Cancer Letters 451 (2019) 79–91

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Adefovir dipivoxil sensitizes colon cancer cells to vemurafenib by disrupting the KCTD12-CDK1 interaction

Jie Yang[a], Wen Wen Xu[b], Pan Hong[a], Fei Ye[a], Xiao-Hui Huang[a], Hui-Fang Hu[a], Qi-Hua Zhang[a], Xin Yan[a], Bin Li[a,**], Qing-Yu He[a,*]

[a] Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering, College of Life Science and Technology, Jinan University, Guangzhou, 510632, China
[b] Institute of Biomedicine, Guangdong Provincial Key Laboratory of Bioengineering Medicine, National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou, 510632, China

ARTICLE INFO

Keywords:
Vemurafenib resistance
Cell cycle
Drug screening
Protein-protein interaction
Drug repurposing

ABSTRACT

Vemurafenib is a B-Raf V600E inhibitor that exerts significant inhibitory effects in melanoma but not in colon cancer, and the mechanism of vemurafenib resistance remains unclear. In this study, bioinformatics analysis of gene profiles in cancer cells treated with vemurafenib or its analog revealed that cell cycle progression is significantly affected by vemurafenib. We found that CDK1 is stably activated in the vemurafenib-resistant (VR) colon cancer sublines that we established, indicating that CDK1 activation is responsible for vemurafenib resistance. As the KCTD12-CDK1 interaction is necessary for CDK1 activation, we screened an FDA-approved drug library consisting of 616 compounds and identified that adefovir dipivoxil (AD), a nucleoside analog for treatment of HBV infections, disrupts the CDK1-KCTD12 interaction and induces G2 phase arrest in the cell cycle. Functional assays demonstrated that AD significantly inhibited colon cancer cell proliferation and tumorigenesis both *in vitro* and *in vivo* with no observed side effects. Furthermore, AD sensitized vemurafenib-resistant colon cancer cells and tumor xenografts to vemurafenib. This study reveals that CDK1 activation induces vemurafenib resistance and that AD is a promising therapeutic strategy for colon cancer both as a single agent and in combination with vemurafenib.

1. Introduction

Colon cancer is the third most common malignancy, and more than 1,800,000 new cases were estimated to have been diagnosed worldwide in 2018. Among these new cases, an increasing incidence of colon cancer are being reported in adolescents and young adults [1–3]. Given that traditional treatment, in surgery and chemoradiotherapy, does not entirely eliminate cancer cells in patients, various targeted therapies, such as cetuximab and regorafenib, have been developed for colon cancer treatment, initially achieving good results in clinical application. However, the emergence of drug resistance after a period of treatment inevitably leads to poor treatment response or cancer recurrence. Recent studies demonstrated that mutations in targeted genes or activation of "front-line proteins" involved in other oncogenic pathways are responsible for drug resistance [4,5]. Therefore, identification of novel therapeutic targets and development of chemosensitizing agents are urgently needed for colon cancer therapy.

Aberrant activation of the Ras/Raf/MEK/ERK signaling pathway promotes tumorigenesis and cancer progression [6]. B-Raf belongs to the Raf family and is widely expressed in different cancers and plays an important role in promoting cancer growth [7,8], indicating that B-Raf is a potential target for anti-tumor drug development [9–11]. Vemurafenib is a B-Raf/B-Raf V600E inhibitor under investigation in phase 3 clinical trials for melanoma therapy [12]. Although clinical trials have demonstrated that vemurafenib is effective for melanoma treatment, the drug has minimal effects on colon cancer [13]. Comprehensive analysis of published databases using the Ingenuity Pathway Analysis (IPA) revealed that expression of CDK1 is significantly inhibited in response to vemurafenib treatment in cancer cells [14,15] but remains unaffected in vemurafenib-resistant (VR) cancer cells, indicating that CDK1 might be responsible for development of vemurafenib resistance in colon cancer [16]. To investigate the mechanisms of action for vemurafenib in cancer cells, we established VR cell lines by treating colon cancer cells with increasing concentrations of vemurafenib. Herein,

* Corresponding author. Institute of Life and Health Engineering, College of Life Science and Technology, Jinan University, Guangzhou, 510632, China.
** Corresponding author. Institute of Life and Health Engineering, College of Life Science and Technology, Jinan University, Guangzhou, 510632, China.
E-mail addresses: libin2015@jnu.edu.cn (B. Li), tqyhe@jnu.edu.cn (Q.-Y. He).

https://doi.org/10.1016/j.canlet.2019.02.050
Received 23 December 2018; Received in revised form 14 February 2019; Accepted 28 February 2019

activation of CDK1 signaling in VR cells led us to hypothesize that cell cycle checkpoints are associated with the sensitivity of cancer cells to vemurafenib.

Cell cycle dysregulation is a common feature in human cancers leading to at least two hallmarks of cancer development: uncontrolled cell proliferation and genomic/chromosomal instability [17,18]. Proper progression through the cell cycle is mediated by members of the cyclin-dependent kinase (CDK) family, and activity of members of this family is regulated by specific activators (cyclins) and inhibitors (Ink 4 and Cip/Kip family members) [19]. Our previous study identified that the interaction between potassium channel tetramerization domain containing 12 (KCTD12) and CDK1 is necessary for CDK1 activation and G2/M transition [20]. Constitutive CDK1 activation causes significant changes in protein phosphorylation and drives tumor cell cycle progression, resulting in dysregulated cell proliferation and tumorigenicity [21,22]. These findings suggest that CDK1 may be a therapeutic target for the treatment of human cancer.

In the field of drug discovery, the concept of “old drugs for new applications” is gaining increasing recognition [23]. When a new function for a clinical drug is identified, it increases the opportunity for that drug to be clinically utilized. In this study, a drug library consisting of 616 Food and Drug Administration (FDA)-approved compounds was used to screen for drugs with the potential to block the CDK1 and KCTD12 interaction. We found that Adefovir dipivoxil (AD), a nucleoside analog approved by the FDA for clinical therapy of hepatitis B virus (HBV) infections [24–26], disrupted the CDK1-KCTD12 interaction. AD is an oral prodrug of adefovir, an analog of AMP [27]. Previous studies demonstrated that AD suppressed viral DNA synthesis by targeting reverse transcriptase in host cells [28,29]. However, the role of AD in cancer therapy remains unclear. In this study, the effects of AD on cell cycle progression, proliferation and tumorigenicity of colon cancer cells were investigated both *in vitro* and *in vivo*. Furthermore, we assessed whether targeting CDK1-KCTD12 with AD enhances the sensitivity of colon cancer cells to vemurafenib.

2. Materials and methods

2.1. Cell lines and drugs

Human colon cancer cell lines HCT116 and HT29 (ATCC, Rockville, MD, USA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in 5% CO2. Vemurafenib-resistant sublines, designated HCT116-vemurafenib resistant (VR) and HT29-VR, were established by treating HCT116 and HT29 cells, respectively, with high concentrations (200 μM) of vemurafenib. All cell lines were authenticated by short tandem repeat profiling and were tested for mycoplasma contamination. Vemurafenib and adefovir dipivoxil were purchased from Selleck Chemicals (Huston, TX, USA) and were dissolved in dimethyl sulfoxide (DMSO).

2.2. Plasmids, transfection and infection

The CDK1 lentiviral expression plasmid was generated by PCR amplification from a colon cancer cDNA library and cloned into the pLVX vector. Primers for generating the CDK1 plasmid were as follows: forward (5′-GCGAATTCATGGAAGATTATACCAAAA-3′) and reverse (5′-GCGGATCCTTACTTATCGTCGTCATCCTTGTAATCCA TCTTCTTAAT CTGAT-3′). Transfection, infection and establishment of stable cell lines were performed as described previously [30,31]. The Lipofectamine™ 3000 reagent (Thermo Fisher Scientific) was used for transfection according to the manufacturer's recommendations.

2.3. Western blotting

Cell pellets suspended in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) was centrifuged at 14,000 g for 30 min at 4 °C, and the supernatant was loaded onto a sodium dodecyl sulfate (SDS) polyacrylamide gel for electrophoresis, after which proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% fat-free milk in Tris-buffered saline-Tween 20 (TBST), membranes were incubated with primary antibodies followed by corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology). Signals were detected using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and visualized by exposure to autoradiographic film. Primary antibodies used included cyclin A2 (Proteintech, Chicago, IL, USA), CDK1, p-CDK1, and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Purification of CDK1-GST and KCTD12-His fusion proteins

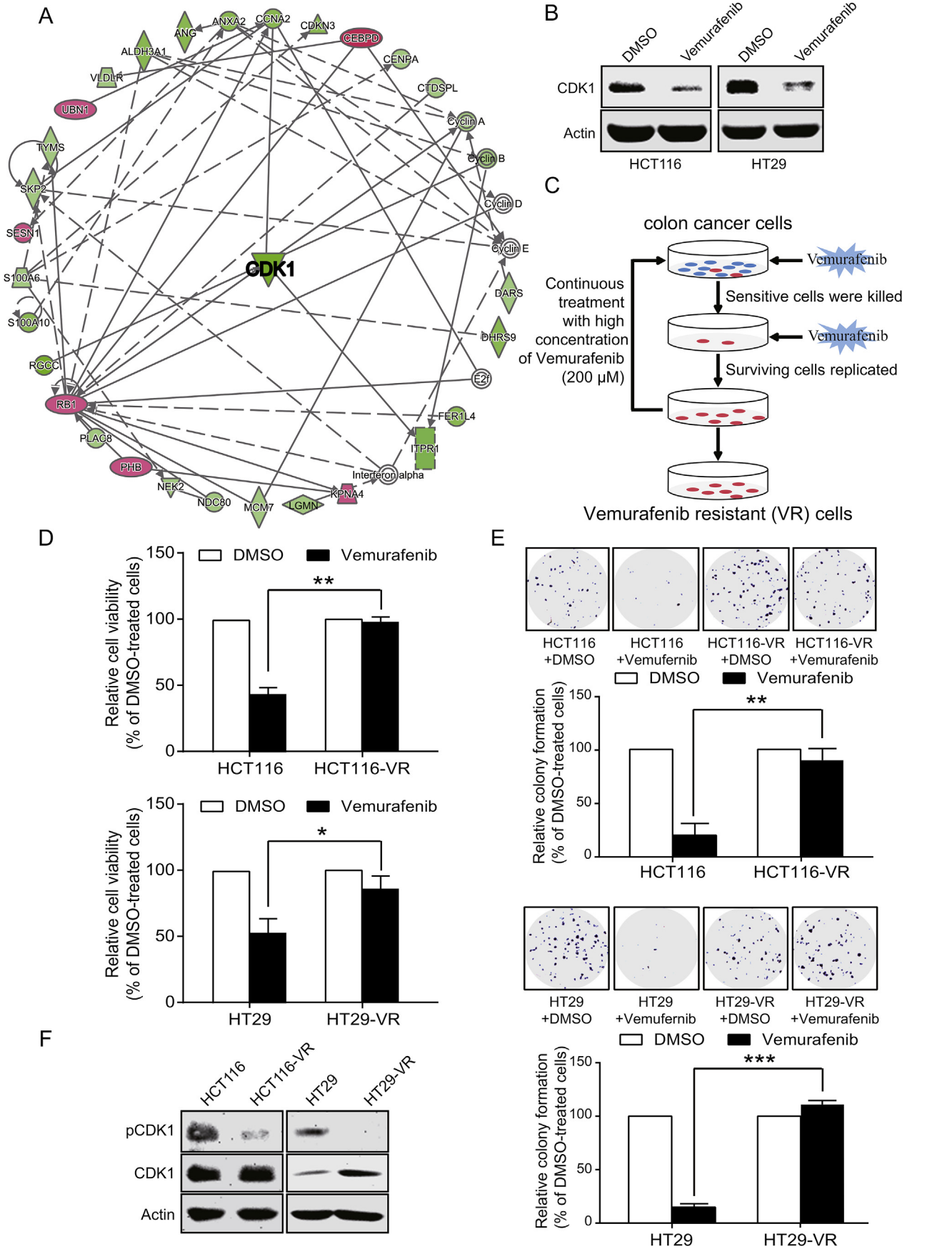
Protein purification was performed as described previously [32]. The KCTD12 gene was amplified using genomic DNA from HCT116 cells by PCR using the forward primer 5′-CGCGGATCC ATGG CACTGGCGGATAGCACACG-3′ and the reverse primer 5′-CGCGAATTC CTCCCTGCATAAGACGTACT-3′ following by cloning into the His-tagged pET-28b vector. The CDK1 gene was amplified using the forward primer 5′-CGCGGATCCATCTACCATACCCATTCACTAACT-3′ and reverse primer 5′-CGCGAATTCCTACATCTTCTTAATCTGATTGTCC-3′ followed by cloning into a GST flag-tagged pGEX-4T-1 vector. KCTD12 and CDK1 expression plasmids were transformed into E. coli BL21 star (DE3) cells for subsequent expression. Bacteria were grown to a 600 nm (OD600) optical density of approximately 1.0 at 37 °C, followed by induction with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 6 h and lysing via sonication. KCTD12-his and CDK1-GST fusion proteins were isolated by loading whole cell lysate onto his-binding and GST-binding columns according to the manufacturer's instructions (Beyotime Biotechnology, Shanghai, China) and were verified by SDS-PAGE and Western blotting.

2.5. Enzyme-linked immunosorbent assay (ELISA) screening system

Experiments were performed using ELISA. Briefly, the first layer antibody GST-tag antibody (Proteintech) in coating buffer (1 ng/μL) was added into 96-well plates and cultured overnight at 4 °C. After being washed with phosphate-buffered saline (PBS) and blocked with 5% bovine serum albumin (BSA), the purified fusion protein CDK1-GST (1 μg) was added and the plate was softly rocked for 5 h at room temperature. The plate was then incubated with 1 μg purified KCTD12-his protein for 3 h at 37 °C, and 616 small molecular inhibitors (10 μM) from the FDA-approved Drug Library (Selleck Chemicals, Huston, TX, USA) were individually added into each well. After incubating with his-tag antibody, the corresponding secondary antibody and tetramethylbenzidine (TMB), absorbance was measured at wavelengths of 450 nm and 630 nm. Levels of KCTD12-CDK1 interaction were determined using the following formula: O.D.450-O.D.630 [33]. Coating buffer, TMB and termination buffer were purchased from Neo-Bioscience (Shenzhen, Guangdong, China).

2.6. Flow cytometric cell cycle analysis

Cells were fixed in 70% alcohol for 1 h at −20 °C followed by addition of propidium iodide (PI) staining buffer (33 μg/ml PI, 0.13 mg/ml RNaseA, 10 mM EDTA, 0.5% TritonX-100) for 10 min at room temperature. Flow cytometry analyses of cells stained with the phospho-H3 AF488 antibody (Cell Signaling Technology) were performed as described previously [20]. Briefly, fixed cells were treated with 0.25% Triton X-100 on ice for 15 min, incubated with phospho-H3 antibody for 3 h at room temperature, and then stained with PI. Cells were analyzed on a BD Accuri™ C6 Analyzer (BD Biosciences, San Jose, CA, USA).



(caption on next page)

Fig. 1. CDK1 is activated in vemurafenib-resistant colon cancer. (**A**) IPA analysis suggested significant changes in the expression of cell cycle checkpoint proteins, including CDK1 signaling, in colon cancer cells treated with PLX4720, an analog of vemurafenib (GSE50791). (**B**) Colon cancer cells, HCT116 and HT29, were treated with vemurafenib (10 μM) for 24 h, and CDK1 expression was detected by Western blotting. (**C**) Diagram depicting the establishment of VR sublines from colon cancer cells. (**D**) HCT116-VR and HT29-VR, as well as parental cells, were treated with vemurafenib (10 μM) or DMSO for 6 days, and cell viability was determined by WST-1 assay. (**E**) Comparison of the ability of vemurafenib-resistant colon cancer cells and parental cells to form colonies upon vemurafenib treatment (10 μM) using colony formation assay. (**F**) Western blot was performed to detect expression levels of p-CDK1 and CDK1 in Vemurafenib-resistant colon cancer cells and parental cells. Bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2.7. WST-1 assay

Cell viability was measured using a WST-1 Cell Proliferation and Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China). Cells were seeded in 96-well plates and treated with drugs at various concentrations and multiple time points. WST-1 reagent was added, and cells were incubated at 37 °C for 4 h. Absorbance was measured as described previously [34].

2.8. Carboxyfluorescein succinimidyl ester (CFSE) assay

Cells were resuspended in CFSE solution (Abcam, Cambridge, MA, USA) at 37 °C for 10 min, and staining was terminated with precooled DMEM medium. Cells were washed twice with DMEM medium, cultured at 37 °C for 48 h and then collected for flow cytometry analysis as described previously [35].

2.9. Colony-formation assay

A colony-formation assay was performed as described previously [36]. Briefly, cells were seeded in 6-well plates at a density of 500 cells per well. After 14 days, cells were fixed with methanol and stained with 0.1% crystal violet. The number of colonies was then quantified for analysis.

2.10. Coimmunoprecipitation (Co-IP)

Co-IP experiments were performed as described previously [37]. Briefly, after prewashing with IgG (Santa Cruz Biotechnology) and protein A/G Sepharose beads (Invitrogen, Gaithersburg, MD, USA) for 1 h at 4 °C, cell lysates were incubated with the appropriate primary antibody overnight at 4 °C followed by 4 h of incubation with protein A/G Sepharose beads. The beads were washed three times with lysis buffer and eluted in 2 x SDS/PAGE loading buffer for immunoblotting.

2.11. Tumorigenicity in nude mice

Tumor xenograft experiments were performed as described previously [20,38,39]. Female BALB/c nude mice aged 6–8 weeks were maintained under standard conditions and cared for according to institutional guidelines for animal care. Briefly, cells were suspended in a 1:1 mixture of PBS:Matrigel and subcutaneously injected into the flanks of mice. Next, mice were randomized into treatment and control groups when tumors reached ~5 mm diameter. Treatment groups received oral gavage of AD (10 mg/kg or 50 mg/kg) or vemurafenib (20 mg/kg) every other day, whereas control groups received vehicle only. An additional treatment group was given vemurafenib (20 mg/kg) combined with AD (10 mg/kg). Tumor size was measured with calipers every two or three days, and tumor volume was calculated using the following equation: $V = (\text{length} \times \text{width}^2)/2$. The body weight of mice was monitored weekly during the experiments to evaluate overall health. All animals were euthanized at the end of the study, and tumors, lungs, liver, and kidneys were collected for further analysis. All animal experiments were approved by the Ethics Committee for Animal Experiments of Jinan University.

2.12. Analysis of gene expression from gene expression omnibus (GEO) database and IPA analysis

Gene expression datasets for melanoma [15,16] and colon cancer cells [14] were downloaded from GEO (accession numbers GSE42872, GSE67088, and GSE50791). Differentially expressed genes were analyzed using IPA analysis as described previously [37].

2.13. Statistical analysis

Data from three independent experiments are expressed as the mean ± SD and were compared using Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. CDK1 acts as a downstream effector of vemurafenib

To explore vemurafenib's mechanisms of action in cancer cells, we analyzed gene expression changes in vemurafenib-treated melanoma cells derived from the GEO repository using Ingenuity Pathway Analysis [15,16]. We also analyzed GEO data derived from colon cancer cells treated with PLX4720, an analog of vemurafenib [14,40]. Bioinformatics analysis indicated that a cluster of differentially expressed genes was enriched in cell cycle-related pathways in both colon cancer and melanoma cells. Among these genes, cyclins and CDK1 were downregulated in colon cancer and melanoma cells treated with PLX4720 or vemurafenib (Fig. 1A and Supplementary Fig. S1A). In addition to a recent study demonstrating that vemurafenib treatment suppresses cell cycle progression, we hypothesized that cell cycle checkpoints, in particular CDK1, were functionally involved in the potential anticancer effects of vemurafenib [41]. Our results showed that CDK1 expression was downregulated in colon cancer cells treated with vemurafenib (Fig. 1B, Supplementary Figs. S1C and S1D) in a time- and dose-dependent manner. However, data mining of public databases indicated that unlike downregulated CDK1 expression in vemurafenib-treated melanoma cells [15] (Supplementary Fig. S1B, left panel), vemurafenib did not exert a significant change in CDK1 expression in VR melanoma cells [16] (Supplementary Fig. S1B, right panel). To investigate the changes in expression and activity of CDK1 in vemurafenib-resistant colon cancer cells, we established two VR cell lines, HCT116-VR and HT29-VR, by continuously incubating parental cells with high concentrations of vemurafenib for further study (Fig. 1C). Although vemurafenib inhibited growth of parental colon cancer cells in a time- and dose-dependent manner (Supplementary Fig. S1E), VR cell lines exhibited no response to vemurafenib treatment (Fig. 1D and E). We next determined expression levels of CDK1 and p-CDK1, the inactive form of CDK1, in the two VR colon cancer cell lines, and Western blot assays revealed that CDK1 activity was enhanced in VR cells (Fig. 1F). These results indicate that CDK1 not only mediates the anticancer effects of vemurafenib but also plays an important role in the resistance of colon cancer cells to vemurafenib.

3.2. CDK1 expression induces vemurafenib resistance in colon cancer

To study the functional role of CDK1 in vemurafenib resistance, two colon cancer cell lines with stable overexpression of CDK1 (Fig. 2A)

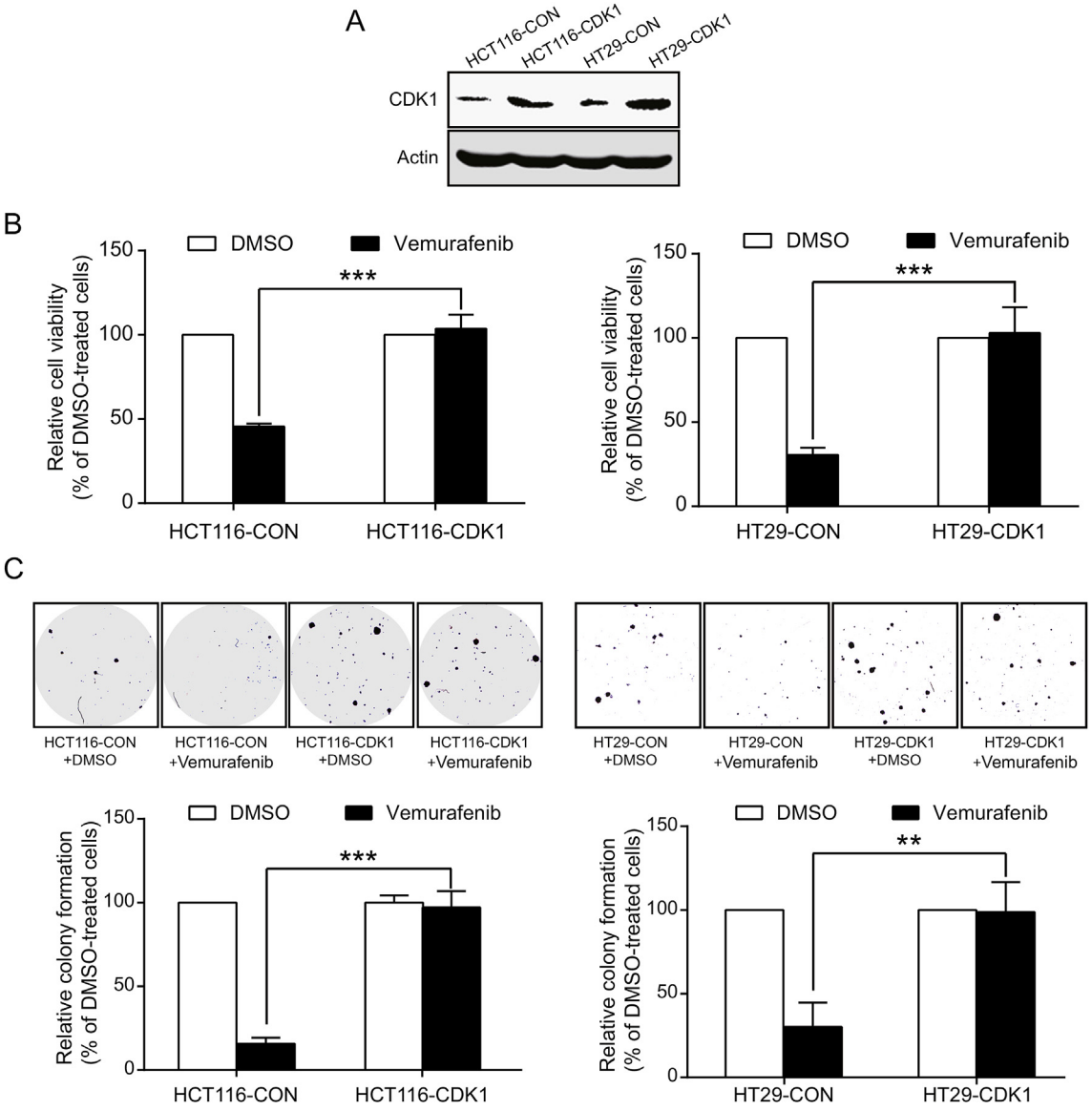


Fig. 2. CDK1 induces vemurafenib resistance in colon cancer cells. (**A**) Successful establishment of CDK1-overexpressing HCT116 and HT29 cells. (**B**) HCT116-CDK1, HT29-CDK1, and control cells were treated with vemurafenib (10 μM) for 6 days, and cell viability was examined by WST-1 assay. (**C**) The ability of CDK1-overexpressing colon cancer cells and control cells to form colonies under vemurafenib treatment (10 μM) was compared by colony formation assay. Note that CDK1 overexpression increased the resistance of colon cancer cells to vemurafenib. Bars, SD; **, $P < 0.01$; ***, $P < 0.001$.

were constructed, HCT116-CDK1 and HT29-CDK1, and vemurafenib sensitivity was assessed. WST-1 assay showed that vemurafenib had no observed effect on CDK1-overexpressing cells. However, cell viability of the parental cells, HCT116-CON and HT29-CON, was markedly reduced (Fig. 2B). Similar results were observed for colony formation assays, demonstrating that vemurafenib does not affect the ability of CDK1-overexpressing colon cancer cells to form colonies compared to control cells (Fig. 2C). These results suggest that CDK1 enhances the resistance of colon cancer cells to vemurafenib.

3.3. Adefovir dipivoxil disrupts the KCTD12-CDK1 interaction

CDK1 activation is a prerequisite for its function in promoting cell cycle progression [20], and some upstream regulators, such as cell division cycle 25 (CDC25) phosphatases, reportedly interact with and activate CDK1 to promote cell division [20,42], indicating that targeting the interaction between CDK1 and its activator represents a promising strategy for the development of anticancer drugs. Our recent study demonstrated that the KCTD12-CDK1 interaction is necessary for CDK1 activation and mitosis, therefore promoting tumorigenesis [20]. In the present study, we aimed to identify small molecule inhibitors that potently inhibited CDK1 activation through disruption of the KCTD12-CDK1 interaction. By screening an FDA-approved library consisting of 616 compounds using the ELISA system described in Fig. 3A, we identified three small molecular inhibitors, Topiramate, Stavudine and Adefovir Dipivoxil (AD), that exhibited inhibitory effects on the KCTD12-CDK1 interaction (Fig. 3B). To confirm the preliminary screening data in cancer cells, we treated HCT116 and HT29 cells separately with these three inhibitors, and coimmunoprecipitation assays were performed to assess the KCTD12-CDK1 interaction. As shown in Fig. 3C, KCTD12-CDK1 interaction was significantly inhibited by AD treatment in both colon cancer cell lines, whereas the other two inhibitors exhibited inconsistent results in these cell lines. Furthermore, our results demonstrated that AD suppressed CDK1 activation in a dose- and time-dependent manner (Fig. 3D and E). AD has been clinically used as an anti-viral drug to prevent viral replication by targeting reverse transcriptase, especially in treating hepatitis B virus (HBV) infection [24–26] (Fig. 3F). These data demonstrate that AD also targets

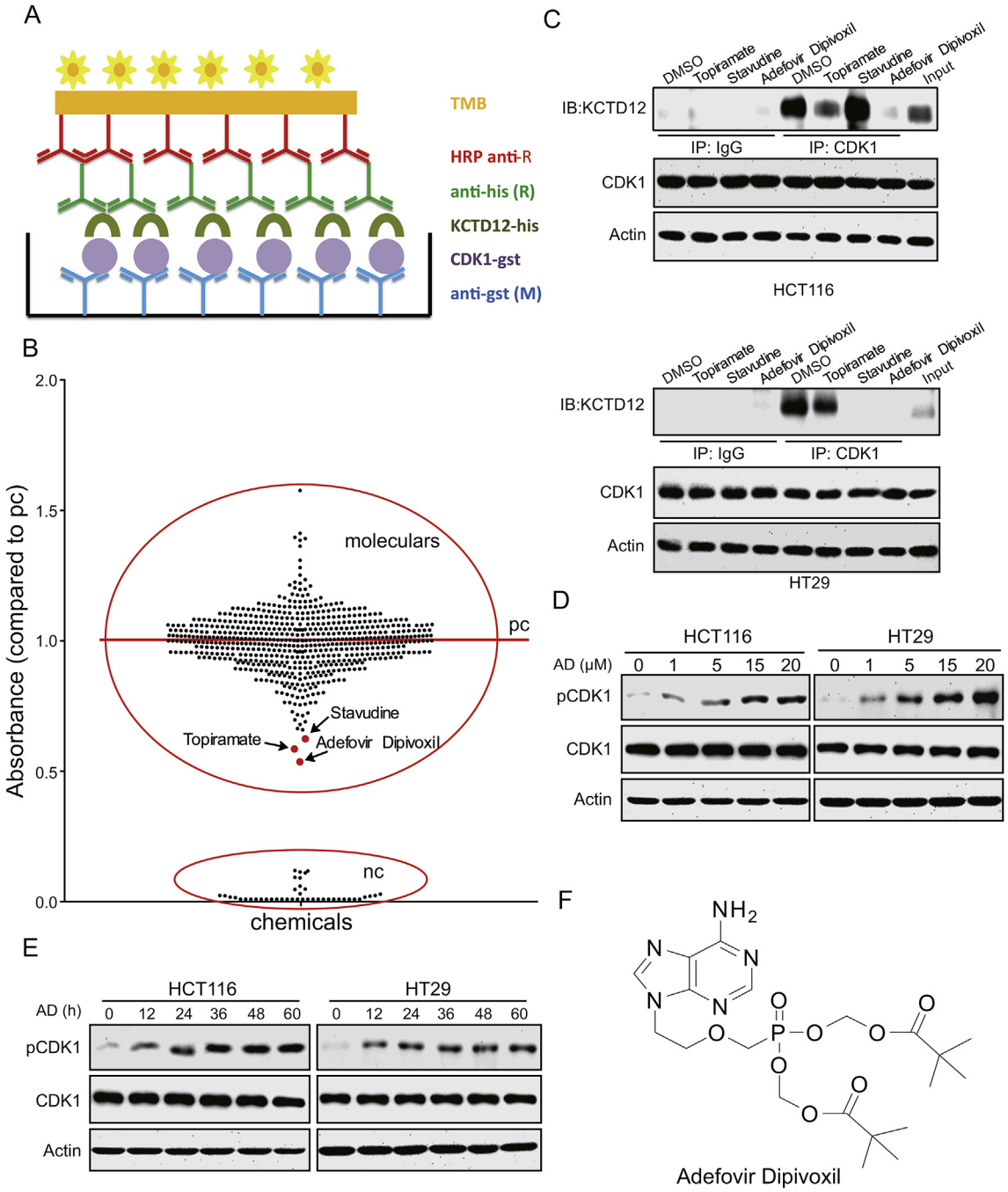
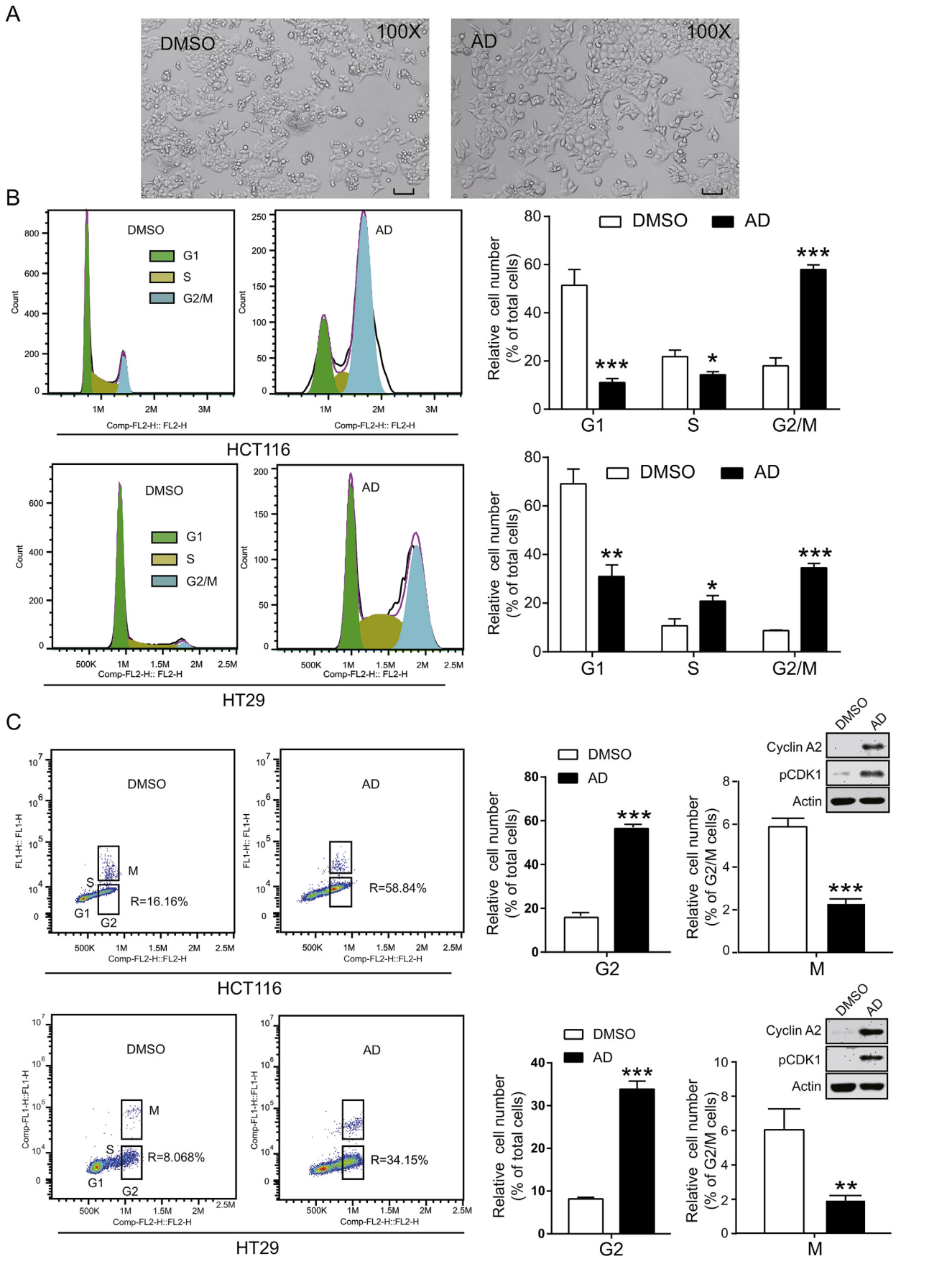


Fig. 3. AD disrupts KCTD12-CDK1 interaction and suppresses CDK1 activation. (**A**) The workflow of the ELISA screening system. Prokaryotic-expressed recombinant protein CDK1-GST was purified and fixed on the plate though interaction with the GST-tag antibody immobilized on the 96-well plate. The other purified recombinant protein, KCTD12-His, was added into the well together with individual small molecule inhibitors derived from an FDA-approved compound library consisting of 616 inhibitors, and interaction levels between CDK1 and KCTD12 were analyzed using the formula "OD450-OD630" based on the reaction of HRP and TMB. (**B**) The absorbance in wells with different inhibitors treatment is presented as a percentage relative to the wells treated with vehicle control. Red dots indicate compounds that exerted the most significant inhibitory effects on KCTD12-CDK1 interaction: Topiramate; Stavudine; Adefovir Dipivoxil. (**C**) A co-IP assay was performed to determine the interaction of KCTD12 and CDK1 in colon cancer cells treated with Topiramate, Stavudine and Adefovir Dipivoxil. Expression of KCTD12 was determined in CDK1 immuonprecipitates by Western blotting. (**D**) HCT116 and HT29 cells were treated with AD in different concentrations for 24 h, and expression levels of p-CDK1 and CDK1 were detected by Western blotting. (**E**) Colon cancer cells were treated with AD (10 μM) for multiple time points, and expression and activation level of CDK1 were detected by Western blotting. (**F**) The structure chart of AD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the KCTD12-CDK1 interaction and inactivates CDK1 in cancer cells.

3.4. Adefovir dipivoxil induces G2 phase cell cycle arrest

Given that the interaction of KCTD12 and CDK1 is necessary for G2-M transition during mitosis [20,43], we postulated that AD might have an important effect on cancer cell division. To test this hypothesis, we treated HCT116 colon cancer cells with AD for 24 h and found that cells became much larger than control cells (Fig. 4A). This phenomenon suggests that AD treatment inhibits cell division. HCT116 and



(caption on next page)

Fig. 4. AD prevents colon cancer cells from entering M phase. (**A**) Images of HCT116 cells treated with AD (10 μM) or DMSO for 24 h. (**B**) Cell cycle distribution was examined by flow cytometry in cells treated with DMSO or AD (10 μM) for 36 h. Data showed that AD induced G2/M cell cycle arrest in HCT116 and HT29 cells. (**C**) Cells at M phase were distinguished from the cells at G2/M phase using a phopho-H3 antibody in flow cytometry analysis. Percentages of G2 phase cells among the whole population and percentages of M phase among the G2/M phase cells were statistically derived. Western blot data indicated increased expression levels of pCDK1 and cyclin A2. R, cells at G2 phase. Bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to DMSO-treated cells.

HT29 cells were treated with AD or DMSO control, and flow cytometry was performed to analyze cell cycle distribution. As shown in Fig. 4B, the ratio of HCT116 cells at G2/M phase was significantly increased in response to AD treatment, and a similar result was observed in HT29 cells. To determine which phase AD was specifically affecting, cells in M phase, indicated by phospho-H3-positive cells, were distinguished from cells at G2 phase. Flow cytometry analysis showed that the ratio of M phase cells was significantly decreased, whereas the ratio of G2 phase cells was significantly increased in HCT116 and HT29 cells treated with AD (Fig. 4C). Moreover, expression of Cyclin A2, an important regulator of G2 phase [44], was markedly upregulated. As expected, CDK1 activity was inhibited in AD-treated colon cancer cells (Fig. 4C). The above experiments were repeated in HCT116 and HT29 cells with CDK1 overexpression, which are resistant to vemurafenib treatment, and a similar change in the percentages of G2 and M phase cells was observed upon AD treatment (Supplementary Fig. S2A). Therefore, the above findings indicate that AD prevents cells from entering M phase in both VR and parental colon cancer cells.

3.5. Adefovir dipivoxil inhibits tumorigenesis of colon cancer cells

Next, we evaluated the effects of AD on cancer cell proliferation. HCT116 and HT29 cells were treated with AD or DMSO control, and data from CFSE assays revealed that cell division was significantly inhibited by AD (Fig. 5A). WST-1 and colony formation assays were also performed to confirm the inhibitory effect of AD on colon cancer cell proliferation. As shown in Fig. 5B and C, the ability of HCT116 and HT29 cells to proliferate and form colonies was markedly reduced when cells were treated with AD. Cell viability assays demonstrated that AD suppressed cell growth in a time- and dose-dependent manner (Supplementary Fig. S2B). Moreover, this finding was confirmed in VR colon cancer cells overexpressing CDK1 (Supplementary Figs. S2C and S2D). Next, we examined the therapeutic potential of AD using a mouse model. HT29 cells were subcutaneously injected into nude mice to establish tumor xenografts, and mice subsequently received oral administration of AD or vehicle control. Results revealed that tumor burden was significantly reduced by AD treatment in a dose-dependent manner, with decreases of 33.1% and 47.1% in groups receiving 10 mg/kg and 50 mg/kg, respectively, compared to vehicle-treated mice (Fig. 5D). Western blot analysis of tumor xenografts showed an obvious inactivation of CDK1 signaling and cell cycle arrest at the G2 phase as indicated by increased expression of p-CDK1 and cyclin A2, respectively (Fig. 5E). These data are consistent with the *in vitro* data shown in Fig. 4. Note, no significant difference was observed between treated and control groups in terms of body weight (Fig. 5F). Histological examination of the vital organs, including lungs, liver and kidneys, did not reveal any overt changes in morphology, suggesting that AD treatment had no toxic effects on animals (Fig. 5G). Collectively, we demonstrated that AD significantly suppresses the growth of colon cancer cells and VR cells *in vitro* and *in vivo* with no observed side effects.

3.6. Adefovir dipivoxil sensitizes vemurafenib-resistant colon cancer cells to vemurafenib

Given that CDK1 activation leads to vemurafenib resistance in colon cancer cells and AD inactivates CDK1 though disrupting the KCTD12-CDK1 interaction, we next investigated whether AD treatment enhances the sensitivity of VR colon cancer cells to vemurafenib. Using WST-1 and colony formation assays, we found that combined use of AD and vemurafenib exerted a more potent effect on suppression of cancer cell proliferation compared to either vemurafenib or AD treatment alone in CDK1-expressing colon cancer cells (Fig. 6A and B). To examine whether AD reverses the resistance of CDK1-overexpressing colon cancer cells to vemurafenib *in vivo*, HT29 cells with ectopic expression of CDK1 were subcutaneously injected into nude mice, and tumor-bearing mice were treated with vemurafenib, AD alone or a combination. Although vemurafenib or AD had no effect or only a modest anti-proliferative effect on tumors, combined vemurafenib and AD exerted a significantly synergistic effect on suppressing the growth of tumor xenografts (Fig. 6C). Moreover, immunohistochemical analysis of Ki-67 proliferation index also provided evidence that tumor cell proliferation was significantly inhibited by combined use of vemurafenib and AD (mean index decreased from 51.7% in the vehicle-treated group to 47.0% in the vemurafenib-treated group and 22.0% in the AD/Vemurafenib combination-treated group) (Fig. 6D). Western blot analysis of tumor xenografts showed that CDK1 activation in VR tumors was significantly inhibited by combined use of vemurafenib and AD (Fig. 6E). Considering the lack of significant changes in body weight (Fig. 6F), our results demonstrate treatment efficacy and safety of AD as a vemurafenib sensitizer in colon cancer cells.

4. Discussion

B-Raf is frequently overexpressed in different cancers and plays an important role in tumorigenesis [7,8]. Previous studies have demonstrated that vemurafenib exhibits significant effects on suppressing melanoma cell proliferation by targeting B-Raf/B-Raf V600E. However, the mechanism by which vemurafenib suppresses cancer cell proliferation remains unclear, and the therapeutic efficacy of vemurafenib on colon cancer is reduced compared to melanoma [13]. Understanding the molecular mechanisms underlying drug resistance can provide clues to identify potential targets for development of anticancer drugs. A recent study demonstrated that colon cancer is unresponsive to vemurafenib treatment due to feedback activation of EGFR [13]. Moreover, cancer cells will also be unresponsive to targeted drugs if certain “front-line proteins”, including direct performers, such as cyclins and epithelial-mesenchymal transition (EMT) factors that receive signals from upstream factors and lead to tumorigenesis and cancer development, are activated [45]. This finding suggests that targeting “front-line proteins” may be a better strategy for overcoming tumor resistance to chemotherapeutic drugs. In this study, we uncovered the mechanism for chemoresistance of colon cancer cells to vemurafenib and aimed to resolve this problem by focusing on “front-line proteins”. Using IPA analysis, we found that cell cycle checkpoint signaling is one of the biological processes affected by vemurafenib, and expression of CDK1, one of the “front-line proteins”, was significantly decreased. Furthermore, CDK1 overexpression significantly increased colon cancer cell resistance to vemurafenib, indicating that CDK1 is an effector of vemurafenib and plays an important role in vemurafenib resistance in colon cancer (Figs. 1–2, Supplementary Fig. S1).

Signal transduction is dependent on specific protein-protein interactions in cells [46,47], and emerging evidence suggests that these protein-protein interactions are potential targets for cancer treatment [48,49]. Modified ELISA has been used to detect protein-protein interactions and facilitates the screening of small molecules targeting protein-protein interaction *in vitro*. For example, researchers identified SLCB050 as a novel anti-tumor compound that inhibits DX2-p14/ARF

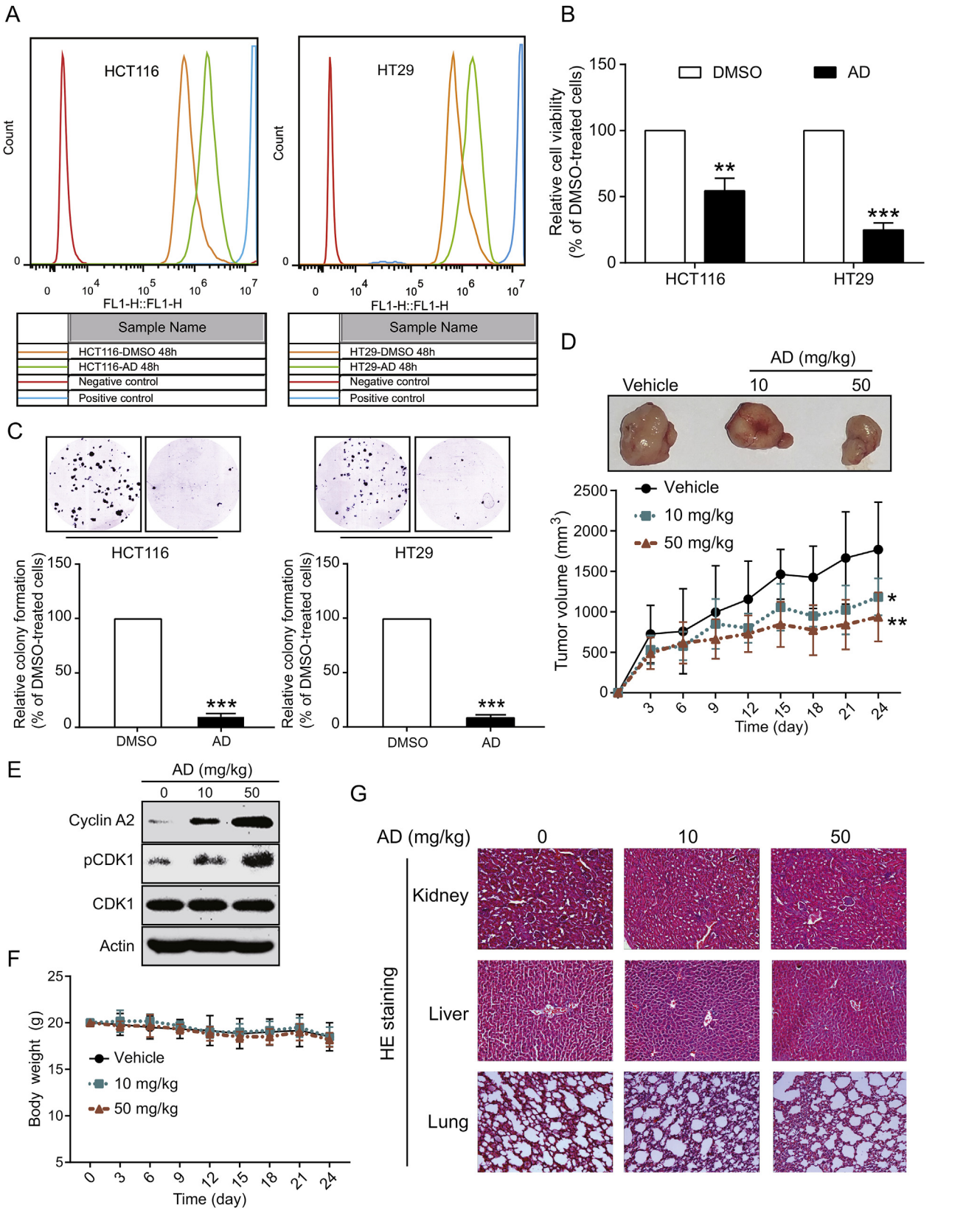
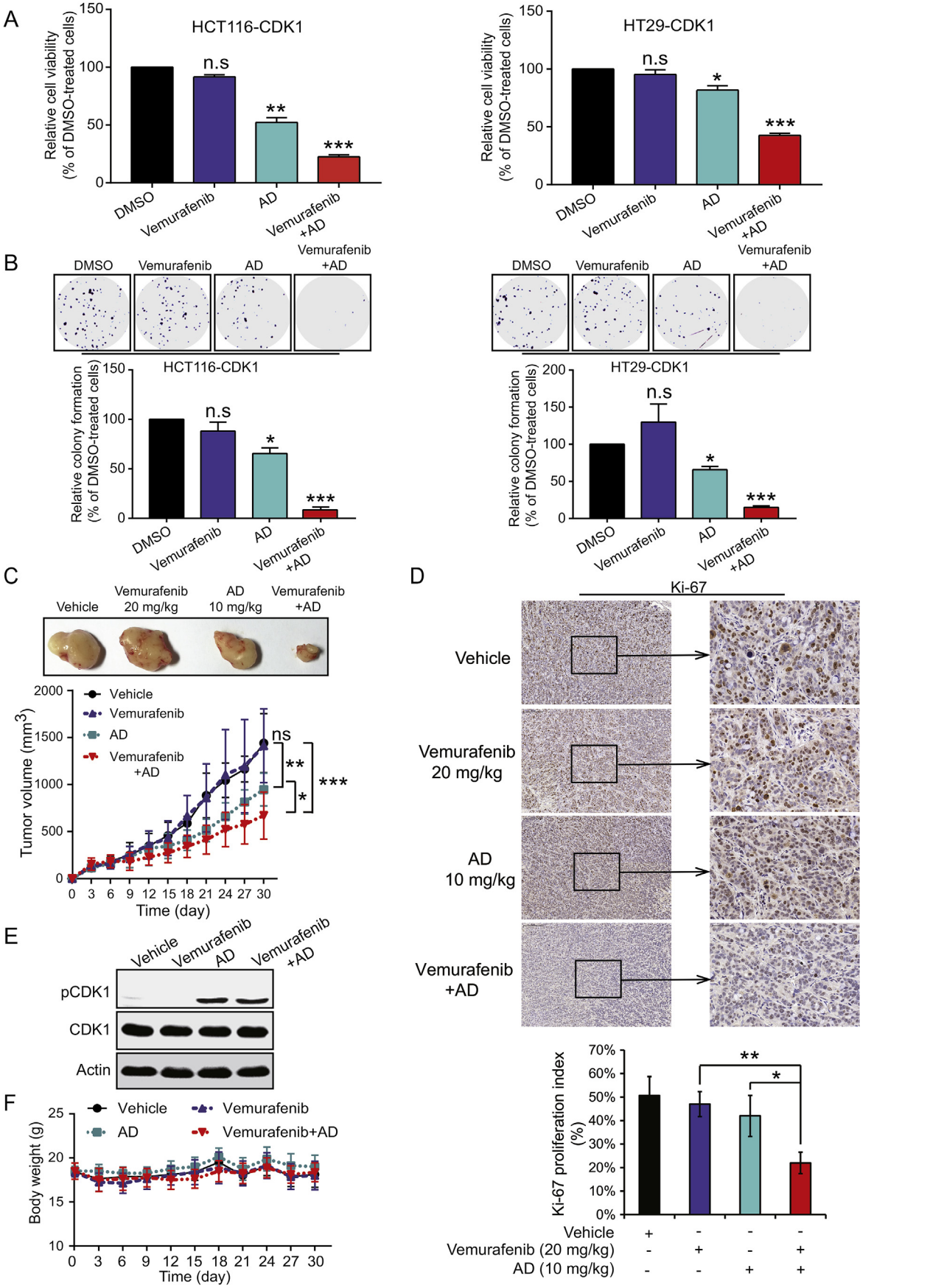


Fig. 5. AD suppresses tumorigenesis of colon cancer cells *in vitro* and *in vivo*. (**A**) HCT116 and HT29 cells treated with DMSO or AD (10 μM) for 48 h were stained with CFSE, and the rate of cell division was analyzed by flow cytometry. (**B**) Comparison of cell viability in colon cancer cells with or without AD treatment by WST-1 assays. (**C**) Colony formation ability of colon cancer cells with or without AD treatment was examined by colony formation assays. (**D**) Nude mice bearing HT29-derived xenografts were orally administered AD (10 mg/kg or 50 mg/kg) or vehicle daily (n = 6 per group). Tumor curves showing that AD significantly suppressed growth of tumor xenografts. (**E**) Expression levels of cyclin A2, pCDK1 and CDK1 were compared between tumors from mice treated with AD and vehicle, by Western blot. (**F**) Body weight of nude mice during the AD treatment period. (**G**) Representative images of liver, kidney and lung specimens stained with hematoxylin and eosin (H&E) indicated no toxic effects. Bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to the control group treated with DMSO.



(caption on next page)

Fig. 6. AD reverses the resistance of VR colon cancer cells to vemurafenib. (**A, B**) HCT116-CDK1 and HT29-CDK1 cells were treated with vemurafenib (10 μM), AD (1 μM), or the combination of both, and cell viability and colony formation ability were measured by WST-1 (**A**) and colony-formation assays (**B**). (**C**) Nude mice bearing HT29-CDK1-derived tumor xenografts were treated with vemurafenib (20 mg/kg), AD (10 mg/kg) or a combination of vemurafenib and AD daily (n = 6 per group). (**D**) Immunohistochemical analysis of Ki-67 proliferative index. (**E**) Expression of p-CDK1 and CDK1 was compared among tumors from mice treated with vemurafenib, AD, or a combination of vemurafenib and AD by Western blot. (**F**) Body weight of nude mice during the experiment period suggested the safety of the single and combined treatments. Bars, SD; n.s., nonsignificance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to control group unless otherwise indicated.

interactions using the ELISA screening system [50]. Our previous study demonstrated that the KCTD12-CDK1 interaction was a prerequisite to CDK1 activation and promotes cell division. During the cell cycle, proper G2/M transition is important for cell division, and CDK1 activation is a precondition for G2/M transition [20]. Although some CDK1 inhibitors have been developed, such as Ro-3306, this compound has not been used for clinical therapy, and the side effects are unclear [20]. By performing ELISA screening and a series of functional assays, we found that AD inhibits the KCTD12-CDK1 interaction and suppresses growth of colon cancer cells *in vitro* and *in vivo* (Figs. 3–5), further suggesting that targeting protein-protein interactions is a promising strategy for developing novel therapeutic agents.

The indication of many FDA-approved drugs includes various diseases other than cancer. Given that the side effects of these "old drugs" and the corresponding countermeasures for clinical treatment are known [51], "old drugs for novel use" is a promising strategy for developing anticancer drugs. AD was the focus of this study. A clinic trial launched in Japan demonstrated that antiviral therapy with AD conveyed longer survival for postoperative HBV-related hepatocellular carcinoma (HBV-HCC) patients [52]. Another study claimed that AD treatment was effective in normalizing liver function, decreasing HBV-HCC recurrence, and improving postoperative survival [53]. In the present study, a series of experiments demonstrated that AD suppresses colon cancer cell proliferation by inactivating CDK1 and inducing G2 phase arrest. Moreover, inactivation of CDK1 by AD reversed CDK1 expression-induced vemurafenib resistance in colon cancer cells, thereby sensitizing VR colon cancer cells to vemurafenib (Figs. 6–7). Furthermore, combined use of AD and vemurafenib exhibited a more potent effect on colon cancer growth inhibition.

Colon cancer is one of the most common malignancies worldwide [1,54]. As most "front-line proteins" are evolutionarily conserved and rarely mutated, these proteins represent more suitable targets for development of novel treatment agents. Our study demonstrated that the activation of CDK1, one of the "front-line proteins", is significantly inhibited by AD, a nucleoside analog approved by the FDA to treat HBV infection. For the first time, our data provide solid evidence that AD used as a single agent or in combination with vemurafenib significantly induces G2 cell cycle arrest to inhibit tumorigenesis of colon cancer cells by disrupting the interaction between CDK1 and KCTD12, thus supporting the hypothesis that AD administration may be a potential strategy for colon cancer therapy.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the National Key R & D Program of China (2017YFA0505100), the National Natural Science Foundation of China (31770888, 31570828, 81672953, 81773085) and the Guangdong Natural Science Research Grant (2016A030313838).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.050.

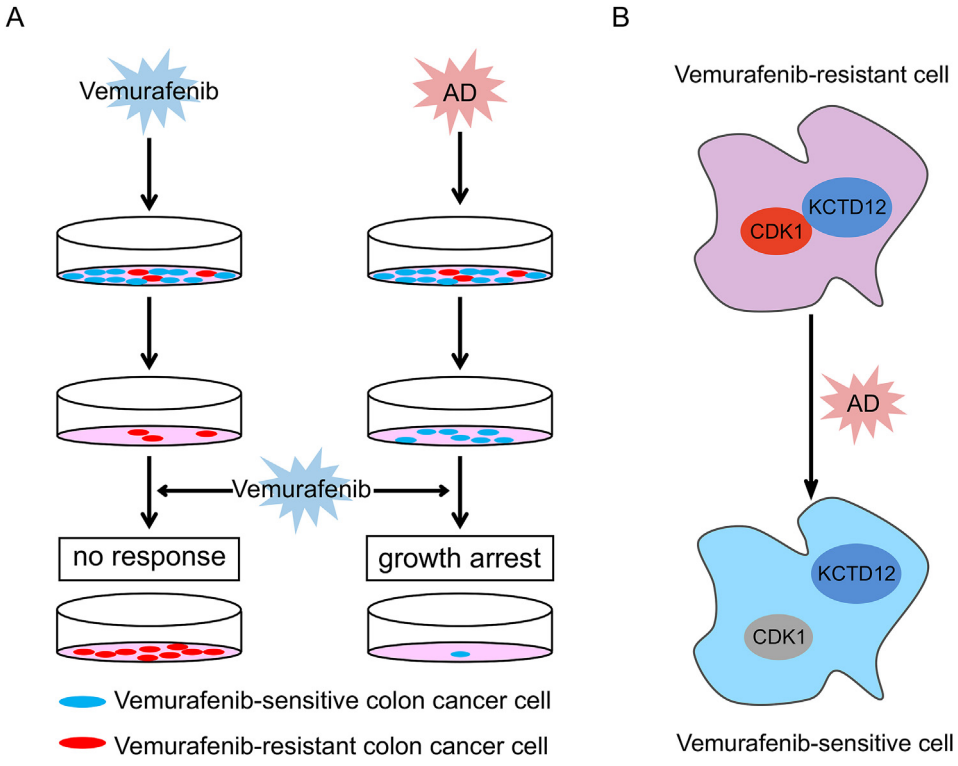


Fig. 7. Schematic diagram summarizing the mechanism for AD sensitizing colon cancer cells to vemurafenib treatment. (**A**) Colon cancer cells show no response to vemurafenib after a period of vemurafenib treatment. AD sensitizes colon cancer cells to vemurafenib, and the combined use of vemurafenib and AD induces growth arrest in colon cancer cells. (**B**) CDK1 activation induces unresponsiveness of colon cancer to vemurafenib, while AD inactivates CDK1 by disrupting the KCTD12-CDK1 interaction, thus sensitizing colon cancer cells to vemurafenib.

References

[1] R.L. Siegel, K.D. Miller, S.A. Fedewa, D.J. Ahnen, R.G. Meester, A. Barzi, A.J.C.a.c.j.f.c. Jemal, Colorectal cancer statistics 67 (2017) 177–193 2017.

[2] H. Brody, Colorectal cancer, Nature 521 (2015) S1.

[3] B.A. Weinberg, J.L. Marshall, M.E. Salem, The growing challenge of young adults with colorectal cancer, Oncology 31 (2017) 381–389.

[4] A. Gautam, K. Chaudhary, R. Kumar, S. Gupta, H. Singh, G.P. Raghava, Managing drug resistance in cancer: role of cancer informatics, Methods Mol. Biol. 1395 (2016) 299–312.

[5] M.S. Feizabadi, Modeling multi-mutation and drug resistance: analysis of some case studies, Theor. Biol. Med. Model. 14 (2017) 6.

[6] A. Lassen, M. Atefi, L. Robert, D.J. Wong, M. Cerniglia, B. Comin-Anduix, A. Ribas, Effects of AKT inhibitor therapy in response and resistance to BRAF inhibition in melanoma, Mol. Canc. 13 (2014) 83.

[7] G. Maurer, B. Tarkowski, M. Baccarini, Raf kinases in cancer-roles and therapeutic opportunities, Oncogene 30 (2011) 3477–3488.

[8] A. Orlandi, A. Calegari, A. Inno, R. Berenato, M. Caporale, M. Niger, I. Bossi, M. Di Bartolomeo, F. de Braud, F. Pietrantonio, BRAF in metastatic colorectal cancer: the future starts now, Pharmacogenomics 16 (2015) 2069–2081.

[9] J.H. Strickler, C. Wu, T. Bekaii-Saab, Targeting BRAF in metastatic colorectal cancer: maximizing molecular approaches, Cancer Treat Rev. 60 (2017) 109–119.

[10] R. Herr, M. Kohler, H. Andrlova, F. Weinberg, Y. Moller, S. Halbach, L. Lutz, J. Mastroianni, M. Klose, N. Bittermann, S. Kowar, R. Zeiser, M.A. Olayioye, S. Lassmann, H. Busch, M. Boerries, T. Brummer, B-Raf inhibitors induce epithelial differentiation in BRAF-mutant colorectal cancer cells, Cancer Res. 75 (2015) 216–229.

[11] C.J. Punt, M. Koopman, L. Vermeulen, From tumour heterogeneity to advances in precision treatment of colorectal cancer, Nat. Rev. Clin. Oncol. 14 (2017) 235–246.

[12] P.B. Chapman, A. Hauschild, C. Robert, J.B. Haanen, P. Ascierto, J. Larkin, R. Dummer, C. Garbe, A. Testori, M. Maio, D. Hogg, P. Lorigan, C. Lebbe, T. Jouary, D. Schadendorf, A. Ribas, S.J. O'Day, J.A. Sosman, J.M. Kirkwood, A.M. Eggermont, B. Dreno, K. Nolop, J. Li, B. Nelson, J. Hou, R.J. Lee, K.T. Flaherty, G.A. McArthur, Improved survival with vemurafenib in melanoma with BRAF V600E mutation, N. Engl. J. Med. 364 (2011) 2507–2516.

[13] A. Prahallad, C. Sun, S. Huang, F. Di Nicolantonio, R. Salazar, D. Zecchin, R.L. Beijersbergen, A. Bardelli, R. Bernards, Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR, Nature 483 (2012) 100–103.

[14] M. Boerries, R. Herr, T. Brummer, H. Busch, Global gene expression profiling analysis reveals reduction of stemness after B-RAF inhibition in colorectal cancer cell lines, Genom Data 4 (2015) 158–161.

[15] T.J. Parmenter, M. Kleinschmidt, K.M. Kinross, S.T. Bond, J. Li, M.R. Kaadige, A. Rao, K.E. Sheppard, W. Hugo, G.M. Pupo, R.B. Pearson, S.L. McGee, G.V. Long, R.A. Scolyer, H. Rizos, R.S. Lo, C. Cullinane, D.E. Ayer, A. Ribas, R.W. Johnstone, R.J. Hicks, G.A. McArthur, Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis, Cancer Discov. 4 (2014) 423–433.

[16] I. Zubrilov, O. Sagi-Assif, S. Izraely, T. Meshel, S. Ben-Menahem, R. Ginat, M. Pasmanik-Chor, C. Nahmias, P.O. Couraud, D.S. Hoon, I.P. Witz, Vemurafenib resistance selects for highly malignant brain and lung-metastasizing melanoma cells, Cancer Lett. 361 (2015) 86–96.

[17] M.B. Ujiki, X.Z. Ding, M.R. Salabat, D.J. Bentrem, L. Golkar, B. Milam, M.S. Talamonti, R.H. Bell Jr., T. Iwamura, T.E. Adrian, Apigenin inhibits pancreatic cancer cell proliferation through G2/M cell cycle arrest, Mol. Canc. 5 (2006) 76.

[18] T. Ji, C. Lin, L.S. Krill, R. Eskander, Y. Guo, X. Zi, B.H. Hoang, Flavokawain B, a kava chalcone, inhibits growth of human osteosarcoma cells through G2/M cell cycle arrest and apoptosis, Mol. Canc. 12 (2013) 55.

[19] M. Malumbres, M. Barbacid, Cell cycle, CDKs and cancer: a changing paradigm, Nat. Rev. Canc. 9 (2009) 153–166.

[20] Y. Zhong, J. Yang, W.W. Xu, Y. Wang, C.C. Zheng, B. Li, Q.Y. He, KCTD12 promotes tumorigenesis by facilitating CDC25B/CDK1/Aurora A-dependent G2/M transition, Oncogene 36 (2017) 6177–6189.

[21] T. Namiki, T. Yaguchi, K. Nakamura, J.C. Valencia, S.G. Coelho, L. Yin, M. Kawaguchi, W.D. Vieira, Y. Kaneko, A. Tanemura, I. Katayama, H. Yokozeki, Y. Kawakami, V.J. Hearing, NUAK2 amplification coupled with PTEN deficiency promotes melanoma development via CDK activation, Cancer Res. 75 (2015) 2708–2715.

[22] D. Milewski, A. Pradhan, X. Wang, Y. Cai, T. Le, B. Turpin, V.V. Kalinichenko, T.V. Kalin, FoxF1 and FoxF2 transcription factors synergistically promote rhabdomyosarcoma carcinogenesis by repressing transcription of p21(Cip1) CDK inhibitor, Oncogene 36 (2017) 850–862.

[23] Q. Liu, Editorial: old drugs learn new tricks: advances and applications for drug repurposing, Curr. Top. Med. Chem. 16 (2016) 3627–3628.

[24] M.R. Khan, M.S. Chowdhury, M. Saha, S.M. Roknuzzaman, M. Mahmuduzzaman, A.R. Miah, P.K. Roy, M.A. Raihan, K.M. Rahman, Efficacy of adefovir dipivoxil therapy in patients with chronic hepatitis B viral infection, Mymensingh Med. J. 23 (2014) 715–719.

[25] P. Marcellin, E.J. Heathcote, M. Buti, E. Gane, R.A. de Man, Z. Krastev, G. Germanidis, S.S. Lee, R. Flisiak, K. Kaita, M. Manns, I. Kotzev, K. Tchernev, P. Buggisch, F. Weilert, O.O. Kurdas, M.L. Shiffman, H. Trinh, M.K. Washington, J. Sorbel, J. Anderson, A. Snow-Lampart, E. Mondou, J. Quinn, F. Rousseau, Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B, N. Engl. J. Med. 359 (2008) 2442–2455.

[26] N. Shahabadi, M. Falsafi, Experimental and molecular docking studies on DNA binding interaction of adefovir dipivoxil: advances toward treatment of hepatitis B virus infections, Spectrochim. Acta Mol. Biomol. Spectrosc. 125 (2014) 154–159.

[27] F. Perez-Roldan, P. Gonzalez-Carro, M.C. Villafanez-Garcia, Adefovir dipivoxil for chemotherapy-induced activation of hepatitis B virus infection, N. Engl. J. Med. 352 (2005) 310–311.

[28] J. Delmas, O. Schorr, C. Jamard, C. Gibbs, C. Trepo, O. Hantz, F. Zoulim, Inhibitory effect of adefovir on viral DNA synthesis and covalently closed circular DNA formation in duck hepatitis B virus-infected hepatocytes in vivo and in vitro, Antimicrob. Agents Chemother. 46 (2002) 425–433.

[29] J.G. Julander, R.W. Sidwell, J.D. Morrey, Characterizing antiviral activity of adefovir dipivoxil in transgenic mice expressing hepatitis B virus, Antivir. Res. 55 (2002) 27–40.

[30] W.W. Xu, B. Li, J.F. Zhao, J.G. Yang, J.Q. Li, S.W. Tsao, Q.Y. He, A.L.M. Cheung, IGF2 induces CD133 expression in esophageal cancer cells to promote cancer stemness, Cancer Lett. 425 (2018) 88–100.

[31] B. Li, S.W. Tsao, K.W. Chan, D.L. Ludwig, R. Novosyadlyy, Y.Y. Li, Q.Y. He, A.L. Cheung, Id1-induced IGF-II and its autocrine/endocrine promotion of esophageal cancer progression and chemoresistance–implications for IGF-II and IGF-IR-targeted therapy, Clin. Cancer Res. : an official journal of the American Association for Cancer Research 20 (2014) 2651–2662.

[32] H. Li, N. Li, Q. Xu, C. Xiao, H. Wang, Z. Guo, J. Zhang, X. Sun, Q.Y. He, Lipoprotein FtsB in Streptococcus pyogenes binds ferrichrome in two steps with residues Tyr137 and Trp 204 as critical ligands, PLoS One 8 (2013) e65682.

[33] B. Li, W.W. Xu, X.Y. Guan, Y.R. Qin, S. Law, N.P. Lee, K.T. Chan, P.Y. Tam, Y.Y. Li, K.W. Chan, H.F. Yuen, S.W. Tsao, Q.Y. He, A.L. Cheung, Competitive binding between Id1 and E2F1 to Cdc20 regulates E2F1 degradation and thymidylate synthase expression to promote esophageal cancer chemoresistance, Clin. Cancer Res. : an official journal of the American Association for Cancer Research 22 (2016) 1243–1255.

[34] Y. Wang, Y.J. Li, X.H. Huang, C.C. Zheng, X.F. Yin, B. Li, Q.Y. He, Liensinine perchlorate inhibits colorectal cancer tumorigenesis by inducing mitochondrial dysfunction and apoptosis, Food & function 9 (2018) 5536–5546.

[35] A.B. Lyons, S.J. Blake, K.V. Doherty, Flow cytometric analysis of cell division by dilution of CFSE and related dyes (Chapter 9), Curr Protoc Cytom (2013) Unit 9.11.1–9.11.12.

[36] B. Li, Y.Y. Li, S.W. Tsao, A.L. Cheung, Targeting NF-kappaB signaling pathway suppresses tumor growth, angiogenesis, and metastasis of human esophageal cancer, Mol. Canc. Therapeut. 8 (2009) 2635–2644.

[37] Y. Wang, R.Y. Yu, J. Zhang, W.X. Zhang, Z.H. Huang, H.F. Hu, Y.L. Li, B. Li, Q.Y. He, Inhibition of Nrf2 enhances the anticancer effect of 6-O-angeloylenolin in lung adenocarcinoma, Biochem. Pharmacol. 129 (2017) 43–53.

[38] W.W. Xu, B. Li, X.Y. Guan, S.K. Chung, Y. Wang, Y.L. Yip, S.Y. Law, K.T. Chan, N.P. Lee, K.W. Chan, L.Y. Xu, E.M. Li, S.W. Tsao, Q.Y. He, A.L. Cheung, Cancer cell-secreted IGF2 instigates fibroblasts and bone marrow-derived vascular progenitor cells to promote cancer progression, Nat. Commun. 8 (2017) 14399.

[39] B. Li, W.W. Xu, L. Han, K.T. Chan, S.W. Tsao, N.P.Y. Lee, S. Law, L.Y. Xu, E.M. Li, K.W. Chan, Y.R. Qin, X.Y. Guan, Q.Y. He, A.L.M. Cheung, MicroRNA-377 suppresses initiation and progression of esophageal cancer by inhibiting CD133 and VEGF, Oncogene 36 (2017) 3986–4000.

[40] I. Theochari, M. Goulielmaki, D. Danino, V. Papadimitriou, A. Pintzas, A. Xenakis, Drug nanocarriers for cancer chemotherapy based on microemulsions: the case of Vemurafenib analog PLX4720, Colloids Surfaces B Biointerfaces 154 (2017) 350–356.

[41] L. Quan, Y. Wang, J. Liang, J. Shi, Y. Zhang, K. Tao, Identification of the interaction network of hub genes for melanoma treated with vemurafenib based on microarray data, Tumori 101 (2015) 368–374.

[42] R. Boutros, V. Lobjois, B. Ducommun, CDC25 phosphatases in cancer cells: key players? Good targets? Nat. Rev. Canc. 7 (2007) 495–507.

[43] N.R. Brown, S. Korolchuk, M.P. Martin, W.A. Stanley, R. Moukhametzianov, M.E. Noble, J.A. Endicott, CDK1 structures reveal conserved and unique features of the essential cell cycle CDK, Nat. Commun. 6 (2015) 6769.

[44] Y. Li, L. Peng, E. Seto, Histone deacetylase 10 regulates the cell cycle G2/M phase transition via a novel let-7-HMGA2-cyclin A2 pathway, Mol. Cell Biol. 35 (2015) 3547–3565.

[45] H. Liang, T. Yu, Y. Han, H. Jiang, C. Wang, T. You, X. Zhao, H. Shan, R. Yang, L. Yang, H. Shan, Y. Gu, LncRNA PTAR promotes EMT and invasion-metastasis in serous ovarian cancer by competitively binding miR-101-3p to regulate ZEB1 expression, Mol. Canc. 17 (2018) 119.

[46] N.S. Latysheva, M.E. Oates, L. Maddox, T. Flock, J. Gough, M. Buljan, R.J. Weatheritt, M.M. Babu, Molecular principles of gene fusion mediated rewiring of protein interaction networks in cancer, Mol. Cell. 63 (2016) 579–592.

[47] Y.D. Cai, Q. Zhang, Y.H. Zhang, L. Chen, T. Huang, Identification of genes associated with breast cancer metastasis to bone on a protein-protein interaction network with a shortest path algorithm, J. Proteome Res. 16 (2017) 1027–1038.

[48] Y. Hao, S. Zhao, Z. Wang, Targeting the protein-protein interaction between IRS1 and mutant p110alpha for cancer therapy, Toxicol. Pathol. 42 (2014) 140–147.

[49] A.A. Ivanov, F.R. Khuri, H. Fu, Targeting protein-protein interactions as an anticancer strategy, Trends Pharmacol. Sci. 34 (2013) 393–400.

[50] A.Y. Oh, Y.S. Jung, J. Kim, J.H. Lee, J.H. Cho, H.Y. Chun, S. Park, H. Park, S. Lim, N.C. Ha, J.S. Park, C.S. Park, G.Y. Song, B.J. Park, Inhibiting DX2-p14/ARF interaction exerts antitumor effects in lung cancer and delays tumor progression, Cancer Res. 76 (2016) 4791–4804.

[51] Y. Li, P.K. Li, M.J. Roberts, R.C. Arend, R.S. Samant, D.J. Buchsbaum, Multi-targeted therapy of cancer by niclosamide: a new application for an old drug, Cancer Lett. 349 (2014) 8–14.

[52] K. Sakamoto, T. Beppu, H. Hayashi, S. Nakagawa, H. Okabe, H. Nitta, K. Imai,

D. Hashimoto, A. Chikamoto, T. Isiko, K. Kikuchi, H. Baba, Antiviral therapy and long-term outcome for hepatitis B virus-related hepatocellular carcinoma after curative liver resection in a Japanese cohort, Anticancer Res. 35 (2015) 1647–1655.

[53] J. Yin, N. Li, Y. Han, J. Xue, Y. Deng, J. Shi, W. Guo, H. Zhang, H. Wang, S. Cheng, G. Cao, Effect of antiviral treatment with nucleotide/nucleoside analogs on postoperative prognosis of hepatitis B virus-related hepatocellular carcinoma: a two-stage longitudinal clinical study, J. Clin. Oncol. 31 (2013) 3647–3655.

[54] W. Chen, R. Zheng, P.D. Baade, S. Zhang, H. Zeng, F. Bray, A. Jemal, X.Q. Yu, J. He, Cancer statistics in China, 2015, Ca - Cancer J. Clin. 66 (2016) 115–132.