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The lethal effect of bis-type azridinylnaphthoquinone derivative on oral cancer cells (OEC-M1) associated with anti-apoptotic protein bcl-2

Yi-Chen Peng,[a] Hsien-Shou Kuo,[b] Hsin-Da Tsai,[b] Yu-Ping Yang[b] and Yuh-Ling Lin[a,*]

[a]*Department of Medicine, College of Medicine, Fu-Jen Catholic University, Taiwan, ROC*
[b]*Department of Biochemistry, College of Medicine, Taipei Medical University, Taiwan, ROC*

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Abstract—Several drugs of aziridinylbenzoquinone analogs have undergone clinical trials as potential antitumor drugs. These bioreductive compounds are designed to kill tumor cells preferentially within the hypoxic microenvironment. From our previous reported data, it was found that the synthesized 2-aziridin-1-yl-3-[(2-{2-[(3-aziridin-1-yl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)thio]ethoxy}ethyl)thio]naphthoquinone (AZ-1) is a bioreductive compound with potent lethal effect on oral cancer cell, OEC-M1. It was found in this study that the lethal effect of the oral cancer cell lines OEC-M1 induced by AZ-1 was mediated through the cell cycle arrest and apoptosis pathway.

The LC_{50} values of OEC-M1 and KB cells induced by AZ-1 compound were 0.72 and 1.02 μM, respectively, which were much lower than that of normal fibroblast cells (SF with LC_{50} = 5.6 μM) with more than 90% of normal fibroblasts surviving as compared to control at a concentration of AZ-1 as high as 2 μM. It was interesting to note that the LC_{50} of monotype diaziridinylbenzoquinone compound, diaziquone (AZQ), was 50 μM on OEC-M1 cells. Comparing the cytotoxicity of AZ-1 and AZQ on OEC-M1 cells, AZ-1 is approximately 70 times more potent than AZQ.

By using Western blot, both G_2/M phase cell cycle arresting protein, cyclin B, and anti-apoptotic protein, bcl-2, were expressed in OEC-M1 cell when the concentrations of AZ-1 were increased from 0.125 to 0.5 μM and then decreased from 1 to 2 μM of AZ-1 treatment as compared with control for 24 h. Both proteins were expressed most abundantly at 0.5 μM AZ-1. However, the expression of bcl-2 protein in OEC-M1 was significantly decreasing in a dose-dependent manner and was only about 50% protein level at 2 μM AZ-1 for 48 h as compared with control. The cell survival check protein p53 increased from 1.72- to 2.8-fold and 1.36- to 2.16-fold at concentrations of AZ-1 from 0.125 to 2.0 μM in a dose-dependently increasing manner on OEC-M1 as compared with control for 24 and48 h treatments, respectively. The apoptotic-related phenomena were observed, which included apoptotic body formation and the enzyme activity change of caspase-3. The apoptotic bodies and caspase-3 activity of OEC-M1 were induced only at 2 μM AZ-1 for a 24 h treatment, yet apoptotic body formation was observed at as low as 0.5 μM AZ-1 and in a dose-dependently increasing manner for a 48 h treatment. The caspase-3 activity was increased 20.6%, 26.8%, and 84.2%, respectively, at 0.5, 1, and 2 μM concentrations of AZ-1 for a 48 h treatment as compared with control. These results indicate that AZ-1 induced the cell death of OEC-M1 through the G_2/M phase arrest of cell cycle and anti-apoptosis first and then apoptosis following a 48 h treatment. All of the pathway might be associated with bcl-2 and p53 protein expression. We propose that the AZ-1 could be used as anti-oral cancer drug for future studies with animal models.

1. Introduction

Oral cancer is a serious problem of public health, more than 300,000 new cases of oral cancer occurring worldwide annually. Of these, approximately 50,000 cases occur in United States and Europe, and two-thirds of which occur in developing countries. In the Nordic countries (Norway, Sweden, Denmark, and Finland), approximately 1500 cases are being diagnosed with oral squamous cell carcinoma annually. The overall mortality rate for intra-oral cancer remains high at approximately 50%, even with modern medical services, probably due to the advanced stage of the disease at the time of discovery.[1–4] According to the statistics of oral cancer from organization of health department in Taiwan, the increasing rate of incidence was 39.98%

Keywords: Bis-type aziridinylnaphthoquinone; Oral cancer cells (OEC-M1); Cell cycle arrest; p53 protein.
* Corresponding author. Tel.: +886 2 29053463; fax: +886 3 3979280; e-mail: med0018@mails.fju.edu.tw

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during the 1996 to 2000 year, which is the highest rate in all kinds of cancers. This aggressive type of oral carcinoma is associated with severe morbidity and high mortality with less than 50% long-term survival. Despite extensive research on treatment modalities toward oral cancer, the 5-year survival rate of this disease has not improved over the last 4–5 decades.[5,6] However, this situation may change drastically during the next decade because the molecular targeting research has brought about a revolution in the understanding of disease processes.[7,8] Chemoprevention is the use of pharmacologic or natural agents that inhibit the development of invasive cancer. These work either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of pre-malignant cells in which such damage has already occurred.[9]

The bioreductive drugs, aziridinylbenzoquinones, are a class of compounds which are designed to exploit one of the features of solid tumor biology caused by an inadequate blood supply to solid tumor, namely tumor hypoxia; such regions generally are resistant to radiation, chemotherapeutic, and other O_2-requiring treatments.[10–13] The ideal bioreductive drug should be administered as an inactive prodrug that is only activated under low-oxygen conditions by one- or two-electron reductase.[14] The aziridine-substituted benzoquinones such as, mitomycin C, triaziquone, RH1, and tirapazamine (TPZ), are three principal aziridinyl quinone classes of hypoxia-specific cytotoxins that are being developed for clinical use.[15–17] These agents are composed of an aziridinyl moiety on a quinone structure and they are converted by reductive metabolism to a bifunctional alkylating species that can cross-link DNA that occurs in the major groove which interacts predominantly at guanine-N7.[18] These agents probably produce their major cytotoxic activities through the formation of DNA cross-link. In the case of diaziridinyl substituted quinone, this highly cytotoxic bifunctional alkylating agent can cross-link DNA in cells that results in inducing complicated cellular mechanisms leading to cell death by apoptosis such as TPZ and CI-1010 or necrosis.[19,20]

The tumor tissue was with lower oxidative reduction (redox) potential, relative to most normal tissues, which could increase reductive activation of these quinone derivatives in tumor.[11] Therefore, the selectivity of bioreductive drugs is governed not only by difference in oxygen tension between tumor and normal tissue but also by levels of enzymes catalyzing bioreductive activation such as DT-diaphorase.[13,21,22] This fact led to publication in 1990 of the concept of 'enzyme-directed bioreductive development' by Workman and Walton.[23] In many cases, the biological activity of quinone is attributed to the ability to accept electrons to form the corresponding radical anion or dianion species. Quinone moiety substituted with aziridine had been shown as a potent alkylating agent due to bioreduction either by the one-electron reducing enzymes (e.g., NADPH cytochrome P450 reductase, cytochrome *b*5 reductase) or by a two-electron reducing enzyme ((NADP)H oxidoreductase, NQO1) to form the corresponding aziridinyl hydroquinones.[24–26] The hydroquinone moiety in the corresponding aziridinyl hydroquinone effectively changes the p*K* of the aziridine ring such that it is protonated and becomes activated toward nucleophilic attack under physiological pH. In our previous report, we had synthesized this bis-type of aziridinylnaphthoquinone series of bioreductive compounds with various lengths of spacer between two of each aziridinylnaphthoquinone structure and evaluated their anticancer activities.[27,28] It was found that one of these compounds, 2-aziridin-1-yl-3-[(2-{2-[(3-aziridin-1-yl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)thio]ethoxy}ethyl)thio]naphthoquinone (AZ-1; Scheme 1), exhibited more potent lethal effect on oral cancer cell (OEC-M1) and less cytotoxicity to normal fibroblast cell (SF). In this study, we investigated the anticancer mechanism of AZ-1 to our local cell line OEC-M1 cell and compared the lethal effect of OEC-M1 with that of another ATCC cell line KB cell induced by AZ-1. We also compared the cytotoxicity of AZ-1 with another bioreductive compound diaziquone (2,5-diaziridinyl-3,6-bis(carboethoxy-amino)-1,4-benzoquinone; AZQ) to OEC-M1 cells in the cell viability. The AZQ is monotype of benzoquinone with two of aziridinyl groups functional in the para-position of structure, herein.

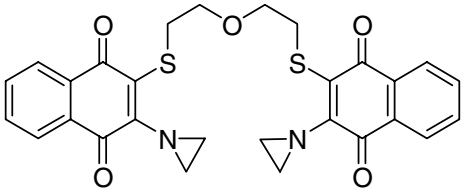
2. Results

2.1. The cytotoxicity of AZ-1 to OEC-M1 and KB cells

The LC_{50}'s of AZ-1 to OEC-M1 and KB cells were 0.72 and 1.02 μM, respectively. The responses of these two cell lines, KB and OEC-M1, to AZ-1 were similar in cell viability in a dose-dependent manner. In normal fibroblast cell (SF), there was an over 90% survival rate at 2 μM AZ-1 (Fig. 1). The cytotoxicity of AZQ as monotype triaziridinylbenzoquinone compound on OEC-M1 cell was compared with that of AZ-1 compound. Its LC_{50} = 50 μM was far less potent than AZ-1 compound to OEC-M1 cells (Fig. 2).

2.2. Analysis of cell cycle arrest and apoptosis by flowcytometry and Hoechst staining

The DNA content of cells was analyzed to determine whether cell cycle arrest or apoptosis was induced by AZ-1. The DNA stained data of cell population distribution and sub-G_1 area formation analyzed by flowcytometry indicated that OEC-M1 cells were arrested at



AZ-1

Scheme 1. The chemical structure of AZ-1.

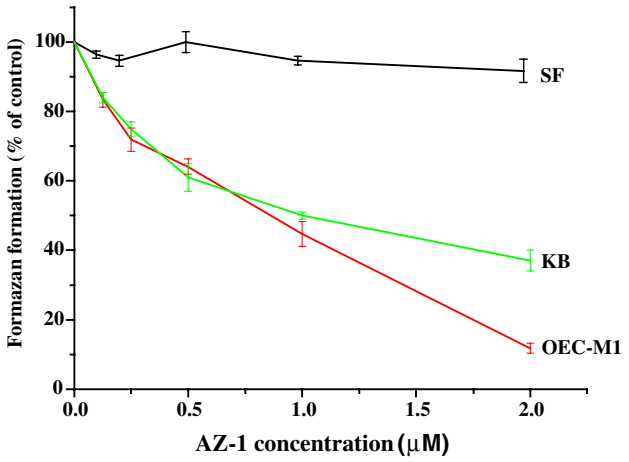


Figure 1. AZ-1 compound inhibited the proliferation of three cell lines SF, OEC-M1, and KB. Cells were seeded for 18 h before the addition of AZ-1 with various concentrations. The death effects of these three cell lines were compared by the MTT assay. The MTT assay was used to determine the cell viability after an additional 24 h of culture. Data were from quadruplet wells and are representative of three separate experiments.

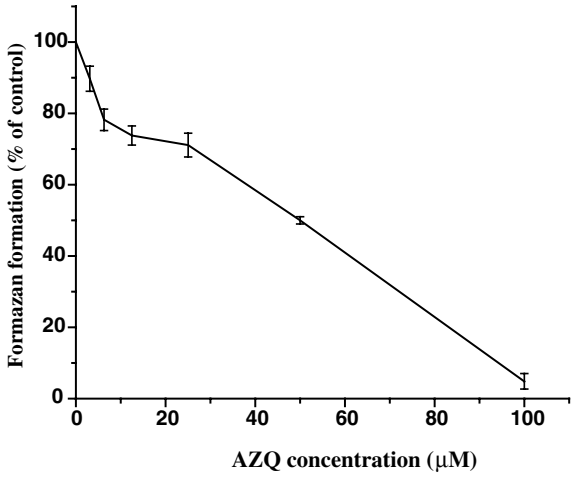


Figure 2. AZQ compound inhibited the proliferation of cell line OEC-M1 cells that were seeded for 18 h before the addition of AZQ with various concentrations. The MTT assay was used to determine the cell viability. The MTT assay was used to determine the cell viability after an additional 24 h of culture. Data were from quadruplet wells and are representative of three separated experiments.

the G_2/M phase of cell cycle at a concentration of AZ-1 as low as 0.5 μM (Fig. 3B). For a small population of cells, apoptotic formation in the sub-G_1 area was 12.3% and 17.5% at 1 and 2 μM AZ-1, respectively, after 24 h treatment (Figs. 3C and D), as compared with control cells (0.1% DMSO treatment only) (6.1%; Fig. 3A). When OEC-M1 cells were treated with 0.5 μM AZ-1 for various time periods, the G_2/M phase arrest was also found in 24 h (Fig. 4C) and apoptotic bodies of the sub-G_1 area were significantly increased with 23.2% area (Fig. 4D) for 48 h treatment as compared with control cells (4.8%; Fig. 4A). These phenomena could also be found in OEC-M1 cells by Hoechst staining at 2 μM AZ-1 for 24 h treatment with a small portion of apoptotic body formation (Fig. 5C, left column), and with apoptotic body formation at as low as 0.5 μM for 48 h treatment (Fig. 5E) and with the highest number at a concentration of 2 μM (Fig. 5F, right column). The Hoechst staining method was used to identify the apoptotic nuclei in OEC-M1 cells. Apoptotic cells contained the apoptotic bodies that showed blue peripherally clumped or fragmented chromatin, as indicated by an arrow in Figure 5. Based on the results of Figures 3–5, it was found that apoptotic bodies could be observed only at the percent of sub-G_1 area at least over 17%.

2.3. The expression of cell cycle arrest and apoptosis associated with proteins, and caspase-3 activity

Next, determination was made to find out whether the induction of OEC-M1 cells apoptosis by AZ-1 was associated with the expression of cell cycle arrest and the apoptosis-related proteins. It was found that the cell survival checkpoint protein p53, anti-apoptotic protein bcl-2, and cell arrest protein cyclin B were all increased in a dose-related manner (Fig. 6). The p53 protein increased the expression from 1.72- to 2.8-fold and 1.36- to 2.16-fold at concentrations of AZ-1 from 0.125 to 2.0 μM on OEC-M1 cell as compared to control for 24 and 48 h treatments, respectively. The cyclin B and bcl-2 proteins increased from 1.08- to 1.25-fold and 1.27- to 1.57-fold at a concentration of 0.125–0.5 μM, respectively, and then decreased from 1 to 2 μM AZ-1 as compared with control for 24 h. Therefore, for 24 h treatment, both cyclin B and bcl-2 proteins were expressed most abundantly at 0.5 μM AZ-1 concentration. However, for 48 h treatment the expression of bcl-2 protein in OEC-M1 cell induced by various concentrations of AZ-1 was significantly decreased in a dose-dependent manner, and only about leave 50% protein level as compared with control was at the highest concentration of AZ-1 2 μM. From Western blot, the values of the relative protein expression-quantifying table revealed that the expression of proteins was affected by various concentrations of AZ-1 in OEC-M1 cells after 24 and 48 h treatment, and this relative protein expression was compared with the control (C, fold = 1) (Table 1). In case of caspase-3 activity in OEC-M1 cells, the solid area was shifted at 2 μM AZ-1 treatment for 24 h with 26.8% increasing (Fig. 7C), whereas for 48 h treatment, it was increased in a dose-dependent manner from 18.9% to 84.2% at concentrations of AZ-1 from 0.5 to 2 μM as compared with the control (gray dot line) (Fig. 7D–F).

3. Discussion

Our previous study on different series of bis-aziridinylnaphthoquinone compounds has identified that these compounds exhibit more potent response toward the solid tumors than the circulation tumors,[27] and these results are supported by some reports indicating that there are differences in the reductive metabolism between the solid tumors and the circulation tumors.[11] Considering the importance of all the cellular reductases (e.g., NADPH cytochrome P450 reductase, cytochrome *b*5 reductase, (NADP)H oxidoreductase, and NQO1) in

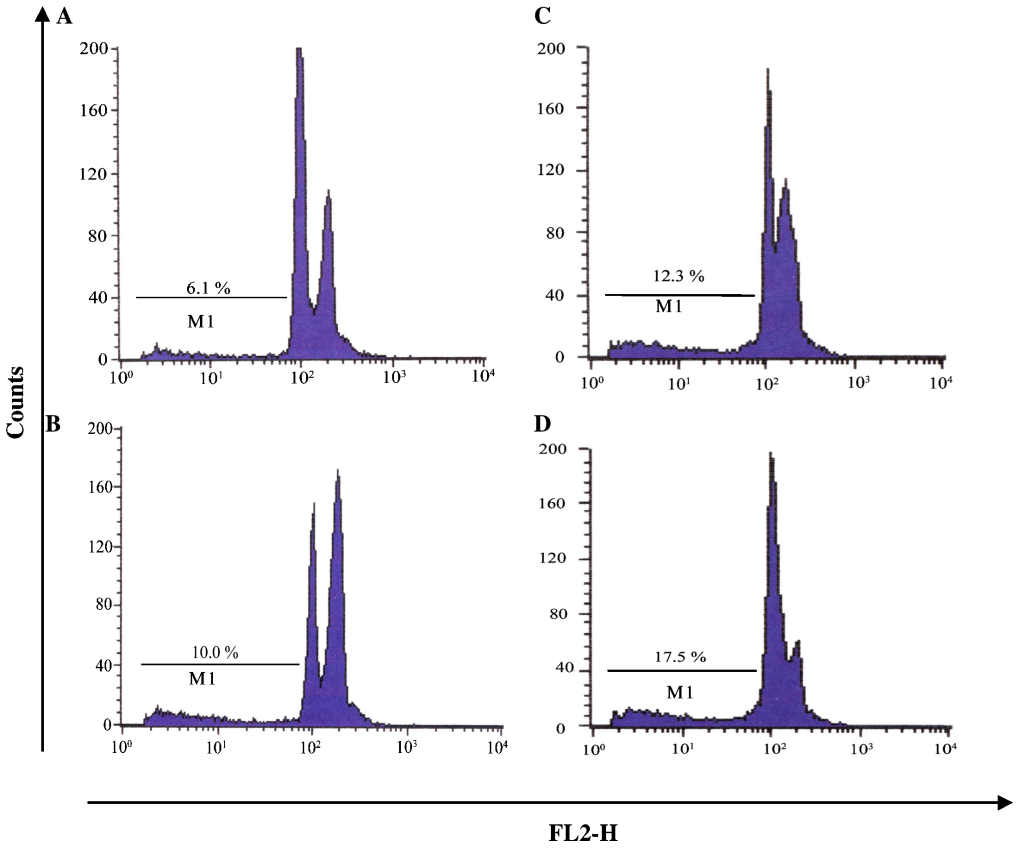


Figure 3. Apoptosis is induced by AZ-1. OEC-M1 cells were treated in 24 h culture with (A) 0.1% DMSO control; (B) 0.5 μM; (C) 1.0 μM; and (D) 2.0 μM of AZ-1 before PI staining and analysis of DNA content. Apoptosis is apparent by the large population of cells with increased DNA content in the sub-G_1 area. (M1-area).

response to the whole cellular reductive metabolism, these reductases are responsible for bioactivation of AZ-1. However, the bioreductive drugs, such as AZQ, mitomycin C, and TPZ, have been developed to exploit the oxygen deficiency in the hypoxic fraction of solid tumors on the premise that hypoxic cells should show a greater propensity for reductive metabolism than well-oxygenated cells.[11,14,29–31]

Oral cancer is one of the most disfiguring types of cancer as the surgical removal of the tumor may result in facial distortion. Oral cancer is also known to exhibit 'field cancerization' resulting in development of a second primary tumor.[32] Prevention and early detection/treatment of oral cancer could significantly improve the quality of life for individuals at risk. Recently, the targeted elimination of oral squamous carcinoma cell by inducing apoptosis has emerged as a valued strategy to combat oral cancer.[33–35] Apoptosis plays an important role in removal of aberrant cells that might otherwise cause the development of tumor.[36] Normally, the processes of cell reproduction take place through an ordered process, known as cell cycle. The tumor suppressor gene p53 is a multifunctional protein that is mainly responsible for maintaining genomic integrity and is the most frequently mutated gene in human tumors.[37] In response to DNA damage, aberrant growth signals, or chemotherapeutic drugs, p53 is stabilized and induces apoptosis and/or cell cycle arrest.[38–40] Therefore, p53 is a tumor suppressor gene with key regulator effects on both cell cycle and apoptosis. P53 exerts its control on apoptosis by interacting with other important apoptotic molecules, such as members of the bcl-2 family.[41] The protein from the bcl-2 gene family which is an anti-apoptotic associated protein plays an important role in the regulation of apoptosis.[42] In the apoptosis pathway, some drugs will activate a large family of cysteine protease family such as caspases which specifically cleave their substrates after aspartic acid, then activate or inactivate their cellular protein targets by a process of limited proteolysis.[43,44] Most of them play central roles in execution of apoptosis.[45]

In our study, AZ-1 induced the lethal effect on OEC-M1 and KB cells (Fig. 1). As shown in Figure 2, AZQ with LC_{50} = 50 μM to OEC-M1 cells is at a much higher concentration than the AZ-1 compound (LC_{50} = 0.72 μM). Whereas, the bis-type of AZ-1 is more potent than compound, AZQ to OEC-M1, and with lower cytotoxicity to normal fibroblast cells. As shown in Fig. 3B, the cell cycle arrested at the G_2/M phase was observed at 0.5 μM AZ-1 and 1 to 2 μM in a small portion of sub-G_1 area for a 24 h treatment. However, changing of the sub-G_1 area in OEC-M1 cell at 0.5 μM AZ-1 treatment was effected in a time-dependent manner, and there was the largest sub-G_1 area at 2 μM AZ-1 for 48 h treatment (Fig. 4D). From Figures 3–5, it was found that some of apoptotic body number at 2 μM for 24 h, but the apoptotic bodies

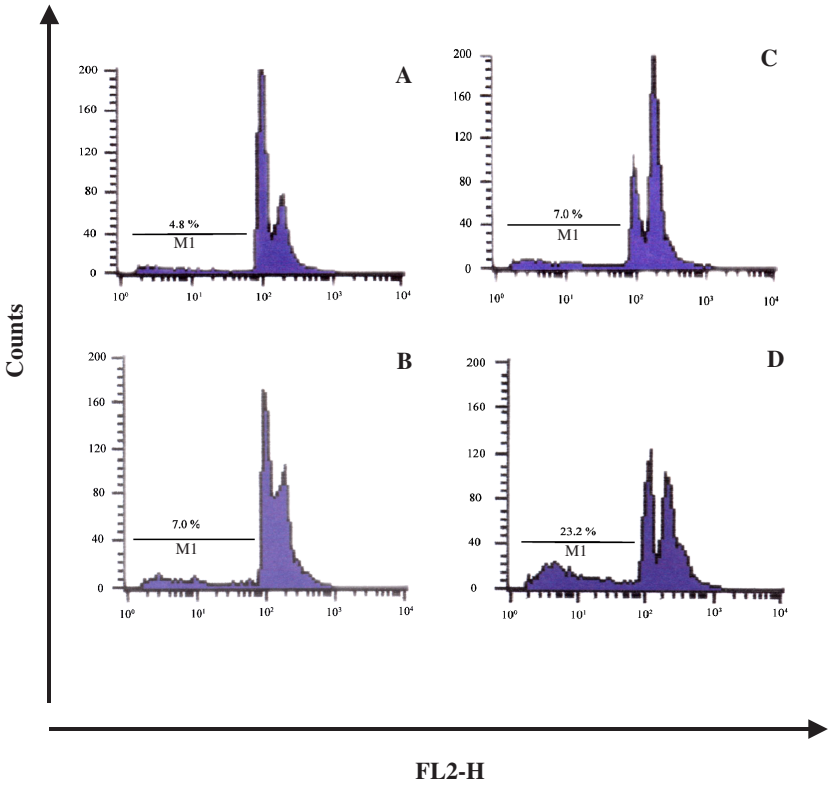


Figure 4. Apoptosis is induced by 0.5 μM AZ-1 compound with various time periods. OEC-M1 cells were treated with 0.5 μM AZ-1 for (A) 0 h; (B) 12 h; (C) 24 h; and (D) 48 h before PI staining and analysis of DNA content. Apoptosis is apparent by the large population of cells with increased DNA content in the sub-G_1 area (M1-area).

increased slightly at 0.5 μM AZ-1 and were most abundant at a concentration of 2.0 μM for 48 h. From Western blot, it was clear that the p53 protein increased in a dose-dependent manner both at 24 and 48 h treatments. The relative fold of p53 protein expression, is a little bit more at 24 h than 48 h. Therefore, the p53 protein produces a more important regulatory role on OEC-M1 cell in early stage after AZ-1 treatment, and the effect lasted for 48 h. The G_2/M arrest protein, cyclin B, and anti-apoptotic protein, bcl-2, were with the same trend that increased from 0.125 to 0.5 μM and decreased at 1–2 μM concentration of AZ-1 treated for 24 h. However, the expression of bcl-2 protein was totally in a decreasing manner as the dose increases at various concentrations of AZ-1 for 48 h. When OEC-M1 cell was arrested at the G_2/M phase in 0.5 μM AZ-1 treated for 24 h, the expression of cyclin B and bcl-2 proteins was most abundant at this concentration from Western blot (Fig. 6). Therefore, OEC-M1 cells were triggered into the cell cycle arrested at the G_2/M phase at 24 h, and then apoptosis followed for 48 h at 0.5 μM AZ-1 (Fig. 5). As shown in Figure 7, the protease caspase-3 was activated by AZ-1 to take part in the apoptosis process for DNA fragmentation in OEC-M1 cell. Caspase-3 activity was induced at 2 μM AZ-1 for 24 h, but with the enzyme activity from 0.5 to 2 μM for 48 h treatment. The expression of anti-apoptotic protein bcl-2 in OEC-M1 induced by AZ-1 was increased at lower concentration (0.125–0.5 μM) and then decreased a little bit at higher concentration (1 and 2 μM) for 24 h. However, for 48 h treatment the expression of the bcl-2 protein is significantly decreased in a dose-dependent manner while in apoptosis pathway. These phenomena were also similar with some other bioreductive quinone-containing compounds such as menadione, AZQ, and adriamycin.[46] The report from Ngo et al. also suggested that the activation of AZQ and its analogues is a prerequisite for p53 induction and moreover the induction of p53 by AZQ requires both the quinone and aziridine moieties of the AZQ molecule.[32] In conclusion, AZ-1 at 0.125–0.5 μM induced arrest of OEC-M1 cell at the G_2/M phase, then toward apoptosis pathway when the concentration of AZ-1 increased to as high as 2 μM. For the time course of 0.5 μM of AZ-1 treatment, the cell was arrested at the G_2/M phase for 24 h, and then into the apoptosis pathway for 48 h. These results could be referred to by the experimental design on animal model testing in the future. We believe that the mechanism of apoptotic pathway in OEC-M1 cells induced by this bioreductive compound AZ-1 is involved in the regulation effect of p53 protein on cell cycle arrest at the G_2/M phase by protein cyclin B. Meanwhile, induction of the expression of anti-apoptotic protein, bcl-2, in the initial stage, then to the apoptosis pathway at a higher concentration or prolonged treatment just as 2 μM or 48 h mediated the bcl-2 protein decrease and increase of caspase-3 activity that led to the apoptotic body formation and to apoptosis in the end.

4. Materials and methods

RPMI 1640 medium, DMEM, fetal bovine serum (FBS), 2 mM L-glutamine, MEM non-essential amino

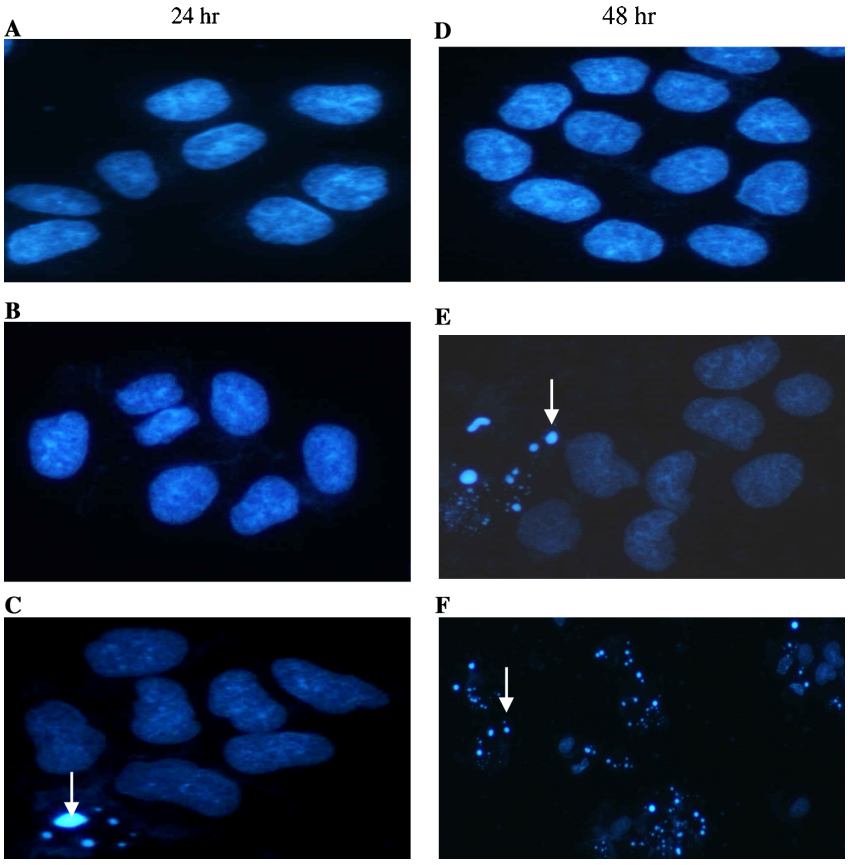


Figure 5. Apoptosis of OEC-M1 cells was induced by various concentrations of AZ-1. The OEC-M1 cells were treated for 24 h (A–C) or 48 h (D–F) cultured with 0.1% DMSO control (A and C), 0.5 μM (B and D), and 2.0 μM (C and F) of AZ-1 before Hoechst staining and analysis of DNA nuclei. Apoptotic cells showed blue peripherally clumped or fragment chromatin as indicated by arrow.

acids, trypsin–EDTA solution, PBS, Hank's balance salt solution (HBSS), penicillin–streptomycin, and fungizone were purchased from Gibco Laboratories (Grand Island, NY). The compounds of $NaHCO_3^-$, MTT, trypan blue, EDTA, propidium iodide (PI), Hoechst 33258, and diaziquone (AZQ) were purchased from Sigma Chemical (St. Louis). The primary antibodies of p53, bcl-2, and cyclin B were all purchased from BD Transduction Lab. (BD Bioscience). The assay kit of caspase-3 enzyme activity PhiPhiLux G1D2 was purchased from OncoImmunin Inc. (MBL, Nagoya, Japan). The second antibody, affinity-purified goat anti-mouse IgG with peroxidase-conjugated, was purchased from Jackson Immuno Research Lab. (West Grove, PA). Chamber slide was purchased from NUNC (Roskilde, Denmark). All other chemicals were purchased from Merck (Darmstadt, Germany). AZ-1 compound was obtained from total synthesis by our laboratory, dissolved in dimethylsulfoxide before experimental use and aliquots were stored in −20 °C, with stability for several years.

4.1. The AZ-1 compound synthesized

The detailed synthesis method of the AZ-1 compound was described in our previous report.[27] Briefly, bisaziridinylnaphthoquinone I was synthesized as described previously and as summarized in Scheme 2.

4.2. The human cell lines cultured

OEC-M1 and KB cells (human oral epidermal adenocarcinoma) were cultured in the RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, and 25 mM Hepes. Skin fibroblast (SF) cells were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and MEM non-essential amino acids. The OEC-M1 cell line was isolated and purified from our local oral cancer patient. The cell culture medium for three cell lines all contained penicillin–streptomycin and fungizone. All cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Cell cultures were subcultured once or twice weekly using trypsin–EDTA to detach the cell from their culture flask. Numbers of cells were counted after trypsinization by a Neubauer hemocytometer (VWR, Scientific Corp., Philadelphia, PA).

4.3. Cytotoxicity determined by MTT assay for cell viability

The MTT assay was performed according to the method of Skehan et al., with minor modifications.[47] One day before drug application, cells were seeded in 96-well flat-bottomed microtiter plates (3000–5000 cells per well). Cells were incubated for 24 h for various dosages or various times with one dosage of AZ-1, applied as serial dilutions (100 μl per well) on various

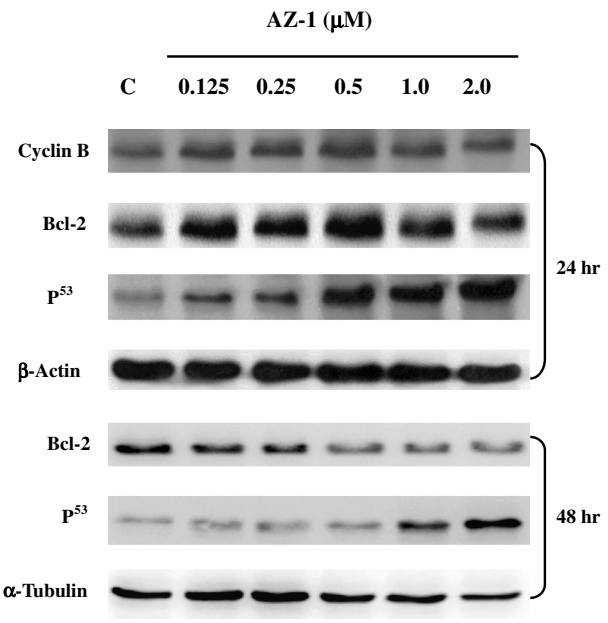


Figure 6. The expression of cell cycle and apoptotic proteins, including cyclin B, bcl-2, and p53 in OEC-M1 induced by AZ-1 for 24 or 48 h treated. Those protein expressions were assessed by Western blot method (Materials and methods). The OEC-M1 cells were treated with various concentrations of AZ-1 for 24 or 48 h. After treatment the cell lysate was prepared for SDS–PAGE separated and analyzed. Lane C is untreated control (only 0.1% DMSO added to OEC-M1 cells). The β-actin and α-tubulin were an internal control on OEC-M1 cells for 24 and 48 h, respectively.

concentrations. Twenty microliters of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The formazan product was dissolved by adding 100 μl DMSO to each well, and the plates were read at 550 nm. All measurements were performed in triplicate and each experiment was repeated at least three times. The LC_{50} was calculated from the 50% formazan formation compared with control without drugs addition.

4.4. Apoptotic bodies stained by Hoechst #33258

The cells were cultured in RPMI 1640 complete medium for OEC-M1 cell at chamber slide (1×10^4 cells/ml). Various concentrations of AZ-1 compound were added and incubated at 37 °C for 24 or 48 h. After incubation, the cultured medium was removed and the cells were fixed by acetic acid/methanol (1:3) solution for 10 min. In following step, the fixed solution was removed and cells were dried in air for another 10 min. The cells were stained by Hoechst 33258 stain solution (0.5 μg/ml in HBSS) at room temperature for 30 min. After the stain solution was removed, the cells were washed for three times by distilled water, then 1 drop of mounting solution (0.1 M citric acid/0.2 M di-sodium phosphate/glycerol = 1:1:2) was added before fixing coverslip. Apoptotic cells showed blue peripherally clumped or fragmented chromatin.

4.5. Apoptosis analysis by flowcytometry

The apoptotic nuclei of OEC-M1 cells induced by AZ-1 were also identified by the method of flowcytometry as described by Dive et al., with minor modification.[48] The OEC-M1 cells were treated with various concentrations of AZ-1 for 24 or 48 h, then cells were harvested and DNA was stained with propidium iodide (PI). The DNA content was measured by a flowcytometer (Becton–Dickinson FACScan).

4.6. Caspase-3 enzyme activity assay

The enzyme activity of caspase-3 on OEC-M1 cell induced by AZ-1 compound for 24 or 48 h was evaluated as the protease activity by the PhiPhiLux G_1D_2 kit. A substrate of PhiPhiLux G_1D_2, which can penetrate into the cell nucleus, is converted to fluorescent form when it is cleaved by protease activity of caspase-3.[49] Assessment was performed according to the manufacturer's recommendations. Briefly, the OEC-M1 cells were harvested after being treated with various concentrations of AZ-1 or 0.1% DMSO only without AZ-1 contained (control cell). The cells were gently washed by PBS buffer once, then 10 μl of the PhiPhiLux G_1D_2 was added to each treatment, mixed very gently, and incubated for 45 min at 37 °C in 5% CO_2. In the following step, the cells were precipitated by centrifugation, after centrifugation the cell pellet was washed twice by PBS buffer. Finally, the enzyme activity of caspase-3 in OEC-M1 cells was measured using the intensity of fluorescence of PhiphiLux G_1D_2 by flowcytometry (Becton–Dickinson, Mountain View, CA).

4.7. Western blot analysis

This method was used according to the method of Bacus et al. slightly modified.[50] Briefly, OEC-M1 cells were

Table 1. The expression of cyclin B, bcl-2, and p53 protein on OEC-M1 cell was induced by AZ-1 compound for 24 or 48 h treatment

Proteins	C	AZ-1 (μM)				
		0.125	0.25	0.5	1	2
24 h						
Cyclin B	1	1.08	1.13	1.25	1.18	1.15
Bcl-2	1	1.27	1.43	1.57	1.32	1.33
P53	1	1.72	1.85	2.52	2.55	2.80
48 h						
Bcl-2	1	0.86	0.74	0.65	0.56	0.57
P53	1	1.36	1.41	1.42	1.89	2.16

The data are expressed as the relative fold of control cell (C = 1-fold; control cell was treated by 0.1% DMSO only).

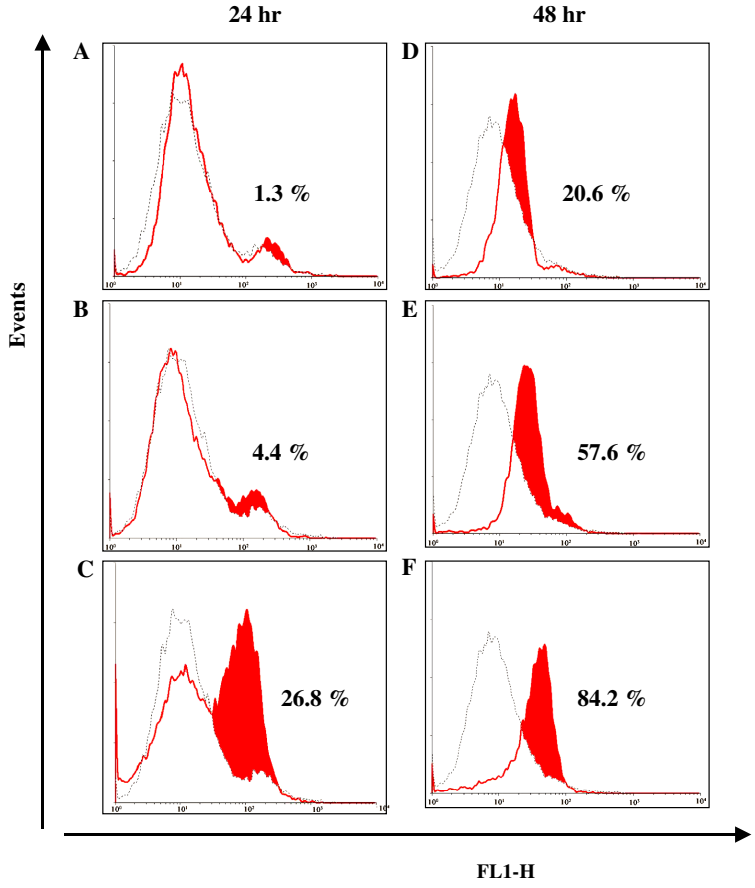
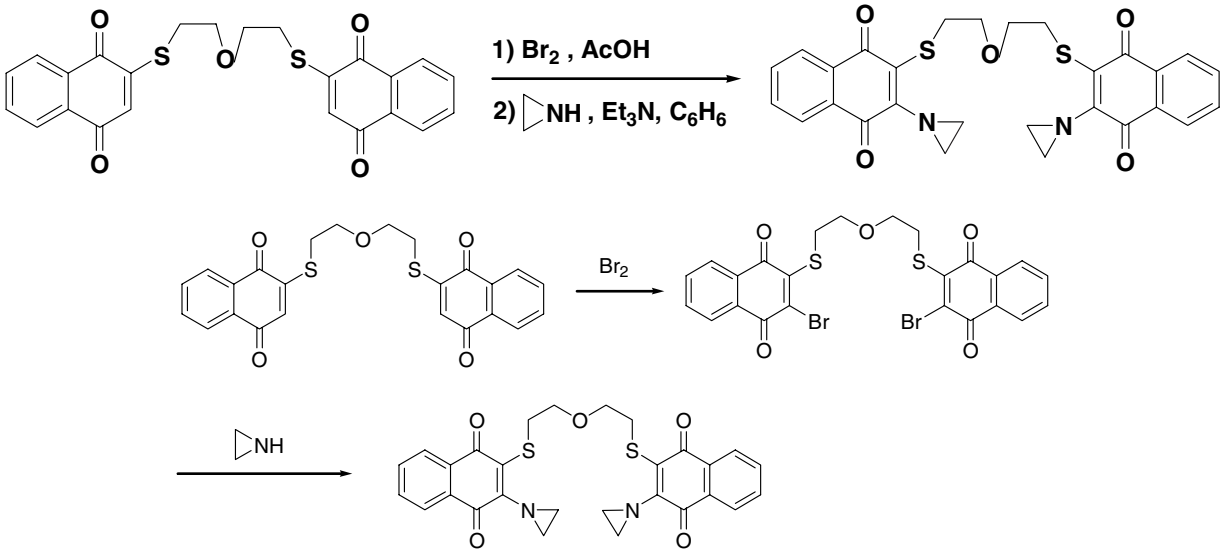


Figure 7. The enzyme activity of caspase-3 in OEC-M1 was induced by AZ-1 compound for 24 h (A–C) or 48 h (D–F) incubated. Cells were seeded for 18 h before the addition of AZ-1 with various concentrations (A and D) 0.5 μM; (B and E) 1 μM; and (C and F) 2 μM. The cell lysate was prepared for analysis by PhiPhiLux G_1D_2 assay kit according to the instructions of the manufacturer. The enzyme activity is shown as percent of shift area (peak portion with solid area) relative to 0.1% DMSO control cell (gray dot-line peak).



Scheme 2. The chemical synthesis of AZ-1.

collected from a 100 mm cultured dish after being challenged by various concentrations of the AZ-1 compound for 24 or 48 h. Cell pellets were spun down by centrifuge (1000$g \times 20$ min). Pellets were resuspended in cold buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride,

1 mM benzamidine, 30 mg/ml leupeptin, 5 mg/ml aprotinin, and 5 mg/ml pepstatin A; all from Sigma), incubated on ice for 5 min, and lysed by sonication. Cell lysate (25 μg) was separated by 12% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Amersham, United Kingdom). Blots were incubated with blocking buffer (11 mM Tris-base, pH 7.4, 154 mM NaCl, and 5% skim milk), washed by washing buffer (11 mM Tris-base, pH 7.4, 154 mM NaCl, and 0.1% Tween 20), and incubated with specific antibodies to probe specific proteins. The primary antibodies were monoclonal antibodies produced from mouse with anti-human cell activity (Imgenex Co., San Diego). The secondary antibody (Jackson Immun-Research Lab. Inc., PA) was conjugated with the enzyme (horseradish peroxidase) and diluted to appropriate concentration with blocking buffer. The dilution factors for the primary and secondary antibodies were various (dependent on different proteins) and 1:5000, respectively. The primary antibody of β-actin used was a mouse monoclonal antibody with the dilution factors 1:10,000 (Calbiochem, San Diego, CA). Immunodetection was carried out using the enhanced chemiluminescence (ECL; from NEN, Boston, MA) detection system. To quantify the amount of protein expression on OEC-M1 cells induced by AZ-1 compound was achieved by measuring the intensity of chemiluminescence of the second antibody concentration using the densitometer (BioRad Gel Doc 2000, software and analyzed using Gel Doc). The values in the relative protein expression quantifying in table represent the relative amount of proteins expression in respect to β-actin or α-tubulin expression. The β-actin and α-tubulin are the internal control of OEC-M1 cells for 24 and 48 h, respectively.

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