PBRM1 Deficiency Sensitizes Renal Cancer Cells to DNMT Inhibitor 5-Fluoro-2’-Deoxycytidine

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PBRM1 is a tumor suppressor frequently mutated in clear cell renal cell carcinoma. However, no effective targeted therapies exist for ccRCC with PBRM1 loss. To identify novel therapeutic approaches to targeting PBRM1-deficient renal cancers, we employed a synthetic lethality compound screening in isogenic PBRM1+/+ and PBRM1-/- 786-O renal tumor cells and found that a DNMT inhibitor 5-Fluoro-2’-deoxycytidine (FdCyd) selectively inhibit PBRM1-deficient tumor growth. RCC cells lacking PBRM1 show enhanced DNA damage response, which leads to sensitivity to DNA toxic drugs. FdCyd treatment not only induces DNA damage, but also re-activated a pro-apoptotic factor XAF1 and further promotes the genotoxic stress-induced PBRM1-deficient cell death. This study shows a novel synthetic lethality interaction between PBRM1 loss and FdCyd treatment and indicates that DNMT inhibitor represents a novel strategy for treating ccRCC with PBRM1 loss-of-function mutations.

Keywords: PBRM1 gene mutation, DNA methyltransferase inhibitor, renal cell carcinoma, FdCyd, synthetic lethality

INTRODUCTION

Worldwide, renal cell carcinoma (RCC) represents the sixth most frequently diagnosed cancer in men and the 10th in women, accounting for 5% and 3% of all oncological diagnoses, respectively (1). Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of kidney cancer and somatic inactivation of VHL occurs in about 70% of sporadic ccRCGs (2, 3). Although loss of VHL is the initiating event in ccRCC, the acquisition of additional mutations have to accumulate to finally give rise to ccRCC. Polybromo 1 (PBRM1), a tumor suppressor gene encoding the BAF180 protein, has been identified by next-generation sequencing as the second most frequently mutated genes in ccRCC (4). PBRM1 functions as a chromatin-targeting subunit of SWI/SNF chromatin remodeling complexes (5). Its mutation further activates the HIF response and cooperates with the VHL mutation to generate ccRCC in mouse models (6–8). The loss of PBRM1 expression was also associated with aggressive features and advanced stage, as well as with worse prognosis (9–11).

Since the fact of the high frequency of mutations and loss of expression of PBRM1 in tumor, one approach for developing new therapy options in ccRCC would be to identify targets that have synthetic lethal relationships with PBRM1 loss. Synthetic lethality is defined as the setting in which inactivation of either of two genes individually does not affect cell viability but loss of function of both genes simultaneously causes lethality. In cancer, this means identifying targeted therapies that
cause selective lethality in the cancer cells that lack a specific tumor suppressor gene but spare normal cells (12). Recent studies have found that PBRM1 has a synthetic lethality interaction with genes involved in some epigenetic regulation, including EZH2 (13, 14) and poly ADP-ribose polymerase 1 (PARP1) (15). These researches suggested that PBRM1 mutant cancer cells will create dependence on other epigenetic machinery component to maintain cellular survival. With this hypothesis, we employed a druggable synthetic lethality screen using an epigenetic compound library and a PBRM1 isogenic ccRCC pair. Among the screen, we identified 5-Fluoro-2′-deoxycytidine (FdCyd), a DNA methyltransferase inhibitors (DNMTi) as synthetic lethality drugs for PBRM1-deficient ccRCC cells.

FdCyd is an antineoplastic agent that inhibits DNA methyltransferase and DNA methylation by acting as cytidine antimetabolite-forming covalent complexes with DNMTis and DNA (16). It is currently under assessment in clinical trials of various advanced solid tumors (17, 18). DNMTis induce global hypomethylation, which results in the re-expression of certain tumor suppressor genes (19, 20). On the other hand, the covalent trapping also causes DNA damage that was shown to be involved in the cytotoxic effects of DNMTi (20).

In this study, we describe that a DNA methyltransferase inhibitor FdCyd induces synthetic lethality in PBRM1-deficient ccRCC. Further mechanical studies reveal that FdCyd causes DNA damage and reactivates the tumor suppressor gene XAF1, leading to an activation of p53 signaling pathways, followed by cell cycle arrest at G2/m phase and apoptosis in PBRM1-deficient cells. Our data provide a strong evidence of novel PBRM1 synthetic lethality targets in human epigenetics modifiers.

MATERIALS AND METHODS

Cell Lines and Culture
Human ccRCC cell lines 786-O, Caki-1 and HEK 293T cell line were cultured from from ATCC (Rockville, MD, USA). 786-O cells were cultured in RPMI-1640 media containing 10% FBS and 1% pen/strep. Caki-1 cells were cultured in McCoy’s 5A media containing 10% FBS and 1% pen/strep. HEK 293T cells were cultured in DMEM media containing 10% FBS and 1% pen/strep. All the cells were incubated in a humidified incubator adjusted with 5% CO2 at 37°C. All the cells were routinely screened for mycoplasma absence.

Generation of PBRM1+/− Cells and Lentivirus Preparation
PBRM1 gene knockout was performed in786-O, Caki-1 cell lines using a CRISPR/Cas9-based gene editing technique. All sgRNAs were cloned into the lenti-CRISPR-V2 plasmids (Addgene plasmid #52961). Cells were transfected with the PBRM1 KO constructs using the lentiviral transduction. Then the transduced cells were selected by puromycin (Solarbio). Isolated KO clones were verified with Western blotting with and Sanger sequencing of the genomic PBRM1 locus. To produce lentiviruses, CRISPR-V2 plasmid, pMD2.G (Addgene plasmid #12559) and psPAX2 (Addgene plasmid #12260) were co-transfected into HEK 293t cells using Lentifit (Hanbio). At 48 h post-transfection, cell supernatants were harvested and virus was concentrated using PEG-8000. The information of the primer sequences is available in Supplementary Table 1.

Epigenetics Compound Library Screen and Cell Viability Measurement
Epigenetics Compound Library (L1200, 773 epigenetics compounds) was purchased from Topscience. After the first round of screening, 107 compounds were prepared in 384-well plates with an eight-dose, interplate titration format, ranging from 14 nM to 30 μM of final concentrations. 786-O PBRM1+/− or PBRM1−/− #1 cells were seeded at 800 cells per well in the 384-well plates containing the compound library and incubated at 37°C in a CO2 incubator for 72 h. All the liquid handling was done with Bravo Automated Liquid Handling Platform (Agilient). For cell viability measurement, cells were incubated with CellTiter-Glo® 2.0 solution (Promega, USA) for 10 min and the fluorescence was measured with a SPECTROstar Nano Microplate Reader (BMG Labtech). The screen was done in duplicated and the half maximal inhibitory concentration (IC50) of each compound for the isogenic cell pair were calculated with GraphPad Prism 8. Synthetic lethality hits were calculated according to the following formula: SI = IC50PBRM1 (+/+)/IC50PBRM1−/−. Drugs with SI > 2 were chose as synthetic lethality candidates.

RNA Isolation and RT-qPCR
Total RNA was harvested from cultured cells using Trizol reagent (Invitrogen, USA). RT was then performed with the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). qPCR was performed using TB Green® Premix Ex Taq™ II (Takara, Japan). The sequences of primers used are included in Supplementary Table 1.

Western Blot Analysis
Total protein from cells was harvested with ice-cold RIPA buffer (Thermo Scientific) plus protease inhibitor cocktail (Topscience). Proteins were fractionated by SDS–PAGE and transferred onto PVDF membrane (Bio-Rad). Membranes were incubated with the indicated primary antibodies overnight at 4°C and further incubated with the corresponding HRP-conjugated secondary antibodies (Epizyme, 1:5000) for 1.5 h at room temperature. Target proteins were detected using ECL Substrate (Epizyme) under an Amersham Imager 600 (GE Healthcare Life Sciences).

Tumor Xenograft Mouse Model
All animal procedures were approved by the Institutional Animal Care and Use Committee at the PLA Naval Medical University and were undertaken in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Five-weeks-old, female BALB/c nude mice were implanted in the left flanks with 786-O PBRM1+/+ and PBRM1−/− #1 cells suspended in Matrigel. When palpable tumors became detectable, animals with each tumor were divided...
randomly into 2 groups (n = 5 mice per group) for treatment with vehicle and Fdcyd. Mice were treated with vehicle (sterile saline containing 0.5% Hydroxy propyl methyl cellulose and 0.2% Tween80) or Fdcyd (25mg/kg, po, daily) for 21 days. The tumor sizes were measured by a Vernier caliper and calculated based on the modified ellipsoid formula (long axis × short axis2 × π/6). The mouse body weight was measured regularly to assess potential compound toxicity. At the end of experiments, mice were sacrificed and the tumors were harvested for weighing and further analyses.

Cell Cycle and Apoptosis Assays
786-O PBRM1+/+ and PBRM1−/− cells grown in 6-well plates were treated with Fdcyd for 24 h. For cell cycle analysis, cells were harvested for propidium iodide (PI) staining. For apoptosis analysis, Annexin V-FITC Apoptosis Detection Kit (Beyotime) was used. The cell fluorescence was analyzed with a BD FACSCalibur flow cytometer and the data was analyzed by FlowJo software.

Immunofluorescence Analyses
Cells were washed twice with PBS and fixed in 4% PFA for 15 min at room temperature. Cells were then treated with PBST (0.1% Triton X-100 in PBS), followed by blocking with 2% goat serum, 5% BSA, 0.5% Tween20 for 1 h. After that, cells were incubated with primary antibodies overnight at 4°C and further incubated with a secondary fluorescence-conjugated antibody for 60 min at room temperature in the dark. The nuclei were stained with DAPI Staining Solution (Abcam 228549).

Bisulfite Modification and Methylation-Specific PCR
Genomic DNA (1 ug), harvested with a TIANamp Genomic DNA Kit (Tiangen), was used for bisulfite modification with the DNA Bisulfite Conversion Kit (Tiangen). Bisulfite modified DNA was used for methylation-specific PCR (MSP) reaction with designed primers. The information of the primer sequences is available in Supplementary Table 1.

Statistical Analysis
All data were expressed as the mean ± standard deviation (s.d.). Statistical significance of differences between control and test groups was determined by Student’s t test or two-way ANOVA using Graphpad Prism 6 (GraphPad Software, La Jolla, CA). P values <0.05 were considered significant.

RESULTS
Epigenetic Compound Library Screen Identifies DNMT Inhibitors as Synthetic Lethal Drugs in PBRM1-Deficient Renal Cancer Cells
To screen and identify PBRM1 synthetic lethality targets, we first generate PBRM1 knockout (KO) 786-O cells with CRISPR-Cas9 gene editing system. The use of PBRM1 isogenic cell pairs will ensure that the identified effects operate in one particular genetic background. PBRM1 KO was verified with qPCR analysis (Figure S1A), Western blot and genomic Sanger sequencing of the sgRNA target regions with PBRM1 gene (Figures 1A, B). The growth rate of 786-O isogenic cell pairs were measured using CellTiter-Glo (Figure S1B). Among the three proved PBRM1 KO clones (PBRM1−/− #1–3), PBRM1−/− #1 was used for the synthetic lethality screen and the other clones were used for hits validation. To screen for clinical actionable vulnerabilities affiliated with PBRM1 deficiency, we used a library of 774 small-molecule targeting most of druggable human epigenetic proteins. The screen was done by two process. First, cells were exposed to epigenetic compounds of 10μM concentration for three days, after which, drug inhibition rate was assessed using CellTiter-Glo (Figure 1C). Next, drugs with inhibition rates greater than 50% were arrayed in 384-well plates with eight-dose to determine the estimated IC50 values for the PBRM1 isogenic pair (PBRM1+/+ and PBRM1−/− #1 cells) (Figure 1D). After two-roads screen, we identified six candidate drugs that showed a selectivity index (SI) >2 for the PBRM1−/− #1 cells. We selected Fdcyd for further study as: (i) the best candidate synthetic lethality hit was Fdcyd; (ii) the parallel small molecule inhibitor screen contained two DNMTi (Fdcyd and 5-Azacytidine) as candidate synthetic lethal drugs; and (iii) Fdcyd is a DNA methyltransferase (DMNT) inhibitor currently used in solid tumor clinical trials. To verify the screening results, we examined Fdcyd in parental 786-O PBRM1+/+ and three PBRM1−/− clones (#1, #2, and #3). All three PBRM1−/− 786-O clones were significantly more sensitive to Fdcyd than the parental PBRM1+/+ cells (Figure 1E). Similar results were obtained with the CAKI1 PBRM1+/+ and two CAKI1−/− clones (Figure 1F). We also tested another DNMTis, 5-Azacytidine and Decitabine, in PBRM1-isogenic 786-O pair for synthetic lethality effect (Figures 1G, H). These results suggested that PBRM1-deficient RCC cells are synthetic lethal with the inhibitors of DNMT.

Fdcyd Induces Apoptosis and G2/S Cell Cycle Arrest Selectively in PBRM1−/− RCC Cells
To further explore the synthetic lethality of Fdcyd, we examined the effect of Fdcyd on the colony forming ability of PBRM1-isogenic 786-O cells and CAKI-1 cells. Treatment of PBRM1−/− RCC cells with Fdcyd for 7days impaired colony formation, whereas wild-type RCC cells were relatively resistant to Fdcyd treatment (Figures 2A, S4A, B). Next, to test whether the synthetic lethality effect accompanied cell apoptosis or cell cycle arrest, we first performed Annexin V/PI double staining and analyzed cells with flow cytometry. Fdcyd selectively induced apoptosis in PBRM1−/− RCC cells (Figures 2B, C, S4C). Similarly, treatment with 1 μM Fdcyd for 24h increased the percentage of cells in the G2/M phase from 22% to 50% (Figures 2D, E, S4D, E). To further substantiate our conclusion, we analyzed the expression of the cleavage of PARP1/caspase-3 by western blot. These two apoptosis markers increased obviously in PBRM1−/− 786-O cells treated with Fdcyd (Figures 2F, S4F). Meanwhile, the anti-apoptosis factor BCL-2 was found down-regulated in PBRM1−/− 786-O cells comparing to PBRM1+/+ 786-O cells, especially after Fdcyd treatment. These results demonstrated that Fdcyd-induced
synthetic lethality in PBRM1−/− RCC cells by inducing apoptosis and cell cycle arrest.

**Fdcyd Altered the Transcription of Genes Involved in P53 Pathway and DNA Damage Responses**

To explore molecular pathways underlying the synthetic lethality, we conducted transcriptome analysis of the PBRM1 isogenic 786-O cells with or without Fdcyd treatment. KEGG pathway analysis showed that gene sets related to p53 pathway were significantly enriched among genes up-regulated in PBRM1-deficient cells compared to 786-O control cells treated with Fdcyd (Figures 3A, B), suggesting that tumor cell death under Fdcyd treatment may due to the activation of p53 signaling pathway. On basis of this observation, we assumed that the cells treated with Fdcyd might evoke DNA damage...
responses. Indeed, several genes related to pressure response and DNA repair were up-regulated in PBRM1-deficient cells compared to 786-O control cells in the absence of exogenous DNA damage (Figure 3C), which means persisting DNA damage or impaired DNA damage repair (DDR) may accumulate in 786-O. PBRM1-deficient cells expressed higher levels of Pro-DNA damage response genes, including BAX, HUS1B, SREPD3, CASP3, EXD2, CLU, BTG2 and USP51. Additionally, gene expression of BCL2, PARP9, MACROD2, EYA4 and TEX15, which were involved in DNA repair and response to genotoxic stress was down-regulated in PBRM1-deficient cells. Because sustained DNA damage usually leads to an increase in tumor mutation burden (TMB), we evaluated the tumor mutational burden (TMB) of PBRM1-mutant ccRCC. Using whole-exome sequencing (WES) data from the Tumor Cancer Genome Atlas (TCGA), we found that PBRM1-mutant ccRCC had a significantly higher TMB than PBRM1-WT tumors (**, P < 0.01, Mann–Whitney U test, Figure S6A). Meanwhile, RCC data mining of GEPIA database demonstrated a positive correlation between expression of PBRM1 and DNA repair genes. (Figures S6B–E). To further test whether PBRM1-defective 786-O cells undergo severer DNA damage response, we monitored the presence of γH2AX and RAD51 in these cells treated with Fdcyd. We found an increase in the number of γH2AX and RAD51 foci after exposure to Fdcyd, an effect that was more pronounced in PBRM1−/− 786-O cells and CAKI-1 cells. (Figures 3D, E, S4G–J). Taken together, these results suggested that Fdcyd selectively induces DNA damage in PBRM1-deficient RCC cells.

Reactivation of XAF1 by Fdcyd Was Associated With Demethylation of Hypermethylated Promoter

Since DNMTi can reactivate tumor suppressor gene through trapping of DNMT and inhibit the methylation mediated by enzymes during the following DNA replication (21), we speculated that the anti-tumor effect of Fdcyd was due to its demethylation process.

To define selection criteria for candidate tumor suppressor genes reactivated by Fdcyd, we first analyzed transcripts differentially regulated in 786-O PBRM1 isogenic pair with or without Fdcyd treatment. Thirty-eight genes were both up-regulated by Fdcyd (Figures 4A–C). Next, on the basis of the Tumor Suppressor Gene database (TSGene, https://bioinfo.uth. uab.edu/tsgene)}
edu/TSGene/) (22), a set of seven candidate TSG genes (ISG15, XAF1, MAPK13, IFI44L, EVI2B, RGS22, REC8) was defined. Hypermethylation of CpG islands in the promoter regions of TSGs represents an important feature of DNA methylation aberrancies in cancer (23). As a third selection criterion for potential TSG genes, genes associated with a promoter hypermethylation phenotype were further investigated (Figures S2A–D). This reduced the number of potential genes to four candidates ISG15, XAF1, MAPK13 and REC8. QPCR confirmed that ISG15 and REC8 mRNA were up-regulated in both isogenic cell lines (Figures 4D, E, S5A). To investigate whether increased gene expression was due to the reversal of promoter methylation, we performed methylation-specific polymerase chain reaction (MSP) and found that reactivation of XAF1 by Fdcyd was associated with demethylation of hypermethylated promoter (Figures 4F, G, S5C). Moreover, the protein expression of XAF1 was verified with western blots, showing that Fdcyd treatment significantly increased its level in both isogenic cell lines (Figures 4H, S5B). We also found increased protein expression of ISG15 in renal tumor cells after Fdcyd treatment (Figure S2E), although it may not due to the demethylation mechanism. XAF1 (XIAP Associated Factor 1) functions as a tumor suppressor by mediating apoptosis stress response of cancer cells (24, 25). In TCGA (The Cancer Genome Atlas) ccRCC data, patients with low expression of XAF1 have significantly worse prognosis than those expressing high levels (Figure S2F).

Demethylation by Fdcyd of the Promoter of Tumor Suppressor Gene XAF1 Promotes Cell Apoptosis

To determine whether silencing of XAF1 conferred resistance to Fdcyd-induced cell death, 786-O isogenic pair cells were treated concurrently with Fdcyd and XAF1 siRNA. Fdcyd selectively

**FIGURE 3** | Fdcyd altered the transcription of genes involved in P53 pathway and DNA damage responses. (A) The KEGG pathway analysis was manipulated to identify the most significantly up-regulated pathways by Fdcyd in PBRM1-deficient 786-O cells. (B, C) Heatmap image of comparing gene expression profiles in p53 pathway and DNA damage is shown. Heatmap of Z scores (range: -2 to +2) displaying relative gene expression across all clusters. (D) Immunofluorescence staining of γH2AX foci in Fdcyd and DMSO treated 786-O PBRM1-isogenic cells. Scale bars, 10 µm. (E) Quantification of the number of γH2AX foci per nuclei in 786-O PBRM1-isogenic cells treated with Fdcyd or DMSO. A minimum of 20 nuclei per condition were analyzed. Data are mean ± SD of three independent experiments. ***P < 0.001, student’s t test.
inhibited the viability of PBRM1-deficient 786-O cells and this effect was significantly reversed by siRNA silencing of XAF1 gene (Figures 5A, B). Moreover, the colony forming assay also showed that silencing of XAF1 did impair the growth inhibition effect of Fdcyd on PBRM1-deficient 786-O cells (Figure 5C). Because XAF1 has been implicated as playing a role in apoptosis pathways, we detected the apoptosis in Fdcyd and XAF siRNA co-treated cells. Results showed that silencing of XAF1 rescued the apoptosis induced by Fdcyd on PBRM1-deficient 786-O cells (Figure 5D). XAF siRNA reduced Fdcyd-induced apoptosis of PBRM1-deficient 786-O cells from 21.8% to 10.7%. This outcome was consistent with the western blot analysis of the expression of the cleavage of PARP1/caspase-3. XAF siRNA also reduced the cleavage of PARP1/caspase-3 expression induced by Fdcyd in PBRM1-deficient 786-O cells (Figure 5E). However, silencing of XAF1 had no effect on cell cycle arrest (Figures S3A–C). To further explore the role of XAF1 in Fdcyd-induced DNA damage, we performed γH2AX and RAD51 staining in these cells treated with Fdcyd and XAF1 siRNA. The number of γH2AX and RAD51 foci increased remarkably in PBRM1-deficient cells after exposure to Fdcyd (Figures 5F–I, S3D–G). Meanwhile, XAF1 siRNA did not seem to alleviate any DNA damage in PBRM1-deficient 786-O cells. Collectively, these data indicated that re-expression of gene
FIGURE 5 | Continued
XAF1 contributed to the cell cytotoxic effect of Fdcyd through apoptosis induction.

Fdcyd Treatment Induces Synthetic Lethality in PBRM1-Deficient Renal Cancer in vivo

To assess synthetic lethality by Fdcyd treatment in vivo, we evaluated the antitumor effect of Fdcyd in mice bearing established xenografts derived from PBRM1 isogenic cell pair. Mice bearing PBRM1 isogenic tumor xenografts were given Fdcyd (25mg/kg, PO) daily for 21 days and tumor growth rate was monitored periodically. Compared with the vehicle group, Fdcyd treatment caused a delay in tumor growth of PBRM1-deficient 786-O xenografts, as measured by tumor volume (Figures 6A, B) and tumor weight (Figure 6C).

Meanwhile, we did not see obvious weight loss in Fdcyd treated mice, suggesting the tolerable toxicity of the drug (Figure 6D). WB analyses of tumor tissues revealed that Fdcyd treatment significantly induced γH2AX levels, as well as PARP1 cleavage in PBRM1-deficient 786-O tumors (Figure 6E).

Together, our data demonstrated that Fdcyd treatment induced synthetic lethality in PBRM1-deficient renal cancer in vivo.

DISCUSSION

Mutations in genes encoding subunits of SWI/SNF chromatin-remodeling complexes are frequently observed in a large variety of human cancers, generally occurring in approximately 25% of all cancers (26). PBRM1, a component of the SWI/SNF complex, contains six acetyl-lysine binding bromodomains (BDs) and regulates gene expression including interferon stimulated gene factor and HIF related genes (27–29). Recently, PBRM1-deficient tumor cells were shown to be sensitized to PARP1 inhibitor (15). PBRM1-defective tumor cells exhibited elevated levels of DNA damage response and PARP inhibitor exposure exacerbated this phenotype.

Here, we conducted an epigenetic compound library screen and identified DNMTis as synthetic lethal compounds in PBRM1-deficient ccRCC cells. All three DNMTis Fdcyd, 5-Azacytidine and Decitabine from the library were found to be synthetic lethal in the test. The synthetic lethality of PBRM1 was verified in two different PBRM1 isogenic ccRCC pairs. Both 5-Azacytidine and Decitabine were FDA approved for the treatment of myelodysplastic syndrome (MDS) in 2004 and 2006 respectively (30, 31). Fdcyd is currently under assessment in clinical trials of various advanced solid tumors, suggesting the finding might have translational utility in kidney cancer types which PBRM1 is mutated. These agents serve as cytidine antimetabolite-forming covalent complexes with DNMTis and DNA (32). DNMTis trapped in this form ultimately lose their function, leading to down-regulation of DNA methylation (33). This covalent trapping, however, also induces DNA damage that was suggested to be involved in the cytotoxic effects of DNMTis. Therefore, it might be reasoned that compounds with DNMTi activity generally induce supernormal toxicity in cells under DNA repair defect or stress response.

The transcriptional profile of the 786-O cells mutated in PBRM1 revealed a dramatic change in expression of genes responsible for DNA damage response (Figure 3C). These genes involved in double-strand breaks resection, homologous recombination and replicative stress response express higher levels in PBRM1-deficient 786-O and CAKI-1 cells without exogenous damage stimulus. Furthermore, nuclear γH2AX and RAD51 foci measurement showed higher preexisting levels of γH2AX and RAD51 foci in PBRM1-/- cells than in PBRM1+/+ cells. These results suggested that loss of PBRM1 gene function impairs the DNA damage response. Therefore, it can be postulated that PBRM1-deficient RCC cells have persistent DNA damage activation and may be sensitive to DNA damage drugs. Phenotypically, Fdcyd treatment induced significant DNA damage in PBRM1-deficient cells (Figures 3D, E). Increased DNA damage cause cellular G2/M arrest and apoptosis in PBRM1-deficient cells. Nonetheless, negligible DNA damage and apoptosis were observed in PBRM1-WT cells upon Fdcyd treatment. This phenotype is in accordance with previous observations that Fdcyd treatment inhibited HCT116 cells at G2/M check point, induced apoptosis and amplified the DNA damage repair signal (34).

Abnormal hypermethylation of DNA may occur in renal cell carcinoma (RCC), resulting in tumor suppressor genes silenced and tumorigenesis (35). Many TSGs have been reported to be partially or completely silenced because of the hypermethylation of their promoter regions leading to drug resistance (36). We wondered whether reactivation of silenced TSGs by Fdcyd might also contribute to the synthetic lethal effect. To answer this question, we found three reactivated tumor suppressor genes XAF1, ISG15 and REC8 in Fdcyd treated 786-O cells. MSP analysis further confirmed that hypermethylation of a CpG Island in the promoter of XAF1 was found in 786-O and CAKI-1 cells and Fdcyd treatment led to XAF1 promoter region demethylation (Figure 4F). This was associated with re-activation of XAF1 mRNA and protein expression (Figure 4H). The X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1) was involved in apoptosis signaling by antagonizing the anti-apoptotic activity of XIAP and the anti-Caspase activity (37, 38). It was also
reported that XAF1 promotes the genotoxic stress-induced cell death response through different activities (39). To further confirm the role of XAF1 in Fdcyd treatment on tumor cells, we assessed whether re-expression of XAF1 could alleviate apoptosis, DNA damage and cell cycle arrest. The results reflect limited effect of XAF1 on DNA damage and cell cycle arrest. However, inhibition of XAF1 by siRNA in Fdcyd treated 786-O cells considerably alleviated apoptosis (Figure 5D). These observations suggested that XAF1 played an important role in apoptosis induction by Fdcyd. These data suggested a direct correlation between XAF1 re-expression and Fdcyd demethylation.

Recently, inactivation of PRBM1 was found to sensitize tumor cells to T cell–mediated killing and increased Type I interferon response (40). Interestingly, an association between PRBM1 loss of function, present in ~60% of ccRCC, and response to immunotherapy has been reported, though the association has not been observed consistently (41–44). On the other side, anti-tumor DNA-demethylation agents were reported to upregulate immune signaling and interferon response pathway (45, 46). Investigating whether DNA-demethylation agents may have clinical relevance for immune modulation in PRBM1-deficient renal tumor cells remains an important question that warrants further studies.

In summary, results of our in vitro and mouse xenograft in vivo studies indicated that Fdcyd treatment was synthetic lethal with PRBM1 loss in ccRCC and Fdcyd could serve as a novel therapeutic agent for renal cancer with PRBM1 deficiency.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.
ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the PLA Naval Medical University.

AUTHOR CONTRIBUTIONS

LW and CY contributed to conception and design of the study. DG and KD performed all of the experiments with assistance from AJ, YB, SJ and FH. DG wrote the paper and prepared diagrams. KD and ZF participated in the material preparation and manuscript review. All authors have read and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.870229/full#supplementary-material

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Supplementary Figure 3 | (A) Flow cytometer analysis propidium iodide (PI)-stained 786-O PBRM1+/+ and 786-O PBRM1−/− cells treated with Fdcyd and XAF1 siRNA. (B, C) Percentage of cell populations in G1, S, and G2/M phases from the flow cytometry analysis. (D, E) Immunofluorescence staining of RAD51 foci in Fdcyd and XAF1 siRNA treated 786-O PBRM1-isogenic cells. Scale bars, 25 µm. (F, G) Quantification of the number of RAD51 foci per nuclei in 786-O PBRM1-isogenic cells treated with Fdcyd and XAF1 siRNA. A minimum of 20 nuclei per condition were analyzed. Data are mean ± SD of three independent experiments. **P < 0.001, student’s t test.

Supplementary Figure 4 | (A, B) Colony formation assay of CAK1-6 PBRM1+/+ and CAK1-1 PBRM1−/− cells treated with indicated concentrations of Fdcyd for 14 days. (C) Flow cytometry analysis Annexin V/PI double stained CAK1-1 PBRM1+/+ and CAK1-1 PBRM1−/− cells treated with Fdcyd. (D, F) Flow cytometry analysis propidium iodide (PI)-stained CAK1-1 PBRM1+/+ and CAK1-1 PBRM1−/− cells treated with Fdcyd. (E) Percentage of cell populations in G1, S, and G2/M phase from the flow cytometry analysis. Data are mean ± SD of three independent experiments. *P < 0.05, student’s t test. (F) Immunoblot analysis of PARP1 and caspase-3. The cleavage of PARP1 and caspase-3 was shown as markers of apoptosis induction. (G, H) Immunofluorescence staining of γH2ax and RAD51 foci in Fdcyd treated CAK1-1 PBRM1-isogenic cells. Scale bars, 25 µm. (I) Quantification of the number of γH2ax and RAD51 foci per nuclei in CAK1-1 PBRM1-isogenic cells treated with Fdcyd. A minimum of 20 nuclei per condition were analyzed. Data are mean ± SD of three independent experiments. ***P < 0.001, student’s t test.

Supplementary Figure 5 | (A) Relative mRNA expression level of gene XAF1 as determined by qPCR. Data are mean ± SD of three independent experiments. **P < 0.01, student’s t test. (B) Immunoblot analysis of XAF1. (C) Methylation-specific polymerase chain reaction confirmed demethylation of hypermethylated promoter region in CAK1-1 isogenic cell pair. (D, E) Persistence of γH2AX foci over time in PBRM1-mutant 786-O cells after exposure to Fdcyd. (F) Immunoblot analysis of PBRM1 knockdown efficiency in CAK1-1 cells. (G) Immunoblot analysis of Knockdown efficiency of siXAF1 in 786-O cells. (H) Relative mRNA expression level of the indicated genes as determined by qPCR. Data are mean ± SD of three independent experiments. **P < 0.01, student’s t test. (I, J) Immunoblot analysis illustrating the c-PARP1 and γH2AX protein levels in 786-O PBRM1+/+ and PBRM1−/− tumor tissues.

Supplementary Figure 6 | (A) Comparison of TMB between PBRM1-WT and PBRM1-mutant renal cell carcinomas in TCGA database. PBRM1-mutant ccRCC had a significantly higher TMB than PBRM1-WT tumors (**, P < 0.01, Mann–Whitney U test). (B–E) Correlation between mRNA expression of PBRM1 and DNA repair genes in the GEPH database. (F) GSEA analysis of ps5 signaling pathway between PBRM1-deficient cells compared to 786-O control cells treated with Fdcyd.
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