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ATR inhibition sensitizes HPV⁻ and HPV⁺ head and neck squamous cell carcinoma to cisplatin

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Abstract

Objectives: Cisplatin is commonly used in the treatment of head and neck squamous cell carcinoma (HNSCC), and the repair of cisplatin-induced DNA damage involves activation of the DNA damage response protein ataxia telangiectasia and Rad3-related (ATR). Resistance to cisplatin therapy exacerbates adverse toxicities and is associated with poor outcomes. Since repair of cisplatin-induced DNA damage contributes to resistance, we hypothesized that inhibition of ATR using AZD6738, a well-tolerated and orally-bioavailable inhibitor, would enhance the sensitivity of HNSCC cells and tumors to cisplatin.

Materials and methods: A panel of human papilloma virus-negative (HPV⁻) and HPV⁺ HNSCC cell lines were treated with cisplatin in the absence or presence of AZD6738, and effects on cell viability, colony formation, apoptosis signaling, and DNA damage were assessed. The impact of co-treatment with cisplatin plus AZD6738 on the growth of HPV⁻ and HPV⁺ cell line- and patient-derived xenograft tumors was also examined.

Results: Inhibition of ATR with AZD6738 enhanced cisplatin-induced growth inhibition of HNSCC cell lines and tumors, in association with increased apoptosis signaling and DNA damage. Both HPV⁻ and HPV⁺ models were sensitized to cisplatin by ATR inhibition.

Conclusion: Inhibition of ATR promotes sensitization to cisplatin in preclinical *in vitro* and *in vivo* models of HPV⁻ and HPV⁺ HNSCC, supporting clinical evaluation of this strategy in this disease.

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Conflict of interest

DEJ and JRG are co-inventors of cyclic STAT3 decoy and have financial interests in STAT3 Therapeutics. STAT3 Therapeutics holds an interest in cyclic STAT3 decoy, which is not a focus of the studies in this manuscript. The remaining authors declare no conflicts.

Keywords

ATR; cisplatin; AZD6738; HNSCC

Introduction

Exposure to tobacco products and an increasing incidence of oropharyngeal infection with human papilloma virus (HPV) have contributed to making head and neck squamous cell carcinoma (HNSCC) one of the leading causes of cancer mortality worldwide (1,2). Historically, surgery, radiation and chemotherapy have served as the primary treatment options for HNSCC, although these approaches are commonly associated with multiple adverse toxicities and effects on speech and swallowing. Three molecular targeting agents have been approved by the US Food and Drug Administration (FDA) for the treatment of HNSCC. Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, was approved in 2006 (3, 4), while pembrolizumab and nivolumab, antibodies targeting the checkpoint receptor PD-1 on effector T cells, were approved in 2016 (5-7). Although the application of these new agents represents a significant advance in the treatment of HNSCC, in each case, only a minority subset of patients respond (3-7). Hence, the use of conventional chemotherapy remains a mainstay of treatment.

The most commonly used chemotherapy drug for HNSCC is cisplatin (8, 9). Cisplatin acts by forming covalent adducts with DNA bases, primarily guanine, promoting the formation of intra-strand DNA crosslinks (and less frequently, inter-strand crosslink) (10-12). Failure to repair these crosslinks blocks the progression of DNA and RNA polymerases, leading to induction of cell death (13). The repair of cisplatin-induced DNA intra-strand crosslinks occurs via nucleotide excision repair and involves activation of the kinase activity of ataxia telangiectasia and Rad3-related (ATR) and the ATR substrate CHK1 (14-18). The development of small molecule inhibitors of ATR, including M6620 (formerly called VX-970 and VE-822) (19, 20) and AZD6738 (21,22), has confirmed the critical role of ATR in the repair of cisplatin-induced damage. Moreover, treatment with ATR inhibitors has been shown to enhance cell death induced by chemotherapy drugs and radiation in cancer cell lines *in vitro* (19-21,23-30). *In vivo* studies have shown that M6620 and AZD6738 sensitize lung cancer and pancreatic ductal adenocarcinoma tumors to cisplatin and gemcitabine, respectively (20, 21, 31). The ability of ATR inhibitors to enhance responsiveness to chemotherapy is amplified in cells and tumors deficient in ataxia telangiectasia mutated (ATM) (21,23, 28, 32). The impact of ATR inhibitors on HNSCC has not been reported.

The profile of HNSCC in the United States is characterized by a dramatically increasing incidence of HPV-positive (HPV⁺) oropharyngeal carcinoma (33, 34). Current estimates indicate that roughly 75% of oropharyngeal HNSCC cases are associated with HPV infection (35, 36). Recent investigations implicate a complex interaction between HPV and DNA damage response proteins, including ATR and ATM (37-39). In particular, HPV recruits and exploits DNA damage response proteins to facilitate viral replication (37-39). However, the impact of HPV on ATR-mediated repair of chemotherapy-induced DNA damage is incompletely understood. HNSCC offers a unique opportunity for investigation of

chemosensitization by ATR inhibitors as both HPV-negative (HPV⁻) and HPV⁺ HNSCC preclinical models exist. In this report we utilized a broad panel of HPV⁻ and HPV⁺ HNSCC cell lines to determine the effects of ATR inhibition on cellular growth and survival following cisplatin treatment. Inhibition of ATR signaling was achieved using AZD6738, an orally bioavailable inhibitor currently being evaluated in clinical trials (40), or by downregulation of ATR mRNA and protein. We further evaluated the *in vivo* anti-tumor activities of AZD6738 and cisplatin, alone and in combination, against both HPV⁻ and HPV⁺ cell line-derived and patient-derived xenograft tumors. Our findings support clinical evaluation of AZD6738/cisplatin co-treatment in patients with either HPV⁻ or HPV⁺ HNSCC.

Materials and methods

Cell lines and cell culture

All cell lines were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. The authenticity of each cell line was validated by Short Tandem Repeat (STR) profiling prior to use and every 6 months thereafter.

Crystal violet assays

Cells were plated in 96-well plates (500-5,000 cells/well depending on growth rate) and incubated with drugs for 48 hours. Treated cells were washed with 50 μ L PBS then incubated in 50 μ L of 0.5% crystal violet in 25% methanol. After 30 minutes the staining solution was removed and the plates washed with water 5 times. After overnight air-drying, crystal violet stain was dissolved using a 1:1 mixture of 200 mM sodium citrate (pH 6.0) and 100% ethanol. Plates were then briefly placed on a shaker and the resulting absorbance of the solution was read at 590 nm. IC₅₀ values were calculated using GraphPad Prism (GraphPad Software).

Annexin V/PI flow cytometry

Annexin V/PI staining was performed using FITC Annexin V Apoptosis Detection kits (BD Biosciences, cat# 556547). Cells were removed from plates by trypsinization, washed twice with PBS, then resuspended in 1x Binding Buffer. The cells (1×10^5 cells in 100 μ L) were incubated with 2 μ L FITC-annexin V and 2 μ L propidium iodide (PI) for 15 minutes at room temperature. Binding buffer (400 μ L) was then added to each tube prior to analysis on a FACS Calibur DXP8 (BD Biosciences). Data was analyzed using FlowJo software (FlowJo, LLC).

Immunoblotting

Immunoblotting was performed as previously described (41). The following primary antibodies were used in this study: rabbit anti-pATR Ser428 (CST, cat# 2853), rabbit anti-ATR (CST, cat# 13934), rabbit anti-ATM (CST, cat# 2873), rabbit anti-PARP-1 (CST, cat# 9542), rabbit anti-pCHK1 Ser345 (CST, cat# 2348), mouse anti-CHK1 (CST, cat# 2360), rabbit anti-pH2AX Ser139 (CST, cat# 9718), mouse anti-TP53 (Santa Cruz, cat# sc-126), and rabbit anti-GAPDH (CST, cat# 5174). Goat anti-mouse HRP (Bio-rad, cat# 1706516)

and goat anti-rabbit HRP (Bio-rad, cat# 1706515) antibodies were used as secondary antibodies.

Colony formation assays

Cells were plated in 6-well plates ($1.7\text{-}2.0 \times 10^5$ cells/well) and incubated with drugs for 24 hours. Afterwards, cells were trypsinized, plated (400-800 cells/well depending on growth rate) in triplicate in new 6-well plates, then allowed to grow for at least 14 days. Colonies were then washed with 1 mL of PBS and incubated in 650 μL of 0.5% crystal violet. Colonies were counted using the ImageJ software and later dissolved with a 1:1 mixture of 200 mM sodium citrate and 100% ethanol. The absorbance (590 nm) of the resulting solution was then measured.

siRNA knockdown

ON-TARGETplus SMARTpool siRNAs were obtained from Dharmacon for ATR (cat# L-003202-00) and ATM (cat# L-003201-00). ON-TARGETplus non-targeting pool siRNA (siNT; cat# D-001810-10) was used as a control. Cells (1×10^6 per 6 cm dish) were transfected with 60 nmol of siRNA using Lipofectamine RNAiMAX transfection reagent (cat# 13778150), followed by incubation for 48 hours prior to analysis.

In vivo xenograft models

To generate xenograft tumors from human HNSCC cell lines, 1×10^6 cells were injected into each flank of nude mice. Patient-derived xenograft (PDX) tumor models were propagated bilaterally in NOD/SCID gamma (NSG) mice (NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}* Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ) as previously described (42). Tumors were grown to an average volume of 100 mm³ before randomization and treatment with the indicated dosing regimens. Tumors were subsequently measured by calipers twice per week. This work was performed in accordance with UCSF IACUC protocol # AN109582.

Statistical analysis

For *in vitro* studies, statistical differences between treatment groups were determined using two-tailed Student's t-test. Figures for *in vitro* studies are representative of at least three independent experiments, except where indicated. For *in vivo* studies, Mann-Whitney test was used to determine statistical differences between treatment groups. Error bars in all figures indicate standard deviations.

Results

ATR inhibition sensitizes HPV⁻ and HPV⁺ HNSCC cells to growth inhibition by cisplatin

We first determined the sensitivity of a panel of 21 human HNSCC cells lines toward cisplatin (Figure 1A and Supplemental Table 1). Cells were treated with a range of cisplatin concentrations for 48 hours, followed by assessment of growth in crystal violet assays. A relatively narrow range of IC₅₀ values was observed, with CAL-33 cells being the most sensitive line (IC₅₀ = 2.0 μM) and SCC-9 being the most resistant (IC₅₀ = 21.7 μM).

Somewhat surprisingly, the 4 HPV⁺ cell lines (UM-SCC-47, UPCI-SCC-090, 93VU147T, UD-SCC-2) in the panel were among the most resistant (IC₅₀'s from 8.9-18.0 μM).

To determine whether potential relationships exist between sensitivity to cisplatin and the expression levels of total ATR, ATM, CHK1, and CHK2 proteins we performed immunoblotting, with β-actin serving as the loading control (Figure 1B). Varying levels of the four proteins were detected among the different HNSCC cell lines, with no absolute correlation with cisplatin sensitivity.

Despite the fact that no clear correlation was established between ATR levels and cisplatin sensitivity, prior studies have indicated that targeted inhibition of ATR may provide a means for enhancing the *in vitro* and *in vivo* sensitivity to cisplatin in lung cancer models (20, 21). To determine the impact of inhibiting ATR on cisplatin-induced growth inhibition, we assessed IC₅₀'s for cisplatin in the absence or presence of 500 nM AZD6738, a highly selective ATR inhibitor (21, 22). At the 500 nM concentration, AZD6738 alone did not impact the growth of the cells in 48-hour assays. Figure 1C (and Supplemental Table 2) depicts the ratio of the IC₅₀ obtained in the presence versus absence of AZD6738. As shown, AZD6738 enhanced sensitivity to cisplatin in all 21 HNSCC cell lines, although a range of sensitization was observed. The greatest sensitization was observed in CAL 27 cells, where AZD6738 caused a roughly 16.7-fold decrease in IC₅₀ for cisplatin. By contrast, BICR 6 cells, the least sensitized cell line, exhibited only a 1.1-fold decrease in IC₅₀ for cisplatin in the presence of the ATR inhibitor. Notably, AZD6738 caused a greater than 2-fold decrease in cisplatin IC₅₀'s in 3 out of 4 HPV⁺ cell lines (UPCI-SCC-090, UM-SCC-47, 93VU147T), but a less than 2-fold decrease in HPV⁺ UD-SCC-2 cells.

To confirm the impact of AZD6738 on cisplatin-induced growth inhibition in HNSCC cells we performed colony formation assays. CAL 27 (highly sensitized in crystal violet assays) and Detroit 562 (weakly sensitized in crystal violet assays) were treated with vehicle, 500 nM AZD6738 alone, 2 μM cisplatin alone, or the combination of AZD6738 and cisplatin. Following 24 hours of treatment, cells were replated and grown for at least 14 days, followed by determination of colony formation (Figure 2A and Supplemental Figure 1). As shown, the combination of AZD6738 and cisplatin resulted in enhanced loss of colony formation in both cell line models, relative to treatment with either agent alone. The combination also resulted in modestly enhanced loss of colony formation, relative to single agents, in the HPV⁺ cell line UM-SCC-47 (Supplemental Figure 1). By contrast, co-treatment with AZD6738 and paclitaxel, which exerts anticancer activity via effects on microtubules, did not result in enhanced loss of colony formation (Figure 2B).

ATR inhibition enhances cisplatin-induced DNA damage and cell death induction in HNSCC cells

To investigate the mechanism whereby AZD6738 enhanced cisplatin-induced growth inhibition of HNSCC cells, we first evaluated cell death induction. For these experiments we utilized the 3 HPV⁻ cell lines that were sensitized to cisplatin to the greatest degree by AZD6738 (CAL 27, BICR 56, PE/CA-PJ49; see Figure 1C) and the 3 HPV⁻ cell lines that were sensitized to the least degree (Detroit 562, SCC-25, BICR 6). Cells were treated for 24 hours with vehicle, 500 nM AZD6738 alone, 2 μM cisplatin alone, or the combination,

followed by flow cytometric analysis of annexin V and PI staining as an indicator of cell death (Figure 3A). Consistent with the cell growth assays in Figure 1C, ATR inhibition with AZD6738 markedly enhanced cisplatin-induced cell death in CAL 27, BICR 56, and PE/CA-PJ49. Similarly, AZD6738 enhanced cisplatin-induced death in HPV⁺ UM-SCC-47 cells (Figure 3A). By contrast, Detroit 562, SCC-25, and BICR 6 failed to show substantially enhanced cell death induction following treatment with the combination.

We next examined the impact of AZD6738, cisplatin, and the combination on apoptosis signaling, DNA damage, and ATR activity in HNSCC cells. Immunoblot analyses revealed increased cleavage of poly(ADP-ribose) polymerase (PARP), a marker of apoptosis signal transduction, in all 6 cell lines (as well as HPV⁺ UM-SCC-47) following treatment with the combination of AZD6738 and cisplatin, relative to cells treated with either agent alone (Figure 3B). Similarly, co-treatment with AZD6738 plus cisplatin resulted in markedly elevated levels of phospho-H2A.X (γ -H2A.X), a marker of DNA damage. Protein lysates were also analyzed for expression of phospho-CHK1 (p-CHK1), the downstream product of ATR kinase activity. Treatment with AZD6738 reduced the baseline levels of p-CHK1 to undetectable levels, consistent with inhibition of ATR activity. As expected, treatment with cisplatin alone led to induction of p-CHK1 in all 6 cell lines. Interestingly, however, p-CHK1 upregulation by cisplatin was markedly greater in the cell lines that were more weakly sensitized by AZD6738 compared to the cell lines that were more strongly sensitized by the ATR inhibitor. Expression of p-CHK1 returned to baseline levels in all 6 HNSCC cell lines following co-treatment with cisplatin and AZD6738.

Inhibition of expression of ATR, but not ATM, potentiates cisplatin-induced growth inhibition

To confirm that abrogation of ATR signaling enhances growth inhibition by cisplatin in HNSCC cells, we used siRNA to prevent ATR expression. Experiments were performed using 2 HNSCC cell lines that were strongly sensitized to cisplatin by AZD6738 (BICR 56, PE/CA-PJ49) and 2 that were weakly sensitized (Detroit 562, BICR 6). Immunoblotting confirmed efficient downregulation of ATR protein by the ATR siRNA (siATR) relative to transfection with nontargeting siRNA (siNT) (Figure 4A). Following transfection of siRNAs, cells were treated with cisplatin and cell growth analyzed by crystal violet assays. Consistent with findings using AZD6738, siATR-mediated downregulation of ATR resulted in strong sensitization to cisplatin in BICR 56 and PE/CA-PJ49 cells, and only weak sensitization in Detroit 562 and BICR 6 cells (Figure 4B).

We also assessed the impact of ATM downregulation, using siRNA directed against ATM mRNA (siATM) (Figure 4). In contrast to ATR downregulation, inhibition of ATM expression had little impact on cisplatin sensitivity in any of the 4 cell lines tested. Similarly, siRNA-mediated downregulation of p53 (data not shown), a key substrate of ATR signaling, had negligible effect on cisplatin sensitivity in the 3 cell lines that expressed basal levels of p53 protein (BICR 6 did not express detectable p53). These findings related to p53 are perhaps not surprising, as p53 has been reported to be mutated, and likely nonfunctional, in BICR 56 cells.

AZD6738 enhances the *in vivo* efficacy of cisplatin against HNSCC cell line-derived and patient-derived xenograft tumors

We next assessed the impact of AZD6738 on the *in vivo* activity of cisplatin against HNSCC xenograft tumors. Nude mice were inoculated subcutaneously with HPV⁻ CAL 27 cells or HPV⁺ UM-SCC-47 cells. Following the development of palpable tumors, mice were randomized, then treated with vehicle alone, cisplatin alone, AZD6738 alone, or the combination of cisplatin and AZD6738 (Figure 5A and 5B). For CAL 27-derived tumors, treatment with the combination resulted in greater inhibition of tumor growth than treatment with either agent alone (cisplatin vs. combination $p=0.0082$; AZD6738 vs. combination $p=0.0041$). Similarly, statistically significant greater inhibition of tumor growth was observed with the combination in mice harboring UM-SCC-47-derived tumors (cisplatin vs. combination $p=0.0026$; AZD6738 vs. combination $p=0.0030$).

To confirm our findings in more clinically relevant *in vivo* models, we evaluated AZD6738 activity against 2 PDX models. NSG mice were implanted with tumor tissue derived from an HPV⁻ PDX (PDX6851) or an HPV⁺ PDX (PDX7157), followed by randomization and treatment with vehicle, cisplatin, AZD6738, or the combination (Figure 5C and 5D). As was seen with the cell line-derived xenografts, treatment with cisplatin plus AZD6738 resulted in greater tumor growth inhibition than treatment with either agent alone. The enhanced anti-tumor activity of the combination was observed in both the HPV⁻ and HPV⁺ models.

Discussion

Despite substantial adverse toxicities associated with its use, cisplatin remains a mainstay treatment for head and neck cancer. Continuing efforts are needed to identify agents that can be used in combination to enhance cisplatin activity against HNSCC tumors, and thereby allow lower doses of cisplatin to be used. Such agents also might be useful for overcoming the inherent or acquired resistance to cisplatin that is commonly seen in HNSCC tumors and markedly impacts the success of treatment. Since cisplatin exerts its killing effects against cancer cells by causing DNA damage, it is reasonable to hypothesize that inhibition of the processes that repair this damage are likely to enhance cisplatin potency. The repair of cisplatin-induced DNA damage is known to occur, in large part, via activation of ATR (43). Small molecule inhibitors of ATR, including orally bioavailable AZD6738, have recently been developed and shown to enhance cisplatin activity in preclinical models of lung cancer and gynecologic cancers (20-22, 30). The impact of AZD6738 on cisplatin sensitivity of HNSCC cell lines and tumors, however, has not been reported. We find that a nontoxic dose of AZD6738 sensitized all 21 HNSCC cell lines tested to cisplatin, although variation in the degree of sensitization was observed. The combination of AZD6738 and cisplatin also produced greater inhibition of HNSCC tumor growth, relative to either agent alone, in *in vivo* experiments. AZD6738 is currently being evaluated in early phase clinical trials; our studies support clinical evaluation of AZD6738 plus cisplatin in HNSCC.

The prevalence of HPV⁺ HNSCC is increasing dramatically, particularly among younger adults (33, 44-48). Greater than 75% of oropharyngeal cancers in the developed world test positive for HPV. HNSCC patients with HPV⁺ disease typically have better prognoses and respond better to therapy than patients with HPV⁻ disease (49-55). However, recurrent and

metastatic HPV⁺ HNSCC remains difficult to treat and is often lethal. Moreover, recent findings indicate that cetuximab therapy is largely ineffective for HPV⁺ HNSCC (56, 57). Thus, improved therapeutic options are needed for both HPV⁻ and HPV⁺ HNSCC. In our studies, the four HPV⁺ HNSCC cell lines studied were moderately more resistant to cisplatin alone compared to most of the 17 HPV⁻ cell lines. None-the-less, AZD6738 sensitized both HPV⁺ and HPV⁻ cell lines to cisplatin. Indeed, 3 of the 4 HPV⁺ cell lines exhibited a greater than two-fold enhancement of sensitivity to cisplatin in the presence of AZD6738. In our *in vivo* studies, combined treatment with cisplatin and AZD6738 exhibited greater anti-tumor effects, relative to either agent alone, against both HPV⁺ and HPV⁻ xenograft models, including PDX models. These findings suggest that inhibition of ATR may be a useful strategy for enhancing response to cisplatin in both HPV⁺ and HPV⁻ HNSCC patients.

Our studies utilized simultaneous treatment with cisplatin and AZD6738. Using this approach, we found that the impact of ATR inhibition varied amongst the 21 cell lines. Although AZD6738 enhanced sensitivity to cisplatin in all 21 cell lines, varying degrees of sensitization were observed. While this variability may be reflective of differing gene expression patterns, the timing of ATR inhibition also may be a factor. As AZD6738 and other inhibitors of ATR advance to clinical testing in combination with cisplatin, it will be important to evaluate the impact of dosing and schedule of administration. Identification of baseline predictive biomarkers, such as loss of ATM or other DNA damage response proteins, will also help to optimize combination regimens incorporating ATR inhibitors and cisplatin. Collectively, these studies may provide an effective therapeutic strategy for enhancing anti-tumor activity and reducing the adverse toxicities of cisplatin in patients with HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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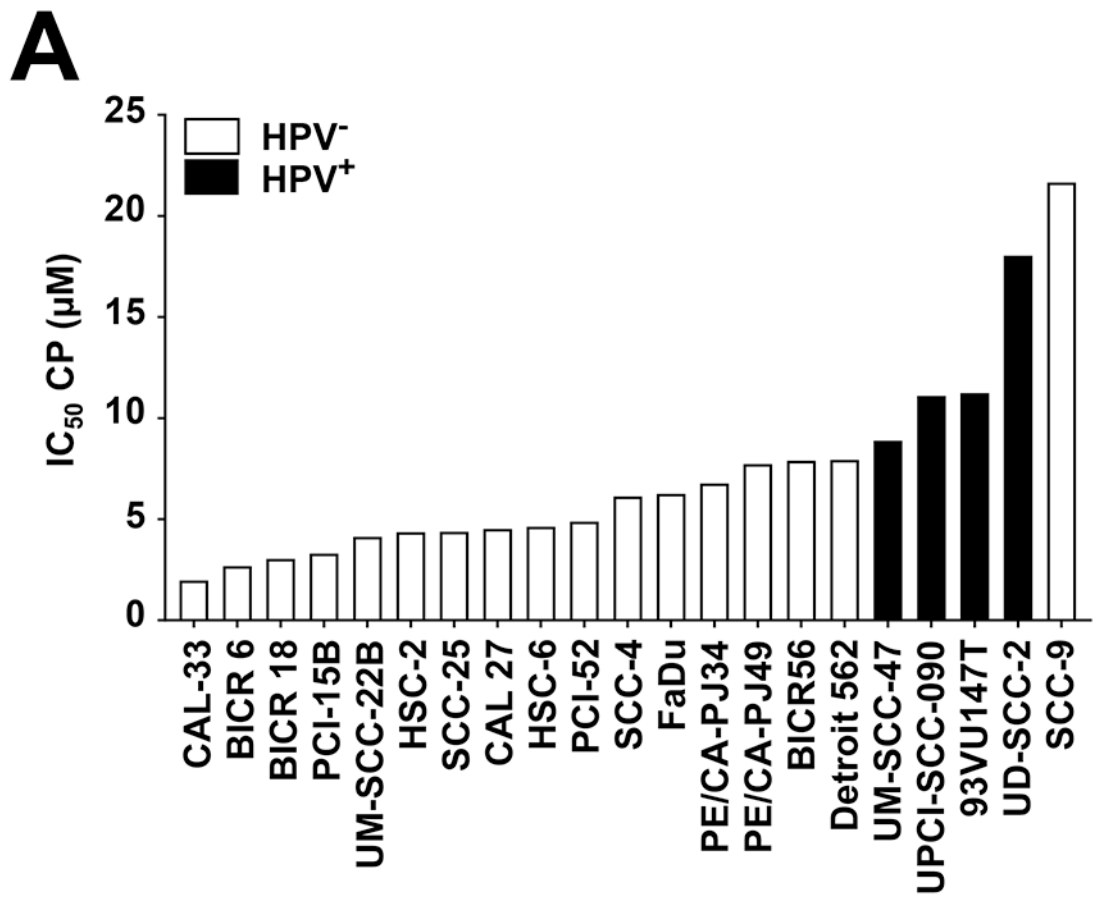
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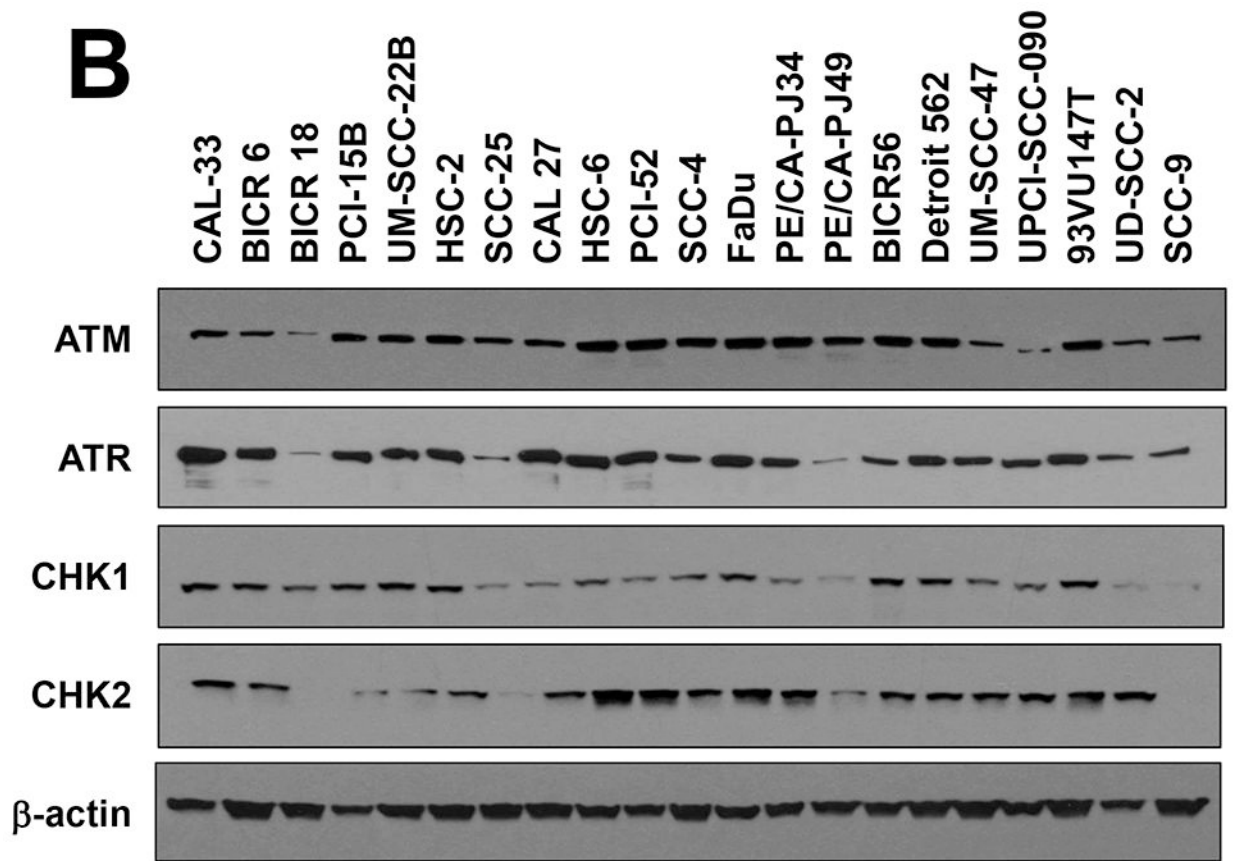
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Highlights

- ATR inhibition with AZD6738 sensitized a panel of 21 HNSCC cell line to cisplatin.
- AZD6738 enhanced tumor growth inhibition by cisplatin *in vivo*.
- AZD6738 enhancement of cisplatin effects was observed in both HPV⁻ and HPV⁺ models.





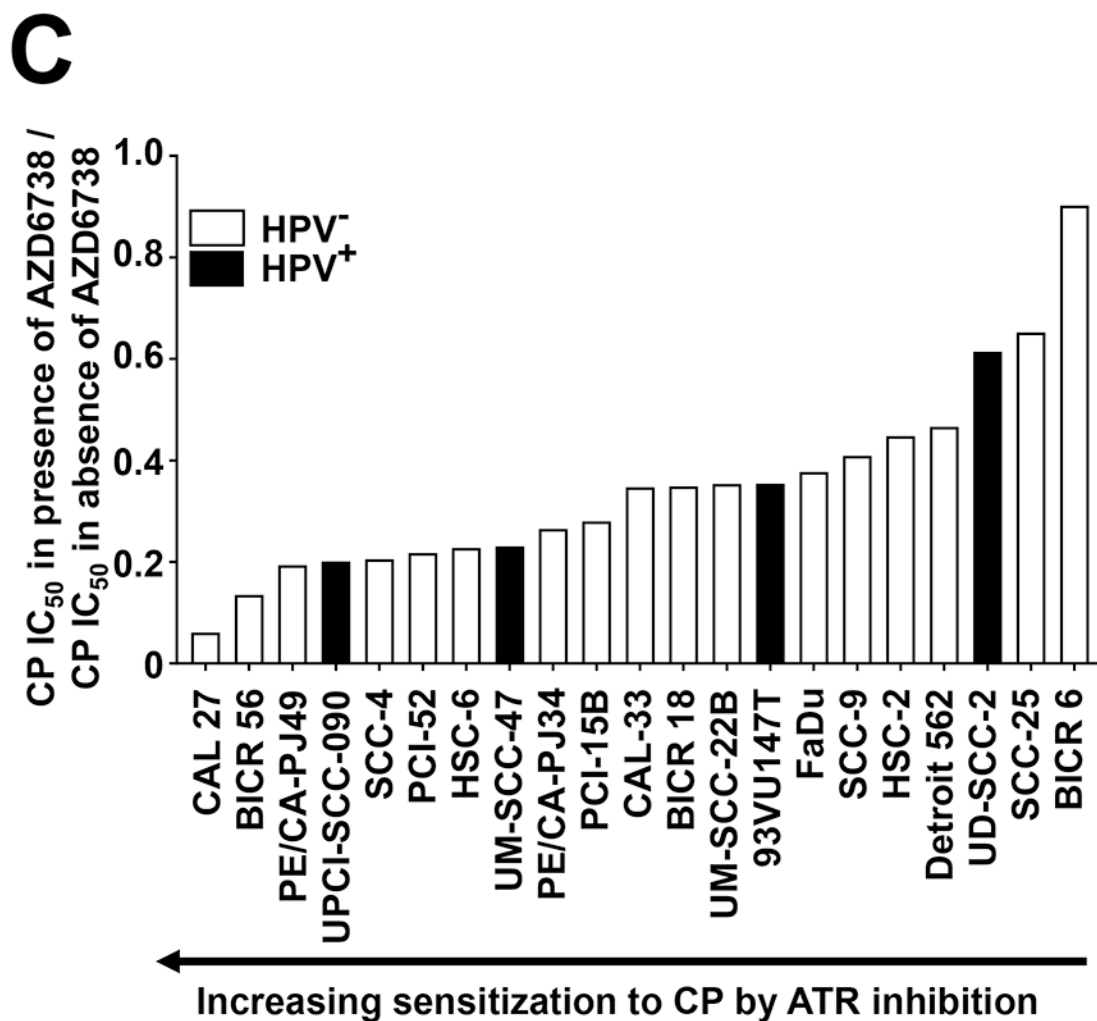
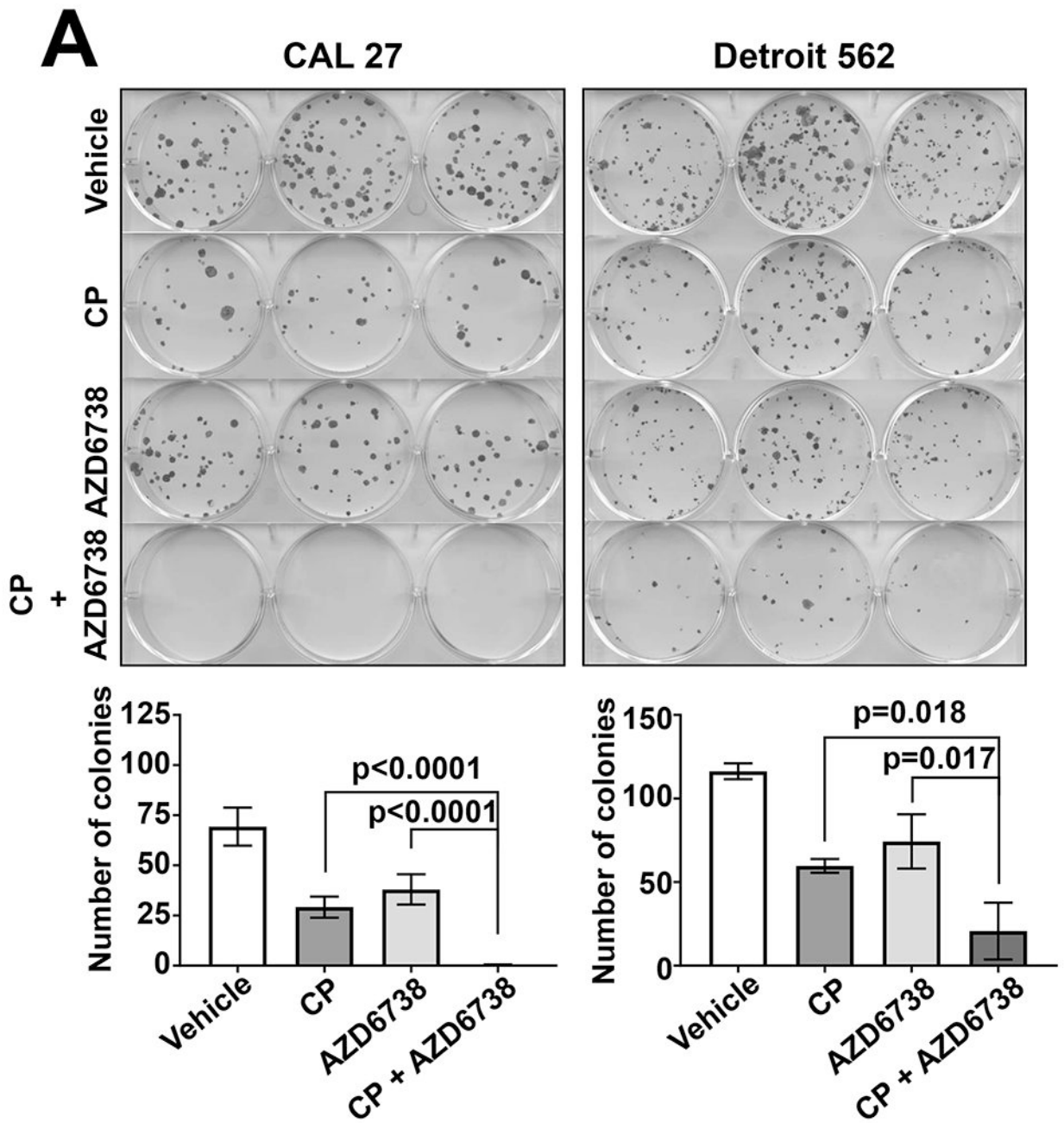


Fig. 1.

ATR inhibition by AZD6738 sensitizes HNSCC cell lines to cisplatin (CP). A, IC₅₀ values for CP are shown for HPV⁻ (open bars) and HPV⁺ (solid bars) HNSCC cell lines. Cells were incubated for 48 hours with varying concentrations of CP, followed by performance of crystal violet assays. IC₅₀ values were extrapolated from titration treatment curves performed in triplicate. B, Whole cell lysates of HNSCC cell lines were subjected to immunoblotting for ATM, ATR, CHK1, or CHK2, with β -actin used as a control for protein loading. C, CP IC₅₀ values determined in the absence (vehicle treatment) or presence of 500 nM AZD6738. The plotted values represent the ratio of CP IC₅₀s in the presence of AZD6738 to CP IC₅₀s in the absence of AZD6738. Solid bars again indicate HPV⁺ cell lines.



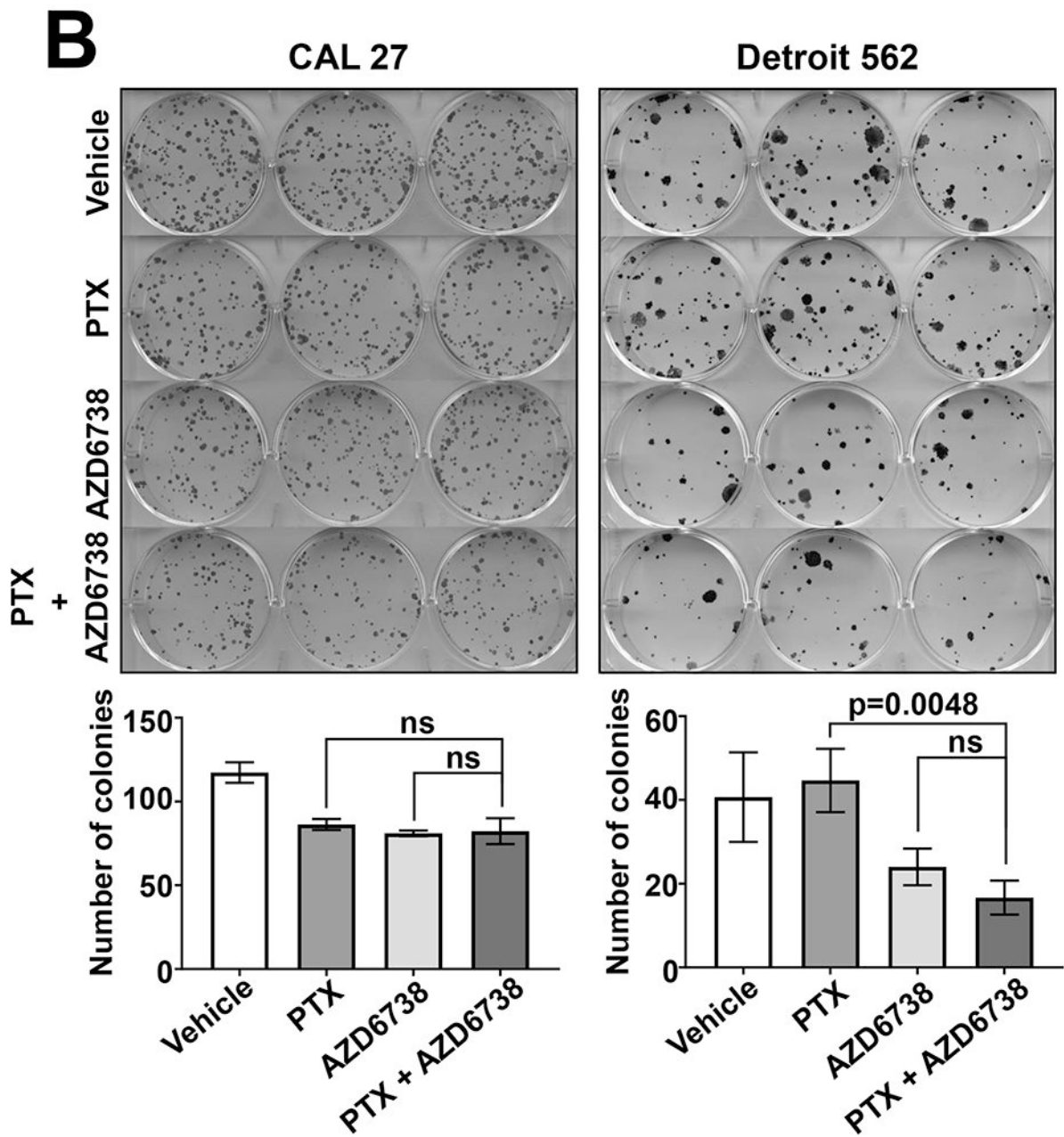


Fig. 2.

AZD6738 enhances loss of colony formation by cisplatin (CP). A, CAL 27 and Detroit 562 cells were treated for 24 hours with vehicle, 2 μ M CP alone, 500 nM AZD6738 alone, or CP plus AZD6738. The treated cells were then replated in 6-well plates, then incubated for an additional 14 days before staining with crystal violet and counting of colonies. Colonies were defined as consisting of 50 cells or more. Bar graphs represent the average number of colonies from triplicate wells and error bars represent standard deviations. B, CAL 27 and Detroit 562 were treated with vehicle, 0.15 nM paclitaxel (PTX) alone, 500 nM AZD6738 alone, or PTX plus AZD6738, then analyzed in colony formation assays as in panel A.

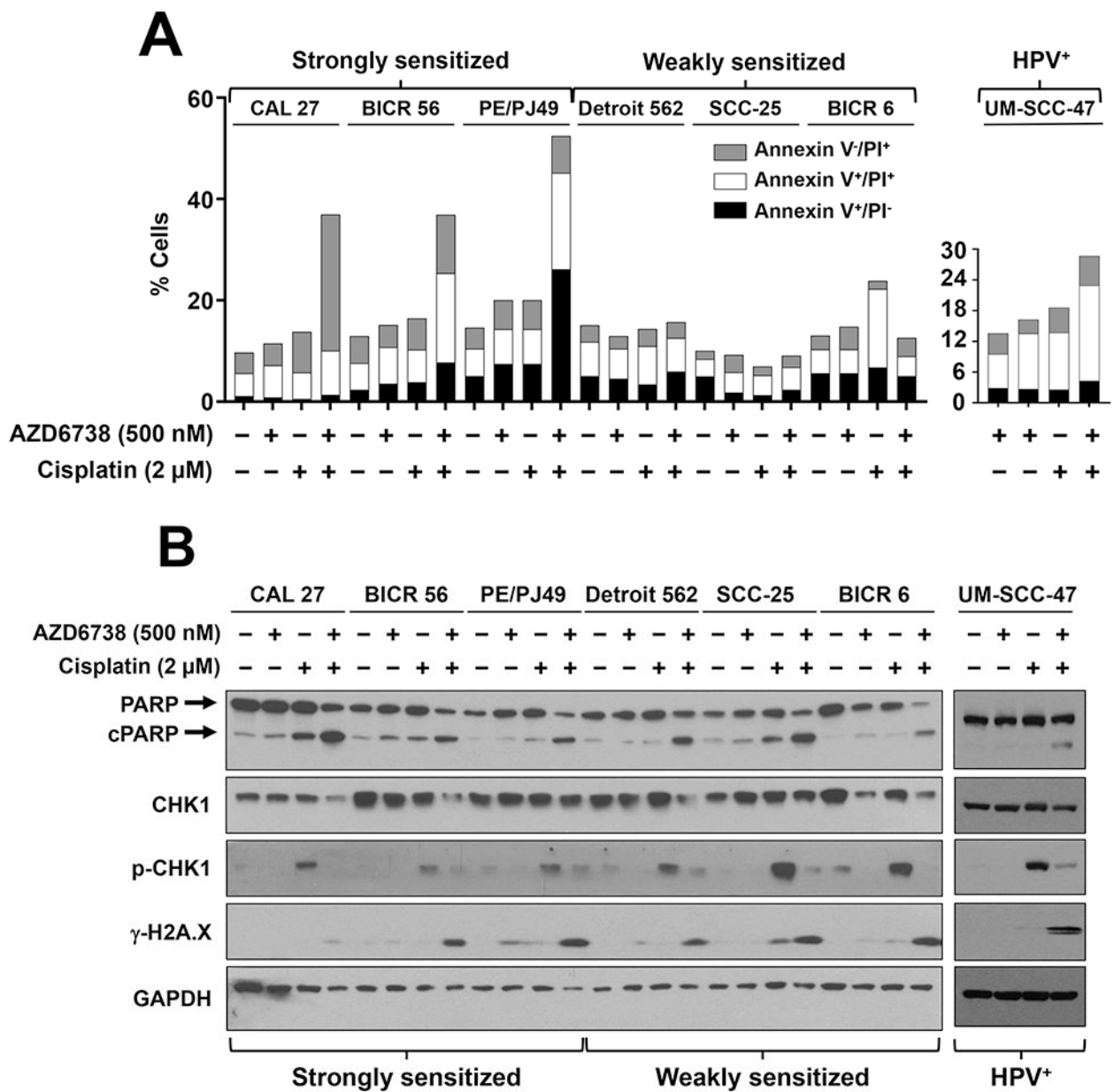


Fig. 3. ATR inhibition enhances cisplatin-induced apoptosis and DNA damage. The indicated cell lines were treated for 24 hours with either vehicle, 500 nM AZD6738, 2 μM cisplatin or 500 nM AZD6738 plus 2 μM cisplatin (A, B). A, Annexin V and PI staining was performed and the percentage of Annexin V-positive and/or PI-positive cells determined by flow cytometry. The figure represents one experiment. B, Immunoblots were performed for common indicators of apoptosis signaling (cleaved PARP (cPARP)), DNA damage (γ-H2A.X), and DNA damage/repair (p-CHK1). PE/CA-PJ49 cells are abbreviated as PE/PJ49.

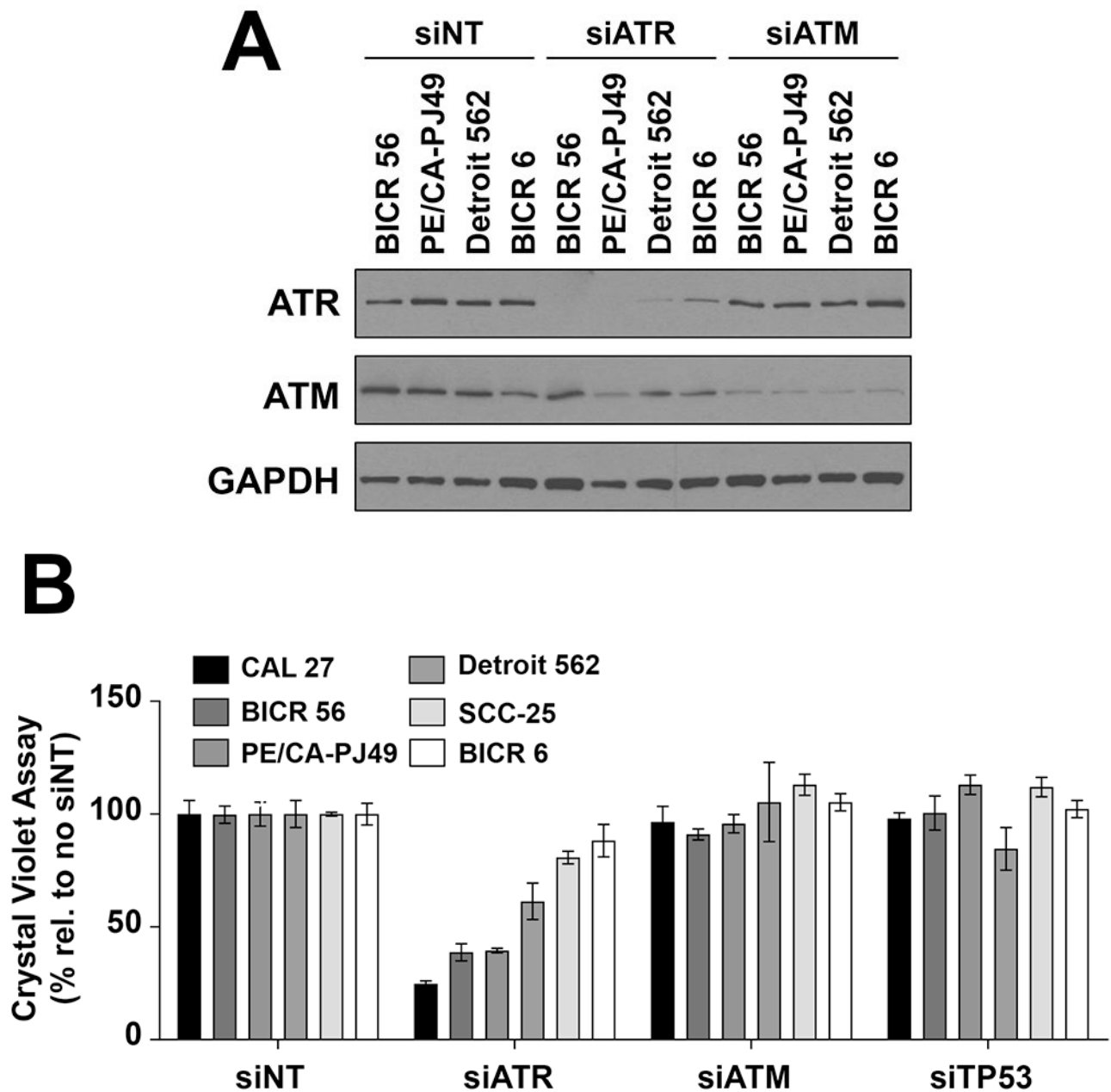


Fig. 4. Inhibition of ATR expression, but not ATM or p53 expression, sensitizes HNSCC cell lines to cisplatin. A, HNSCC cell lines were transfected with nontargeting siRNA (siNT) or siRNAs directed against ATR, ATM, or p53 mRNAs, as described in Materials and Methods. After 48 hours, whole cell lysates were prepared and subjected to immunoblotting to confirm effective knockdown of the intended targets. B) Cells transfected for 48 hours with the indicated siRNAs were treated with 10 μ M cisplatin for an additional 48 hours, followed by performance of crystal violet assays. Data points were normalized to treatment with siNT for each cell line. Columns and error bars represent the average and standard deviation of triplicate wells, respectively.

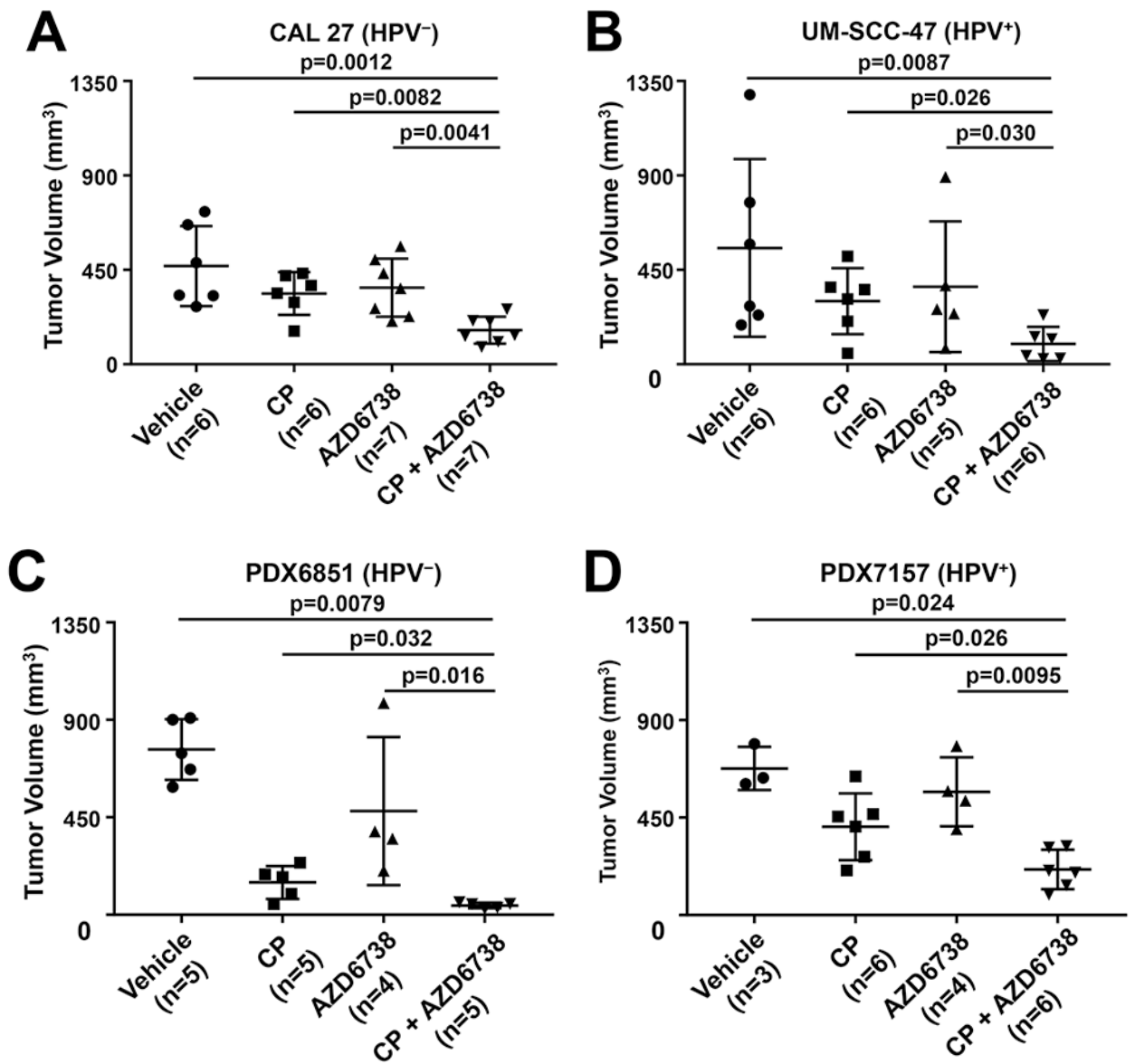


Fig. 5. Co-treatment with AZD6738 and cisplatin enhances growth inhibition of HPV⁻ and HPV⁺ HNSCC cell line and PDX tumors. A-D, Mice harboring tumors derived from HPV⁻ (CAL 27) or HPV⁺ (UM-SCC-47) cell lines, or HPV⁻ (PDX6851) or HPV⁺ (PDX7157) PDX models were treated with vehicle, AZD6738 (25 mg/kg, PO, 5 days on, 2 days off), cisplatin (5 mg/kg, IP, once per week), or the combination. Tumor volumes for CAL 27 (A), UM-SCC-47 (B), PDX6851 (C), and PDX7157 (D) are reported upon experiment completion on days 29, 16, 24, and 25 post-initial treatment, respectively. The number of tumors in each group is indicated by “n”. P values were calculated using Mann-Whitney t tests.