PARP1 Is Overexpressed in Nasopharyngeal Carcinoma and Its Inhibition Enhances Radiotherapy

Jeremy P.H. Chow¹, Wing Yu Man¹, Mao Mao¹, Han Chen¹, Florence Cheung², John Nicholls³, Sai Wah Tsoo⁴, Maria Li Lung⁵, and Randy Y.C. Poon¹

Abstract
Nasopharyngeal carcinoma is a rare but highly invasive cancer. As options of agents for effective combination chemoradiotherapy for advanced nasopharyngeal carcinoma are limited, novel therapeutic approaches are desperately needed. The ubiquitin ligase CHFR is known to target PARP1 for degradation and is epigenetically inactivated in nasopharyngeal carcinoma. We present evidence that PARP1 protein is indeed overexpressed in nasopharyngeal carcinoma cells in comparison with immortalized normal nasopharyngeal epithelial cells. Tissue microarray analysis also indicated that PARP1 protein is significantly elevated in primary nasopharyngeal carcinoma tissues, with strong correlation with all stages of nasopharyngeal carcinoma development. We found that the PARP inhibitor AZD2281 (olaparib) increased DNA damage, cell-cycle arrest, and apoptosis in nasopharyngeal carcinoma cells challenged with ionizing radiation or temozolomide. Isobologram analysis confirmed that the cytotoxicity triggered by AZD2281 and DNA-damaging agents was synergistic. Finally, AZD2281 also enhanced the tumor-inhibitory effects of ionizing radiation in animal xenograft models. These observations implicate that PARP1 overexpression is an early event in nasopharyngeal carcinoma development and provide a molecular basis of using PARP inhibitors to potentiate treatment of nasopharyngeal carcinoma with radio- and chemotherapy.

Introduction
Nasopharyngeal carcinoma is a highly invasive cancer. Although nasopharyngeal carcinoma is relatively rare in most parts of the world (annual incidence rate generally less than 1 per 100,000 population), high incidence rates are found in southern China and Southeast Asia (1). For example, nasopharyngeal carcinoma among women and men in Hong Kong is 7 and 18 per 100,000, respectively (2). The prevailing view is that infection by Epstein–Barr virus (EBV) is a significant etiologic factor for nasopharyngeal carcinoma (reviewed in ref. 3). Epidemiologic evidence also links the consumption of dietary nitrosamines (such as salted fish) to nasopharyngeal carcinoma (reviewed in ref. 4).

Radiotherapy has been used as the primary treatment of nasopharyngeal carcinoma (5). This reflects both the inaccessible location of the nasopharynx as well as the radiosensitivity of nasopharyngeal carcinoma cells. More recent advances in radiotherapy such as three-dimensional conformal radiotherapy and intensity-modulated radiotherapy have helped to achieve good prognosis, in particular for early-stage nasopharyngeal carcinoma (6, 7). For advanced nasopharyngeal carcinoma, however, treatment outcomes have been poor due to distant metastases and recurrence (8). Chemotherapy is used in combination with radiotherapy to control advanced nasopharyngeal carcinoma. Commonly used chemotherapeutic agents are restricted to standard compounds such as the DNA-damaging agent cisplatin and the thymidylate synthase inhibitor 5-fluorouracil. Effective agents for combination chemoradiotherapy for nasopharyngeal carcinoma are still to be established.

One of the earliest responses to DNA damage is the binding of PARP to damaged sites. PARP catalyzes the transfer and polymerization of ADP ribose units from NAD⁺ to form branched polymers of ADP ribose covalently linked to heterologous acceptor proteins or PARP itself (reviewed in ref. 9). PARP-catalyzed poly(ADP-ribosyl)ation plays pivotal roles in the processing and resolution of DNA breaks. The binding of PARP to
the site of DNA damage activates its catalytic activity and promotes local poly(ADP-ribose)-dependent recruitment of DNA repair enzymes. Poly(ADP-ribosyl)ation of histones and other proteins may also mediate the decondensation of chromatin structure required for efficient DNA repair. PARP1 is the most abundant and active enzyme of the PARP family, but roles of other members including PARP2 and PARP3 in DNA damage responses are emerging (10).

PARP inhibitors have been evaluated in clinical trials either as single agents or in combination with DNA-damaging agents (reviewed in ref. 11). As stand-alone agents, PARP inhibitors have been used to induce synthetic lethality in homologous repair-deficient tumors. For example, PARP inhibitors are effective in targeting BRCA1- or BRCA2-deficient breast and ovarian cancer. The rationale of the approach is that inhibition of PARP blocks the repair of spontaneous single-strand lesions, leading to the formation of double-strand breaks through mechanisms such as stalled replication forks. These double-strand breaks become lethal in cells that are deficient in double-strand break repair (such as those with mutations in BRCA1 or BRCA2). Synthetic lethality of PARP inhibitors is not limited to BRCA1 or BRCA2 deficiency. Recently, it was discovered that PARP inhibitors markedly sensitize Ewing’s sarcoma cells harboring the EWS-FLI1 gene translocation (12). More importantly, PARP inhibitors could potentially be used as agents that enhance chemo- or radiotherapy-induced DNA damage in patients without defined gene mutations. This remains an emerging field with many parameters to be deciphered (reviewed in ref. 11).

Although PARP1 has been implicated as a therapeutic target in several types of cancer, its role has not been rigorously investigated in nasopharyngeal carcinoma. Here, we present evidence that PARP1 is overexpressed in nasopharyngeal carcinoma in comparison with normal nasopharyngeal cells. Importantly, nasopharyngeal carcinoma cell growth was inhibited synergistically by DNA-damaging agents and an inhibitor of PARP.

Materials and Methods

Materials

Olaparib (AZD2281; Selleck Chemicals) and temozolomide (Sigma-Aldrich) were obtained from the indicated suppliers.

Cell culture

IMR90 (normal fibroblasts) were obtained from the American Type Culture Collection. Nasopharyngeal carcinoma cell lines C666-1 (13), CNE2 (14), HNE1 (15), and HONE1 (15) were obtained from nasopharyngeal carcinoma AoC Cell Line Repository and were propagated in RPMI-1640 (for C666-1) or Dulbecco’s Modified Eagle Medium (DMEM, for other nasopharyngeal carcinoma cells) supplemented with 10% (v/v) FBS (Life Technologies) and 50 U/mL penicillin-streptomycin (Life Technologies). No authentication was carried out by the authors. Telomerase-immortalized nasopharyngeal epithelial cell lines NP361, NP460, and NP550 (16) were propagated in keratinocyte serum-free medium supplemented (Life Technologies) with 50% v/v Epilife (Sigma). Cells were grown in humidified incubators at 37°C in 5% CO2.

Trypan blue analysis was conducted as described previously (17). For clonogenic survival assays, 500 cells were seeded onto 60-mm dishes. After 16 hours, the cells were irradiated with different doses of ionizing radiation and either mock-treated or exposed to the different concentrations of AZD2281 for another 24 hours. After 10 days, colonies were fixed with methanol/acetic acid (2:1 v:v) and visualized by staining with 2% (w/v) crystal violet in 20% methanol.

Flow cytometry

Flow cytometry analysis after propidium iodide staining was conducted as described previously (17).

Ionizing radiation

Ionizing radiation was delivered with a caesium137 source from a MDS Nordion Gammacell 1000 Elite Irradiator. Unless stated otherwise, cells were irradiated with a dose of 15 Gy.

γ-H2AX staining

Cells grown on poly-l-lysine–treated coverslips were fixed by ice-cold methanol for 10 minutes. The cells were then washed twice with PBS, 5 minutes each, before permeabilized and blocked with 3% bovine serum albumin (BSA) and 0.2% Tween-20 in PBS at room temperature for 30 minutes. The cells were washed twice with wash buffer (0.2% Tween-20 in PBS) before incubated with phosphor-histone H2AXSer139 antibody (Bethyl Laboratory; diluted in 3% BSA, 0.2% Tween-20 with PBS) at 4°C for 16 hours. The cells were then washed with wash buffer for four times, 5 minutes each, and incubated in dark with Alexa Fluor 594 goat anti-rabbit immunoglobulin G (IgG) secondary antibodies (Life Technologies) at 25°C for 2 hours. After washing four times with wash buffer for 5 minutes each, the cells were stained with Hoechst 33342 (5 μg/mL in wash buffer) for 5 minutes, washed three times with wash buffer, before mounted with 100 mmol/L N-propyl-gallate in 9:1 v/v glycerol:PBS.

Isobologram analysis

The combination effects of two treatments were analyzed according to the median-effect method of Chou and Talalay (18). Each dose–response curve was used to calculate the isobologram, the value of median-effect dose (D50), the sigmoidicity of the dose–effect curve (m), and the linear correlation coefficient of the median-effect plot (r). The combination index (CI) at different effective dose (ED) ED50, ED5, and ED75 was calculated according to Chou and Talalay (18) using CalcuSyn Version 2.1 (Biosoft). CI value < 1 indicates synergistic effect (0.1–0.5 strong
synergism; <0.1 very strong synergism); CI value of 1 indicates additive effect; and CI value > 1 indicates antagonistic effect.

**Real-time PCR**

Total RNA was extracted from approximately 3 × 10^6 cells using Nucleospin RNA (Macherey-Nagel). The total RNA (1 µg) was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer’s instruction. Semiquantitative real-time PCR was conducted by mixing the cDNA with SYBR Green Real-Time PCR Master Mixes in a 7500 Fast Real-Time PCR System (Life Technologies) using the following primers for actin (5′-GGGAAATCGTGCGTGACATT-3′; 5′-GGAACCCGCTCATTGCCAAT-3′), CHFR (5′-GGCAACCCAGGTTTGCACAT-3′; 5′-AGTCAGGACGGGATGTTACG-3′), and PARP1 (5′-GATGGGTTCTCTGAGCTTCG-3′; 5′-ACACCCCTTGACGCTTTCC-3′).

**Antibodies and immunoblotting**

Antibodies against β-actin (Sigma-Aldrich), phospho-histone H2AXSer139 (Bethyl Laboratory), and cleaved PARP1 (Abcam) were used to detect antibodies against PARP1 at 1:200 dilution. Similar to what was previously reported (20), slides were incubated with antibodies against PARP1 and stained with hematoxylin. The immunohistochemically stained tissue microarray was reviewed by two independent pathologists.

**Nasopharyngeal carcinoma tissue microarray analysis**

A tissue microarray containing normal nasopharyngeal epithelium and nasopharyngeal carcinoma specimens was constructed. The nasopharyngeal carcinoma specimens were obtained from patients with nasopharyngeal carcinoma before treatment at Queen Mary Hospital (Hong Kong) and Pamela Youde Nethersole Eastern Hospital (Hong Kong). Construction of the nasopharyngeal carcinoma tissue microarray and immunohistochemical staining using the standard streptavidin–biotin–peroxidase complex method was similar to that previously reported (20). Slides were incubated with antibodies against PARP1 at 1:200 dilution and counterstained with hematoxyn. Immunohistochemically stained tissue microarray was reviewed by two independent pathologists. For evaluation of the staining, the normal malignant and malignant tissues were scored by assessing the PARP1 staining in the nucleus. A random four views were counted under ×400 microscopy. The intensity of staining was graded by an arbitrary scale that ranged from 0 to 3, representing negative (0′), weak (1′), moderate (2′), and strong (3′) staining, respectively. Staining values of 0 and 1 were classified as low expression, whereas 2 and 3 were classified as high expression. The Pearson χ² test was used to assess the association between PARP1 expression and several clinicopathologic variables. A P value less than 0.05 was regarded as statistically significant.

**Tumor xenografts**

The experimental protocol was evaluated and approved by the Animal Care Committee, Hong Kong University of Science and Technology (Clear Water Bay, Hong Kong). HONE1 cells (2 × 10⁶) were injected subcutaneously into both sides of the dorsa of 4- to 6-week-old female BALB/c nude mice. Four animals (n = 8) per group were used in control and combination treatment and 3 animals were used in the single treatments. Tumors were regularly measured using a Vernier caliper. Volume was calculated according to the formula: \( \pi/6 \times \text{length} \times \text{width}^2 \). Day = 0 was designated as when the tumor volume was approximately 100 mm³. AZD2281 (50 mg/kg) was administrated with intraperitoneal injection into the mice daily from day 1 to 5. Ionizing radiation (2 Gy) was delivered at day 1 and 4. In the combination treatment, AZD2281 was administrated 30 minutes before ionizing radiation delivery. Mice were killed when the tumors in the control group reached 1,000 mm³.

**Results**

**Overexpression of PARP1 protein in nasopharyngeal carcinoma cell lines**

Frequent alterations of gene expression have been reported in nasopharyngeal carcinoma. However, their implication in tumorigenesis or treatment is not always immediately apparent. Among these, transcription of the ubiquitin ligase CHFR was found to be epigenetically inactivated in nasopharyngeal carcinoma through promoter methylation (21). To study whether CHFR protein is altered in nasopharyngeal carcinoma cells, antibodies against CHFR were generated. Lysates from different nasopharyngeal carcinoma cell lines (C666-1, CNE2, HNE1, and HONE1) were prepared and analyzed with immunoblotting using antibodies specific for CHFR. Normal nasopharyngeal epithelial cells that were immortalized with telomerase were used as a comparison. CHFR was found to be downregulated in all the nasopharyngeal carcinoma cell lines (Fig. 1A). In agreement with this, CHFR was also present at a relatively high level in three different immortalized nasopharyngeal epithelial cell lines (NP361, NP460, and NP550; Fig. 1B). As a control, the normal human fibroblasts strain (IMR90) also contained a high abundance of CHFR.

Recently, CHFR was reported to be able to regulate PARP1 by targeting PARP1 for ubiquitination and degradation (22). We also found that PARP1 expression could be reduced by overexpression of CHFR and increased by knockdown of CHFR (our unpublished data). This prompted us to examine the expression of PARP1 in nasopharyngeal carcinoma cells. Significantly, PARP1 displayed an inverse relationship with CHFR, expressing at high levels in all the nasopharyngeal carcinoma cell lines and at low levels in the nasopharyngeal epithelial...
Figure 1. Nasopharyngeal carcinoma cell lines overexpress PARP1 and are sensitive to PARP1 inhibition. A, overexpression of PARP1 and downregulation of CHFR in nasopharyngeal carcinoma cell lines. Several nasopharyngeal carcinoma cell lines (C666-1, CNE2, HNE1, and HONE1) and an immortalized normal nasopharyngeal cell line (NP460) were analyzed. Cell-free extracts were prepared and the indicated proteins were detected by immunoblotting. Actin analysis was included to assess protein loading and transfer. B, the expression of PARP1 and CHFR in several immortalized normal nasopharyngeal cell lines (NP361, NP460, and NP550) and nasopharyngeal carcinoma cell lines (C666-1 and HONE1) were analyzed. Normal human fibroblasts strain IMR90 was also included as a control. Actin analysis was included to assess protein loading and transfer. C, CHFR but not PARP1 is regulated at the transcriptional level in nasopharyngeal carcinoma cell lines. The relative expression of the mRNA of PARP1 and CHFR in nasopharyngeal carcinoma cell lines (CNE2, C666-1, and HNE1) and immortalized normal nasopharyngeal cell lines (NP361, NP460, and NP550) was quantified with semiquantitative real-time PCR and normalized with actin mRNA (mean ± SD of three independent experiments). D, AZD2281 promotes apoptotic cell death in nasopharyngeal carcinoma cells. Nasopharyngeal carcinoma (HONE1 and HNE1) and normal nasopharyngeal epithelial cells (NP460) were incubated with either control buffer or the indicated concentrations of AZD2281 for 48 hours. The cells were harvested, fixed, and their DNA contents were analyzed with flow cytometry. The positions of 2N and 4N DNA contents are indicated. E, AZD2281 reduces cell viability in nasopharyngeal carcinoma cells. Nasopharyngeal carcinoma and normal nasopharyngeal epithelial cells were treated as in D. The cells were stained with Trypan blue and the number of viable cells was analyzed with a hemocytometer. F, AZD2281 promotes PARP1 cleavage in HONE1. HONE1 cells were treated as in D. Lysates were prepared and subjected to immunoblotting analysis. Equal loading of lysates was confirmed by immunoblotting for actin. G, AZD2281 reduces clonogenic survival in HONE1 cells. HONE1 cells were incubated with different concentrations of AZD2281 for 48 hours. The cells were washed and replated for clonogenic survival assays.
cell lines (Fig. 1A and B). As expected, CHFR mRNA was present at a lower level in nasopharyngeal carcinoma cell lines than in the nasopharyngeal epithelial cell lines (Fig. 1C). Consistent with regulation by posttranslational mechanisms, we found that PARP1 mRNA was expressed at similar levels between nasopharyngeal carcinoma and nasopharyngeal cell lines. Taken together, these results indicate that PARP1 is present at a significantly higher level in nasopharyngeal carcinoma cell lines than in normal nasopharyngeal epithelial cells.

**Inhibition of PARP enhances the cytotoxicity induced by DNA-damaging agents in nasopharyngeal carcinoma cells**

Given that PARP1 is overexpressed in nasopharyngeal carcinoma cells, we next addressed whether this translates into alterations in the sensitivity to PARP inhibitors. Olaparib (AZD2281; ref. 23) is an orally active PARP inhibitor undergoing phase I and II clinical trials (11). Incubation of nasopharyngeal carcinoma and normal nasopharyngeal epithelial cells with AZD2281 alone induced a stress response. In the nasopharyngeal carcinoma cell lines HONE1 and HNE1, a relatively high concentration of AZD2281 (10 μmol/L) induced apoptotic cell death (as indicated by the appearance of sub-G1 cells; Fig. 1D). An increase in 4N DNA contents was also apparent, in particular for HONE1, suggesting a robust activation of cell-cycle progression (see also Fig. 1D). Moreover, an increase in apoptotic cell death in nasopharyngeal carcinoma cells but not in normal nasopharyngeal epithelial cells was challenged with the same concentrations of AZD2281. In agreement with this, Trypan blue analysis indicated that a dose-dependent reduction of cell number for nasopharyngeal carcinoma cells (Fig. 1E). In contrast, only a marginal decrease in the number of viable cells was observed after normal nasopharyngeal epithelial cells were incubated with AZD2281. Consistent with an increase in apoptotic cell death in nasopharyngeal carcinoma cells, we found that AZD2281 stimulated an accumulation of cleaved PARP1 and p53 in a dose-dependent manner (Fig. 1F). The long-term clonogenic survival of HONE1 cells was likewise reduced by AZD2281 treatment (Fig. 1G).

We next examined whether inhibition of PARP could potentiate the cytotoxic effects of DNA-damaging agents. A partial G2 arrest was stimulated in both nasopharyngeal carcinoma and nasopharyngeal epithelial cells after irradiated with 10 Gy of ionizing radiation (Fig. 2A). Significantly, more nasopharyngeal carcinoma cells were arrested in G2 when they were irradiated in the presence of 1 μmol/L of AZD2281. In marked contrast, normal nasopharyngeal epithelial cells seemed to be impervious to AZD2281. As a control, exposure of nasopharyngeal carcinoma cells to AZD2281 alone did not affect normal cell-cycle progression (see also Fig. 1D). Moreover, an extensive sub-G1 population was induced when HONE1 were treated with both ionizing radiation and AZD2281. Although sub-G1 population was not apparent for HNE1 and CNE2 cells, Trypan blue exclusion analysis confirmed that substantial cell death was triggered when the cells were exposed to both ionizing radiation and AZD2281 (Fig. 2B).

To ensure that the stimulation of nasopharyngeal carcinoma cytotoxicity by AZD2281 was not limited to ionizing radiation, cells were also treated with the DNA-methylating agent temozolomide. Incubation with either AZD2281 or temozolomide (125 μmol/L) alone did not significantly stimulate apoptosis. Massive apoptosis was triggered when nasopharyngeal carcinoma cells were incubated with both temozolomide and AZD2281 together (Fig. 3A). Although normal nasopharyngeal epithelial cells also responded to the combined AZD2281 and temozolomide treatment by displaying more G2-arrested cells, there was no increase in sub-G1 population. Consistent with the results from the flow cytometry, high level of γ-H2AX was induced after incubation with AZD2281 and temozolomide in HONE1, but not in NP460 (Fig. 3B).

To investigate more rigorously whether the effects of PARP inhibition and DNA damage on nasopharyngeal carcinoma cells represent synergism, isobologram analysis was carried out according to the median-effect method of Chou and Talalay (18). Using Trypan blue exclusion assays to monitor cytotoxicity, we found that AZD2281 and ionizing radiation acted synergistically on nasopharyngeal carcinoma (HONE1 and nasopharyngeal epithelial (NP460) cells. The γ-H2AX foci staining returned to a background level after 24 hours, suggesting that the damaged DNA was repaired in both nasopharyngeal carcinoma and nasopharyngeal cells (Fig. 4B). Significantly, efficient DNA repair in HONE1 cells was attenuated in the presence of AZD2281. In contrast, the DNA repair in NP460 cells was resistant to AZD2281.

Collectively, these results indicate that inhibition of PARP enhances ionizing radiation- or temozolomide-mediated DNA damage and apoptosis in nasopharyngeal carcinoma cells but not in normal nasopharyngeal epithelial cells.

**PARP inhibition and ionizing radiation act synergistically to promote cytotoxicity**

To investigate more rigorously whether the effects of PARP inhibition and DNA damage on nasopharyngeal carcinoma cells represent synergism, isobologram analysis was carried out according to the median-effect method of Chou and Talalay (18). Using Trypan blue exclusion assays to monitor cytotoxicity, we found that AZD2281 and ionizing radiation acted synergistically on nasopharyngeal carcinoma cells (HONE1 and HNE1; Fig. 5A and Table 1). The CI was less than 1, which indicated synergism, over a range of effective dose (ED50, ED75, and ED90). In contrast, synergism between AZD2281 and ionizing radiation were less effective on nasopharyngeal epithelial cells (NP460), with the CI values actually exceeding 1 at ED50 (indicating antagonism). We also examined the long-term survival of HONE1 cells using clonogenic survival assays (Fig. 5B and Table 2). Similar analysis indicated a synergism between AZD2281 and
ionizing radiation at ED_{75} and ED_{90} for long-term clonogenic survival. Collectively, these data showed that AZD2281 and ionizing radiation act synergistically to induce cytotoxic effects on nasopharyngeal carcinoma cells.

**Combined AZD2281 and ionizing radiation treatment reduces tumor growth in tumor xenograft models**

We next evaluated the effects of PARP inhibition on nasopharyngeal carcinoma growth in animal tumor models. HONE1 cells were injected subcutaneously into nude mice. AZD2281, ionizing radiation, or a combination of both were delivered using a fractionated dose approach (Fig. 6A). Treatment with AZD2281 or ionizing radiation individually reduced the rate of tumor growth. But more significant reduction of tumor growth was induced in the group treated with both AZD2281 and ionizing radiation. More importantly, there was in fact a decrease in tumor volume during the period of combined treatment (Fig. 6A and B). Tumor size increased again only after the treatment ceased. These results indicated that AZD2281 and ionizing radiation exerted a strong tumor inhibitory effect in xenograft models.

**Overexpression of PARP1 in primary human nasopharyngeal carcinoma**

To confirm that the overexpression of PARP1 observed in nasopharyngeal carcinoma cell lines are relevant in primary nasopharyngeal carcinoma tumors, we next examined the expression of PARP1 in primary human nasopharyngeal carcinoma using tissue microarray analysis (Fig. 6C). Normal nasopharyngeal and nasopharyngeal carcinoma tissues were scored for low or high expression of PARP1 by assessing PARP1...
staining in the nucleus (Table 3). High expression of PARP1 was found in nasopharyngeal carcinoma tissues. Correlation was observed between PARP1 expression and several clinicopathologic parameters, including patient age and survival. Importantly, an increase in PARP1 expression could be observed in all nasopharyngeal carcinoma stages, suggesting that the alteration of PARP1 expression is an early event in nasopharyngeal carcinoma development.

**Discussion**

In this study, we found that PARP1 protein is highly expressed in nasopharyngeal carcinoma cell lines and primary nasopharyngeal carcinoma tissues in comparison with nasopharyngeal epithelial cells. The PARP inhibitor AZD2281 increased DNA damage, cell-cycle arrest, and apoptosis in nasopharyngeal carcinoma cell lines treated with ionizing radiation or temozolomide. Finally, AZD2281 also enhanced the tumor-inhibitory effects of ionizing radiation in mouse xenograft models.

A major focus of the clinical development of PARP inhibitors is to determine whether they can potentiate radio-/chemotherapy-mediated DNA damage, especially for tumors that lack intrinsic defects in DNA damage repair (reviewed in ref. 11). For nasopharyngeal carcinoma, we found that PARP inhibition was indeed effective in potentiating the cytotoxicity of DNA-damaging agents using both cell line (Figs. 2 and 3) and animal models (Fig. 6). Isobologram analysis indicated...
that at least for the cell line models, the effects of AZD2281 and ionizing radiation were synergistic rather than simply additive (Fig. 5). AZD2281 and ionizing radiation displayed synergism on nasopharyngeal carcinoma cells over the range of effective doses in both short-term growth assay (Fig. 5A and Table 1) and long-term cell survival (Fig. 5B and Table 2). Results from the xenograft studies further indicated that combinational treatment of AZD2281 and ionizing radiation could further lead to shrinkage of tumor size (Fig. 6). However, the treatment also has synergistic effects on normal nasopharyngeal cells (for ED₅₀ and ED₇₅; Fig. 5A and Table 1). Moreover, synergism was found at all effective doses for long-term nasopharyngeal carcinoma cell growth (Fig. 5B and Table 2). Furthermore, the combined AZD2281 and ionizing radiation treatment resulted in a approximately 15% weight loss of the animals (Supplementary Fig. S1), suggesting potential high toxicity to normal cells.

Hence, although our findings provide a rationale to support testing of PARP inhibitors and DNA-damaging agents on nasopharyngeal carcinoma, further research is required to uncover the precise clinical responses of these treatments.

Conceptually, PARP inhibition should only enhance radio-/chemotherapy if DNA damage is selectively increased in tumor compared with normal tissues. Tumors that already harbor DNA repair defects such as mutations in BRCA1 or BRCA2 genes serve as classic examples. So far, there is no evidence of impairment of the components of the DNA repair pathways such as BRCA2 in nasopharyngeal carcinoma (24, 25). As implied by our study, however, the abundance of PARP1 in tumors may also play a role in selectively sensitizing the cells to PARP inhibitors. Nasopharyngeal carcinoma cell lines contained significantly more PARP1 than nasopharyngeal epithelial cells (Fig. 1A and B) and were more sensitive to AZD2281 alone (Fig. 1D and E) or when AZD2281 was added together with DNA-damaging agents (Figs. 2 and 3).

The observations that PARP1 was overexpressed even at early stages of nasopharyngeal carcinoma development (Table 3) imply that alteration of PARP1 could be a driver of tumorigenesis. Consistent with our observations, overexpression of PARP1 has also been reported in other tumors, including colorectal cancer (26), endometrial cancer (27), hepatocellular carcinoma (28), and melanomas (27). Several studies have reported the association of PARP1 protein expression with a worse prognosis. For example, PARP1 overexpression is associated with overall poor prognosis, higher tumor grade, BRCA1-mutated and triple-negative phenotype breast cancers (29–31). In nasopharyngeal carcinoma, PARP1 expression was correlated with increase in patient age and poorer survival (Table 3).

What are the mechanisms that lead to PARP1 overexpression in nasopharyngeal carcinoma and other cancers? Transcription of the PARP1 gene relies on
transcription factors such as Sp1, Sp3 (32), and NF1 (33). However, the increase in PARP1 protein in nasopharyngeal carcinoma was unlikely to be due to an increase in transcription because no significant variation in PARP1 mRNA between nasopharyngeal carcinoma and nasopharyngeal epithelial cell lines was observed (Fig. 1C). This is also consistent with the lack of report on an alteration of PARP1 mRNA expression from various microarray studies. Instead, we believe that the protein level of PARP1 is usually kept at a low level by CHFR in normal nasopharyngeal cells (22). The epigenetic inactivation of CHFR in nasopharyngeal carcinoma (21) provides a molecular basis of how PARP1 is upregulated at the protein level (see Fig. 6D for a model). In this connection, it is possible that methylation of the CHFR promoter can be used as a marker for PAPR1 expression, which in turn can be used as a prediction for sensitivity to PARP inhibitors.

An interesting question is why overexpression of PARP1 would lead to an increase in sensitivity to PARP inhibition. We believe that “PARP1 addiction” may play a role on the reliance of nasopharyngeal carcinoma cells on PARP1 for DNA damage responses. As the DNA repair circuitry gets rewired by the constitutive overexpression of PARP1, the cell may become exquisitely dependent on PARP1 activity. Nasopharyngeal carcinoma cells were clearly more dependent on PARP activity for effective repair than nasopharyngeal epithelial cells (Fig. 4). The PARP1 addiction may be analogous to the addiction to many oncogenes in cancer cells. Another possible reason why PARP1-overexpressing cells were sensitive to PARP inhibitor is the recent finding that the cytotoxicity of PARP

| Table 1. AZD2281 and ionizing radiation act synergistically to induce cytotoxicity in nasopharyngeal carcinoma cells |
|-----------------|--------|--------|--------|--------|--------|--------|
|                | ED50  | ED75  | ED90  | Dm    | m     | r       |
| HONE1          | 0.510 | 0.633 | 0.786 | 1.037 | 0.940 | 0.998   |
| HNE1           | 0.199 | 0.181 | 0.165 | 0.707 | 0.764 | 0.961   |
| NP460          | 0.468 | 0.740 | 1.372 | 0.860 | 0.538 | 0.943   |

NOTE: Nasopharyngeal carcinoma (HONE1 and HNE1) and nasopharyngeal epithelial cells (NP460) were treated with ionizing radiation and AZD2281 for 48 hours and analyzed with Trypan blue analysis. The CI at ED50, ED75, and ED90 was calculated. The value of Dm, m, and r are indicated. A CI < 1 indicates synergism.
Results from this study introduced further questions about PARP1 and nasopharyngeal carcinoma. PARP inhibitors in development mimic the nicotinamide moiety of NAD$^+$ and bind PARP’s catalytic domain. In addition to AZD2281, several PARP inhibitors are in the development pipeline. It would be interesting to see whether they offer better efficacy when combined with ionizing radiation in treatment of nasopharyngeal carcinoma. Although PARP1 is believed to be the most abundant isoform of the PARP family, it is possible that other family members may also play a role in nasopharyngeal carcinoma. Inhibitors that target specific isoforms may be useful. Eventually, randomized studies will be needed to validate PARP inhibitors as therapeutic agents for patients with nasopharyngeal carcinoma. Another unresolved issue is although we know that PARP1 is overexpressed in nasopharyngeal carcinoma and other cancers, little is known about its activity. It would be interesting to assay PARP1 activity in cell lines and tumor specimens from patients.

Table 2. AZD2281 and ionizing radiation act synergistically on clonogenic survival of nasopharyngeal carcinoma cells

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<th>CI</th>
<th>ED$_{50}$</th>
<th>ED$_{75}$</th>
<th>ED$_{90}$</th>
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<td>HONE1</td>
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<td>0.606</td>
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NOTE: HONE1 cells were treated with ionizing radiation and AZD2281 and analyzed with clonogenic survival assays. The CI at ED$_{50}$, ED$_{75}$, and ED$_{90}$ was calculated. A CI < 1 indicates synergism.

Figure 6. AZD2281 and ionizing radiation (IR) inhibit tumor growth in mouse xenograft models. A, HONE1 cells were injected subcutaneously into nude mice. AZD2281 (closed arrowhead) and ionizing radiation (open arrowhead) were delivered at the indicated time points as described in Materials and Methods. The volume of the tumor was measured on different days (control, white square, $n = 8$; AZD2281, white circle, $n = 6$; ionizing radiation, gray circle, $n = 6$; ionizing radiation and AZD2281, dark circle, $n = 8$; mean ± SD). B, representative mice with different treatments at day 28 are shown. C, PARP1 is overexpressed in human primary nasopharyngeal carcinoma (NPC). The expression of PARP1 in normal nasopharyngeal and nasopharyngeal carcinoma tissues was analyzed using tissue microarray and immunohistochemistry. Representative images of PARP1 and hematoxylin and eosin staining in normal nasopharyngeal and nasopharyngeal carcinoma tissues are shown. D, model of the roles of CHFR and PARP1 in nasopharyngeal carcinoma. In nasopharyngeal carcinoma, CHFR is epigenetically inactivated by promoter hypermethylation. The decrease in CHFR protein leads to a stabilization of PARP1 protein. As PARP1 is increased in early stages of nasopharyngeal carcinoma, the sustained increase in PARP1 may contribute to tumor progression of this cancer. Moreover, PARP1 overexpression may lead to “PARP1 addiction,” in which DNA repair becomes more reliant on PARP1 than in normal cells.
PARP in Nasopharyngeal Carcinoma

Table 3. PARP1 is overexpressed in human primary nasopharyngeal carcinoma

<table>
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<th>Variable</th>
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<td></td>
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<td>4 (80)</td>
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<td>5 (62)</td>
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<td>55 (60)</td>
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NOTE: The expression of PARP1 in normal nasopharyngeal and nasopharyngeal carcinoma tissues was analyzed using tissue microarray and immunohistochemistry. Nonmalignant and malignant tissues were scored for low or high expression of PARP1 by assessing PARP1 staining in the nucleus. The Pearson χ² test was used to assess the association between PARP1 expression and several clinicopathologic variables. aVariables with statistical significance (P < 0.05) by Pearson χ² test.

treated with PARP inhibitors. In addition to DNA repair, PARP family also participates in other processes, includ-

References

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PARP1 Is Overexpressed in Nasopharyngeal Carcinoma and Its Inhibition Enhances Radiotherapy


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