A high-throughput small molecule screen identifies synergism between DNA methylation and Aurora kinase pathways for X reactivation

Derek Lessing,a,b,c,1 Thomas O. Diala,b,c,1 Chunyao Weia,b,c,1 Bernhard Payerd, Lieselot L. G. Carretta,b,c,1,2 Barry Kesnera,b,c,1 Attila Szentob,c,1 Ajit Jadhavd, David J. Maloneye, Anton Simeonovf, Jimmy Theriaultg, Thomas Hasakag, Antonio Bedalovh, Marisa S. Bartolomei, and Jeannie T. Leea,b,c,2

Howard Hughes Medical Institute, Massachusetts General Hospital, Boston, MA 02114; aDepartment of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114; bDepartment of Genetics, Harvard Medical School, Boston, MA 02115; cCentre for Genomic Regulation, Barcelona, Spain; dHoward Hughes Medical Institute, University College, London, WC1E 6BT, UK; eHoward Hughes Medical Institute, Massachusetts General Hospital, Boston, MA 02114; fClinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; and gDepartment of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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Female mammals are subject to a form of epigenetic regulation termed X-chromosome inactivation (XCI), in which one of the two X chromosomes is transcriptionally silenced. Once established, silencing of the inactive X (Xi) is robust and difficult to reverse pharmacologically. However, the Xi is a reservoir of ~1,000 functional genes that could be potentially tapped to treat X-linked disease. To identify compounds that could reactivate the Xi, here we screened ~367,000 small molecules in an automated high-content screen using an X-linked GFP reporter in mouse fibroblasts. Given the robust nature of silencing, we sensitized the screen by “priming” cells with the DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine (5azadC). Compounds that elicited GFP activity include VX680, MLN8237, and 5azadC, which are known to target the Aurora kinase and DNA methylation pathways. We demonstrate that the combinations of VX680 and 5azadC, as well as MLN8237 and 5azadC, synergistically up-regulate genes on the Xi. Thus, our work identifies a synergism between the DNA methylation and Aurora kinase pathways as being one of interest for possible pharmacological reactivation of the Xi.

Significance

In mammalian female cells, nearly all genes are silenced on one of two X chromosomes. Heterozygous females with “dominant” X-linked diseases, such as Rett syndrome, may benefit from pharmacological reactivation of the silent, healthy allele in affected organs. Toward establishing proof of concept, here we carry out a primed screen of a large library of small molecules for compounds that can reactivate expression from the inactive X (Xi). We identify a combination of compounds that inhibits the DNA methylation and Aurora kinase pathways and demonstrate that the two pathways act synergistically to repress genes on the Xi, including genes involved in X-linked disease.


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Data deposition: The complete primary screen data have been deposited in the PubChem database, pubchem.ncbi.nlm.nih.gov (accession no. AID 743238). The sequence read data have been deposited into the Gene Expression Omnibus (GEO) database (accession no. GSE85103). 1D.L. and T.O.D. contributed equally to this work.

2To whom correspondence should be addressed. Email: lee/molbio.mgh.harvard.edu.

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respectively (19, 20). In recent years, several approaches have been taken to define a pharmacological means of reactivating the Xi in somatic cells. Two siRNA screens led to identification of a number of factors, but the screens obtained divergent results with no overlap (21, 22). Possible explanations for this are that the screens might not have been fully comprehensive or that XCI cannot be robustly overcome by disrupting a single factor. Another screen involving siRNAs and a limited collection of small molecules identified ribonucleoside-diphosphate reductase subunit M2 (RRM2) as being synergistic with 5-aza-2′-deoxycytidine for reactivation of the Xi (23). In yet another approach, Xist RNA was used as bait to pull down interacting proteins, a number of which could be targeted using small molecules to reactivate the Xi (15). It was demonstrated that derepression of the Xi can be achieved robustly only when two or more interactors were targeted. Although >100 interacting proteins were identified, most of the interactors are not druggable with small molecules. Thus, additional approaches are needed to maximize the potential for pharmaceutical intervention. With this in mind, here we undertake an unbiased approach and perform a high-throughput small molecule screen to identify compounds that will reactivate a reporter transgene on the Xi.

**Results**

**A High-Throughput Screen for X Reactivation.** We developed a female mouse fibroblast cell line, Xi-TgGFP, in which the Xi carries a silent GFP transgene (24) as a reporter for reactivation (Fig. 1A). Using the Xi-TgGFP cell line, we screened >367,000 molecules, combining compounds from the NIH’s Molecular Libraries Program, the Broad Institute’s Diversity-Oriented Synthesis Library, and a panel of kinase and epigenetic inhibitors from the National Center for Advancing Translational Sciences (NCATS) (Fig. 1B). Because previous work demonstrated that the Xi is repressed by multiple synergistic mechanisms (15, 18), we reasoned that the odds of success would be increased by performing a primed screen in which cells were sensitized to derepression with the DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5azaC), a compound shown previously to elicit a very low level of Xi reactivation (18). We chose a priming concentration of 0.5 μM 5azaC, empirically determined to yield ~1% GFP+ cells, a value that was just above background levels (Fig. S1).

GFP reactivation in the Xi-TgGFP cells was scored in the high-throughput, primed screen via automated microscopy, after a 3-day treatment with each compound tested in duplicate at 7.5 μM with 5azaC priming. We found ~1,900 compounds that reactivated GFP in at least 10% of cells (on a normalized scale) (Methods). We resourced ~1,400 of these and repeated the GFP reactivation assay at 5.0 μM and 0.5 μM (both concentrations with 0.5 μM 5azaC) alongside a counterscreen for autofluorescence (i.e., false-positive GFP signal). Almost all were either autofluorescent or too toxic. The Aurora kinase inhibitors VX680 (25) and MLN8237 (26) were chosen for further studies, as detailed below.

**Synergism Between VX680, MLN8237, and 5azaC.** The Aurora kinase family consists of Aurora kinase A (AURKA), B (AURKB), and C (AURKC). Whereas AURKA and AURKB are ubiquitously expressed, AURKC is expressed only in the testis (27) and was therefore not likely to be relevant as a target here. AURKA was also recently identified in an shRNA screen for X reactivators using a similar X-reactivation assay (22), and AURKB was identified as a protein that directly interacts with Xist RNA (15).

Application of VX680 in the screen led to a 23.5% GFP-reactivation average of two replicates (Fig. S1C). We reproduced its reactivation via an independent assay, in which we used quantitative RT-PCR (qRT-PCR) to measure GFP expression (Fig. 2A). VX680 alone boosted GFP expression by 6.6-fold and, when combined with 5azaC, by 4.3-fold over treatment with 5azaC alone. We compared this expression to that of a male fibroblast line carrying the GFP transgene on the single, active X chromosome (Xa-TgGFP), which represents the theoretical maximum for GFP activity on the X chromosome. When normalized to male Xa-TgGFP expression, the response of VX680 and 5azaC reached 13% of the theoretical maximum (15, 18).
AURKA and AURKB Knockdown Partially Recapitulates VX680- and MLN8237-Induced Reactivation of GFP. Next, we sought to determine whether AURKA and AURKB are the relevant targets of VX680 and MLN8237 for X reactivation. VX680 and MLN8237 (28, 29) can affect other protein kinases as well (30–32) (Table S2). We directly tested the roles of Aurora and Aurok in reactivation by knocking down their expression with siRNAs alone or together. Each was efficiently knocked down to ~10% of normal levels (Fig. 2C). Aurora kinase knockdown alone led to no increase in GFP expression. In the presence of 0.5 μM 5azadC, knockdown of either AURKA or AURKB individually also did not result in increased GFP transcription. However, with simultaneous knockdown, GFP expression increased 4.8-fold relative to 5azadC treatment by itself. Because this level was just 4% of Xa-TgGFP levels, compared with 13% for VX680 or 83% for MLN8237, VX680- and MLN8237-mediated reactivation can be attributed only in part to AURKA and AURKB. Whereas residual Aurora kinase activity after knockdown may be greater than after inhibition by VX680 or MLN8237, it is also possible that these compounds target additional kinases to achieve their full effect on X reactivation.

Additional Effects of VX680. During mitosis, AURKA is necessary for proper centrosome maturation, spindle assembly, and centrosome separation. AURKB is a member of the chromosomal passenger complex, which phosphorylates histone H3 and other substrates for proper cytokinesis (27). Lack of AURKA function is known to have a severe effect on cell cycle progression and to cause lethality before implantation, whereas lack of AURKB is known to be lethal after implantation and to cause errors in chromosome segregation (33). Therefore, we further examined effects of Aurora kinase inhibition on general cellular processes. As expected, cell proliferation was inhibited by VX680 in a dose-dependent manner; this toxicity was similar with or without 5azadC, consistent with the known effects of Aurora kinase inhibition on cell division (Fig. 3A).

Work on Aurora kinases has shown that dividing cells lacking both kinases exit mitosis before anaphase and give rise to aneuploid daughters (34). We therefore considered the possibility that apparent GFP reactivation might be an artifact of this. We first looked at Xist expression and localization by FISH after treating cells with VX680 alone to focus on its mitotic effects. Interestingly, cells treated with 1 μM VX680 developed nuclei more than five times the size of control-treated nuclei (Fig. 3B). Furthermore, the VX680-treated cells exhibited an excessive number of abnormally large nuclei.

In our screen were 28 other Aurora kinase inhibitors apart from VX680. Whereas none elicited GFP-reactivation activity within an acceptable level of cell toxicity (Table S1), MLN8237 was confirmed with even greater reactivation activity than VX680 in the GFP qRT-PCR assay (Fig. 2B). MLN8237 at 1 μM elicited a 13.9-fold activity by itself. When combined with 5azadC, it yielded a 16.6-fold reactivation level over 5azadC alone; this was 83.5% of the male Xa-TgGFP control (Fig. 2B). MLN8237 is a more specific AURKA inhibitor than VX680 (IC50 = 7 nM for AURKA in a cell culture assay, vs. 1,500 nM for AURKB) (28). Combined, our findings implicate the Aurora kinase pathway as one of potential significance for pharmacological X reactivation.
number of Xist RNA clouds. By performing DNA FISH to detect the GFP transgenic locus, control cells, which were tetraploid due to immortalization with SV40 large T antigen, showed an average of 1.9 ± 0.75 Xi per cell (n = 158), whereas VX680-treated cells showed 7.6 ± 5.2 Xi per cell (n = 58) (Fig. 3C). There was, however, no qualitative difference in the Xist clouds of the control and VX680-treated cells, with Xist properly colocalizing with the GFP transgene probe for both. We also examined steady-state Xist RNA levels by qRT-PCR (Fig. 3D). Nonsignificant differences were observed (P = 0.07); however, the downward trend in Xist expression upon drug treatment was confirmed by RNA sequencing (RNA-seq) (Fig. 3D and Fig. S2, and see below), which showed Xist levels at between 45% (5azadC only) and 53% (5azadC + VX680) of the control samples.

We then examined the DNA content of treated Xi-TgGFP cells by FACS. Whereas most 5azadC-treated cells fell within peaks corresponding to stages G1 and G2 of mitosis (Fig. 4A, Center; ~33K and 55K on x axis), VX680-treated cells had higher DNA content on average and a wider range of ploidies (Fig. 4B, Center, and C, Center). Gating for GFP+ cells using side scatter (SSC) vs. GFP fluorescence corrected for the increased autofluorescence of the VX680-treated cells (Fig. 4, Left, Fig. S3, and Methods). Treatment with 5 μM 5azadC resulted in 15.9% GFP+ cells (Fig. 4A, Left), compared with 0.0021% GFP+ mock-treated cells and 0.40% GFP+ 0.5 μM 5azadC-treated cells (Fig. S3). After 1 μM VX680 treatment, 1.19% of the cells were GFP+ (Fig. 4B, Left). The combination of 0.5 μM 5azadC and 1 μM VX680 again proved synergistic for GFP reactivation, with 3.62% of treated cells GFP+ (Fig. 4C, Left). We next asked whether VX680-treated GFP+ cells skewed toward higher DNA content, which would suggest that aneuploidy played a significant role in GFP reactivation. Notably, DNA content of GFP+ cells was very similar to the profile of all cells after VX680 treatment (Fig. 4B, Center and Right, and C, Center and Right). Most of these cells were not GFP+ despite having DNA content much greater than nontreated or 5azadC-treated cells. Collectively, our data show that GFP reactivation observed with VX680 treatment is unlikely to be due to aneuploidy. Furthermore, the modest reduction in Xist expression does not perturb localization to the Xi.

**Reactivation of Native Xi Genes.** To further explore the effects of 5azadC and VX680 on native X-linked genes, we performed

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**Fig. 5.** Allele-specific analysis of X-linked gene expression. (A) RNA-seq, GFP aligned with its cDNA sequence. (B) Meq2 expression assayed by luciferase. (Left) Normalized activity from Xi-linked Meq2-Luc (Xi-B cell line) after 3-d treatment with 1 μM VX680 (VX) or 1 μM MLN8237 (MLN) treatments ≥0.5 μM 5azadC. Ctrl, control (DMSO) treatment. (Right) Comparison with a control line, Xa-3, where Meq2-Luc is on the Xa (note logarithmic scale). Error bars indicate SD. Means of at least three biological replicates are shown. **P = 0.005 for 5azadC, 5azadC + VX, and 5azadC + MLN each compared with the control without 5azadC.) ***P = 4.4 × 10⁻⁵. (C) Lamp2, (D) Fhl1, and (E) Msx aligned with mm9 in IGV (Top track for each panel). Note that, for the allelic analysis, reads appear only where there are polymorphisms that enable distinction between cas (Xa) and mus (Xi). For B–D, normalized cas and mus reads are shown. The scale is indicated at Right; note that it is smaller for mus reads. Xi-TgGFP cells were treated as indicated: 5azadC, VX680 (VX), or 5azadC + VX680 as in Fig. 2.
reactivated by the combination (Fig. 5C and Fig. S4). Meanwhile, for four-and-a-half LIM domains protein 1 (Fhl1) (Fig. 5D) and moesin (Msn) (Fig. 5E), the combination treatment resulted in a synergistic boost of expression from the Xi. We also observed up-regulation from the Xa (cas) allele in several cases, including Fhl1, Msn, and solute carrier family 25 member 5 (Slc25a5) (Fig. S4).

Changes in expression of the Rett syndrome disease gene, Mecp2, were not detected by RNA-seq. We therefore turned to a more sensitive system, using a mouse fibroblast clonal cell line with a luciferase reporter knocked into the endogenous Mecp2 locus. Xi-8 cells contain this reporter on the Xi. We found that 5azadC incubation resulted in measurable Mecp2 reactivation, and the addition of VX680, but not MLN8237, enhanced this reactivation significantly (Fig. 5B). This activity, however, was three orders of magnitude less than Xa-linked Mecp2-Luc activity (Xa-3 cells; Fig. 5B). Longer treatment with 5azadC + VX680 or MLN8237 at lower concentrations did not lead to significant increase over 5azadC alone (Fig. S5).

Whereas most Xi genes were not changed by more than two- to threefold either up or down, some 12 to 24 genes on the Xi were reactivated by at least threefold with an FDR of <0.05, upon treatment with 5azadC, VX680, or their combination (Fig. 6). We compared Xi linked to autosomal responses (Fig. 6C and Fig. S6). Chr13 is of similar size to the X chromosome and also showed upward and downward gene expression changes of the mus allele after treatment (Fig. 6F). However, Xi genes showed a greater overall magnitude of fold change compared with Chr13 genes (mus allele), both considering all genes on each chromosome, or only those on each with a significant change (Fig. S6). Taken together, our data demonstrate that different treatments elicited reactivation of distinct sets of Xi genes, raising the possibility that defined drugs could be tailored to select genes or regions on the Xi.

**Discussion**

Our work demonstrates that treatment of female cells with inhibitors of DNA methylation (DNMT) and Aurora kinases leads to a synergistic reactivation of select genes on the Xi. The DNA methyltransferase inhibitor 5azadC has long been known to have a small but significant effect on reactivating the Xi. Here we show that it can do so synergistically with two Aurora kinase inhibitors. We find that when the endogenous genes of the Xi are considered as a whole, three different compound treatments (5azadC alone, VX680 alone, or 5azadC + VX680) reactivate distinct subsets of genes on the Xi. The affected genes are distributed across the length of the X chromosome (Fig. 6B); interestingly, a cluster of 11 reactivated genes is located adjacent to the macrosatellite locus DXZ4, a boundary that separates two large megadomains of the Xi (15, 36, 37).

Whereas we do not fully understand the mechanisms of reactivation, our data suggest several factors may be in play. First, we observe that drug treatment results in a 50% down-regulation of Xist RNA levels (Fig. 3D and Fig. S2). Although Xist localization is unaffected, the reduced levels could potentially play a role in Xi reactivation. Reduced Xist levels were also observed by Bhatnagar et al. (22) upon knockdown of Aurora. The action of 5azadC on DNA methylation and derepression of Xi genes is well established (18, 38); however, its synergism with Aurora kinase inhibition has not been previously reported. Our data indicate that direct inhibition of AURKA and AURKB contribute to reactivation, as knockdown of both Aurora and Aurkb accounts for some but not all of the GFP reactivation resulting from VX680 or MLN8237 treatment (Fig. 2C). Our data are consistent with recent findings regarding the Aurora kinases for maintaining silencing of the Xi. In the findings of Bhatnagar et al. (22), shRNAs targeting Aurora resulted in reactivation of various X-linked genes without obvious aneuploidy. AURKB was also pulled down as a protein associating with Xist RNA (15), and its inhibition in combination with 5azadC and etoposide treatments resulted in a synergistic reactivation of Xi genes. AURKB has also been implicated in regulating XIST RNA expression.
adherence to the Xi in human cells undergoing mitosis (39). Significantly, our unbiased approach via a high-throughput screening assay independently identified AURKA and AURKB as relevant targets. Beyond the finding that the DNA methylation and Aurora kinase pathways act in suppressing the Xi, it is clear that application of VX680 or MLN8237 would be toxic as a therapeutic in the setting of mitotically active cells. Future efforts toward developing a drug based on the synergy between DNMT1 and the Aurora kinases must tease apart the Xi-reactivation effects of VX680/MLN8237 from the effects on the cell cycle. Tests in nondividing cells, such as neurons, would be of particular interest for neurological disorders such as Rett syndrome. Evidence that the Xi reactivation is distinct from cell cycle effects includes the fact that VX680 and MLN8237 both affect the cell cycle, but their effects on Xi reactivation are not identical. It is possible that other kinases inhibited by VX680 and MLN8237 contribute their effects on Xi reactivation are not identical. It is possible for neurological disorders such as Rett syndrome. Evidence that nondividing cells, such as neurons, would be of particular interest for developing a drug based on the synergy between DNMT1 and the Aurora kinases must tease apart the Xi-reactivation effects of VX680/MLN8237.

Methods

Work involving mice adhered to the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC) protocol no. 2004N000100. Immortalized Xi-GFP tail tip fibroblasts (TTFs) for screening were derived from a cross between a M. musculus strain carrying an X-linked GFP marker (24) and a M. m. castaneus WT mouse. The high-throughput small molecule screen was performed on the Thermo CRT Catalyst Express with automated high-content imaging via ImageXpress Micro. FISH to Xist RNA was performed as described (40). Knockdowns of Aurka and Aurkb were performed using siRNAs from Dharmacon lipofected into Xi-GFP fibroblasts. FACS was performed on the BD LSR II and results were analyzed using Flowjo. RNA-seq analysis was performed as previously described (15) on two biological replicates. Details can be found in SI Methods.

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