of the human G9a histone H3K9 methyltransferase, as described in refs 31 and 32. AdoMet competition experiments on Dnmt3A/3L were carried out as described in refs 31 and 32.

DNMT1 Inhibition Assays. DNMT1 was expressed and purified basically as described in ref 33. DNMT1 activity was measured by following the transfer of radioactively labeled methyl from SAM to a biotinylated oligonucleotide containing one hemimethylated CpG site as described in ref 34.

MTS Assays. DU145, a human prostate carcinoma cell line was obtained from ATCC (Rockville, USA). The DU145 cell line was maintained in RPMI (Invitrogen, France) supplemented with 10% fetal bovine serum (Perbio, Belgium) and 2 mM glutamine. Cells were

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incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. DU145 cells, 3×10^4, were seeded in 96 well plates in 80 μL medium/well and incubated after seeding with 20 μL of increasing concentrations of inhibitor. Absorbance at 490 nm was determined after 72 h of incubation using CellTiter96. Aqueous Non-Radioactive Cell Proliferation Assay was conducted following the manufacturer's instructions (Promega, Germany).

***In Vivo* DNA Methylation Assay.** The CMV-YFP cassette was isolated from the Clontech plasmid pEYFPN1, methylated *in vitro* with M. SssI, ligated with unmethylated Neomycine resistance cassette from the same plasmid, and directly transfected into HEK293 cells. Complete methylation of the YFP cassette was confirmed by bisulfite sequencing. After antibiotic selection, several clones were isolated and analyzed for their background reporter gene activity (YFP fluorescence) as well as for the response to demethylation induced by 5-aza-deoxycytidine, a well-known DNA demethylation agent. We selected one clone that showed the greatest dynamic range in fluorescence response upon demethylating treatment. DNA methylation was studied by bisulfite sequences essentially as described in ref 29. The level of CpGs methylation in the promoter of the YFP reporter gene was 60−80%, which was sufficient to suppress gene expression; after treatment with 5-aza-deoxycytidine, DNA methylation was drastically reduced.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Screening of 80 compounds at 100 μM on the Dnmt3a/3L C-terminal domains complex in the presence of SAM; 12 hits tested at 10 μM for inhibition of M. SssI; AdoMet competition experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(P.B.A.) Tel: +33534506492. Fax: +33534503492. E-mail: paola.arimondo@etac.cnrs.fr. (A.J.) Tel: +4971168564390. Fax: +4971168564392. E-mail: albert.jeltsch@ibc.uni-stuttgart.de.

Present Address

▽USR3388 CNRS-Pierre Fabre ETaC, CRDPF, 3 avenue H. Curien, 31035 Toulouse cedex 01, France.

Author Contributions

○These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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