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

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X-chromosome inactivation is a mechanism of dosage compensation in which one of the two X chromosomes in female mammals 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 a high-throughput 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 (5azaCdC). Compounds that elicited GFP activity include VX680, MLN8237, and 5azaCdC, which are known to target the Aurora kinase and DNA methylation pathways. We demonstrate that the combinations of VX680 and 5azaCdC, as well as MLN8237 and 5azaCdC, 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.

X reactivation | high-throughput screen | small molecules | Aurora kinase | DNA methyltransferase

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 to avoid gene dosage imbalance compared with males (1–3). As a result of this process, in any particular female cell an X-linked gene’s function is provided solely by one of its two alleles. XCI has been intensively investigated over the past 55 years and a number of regulatory factors have now been identified. These factors include long noncoding RNAs (lncRNAs) that mediate X-chromosome counting, allelic choice, and initiation of silencing, as well as protein factors that interact with the lncRNAs to effect each of these steps of silencing. XCI is observed in three contexts in vivo in mice (4). In the male germline, the X and Y chromosomes are inactivated together during pachytene of the first meiotic prophase (5). In the preimplantation embryo, the paternally inherited X chromosome is inactive as a consequence of imprinting (6). Finally, in the epiblast lineage, a random XCI process occurs after the paternal X is reactivated (7).

Whereas XCI has been studied extensively, X-chromosome reactivation (XCR) has been less amenable to molecular analysis and its underlying mechanisms remain poorly understood (8–10). XCR occurs naturally in two contexts. At embryonic day 4.0 (E4.0), the imprinted form of XCI is reversed and this paternal X reactivation results in a transient state in which two active X chromosomes (Xa’s) are present in the epiblast lineage (7). As the embryo differentiates into three germ layers, random XCI initiates for one of the two X chromosomes (11), a process that begins with the expression of the noncoding RNA, “X-inactive specific transcript” or Xist, from the future inactive X chromosome (Xi) (12) and continues with the recruitment of chromatin modification factors such as Polycomb repressive complex 2 (13–15). The same Xi then remains silenced for all subsequent cell divisions throughout the life of the mouse—with the exception of the germ cell lineage, in which XCI is once again reversed before female meiosis (16, 17). Thus, during mouse development, multiple rounds of XCI and XCR occur.

Once silencing is established, the Xi is extremely robust and becomes difficult to reactivate outside of the normal developmental context, due to multiple parallel mechanisms of silencing involving Polycomb complexes and histone H3 lysine 27 trimethylation, incorporation of variant histones, hypoacetylation of histone tails, and increased DNA methylation (1–3, 18). However, the Xi harbors genes that could in principle be reactivated to treat X-linked diseases, such as Rett syndrome and cyclin-dependent kinase-like 5 (CDKL5) syndrome, two neurodevelopmental disorders affecting girls who are heterozygous for mutations in methyl CpG binding protein 2 (MECP2) and CDKL5.

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.
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 (5azadC), a compound shown previously to elicit a very low level of Xi reactivation (18). We chose a priming concentration of 0.5 μM 5azadC, 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 5azadC 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 5azadC) 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 5azadC. 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 siRNA 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 to Xi-TgGFP in a screen led to a 23.5% GFPR-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 5azadC, by 4.3-fold over treatment with 5azadC alone. We compared this expression to that of a male fibroblast line carrying the GFP transgene on the 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 5azadC reached 13% of the theoretical maximum (15, 18).

Fig. 2. GFP reactivation by Aurora kinase inhibitors, VX680 and MLN8237. (A) Xi-TgGFP female fibroblasts were treated for 3 d with VX680 at 1 μM, 5azadC at 0.5 μM, or both. Expression is relative to X-TgGFP/⁺/+ male cells. Means ± SD of three to five biological replicates are shown. Note that the y axis is a logarithmic scale. Fold differences vs. controls are indicated in red; *P = 0.02, ***P < 0.001. (B) The same cells were treated with 1 μM MLN8237 and tested as in A. **P = 0.01; ***P < 0.001. (C) Xi-TgGFP cells treated with control, Aurora, Aurora, or both Aurora and Aurora siRNAs. (Left) qRT-PCR of GFP expression, mean RNA levels ± SD, *P = 0.02. Note logarithmic scale on y axis. (Right) Knockdown efficiency of each Aurora gene assessed by qRT-PCR, ***P < 0.001.
Included 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 13.9-fold activity by itself. When combined with 5azaC, it yielded a 16.6-fold reactivation level over 5azaC 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 division (Fig. 3A)). 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 cell toxicity, 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 5azaC, 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 anucleated 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 cell toxicity, 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 5azaC, consistent with the known effects of Aurora kinase inhibition on cell division (Fig. 3A).

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 AURKA and AURKB 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 5azaC, 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 5azaC 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 5azaC, consistent with the known effects of Aurora kinase inhibition on cell division (Fig. 3A).

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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 synergetic 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
Reactivated genes on the Xi due to compound treatments. (A) Venn diagram of reactivated Xi-linked genes following indicated treatment. Expression of each gene increased at least threefold with an FDR of <0.05. (B) Distribution of reactivated genes along the Xi. Nucleotide coordinates in megabases are indicated at left; X-linked RefSeq genes are in blue to the right of the coordinates. Of these, reactivated genes are named with the color indicated by the small Venn diagram. Locations of Dzx4 and Xist are indicated in gray. (C) RNA-seq results summarized for the three compound treatments for the Xi (mus allelic). In each panel, log(fold-change of drug treatment vs. control) on the y axis is plotted against average expression levels across the samples, expressed as log2(counts per million) on the x axis. Each dot represents one gene. Red dots represent genes where the log(fold-change) is significant (FDR < 0.05); the black lines represent a threshold of a threefold change in either direction, i.e., log(fold-change) > 1.6 or < -1.6.

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.

Whereas most Xi genes were not changed by more than twofold 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 mouse allele after treatment (Fig. S6). 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.
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 DNM1L and the Aurora kinases must tease apart the Xi-reactivation effects of VX680/MLN8237 from the effects on the cell cycle. Tests in nondenuding 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 to the reactivation that may not be cell cycle dependent (Table S2) (30, 32). In the future, it may also be possible to use medicinal chemistry to enhance VX680’s Xi-reactivation potential while reducing the effects on cell cycle and other pathways.

Methods

Work involving mice adhered to the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC) protocol no. 2004N00100. Immortalized Xi-GFP tail 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 CRS Catalyst Express with automated high-content imaging via ImageXpress Micro. FISH to Xi RNA was performed as described (40). Knockdowns of Aurora 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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