The Role and Mechanism of CRT0066101 as an Effective Drug for Treatment of Triple-Negative Breast Cancer

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Key Words
Triple-negative breast cancer • CRT0066101 • PRKD • Phosphoproteome

Abstract
Background/Aims: Breast cancer is clinically classified into three main subtypes: estrogen receptor-positive (ER+) breast cancer, human epidermal growth factor receptor 2-positive (HER2+) breast cancer, and triple-negative breast cancer (TNBC). Without specific targeted therapies, patients with TNBC have poorer prognosis compared with those with ER+ and HER2+ breast cancer. Protein kinase D (PRKD) family members play crucial roles in cancer progression. CRT0066101, a PRKD inhibitor, has been reported to have anticancer activity in many cancer types. Nevertheless, the role and mechanism of CRT0066101 in TNBC have not been well investigated. Methods: The expression level of PRKDs was analyzed in breast cancer samples and breast cancer cell lines. The effects of inhibiting PRKD activity with CRT0066101 on TNBC cell proliferation, cell cycle, apoptosis, and tumor growth were studied by Cell Counting Kit-8 assay, cell cycle assay, propidium iodide/annexin-V assay, and a xenograft mouse model, respectively. To uncover the molecular mechanism of CRT0066101 in TNBC, comparative phosphoproteomic analysis using iTRAQ was employed. Results: We found that PRKD2 and PRKD3 were preferentially expressed in breast cancers. Immunohistochemistry confirmed the overexpression of PRKD2 and PRKD3 in TNBC. CRT0066101, which inhibited the activity of PRKDs, dramatically inhibited proliferation, increased apoptosis and the G1-phase population of TNBC cells in vitro, and reduced breast tumor volume in vivo. Comparative phosphoproteomic
analysis between breast cancer cells with and without CRT0066101 treatment revealed that the anti-breast cancer effects involved regulation of a complex network containing multiple enriched pathways and several hub-nodes contributing to multiple cancer-related processes, thus explaining the described effects of CRT0066101 on TNBC \textit{in vitro} and \textit{in vivo}. Finally, we validated several roles of PRKD inhibition by treatment with CRT0066101 and small interfering RNAs against PRKD2 and PRKD3 (siPRKD2 and siPRKD3), including p-MYC(T58/S62), p-MAPK1/3(T202/Y204), p-AKT(S473), p-YAP(S127), and p-CDC2(T14). 

**Conclusion:** PRKD inhibitor CRT0066101 exhibits anti-TNBC effects via modulating a phosphor-signaling network and inhibiting the phosphorylation of many cancer-driving factors, including MYC, MAPK1/3, AKT, YAP, and CDC2, providing insight into the important roles as well as the molecular mechanism of CRT0066101 as an effective drug for TNBC.

**Introduction**

Breast cancer, the most common type of cancer in women, is clinically classified into estrogen receptor-positive (ER\(^+\)) breast cancer, human epidermal growth factor receptor 2-positive (HER2\(^+\)) breast cancer, and triple-negative breast cancer (TNBC). TNBC is characterized as ER\(^-\), progesterone receptor-negative, and HER2\(^-\). Owing to the lack of a molecular target, cytotoxic radiotherapy and chemotherapy are the only choices for TNBC patients [1]. However, the recurrence rate is high after cytotoxic chemotherapy and radiotherapy [2]. TNBC's high resistance to therapy has been attributed to tumors showing mesenchymal and stem cell features, and there are no specific targeted therapies [3-5]. TNBC is resistant to standard hormone therapy and HER2-directed agents such as trastuzumab, due to the loss of expression of hormone receptors and HER2 [6, 7]. The present methods for treating TNBC mainly consist of surgical resection, auxiliary assist, and localized chemotherapy, but postoperative recurrence is difficult to control. Thus, development of new therapeutic drugs for TNBC is important.

The protein kinase D (PRKD) family consists of serine/threonine kinases that belong to the calcium/calmodulin-dependent kinase (CaMK) superfamily. It contains three members: PRKD1, PRKD2, and PRKD3. PRKDs have been reported in multiple cancer-promoting processes such as cell proliferation [8, 9], cell migration [10, 11], epithelial-mesenchymal transition [12], and angiogenesis [13]. Differences in function between PRKD isoforms have been described in breast, colon, gastric, and prostate cancers [10, 14-20]. In breast cancer, PRKD1 has been shown to promote cell growth [21] and inhibit invasiveness [22, 23]. By comparison, PRKD2 and PRKD3 promote proliferation, metastasis, and chemoresistance in breast cancer progression [14, 24, 25]. In TNBC, PRKD1 is silenced by hypermethylation [26]. In contrast to PRKD1, PRKD2 and PRKD3 have been found by our and other research groups to play important roles in promoting oncogenic transformation of TNBC [14, 24, 27, 28]. Until now, several small-molecule inhibitors have been shown to inhibit PRKD activity \textit{in vitro}, such as CID755673 and its analogs [15, 29], 3, 5-diarylazoles [30], 2, 6-naphthyridine and bipyridyl inhibitors and their analogs [31], CRT5 [32], and CRT0066101 [33]. These compounds effectively block proliferation, migration, and invasion \textit{in vitro} [34]. CRT0066101, a small-molecule PRKD inhibitor, was reported to show potential anticancer effects in xenograft models of pancreatic [33], colorectal [35], and breast cancer [27]. According to these reports, CRT0066101 was well tolerated and had no significant side effects in mice, therefore suggesting CRT0066101 as a promising agent for clinical trials. However, a thorough understanding of the anticancer effects and underlying mechanism of CRT0066101 in TNBC is needed for further validation and exploration to facilitate its clinical use.

A more comprehensive understanding of the identity of the candidate phosphoproteins affected by PRKD inhibition by CRT0066101 will be important to understand the role of PRKDs and CRT0066101 in cancer. We therefore used iTRAQ comparative phosphoproteomic
analysis to uncover global effects of CRT0066101 in TNBC. The results provide new insight into the functions of PRKDs and clues for combating TNBC with CRT0066101.

Materials and Methods

Breast cancer samples

We obtained all the breast cancer samples from Nanjing General Hospital. After resection, samples were immediately cryopreserved in liquid nitrogen. Hematoxylin and eosin-stained frozen sections were prepared from each mammary gland specimen to confirm the diagnosis and to obtain histopathological classifications and molecular subtypes. All the breast cancer specimens were analyzed anonymously after obtaining the patients’ written consent. This study was approved by the medical ethics committee of Suzhou Institute of Biomedical Engineering and Technology.

Cell culture

MCF7, MDA-MB-468, BT549, and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 1% penicillin-streptomycin solution (Life Technologies) and 10% fetal bovine serum (HyClone). T47D and HS578T cells were cultured in RPMI 1640 medium (Corning Cellgro) supplemented with 1% penicillin-streptomycin solution and 10% fetal bovine serum. All the cell lines used in this study were from the American Type Culture Collection and used within 6 months.

Western blot

For western blot analysis, primary antibodies against PRKD1, PRKD2, PRKD3, p-PRKD(Ser744/748), AKT, p-AKT(S473), YAP, p-YAP(S127), MAPK1/3, p-MAPK1/3(T202/Y204), CDC2, MYC, and p-MYC(S62) were purchased from Cell Signaling Technology. p-CDC2(T14) antibody was purchased from Signalway Antibody LLC. p-MYC(T58) antibody was purchased from ABM. Anti-rabbit secondary antibody, anti-mouse secondary antibody, and β-actin primary antibody were purchased from Santa Cruz Biotechnology. Western blotting was carried out following standard protocols.

Cell proliferation, cell cycle, and cell apoptosis assays

Cell proliferation was determined by using Cell Counting Kit-8 reagent (Dojindo Laboratories) according to the manufacturer’s instructions. For cell cycle analysis, unsynchronized cells were digested with trypsin and fixed with 70% ethanol. DNA was stained with propidium iodide and the cell cycle distribution was analyzed by flow cytometry using a BD FACSCholor (Becton Dickinson). For the cellular apoptosis experiments, an Annexin-V/Dead Cell Apoptosis Kit (Invitrogen) was used to stain the cells according to the manufacturer’s instructions and analyzed on the BD FACSCholor flow cytometer.

In vivo mouse model

Female athymic STOCK-Fixn1nu/Nju 4-week-old mice were obtained from Shanghai Sushang Biology Technology Co., Ltd. Breast cancer cells (5 × 10⁶ cells) were subcutaneously injected into the armpit of mice. Upon CRT0066101 treatment, nude mice bearing MDA-MB-231 and MDA-MB-468 tumor xenografts were administered daily vehicle control (dextrose) or CRT0066101 at 80 mg/kg orally. A Vernier caliper was used to calculate tumor volume by the following formula: volume = 0.5 × length × width². All of the experiments were conducted in accordance with the instructional standard guidelines of Suzhou Institute of Biomedical Engineering and Technology for animal experiments.

PRKD-regulated phosphoproteomic analysis using iTRAQ

Cells were prepared using a ReadyPrep Protein Extraction kit (Bio-Rad Laboratories). Extracted protein concentration was determined by a BioSpec-nano (Shimadzu Biotech, Kyoto, Japan). Approximately 4 mg of protein/sample was used for quantitative phosphoproteomic profiling. Each protein sample was reduced and alkylated and digested with trypsin (Promega). Following tryptic digestion, peptide samples were desalted using a MonoTip C18 (Shimadzu Biotech). The eluted peptides were dried in a SpeedVac and then labeled with iTRAQ 8-plex reagents according to the manufacturer’s instructions.
Phosphopeptide enrichment was performed using a Titansphere Phos-TiO Kit (Shimadzu Biotech) according to the manufacturer’s instructions. Eluted phosphopeptides were combined, acidified with 100 mL of 2.5% trifluoroacetic acid, desalted with the MonoTip C18, and resuspended in 0.1% formic acid. Samples were analyzed using a Prominence nanoflow liquid chromatography system (Shimadzu Biotech) connected to a liquid chromatography-mass spectrometry ion-trap time-of-flight mass spectrometer (Shimadzu Biotech). The detected fragments were analyzed with ProteinLayer software using the Swiss-Prot human database and the phosphorylation sites were determined using PTM Finder Software (Shimadzu Biotech).

Abundance ratios between samples were quantified by LabSolution Software (Shimadzu Biotech) via the quantification of iTRAQ-labeled peptides, with a synthetic peptide corresponding to the residues between 14 and 38 (TQCPDDSTCCELPTGK) of mouse granulin-3 labeled with [d0]/[d6]-DMPITC used as an internal standard for quantification (BioworldInc, Minneapolis, MN). To minimize contamination from isobaric ions, only the peptides with isolation specificity greater than 75% were quantified.

Statistical analysis
GraphPad Prism version 5.0 software was used for all statistical analyses. Data were expressed as the mean ± standard error of the mean (SEM).

Results

**PRKD2 and PRKD3 are the two major PRKDs expressed in breast cancer**

To test the role of PRKDs in breast cancer, we first examined the gene expression data for 1888 breast cancer samples from The Cancer Genome Atlas (TCGA) and found that PRKD2 and PRKD3 were the two major PRKD isoforms expressed relative to PRKD1 (Fig. 1A and Supplementary Table 1 - all supplementary material available online at www.cellphysiolbiochem.com). Second, we explored the PRKD1, PRKD2, and PRKD3 gene expression data in breast cancer cell lines [36] and found that PRKD2 and PRKD3 were the two major isoforms expressed among all PRKDs in TNBC cell lines (Fig. 1B and Supplementary Table 2). Third, we collected 34 breast tumor samples and verified that PRKD2 and PRKD3 expression was higher than PRKD1 according to quantitative reverse transcription PCR (RT-qPCR) (Fig. 1C and Supplementary Table 3). Fourth, analysis of TNBC by immunohistochemistry also confirmed that PRKD2 and PRKD3 levels were elevated in TNBC tissues compared with precancerous tissues (Fig. 1D). To select suitable cell models, we carried out western blotting on the breast cancer cell lines and selected MDA-MB-231 and MDA-MB-468, which express PRKD2 and PRKD3 and not PRKD1, as model TNBC cell lines (Fig. 1E).

**Anti-TNBC effects of PRKD inhibitor CRT0066101 in vivo and in vitro**

To test the anti-TNBC effects of CRT0066101, we first carried out in vitro assays, including proliferation, cell cycle, and apoptosis assays. The results show that CRT0066101 induced a marked reduction in proliferation in the investigated TNBC cell lines (Fig. 2A), an increase in the population of cells in G1 phase and a decrease in S-phase cells (Fig. 2B), and increased apoptosis (Fig. 2C), suggesting that CRT0066101 exhibited anti-TNBC effects in vitro. However, it did not have a discernible toxic effect on the noncancerous breast cell line MCF10A compared with PRKD2- or PRKD3-overexpressing breast cancer cell lines (Supplementary Fig. S1). To further validate the anti-TNBC effects of CRT0066101 using in vivo xenograft mouse models, we confirmed that CRT0066101 significantly reduced TNBC tumor volume in vivo (Fig. 3). Taken together, our results suggest that CRT0066101 is effective in the treatment of TNBC.

**Identification of phosphoproteomes after treating MDA-MB-231 with CRT0066101**

Since PRKDs are serine/threonine kinases, iTRAQ comparative phosphoproteomic analysis was used to reveal phosphorylation events affected by CRT0066101 in TNBC cells. Fig. 4A shows the workflow of analysis of MDA-MB-231 cells treated with phosphate-
buffered saline or CRT0066101. Each group contains two experimental duplicates. In total, 2252 phosphopeptides matching 3034 phosphosites from 1353 phosphoproteins were identified with a $P$ value < 0.005 (Fig. 4B and Supplementary Table 4).

We regarded a phosphosite in a phosphopeptide of a particular phosphoprotein as being PRKD regulated if we detected a 1.5-fold or greater change between the sample and control. In total, 2232 phosphopeptides matching 3002 phosphosites from 1347 phosphoproteins were identified as being PRKD regulated (Fig. 4B and Supplementary Table 5).
Fig. 2. CRT0066101 alters the biological behaviors of TNBC. TNBC cells were treated with 0, 1, and 3 μM CRT0066101 and cell proliferation was determined via (A) Cell Counting Kit-8 assay, and (B) cell cycle and (C) cell apoptosis were analyzed by flow cytometry. Data represent the mean ± SEM. *P<0.05, **P<0.01, and ***P<0.001 by t test.
Systematic analysis of CRT0066101-related phosphoproteome

To further characterize the mechanism of CRT0066101 in TNBC, the derived comparative phosphoproteomic data first underwent reactome analysis [37]. The results show enriched reactomes involved in various biological events, such as RNA metabolism, cell cycle, gene expression, protein metabolism, disease, programmed cell death, immune system, and DNA repair (Supplementary Fig. S2). There were 52 pathways with \( P < 0.01 \) and FDR < 0.01 enriched in the reactomes of CRT0066101-related phosphoproteins (Supplementary Table 6). Among these 52 pathways, 12 are involved in RNA metabolism, 11 are involved in cell cycle, 8 are involved in protein metabolism, 7 are involved in disease, and 6 are involved in gene expression (Fig. 5A).

The networks of CRT0066101-related phosphoproteins were further investigated using the IMEx and STRING interactome databases. The nodes with the top 15 largest number of degrees or betweenness were suggested to be the hub-nodes in the networks. In the CRT0066101-related phosphoprotein network analyzed using the IMEx interactome database, the 15 nodes identified as hub-nodes were ELAVL1, SUMO2, UBC, HSP90AA1, MYC, CUL1, HSP90AB1, HDAC1, VCP, BRCA1, VHL, IKBKG, HDAC2, SRC, and CTNNB1 (Fig. 5B and Supplementary Table 7). In the CRT0066101-related phosphoprotein network analyzed...
using the STRING interactome database, the 15 nodes identified as hub-nodes were UBC, SRC, HSP90AA1, HDAC1, CTNNB1, MAPK1, MAPK14, HDAC2, JUN, RPS14, SHP90AB1, MYC, MAPK3, and CUL1 (Fig. 5C and Supplementary Table 8).

**CRT0066101 suppresses the phosphorylation of MYC, MAPK1/3, AKT, YAP and CDC2**

Since PRKD activity is suppressed by CRT0066101, it is plausible that the phosphorylation levels of downstream signaling proteins regulated by PRKDs decrease. To further explore the mechanism of CRT0066101 in breast cancer, we intended to validate several important targets of CRT0066101. Via integrating the result of NetworkAnalyst and PRKD-regulated phosphoproteins, we chose a series of significantly downregulated phosphoproteins as well as potential functionally important targets for further validation.

As described above, CRT0066101 inhibited proliferation, increased apoptosis, and increased the G1-phase population of TNBC cells. In the current study, MYC was found as a most important hub-node in PRKD-regulated pathways using the IMEx interactome database and STRING interactome database; MAPK1 and MAPK3 as CRT0066101-regulated phosphoproteins were two hub-nodes in the CRT0066101-related network. Although YAP, AKT, and CDC2 were not hub-nodes in the CRT0066101-related network, we observed that the phosphorylation levels of YAP, AKT, and CDC2 were significantly decreased in the...
CRT0066101-related table (Supplementary Table 5). It has been reported that CRT0066101 inhibits the phosphorylation level of YAP, AKT, and CDC2 in multiple cancer types [35, 38, 39]. MYC, MAPK1/3, AKT, and YAP play important roles in cancer progression. MYC is overexpressed in 30–50% of high-grade breast cancer [40, 41]. In addition, MYC plays critical roles in multiple cellular pathways that promote cell survival and proliferation [42, 43]. MAPK1 and MAPK3 are major determinants in the control of diverse cellular processes such as tumor growth and survival [44]. AKT and YAP promote tumorigenesis via several oncogenic events such as cell apoptosis and cell growth [45, 46]. Cyclin-dependent kinases
(CDKs) regulate the cell cycle at several checkpoints, including G1 and G2/M [47]. The G1 checkpoint determines whether cells progress into S phase, and CDC2 plays a role in G1/S progression.

To confirm that CRT0066101 regulates several targets in TNBC cells, we examined MYC, MAPK1/3, AKT, YAP, and CDC2 in selected model TNBC cell lines that were exposed to increasing concentrations (0, 1, and 3 µM) of CRT0066101 for 1 day. The results show that CRT0066101 treatment decreased p-MYC, p-MAPK1/3, p-AKT, p-YAP, and p-CDC2 levels (Fig. 6). Similar results were also found by combined treatment with small interfering RNAs (siRNAs) against PRKD2 and PRKD3 (siPRKD2 and siPRKD3) (Supplementary Fig. S3).

**Discussion**

PRKDs are involved in a series of cancer-related processes including cell proliferation, apoptosis, differentiation, and cell cycle. Abnormal expression of PRKDs has been reported in various types of cancers, making them promising drug targets for cancer. In the present study, our results show that PRKD2 and PRKD3 are the two major PRKD isoforms expressed in breast cancer (Fig. 1). We also show that the PRKD pan-inhibitor CRT0066101 decreased TNBC cell proliferation *in vitro* and *in vivo* (Fig. 2 and 3), suggesting that CRT0066101 has

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**Fig. 6.** Effect of CRT0066101 on the protein and phosphorylation levels of key regulators in TNBC. (A) MDA-MB-231 and (B) MDA-MB-468 cell lines were exposed to increasing concentrations (0, 1, and 3 µM) of CRT0066101 for 1 day. The protein and phosphorylation levels of PRKD, MYC, MAPK1/3, AKT, YAP, and CDC2 were examined by western blot.
antitumor activity in TNBC. These results are in accord with the effects of CRT0066101 reported in many other cancers including colon and pancreatic cancers [33, 35]. In addition, our study revealed that CRT0066101 treatment induced TNBC cell arrest at the G1 phase of the cell cycle and increased TNBC cell apoptosis (Fig. 2). Taken together, these results suggest that CRT0066101's inhibition of TNBC proliferation is mediated via induction of G1-phase arrest and increasing the proportion of apoptotic cells.

Recently, several PRKD inhibitors, including CRT0066101, have been identified. Harikumar and colleagues found that CRT0066101 given orally significantly inhibits pancreatic tumor growth in vivo [33]. Wei and colleagues showed that CRT0066101 given orally suppresses colorectal cancer development in vivo [35]. Meanwhile, Borges et al. [27] demonstrated that CRT0066101 effectively targets estrogen-receptor-negative breast cancers. In our study, we show that CRT0066101 potently inhibited TNBC growth in vivo (Fig. 3). Our results, together with results from other groups, strongly indicate that CRT0066101 is an effective drug for the treatment of TNBC.

Although many reports have shown that CRT0066101 is an effective therapeutic in many different cancers, the mechanisms underlying this compound’s anticancer effects are unknown. Since PRKDs are serine/threonine kinases, phosphoproteomic analysis was used to uncover the mechanism of CRT0066101's effects in breast cancer. Through reactome and network analyses, we found multiple hub-nodes related to cell proliferation, apoptosis, and the cell cycle.

In our study, we observed that the PRKD inhibitor CRT0066101 decreased breast cancer growth in vitro and in vivo, and increased breast cancer cell apoptosis and accumulation of cells in G1 phase. MYC plays key roles in cell growth [43], cell cycle [48], and apoptosis [49], and its aberrant expression has been shown to be involved in nearly all human cancers [43]. Our study showed that CRT0066101 significantly suppressed the phosphorylation of MYC on Thr58 and Ser62 in the investigated breast cancer cell lines. These sites are well documented to regulate MYC stability. Phosphorylation at Ser62 is known to stabilize c-MYC, whereas phosphorylation at Thr58 and Ser62 leads to its degradation by the proteasome [50, 51]. Our results show that when CRT0066101 or siPRKD2&3 suppressed the phosphorylation of MYC on Thr58 and Ser62 in MDA-MB-468 cells, the level of MYC protein significantly decreased (Fig. 6B and Supplementary Fig. S3B). MYC protein levels were slightly decreased by CRT0066101 or siPRKD2&3 in MDA-MB-231 cells (Fig. 6A and Supplementary Fig. S3A). These results were consistent with recently work reported by Xiaoxin Sun [52]. The decreased MYC protein level might due to other regulation mechanisms, for example, transcription repression upon treatment of CRT0066101 or siPRKD2&3. The detail mechanism is worthy for further exploration. Meanwhile, PRKD inhibition restrained the MAPK1/3 and AKT signaling pathways. PRKD has been reported to regulate the MAPK pathway to promote cell growth [53]. In the current study, our results showed that CRT0066101 strongly decreased the phosphorylation of MAPK1/3 on Thr202 and Tyr204 and phosphor-AKT on Ser473. Taken together, these results show that the PRKD inhibitor CRT0066101 inhibits breast cancer progression via inhibition of the phosphorylation of MAPK1/3 and AKT. The Hippo-YAP pathway has been reported to mediate cell proliferation [54]. Our results show that PRKD inhibition led to a decrease in phosphorylation of YAP on Ser127. A related report showed that CRT0066101 prevented an increase in YAP phosphorylation on Ser127, YAP cytoplasmic accumulation, and increase in the mRNA levels of YAP/TEAD-regulated genes in intestinal epithelial cells. Furthermore, siRNA-mediated knockdown of PRKDs markedly attenuated YAP nuclear-cytoplasmic shuttling and phosphorylation at Ser127 [39, 55].

Cell cycle checkpoint loss is a hallmark of cancer. CDKs regulate the cell cycle at several checkpoints, including G1 and G2/M [47]. The G1 checkpoint determines whether cells go into S phase, and CDC2 plays a role in G1/S procession. In our study, the level of phosphor-CDC2 (Thr14) was decreased, whereas the level of total CDC2 did not change in the CRT0066101-treated breast cancer cells. These results suggest that the G1 arrest by CRT0066101 in breast cancer cells is due to suppression of the phosphorylation of CDC2 on
Thr14. This phosphorylation change was also confirmed using siPRKD2 and siPRKD3 (Supplementary Fig. S3).

**Conclusion**

Taken together, the results of the current study analyzing the phosphoproteome show that CRT0066101 is an effective drug for inhibiting proliferation, apoptosis, and cell cycle of TNBC via decreasing phosphorylation of MYC, MAPK1/3, AKT, YAP, and CDC2. The molecular mechanism underlying CRT0066101’s effects in breast cancer is depicted schematically in Fig. 7, and provides new insights into the functions of PRKDs and supports the use of CRT0066101 for combating TNBC.

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**Disclosure Statement**

The authors declare that they have no conflict of interests.

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