Altered RNA splicing initiates the viral mimicry response from inverted SINEs following type I PRMT inhibition in Triple-Negative Breast Cancer

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Altered RNA splicing initiates the viral mimicry response from inverted SINEs following type I PRMT inhibition in Triple-Negative Breast Cancer

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Abstract

Triple negative breast cancer (TNBC) is the most aggressive breast cancer subtype with the worst prognosis and few effective therapies. Here, we undertook a screen of epigenetic chemical probes to systematically uncover the epigenetic regulators critical for TNBC growth. We identified MS023, an inhibitor of type I protein arginine methyltransferases (PRMTs), as having anti-tumor growth activity in TNBC *in vitro* and *in vivo*. Pathway analysis of TNBC cell lines indicates that the activation of interferon responses pre- and post-MS023 treatment is a functional biomarker and determinant of response; and these observations extend to a panel of patient-derived organoids. Inhibition of type I PRMT triggers an interferon response through the antiviral defense pathway with the induction of double-stranded RNA (dsRNA). The observed dsRNA accumulation is derived, at least in part, from inverted-repeat Alus (IR-Alus), many of which are expressed from retained introns induced by MS023, which inhibits arginine methylation of RNA-binding proteins and alters mRNA splicing machinery. Together, our results represent a shift in understanding the anti-tumor mechanism of type I PRMT inhibitors and provide a novel rationale and biomarker approach for the clinical development of type I PRMT inhibitors.

Introduction

Breast cancer is the most frequently diagnosed cancer worldwide¹. Triple negative breast cancer (TNBC) is a heterogeneous breast cancer subtype characterized by the absence of expression of estrogen (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)^{2,3}. TNBC represents 15-20% of breast cancer cases but accounts for 25% of deaths³. Patients with TNBC experience have a worse prognosis and poorer overall survival rate than other subtypes due to both higher rates of recurrence (over 30%) and shorter survival following metastatic recurrence⁴. Due to the lack of the targetable receptors that define other subtypes, options for TNBC treatment are limited, with chemotherapy representing the mainstay of current treatment. Despite some recent progress, new and effective targeted therapies for TNBC are urgently needed.

Alterations in epigenetic machinery, including mutations or overexpression of chromatin modulators as well as DNA hypermethylation, can disrupt normal development, resulting in pathologies, including cancer⁵. Such evidence, coupled with recent progress in the development of drug-like small molecules capable of modulating epigenetic, has fueled interest in exploiting the therapeutic potential of epigenetic targets⁶. Targeting epigenetic regulators such as histone deacetylase inhibitors, BET Bromodomain inhibitors, and protein arginine methyltransferase 5 (PRMT5) inhibitors, have shown anti-tumor effects in TNBC and other cancers⁷⁻¹⁰. Despite these advances, the systematic understanding of epigenetic vulnerabilities in TNBC still remains unclear.

To better understand how epigenetic regulators contribute to TNBC growth and survival, we screened a collection of TNBC cell lines with a focused library of validated chemical probes that selectively target specific epigenetic regulators. Our screen revealed several previously reported epigenetic targets for TNBC, including enhancer of zeste homolog 2 (EZH2), BET bromodomains and PRMT5^{7,8,11}. In addition to these, our results also revealed that type I PRMT, the enzymes that catalyze asymmetric ω -N^G, N^G-dimethylarginine (ADMA) of histones and non-histone proteins, are important for TNBC growth. Among type I PRMT, the activity of PRMT1 accounts for ~85% ADMA levels¹². PRMT1-mediated arginine methylation is required for multiple cellular processes, including transcription, cell signaling, RNA splicing, and DNA repair¹³. Increasing evidence demonstrates the functional roles of PRMT1 in mRNA splicing by directly methylating RNA-binding proteins and splicing factors and thus regulating their localization and function¹⁴⁻¹⁶. DNA repair proteins, such as BRCA1, MRE11, and 53BP1, have also been identified as PRMT1 substrates, thereby revealing a role of PRMT1-dependent methylation in regulating the DNA damage response¹⁷⁻¹⁹. Although cancer-associated mutations in PRMT1 are rare, its aberrant expression often correlates with poor outcome in various cancer types, including breast cancer^{20,21}. Hence, the oncogenic potential of PRMT1 has been recognized and it has recently received considerable attention as a potential therapeutic target in cancer²². Notably, GSK3368715, a type I PRMT inhibitor, has entered human clinical trials (NCT03666988), potentially opening up new avenues for the treatment of solid and hematological malignancies²². However, little is known about the therapeutic potential of type I PRMT inhibition in TNBC, and reliable biomarkers to identify tumors susceptible to type I PRMT inhibition are not known.

In this study, we developed a chemical screening approach to systematically investigate the epigenetic dependencies of TNBC and identified a specific vulnerability to the inhibition of type I PRMT. We then examined the molecular factors underlying the cell-line specific dependencies and validated our findings in patient-derived models. Our data show that type I PRMT inhibition alters mRNA splicing, which leads to the expression of Alu sequences that can form cytosolic dsRNA. This in turn triggers an antiviral interferon-mediated response, that push TNBC cells that are already stressed due to the elevated pre-existing interferon response signature over a threshold to induce cell death.

Results

A chemical probe screen identifies type I PRMT as key mediators of TNBC growth

To identify epigenetic regulators of TNBC cell proliferation, we performed a cell-based chemical screen using a collection of 36 epigenetic chemical probes from the Structural Genomics Consortium collection (SGC: <https://www.thesgc.org/chemical-probes>)^{10,23}. This chemical probe library includes tool compounds that selectively target key epigenetic regulatory proteins, including several epigenetic targets for which there are drugs currently in preclinical or clinical development. Each compound was evaluated in 15 TNBC cell lines, and the cell confluency using a live-cell imaging platform was assessed over 6 days (Figure 1a). The primary screen identified 6 inhibitors that significantly and substantially reduced cell proliferation by more than 50% in at least one-third of the TNBC cell lines screened (Figure 1b). BET Bromodomain inhibitors (JQ1 and PFI-1) showed sensitivity across almost all the TNBC cell lines as previously reported⁷. H3K27me3 methyltransferase and demethylase inhibitors (UNC1999 and GSKJ4), as well as protein arginine methyltransferase 5 (PRMT5) inhibitor (GSK591), also had significant growth inhibitory properties on a wide variety of cancer cells, including TNBC^{8,24,25}. Interestingly, a selective and cell-active inhibitor of type I PRMT, MS023, also showed high potency across many TNBC cell lines, whereas its chemically similar but inactive compound MS094 showed no effect (Figure 1b, c)²⁶. Since type I PRMT inhibitors have potential broad applicability in diverse human cancers²⁷ with at least one drug currently in clinical trial (NCT03666988), we sought to further characterize the action of type I PRMT inhibition in TNBC.

PRMT1 is a major target among type I PRMT in TNBC.

Given the potency of MS023 against all type I PRMT members, including PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8²⁶, we sought to understand whether any specific PRMTs are the dominant target in TNBC. As illustrated in the screening heatmap of cell confluence (Figure 1b), inhibitors specifically targeting PRMT3 (SGC707) or PRMT4 (TP-064) and PRMT6 (MS049) showed no significant anti-proliferative effect across the TNBC cell lines (Supplementary Table 1). Likewise, assessment of cell viability by CellTiter-Glo luminescent confirmed the profound inhibitory effect of MS023 on the representative TNBC cell lines, which was not observed with the other three inhibitors (Figure 1d). These data rule out a major role for PRMT3, PRMT4 and PRMT6 in sustaining TNBC cell proliferation, individually.

Analysis of the public human essential gene database²⁸ revealed that, among type I PRMT, PRMT1 has the highest essential gene score across all the TNBC cell lines, suggesting a critical role for PRMT1 in TNBC (Figure 1e). We then compared the expression level of type I PRMT in our 15 TNBC cell lines and found that PRMT1 was expressed at a higher level compared to other type I PRMT (Figure 1f). Notably, PRMT8 is barely expressed in TNBC, which is consistent with the evidence of PRMT8 having brain-specific expression patterns²⁹. Taken together, we summarized that the observed efficacy of MS023 inhibitor in TNBC is primarily due to the inhibition of PRMT1 catalytic activity.

PRMT1 inhibition suppress tumor growth *in vitro* and *in vivo*

To further assess the PRMT1 dependency in TNBC, we investigated whether the expression level of PRMT1 was increased in TNBC by interrogating two large, independent, publicly available clinical cohorts, the TCGA and METABRIC^{30,31}. In both cohorts examined, PRMT1 mRNA expression is significantly elevated in the basal-like subtype (corresponding to the most common subtype of TNBC¹³) compared to non-basal breast tumors (Figure 2a-b). Likewise, PRMT1 expression was found to be higher in basal-like subtype in a smaller,

independent breast cancer patient-derived xenograft (PDX) cohort from the Princess Margaret Cancer Centre (PM-PDX) (Figure 2c). Similarly, elevated PRMT1 expression was observed in TNBC cell lines compared to non-TNBC lines (Figure 2d).

To validate PRMT1 as a potential therapeutic target in TNBC, we performed dose-response assays with nine concentrations (ranging from 40 nM to 10 μ M) of MS023 across 17 TNBC cell lines and assayed the response using a live-cell imaging platform to monitor cell confluence over time (Supplementary Figure 1a). We calculated the area above area (AAC) to capture the efficacy and potency of the inhibitor, with a higher AAC value indicating greater sensitivity to MS023 treatment (Figure 2e). These highly reproducible responses identified both sensitive (e.g. MDA-MB-468) and resistant (e.g. Hs578-T) cell lines, suggesting that MS023 is not universally cytostatic, and that specific determinants of sensitivity exist within TNBC models. A representative experiment shows the effects of MS023 on TNBC cell growth in both sensitive and resistant cell lines over 5 days (Figure 2f).

Given that PRMT1 is the main asymmetric dimethylarginine (ADMA) methyltransferase³², we investigated whether the anti-proliferative effect of MS023 coincides with the reduction of the ADMA mark. To address this, we measured the ADMA levels in a sensitive cell line (MDA-MB-468) upon MS023 treatment for 5 days (Figure 2g). We observed a decreased ADMA level in a dose-dependent manner over the dose range from 0.1 μ M to 3 μ M, which had a significant suppressive effect on proliferation the MDA-MB-468 cell line. The observed PRMT1 dependent effect of MS023 inhibition was not due to changes in PRMT levels as its expression was not altered upon the treatment (Figure 2g). MS023 treatment also reduced the ADMA mark in the resistant cell line (Hs578-T), ruling out the possibility that differential sensitivity was attributable to drug-efflux pumps or other mechanisms, which might simply prevent target modulation by MS023.

To further confirm that the observed effects were due to inhibition of PRMT1 activity, we performed genetic knockdown assays in both sensitive and resistant cell lines. Knockdown of PRMT1 using inducible shRNA reduced both PRMT1 expression and ADMA marks in both cell lines (Figure 2h). As observed with MS023 treatment, PRMT1 knockdown significantly suppressed cell growth in the sensitive cell line but not in the resistant cell line (Figure 2i). Taken together, these data confirm that the anti-proliferative effect of MS023 in a subset of TNBC cell lines is due to the inhibition of PRMT1 methyltransferase activity, but that PRMT1 methyltransferase activity is not in itself essential for all TNBC lines.

To determine whether the *in vitro* effects observed can translate to antitumor activity *in vivo*, we next evaluated the efficacy and tolerability of MS023 *in vivo*. Mice bearing MDA-MB-468 xenografts were randomized into two groups, which were treated with control (water) or MS023, respectively. MS023 was well-tolerated, with no difference in the bodyweight of animals dosed with MS023 versus control over 35 days (Figure 2j). Once-daily dosing of 60 mg/kg of MS023 significantly reduced tumor growth and final tumor weight (Figure 2k-2l). Notably, the ADMA mark was significantly reduced in the xenograft tissue of mice treated with MS023 compared to the control mice, suggesting that the reduction of tumor size coincides with the disruption of the enzymatic function of PRMT1 (Figure 2m). Collectively, these data suggest that type I PRMT inhibition, primarily through PRMT1 inhibition, is a promising therapeutic strategy for the treatment of some TNBC.

Pre-existing interferon signaling underlies the sensitivity to type I PRMT inhibition

PRMT1 has been implicated in multiple biological processes, including transcriptional modulation, pre-mRNA splicing, cell cycle regulation and receptor signaling^{13,33}. However, the extent to which these pathways contribute the PRMT1 dependency of TNBC tumor cells is not

well understood. Previous studies have shown that the sensitivity to PRMT1 genetic ablation or chemical inhibition has been linked to MTAP (methylthioadenosine phosphorylase) loss, although the correlation in breast cancer is not as strong as in lymphoma²². Our data show no correlation between the response to PRMT1 inhibition and MTAP deficiency (Supplementary Figure 1b). A recent report showed that mutation of the splicing factor SRSF2 sensitizes cells to type I PRMT inhibition in leukemia¹⁶. However, we found that SRSF status appears unrelated to PRMT1 sensitivity in TNBC (Supplementary Figure 1c). Additionally, neither the status of TP53, a common modulator of chemotherapeutic response which is lost or mutated in most TNBC, nor the expression of any type I PRMT, was predictive of MS023 response (Supplementary Figure 1c). Having ruled out these candidates, we performed systematic global profiling of basal gene expression for our panel of 17 TNBC cell lines³⁴ and calculated the Pearson correlation coefficients of MS023 activity (as measured by the AAC value of the MS023 dose-response curve in Figure 2e) with individual gene expression levels (Figure 3a). We then subjected these rank-ordered gene lists to gene set enrichment analysis (GSEA). Gene sets associated with the interferon response α pathway stood out as the most enriched pathway, with several additional immune-related pathways, including interferon γ response and TNF α signaling via NF- κ B pathways, also among the most enriched in MS023-sensitive lines (Figure 3b-3c). These data suggest that a pre-existing elevated expression of genes involved in the interferon signaling pathway might predict the responsiveness to MS023 (Figure 3b).

To better address this hypothesis in more clinically relevant TNBC models, we tested whether the correlation of interferon signaling gene signatures and MS023 sensitivity was also evident across a panel of TNBC patient-derived organoids. 3D cancer organoid models are thought to better recapitulate clinical disease and epithelial heterogeneity in TNBC and are therefore regarded as a superior tool for the evaluation of drug responses^{35,36}. Four different organoid models were treated with either DMSO or MS023 at dose ranges from 0 μ M to 10 μ M, and assessed by microscopic evaluation and PrestoBlue staining. Representative images of these organoid models (Figure 3d) and the quantified percent cell viability of each model and treatment conditions are shown (Figure 3e). Using the transcriptomic data of tumors matching these organoid models, the differential expression of IFN response genes was studied between the MS023-sensitive and resistant organoid models (Figure 3f). Consistently, organoids with higher basal interferon gene expression were more sensitive to MS023 treatment, while organoids with lower interferon gene expression were more resistant. RT-qPCR of RNA extracted from organoids, further confirmed the upregulation of TH1 chemokines (e.g. CCL2 and CCL5) and antigen presentation genes (e.g. B2M and BTG1) in sensitive models (Figure 3g). Taken together, our findings across a range of patient-derived models suggest that the pre-existing levels of IFN response signature are correlated with the degree of sensitivity to type I PRMT inhibition.

Type I PRMT inhibition triggers interferon responses.

To better understand the potential mechanisms underlying the effects of type I PRMT inhibition in TNBC, we performed RNA sequencing (RNA-seq) on MDA-MB-468 cells following 5 days of treatment with MS023 and identified 2085 genes to be significantly differentially expressed ($p < 0.05$, FDR < 0.05) (Figure 4a). Hallmark enrichment analysis revealed that E2F targets and G2M checkpoint pathways were downregulated after MS023 treatment, suggesting that type I PRMT inhibition affects the expression of genes that function in cell-cycle regulation (Figure 4b). In agreement, MS023 treatment leads to a modest but significant decrease in S phase and a concurrent increase in G1 phase (Supplementary Figure 2a)²². Also consistent with the anti-tumor effect of MS023 observed in sensitive cell lines,

GSEA revealed induction of the apoptosis pathway upon MS023 treatment (Supplementary Figure 1d), as observed by an increase in the proportion of cells that stained positive for the apoptotic marker caspase 3/7 (Supplementary Figure 2b). In addition to the above noted effects, which likely reflect the downstream consequences of type I PRMT inhibition, a comparison of the differential gene expression patterns in both MDA-MB-468 (sensitive) and Hs578-T (resistant) lines showed that MS023 treatment significantly decreased the expression of DNA repair genes in the sensitive line, but not in the resistant line (Figure 4c). Western blot analysis revealed that the phosphorylation of histone H2AX at serine 139 (γ H2AX), a marker of DNA damage, was significantly enhanced in response to MS023 treatment in the MDA-MB-468 cell line (Figure 4d). Thus, we conclude that the effects of MS023 in the sensitive TNBC cells result in both cell cycle arrest and induction of apoptosis, possibly in response to DNA damage.

GSEA further suggested an innate immune response mechanism, which could contribute to the antiproliferative phenotype. Upregulation of the interferon α (IFN α) and interferon γ (IFN γ) innate immune response pathways were prominent GSEA signatures triggered by type I PRMT inhibition in MDA-MB-468 cells (Figure 4b, Supplementary Figure 2c). Among the top upregulated genes after MS023 treatment, we observed increased expression of genes such as STING1, OAS1 and TLR-3, which are involved in recognition and signaling of intracellular dsRNAs. These markers of an antiviral stress response were observed both in RNA-seq and subsequent qPCR validation (Figure 4e-4f). Together, these results identified a stress response to elevated dsRNA as a potential result of type I PRMT inhibition, which could underlie its antiproliferative and cytotoxic effects.

Type I PRMT inhibition induces dsRNA accumulation from intron-retained RNAs

To investigate whether and how MS023 treatment may trigger a viral mimicry response in TNBCs, gene ontology (GO) analysis was performed in both MDA-MB-468 (sensitive) and Hs578-T (resistant) cell lines. MS023 induced immune related responses in sensitive lines to a much greater extent than the resistant one (Figure 5a and Supplementary Figure 3a). Gene sets, including cellular response to dsRNA and the dsRNA sensing signaling (endosomal vacuolar pathway), were among the most significant positively enriched pathways (Figure 5b-5c). To further validate the dsRNA formation, immunofluorescence staining using a dsRNA-specific antibody (J2 antibody), a gold standard for dsRNA detection³⁷, identified a robust increase of cytoplasmic dsRNA in response to MS023 treatment in MDA-MB-468 cell line (Figure 5d-5e), but not in the resistant MS023 line (Supplementary Figure 3b-c).

Having observed cytoplasmic dsRNA accumulation in response to type I PRMT inhibition, we then sought to identify the source of dsRNA and its relationship to inhibition of type I PRMT. It was recently reported that disruption of mRNA splicing through inhibition of splicing regulatory kinases could trigger an antiviral response through the induction of mis-spliced RNAs. In particular, mis-spliced mRNAs with retained introns formed double-stranded structures that accumulated in the cytoplasm³⁸. Given the broad effects of type I PRMT on mRNA splicing^{13,16,39,40}, we hypothesized that induction of dsRNA formation in response to MS023 treatment was due to direct deregulation of RNA splicing^{41,42}. To test this hypothesis, RNA-seq data were analyzed to identify the MS023-associated disruptive alternative splicing events (ASEs). In total, 546 statistically significant differentially spliced events, including exon skipping, alternative splicing at the 3' or 5' site (A3/5SS), and retained introns (RIs), distributed across 422 genes were identified following MS023 treatment (Figure 5f). Importantly, RIs are one of the major ASEs, suggesting that the dominant effect of type I PRMT inhibition on splicing is to induce intron retention.

Recent studies have shown that intronic SINE elements, specifically inverted-repeat Alus (IR-Alus), induced after treatment with DNA-hypomethylating agents, lead to immunogenic dsRNA⁴³. We asked whether IR-Alus within retained introns could be the source

of dsRNA induced by MS023 treatment. A search for sequences capable of forming IR-Alus within the MS023-associated retained introns revealed that 62% of the total 152 retained introns had sequences that intersected with IR-Alus, and about 50% of the retained introns had IR-Alu sequences pairs that were bidirectionally transcribed in both sense and antisense directions (Figure 5g). Immunopurification of dsRNA followed by qRT-PCR revealed enrichment for selected retained intron mRNAs, but not two representative host mRNAs (actin, GAPDH) (Figure 5h), confirming their contributions to the pool of dsRNA in response to type I PRMT inhibition.

Discussion

TNBC is an aggressive breast cancer subtype that lacks effective therapeutics due to, at least in part, the genetic and epigenetic complexity that has limited the development of targeted therapies^{44,45}. In this study, we took an unbiased epigenetic chemical screening approach and identified type I PRMT inhibitor MS023 as capable of suppressing the proliferation of a subset of TNBC cells. Type I PRMT have recently come into focus as promising targets due to their overexpression in many cancer types and the finding that their inhibition is tumor suppressive in many of these settings^{13,22}. Specifically, in TNBC, our studies show that genetic and pharmacological inhibition of PRMT1, the main target among type I PRMT, impairs a subset of TNBC growth through induction cell cycle arrest and apoptosis. Moreover, preclinical models showed that type I PRMT inhibition significantly suppressed tumor growth in patient-derived tumor models *in vitro* and *in vivo*.

Molecular-based prediction of drug response is a major goal of precision oncology^{46,47}. Given the complexity and heterogeneity of TNBC, the development of robust molecular predictors of drug response represents both an opportunity and a challenge. Although several factors, such as genomic deletion or epigenetic silencing MTAP, or SRSF mutation, have been linked to the sensitivity of cancer cells to type I PRMT inhibition, none of these molecular markers correlated with response to MS023 in the context of TNBC^{16,22}. On the other hand, our gene expression data revealed that TNBC cells that are most sensitive to MS023 treatment have gene expression signatures enriched for interferon response and antiviral signaling. This correlation, which was confirmed in patient-derived organoids, identifies an opportunity for the development of markers of innate immune response to select patients most likely to respond to type I PRMT inhibition.

Emerging evidence supports that some epigenetic targeting molecules, such as inhibitors targeting DNMT^{48,49}, EZH2⁵⁰, LSD1⁵¹, HDAC⁵², SETDB1⁵³, PRMT5⁵⁴, CARM1⁵⁵ and BET proteins⁵⁶, are capable of inducing robust anti-tumor immune responses. These effects are the result of induced “viral mimicry effects”, enhanced tumor antigen expression and/or presentation, suppression of T cell exhaustion, remodeled tumor microenvironment, or combinations thereof. Here, we discovered a new link between type I PRMT inhibition and innate anti-tumor immunity. Pharmacologic inhibition of type I PRMT amplifies pre-existing interferon responses by inducing cytosolic dsRNA accumulation in sensitive TNBC lines. Unlike many of the other epigenetic inhibitors that induce ERV expression, MS023 treatment triggers dsRNA accumulation from intron retained RNAs. While PRMT enzymes are often classified as epigenetic targets based on their histone substrates, PRMTs have a much broader set of targets, especially RNA-binding proteins involved in mRNA splicing^{39,40}. Given these broad roles of type I PRMT on RNA splicing, our discovery is consistent with the recent report showing that spliceosome-targeted therapies result in intron-containing transcripts that form double-stranded RNAs³⁸.

In summary, our results show that type I PRMT inhibition elicits potent anti-tumor activity associated with increased interferon responses and dsRNA accumulation derived from mis-spliced RNAs. Notably, the innate immune effects of type I PRMT inhibition are restricted

to TNBC cells with pre-existing elevated interferon response gene expression signatures, suggesting that MS023 treatment may push TNBC cells that are already stressed over a threshold to induce cell death. Further work is needed to elucidate the factors contributing to the basal interferon response gene expression signature and how these factors may prime TNBC cells to MS023 sensitivity. The identification of these biomarkers of sensitivity provides important insights into the targeted development of this class of therapy in TNBC and other cancers. Furthermore, given the general role of type I PRMT inhibitors in stimulating dsRNA and interferon response signals, targeting type I PRMT in combination with anti-PD-(L)1 may offer potential new opportunities for cancer immunotherapy.

Material and methods

Cell culture. Human breast cancer cell lines were obtained from American Type Culture Collection (ATCC, Teddington, UK). All cells were routinely cultured in RPMI 1640 (Life Technologies 11965), or Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS recommended by suppliers. The cell lines were authenticated by short-tandem-repeat (STR) analysis and matched to the German Collection of Microorganisms and Cell Cultures (DSMZ) database, and they were used for no more than 25 passages after STR typing. Mycoplasma tests were routinely performed using MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Epigenetic chemical screen. TNBC cells were plated adherently in 384- well plates with 500 cells/well and imaged using IncuCyte® ZOOM Live-Cell Analysis System (Essen492 Biosciences). Growth profile was monitored by 10X objective every 6h using Incucyte software 2016 A with an integrated confluence algorithm until 72h. Standard mode per well was used to collect images in phase-contrast mode and averaged to provide a representative statistical measure of the well confluency. The data represented in the heatmap is log2 of the average confluency of triplicates in the probe wells normalized against the average confluency in the DMSO control wells.

Western blot analysis. Cells were lysed directly in 1× lysis buffer (50 mmol/L Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol and 0.1% bromophenol blue). Alternatively, cells were lysed by scraping into a pH 7.4 lysis buffer containing 1% NP-40 (Sigma-Aldrich, Gillingham, UK), 50 mmol/L Tris, 10% glycerol, 0.02% NaN₃, 150 mmol/L NaCl, and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich, Gillingham, UK). Snap-frozen tumor tissues were suspended in a pH 7.4 lysis buffer containing 50 mmol/L Tris base, 150 mmol/L NaCl, 2% TritonX-100, 1% SDS, 10 mmol/L EDTA, and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich, Gillingham, UK). Tissue destruction was done with the bullet blender homogenizer (Next Advance, New York, USA). 20–100 μg of proteins were separated in reducing conditions (2.5% β-mercaptoethanol) by SDS–PAGE (SDS–polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Bio-Rad, Hemel Hempstead, UK) for further processing, following standard western blotting procedures. Primary antibodies used are anti-PRMT1 (Milipore-7404), anti-ADMA (Cell signaling, 13522S), anti GAPDH (Santa Cruz Biotechnology, sc-32233), anti-pH2A.X (Cell signaling 9718S), anti-actin (Santa Cruz Biotechnology, sc-47778), anti-dsRNA(J2) (Scicons 10010200).

Cell cycle assay. Exponentially growing cells in six-well plates were treated with 3 μM MS023 or DMSO for 24h before cell cycle analysis using allophycocyanin (APC) BrdU Flow kit (BD Pharmingen). Briefly, cells were incubated with 10 μM BrdU for 6 h before fixation, permeabilization, and staining with APClabeled anti-BrdU antibody and 7-aminoactinomycin

D (7- AAD) according to the manufacturer's instructions. Cells were then analyzed using a BD FACScan flow cytometer and the percentage of live cells in each cell cycle stage was determined using FlowJo software (version 9.3.1). Experiments were performed in duplicate and the percentage of cells in each stage was compared between the treated and untreated samples for each cell line using a two-tailed t-test. P values ≤ 0.05 were considered significant.

Cell apoptosis assay. For cell death assays, TNBC cells (3000 cells/ well) were seeded in 96-well plates and incubated for 24h at 37°C. The cells were then treated either with DMSO or MS023 for 5 days. Post-treatment, Caspase-3/7 green apoptosis assay reagent (Sartorius #4440) was added to the cells and transferred to Incucyte® ZOOM 2FLR system and analyzed using 2016 integrated software.

Gene set enrichment analysis. Transcriptome data available for our tested 17 cell lines were processed using the Kallisto pipeline³⁴ (Marcotte et al. 2016, 10.1038/nbt.3519). Drug activity was extracted from growth curves produced by IncuCyte assay. The IncucyteDRC R package was used to process the growth curves and obtain concentration and viability normalized to control near confluence point⁵⁷ and PharmacoGx R package was then used to obtain drug activity measures such IC₅₀ and Area-above-the-dose-response curve (AAC)⁵⁸. Genes were ranked based on the Pearson correlation coefficients between the measured drug activity (IC₅₀) and individual gene expression levels over all 17 cell lines. Hallmarks gene sets were downloaded from MsigDB⁵⁹ and the piano R package was used to produce the GSEA results. Pathways enrichment plots were generated using fgsea R package⁶⁰.

Proteomics analysis. MD Anderson protein expression data was downloaded from ref.⁶¹ Princess Margaret Cancer Centre protein expression data was downloaded from ref.³⁴ Pearson correlation coefficients were calculated to measure the degree of association between the measured drug activity (IC₅₀) and individual protein expression levels over all samples.

Splicing data analysis. Alternative splicing events (ASEs) were identified for the human genome (hg38) using vast-tools pipeline^{62,63} with $|dPSI| \geq 0.2$ and $MV|dPSI_at_95| \geq 0.05$ for significance, additional non-default parameters for vast-tools diff module include: -S 3, -e 10, -m 0.01. ASEs were further categorized into functional classifications according to their predicted impact on the ORF: “neutral” for events which generate known functional isoforms or which do not alter the protein sequence (ex. an alternative exon); “protective” for events that reduce the occurrence of deleterious nucleotide sequences and therefore generate a functional protein (ex. removal of an intron/exon containing a premature stop codon) and “deleterious” which denote events that increase the frequency of disruptive sequences in the ORF (ex. inclusion of introns/exons containing premature stop codons or removal of essential exons for protein function)⁶⁴. All post-processing data analysis and figure generation were conducted using custom python3.7 scripts which are available upon request.

Lentiviral mRNA targets. shRNA vector targeting PRMT1 was a gift from Craig Strahdee; the sequence of shRNA is CCGGCAGTACAAAGACTACAA. Lentivirus was produced using standard virus production methods by co-transfecting target and packaging plasmids into HEK293T cells. Cell lines were then transduced with 0.45 μ M filtered and ultracentrifuge-concentrated viral particles with Polybrene (8 μ g ml⁻¹). After 16 h of transduction, the media was changed for fresh regular growth media, and 48 h later, selection started using puromycin (0.2–0.6 μ g ml⁻¹). After the selection was complete in 72 h, cells were termed stably transduced.

qRT-PCR. Total RNA was isolated using Trizol RNA extraction, and dsRNA was isolated by immunoprecipitation. For each of the total RNA and dsRNA, 1 ug of RNA was used to synthesize cDNA using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad) in 20 µl final volume of the reaction mixture. Upon completing the reaction, 180 µl of ddH₂O was added, reaching a final volume of 200 µl (10X dilution). RT-qPCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems) on 2 µl of synthesized cDNA per reaction. The relative abundance of transcripts was normalized to GAPDH and assessed using CFX Maestro (Bio-Rad). Primer designs were set to measure fully spliced transcripts and intron-containing transcripts. The ratio of intron-containing transcripts to fully spliced transcripts represent intron retention. Data were calculated using the Ct method and represented as fold change relative to control data. All experiments were performed in biological duplicates.

dsRNA immunoprecipitation. Protein A Dynabeads were washed and resuspended in NT-2 buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.1% Igepal CA-630). For each sample, 100 µl of beads were pre-bound to 5 ug of anti-dsRNA mAb (J2) overnight at 4 °C. MDA-MB-468 cells (cultured in 10 cm dishes) were treated with 1uM MS023 or DMSO for 5 days, then collected in biological duplicate in ice-cold PBS and spun down at 180 g for 5 minutes at 4 °C. The supernatant was subsequently removed, and cells were then lysed in 1mL RIP buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 5 mM MgCl₂, 0.1% Igepal CA-630, 1 U/µl RNasin Plus) for 5 minutes on ice. The supernatant was transferred to a new tube. An aliquot of 10% of the total collected supernatant was collected and was used for total RNA extraction using Trizol; the remaining 90% was used for dsRNA immunoprecipitation. For immunoprecipitation, 100 µl of J2-bound Protein A Dynabeads was added to the lysate and incubated for 3 hours at 4 °C with constant mixing. Beads were subsequently washed three times with NT-2 buffer, transferred to a new tube, and then washed three times with high salt wash buffer (50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1mM MgCl₂, 0.5% Igepal CA-630, 0.1% SDS). J2-bound dsRNA was harvested directly from beads with Trizol. Chloroform was added at a ratio of 1:5, and RNA was isolated from the aqueous phase using the RNA Clean and Concentrator columns (Zymo).

IF dsRNA Staining. 18 mm glass coverslips (VWR 48382041) were placed in each needed well of a 12 well plate. 1 ml of 100% ethanol was added to each well with a coverslip and incubate for 5 minutes at room temperature. The ethanol was subsequently removed, and the coverslip was washed with PBS. Upon removing PBS, 1 ml of diluted poly-l-ornithine was added to each well with coverslip and was incubated at 37 °C for 30 minutes. Poly-l-ornithine was subsequently removed, and wells were washed with H₂O. Upon the removal of H₂O, the plate was incubated at 37 °C for 2 hours. After the incubation, 1 ml of cells (either 20,000 cells of Hs578-T or 200,000 of MDA-MB-468) were added. The cells to allowed to adhere overnight before treatment. Upon cell adherence to the coverslip, cells were treated by either 1uM MS023 or DMSO for 5 days. Once the treatment is completed, the cells were fixed by 4% formaldehyde for 15 min at room temperature, then washed 3x with PBS and permeabilized with 0.5% (v/v) Triton-X for 10 minutes at room temperature. Once permeabilized, the cells are washed 3x with PBS and blocked in 5% BSA for 1 hour, before incubated by primary anti-dsRNA J2 (Scicons 10010200) at 1:500 dilution at 4 °C overnight. The next day, primary antibodies were removed, and the plate was washed 3x by PBS, then stained by Anti-mouse IgG Alexa 647 (Cell Signaling Technology 4410S) at 1:1000 dilution at room temperature, in the dark, for 1 hour. The slides were mounted onto microscope slides (Fisherbrand Superfrost Plus 1255015) using DAPI containing mountant (Invitrogen ProLong™ Gold Antifade Mountant P36930)

Organoid assay. BXTO.64, DCBXTO.28, DCBXTO.58, and DCBXTO.132 organoids were derived from PDX tumors. Patient tumors were collected with informed patient consent according to UHN approved Research Ethics Board protocols (14-8358). Tumor tissue was minced and digested in 5-10 mL Advanced DMEM containing 1X GlutaMAX, 10 mM HEPES, 1X Antibiotic-antimycotic (AdDF+++), and 250-500 ug/mL Liberase TH for 45 minutes at 37°C with gentle rocking. Tissue was filtered over a 100 µm cell strainer and pelleted by centrifugation at 400 x g for 10 minutes, 4°C. Pellets were washed once with AdDF+++, repelleted and treated with Red Cell Lysis Buffer Hybri-Max for 5 minutes on ice prior to cell counting. Organoids were cultured in media previously described by³⁶. PDX-derived organoids were regularly evaluated for human and mouse cell content by flow cytometry to ensure purity. STR analysis was used to confirm that the organoids matched their tumor of origin, and mycoplasma testing was performed as a quality control step.

For drug assays, organoids were dissociated into single cells, and 2000 cells were plated per well in duplicate in 25 µl of BME/well in 48-well plates. Once the BME had solidified, the organoid/BME domes were overlaid with 475 µl of media +/- drug. Fresh media and drug were applied every 5 days. A well containing BME only (no cells) was included as a media-only control. The cells were culture for 12-21 days (depending on the growth rate of the model) until the untreated controls had formed organoids >50 µm in diameter. Media was removed and the organoids were incubated with 1X PrestoBlue HS Reagent (ThermoFisher) in Breast Organoid Media³⁶ overnight at 37°C. The following day, aliquots of the media supernatant were transferred to a 384-well plate and fluorescence readings were taken at 560/590 nm excitation/emission wavelength using a CLARIOstar Plus microplate reader. The media-only control was used for background correction, and cell viability for each organoid model was normalized to its respective no drug control. Three independent assays were done per organoid model.

Mouse studies. All animal experiments were reviewed and approved by the Animal Care Committee at the University Health Network in Toronto. For in vivo dosing experiments, 1 million MDA-MB-468 cells were subcutaneously implanted into SCID mice following standard procedure. MS023 was prepared at 5% NMP, 20% captisol (20% w/v), 20% of PEG-400, 55% normal saline, and administered to the mice by IP injection, beginning when tumors reached 100 mm³ in volume. The treatments were performed at 60mg/kg for 3 days on and 4 days off, for a total of 5 weeks. Body weights and tumor growth were measured once a week over the course of treatment until the endpoint was reached.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 8.0. A two-tailed Student's t-test was used, and P-value < 0.05 was considered statistically significant.

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Author Contributions

Q.W., C.H.A. and M.L. conceived the project and Q.W. designed the experiments. Q.W and D.Y.N. performed the bulk of the assays. W.B. and Y.S.J. undertook RNA-seq analysis. F.E.C. performed splicing analysis. J.Chen performed dsRNA staining assays. qRT-PCR studies were carried out by S. D., S.A.M. and P.M. conducted Alu analysis. Y.D. and J.L. provided MS023 compound. Organoid assays were done by J.Cruickshank. J.H. performed the mouse studies. N.D.A., W.J.C. and L.X.Z. contributed to cell growing, immunoblotting, knockdown assays. D.W.C., B.H. K., G. D., P.P., J.J., K.B.M., D.B.L., D.D.D.C., M.L. and C.H.A. supervised the research. M.L. and C.H.A. oversaw the project. Figures were designed and prepared by Q.W. The manuscript was written by Q.W., M.L. and C.H.A. with assistance from all authors. All authors reviewed the final version of the manuscript and agreed with its content and submission.

Competing interests

D.D.D.C. received research funds unrelated to this project from Pfizer and Nektar Therapeutics. D.D.D.C. is a co-founder, employee and shareholder of DNAMx, Inc. Other authors declare no competing interests.

Data availability

The RNA expression data referenced during the study are available in a public repository from the website: <http://neellab.github.io/bfg/>. All the other data supporting the findings of this study are available within the article and its supplementary information files.

Code availability

Code to reproduce the RNA-seq bioinformatics analyses and their related data is available at https://github.com/bhklab/TNBC_PRMT1i, code to reproduce the RNA splicing analyses is available at <https://gist.github.com/Fciamponi/ef8aae813575a4947c1f9cb87a1c7330>.

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Figures

Figure 1

Chemical Screen of 36 epigenetic probes identifies type I PRMT as therapeutic targets in TNBC. (a) Flow chart of the epigenetic-focused chemical screen: 36 epigenetic probes were tested in the indicated cell lines and images were recorded by an Incucyte Zoom System in order to calculate cell proliferation rate.

(b) Heatmap showing the average of cell proliferation values of the indicated epigenetic chemical probes at 5 days in 15 TNBC cell lines. Data are represented as percentage of DMSO control. (c) A representative experiment showing effects of in vitro treatment of MDA-MB-436 cells with indicated compound. Images were captured by an Incucyte Zoom. Scale bar represents 300 μ M. (d) Viable cell counts of three TNBC cell lines treated with indicated chemical probes for 7 days. Data shown are mean calculated as percentage of DMSO control. (e) Essential score of type I PRMT across TNBC cell lines. (f) Type I PRMT mRNA expression in TNBC cell lines. P values were determined using one-way ANOVA. **** $p < 0.0001$.

Figure 2

Type I PRMT inhibition suppress tumor growth in vitro and in vivo. PRMT1 gene expression in the (a) TCGA Breast cancer datasets, (b) METABRIC Breast cancer datasets, (c) Princess Margaret Hospital PDX datasets (PM-PDXs) and (d) Princess Margaret Hospital cell line datasets (PM-Cell lines). According to PAM50 classification, the cohorts were designated as basal and non-basal subtypes. Gene expression is reported as $\log_2(\text{TPM}+0.001)$. The number of patients (n) per group is indicated. P values were determined using a Wilcoxon rank sum test. * $p<0.05$, **** $p<0.0001$. (e) Heatmap of responsiveness of MS023 for the indicated cell line. Area above the curve (AAC) calculated from dose response assays across 17 TNBC cell lines over a concentration from 40nM to 10 μM . Data are normalized to DMSO. A higher AAC indicates greater sensitivity. Colored lines are studied in more detailed in this paper. Data shown are mean $\pm$ s.d. of $n = 4$ independent biological replicates. A two-sided Student's t-test was used to derive the p values. (f) A representative experiment showing effects of Hs578T and MDA-MB-468 cells with MS023 at indicated concentrations for 5 days treatment. Bars show the average of four replicates. (g) Representative immunoblots of the ADMA mark of both MS023 sensitive (Hs578T) and MS023 resistant line (MDA-MD-468) following 5-day treatment with the indicated doses of MS023 and the negative control MS094. (h) Representative immunoblots showing the doxycycline (DOX) inducible shRNA knockdown of PRMT1 or luciferase control in MS023 sensitive line (MDA-MB-468) and MS023 resistant line (Hs578-T). (i) Normalized cell confluence of PRMT1 knockdown cells or shRNA luciferase control cells for the indicated time post doxycycline induction. Cell confluence is normalized to T0 time point. Data shown are mean $\pm$ s.d. of $n = 4$ biological replicates. A two-way ANOVA was used to derive the p values. * $p<0.05$; **** $p<0.0001$; n.s means not significant. (j) Body weights measurement of individual mouse over the course of treatment with control or MS023. (k) Individual tumor growth of MDA-MB-468 xenograft model with once-daily administration of 80mg/kg MS023 ($n=3$ per group). (l) Tumor weight was measured as a surrogate for tumor burden from the control and MS023-treated mice. (m) Representative immunoblot of asymmetric dimethyl arginine mark and PRMT1 in tumor tissue from mice treated with control or MS023 at experimental endpoint. T-test was used to derive the p values. * $p<0.05$.

Figure 3

Increased interferon responses underlie the responsiveness to Type I PRMT inhibition. (a) Pearson correlation analysis of individual gene expression and MS023 activity. Volcano plot of log₂ fold change for all gene significantly upregulated (red; left) in sensitive line or in resistant lines (blue; right). (b) Top 9 pathways significantly correlating with MS023 sensitivity. (c) GSEA analysis for gene sets associated with interferon responses enriched in sensitive TNBC lines. (d) Representative images of different

organoids models with either DMSO or MS023 treatment. (e) Differential response of organoids to MS023 treatment. (f) Heatmap showing the differential expression of IFN responsive genes between sensitive and resistant models. (g) qRT-PCR validation of indicated genes expression in both MS023 sensitive line (MDA-MB- 468) and MS023 resistant line (Hs578-T) organoids models.

Figure 4

MS023 treatment triggers interferon responses. (a) Volcano plot of log₂ fold change for genes significantly upregulated (red in the right panel) or downregulated (blue in left panel) upon MS023 treatment (n=3) in MDA-MB-468 cell line. (b) Gene set enrichment analysis (GSEA) of all ranked differentially expressed genes. (c) Heatmap showing the genes associated with DNA repair induced by MS023 treatment in both MDA-MB-468 and Hs578-T cell lines. (d) Representative immunoblots showing the expression level of p_{H2AX}, a DNA damage marker, with either DMSO or MS023 treatment in MS023 sensitive line (MDA-MB-468) and MS023 resistant line (Hs578-T). (e) Heatmap showing RNA-seq data for indicated genes in MDA-MB-468 cells with either DMSO or MS023 treatment. (f) The expression of indicated interferon responsive genes in MDA-MB-468 cells with either DMSO or MS023 treatment analyzed by real-time qPCR.

Figure 5

Type I PRMT inhibition induces cytoplasmic dsRNA formation due to the increased intron retention. (a-c) Type I PRMT inhibition leads to activation of immune signatures in MS023 sensitive cells. (a) Scatterplot of upregulated gene sets enriched in MDA-MB-468 cell after MS023 treatment. Gene sets associated with immune response are red. (b) GSEA analysis for gene sets associated with dsRNA sensing pathway: endosomal vacuolar pathway. (c) GSEA analysis for gene sets associated with cellular response to

dsRNA. (d) Cellular dsRNA was evaluated with anti-dsRNA (J2) immunofluorescence. (e) Quantification of cytoplasmic dsRNA signal intensity. (f) Barplot showing the number of alternative splicing events belonging to each of the main alternative splicing categories. (g) Barplot showing the count of inverted-repeat (IR)-pairs in which the intron intersects with only the first Alu in the pair (1; white), or the only the second Alu in the pair (2; grey), or the two Alus in the pair (1/2; red). (h) Retained intron induced by MS023 treatment forms dsRNA structure. Lysates from MDA-MB-468 cells with either DMSO or MS023 treatment were immunoprecipitated with J2 anti-dsRNA antibody. After treating with RNase III, isolated RNA was analyzed by RT-qPCR using the indicated mRNA primers.

Supplementary Files

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