Novel poly (ADP-ribose) polymerase inhibitor, AZD2281, enhances radiosensitivity of both normoxic and hypoxic esophageal squamous cancer cells

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Summary. Radiotherapy plays an important role in the treatment of esophageal squamous cell carcinoma (ESCC). However, the outcome of radiotherapy in ESCC remains unsatisfactory because esophageal squamous cancer cells, particularly those under hypoxic condition, exhibit radioresistance. The aim of this study was to determine whether or not AZD2281, a potent poly (ADP-ribose) polymerase (PARP) inhibitor, could enhance the radiation sensitivity of two ESCC cell lines, namely ECA109 and TE13. The radiosensitizing effect of AZD2281 was evaluated on the basis of cell death, clonogenic survival and tumor xenograft progression. AZD2281 alone was slightly toxic to ESCC cell lines. Apoptosis was increased and clonogenic survival was decreased in both cell lines when AZD2281 was combined with ionizing radiation (IR) under normoxic condition. AZD2281 enhanced IR-induced apoptosis to a more significant level under chronic hypoxic condition (0.2% O2, 48 hour) than under normoxic condition. AZD2281 also slightly enhanced clonogenic cell death under chronic hypoxic condition compared with that under normoxic condition. This result could be associated with increased radiation-induced DNA double-strand breaks (DSB), decreased DSB repair and increased apoptosis of ESCC cells. Furthermore, homologous recombination (HR) protein Rad51 expression and focus formation were decreased in ESCC cells exposed to moderate chronic hypoxic condition (0.2% O2, 48 hour); this result indicated that chronic hypoxic ESCC cells were HR deficient, possibly causing contextual synthetic lethality with PARP inhibitor in radiation sensitization. AZD2281 was also a radiation sensitizer in ESCC tumor xenograft models. Hence, in vitro and in vivo findings provide evidence that AZD2281 potently sensitizes ESCC cells to X-ray irradiation. The selective cell killing of HR-defective hypoxic cells contributes to radiosensitization by PARP inhibitor in ESCC cells under hypoxic condition.

KEY WORDS: AZD2281, DNA repair, esophageal squamous cell carcinoma, hypoxia, radiosensitivity.

INTRODUCTION

Esophageal cancer is a highly lethal and aggressive disease. This disease has also been considered as one of the most threatening malignancies to human health. One of the major histopathological subtypes of esophageal malignancies is esophageal squamous cell carcinoma (ESCC). In general, ESCC can be effectively treated by radiotherapy and chemoradiotherapy with conventional chemotherapy agents. However, intrinsic tumor radioresistance leads to the high recurrence and poor 5-year survival of ESCC patients.1

Ionizing irradiation exerts its therapeutic effect mainly by causing DNA double-strand breaks (DSB)
followed by apoptosis induction in cancer cells. DSB repair efficiency is a major determinant of cellular radiation sensitivity. Therefore, the manipulation of DSB repair or precursor DNA lesion may influence the efficacy of radiotherapy treatment for cancer. One of the earliest responses to DNA damage is the binding of poly (ADP-ribose) polymerase (PARP) to damaged sites. PARP is a nuclear chromatin-associated protein involved in sensing and relaying the presence of DNA breaks. In response to DNA breaks, PARP catalyzes the addition of poly (ADP-ribose) polymers from NAD onto nuclear acceptor proteins to attract repair proteins and change chromatin conformation. Thus, PARP plays important roles in multiple DNA damage response pathways; as such, this protein is a promising target of cancer therapy.

PARP inhibitors have been evaluated in clinical trials as either single agents or combined with DNA-damaging agents. As single agents, PARP inhibitors effectively target BRCA1- or BRCA2-deficient breast and ovarian cancers. In addition, PARP inhibitors can be used to treat tumors in combination with chemotherapeutic agents. As ionizing irradiation can induce DNA damage, PARP inhibitors may be potential agents to enhance radiation-induced DNA damage to radiosensitize several tumors.

Tumor hypoxia is a common characteristic of solid tumors. This condition is an important factor leading to radiation resistance and poor clinical outcomes. The oxygen occurred during radiotherapy generates free oxygen radicals that induce DNA damage and kill tumor cells. A hypoxic environment can stimulate the selection of locally or systemically aggressive tumor cell phenotypes by increased genomic instability, mutation and downregulation of DNA repair. Under hypoxia, the homologous recombination (HR) pathway involved in DSB repair is compromised. Previous research showed that chronic hypoxia (>48 hour) could reduce HR protein Rad51 levels. Severe acute hypoxia (16 hour with 0.02% O2) followed by reoxygenation or moderate chronic hypoxia (72 hour with 0.2% O2) treatments also decreases Rad51 expression and function. Furthermore, HR-defective hypoxic cells may be sensitized to PARP inhibitors. All solid tumors contain aggressive hypoxic cells; as such, these observations may increase the clinical utility of PARP inhibitors combined with radiotherapy to target treatment-resistant hypoxic tumor cells. ABT-888, a kind of PARP inhibitor, radiosensitizes malignant human cell lines under acute hypoxia.

AZD2281, also known as olaparib, is a potent inhibitor of both PARP-1 and PARP-2. This agent has been used successfully in the context of synthetic lethality to treat tumors with BRCA mutations and combined with platinum-based drugs. Furthermore, AZD2281 can potentiate radiation sensitization in glioblastoma multiforme cells and cells in deficient DSB repair. However, the potential of AZD2281 to radiosensitize ESCC has not been investigated. Our study aimed to examine the radiosensitizing effect of AZD2281 on human ESCC cells under hypoxic and normoxic conditions.

MATERIALS AND METHODS

Reagents and antibodies
AZD2281 (olaparib) was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 10 mmol/L. Rabbit antibodies against PARP, Rad51 and HIF-1α were purchased from Cell Signaling Technology (Boston, MA, USA). Mouse antibodies against β-actin and γH2AX were procured from Millipore (Billerica, MA, USA).

Cell culture
ESCC cell lines ECA109 and TE13 were obtained from Shanghai Institute of Cell Biology (Shanghai, China). These cells were propagated in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin in an incubator with 5% CO2 at 37°C. To induce cell growth under hypoxic condition, we incubated the cells in a modular chamber flushed with air composed of 0.2% O2, 94.8% N2 and 5% CO2 at 37°C.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay
The cells at a density of 4 × 10^5 cells/well were seeded in 96-well plates and allowed to grow for 1 day. These cells were then treated with different doses of AZD2281. After 24 and 48 hours of exposure to AZD2281, 10 μL of 5 mg/mL MTT reagent was added to each well. After these cells were incubated for 4 hours, the supernatant was removed and 150 μL DMSO was added to dissolve formazan crystals. The absorbance of lysate was determined at 490 nm by using a microplate reader. Each experiment was performed thrice.

Clonogenic survival assay
ESCC cells were seeded at serial densities in six-well plates. After cell adhesion occurred, the cells were treated with either DMSO (control) or AZD2281 (5 μmol/L) under normoxic or hypoxic conditions and then subjected to different doses of radiation (0, 2, 4, 6, 8 Gy). After irradiation was performed, the cells were cultured in an incubator with 5% CO2 at 37°C for 10 to 14 days. The colonies were fixed and stained with crystal violet stain and counted using a microscope.
stained with crystal violet. The number of colonies (>50 cells/colony) was counted. Cell survival curves were fitted according to a single-hit multi-target model. Survival enhancement ratio (SER) was calculated as the ratio of the mean inactivation dose in control cells divided by the mean inactivation dose in AZD2281-treated cells.

**Apoptosis assay**
Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide dual staining was performed to assess the fraction of apoptotic cells. The cells were plated in six-well plates and treated with DMSO or AZD2281 under normoxic or hypoxic condition. The treated cells were then irradiated at 8 Gy. After 48 hours, detached and attached cells were collected and washed with cold phosphate buffered saline (PBS). The cells were resuspended in binding buffer and incubated with propidium iodide (PI) and Annexin V-FITC in the dark for 15 minutes at room temperature. The samples were then immediately analyzed by flow cytometry using a FACSan with Cell-Quest software (BD Bioscience, Franklin Lakes, NJ, USA). These experiments were repeated thrice.

**Western blotting**
Cells were harvested and homogenized in radioimmunoprecipitation assay lysis buffer and centrifuged at 12,000 rpm for 20 minute at 4°C. The protein concentrations of the supernatants were determined using a bichinchoninic acid kit (P0010S; Beyotime Biotechnology, Nantong, China). Equal protein amounts were loaded in each well, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted on poly(vinylidene fluoride) membranes. These membranes were blocked and then probed with primary antibodies at 4°C overnight. The washed blot was incubated with conjugated secondary antibodies for 1 hour at 37°C. Immunoblotted proteins were visualized by electrochemi-luminescence reagents, and signals were detected using Chemidoc XRS imaging system (Quantity One Quantitation software, BioRad Laboratories, Hercules, CA, USA).

**Immunofluorescence analysis**

**γH2AX staining**
Cells were grown on glass coverslips and then treated with either DMSO or AZD2281 under hypoxic condition. The cells were then irradiated at 4 Gy. At 1 and 24 hours after irradiation, the cells were fixed with 4% paraformaldehyde for 20 minutes and stained with phosphor H2AX antibody overnight at 4°C followed by staining with FITC-conjugated secondary antibody for 1.5 hour at room temperature. Then the cells were washed with PBS; afterward, the nuclei were counterstained with 2 μg/mL 4’, 6-diamidino-2-phenylindole (DAPI). The images were examined using a laser scanning confocal microscope (Zeiss LSM510; Carl Zeiss Jena, Oberkochen, Germany).

**Rad51 staining**
Cells were grown on glass coverslips and then cultured under normoxic or hypoxic condition. After 0, 4, 12, 24 and 48 hours hypoxia, the cells were fixed with 4% paraformaldehyde for 20 minutes. These cells were initially stained with Rad51 antibody over-night at 4°C and then stained with tetramethyl rhodamine isothiocynate-conjugated secondary antibody for 1.5 hour at room temperature. Afterward, the cells were washed with PBS and the nuclei were counterstained with 2 μg/mL DAPI. The images were examined using a laser scanning confocal microscope (Zeiss LSM510).

**Tumor xenograft mouse models**
Animal experiments were approved by the Ethics Committee of Nanjing Medical University. Five to six-week-old male BALB/c nude mice were provided by Nanjing Medical University Animal Center. ECA109 cells (1 × 10⁷) were injected subcutaneously into one site of the right leg. When the tumors grew to approximately 0.2 cm³ after inoculation on day 9, the mice were randomly divided into four groups (n = 6): (i) vehicle control; (ii) AZD2281 (50 mg/kg intraperitoneally injected daily for continuous 5 days); (iii) 10 Gy fractionated radiotherapy (2 Gy daily for continuous 5 days); and (iv) AZD2281 and 10 Gy (5 × 2 Gy) fractionated radiotherapy (AZD2281 administered 30 minutes before daily dose of 2 Gy radiation). All of the treatments were initially administered at day 9. Tumor growth was measured at an interval of 2 days until day 25. Tumor volume was calculated using the formula: tumor volume = length × (width)²/2.

**Statistical testing**
The GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used to test for statistically significant differences between means (P < 0.05), using the two-tailed t-test.

**RESULTS**

**AZD2281 inhibits ESCC cell proliferation**
To determine the sensitivity of different esophageal carcinoma cell lines to AZD2281 as a single agent, we
performed MTT assay at 24 and 48 hours after the cells were exposed to various concentrations of AZD2281 up to 50 μmol/L. After 24-hour treatment, AZD2281 showed no significant effect on cell proliferation. After 48-hour treatment, however, limited inhibitory effects were observed in both cell lines (Fig. 1). These data indicate that AZD2281 is not a cytotoxic agent and only elicits a limited inhibitory effect on ESCC cell proliferation.

AZD2281 promotes radiosensitivity of ESCC cell lines

Clonogenic assay was performed to investigate the potential radiosensitization activity of AZD2281 on ESCC cell lines under normoxic and hypoxic conditions. Under normoxia, AZD2281 potentiated the cytotoxicity of radiation treatment, in which the SERs at 5 μmol/L were 1.4 and 1.6 for ECA109 and TE13 cells, respectively (Fig. 2A). Under hypoxia, AZD2281 also showed radiosensitization activity with SERs of 1.5 and 1.7 for ECA109 and TE13 cells, respectively (Fig. 2B). These data demonstrate that AZD2281 promotes radiosensitivity of normoxic and hypoxic ESCC cells.

AZD2281 enhances radiation-induced apoptosis in ESCC cells

Annexin V-PI staining was performed to quantify apoptosis after treatment of ESCC cell lines with irradiation and/or AZD2281. The results showed that single-agent treatment of 5 μmol/L AZD2281 elicited a limited pro-apoptosis effect on ECA109 and TE13 cells; by contrast, radiation exposure alone induced a moderate level of apoptosis. The combined treatment of 8 Gy irradiation and 5 μmol/L AZD2281 largely increased apoptotic cells in these cell lines under hypoxia (Fig. 3A). The difference between groups of combined treatment and radiation alone treatment was statistically significant (P < 0.01) under hypoxia. A similar but not statistically significant result was observed in normoxic ESCC cells (Fig. 3B).

AZD2281 impairs DSB repair after irradiation in hypoxic ESCC cells

To examine whether or not AZD2281 could functionally impair DSB repair, we incubated hypoxic ECA109 cells with or without 5 μmol/L AZD2281 and irradiated these cells at 4 Gy of ionizing radiation (IR). The cells were fixed and stained to form γH2AX foci at indicated times after irradiation was performed. The combination of AZD2281 treatment with 4 Gy X-ray increased the formation of phospho-γH2AX foci at 1 hour. At 24 hours after irradiation, AZD2281-treated cells showed a significantly slower decay of γH2AX foci (Fig. 4). These results indicate that the presence of AZD2281 enhances X-ray radiation-induced DNA DSB and impairs DSB repair following IR; this process may be a possible mechanism by which AZD2281 radiosensitizes hypoxic cells.

Exposure to chronic hypoxia leads to decreased Rad51 expression in ESCC cells

It has been reported that hypoxia can decrease the expression of numerous HR proteins. To evaluate how hypoxic condition could alter HR protein expression in ex vivo ESCC cells, we assessed the foci assembly and expression of HR protein Rad51 in ESCC under normoxic and hypoxic conditions (0.2% O2) at indicated time points. Unlike Rad51 foci in normoxic cells, Rad51 foci in hypoxic ECA109 cells failed to assemble (Fig. 5A). Consistently, Rad51 expression was downregulated after the cells were exposed to hypoxic condition for 48 hour
PARP inhibitor AZD2281 did not alter Rad51 expression under either normoxic or hypoxic conditions (Fig. 5C). Hence, we conclude that moderate chronic hypoxia leads to defective HR function.

Combined AZD2281 and IR treatment reduces tumor growth in tumor xenograft models

We evaluated the effect of PARP inhibitor on ESCC growth in tumor xenograft models. Treatment with AZD2281 or IR individually reduced tumor growth. More significant reduction of tumor growth was induced in the group treated with both AZD2281 and IR (Fig. 6). These results indicate that AZD2281 and IR elicit a strong tumor inhibitory effect on xenograft mouse models.

Moreover, we calculated the doubling time required for the tumor to grow twice in size under different treatments. The doubling time for ECA109 tumor in each group is shown in Table 1. AZD2281 enhanced the response of ECA109 xenografts to irradiation with an enhancement factor of 2.5 (Table 1).

**DISCUSSION**

Radiotherapy plays an important role in ESCC treatment. However, the outcome of radiotherapy in advanced ESCC remains unsatisfactory with a 5-year survival rate of 20% to 30% and a locoregional control rate of only 45%. Radioresistance is induced by several factors, such as tumor hypoxia and repair of DNA damage; these factors have been considered as radiosensitive targets of ESCC. PARP-1 and its related family member PARP-2 are nuclear proteins involved in sensing and relaying the presence of DNA breaks. PARP inhibitors can be potentially used in combined therapy, as observed in preclinical models.
Fig. 3. AZD2281 promotes the apoptosis of esophageal squamous cell carcinoma (ESCC) cells under hypoxia (A) and normoxia (B). Two ESCC cell lines were treated, as indicated in the Methods section, stained with Annexin V/PI, and subjected to fluorescence-activated cell sorter (FACS) analysis. Data were expressed as mean ± standard error (SE); n = 3. * P < 0.05; ** P < 0.01.

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with improved growth delay of tumors treated with radiation, temozolomide, cisplatin, carboplatin or cyclophosphamide.\textsuperscript{19,20}

AZD2281 is a potent inhibitor of PARP-1 and PARP-2. AZD2281 exhibits monotherapy activity against tumor cells harboring BRCA1 or BRCA2 mutations via a synthetic lethality interaction.\textsuperscript{21} Radiosensitizing properties of AZD2281 have been described in several kinds of cancer cells. This study is the first to demonstrate that the PARP inhibitor AZD2281 sensitizes normoxic and HR-defective hypoxic ESCC cells to radiation therapy by suppressing DSB repair. In addition, AZD2281 enhances tumor inhibitory effects of IR in xenograft mouse models.

We observed that AZD2281 was not a cytotoxic compound against ESCC cells; by contrast, 5 μmol/L AZD2281 significantly enhanced the sensitivity of ESCC cells to radiation under normoxia and hypoxia. In hypoxic ESCC cells, radiosensitization by AZD2281 was associated with enhanced apoptosis and impaired DSB repair. γ-H2AX foci were detected by immunofluorescence staining; the results verified that AZD2281 could functionally impair DSB repair in irradiated hypoxic ESCC cells, resulting in enhanced cell apoptosis. Previous reports showed that in different cell lines, PARP inhibitors exhibited different pro-apoptosis properties following IR. They conjectured that might be a cell line-dependent phenomenon.\textsuperscript{22,23} In our study, however,
radiation combined with AZD2281 treatment elicited a synergetic effect on the induction of ESCC cell apoptosis, indicating that PARP inhibitor affected the apoptotic pathway in these cell lines under these treatment conditions. Nevertheless, mechanisms other than apoptosis should be further investigated. HR protein expression in hypoxic cells was also decreased. In a previous study, ABT888, another PARP inhibitor, radiosensitized malignant human cell lines under acute hypoxia. However, no alteration was observed in the expression of HR protein Rad51 under acute hypoxia.14 In the present study, Rad51 expression was evidently decreased under chronic hypoxia (0.2% O₂, 48 hours); this result was consistent with attenuated Rad51 foci signaling. Although PARP inhibitor did not affect HR protein expression, clonogenic radiation-induced killing of HR-deficient hypoxic cells was increased after PARP was chemically inhibited. Thus, hypoxia-induced HR defects may lead to synthetic lethality with PARP inhibitor in the selective radiosensitization of hypoxic ESCC cells. These repair-defective hypoxic cells are more sensitive to PARP inhibitors and radiation combined modality as a consequence of ‘synthetic lethality effect’. In other words, PARP inhibitors should show a stronger radiosensitizing

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**Fig. 5** Exposure to chronic hypoxia leads to decreased Rad51 expression in esophageal squamous cell carcinoma (ESCC) cells. (A) Rad51 staining was performed to observe the assembly of homologous recombination protein Rad51 in ECA109 cells under normoxic and hypoxic conditions (0.2% O₂) at indicated time points. The Rad51 assembly was stained as red and the nuclei were stained as blue. (B) Rad51 protein expression was downregulated after ECA109 cells were exposed to hypoxic condition for 48 hours. HIF-1α expression was evaluated at different time points as an indicator of hypoxia. No difference was observed in poly (ADP-ribose) polymerase (PARP) expressions at different hypoxia time points. (C) AZD2281 did not alter Rad51 expression under normoxic or hypoxic condition in ECA109 and TE13 cells.

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**Table 1** Effect of AZD2281 on response of ECA109 xenografted tumor to irradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Doubling time (days)</th>
<th>Absolute growth delay (days)†</th>
<th>Normalized growth delay (days)‡</th>
<th>Enhancement factor</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>6.6 ± 0.7</td>
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<tr>
<td>AZD2281</td>
<td>7.3 ± 1.2</td>
<td>0.7 (7.3–6.6)</td>
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<tr>
<td>IR</td>
<td>9.4 ± 1.7</td>
<td>2.8 (9.4–6.6)</td>
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<tr>
<td>IR+AZD2281</td>
<td>14.3 ± 2.0</td>
<td>7.7 (14.3–6.6)</td>
<td>7.0 (7.7–0.7)</td>
<td>2.5 (7.0/2.8)</td>
</tr>
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†Absolute growth delay: The doubling tumor time of treatment group minus that of the control group. ‡Normalized growth delay: The time of absolute growth delay of tumor in IR combined with AZD2281 group minus that of the AZD2281 group.
AZD2281 enhances radiosensitivity of ESCC

property in hypoxic cancer cells than normoxic cancer cells. The results of clonogenic assays and apoptosis detection provided strong evidence supporting our hypothesis.

In summary, our findings provide evidence that AZD2281 potently sensitizes ESCC cells to X-ray irradiation not only under normoxic condition but also under hypoxic condition; this result is associated with the attenuation of DSB repair. We specifically present a model of hypoxia-mediated contextual synthetic lethality with PARP inhibitor-mediated radiosensitization. The potential for AZD2281 to radiosensitize both normoxic and hypoxic ESCC cells makes it a promising drug to improve the therapeutic ratio of radiotherapy in patients with ESCC.

References


