World Journal of Surgery and Surgical Research

Research Article
Published: 28 Jun, 2023

An Integrated Mechanism of CDK2 and CDK4/6 Inhibition in Gastric Cancer Cells

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Abstract

Clinical studies demonstrate that cyclin-dependent kinase 4/6 inhibitors exhibit limited efficacy in gastric cancer. Cyclin E1, encoded by *CCNE1* gene, contributes to intrinsic resistance to CDK4/6 inhibition in gastric cancer. In this study, we aimed to interrogate the potential application of CDK2/4/6i in gastric cancer. We found that CDK4/6 inhibition showed little to moderate potency in gastric tumor cell lines. In contrast, targeting CDK2/4/6 more potently inhibited cell proliferation and Rb-E2F signaling pathway. Mechanistically, a CDK2/4/6 inhibitor, PF-06873600, exert its effects on promoting cellular senescence *via* an integrated mechanism of cell cycle arrest and cellular senescence by upregulating nuclear factor kappa B (NF-κB) activity. Moreover, PF-06873600 effectively inhibited Rb signaling and cell growth in GC cells over expressing *CCNE1*. Our preclinical data provide scientific rationale for co-targeting CDK2 and CDK4/6 in gastric cancer.

Keywords: Cyclin-dependent kinase; Cell cycle arrest; NF-κB; Senescence

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Received Date: *01 Jun 2023*
Accepted Date: *22 Jun 2023*
Published Date: *28 Jun 2023*

Citation:
Xu L, Wu S, Huang J, Guo R, Zheng M, Zhang Y, et al. An Integrated Mechanism of CDK2 and CDK4/6 Inhibition in Gastric Cancer Cells. World J Surg Surgical Res. 2023; 6: 1483.

Introduction

Gastric Cancer (GC) is the fifth most common cancer and the third leading cause of cancer mortality worldwide [1]. Current standard of care for a majority of GC patients includes surgery and chemotherapy. However, survival rates have not been substantially improved when treated with chemo drugs. The approved targeted therapies for 15% to 20% GC patients with HER2 amplification are anti-HER2 antibody trastuzumab and its antibody-drug conjugate (ADC) [2-4]. Of note, anti-PD1 immunotherapy was FDA-approved for GC in different settings in 2021 [5,6]. Despite this, there are no effective targeted therapies for most GC patients containing other genomic alterations. Thus, there is a need for developing novel therapeutics.

Deregulated cell cycle, such as, amplification of the genes encoding CCNDs, CDK4 and CDK6, loss of CDKN2A and RB1, is a hallmark of cancer [7]. Cyclin-Dependent Kinase 4/6 (CDK4/6) are master regulators of cell cycle entry by phosphorylating and inactivating Retinoblastoma protein (Rb). Hyper-phosphorylated Rb dissociates from transcription factor E2F family, which become active to initiate DNA synthesis and mitosis-related gene transcription, including CCNA, CCNEs and CDK2 [7,8]. Due to the critical role of CDK4/6 in regulating cell cycle, efforts have been made to selectively target these two closely related kinases. CDK4/6 inhibitor (CDK4/6i), for example, Palbociclib (here referred as PAL), Ribociclib and Abemaciclib, have emerged as compelling drugs approved by the FDA in combination with endocrine therapy for the treatment of patients with Hormone Receptor-Positive (HR+) breast cancer [9-11]. However, it only displays limited clinical activity in many tumor types [12]. A recent phase II clinical trial (NCT01037790) demonstrates that PAL fails to show activity in patients with gastric adenocarcinoma [13]. A mechanism of resistance underlying the observation remains elusive.

CCNE1 amplification accounts for approximately 12% GC [3,14]. It is well-established that CDK2/cyclin E1 plays pivotal roles in cell cycle progression. Cyclin E1 itself is not a druggable target, while inhibitors targeting pan CDKs, including CDK2, have been extensively investigated

[15-17]. A CRISPR a screen identifies CDK2 as one of resistance mechanisms in tumor cells treated with CDK4/6i, eventually leading to the development of a CDK2/4/6i, PF-06873600 (hereafter referred to collectively as PF-3600) [18,19]. It is currently in clinical trial for solid tumors. Here, we compared the potency of CDK4/6i and CDK2/4/6i in gastric cancer cell lines. Superiority of co-targeting CDK2 and CDK4/6 may provide rational therapeutic strategies for GC with *CCNE1* amplification.

Material and Methods

Compounds

Palbociclib and PF-06873600 were purchased from Medchem Express. Palbociclib and PF-06873600 were dissolved in water and DMSO, respectively and store at - 80°C.

Cell lines

AGS, HGC-27, MKN45, NCI-N87 and SNU-638 cell lines were purchased from National Collection of Authenticated Cell Cultures (Shanghai, China). All the cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin (Thermo Fisher).

Plasmids

Tet-pLKO-puro was a gift from Dmitri Wiederschain (Addgene plasmid #21915). shCDK2 (5'-GCCCTCTGAACTTGCCTTAAA-3') and sh*CCNE1* (5'-CCTCCAAAGTTGCACCAGTTT-3') were subclone into Tet-pLKO digested with AgeI and EcoRI (New England Biolabs). psPAX2 and pMD.G were purchased from Addgene. pCMV3-N-HA-cyclin E1 was purchased from Sino Biological. HA-cyclin E1 was PCR-amplified and subcloned into pLenti-puro (Addgene plasmid #39481).

Luciferase reporter assay

AGS cells were transfected with FuGENE HD (Promega). Twenty-four hours after co-transfection with pGL-3-NF-κB-Luc and pRL Renilla luciferase control, cells were treated with Palbociclib and PF-06873600 for 24 h. Firefly and Renilla luciferase activities were assayed using Dual-Luciferase assay kit (Promega).

Quantitative Real-time qPCR.AGS cells were treated with DMSO or palbociclib or PF-06873600 for 24 h, and total RNA was extracted with Trizol Reagent (Thermo Fisher). Complementary DNA was synthesized with a SuperScript IV First-Strand kit (Thermo Fisher). Quantitative PCR was performed using SYBR Green PCR Master Mix with a 7500 Fast real-time PCR system (Applied Biosystems). PCR primers are as follows: IL-6forward: 5'-ACT CAC CTC TTC AGA ACG AAT TG-3', reverse: 5'-CCA TCT TTG GAA GGT TCA GGT TG-3'; IL-8 forward: 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3', reverse: 5'-TCT CAG CCC TCT TCA AAA ACT TCT C-3'; Cxcl1 forward: 5'-GCG CCC AAA CCG AAG TCA TA-3', reverse: 5'-ATG GGG GAT GCA GGA TTG AG-3'; GAPDH forward: 5'-GGA GCG AGA TCC CTC CAA AAT-3', reverse: 5'-GGC TGT TGT CAT ACT TCT CAT GG-3'. Differences in mRNA expression levels were normalized with GAPDH by the ΔΔCt method.

Western blotting

Cells were washed with ice-cold PBS twice and lysed with NP-40 lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). The whole cell lysates were centrifuged at 14,000 rpm for 10 min and supernatant was collected. Proteins were subjected to SDS-PAGE followed by immunoblotting with the antibody according to standard protocols. The image was developed either by X-ray film or iBright CL1500 Imaging System (Thermo Fisher). Antibodies used in this study included phospho-Rb S807/811 (Cell Signaling), phospho-NF-κB p65 (Ser536) (Cell Signaling), cyclin A (B-8, Santa Cruz), cyclin E1 (HE12, Santa Cruz), CDK2 (D-12, Santa Cruz), CDK4 (DCS-31, Santa Cruz), CDK6 (B-10, Santa Cruz), NFκB p65 (F-6, Santa Cruz), p21 (Abclonal), HA (Biolegend) and β-actin (Cell Signaling).

Generation of stable cell lines

Lentivirus was produced by transfecting HEK293T cells with lentiviral vectors, including Tet-pLKO-shCDK2, Tet-pLKO-sh*CCNE1* and pLenti-HA-*CCNE1*, together with psPAX2 and pMD.G at a 5:4:1 ratio using calcium phosphate method. The virus was harvested 72 h after transfection and filtered through a 0.45-μm filter unit (Millipore). AGS cells were transduced with inducible shCDK2 virus in the presence of 8 μg/ml polybrene (EMD Millipore) and selected with 2 μg/ml puromycin (Thermo Fisher) for 5 d. To induce shRNA expression, 0.1 μg/ml doxycycline was added to the cells for 72 h. For *CCNE1* stable cell line, AGS cells were transduced with HA-*CCNE1* lentivirus in the presence of 8 μg/ml polybrene and selected with 2 μg/ml puromycin for 5 d.

Colony formation assay

Cells were seeded at 1,000 to 2,000 cells per ml in 6-well plates. Next day after plating, Palbociclib and PF-06873600 were added at increasing concentrations from 0.01 μM to 0.25 μM. Control wells were treated with equivalent amount of either water or DMSO. Medium was replaced every 3 days. After 10 to 14 days, cells were stained with 0.05% crystal violet for 30 min at RT. Cells were de-stained with tap water, air-dried and scanned with a digital scanner. To quantify the data, the crystal violet was extracted with 10% acetic acid and read at 570 nM using a microplate reader. The dose-response curve was plotted with Prism 8 software. Two independent experiments were performed.

β-galactosidase staining

Senescence Associated (SA)-β-gal staining was performed according to the instructions for Senescence β-galactosidase staining kit (Cell Signaling). Briefly, cells were seeded in 6-well plates. Next day, cells were treated with DMSO or Palbociclib or PF-06873600 for 3 days. Image cells under a microscope with 200x magnification. SA-β-gal positive cells were counted manually from 5 different fields of view, from images containing ≥ 100 cells. The experiments were repeated twice and one of the representative results was shown.

Cell-cycle analysis

AGS cells were treated with either DMSO or Palbociclib or PF-06873600 for 3 days. Cells were collected and washed with PBS, fixed with ice-cold 70% ethanol in a drop wise manner while mixing gently on a vortex and stored at 4°C for 2 h. Cells were then pelleted, washed with PBS twice followed by incubation with DAPI (1 μg/ml) in the dark for 30 min at RT. Samples were subjected to flow cytometry by a Beckman CytoFlex LX flow cytometer, and the data were analyzed using CytExpert Software.

Statistical analysis

All data were presented as the mean ± Standard Deviation (S.D.). qPCR assay was performed using Student's t-test. P-values for colony formation assay, luciferase assay, Western blot and β-gal

staining assay were calculated using the one-way analysis of variance (ANOVA), and the cell cycle analysis was analyzed with the two-way ANOVA. Both ANOVAs were carried out using GraphPad Prism. **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001.

Results

CDK2/cyclin E1 confers resistance to CDK4/6i in gastric cancer cell line

To interrogate whether CDK2/cyclin E1 might be associated with resistance to CDK4/6i in gastric tumor cells, we first treated AGS cell line, which was reported to be resistant to PAL [20], with increasing concentrations of PAL. Indeed, AGS tumor cells are resistant to CDK4/6 inhibition although there is statistically significant at higher concentrations (Figure 1A, 1B). We then generated Dox-inducible shRNA-mediated knockdown of CDK2 or *CCNE1* in AGS cells. To rule out the possibility that Dox alone might affect cell fitness in parental cells, AGS cells were incubated with PAL and Dox. Similar cell growth inhibition was obtained with or without Dox (Figure 1A, 1B). As expected, PAL exhibited mild inhibitory effect on cell proliferation even at 0.5 μM concentration in the absence of Dox, comparable to parental AGS cells treated with PAL. However, silencing of CDK2 in the presence of Dox sensitized AGS cells to CDK4/6 inhibition (Figure 1C, 1D). Consistently, significant suppression of Rb phosphorylation and E2F target gene, cyclin A2 was observed upon 1 μM PAL treatment by 24 h in wild-type cells, whereas 10 times less concentration of PAL, i.e., 0.1 μM PAL, completely led to loss of phospho-Rb and cyclin A2 in the presence of Dox suggesting CDK2 is associated with resistance to CDK4/6i in gastric tumor cell (Figure 1E).

It is known that CDK2 typically complexes with cyclin E or cyclin A for full kinase activity [7]. To confirm whether cyclin E1 confers resistance to PAL, we established inducible depletion of *CCNE1* followed by treatment with increasing concentrations of PAL in the presence or absence of Dox. Indeed, reduced expression of cyclin E1 rendered tumor cells sensitive to PAL in cell proliferation as well as inhibition of Rb phosphorylation and cyclin A2 protein levels (Figures 1F-1H). Therefore, these data suggest that CDK2 kinase activity confers drug resistance to CDK4/6 inhibition.

CDK2/4/6i is superior to CDK4/6i in inhibiting cell proliferation and Rb signaling

Having shown that CDK2 activity contributes to CDK4/6i resistance, pharmacological inhibition of CDK2 and CDK4/6 might be an effective therapeutic strategy to overcome resistance to CDK4/6. To assess whether tumor cells were more sensitive to a CDK2/4/6i, PF-06873600 (PF-3600), which demonstrates selectivity for CDK2 (Ki=0.13 nM), CDK4 (Ki=1.25 nM) and CDK6 (Ki=0.11 nM) [19] and is currently in phase I clinical trial, compared to PAL, we treated a panel of gastric cancer cell lines with increasing concentrations of two inhibitors. In accordance with genetic data, co-targeting CDK2 and CDK4/6 markedly inhibited cell proliferation with IC_{50} lower than 30 nM, whereas PAL only displayed weak anti-proliferative activity even at 250 nM in all cell lines tested indicating inhibition of CDK2/4/6 results in increased potency than CDK4/6 inhibition in cell growth (Figure 2A, 2B).

We next evaluated whether the observed differences of sensitivity

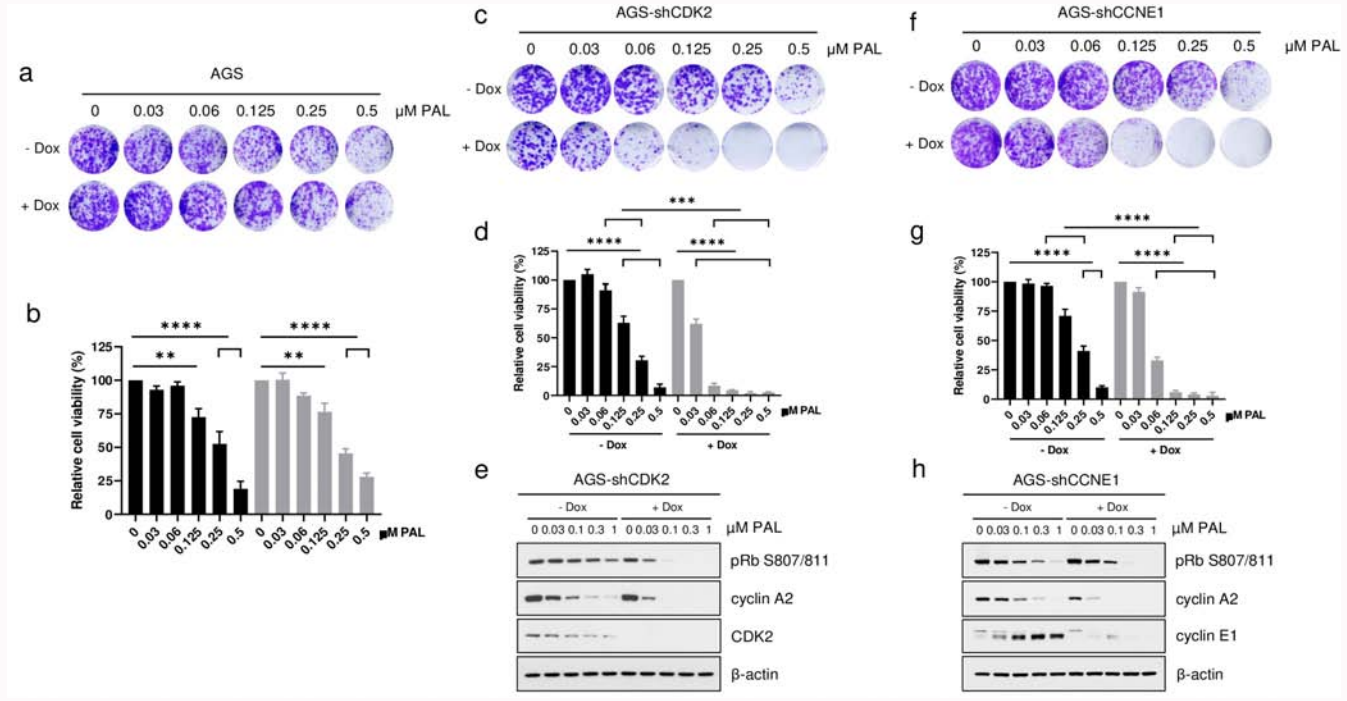

Figure 1: CDK2 and cyclin E1 promote resistance to CDK4/6 inhibition. (a) Colony formation assay in AGS cells treated with various concentrations of PAL as indicated for 10 days in the presence or absence of 0.1 μg/ml Dox; (b) Relative cell viability of the colony formation assay; (c) Colony formation assay for AGS cells with inducible knockdown of CDK2 with or without 0.1 μg/ml Dox and increasing concentrations of PAL for 10 days; (d) Relative cell viability of the colony formation assay; (e) Protein expression levels in inducible AGS-shCDK2 cells in the presence or absence of 0.1 μg/ml Dox for 48 h followed by PAL for 24 h; (f) Clonogenic assay for AGS cells with inducible knockdown of *CCNE1* with or without 0.1 μg/ml Dox and increasing concentrations of PAL for 10 days; (g) Relative cell viability of the colony formation assay; (h) Protein expression levels in inducible AGS-sh*CCNE1* cells in the presence or absence of 0.1 μg/ml Dox for 48 h followed by PAL for 24 h. Data are mean ± S.D. (n=2 technical replicates). Representative data from one experiment are shown. All experiments were performed twice. One-way ANOVA with Tukey's multiple comparison was performed.
****P*<0.001, *****P*<0.0001

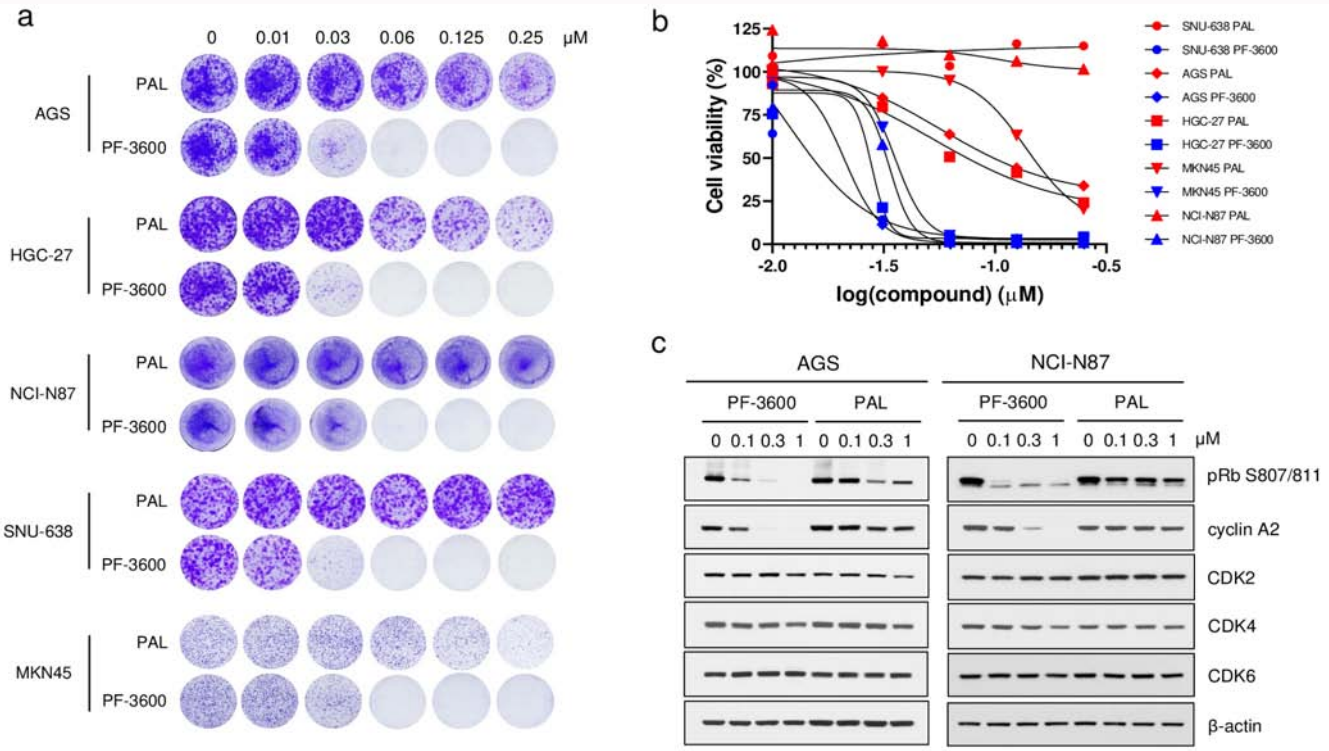

Figure 2: PF-3600 more potently inhibits cell proliferation and Rb signaling in GC. (a) Colony formation assay for a panel of GC cell lines treated with increasing concentrations of PAL and PF-3600 for 8 to 12 days; (b) Quantitative data for clonogenic assay using Prism 8; (c) Protein expression levels of phospho-Rb, cyclin A2 and CDKs in AGS and NCI-N87 cells treated with the indicated concentrations of PAL and PF-3600 for 24 h. Representative data from one experiment are shown. All experiments were performed twice.

were correlated with the degree of Rb signaling suppression. Consistently, we observed that PF-3600 was more potent in suppressing Rb phosphorylation and cyclin A2 in AGS cells treated for 24 h, while PAL showed mild inhibition of pRb (Figure 2C). To validate our findings, we conducted the experiment in another cell line, NCI-N87. Similar results were obtained suggesting targeting CDK2/4/6 is superior to CDK4/6 inhibition in gastric cancer cells (Figure 2C).

CDK2/4/6i induces cell cycle arrest and cellular senescence in GC cells

To gain insight into the consequences mediated by dual CDK4/6i and triple CDK2/4/6i, we then assessed cell cycle profile after 72 h drug treatment. As shown in Figure 3A, PAL induced marginal G1 arrest up to 1 μM. In contrast, AGS cells elicited drastic G2 arrest at 0.3 μM to 1 μM PF-3600. Next, we examined a well-established cellular senescence marker, p21, [21] in AGS cells treated with different concentrations of two inhibitors by immunoblotting. Consistent with the cell cycle data, induction of p21 was of greater magnitude when exposed to CDK2/4/6i even at low concentration. However, PAL only mildly induced p21 expression (Figure 3B, 3C). To further evaluate the phenotypic effects of PAL and PF-3600, we treated two gastric tumor cells with both drugs and found that increasing enlarged and flat cell morphology, characteristics of senescence [21,22], was detected in PF-3600-exposed tumor cells, while few cells underwent senescence with PAL treatment. In agreement with this, PF-3600 triggered strong SA-β-galactosidase activity, another marker for cellular senescence in AGS and, to a lesser extent, HGC-27 cells (Figure 3D, 3E). Again, this was not the case for PAL (Figure 3D, 3E). Thus, these results suggest that targeting CDK2/4/6 more effectively induces cell cycle arrest and cellular senescence compared to CDK4/6 inhibitor.

CDK2/4/6i more potently enhances NF-κB-mediated senescence phenotypes

It has been demonstrated that nuclear NF-κB signaling pathway is activated in senescent cells by inducing SASP [23-25]. Emerging evidence suggests that CDK2 and CDK4/6 are involved in the suppression of NF-κB activation through different mechanisms [26,27]. To elucidate whether co-targeting CDK2 and CDK4/6 could potentiate NF-κB signaling leading to cellular senescence, we accessed the effect of CDK2/4/6i on NF-κB activation using κB-luciferase reporter. Cell cycle arrest and senescence induced by PAL was moderate even up to 1 μM, thereafter, we only included 1 μM PAL to compare with PF-3600. Transcriptional activation of NF-κB was enhanced by PF-3600 in a dose-dependent fashion, whereas only marginal induction of NF-κB activity was observed with 1 μM PAL (Figure 4A). Consistently, treatment of AGS cells with CDK2/4/6i, as low as 0.1 μM, significantly upregulated IL-6, IL-8 and *CXCL1* gene expression (Figure 4B), and known components of SASP [23]. Again, PAL had little effect on increasing SASP-related gene expression indicating CDK2 inhibition greatly synergizes with CDK4/6 inhibition for enhancing NF-κB transcriptional activation (Figure 4B). Furthermore, 24-h treatment of AGS cells with PF-3600 dose-dependently induced phosphorylation of RelA (p65) at Serine 536 (Figure 4C), a marker of NF-κB activation. These data suggest that co-inhibition of CDK2/4/6 more efficiently induces NF-κB-mediated senescence phenotype.

CDK2/4/6i effectively suppresses cell proliferation of *CCNE1*-overexpressing tumor cells

Amplification of *CCNE1* gene is commonly detected in gastric cancer [28,29], where high cyclin E1 protein levels are associated with Chromosomal Instability (CIN) [30,31], immune cell exclusion [32]

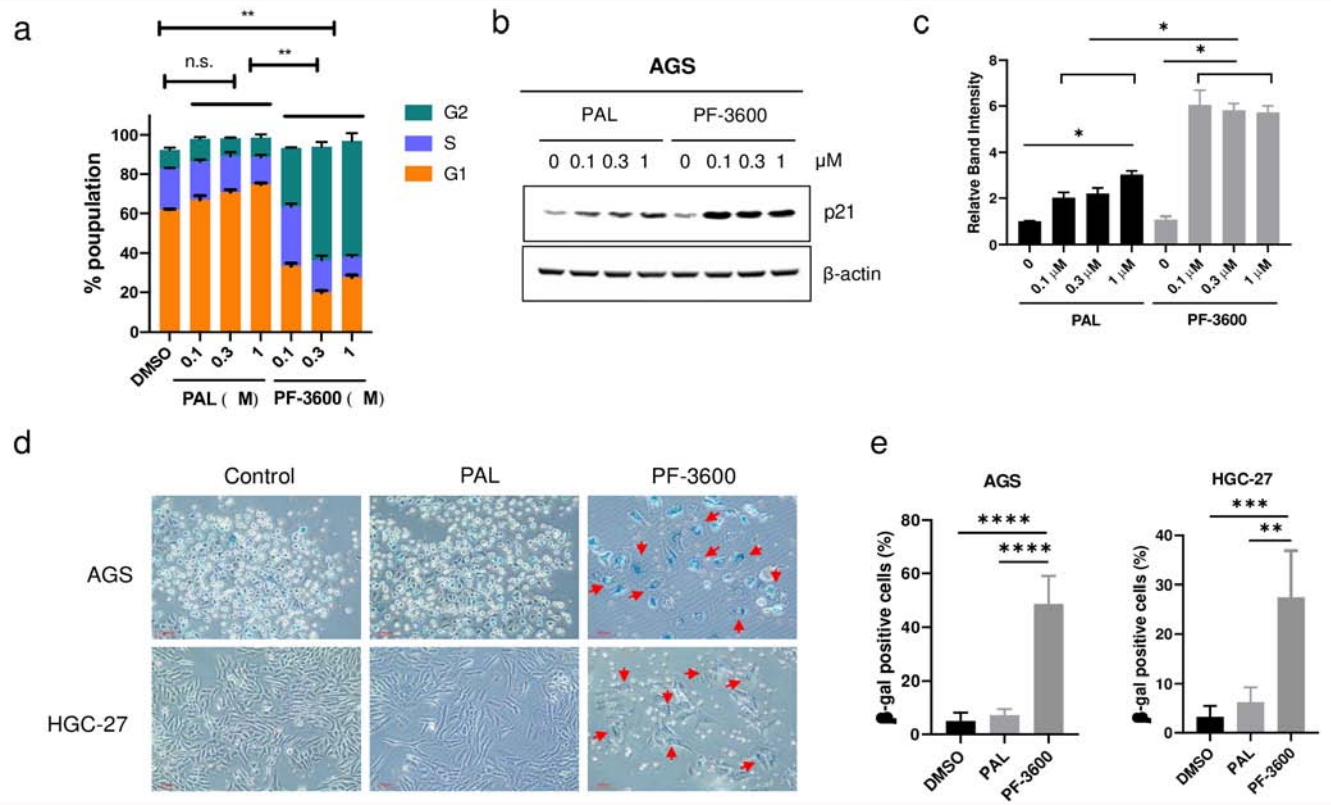

Figure 3: CDK2/4/6i induces robust cell cycle arrest and cellular senescence. (a) Cell cycle analysis in AGS cells treated with the indicated concentrations of PAL and PF-3600 for 3 days; (b) Expression of senescence marker p21 in AGS treated with PAL and PF-3600 for 3 days; (c) Relative band intensity of Western blot by Image J; (d) Senescence-associated β-gal staining in AGS and HGC-27 treated with 0.3 μM PAL and PF-3600 for 3 days. Arrows indicate senescent cells; (e) Percentage of cells with positive β-gal staining. Representative data from one experiment are shown. All experiments were performed twice. One-way ANOVA with Tukey's multiple comparison was performed.
**P<0.01, ***P<0.001, and ****P<0.0001

and resistance to targeted therapies, such as HER2 [33]. Moreover, *CCNE1* amplification inversely correlates with overall survival in patients with gastric cancer [28,34]. Based on this fact, Co-targeting CDK2/4/6 might be effective in this subtype.

We first confirmed that around 12% (54/434) of stomach adenocarcinoma in TCGA possessed *CCNE1* amplification using cBioPortal (Figure 5A). However, *CCNE1*-amplified gastric cancer cell lines are underrepresented in the public domain. Due to lack of availability of cell lines, we developed AGS cell lines that overexpress HA-tagged cyclin E1 stably, i.e., *CCNE1*-high. Western blotting showed high expression of cyclin E1 in stable cell lines compared to an Empty vector (Ev) control (Figure 5B). Treatment of AGS-Ev with PAL at relatively high dose induced significant cell growth inhibition, while ectopic expression of cyclin E1 conferred resistance to PAL at 0.25 μM to 0.5 μM (Figure 5C, 5D). However, PF-3600 equipotently inhibited cell proliferation regardless of cyclin E1 overexpression (Figure 5C, 5D). In line with the cell growth data, PAL insufficiently suppressed phospho-Rb and cyclin A2 in *CCNE1*-high tumor cells than control cells. In addition, CDK2/4/6i exhibited a similar magnitude of inhibition of Rb phosphorylation indicating triple CDK2/4/6 inhibition could serve as a new therapeutic strategy for *CCNE1*-amplified gastric cancer (Figure 5E).

Discussion

Amplification of the CCNE1 gene is frequently observed in various tumor types, for example, uterine cancer, ovarian cancer, and gastroesophageal cancers [31]. High *CCNE1* expression predicts for poor survival in patients with ovarian and gastric cancer [28,35,36]. Hitherto, there is no approved targeted therapy for this disease. Thus, *CCNE1* amplification becomes a significant unmet need.

CDK4/6i have demonstrated remarkable clinical activity in HR+ breast cancer in combination with hormonal therapy. There is great interest in exploring CDK4/6 inhibitors in other tumor types. However, adaptive or intrinsic resistance to these drugs has been reported in melanoma, colorectal cancer, glioblastoma and stomach cancer. The mechanisms of resistance are not fully understood. CDK4/6-cyclin Ds phosphorylates Rb to initiate G1 progression. CDK2-cyclin E/A subsequently hyperphosphorylate Rb for G1/S transition. A wealthy of evidence indicates that CDK2 can compensate loss of CDK4/6 activity resulting in intrinsic or acquired resistance to CDK4/6i in lung and breast cancer [37-40]. In PALOMA-3 clinical trials, by analyzing 302 tumor samples, it has been shown that high *CCNE1* mRNA levels were associated with poor outcomes in PAL-treated HR+ breast cancer patients [41]. In this study, we confirm that silencing or ectopic expression of *CCNE1* in GC cells recapitulates resistance to PAL in patients. Together, the data point to inhibition of CDK2-cyclin E1 as a mechanism of resistance to CDK4/6i and imply that inhibitors against CDK2/4/6 may be valuable as strategies for overcoming drug resistance.

Many efforts have been made to develop CDK2 inhibitors, such as dinaciclib, SNS-032 and AT7519 and Fadraciclib [15,16,42,43]. However, off-targeting other CDKs-induced toxicity limits their

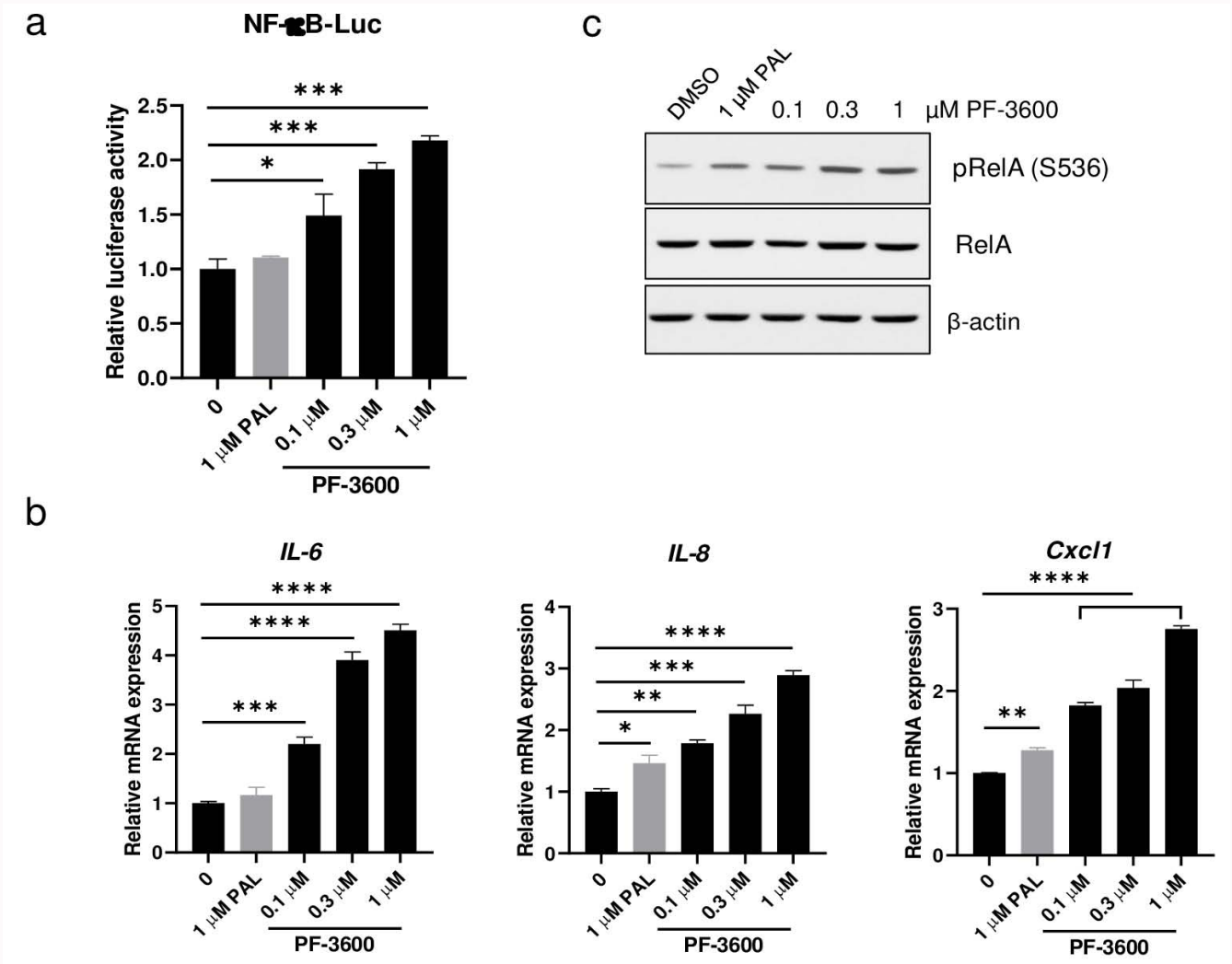

Figure 4: CDK2/4/6i more potently induces NF-κB-mediated SASP. (a) NF-κB luciferase reporter assay in AGS cells treated with the indicated inhibitors for 24 h; (b) Real-time qPCR analysis of SASP-related genes in AGS cells treated with the indicated inhibitors for 24 h; (c) Protein levels of pRelA, total RelA in AGS cells treated with the indicated inhibitors for 24 h. Data are represented as the mean ± S.D. Representative data from one experiment are shown. All experiments were performed twice. Student t-test was performed.
*P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001

applications in the clinic. On the basis of chemical structure of CDK4/6i PAL, a CDK2/4/6i, PF-3600, developed by Pfizer, has shown efficacy in multiple preclinical cancer models including *CCNE1*-amplifed ovarian cancer and Triple Negative Breast Cancer (TNBC) and is currently in clinical trial [18,19].

In this study, we found PF-3600 more effectively suppresses Rb signaling and cell proliferation than PAL not only in *CCNE1*-low GC cell lines, but in *CCNE1*-high cells. In addition, it exhibits a strong inducer of cell cycle arrest and senescence. More intriguingly, here we show that CDK2/4/6 inhibitor exerts its effects as the combined outcome, i.e., cellular senescence, of two different mechanisms, which was not reported to our best understanding. On one hand, the triple CDK inhibitor induces expression of p21, a known senescence marker, and cell cycle arrest. On the other hand, CDK2/4/6 inhibition activates NF-κB-mediated SASP. It has been demonstrated that CDK4/6i arrest cells at G1 phase in breast cancer, Gastrointestinal Stromal Tumor (GIST) and other tumor types [18,44,45]. However, CDK2/4/6i is able to arrest cells at either G1 and/or G2/M phases in a context-dependent manner [18,45,46]. In this work, we observed G2 arrest in AGS cells in the presence of PF-3600. Similar phenotypes have been observed in PF-3600-treated breast cancer cell lines in part due to off-target CDK1 [46]. Further investigation is warranted.

Our findings suggest that co-targeting CDK2 and CDK4/6 induces more profound cell death *via* an integrate mechanism of action in gastric cancer cells, including *CCNE1*-high tumor cells. This study provides a potential therapeutic strategy for targeting CDK2/4/6 in GC patients.

Acknowledgement

We are grateful to Dmitri Wiederschain and Didier Trono for sharing reagents. L.X. is supported by Fujian Provincial Natural Science Foundation (2016J01403) and Fujian Provincial Department of Finance.

Author Contributions

LX, SW, YZ and XW conceived and designed the study. LX, SW,

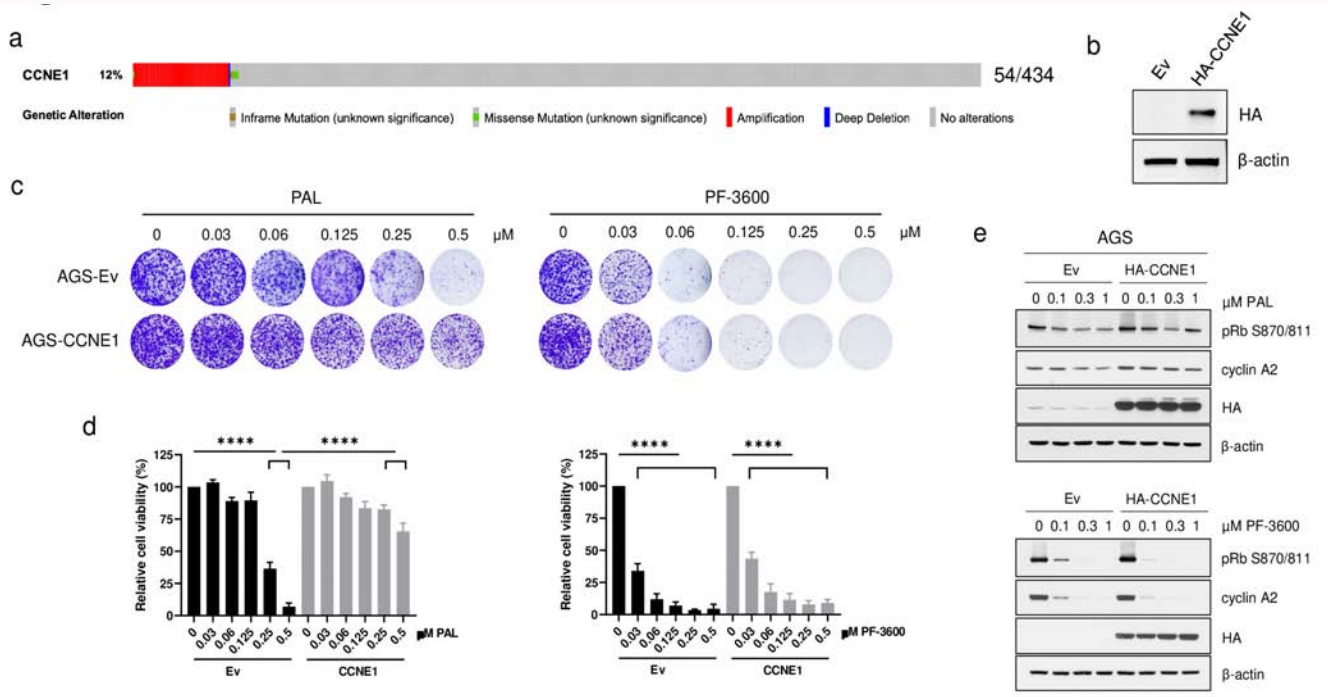

Figure 5: CDK2/4/6i is more effective in *CCNE1*-high GC cells. (a) Analysis of *CCNE1* amplification in GC patients; (b) Overexpression of cyclin E1 in AGS cells; (c) Colony formation assay for AGS-Ev and AGS-*CCNE1* treated with PAL and PF-3600 for 10 days; (d) Relative cell viability of the colony formation assay; (e) Rb-E2F protein expression in AGS-Ev and AGS-*CCNE1* treated with PAL and PF-3600 for 24 h. Data are mean ± S.D. Representative data from one experiment are shown. All experiments were performed twice. One-way ANOVA with Tukey's multiple comparison was performed.
****P<0.0001

and JH performed the experiments and collected the data. LX, SW, JH, RG, and MZ analyzed the data. LX, SW, YZ and XW wrote the manuscript. All authors approved the final version of the manuscript.

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