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Targeting Transcription-Regulating Cyclin Dependent Kinase 12 for the Treatment of Breast Cancer

Thesis presented by

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for the degree of

Master of Research in Biochemistry and Cell Biology

University College Cork

School of Biochemistry and Cell Biology

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Supervisor: Dr. Malgorzata Krajewska

2022

Declaration

The MSc dissertation, targeting transcription-regulating cyclin-dependent kinase 12 for the treatment of breast cancer submitted for the degree of the MRes Biochemistry and Cell Biology is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Candidate's signature _____Martha Burns_____

Date ___27/11/22_____

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Abbreviations

Abbreviation	Explanation
ATR	Ataxia telangiectasia and rad3-related
CDK12	Cyclin Dependent Kinase 12
CHD2	Chromodomain helicase DNA binding protein 2
Chk1	DNA damage checkpoint kinase 1
CycK	Cyclin K
DDR	DNA Damage Response
DSBs	Double-Stranded Breaks
FACS	Fluorescence assisted cell sorting
GI	Genome Instability
PARG	poly(ADP-ribose) glycohydrolase 1
PARP	Poly (ADP-ribose) polymerase
PCPA	Premature Cleavage and Polyadenylation
Pol II S2	RNA Polymerase II p-Serine 2
P-TEFb	positive transcription elongation factor b
RNA Pol II	RNA Polymerase II
TNBC	Triple-Negative Breast Cancer

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Abstract

Cyclin-dependent Kinase 12 (CDK12) with its binding partner Cyclin K regulates transcription through phosphorylation of RNA Polymerase II (RNA Pol II) at serine 2. Previous work showed that inhibition of CDK12 leads to decreased expression of DNA damage response (DDR) genes and sensitizes cancer cells to DNA damage-inducing agents. However, the exact mechanism by which this is achieved remains unclear. This study aimed to investigate CDK12 as a therapeutic target in breast cancer and to identify new drug combinations involving the inhibition of CDK12 and clinically relevant inhibitors of DNA repair. We demonstrated that CDK12 inhibitor SR-4835 is cytotoxic in MCF-7 breast cancer cells at a low nanomolar concentration. The observed toxicity was associated with G2-M cell cycle arrest, increased apoptosis, and DNA damage. We showed that CDK12 inhibition had a minor effect on the phosphorylation of RNA Pol II at serine 2 indicating that global transcription was not affected. Interestingly, we observed that CDK12 inhibition resulted in decreased expression of chromodomain helicase DNA binding protein 2 (CHD2), a potentially new target of CDK12. Next, we tested the combination of CDK12 inhibition with inhibitors of DNA repair including inhibitors of PARP (olaparib) and CHK1 (AZD7762) using a colony formation assay. The preliminary results indicate that combining CDK12 and CHK1 inhibition will likely have greater therapeutic potential than the combination of CDK12 and PARP inhibition. Future studies are required to establish the exact role of CDK12 in transcription and DDR as well as to

investigate further the potential of combining CDK12 and CHK1 inhibition in breast cancer.

Introduction

Breast cancer is the dominant type of cancer in women worldwide (Fig. 1), accounting for 24.5% of cancer diagnoses in women and 11.7% overall. In 2020, over 2 million new cases worldwide were recorded, and deaths accounted for 6.9% of all cancer deaths in that year (Sung *et al.*, 2021). There are 3 clinical subtypes of breast cancer: Human Epidermal growth factor Receptor 2 – positive (HER2+), oestrogen receptor-positive (ER+), and Triple Negative Breast Cancer (TNBC). As its name indicates, TNBC is deficient in all three receptors that are potential targets of treatment, i.e ER negative, HER2 negative, and Progesterone receptor (PR) negative (Perou *et al.*, 2000). There are 5 molecular subtypes of breast cancer: Basal-like, Luminal A, Luminal B, Claudin-Low, and HER2-enriched (Perou *et al.*, 2000) (Sorlie *et al.*, 2003). In terms of current breast cancer landscapes, a 2017 study identified a pattern of increasing incidence of ER+ cancers across Denmark, the United States, and Ireland and decreasing incidence of oestrogen receptor-negative cancers (Mullooly *et al.*, 2017). Breast cancer incidence is most prevalent in countries that have a higher Human Development Index, with a number of lifestyle and hormonal risk factors being especially prevalent, such as alcohol intake and oral contraceptives (Thun *et al.*, 2017). There are hereditary factors that also influence breast cancer development such as the inheritance of mutated BRCA1 and BRCA2

genes which confer a higher risk of developing breast cancer (as well as other cancers) at a younger age (van der Kolk *et al.*, 2010).

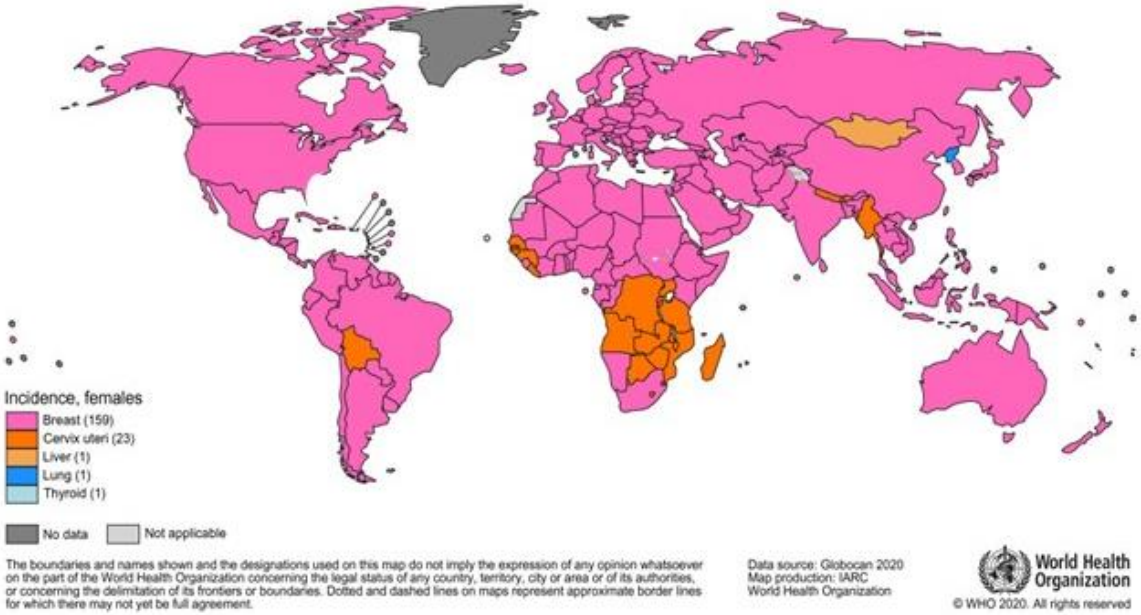


Figure 1. The most common type of cancer Incidence among women in 2020 in each country*. "The numbers of countries represented in each ranking group are included in the legend. However, nonmelanoma skin cancer (excluding introduction basal cell carcinoma), the most common type of cancer in Australia and New Zealand among men and women and in the United States among men, was excluded when constructing the global maps". Source: GLOBOCAN 2020. from (Sung et al., 2021).*

Some of the most common therapies for breast cancer include chemotherapy, surgery and radiotherapy (Nofech-Mozes *et al.*, 2009). These conventional treatments are non-specific and have harsh side effects for patients and can lead to adaptation and resistance in cancer cells (Gilreath *et al.*, 2020). For a subset of breast cancer patients, depending on subtype, these therapies can also be given in conjunction with targeted therapies. An example of this is HER2-positive cancers, where anti-HER2 antibodies such as trastuzumab and HER kinase inhibitors were developed to target HER2 overexpression for an anti-cancer therapeutic effect (Carter *et al.*, 1992; Wainberg *et al.*, 2010). However, resistance to trastuzumab has

been observed which highlights a need for the identification of more viable targets and the development of a wider arsenal of treatments.

A feature of breast cancer and a hallmark of cancer that is a frequent target of treatment is genome instability (GI). GI refers to defects in the processes that control cell division that then lead to the development of copy number variants (CNVs) and mutations. (Draviam, Xie and Sorger, 2004). To compensate for this lack of genomic stability and to continue to be able to proliferate, cancers rely on the DNA damage response (DDR). The DDR is a crucial network of pathways that maintains genome stability by detecting and repairing different types of DNA damage; or at times, triggering apoptosis or cell cycle arrest. Some familial breast cancers develop from the high mutation rate of an impaired DDR due to germline mutations in genes such as BRCA1 or BRCA2 (Lhota *et al.*, 2016). As cancers become more genomically unstable, there is an increased need for a fully functional DNA Damage Response (DDR). For this reason, CDK12 and other DDR-associated proteins have become therapeutic targets. In order to combat resistance to treatments, a wider arsenal of targets must be built that targets several aspects of the DDR and which acts in synergy.

Cyclin-dependent kinase 12, a transcription-regulating kinase

Cyclin-dependent kinases (CDKs) are key regulators of transcription and cell division and work with their respective cyclins to form complexes. CDKs can fall into one of two categories: cell cycle-regulating kinases and transcription-regulating kinases, CDK12 falls into the latter category (Bartkowiak and Greenleaf, 2011). Discovered

during cDNA screens, CDK12 was first called CRKRS before its cyclin partner Cyclin K (CycK) was elucidated (Ko, Kelly and Pines, 2001). Ctk1 was identified in metazoans as a CDK12 ortholog (Bartkowiak *et al.*, 2010). At 1,490 residues in length, CDK12 features N-terminal and C-terminal regions which contain a CTD kinase domain and 20 arginine/serine (RS) domains (Fig. 2). CDK12 shares more than 93% sequence identity with CDK13 and it is thought that much of their functions overlap (Ko, Kelly and Pines, 2001). However, it was observed that CycK forms separate CycK/CDK12 and Cyck/CDK13 complexes and the depletion of CDK12 downregulated the expression of DDR genes and the depletion of CDK13 did not (Blazek *et al.*, 2011).

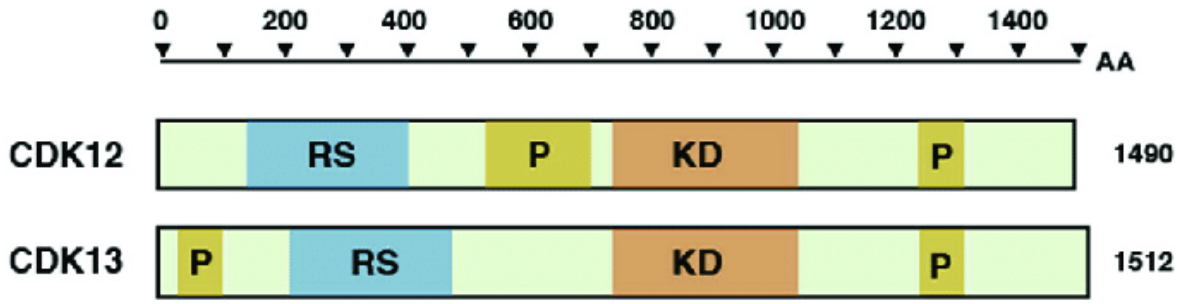


Figure 2. Comparison of the primary structures of CDK12, and CDK13.*"KD kinase domain, RS arginine/serine-rich motifs, P proline-rich motifs, CB cyclin-box, TRM TAT/TAR recognition motif, H histidine-rich segment, and PEST PEST domain. The scale indicates the length of the amino acids (AA) and proteins". Adapted from (Choi, Kim and Jones, 2020)*

CycK/CDK12 complex

A study by Bösken *et al.*, 2014 determined the crystal structure of the CycK/CDK12 complex was determined to be comparable to that of other CDK/Cyc complexes. They observed that the key difference which sets the CycK/CDK12 complex apart from other CDK/Cyc complexes is the kinase C-terminal extension formation with which the ATP substrate associates. The assembly of the two subunits also differed from other observed complexes, involving solely the N-terminal lobe of CDK12 and

the N-terminal region of CycK (Fig. 3). More specifically these interactions occur between E108 of the cyclin in helix H3 and R773 in the kinase helix motif PITAIRE, involved also is helix H5 R145. This N-terminal loop of CDK12 along with the C-terminal loop comprises the kinase fold. CDK12/Cyck is activated by CDK-activating kinase by phosphorylation of the T-loop of CDK12. This study demonstrated that substrates of CDK12 before interaction with the kinase are preferably already prephosphorylated at serine 7 in order to maximise the kinase activity of CDK12.

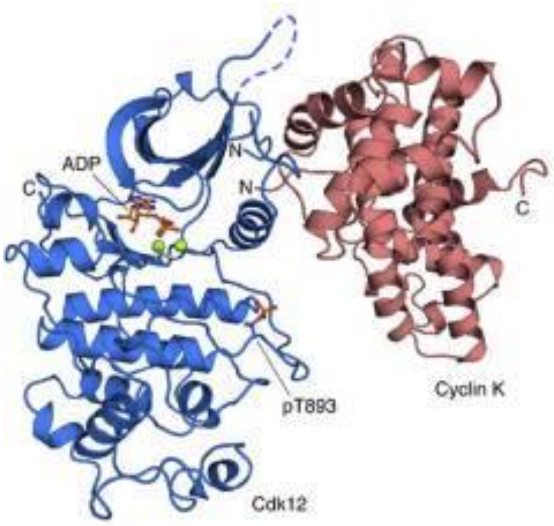


Figure 3 Structure of Cdk12 (blue)/CycK (red) complex. *"The bound nucleotide and the phospho-threonine residue in the activation segment are highlighted". Adapted from (Bösken et al., 2014).*

CDK12 function in transcription regulation

CDKs are involved in transcription beginning with the pre-initiation complex: CDK7, paused RNA Pol II: CDK9, and elongation: CDK12/13(Coin and Egly, 2015; Compe and Egly, 2012). The function of the CycK/CDK12 complex in transcription is the phosphorylation of serine 2 of RNA Polymerase II (Fig. 4)(Bartkowiak *et al.*, 2010). CycK/CDK12 is recruited to a paused RNA Pol II by PAF1C where it phosphorylates serine 2 in the CTD of RNA Pol II, releasing it from stall and initiating elongation. The

CycK/CDK12 complex acts in concert with positive transcription elongation factor b (P-TEFb) to accumulate RNA Polymerase II p-Serine 2 (Pol II S2) and to properly terminate elongation by RNA Pol II by dephosphorylation of serine 2 (Yu *et al.*, 2015; Sansó *et al.*, 2016). The CycK/CDK12 complex does not regulate the transcription of all genes, but rather a minuscule subset of long genes which take part in the DNA Damage Response (DDR) as previously mentioned. Some of the key DDR genes found to be affected by knockdown of CDK12 and CycK were ATR, FANCI, FANCD2, RAD51 and BRCA1. Therefore, CDK12 maintains genome stability through the regulation of DDR gene transcription (Blazek *et al.*, 2011). CDK12 also plays a role in mRNA processing and the expression of RNA processing factors. snoRNA genes were also downregulated when CDK12 was depleted and CDK12 was found to associate with RNA processing factors (Liang *et al.*, 2015).

On the mechanistic level inactivation of CDK12 in cancer cells was shown to cause elongation defects and premature cleavage and polyadenylation (PCPA) leading to decreased expression of long genes including DDR genes (Krajewska *et al.*, 2019). This study demonstrated that CDK12 inhibition caused elongation defects in a length-dependent matter, with longer genes being primarily affected, DDR genes comprising the top category.

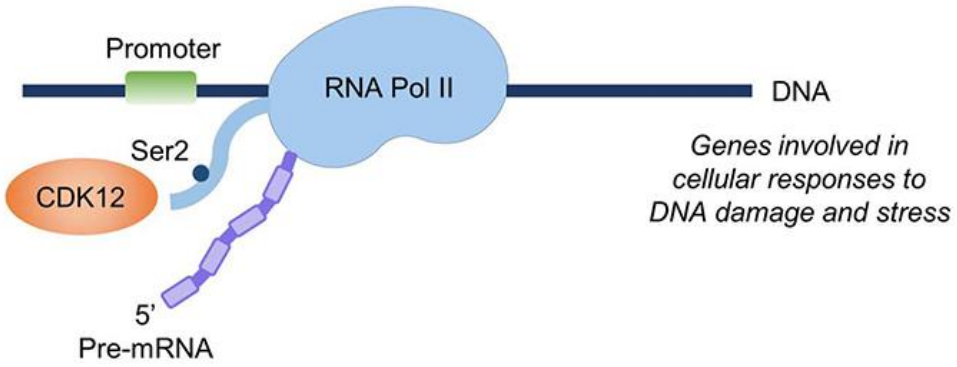


Figure 4. CycK/Cdk12 regulates transcription and phosphorylates Ser2 in the CTD of RNAPII. *"CDK12 phosphorylates RNA polymerase II (RNA Pol II) at Ser2, which promotes transcriptional elongation". Adapted from (Lui, Grandori and Kemp, 2018)*

CHD2 A potential protein partner of CDK12:

Previous studies identified chromodomain helicase DNA binding protein 2 (CHD2) as a potential substrate for CDK12 (Fig. 5) (Krajewska *et al.*, 2019). CHD2 is a chromatin remodeller that is a member of a family of SNF2-like ATPases (Flaus *et al.*, 2006). Although much is yet to be elucidated about its function, it is known that it is essential for proper development (Marfella *et al.*, 2006). CHD2 participates in non-homologous end joining (NHEJ) and is recruited by Poly (ADP-ribose) polymerase 1 (PARP1) to sites of double-stranded breaks (DSBs) by binding to PAR chains. After recruitment to the site of DNA damage by PARP1, CHD2 induces chromatin expansion and histone H3.3 deposition. These proteins then recruit and regulate NHEJ factors. Interestingly, it was found that CHD2 recruitment to DSBs was suppressed by the expression of a catalytically dead mutant PARG due to the previously mentioned competition binding for PAR chains (Luijsterburg *et al.*, 2016). CHD2 has been previously implicated in cancer as a driver gene in chronic

lymphocytic leukaemia and breast implant-associated large cell lymphoma in its mutant form (Rodríguez *et al.*, 2015; Laurent *et al.*, 2020).

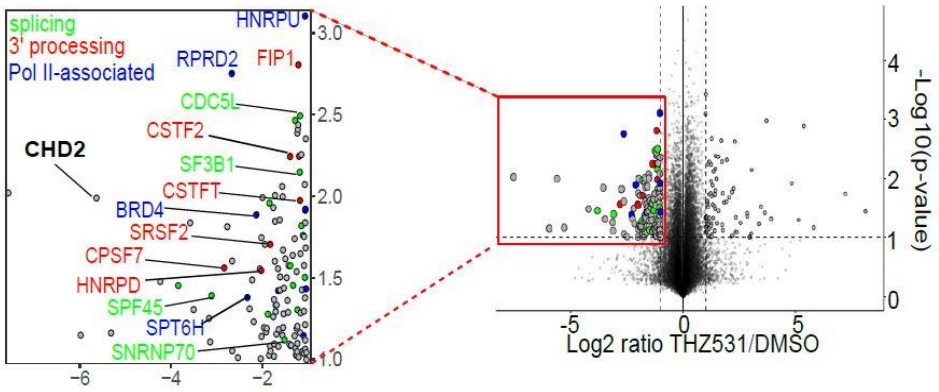


Figure 5. Volcano plot of proteome-wide changes in phosphorylation site occupancy identified through SILAC analysis of NB cells treated with CDK12, inhibitor, THZ531, 400 nM for 2 h. *"Expanded box shows selected co-transcriptional RNA processing proteins". Adapted from (Krajewska et al., 2019)*

CDK12 as a therapeutic target in breast cancer

CDK12 has been observed to promote cancer progression in breast cancer. High expression of CDK12 was associated with the maintenance of stemness in breast cancer cells, increased tumourgenicity and other such traits associated with progression (Peng *et al.*, 2020). It was also found to play an active role in HER2-positive tumours (Mertins *et al.*, 2016). In normal breast cells, CDK12 expression was absent, further highlighting the contrast with the aggressive breast cancer infiltrating ductal carcinomas in which CDK12 is overexpressed (Capra *et al.*, 2006). Another study also points to amplification of CDK12 as a possible biomarker for breast cancer prediction. This study not only confirmed the mentioned tumourgenicity of high CDK12 expression but revealed CDK12 as a driver of trastuzumab resistance in HER2-positive cancers (Choi *et al.*, 2019; Spector and Blackwell, 2009; Vogel *et al.*, 2002). Therefore, CDK12 inhibitors may be highly beneficial in breast cancer with amplification of both CDK12 and HER2 amplification as an alternative to antibody-based therapies which have a lower than 50%

response rate and to which cancers may acquire resistance (Spector and Blackwell, 2009; Vogel *et al.*, 2002). In fact, CDK12 inhibition has already been shown to sensitise HER2-positive breast cancers to a tyrosine-kinase inhibitor (Li *et al.*, 2021).

In addition to HER2-positive cancers, CDK12 inhibition has also been observed to increase sensitivity to the PARP inhibitor olaparib in BRCA1 and BRCA2 mutated cancers. This study targeted a CDK12 inhibitor as a potential combination therapy due to the DDR status being an important factor in determining olaparib cytotoxicity (Bajrami *et al.*, 2014). CDK12 inhibition was also found to be effective in the treatment of TNBC. CDK12 inhibitors create a new avenue for targeted treatment in this genomically unstable cancer (Boyle, 2012). CDK12 inhibition was found to reverse olaparib resistance (Johnson *et al.*, 2016). In another study, CDK12/13 inhibitor SR-4835 was shown to cause TNBC cell death by itself and was also observed to act in synergy in combination with olaparib (Quereda et al., 2019). To enhance the effects of PARP inhibition further, combination therapies of the inhibition of other DDR proteins are being explored. DDR protein Ataxia telangiectasia and rad3-related (ATR), which is also an effective monotherapy, showed tumour regression in combination with olaparib (Wilson et al., 2022).

In ER+ metastatic breast cancer, CDK4/6 inhibitor Palbociclib has been shown to inhibit proliferation and act synergistically in combination with endocrine therapy (Finn *et al.*, 2009). However, in another study that investigated the continued treatment of patients with the aforementioned inhibitor, the median progression-free survival was found to be 5.3 months (Seki *et al.*, 2022). This warrants further

exploration into the treatment of ER+ breast cancer with other CDK inhibitors to produce better outcomes for patients.

Although, its observed potential for several subtypes of breast cancer as mentioned above as both a monotherapy and synergistic partner, the exact mechanism of CDK12 in transcription and DNA damage response remains unclear. Importantly, most of the currently available studies established the significance of targeting CDK12 in HER-positive and triple-negative breast cancer. In this study, we aim to assess the therapeutic significance of targeting CDK12 in ER-positive breast cancer using MCF-7 cells as a model. This overarching aim will be addressed in the following objectives:

1. Investigate the potential of CDK12 as a therapeutic target in breast cancer using publicly available databases including cBioPortal and R2.

2. Assess the effect of CDK12 inhibitor SR-4835 on cell viability, transcription and DNA damage in MCF-7 breast cancer cells.

3. Test the combination of CDK12 inhibition with inhibitors of DNA repair currently in clinical studies including PARP inhibitor (olaparib) and CHK1 inhibitor.

Materials and Methods

Online Data Analysis

cBioPortal

From the list of studies available to be explored, breast cancer studies were selected that were not subtype-specific. Only unique datasets were selected for further analysis. Query by gene was used and CDK12 was then entered on a user-defined list. The genomic profiles chosen were: mutations, structural variant and

putative copy-number alterations which were identified by Genomic Identification of Significant Targets in Cancer (GISTIC). The patient/case set selected was samples with mutation and CNA data. Graphs shown in Fig 7 A and B were generated in the 'Cancer Types Summary' tab.

R2

The Kaplan-Meier plot was generated in the R2 database. The dataset selected was Mixed Tumor Breast - Clynes - 121 - MAS5.0 - u133p2. The gene was entered, and the type of survival was selected as overall survival, the minimum group size entered was set at default, 8.

Cell Culture

Breast cancer cells, MCF-7 were maintained in RPMI (Sigma, #R8758) media supplemented with 10% Fetal Bovine Serum (FBS) (SIGMA F9665) and 1% PS (Penicillin – Streptomycin, Sigma # P4333). MDA-MB-231 cells were maintained in DMEM (MDA-MB-231, Sigma, #D6429) media supplemented with 10% FBS and 1% PS (Penicillin – Streptomycin, Sigma #P4333). Cells were cultured at 37^0C and 5% CO2. All cell lines were tested for mycoplasma.

Cell Viability Assay

MCF-7 cells were plated in a 96-well plate at 2,000 cells/well. After 24 h, cells were treated with increasing concentrations of CDK12 inhibitor (SR-4835) ATR inhibitor (AZD6738), PARP1 inhibitor (AZD2281), and Chk1 inhibitor (AZD7762). After 72 h 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Millipore, #475989 EMD, at 5mg/ml) reagent was added, and the plate was incubated in the

dark for 4 h. DMSO was then added to dissolve formazan crystals which formed as a product of the reduction of the tetrazolium salts contained in the MTT by reductases and dehydrogenases and which would cause inaccurate measurement of absorbance. Absorbance at 570 nm and 630 nm was measured using a plate reader (Thermo Scientific Multiskan GO). Further analysis using Graphpad PRISM 8.4.3 was performed using linear regression analysis to determine the concentration at which there was 50 % cell viability (IC50).

Western Blot sample preparation

MCF-7 cells were plated in 10cm cell culture dishes at 2-3 million cells/plate. Cells were treated after 24 h with DMSO control or SR-4835 at the concentrations indicated. Samples were collected by trysinisation (Trypsin, Sigma #T3924) at the time points indicated. Cells were pelleted by centrifugation at 3500 rpm for 3 mins at 4^0C and stored at -80**°**C until use**.** Cells were lysed at 4°C in lysis buffer containing Radio-Immunoprecipitation Assay (RIPA) buffer (25 nM Tris-HCl, 150 nM NaCl, 1% NP-40, 1% SDS, dH_2O), 20% cOmplete protease inhibitor (Roche, #05056489001), 20% phOsSTOP protease inhibitor (Roche, #04906837001) and 1% Phenylmethylsulfonyl fluoride (PMSF) (stock concentration of 100 nM).

Bradford assay

A Bradford assay was used to determine the protein concentration of each sample. Dilutions (listed below) of Bovine Serum Albumin (BSA) using a stock concentration of 1 mg/ml (Sigma, #A8022) were prepared as a calibration curve (BSA µg/ ml: 0, 10, 50, 100, 150, 200, 250, 300,350,400. Cell lysate samples were diluted 1:25 in distilled water.

20 µL of protein sample was added to a 96-well plate in duplicate and 180 µL Bradford reagent (Sigma, #B6916) was added to each well. The plate was incubated in the dark for 5 mins before absorbance at 595 nm was measured using an 800 TS BIOTEK absorbance reader. The protein concentration of samples was determined using the BSA calibration line equation. 50-65 ug protein samples were then prepared with distilled water and dye (4X protein loading buffer LI-COR, #928-40004) supplemented with β-mercaptoethanol (Sigma, #BCCB9882) at 10%. Samples were boiled at 95°C in a heating block and stored at room temperature until loaded onto the gel.

Western Blotting

Protein lysates were resolved on 6-12% (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) SDS-PAGE gels. Chameleon Duo Pre-Stained Protein Ladder (LI-COR, 928-60000) was used to estimate the molecular weight of proteins. Next, samples were transferred to nitrocellulose membranes (AmershamTM ProtranTM 0.2µm NC (GE Healthcare #10600001)) with transfer buffer (10X stock concentration 25 nM Tris base **(**Sigma, T6791) 192 mM Glycine (Sigma #SLCH2838**)** (diluted to 1X before use) supplemented with 0.1% sodium dodecyl sulfate (SDS) at 100V for 1 h or at 30V overnight (16 h at 4^0C). Following this, membranes were blocked with 5% BSA/Milk Tris-Buffered Saline-Tween 20 (TBS-T) or Intercept Blocking Buffer (LICOR, #.927-60001). Membranes were incubated at 4 °C overnight with primary antibodies. Membranes were incubated with secondary antibodies in the dark for 1 h in order to detect protein use. Antibodies used in the study are listed in Table 1. Membranes were scanned with LiCor Odyssey IR Gel Scanner, quantified using Image Studio Lite Ver 8.2 and analysed in Excel**.**

Table 1. List of antibodies used in the study

Primary Antibodies

Antibody	Company	Catalogue #	Dilution
CHD2	EMD Millipore	MABE873	1:1000
CDK12	Cell Signaling	11973	1:1000
RNA Pol II	Santa Cruz Biotechnology	Sc-55492	1:500
RNA Pol II S2	EMD Millipore	04-1571-I	1:1000
GAPDH	EMD Millipore	2118	1:1000
H2AX	Cell Signaling	9718	1:1000
cPARP	Cell Signaling	9541	1:1000

Secondary Antibodies

Antibody	Company	Catalogue #	Dilution
IRDye 800 CW Goat anti-rat	LI-COR	925-32219	1:10000
IRDye 800 CW Goat anti-rabbit	LI-COR	926-32211	1:10000
IRDye 680 RD Goat anti-mouse	LI-COR	926-68070	1:10000

Colony Formation Assay

1,000 MCF-7 cells or 4,000 MDA-MB-231 cells were plated in a 6-well plate in 2 mL of cell culture media. Cells were treated the following morning with the indicated drug doses for a total volume of 4 mL media in each well, with a DMSO control and treatment of CDK12 inhibitor SR-4835 at 25 nM. Plates were incubated for 10-14 days, washed with PBS, permeabilized with ice-cold methanol and stained with crystal violet (0.2% crystal violet: Sigma #C6158, methanol). Plates were scanned with an Epson Perfection v750 pro scanner and quantified using a fisherbrand counter pen. Colonies containing 50 cells, or more were counted. Data were analysed using Graphpad PRISM 8.4.3. Plates were also quantified using ImageJ. Images were converted to 8 bit and the relevant well to be counted, selected. The threshold was

then set at an appropriate level and particles analysed which gave a count which were further analysed using Graphpad PRISM 8.4.3

Table 2. List of inhibitors used in the study

Drug	Target	Company	Catalogue #
SR-4835	CDK12	Axon medchem	3184
AZD2281	PARP1/2	Axon medchem	1464
AZD7762	Chk1	Axon medchem	1399

RNA isolation and cDNA synthesis

2 million MCF-7 cells were plated in 10cm dishes. After 24h cells were treated with SR-4835 (100 nM) and collected by trypsinisation. Cells were pelleted by centrifugation at 3500 rpm for 3 mins at 4^0C and stored at -80°C if not immediately used for RNA isolation. RNA was isolated using GeneJET RNA Purification Kit (Thermo Scientific, #K0731) as per manufacturer protocol. Briefly, pelleted cell pellets were resuspended in lysis buffer and passed through the supplied purification column and centrifuged. Wash buffers were then passed through the column and centrifuged before purified water was passed through the column and centrifuged to elute RNA. RNA was measured using DeNovix DS-11 Spectrophotometer.

1 µg of purified RNA was reverse transcribed to yield cDNA using Kit: QuantiTect Reverse Transcription Kit (Qiagen, #205311) using a Thermal cycler (Eppendorf vapo.protect mastercycler pro 6321AN014319 or MJ Research DNA Engine PTC-200 Peltier Thermal Cycler). Briefly, template RNA was incubated with gDNA wipeout buffer and RNase-free water and incubated at 42^0C for 2 mins. Reverse transcriptase, buffer and primers were added to the template RNA. Using a thermal

cycler, samples were incubated at 42^{0}C for 15 mins, then 95^{0}C for 3 mins and stored at -20^{0}C.

Quantitative Reverse Transcription PCR (RT-qPCR)

RT-qPCR was performed using FastStart Essential DNA Green Master used at 1x (from FastStart Essential DNA Green Master kit #06 402 712 001 Version 07 Roche). Primers for BRCA1, CHD2 and GAPDH were used at a concentration of 250 nM from 5 µM working stock.

Table 3. Primer Sequences

	Forward (5'-3')	Reverse (3'-5')
BRCA1	TTGTTGATGTGGAGGAGCAA	CAGATTCCAGGTAAGGGGTTC
CHD2	CGAAAACAGGCACTGGACCACT	GATGACGACTGTGTCCGCTGAA
GAPDH	CACCCACTCCTCCACCTTTG	CCACCACCCTGTTGCTGTAG

Analysis was carried out using LightCycler96 machine and quantified using Light Cycler96 SW 1.1 Software and analyzed using Graphpad PRISM 8.4.3, error bars representing the standard deviation between replicates.

Cycles were carried out as below:

a. Preincubation: 1 cycle
b. Step: 95°C for 600s; Acq. 3 Step Amplification: 45 cycles
    i. Step: 95°C for 10s; Acq.
    ii. Step: 67°C for 60s; Acq.
    iii. Step: 72°C for 30s; Acq.
c. Melting: 1 cycle
    i. 95°C for 10s; Acq.
    ii. 65°C for 60s; Acq.
    iii. 97°C for 1s; Acq.

Flow Cytometry Cell Cycle Analysis

Cells were plated and after 24 h treated with 100 nM SR-4835 or DMSO and collected at 24 h by trypsinization. Cells were pelleted by centrifugation at 1500 rpm for 3 mins, resuspended in 5 mL ice-cold 70% ethanol added dropwise on a vortex. Samples were then stored at -20^{0}C for a maximum of 2 weeks. To prepare samples for Fluorescence Assisted Cell Sorting (FACS), cells were centrifuged at 5 mins at 1,500 rpm and ethanol removed. Cells were resuspended in 1 mL PBS and centrifuged for 5 mins at 3500-5000 rpm and PBS was removed. Cells were resuspended in 1 mL PBS-Tween (PBS, 0.05% Tween20 Sigma P1379) and centrifuged for 5 mins at 3500-5000 rpm and PBS-tween removed. Cells were finally resuspended in 700 µL of PBS-tween buffer supplemented with –BSA (2%), RNAseA (50µg), propidium iodide (PI) (5µg) (PBS, 0.05% Tween, 2-3% BSA,1.92% PI,1.92% RNAse A Sigma #R6513). Cells were added to the FACS tube by passing through a filter into the FACS tube. Samples were analysed using a BD FACSCalibur flow cytometer.

FACS data analysis and gating strategy.

Data was analysed using flowjo v10.8.0 software and Graphpad PRISM 8.4. Viable cells and single cells were selected by size. Live single cells were selected on FSC-H vs. SSC-H scatter plot on which a drawing tool was used to encapsulate such cells and eliminate non-viable cells (Fig. 6 A). Single cells were selected on the FL2-A vs. FL2-W plot by using a drawing tool to exclude non-singular cells (Fig. 6 B). An example of gating strategy is indicated below:

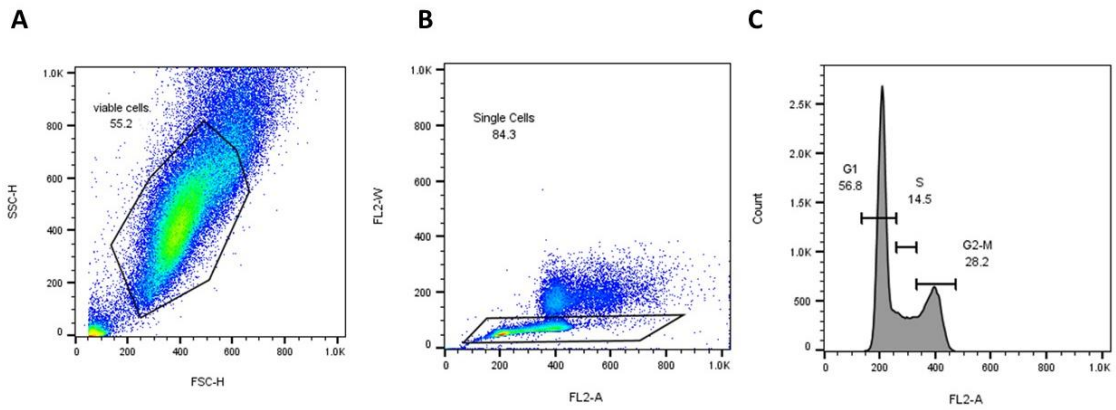


Figure 6. Gating strategy for FACS analysis *(A) Selection of viable cells encapsulated by the drawing tool on FSC-H vs SSC-H plot indicates all viable cells in the samples. (B) Selection of single cells encapsulated by the drawing tool on FL2-A vs FL2-W plot. Indicates single cells(C) Histogram of proportion of cells in each stage of the cell cycle based on the single cell population depicted in B.*

Results

Amplification of CDK12 is associated with lower rates of overall survival in breast cancer.

The preliminary step in the investigation into CDK12 as a therapeutic target for breast cancer treatment was assessed through online resources and databases. CDK12 alterations were assessed in breast cancer using the CBioportal database. Analysis of different breast cancer datasets revealed that CDK12 amplification is the most common CDK12 alteration (up to 14%) occurring in breast cancer overall in comparison to structural variants, deep deletion and multiple alterations (Fig. 7A). This is compared with HER2 which shows very similar levels of amplification (Fig. 7B). Next, we found that amplification of CDK12 correlates with worse overall survival of breast cancer patients (Fig. 7C). Together these analyses indicate that CDK12 is promising therapeutic target for breast cancer treatment.

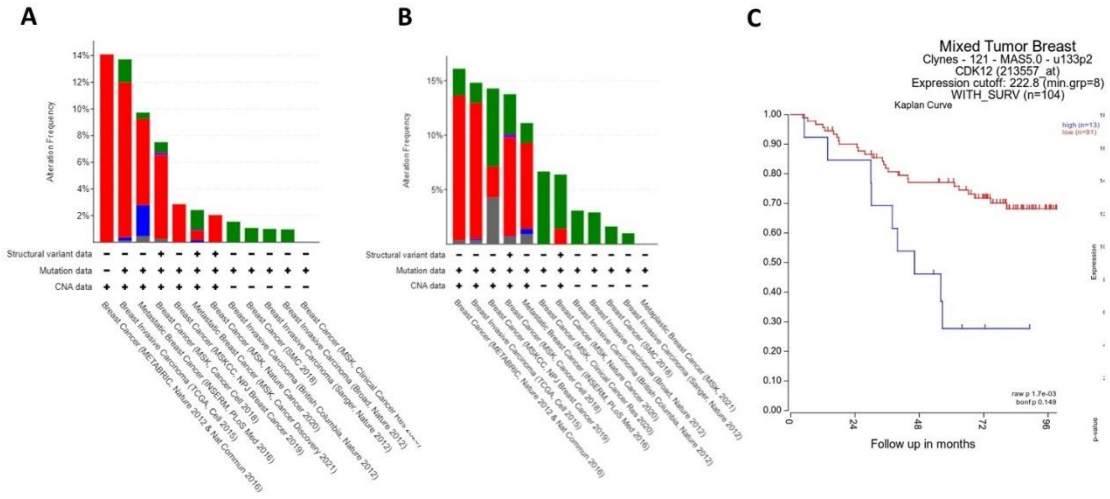


Figure 7. Higher CDK12 expression is associated with lower survival in breast cancer *(A) A plot of alterations (mutation-green, structural variant-purple, amplification-red, deep deletion-blue and multiple alterations-grey) of CDK12 in several listed breast cancer studies adapted from CBioPortal (https://www.cbioportal.org/) (B) A plot of alterations (mutation-green, structural variant-purple, amplification-red, deep deletion-blue and multiple alterations-grey) of HER2 in several listed breast cancer studies adapted from CBioPortal (https://www.cbioportal.org/) (C) Kaplan-Meier plot of CDK12 where blue corresponds to high expression of CDK12 and red corresponds to low expression of CDK12 in the mixed tumour breast dataset. (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi)*

CDK12 inhibitor SR-4835 is cytotoxic to breast cancer cells.

The cytotoxicity of CDK12 inhibitor SR-4835 (Table 2) at which 50% cell death was observed (IC50) in MCF-7 cells was assessed. MCF-7 cells are an ER-positive invasive breast adenocarcinoma cell line with wild-type p53 status and fall into the molecular category of Luminal A (Brooks, Locke and Soule, 1973; Soule *et al.*, 1973). The IC50 was determined after a treatment period of 72 h. The IC50 of SR-4835 was determined to be 160.3nM (average of three replicates, with a standard deviation of 34.9) (Fig. 8 A). To further assess the cytotoxicity of the CDK12 inhibitor, a lengthier colony formation assay was performed in MCF-7 cells. Cells were treated in a 6-well plate for 10 days with concentrations ranging from 10 nM-200 nM before permeabilising with methanol and staining with crystal violet (Fig. 8 B, C). Survival in both assays was significantly diminished at concentrations of over 100

nM, showing the efficacy and potential of CDK12 inhibition in a breast cancer cell line.

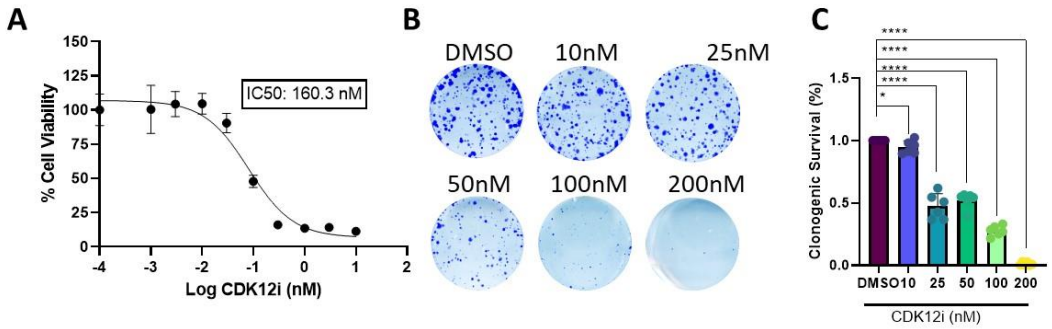


Figure 8. CDK12 inhibition is cytotoxic to MCF-7 cells *(A) Dose-response curve for MCF-7 cells treated with increasing concentrations of CDK12 inhibitor SR-4835 for 72 h. Cytotoxicity is reported as percent cell viability relative to DMSO-treated cells (B)Clonogenic formation assay of MCF-7 cells treated with indicated concentrations of SR-4835. (C)Quantification of (B) where * represents a p-value of 0.0310 and **** represents a p-value of <0.0001 using unpaired t-test analysis (average of 2 replicates).*

CDK12 inhibition causes G2-M cell cycle arrest

To further characterise the effects of CDK12 inhibition on the breast cancer cell line MCF-7, cell cycle analysis was carried out. MCF-7 cells were treated with 100nM SR-4835 and harvested at 24hr and 48hr time points. Cells were stained with propidium iodide (PI), an analogue of ethidium bromide which binds to DNA or RNA and is not permeable to the membrane. Fluorescence Activated Cell Sorting (FACS) was used to analyse the distribution of a cell population in each phase of the cell cycle: G1, S, and G2-M. This system also distinguishes between live and dead cells, the latter of which were eliminated from the cell cycle histogram through gating strategy (Fig. 6). Gating strategy also isolated live, single cells for analysis by their mass. After treatment with CDK12 inhibitor SR-4835 for 48 h, the proportion of cells in the G1 and S phases have decreased and we observed an accumulation of cells in G2-M indicating G2-m cell cycle arrest (Fig. 9 A,B). After 24 h, the proportion

of cells in the G1 and S phases did not significantly differ between treated and untreated cells, showing that this effect of CDK12 inhibition on cell cycle progression is time-dependent.

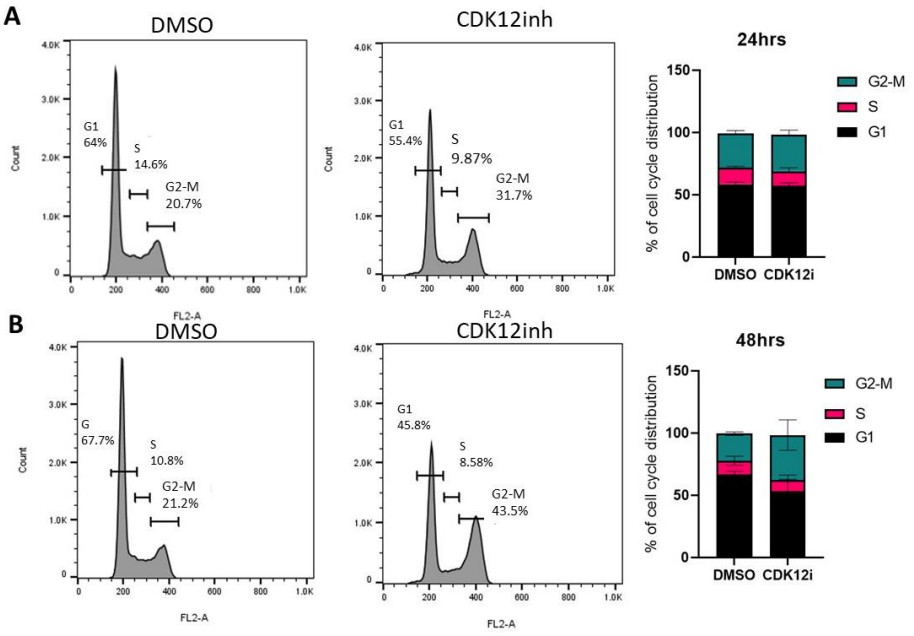


Figure 9. CDK12 inhibition induces G2-M cell cycle arrest *(A)Cell cycle analysis of MCF-7 cells treated with 100nM CDK12 inhibitor for 24 h and quantification of cell cycle analysis (left) (B) Cell cycle analysis of MCF-7 cells treated with 100nM CDK12 inhibitor for 48 h and quantification of cell cycle analysis(average of 2 replicates) (left).*

CDK12 inhibition induces apoptosis and DNA damage.

To evaluate the effects of CDK12 inhibition on apoptosis, the expression of an apoptosis marker was measured using a western blot. DNA repair pathway protein PARP is cleaved by caspase-3 or TGF-β1 to form cPARP in the process of apoptosis (Yang, Zhao and Song, 2004). MCF-7 cells were treated with the indicated concentrations of CDK12 inhibitor and collected at 6hr and 24hr time points. cPARP was detected after 24 h and increased in a dose-dependent manner, revealing that CDK12 inhibition induced apoptosis (Fig. 10 A,B). In order to assess whether CDK12

inhibition induces DNA damage, a western blot was done to determine the expression of phospho-H2AX (p-H2AX) after treatment with a CDK12 inhibitor. H2AX is a member of the H2A histone family which becomes phosphorylated in the presence of DNA damage namely, the formation of double-stranded breaks (DSBs) (Rogakou *et al.*, 1998). An increase in expression of p-H2AX was observed after 24hr treatment, in a dose-dependent manner (Fig. 10 C,D). Together these results indicate that CDK12 inhibition leads to increased DNA damage and apoptosis in breast cancer cells.

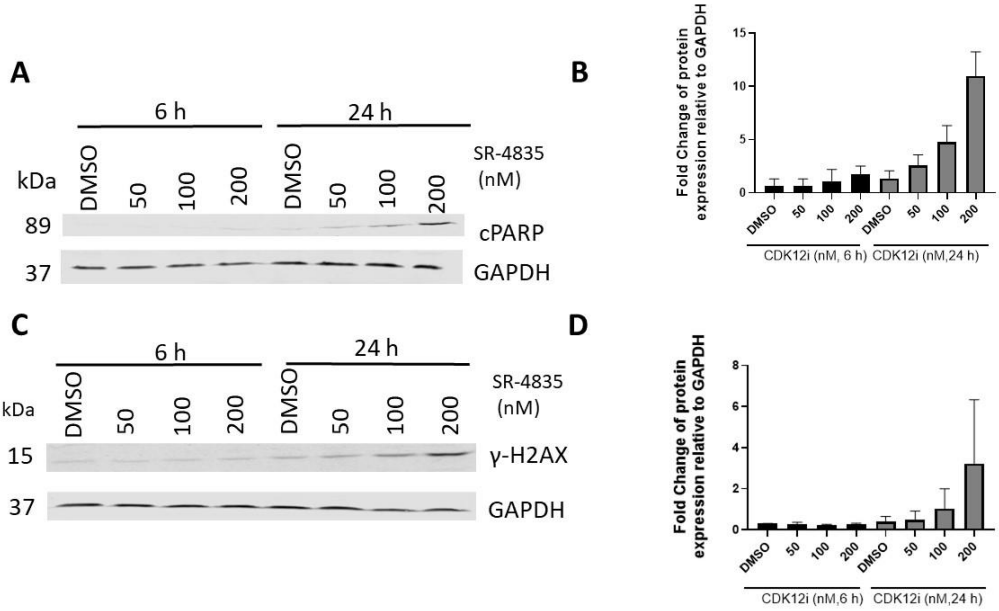


Figure 10. CDK12 inhibition causes apoptosis and DNA damage in breast cancer cells. *(A) Western blot analysis of cleaved PARP (cPARP) in MCF-7 cells treated with CDK12 inhibitor at the indicated concentrations and for the indicated times. GAPDH was used as loading control. (B) Quantification of western blot (left) (average of 2 replicates) (C) Western blot analysis of p-H2AX in MCF-7 cells treated with CDK12 inhibitor at the indicated concentrations for the indicated times. GAPDH was used as loading control. (D) Quantification of (C) (average of 2 replicates).*

CDK12 inhibition does not significantly affect global transcription

CDK12 is known to function in the regulation of transcription of DDR genes through the phosphorylation of serine 2 of RNA Polymerase II (RNA Pol II) (Blazek *et al.*, 2011). The next step in this investigation was to measure the effects of CDK12 inhibition on the expression of RNA Pol II and RNA Pol II Serine 2 (RNA Pol II S2), a marker of transcription elongation, through western blot. MCF-7 cells were treated and collected as previously described before western blot was undertaken. The effect observed on the expression of phosphorylated RNA Pol II was minimal at 6 h post-treatment and more pronounced but not completely suppressed by the 200 nM 24 h timepoint (Fig. 11 A, B), relative to RNA Pol II protein levels. These results indicate that CDK12 does not majorly affect global transcription elongation.

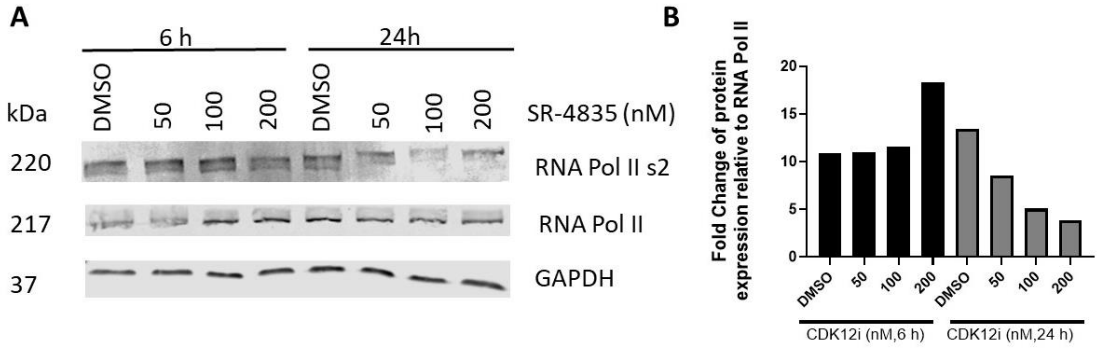


Figure 11. CDK12 inhibition has minimal effect on RNA Pol II phosphorylation. *(A) Western blot analysis of RNA Polymerase II and RNA Polymerase II Serine 2 (S2) in MCF-7 cells treated with CDK12 inhibitor SR-4835 at indicated concentrations for 6 h and 24 h. (B) Quantification of (A).*

CDK12 inhibition affects expression of BRCA1 and CHD2

Chromodomain helicase DNA binding protein 2 (CHD2) is a chromatin remodeler that participates in non-homologous end joining (NHEJ) through recruitment by PARP1. CHD2 was also identified as a potential protein partner

or substrate of CDK12 (Krajewska *et al.*, 2019). We first assess the effect of CDK12 inhibition on CHD2 expression. In these experiments, we also assess the level of BRCA1 expression (positive control) which is a known transcriptional target of CDK12. To investigate CHD2 expression after CDK12 inhibition, qPCR was performed with BRCA1 expression also being measured for comparison. As expected, BRCA1 expression was repressed while the effect of the expression of CHD2 was minimal at lower concentrations (Fig. 12 A). However, the expression of CHD2 was decreased at the highest concentration of 100-200 nM of SR-4835. This decrease was not due to the cytotoxic effect of the CDK12 inhibitor as can be seen by the GAPDH levels. Subsequently, western blot analysis revealed that CHD2 protein expression was decreased by approximately 50% in cells treated with SR-4835, compared to DMSO-treated cells. Expression of BRCA1, used as a positive control was inhibited after 24 h in a dose-dependent manner (Fig 12. B and C). Together, these results indicate that CHD2 is a possible transcriptional target of CDK12 (Fig. 12 B,C).

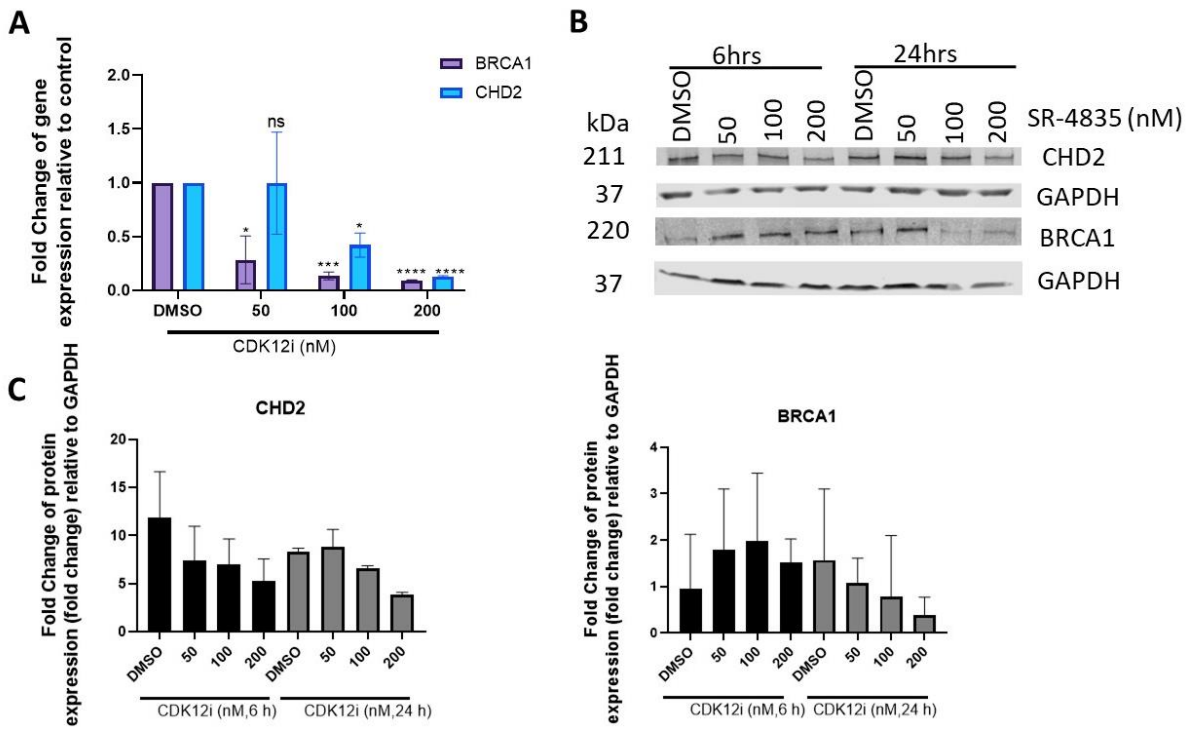


Figure 12. CDK12 inhibition affects BRCA1 expression. *(A) RT-qPCR analysis of MCF-7 cells treated with CDK12 inhibitor SR-4835 at indicated concentrations for 6 h, where ns represents a P-value of >0.05, * represents a p-value of 0.0183 and 0.0049, *** represents a P-value of 0.0009, and **** represents a P-value of <0.0001; unpaired t-test. (B) Western blot analysis of CHD2 and BRCA1 in MCF-7 cells treated with SR-4835 at indicated concentrations for 6 h and 24 h. (C) Quantifications of (B).*

Combination of CDK12 inhibitor and PARP1/2 did not significantly decrease clonogenic survival of breast cancer cells.

As using combinations of inhibitors can lead to a more favourable response to treatment and reduce the likelihood of resistance, targeting other proteins in combination with CDK12 inhibition was investigated. We also observed a minor increase in cPARP expression, demonstrating the CDK12 inhibition did not induce strong apoptosis so a synergistic combination would be beneficial (Fig 10 A and B). Given the role of CDK12 in regulating the expression of DNA repair genes and previous studies, we focused on inhibitors of DNA repair replication in combination experiments. To assess if the combination of CDK12 inhibition and DNA repair inhibition using PARP inhibitor would significantly decrease the survival of MCF-7

cells, colony formation assays were performed. The selection of the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib, AZD-2281 (Table 2) was due to the evidence of synergy with SR-4835 in MDA-MB-231 cells, demonstrated also in (Quereda *et al.*, 2019). The IC50 of olaparib in MCF-7 cells was first determined to be 159.4 μM (Fig. 13 A). A clonogenic assay was then performed with a 10-day treatment with a PARP inhibitor to establish a concentration that would be suitable for a combination assay with CDK12 and to observe the cytotoxic effects of PARP inhibitor olaparib in MCF-7 cells (Fig. 13. B). Once established, the combination assay was performed, and a significant decrease in clonogenic survival was not observed in the combination (Fig. 13 C). To assess the clonogenic survival of PARP inhibition and CDK12 inhibition combination, the assay was repeated in MDA-MB-231 cells, a triple negative cell line and once again, a significant decrease of survival in the combination was observed at the concentrations used, however, this may be an additive effect of the use of two inhibitors (Fig. 13 D). Further optimisation and a wider number of proliferation and cytotoxic assays are needed in order to continue to investigate the lack of significant difference in clonogenic survival between the combination assays and CDK12 inhibition on its
own.

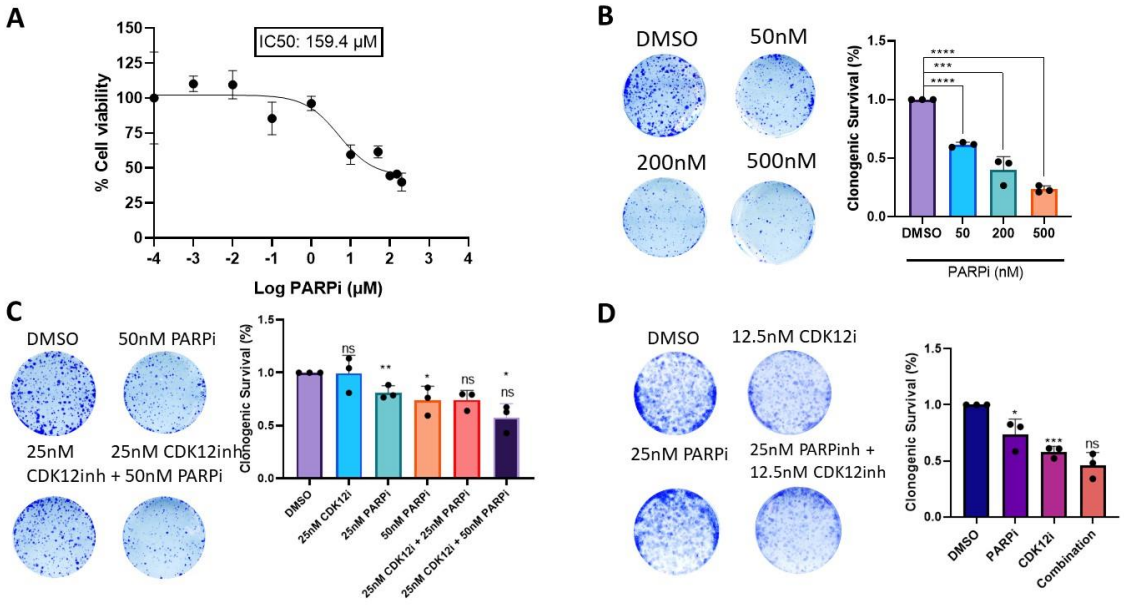


Figure 13 CDK12 inhibition combined with PARP inhibition did not have a significant effect on clonogenic survival *(A) Dose-response curve for MCF-7 cells treated with increasing concentrations of PARP inhibitor olaparib for 72 h. Cytotoxicity is reported as percent cell viability relative to DMSO-treated (B) Long-term clonogenic survival assay of MCF-7 cells treated with DMSO, 50nM, 200nM and 0.5uM of PARP inhibitor for 10 days and quantification (right) where *** represents a p-value of 0.0008 and **** represents a p-value of <0.0001; unpaired t-test. (C) Colony formation assay of MCF-7 cells treated with DMSO, 25nM CDK12inh, 50nM PARPi, 25nM PARP inhibitor and a combination of 25nM CDK12inhibitor and 25nM or 50nM PARPi and quantification (right) where ns represents a p-value of 0.9962 between DMSO and 25nM CDK12i, and ** represents a p-value of 0.0068 between DMSO, * represents a p-value of 0.0281 between DMSO and 50nM PARPi, where ns represents the p-value of 0.0827 between 25nM PARPi and 25nM CDK12i + 25nM PARPi and p-value of 0.0827 in 25nM CDK12i vs 25nM CDK12i + 25nM PARPi, and where * represents a p-value of 0.0269 between 25nM CDK12 and 25nM CDK12i + 50nM PARPi and ns represents a p-value of 0.2073 between 50nM PARPi and 25nm CDK12i + 50nM PARPi. ; unpaired t-test. (D) Colony formation assay of MDA-MB-231 cells treated with DMSO, 12.5nM CDK12i, 25nm PARPi and combination 25nM PARPi and 12.5nM CDK12inh and quantification (right) where * represents a p-value of 0.0275 between DMSO and PARPi, ** represents a p-value of 0.0012 between DMSO and CDK12i and ns represents a p-value of 0.0521 between PARPi and combination and a p-value of 0.1689 between CDK12i and combination; unpaired t-test*

ATR is a DNA damage response (DDR) protein whose expression is regulated by CDK12 and Cyclin K (Blazek *et al.*, 2011)(King *et al.*, 2021). DNA damage checkpoint kinase 1 (CHK1) is a target of ATR (Wang *et al.*, 2006) and has already been shown to be cytotoxic in breast cancer cells. In MCF7 cells, we determined the IC50 of Chk1 inhibitor AZD7762 (Table 2) to be 559nM (Fig. 14 A). A Chk1 inhibitor was used in a combination assay and quantification determined that the inhibition of the MCF-7 colony formation in the combination condition was significant between both CDK12 inhibitor and CHK1 treatments (Fig. 14 B). Future investigations would see

further optimisation of the inhibitors tested as well as a wider array of assays and protein targets.

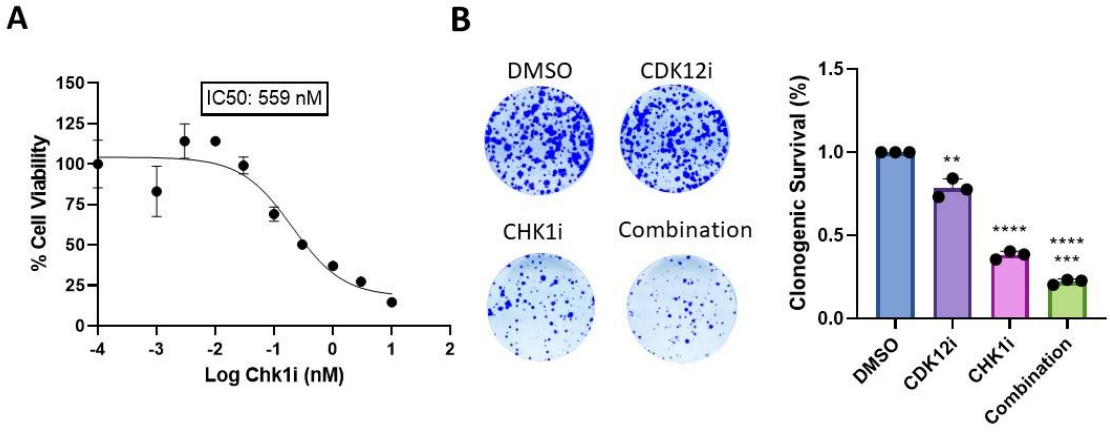


Figure 14 Combined CDK12 and CHK1 inhibition has an additive effect on colony formation. *(A) I Dose-response curve for MCF-7 cells treated with increasing concentrations of Chk1 inhibitor AZD7762 for 72 h. Cytotoxicity is reported as percent cell viability relative to DMSO-treated (B)Combination colony formation assay of MCF-7 cells treated with DMSO, 25nM SR-4835, 1uM Chk1i and a combination for 10 days and quantification (right) where ** represents a p-value of 0.0026 between DMSO and CDK12i, **** represents a p-value of <0.0001 between DMSO and CHK1i and CDK12i and combination, *** represents a p-value of 0.0007 between CHK1 and combination; unpaired t-test*

Discussion

In this study, we explored the effects of an ATP-competitive, selective CDK12/13 inhibitor drug: SR-4835 (Dieter *et al.*, 2021). We measured it effect of CDK12 inhibition on the cell cycle, apoptosis, RNA Pol II phosphorylation, and DDR protein expression and tested the combination of CDK12 inhibition with inhibitors of DNA repair and replication checkpoint. We established and quantified the cytotoxic effects of CDK12 inhibition in MCF-7 cells and demonstrated that DDR gene expression is inhibited in the absence of CDK12, leading to increased DNA damage

likely due to impaired DNA repair. Accumulation of DNA damage observed with CDK12 inhibition leads to cell cycle arrest and in consequence cell death.

When using online datasets to establish CDK12 as a potential target for breast cancer treatment, amplification of CDK12 was found to be the most common alteration in the datasets, providing a large target population. One of the datasets included in the results, METABRIC, had amplification of CDK12 in up to 14% of breast cancers in this study (Fig. 7 A). This is a significant proportion of the cohort for which the inhibition of CDK12 could be beneficial. As previous studies have shown, CDK12 amplification drives tumour progression and is a possible biomarker for breast cancer prediction (Peng *et al.*, 2020; Choi *et al.*, 2019). Therefore, targeting CDK12 overexpression by means of CDK12 inhibitors could provide significant therapeutic benefits.

CDK12 regulates the expression of its target genes by phosphorylating serine 2 of RNA Pol II (Bartkowiak *et al.*, 2010), therefore western blot was used to measure the expression of RNA Pol II S2 after CDK12 inhibition in comparison to RNA Pol II overall levels. RNA Pol II S2 was not observed to be significantly downregulated. This can be accounted for by serine 2 of RNA Pol II also being also phosphorylated by other transcriptional CDKs known to phosphorylate RNA Pol II at serine 2 such as CDK9. A CDK9 inhibitor in combination with the CDK12 may be required to observe the absence or downregulation of RNA Pol II S2(Ramanathan *et al.*, 2001). The other limitations of this western blot included weak signal in RNA Pol II S2 making quantification challenging, with repeats having varying degrees of weaker or stronger bands.

We demonstrated that CDK12 inhibition induces DNA double-strand breaks and downregulates DDR proteins in a breast cancer cell line. BRCA1 expression was shown to be downregulated after 6 h treatment with CDK12 inhibitor through qPCR and further confirmed by western blotting after 24 h treatment. The decrease in expression of DDR genes and the subsequent hindrance of DNA damage repair may not fully account for the cytotoxic effect observed and other proteins may be involved which facilitate CDK12 function in transcription and DDR. We next investigated CHD2 which was identified in previous studies as a potential phospho substrate of CDK12 (Krajewska *et al.*, 2019). CDK12 inhibition had a minor effect on CHD2 mRNA and protein level indicating that CHD2 is a possible transcriptional target of CDK12. To verify CHD2 as a direct phospho-target of CDK12, further investigation using assays such as Co-Immunoprecipitation (Co-IP) or in vitro kinase assays are required. Co-IP was attempted but not successful, however, future research would explore this possibility. Future research would also explore the relationship between CDK12 inhibition and PARP inhibition and their common link-CHD2 (Luijsterburg *et al.*, 2016).

Although clonogenic assays did not reveal a significant difference in clonogenic survival between SR-4835 on its own and in combination with inhibitors of PARP1 or Chk1. We were also not able to reproduce the synergy between the SR-4835 and PARP inhibitor olaparib demonstrated in triple-negative breast cancer cells (Quereda *et al.*, 2019) therefore future studies should determine the optimal drug concentration and short-term cell viability assays in addition to the colony formation assay we could not reproduce.

A limitation of this study is the online data analysis which is not subtype-specific and relates to breast cancer overall. The most significant limitation of this study is the use of a single ER-positive cell line for the majority of experiments and future efforts would include the repetition of experiments with a number of breast cancer cell lines of different subtypes. CDK12 overexpression has been found to be a driver of tumour formation and trastuzumab resistance in HER2-positive cancers (Choi *et al.*, 2019), it would be desirable to repeat experiments in a HER2-positive cell line. The CDK12 inhibitor used throughout this study, SR-4835, is not the only inhibitor available and these assays could be repeated with THZ531(Zhang *et al.*, 2016). As SR-4835 inhibits both CDK12 and CDK13, a CDK12-selective degrader would be necessary for future steps. There are CDK12-selective degraders already available such as BSJ-4-116 which have been effective therapeutically by itself and in combination with PARP inhibitors (Jiang *et al.*, 2021). The promising effect of CDK12 inhibition on TNBC in vitro adds to and widens the arsenal of effective targeted therapies for this cancer subtype which lacks targetable receptors (Quereda *et al.*, 2019).

CDK12 has also been noted as an effective sensitiser of cancer cells to olaparib and trastuzumab which begins to tackle the problem of therapy resistance (Bajrami et al., 2014) (Choi et al., 2019). Although previous work showed CDK12 inhibition causes premature cleavage and polyadenylation and the downregulation of DDR genes (Krajewska et al., 2019), the mechanism of this potent target in genome stability and transcription have yet to be fully determined (Blazek et al., 2011).

Conclusions and Future work

In conclusion, we quantified and characterised the effects of CDK12 inhibition in MCF-7 cells. We showed that CDK12 inhibition induces apoptosis, causes G2-M cell cycle arrest, causes DNA damage and decreases the expression of DDR genes which contributes to its cytotoxic effect. Online analysis of data also told that CDK12 overexpression was associated with poorer outcomes for patients, which agrees with studies that point to CDK12 overexpression as a biomarker for cancer prediction and progression (Peng et al., 2020; Choi et al., 2019). This along with previous work demonstrates the significance of CDK12 as a therapeutic target in cancer and begins to shed light on the mechanisms which allow this effect. Future work would involve replicating the above experiment in a panel of breast cancer cell lines representing different breast cancer subsets would provide a better understanding of CDK12 potential as a therapeutic target in breast cancer. We were unable to reproduce the synergy between the CDK12 inhibitor and PARP inhibitor in TNBC cell line, MDA-MB-231 previously demonstrated in another study (Quereda et al., 2019). Based on our preliminary data combination of CDK12 and CHK1 inhibition would likely provide therapeutic benefit and should be investigated further using a combination of short-term viability/ apoptosis assays and long-term colony formation assays. Ultimately, dissecting the molecular mechanism underlying the most effective combination is required to establish the therapeutic potential of targeting CDK12 in combination with other agents in pre-clinical models.

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