A Novel Bis-aziridinylnaphthoquinone with Anti-Solid Tumor Activity in which Induced Apoptosis is Associated with Altered Expression Of Bcl-2 Protein

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

*Aziridine-containing compounds have been of interest as anticancer agents since the late 1970s. The design, synthesis, and study of aziridinylnaphthoquinone analogues to obtain compounds with enhanced activity/toxicity profiles are an ongoing research effort in our group. A series of bis-aziridinylnaphthoquinone derivatives has been prepared, and the cytotoxic activities of these synthetic bis-aziridinylnaphthoquinone derivatives has been investigated. The synthetic derivatives displayed significant cytotoxicity against human carcinoma cell lines and weak cytotoxic activities against skin fibroblasts (SF). The bis-aziridinylnaphthoquinone **1c** was the most effective of the tested analogues at reducing the viability of Hep2 cells, with an LD_{50} value of 5.23 μM, and also exhibited weak cytotoxic activity against SF cells, with an LD_{50} value of 54.12 μM. The DNA alkylation and DNA interstrand cross-linking abilities of **1c** were also investigated. Bis-aziridinylnaphthoquinone **1c** was an effective agent for alkylation of DNA after chemical reduction in vitro, and its bifunctional alkylating moieties were able to cross-link DNA. We also report here our efforts to determine direct antitumor effects of **1c** on Hep2 cells. Growth arrest in Hep2 cells was preceded by early induction of G_2-M cell cycle arrest at 0.75 μM of **1c** after culture for 24 h, and was then followed by apoptosis after 60 h. This was associated with decreased expression of antiapoptotic bcl2 protein (by 78%) upon culture with 3.0 μM of **1c** after 60 h. Our results suggest that **1c** is a novel antitumor aziridinylnaphthoquinone with therapeutic potential against solid tumors.*

Introduction

The quinone structure is common in numerous natural products associated with antitumor, antibacterial, antimalarial, and antifungal activities.[1,2] In many cases the biological activity of the quinone is attributed to the ability to accept electrons to form the corresponding radical anion or dianion species. A quinone moiety substituted with aziridine had been shown to be a potent alkylating agent as a result of bioreduction by the one-electron reducing enzymes (e.g., NADPH cytochrome P450 reductase, cytochrome b5 reductase) or by a two-electron reducing enzyme ((NADP)H oxidoreductase, NQO1) to form the corresponding aziridinyl hydroquinone.[3–5] 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 at physiological pH values. In the case of a bis-aziridinyl-substituted quinone, this highly cytotoxic bifunctional alkylating agent can cross-link DNA in cells, which results in induction complex cellular mechanism leading to cell death by apoptosis or necrosis.[6]

Hypoxia is a common feature of human tumors.[7,8] Because of their unrestrained growth, tumor cells are forced away from vessels beyond the effective diffusion distance of oxygen in respiring tissue, thereby becoming hypoxic.[9,10] An important consequence of tumor hypoxia is that the effectiveness of anticancer drugs would be expected to fall off as a function of distance from blood vessels. Tumor hypoxia would thus be expected to be an important factor leading to resistance to chemotherapy (because of lower proliferation and lower drug concentrations in the hypoxic cells).[11] Development of effective cancer therapies to combat the hypoxic fraction of solid tumors has always been a challenge.[12] The aziridine-substituted benzoquinones mitomycin C, porfiromycin, and E09 are three principal aziridinylquinone class hypoxia-specific cytotoxins that are being developed for clinical use. These agents are made up of aziridinyl moieties on quinone structures, and on reductive metabolism they convert into bifunctional alkylating species capable of cross-linking DNA in the major groove, interacting predominantly on guanine-N7. These agents probably produce their major cytotoxic activities through the formation of DNA cross-links.[12–15]

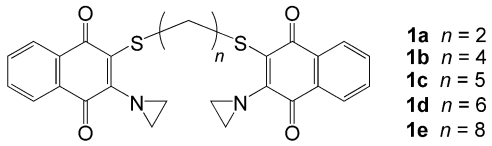
[a] *Prof. Y.-L. Lin*
College of Medicine, Fu-Jen Catholic University
510 Chung Cheng Road, Hsin-chuang
Taipei Hsien 24205, Taiwan (R.O.C.)
Fax: (+886) 33-979-280
E-mail: med0018@mails.fju.edu.tw

[b] *Prof. S.-T. Huang, H.-D. Tsai, Prof. H.-S. Kuo, Y.-P. Yang, Y.-C. Peng*
College of Medicine, Department of Biochemistry, Taipei Medical University
250 Wu Hsing Street, Taipei, Taiwan 110 (R.O.C)

Supporting information for this article is available on the WWW under http://www.chembiochem.com or from the author.

 DOI: 10.1002/cbic.200300825

The design and synthesis of bis-aziridinylnaphthoquinone analogues, to obtain compounds with enhanced activity/toxicity profiles, is an ongoing research effort in our group.[16–18] Examination of the literature showed that the 1,4-naphthoquinone 2,2′-(ethylenedithio)bis[3-(1-aziridinyl)] (**1a**) had been reported as cytotoxic towards Yoshida's and Ehrlich's sarcomas, but there was little molecular and cellular pharmacological information concerning its direct effect on solid tumors.[19] Syntheses of **1a** and its analogues are compelling, due to such cytotoxic activity and their status as potential new leads in antitumor drug discovery efforts toward hypoxic fraction of solid tumors. We therefore synthesized a series of analogues of **1a** and initiated a study to determine the relevance of direct antitumor activities of our synthetic bis-aziridinylnaphthoquinone analogues.



1a $n = 2$
1b $n = 4$
1c $n = 5$
1d $n = 6$
1e $n = 8$

In this paper we report the synthesis of bis-aziridinylnaphthoquinone **1b–e** and investigation of the cytotoxic activities of **1a–e**. We showed that **1c** was an effective agent for induction of cell cycle arrest in Hep2 cells. This cytotoxic activity was associated with alterations in the balance of antiapoptotic bcl-2 proteins. Several biochemical techniques, DNA content analysis, apoptotic nuclei detection, and investigation on the expression of antiapoptotic bcl-2 protein were used to characterize the cellular pharmacological events induced by **1c** on Hep2 cells. We believe that the synthetic route disclosed here provides an efficient route for the preparation of **1b–e**, and that the cytotoxic mechanism study holds promise for the development of new-generation hypoxia-specific cytotoxins.

Results and Discussion

Synthesis of 1b–e

A series of bis-aziridinylnaphthoquinones **1b–e** was prepared from naphthoquinone (**2**) by the route outlined in Scheme 1. Nucleophilic 1,4-addition-elimination of the quinone moiety of 2,3-dichloronaphthoquinone **2** with dithiols **3b–e** under basic condition gave **4b–e** in moderate yields (55–70%). Treatment of **4b–e** with excess aziridine yielded the bis-aziridinylnaphthoquinones **1b–e**. These two-step syntheses gave **1b–e** in overall yields of 20 to 40%.

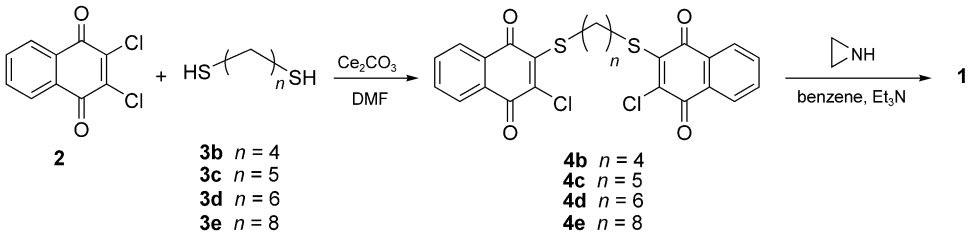
Since most of the aziridinylquinones currently in use have closely adjacent bisfunctional alkylating sites, alkylation on DNA by one of the alkylating sites might hinder the activity of the second, due to steric effects. The bis-aziridinylnaphthoquinones **1a–e** designed here each have two independent DNA-alkylating aziridinylnaphthoquinone separated by a linear spacer, and so alkylation on DNA by the first aziridinylnaphthoquinone should not interfere with the activity of the second aziridinylnaphthoquinone toward alkylation. One of the aims of this study is to develop an efficient synthetic route through which to construct series of bis-aziridine-substituted naphthoquinone derivatives for evolution of cytotoxic activities. The efficient two-step synthesis developed here allows speedy creation of a series of bis-aziridinylnaphthoquinone analogues for cytotoxic activity evaluation.

Cytotoxic activity evaluation

The cytotoxic activities of **1a–e** against three human epithelium solid carcinoma cell lines—larynx Hep2, oral CT-5′, and breast BC-M1 cells—were investigated; the normal skin fibroblast (SF) was used as a control, and the LD_{50} values are listed in Table 1. The bis-aziridinylnaphthoquinones **1a–e** were effective cytotoxic agents against Hep2 cells, with LD_{50} values ranging from 4 to 12 μM, but they were less effective at reducing the viability of CT-5′ and BC-M1 cells, with LD_{50} values ranging from 9 to 25 μM. SF cells were also less sensitive to these analogues, except for **1a** and **1b**. There seem to be a correlation between the cytotoxic activities and the linker distance between the aziridinylnaphthoquinone moieties. As the number of the bridge carbons between the aziridinylnaphthoquinone moieties increased from **1a** to **1e**, the cytotoxic activities of **1a** to **1e** against Hep2 and CT5′ cells diminished, but this trend was reversed in the case of BC-M1 cells. Initial characterization indicated that **1c** was the most effective cytotoxin of the tested analogues against the tumor cells lines. Bis-aziridinylnaphtho-

Table 1. *The cytotoxicities of* ***1a–e*** *(LD_{50}, μM) by MTT assay in three cancer cell lines and normal skin fibroblast (SF).*

Compound	Hep2	CT-5′	BC-M1	SF
1a	4.05	8.52	24.87	7.54
1b	5.55	10.02	23.14	10.50
1c	5.23	14.87	12.42	54.12
1d	10.97	19.82	11.17	35.44
1e	11.52	17.84	8.92	30.12



Scheme 1. *The chemical synthesis of* ***1b–e****.*

quinone **1c** exhibited potent cytotoxic activity against Hep2 cells with LD_{50} values of 5.23 μM, while **1c** was the weakest cytotoxic agent against SF cells, with LD_{50} values of 54.12 μM. Further characterization indicated that **1c** was effective at inhibiting the growth of Hep2 in vitro, whilst treatment of SF cells with **1c** had little effect on cell viability (Figure 1).

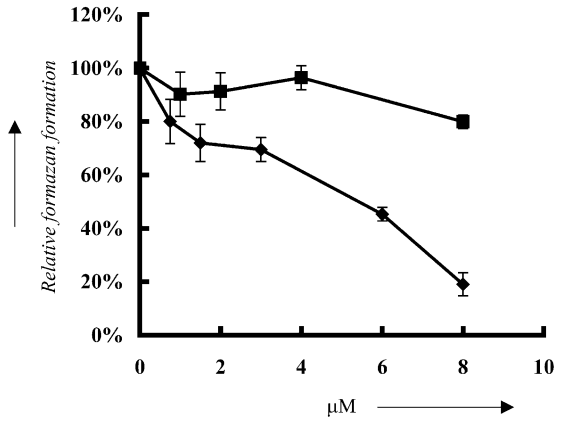


Figure 1. *Bis-aziridinylnaphthoquinone **1c** inhibited the proliferation of cell lines (■, SF; ◆, Hep2). Hep2 and SF cells were seeded for 24 h before the addition of **1c**. MTT assay was used to assess cell viability after an additional 24 h of culture. Data are from quadruplicate wells and are representative of three separated experiments.*

The bioreductive drugs AZQ, mitomycin C, and E09 have been developed to exploit the oxygen deficiency in hypoxic fractions of solid tumors on the premise that hypoxic cells should show a greater propensity than well oxygenated cells for reductive metabolism.[12–15] Our previous study on the different series of bis-aziridinylnaphthoquinone compounds found that they exhibit more potent responses toward the solid tumors than the circulation tumors,[18] and these results are supported by other reports of differences in reductive metabolism between the solid tumors and the circulation tumors.[21] Considering the importance of all the cellular reductases (e.g., NADPH cytochrome P450 reductase, cytochrome b5 reductase, (NADP)H oxidoreductase, NQO1) in response to the whole cellular reductive metabolism, these reductases are probably involved in bioactivation of **1c**. The results from this study suggest that **1c** is a novel class of bis-aziridinylnaphthoquinone cytotoxin against Hep2 cells.

DNA alkylation and interstrand cross-linking

The cytotoxic effects induced by many known aziridinylquinones are attributable to their ability to alkylate DNA. The DNA alkylation and interstrand cross-linking capabilities of the bis-aziridinylnaphthoquinone **1c** were examined. To detect whether **1c** alkylated DNA, piperidine-catalyzed shearing of DNA was used as a measure of DNA alkylation, and the results are shown in Figure 2a. Plasmid DNA was incubated with various concentrations of **1c** or 200 μM of AZQ (2,5-diaziridinyl-3,6-bis-(carboethoxyamino)-1,4-benzoquinone) as positive control, all in the presence of 2 mM of DTT. The DNA was then treated with piperidine, a procedure that uniformly induces strand breakage at sites of base alkylation, resulting in the disappearance of the distinct plasmid band. As shown in Figure 2a, the DNA integrity started to fragment after incubation as low as 2 μM of **1c** (lane 5), but the presence of up to 200 μM of **1c** in the absence of DTT did not induce any change in the integrity of the plasmid DNA after piperidine treatment (data not shown), whilst the DNA integrity was lost completely after incubation with AZQ (lane 6). These results suggested that significant DNA alkylation is induced by bis-aziridinylnaphthoquinone **1c** under reduction conditions.

DNA interstrand cross-linking by **1c** was explored by use of a sensitive agarose gel-base assay, and a cross-link gel is shown in Figure 2b. Plasmid DNA was incubated with various concentrations of **1c** or 200 μM of AZQ as a positive control, all in the presence of 2 mM of DTT. The DNA sample was heated to denature it and was then chilled immediately in ice-water in order to prevent DNA renaturing. Plasmid DNA with cross-linking agents would resist denaturing, resulting in less mobility on the agarose gel. As shown in Figure 2b, the double-strand DNA (DS) band intensity became more intense as the concentration of **1c** was increased (lanes 6 to 3). Plasmid DNA incubated with AZQ was totally resistant to denaturization under these harsh conditions (lane 7). This result thus suggested that bis-aziridinylnaphthoquinone **1c** could cross-link DNA, but was less efficient than AZQ.

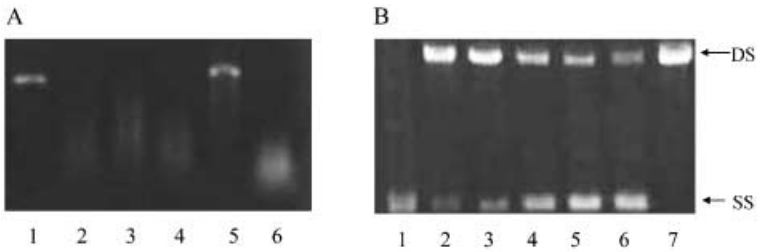


Figure 2. *A) DNA alkylation by **1c**, determined by the agarose gel assay. Plasmid DNA pBR322 was treated with various concentrations of **1c** (lanes 2 to 5: 200, 100, 50, and 2 mM) or 200 mM of AZQ (lane 6) in 2 mM of DTT for 48 h at 37°C and then incubated with 1 M piperidine for 15 min. Lane 1 is pBR322 DNA in 2 mM DTT control. The data shown are representative of four separate experiments. B) DNA inter-stand cross-linking by **1c** determined by the agarose gel assay. Plasmid DNA was treated with various concentrations of **1c** (lanes 3 to 6: 200, 100, 50, and 2 mM) or 200 mM of AZQ (lane 7) in 2 mM of DTT for 2 h at 37°C. Lanes 1 and 2 are denatured and nondenatured controls, respectively. DS is double-strand DNA, and SS is single-strand DNA. The data shown are representative of four separate experiments.*

AZQ and bis-aziridinylnaphthoquinone **1c** are both symmetrical molecules, each possessing two reactive moieties, with the potential to damage DNA (Figure 2). Under reductive conditions, they can alkylate plasmid DNA to form a DNA cross-linking structure. The result shown in Figure 2b showed that **1c** could cross-link DNA, but was less effective than AZQ, since

plasmid DNA was unable to resist denaturization totally upon treatment with 200 μM of **1c** (Figure 2b, lane 3). The insufficient interstrand DNA cross-linking caused by **1c** might due to its being able to form intra-strand DNA cross-linking. The chemical structure of AZQ involves a benzoquinone substituted with two aziridines at the 2- and 5-positions. Various studies have proposed an interstrand DNA-crossing structure model for AZQ and its analogues.[22,23] The corresponding hydroquinone moiety should fit into the major groove of DNA, and the two diagonal aziridinyl groups should alkylate two different strands of DNA, predominately on guanine-N7. The chemical structure of **1c** is much larger than AZQ, and so it is probably unable to fit properly into a DNA major groove for interstrand DNA cross-linking as in the proposed AZQ–DNA interstrand crossing structure model. Bis-aziridinylnaphthoquinone **1c** is a bifunctional alkylating agent with a linear spacer; the two resulting hydronaphthoquinone moieties might fit into two different major grooves of DNA, and the two aziridinyl moieties might alkylate on the same strand of DNA to form intra-strand DNA cross-linking. In this report, we have established that **1c** can alkylate DNA and form interstrand and possibly intra-strand DNA cross-linking.

Induction of G_2-M cell cycle arrest by bis-aziridinylnaphthoquinone 1c and tumor cell apoptosis is associated with altered expression of anti-apoptotic bcl-2

Analysis of DNA content was used to determine whether cell arrest was induced by bis-aziridinylnaphthoquinone **1c**. Cell cycle data indicated that Hep2 cells had a significant population of cells arrested in G_2-M after treatment with **1c** over 24 h compared with DMSO alone (Figure 3). The peak area under G_2-M phase started to ascend at concentrations of **1c** as low as 0.75 μM. We then investigated whether cell cycle arrest was apparent before the apoptosis of **1c**-treated cells. Figure 4 shows that Hep2 cells treated with **1c** for 60 h exhibited large increases in apoptotic cells identified as subcellular populations with decreased DNA, and Hep2 cells treated with 3.0 μM of **1c** exhibited the largest apoptotic area, with 37.8%. This is similar to the apoptotic bodies observed with the Hoechst stain (Figure 5). The Hoechst staining method was used to identify the apoptotic nuclei in Hep2 cells. Apoptotic cells containing apoptotic bodies showed blue peripherally clumped or fragmented chromatin, as indicated by arrows in Figure 5. From the visual observation on the Hoechst staining results in Figure 5, the Hep2 cells treated with 3.0 μM had the highest number of apoptotic bodies.

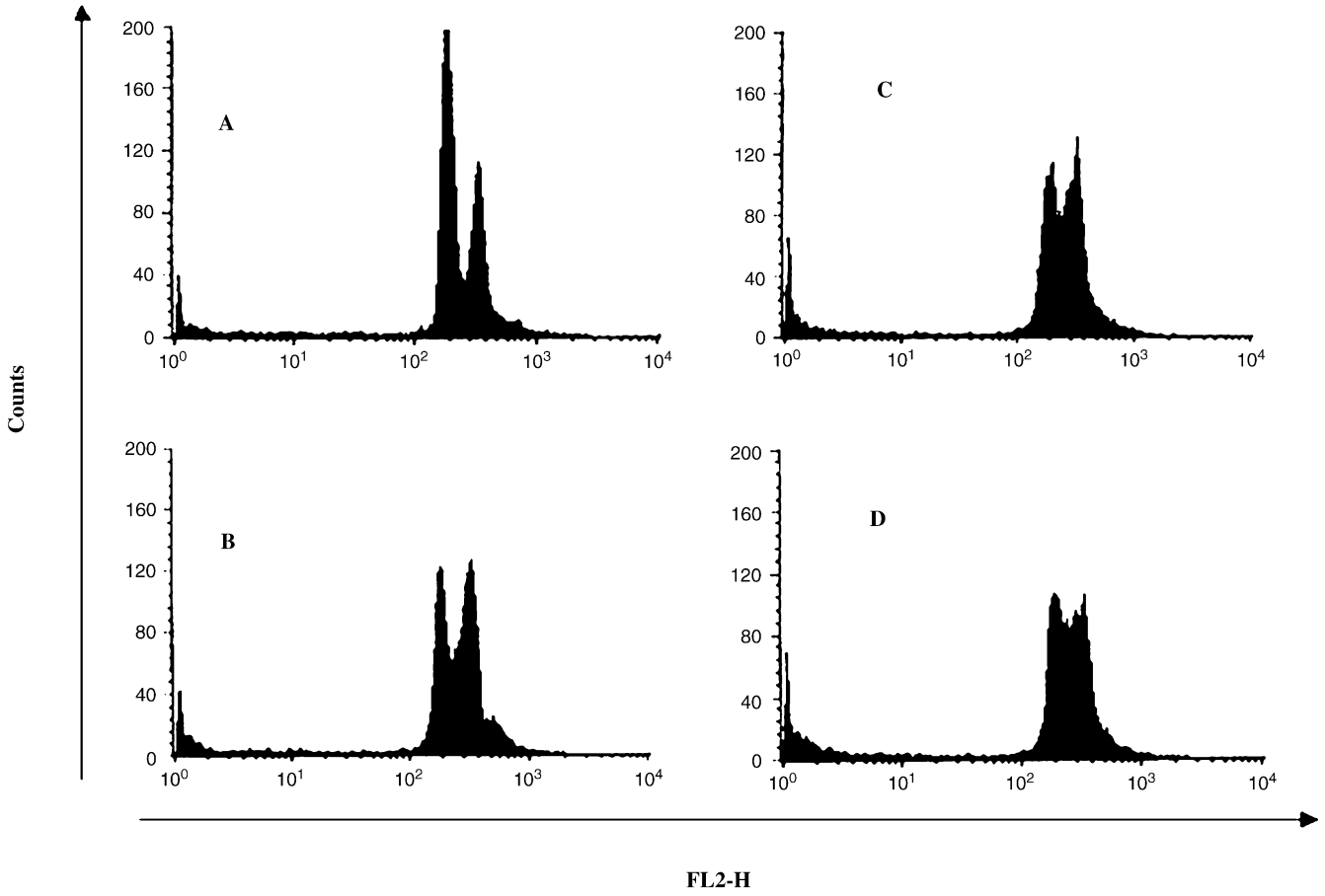


Figure 3. *G_2-M cell cycle arrest is induced by **1c**. Hep2 cells were synchronized and treated in 24 h culture with: A) DMSO control, B) 0.75 μM, C) 1.50 μM, or D) 3.0 μM of **1c** prior to PI staining and analysis of DNA content. Cell cycle arrest in G_2-M is apparent from the large populations of cells with increased DNA content. The data shown are representative of three separate experiments.*

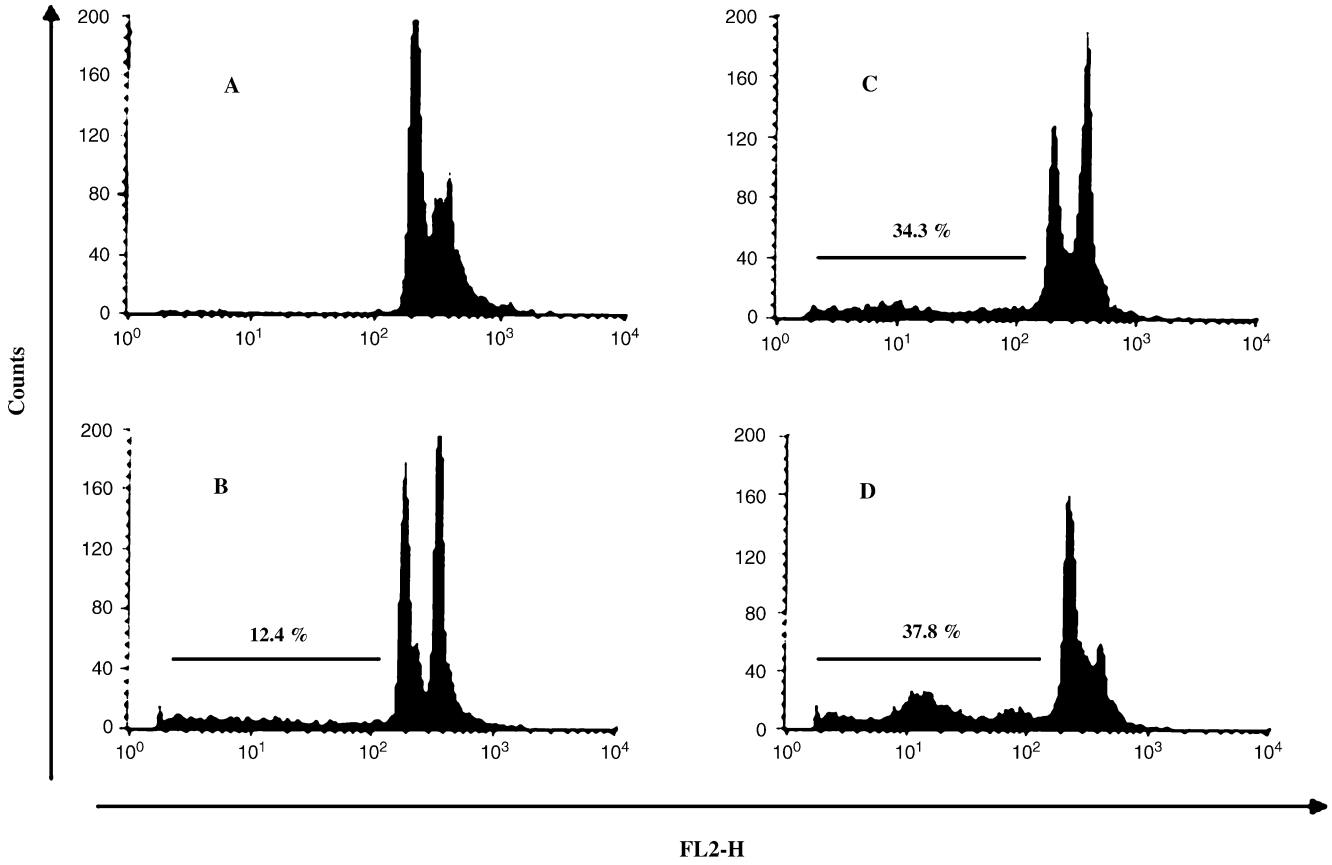


Figure 4. *Apoptosis is induced by* ***1c****. Hep2 cells were treated in 60 h culture with: A) DMSO control, B) 0.75 μM, C) 1.50 μM, or D) 3.0 μM of* ***1c*** *prior to PI staining and analysis of DNA content. Apoptosis is apparent by the large population of cells with increased DNA content in sub G_1. The data shown are representative of three separate experiments.*

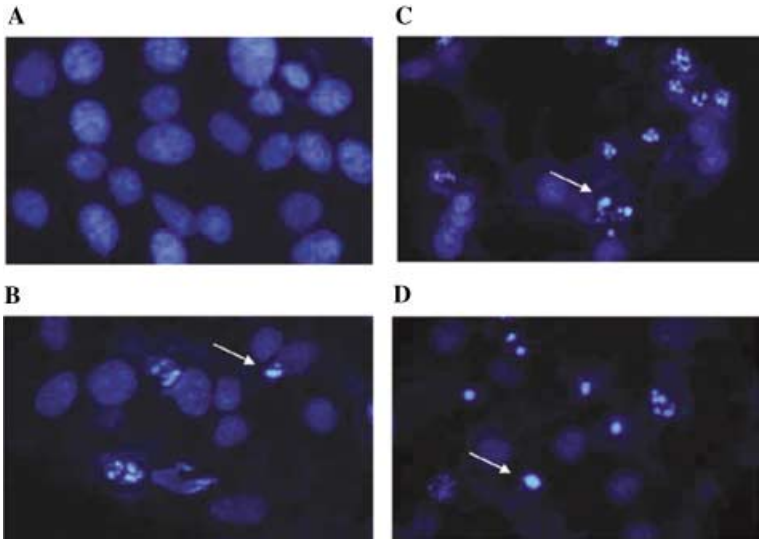
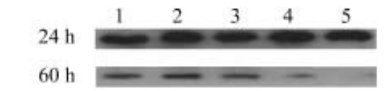


Figure 5. *Apoptosis is induced by* ***1c****. Hep2 cells were treated in 60 h culture with: A) DMSO control, B) 0.75 μM, C) 1.50 μM, or D) 3.0 μM of* ***1c*** *prior to Hoechst staining and analysis of DNA nuclei. Apoptotic cells showed blue peripherally clumped or fragment chromatin as indicated by arrows. The data shown are representative of three separate experiments.*



Time	1	2	3	4	5
24 h	1	1.05	1.12	1.31	1.24
60 h	1	0.98	0.83	0.52	0.22

Figure 6. *Bis-aziridinylnaphthoquinone* ***1c*** *alters the expression of antiapoptotic bcl-2 proteins. The expression of bcl-2 proteins was assessed by immunoblotting, and a relative bcl-2 protein expression quantization table is provided below. The Hep2 cells were treated with various concentrations of* ***1c*** *(lanes 2 to 5: 0.38, 0.75, 1.5, and 3.0 mM) for 24 h or 60 h, and cell lysate was prepared for analysis. Lane 1 is the untreated control. The immunoblots shown have been equilibrated for protein loading; the data shown are representative of three separate experiments. A relative bcl-2 protein expression-quantifying table is shown below the immunoblot.*

We next set out to determine whether the induction of Hep2 cells apoptosis by **1c** was associated with expression of anti-apoptotic bcl-2 protein. We found that **1c** induced major changes in Hep2 cells' expression of bcl-2 protein (Figure 6). From the Western Blot results and the values in the relative bcl-2 protein expression quantifying table (shown below the immunoblots), the expression of bcl-2 protein was unaffected by various concentrations of **1c** in Hep2 cells after 24 h, and the relative bcl-2 protein expression was comparable with the control (lane 1). In

contrast, the bcl-2 protein expression was strongly reduced, by 48% and 78%, with Hep2 cells cultured with 1.5 μM and 3 μM of **1c**, respectively, after 60 h (lanes 4 and 5).

Normally, the processes of cell reproduction taken place through an ordered process, generally known as a cell cycle. The coordination of these is divided into four phases: S, G_2, M, and G1. The chromosomal replication and segregation occur only during the S and M phases. It is typically the G_1 and G_2 phases, when the cells are responding to the proliferative and anti-proliferative signals, that determine where the cell cycle proceeds or comes to a halt. Cell cycle checkpoints relate to genetic damage phenomena, causing the cell to pause in G_1 or G_2, allowing time for repair enzymes to correct the lesion.[24] Some anticancer drugs such as cisplatin induce apoptosis in neoplastic cells, which suggests that the normal occurrence of condensed chromatin during the G_2 phase of a cell cycle may increase a cell's susceptibility to damage during the G_2 phase.[25,26] The capability for DNA alkylation shown by **1c** suggests that the cytotoxic mechanism of **1c** does involve substantial nuclear DNA damage. The nuclear DNA damage by **1c** may be closely related to induction G_2-M cell cycle arrest properties. The Hep2 cells proliferation was inhibited by the early induction of G_2-M cell cycle arrest within 24 h of treatment with **1c**. The prolonged effect of the G_2-M cell cycle arrest on Hep2 cell induced by **1c** results in the cells' being steered into the apoptotic pathway. We observed the appearance of apoptotic bodies after 60 h of treatment with **1c**. The DNA content analysis and reduction in the expression of the bcl-2 protein also support the speculation that the Hep2 cells were forced into an apoptotic pathway with prolonged treatment with **1c**.

Conclusion

A series of bis-aziridinylnaphthoquinone prodrugs has been prepared as potential anticancer agents, and we have identified a novel bis-aziridinylnaphthoquinone, **1c**, with selective cytotoxic activity against Hep2 cells and low cytotoxicity when cultured with non-neoplastic human adherent fibroblast. Furthermore, **1c** is an effective DNA alkylation agent with the capability for interstrand and possible intrastrand DNA cross-linking in vitro. The cytotoxic effect induced by **1c** on Hep2 cells may correlate with early induction G_2-M cell cycle arrest properties. The prolonged effect on the G_2-M cell cycle arrest in Hep2 cells induced by **1c** results in the cells' being steered into the apoptotic pathway with inhibition of antiapoptotic bcl-2 expression. Future detailed molecular pharmacological studies of bis-aziridinylnaphthoquinone **1c** are underway. We believe that the efficient synthetic approach disclosed here has the potential for quick output of a series of bis-aziridinylnaphthoquinones for evaluation and for cytotoxic mechanism studies, and that the cytotoxic mechanism study holds promise for the development of a new generation of potent antitumor agents.

Experimental Section

Synthesis of 1b–e: See the Supporting Information.

Cell culture: The cell lines CT-5′ (human oral epidermal carcinoma), Hep2 (human larynx epidermal carcinoma), and BC-M1 (human breast adenocarcinoma) were cultured in the RPMI 1640 medium with 10% fetal bovine serum (FBS), L-glutamine (2 mM), and Hepes (25 mM). Skin fibroblast (SF) was cultured in DMEM medium with 10% fetal bovine serum (FBS), L-glutamine (2 mM), and MEM non-essential amino acid. The cell culture media for four cell lines all contained pencillin-streptomycine and fungizone. All the media and supplements were purchased from Gibico Laboratories (Grand Island, NY). All cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Numbers of cells were counted after trypsinization with a Neubauer hemocytometer (VWR, Scientific Corp. Philadelphia, PA.)

Cytotoxicity assay (MTT assay and trypan blue assay): The MTT assay was performed by the method of Skehan et al.[27] One day before drug application, cells were seeded in 96-well, flat-bottomed microtiter plates (3000–5000 cells per well). Epithelium carcinoma cells were incubated for 24 h with drugs, applied as serial 1:2 dilutions (100 μL per well) ranging from 20 μM down to 0.2 μM. MTT (20 μL, 5 mg mL^{-1}) was added to each well, and incubation was carried out for 4 h at 37°C. The formazan product was dissolved by addition of dimethyl sulfoxide (DMSO, 100 μL) 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 LD_{50} value was calculated from the 50% formazan formation, in relation to a without drugs addition.

DNA alkylation: Plasmid pBR322 DNA (1 μg) was incubated with various concentrations of **1c**, or AZQ (200 μM, as the positive control), in the presence of DTT (dithiothreitol, 2 mM) in Tris-EDTA buffer (50 μL, 10 mM Tris, 1 mM EDTA, pH 7.4) for 48 h at 37°C. Treated DNA was then incubated for 15 min with piperidine at a final concentration of 1 M, precipitated, and electrophoresed through a 1% agarose gel.[28]

DNA interstrand cross-linking: This procedure is a slight modification of one previous published by Hartley et al.[29] Plasmid DNA was incubated with various concentrations of **1c** in triethanolamine (25 mM), EDTA (1 mM, pH 4), and DTT (2 mM) at 37°C for 2 h. The reactions were terminated by addition of an equal volume of sodium acetate (0.6 M), EDTA (20 mM), and tRNA (100 μg mL^{-1}), and the DNA was immediately precipitated by the addition of 3 volumes of 95% ethanol. After centrifugation and removal of supernatant, the DNA pellet was dried by lyophilization. Samples were dissolved in DMSO (10 mL, 30%), EDTA (1 mM), bromophenol blue (0.04%), and xylene cylanol (0.04%), heated at 90°C for 2 min, and chilled immediately in an ice-water bath prior to gel loading. Electrophoresis was performed on 20 cm 0.8% submerged horizontal agarose gel at 40 V for 16 h with Tris-acetate running buffer. A final concentration of 1 μg mL^{-1} of plasmid was loaded onto each lane of the gel.

Cell cycle analysis: Cultured Hep2 cells were first subjected to serum starvation without FBS overnight to synchronize cell phase, and the cells were then treated with various concentrations of **1c** for 24 h. The cells were harvested, and the cell DNA was stained with hypotonic staining buffer (0.1% sodium citrate, 0.3% triton X-100, 0.01% propidium iodide (PI), and 0.01% ribonuclease A) for 15 min on ice, in the dark. The statistics analysis of DNA content in sub-G_1 was analyzed by two parameters, cell counts versus FL-2. Ten thousand gated events per sample were acquired on a Becton

Dickinson FACSan by use of CellQuest software and analyzed with the aid of EXPO 32 and MultiCycle software (Beckman–Coulter, High Wycombe, United Kingdom). The DNA content was measured by a Becton Dickinson FACScan using CellQuest software and analyzed with the aid of EXPO 32 and MultiCycle software.

Apoptosis analysis: The apoptotic nuclei of Hep2 cells induced by **1c** were identified by two methods: flow cytometry analysis and Hoechst #33258 staining as described by Dive et al., with minor modification.[30] The Hep2 cells were treated with various concentrations of **1c** for 60 h. The cells were harvested, and DNA was stained with PI. The DNA content was measured by flow cytometry (Becton Dickinson FACScan). In the Hoechst staining, cells treated with **1c** were also cytospun onto slides for morphological analysis, fixed with acetic acid/methanol 1:3, air-dried, and stained with Hoechst solution (50 $\mu g\,mL^{-1}$ final concentration). Morphology and effects of **1c** on cell organelles were observed by fluorescence microscopy. Cells were considered non-apoptotic when nuclei were morphologically normal with homogeneously distributed blue-stained chromatin. Apoptotic cells showed blue peripherally clumped or fragmented chromatin.

Western Blot analysis of bcl-2 protein: This method was slightly modified from that described by Bacus et al.[31] Briefly, cells were collected from 100 mm culture dishes after having been challenged by various concentrations of compound **1c** for 24 and 60 h. Cell pellets were spun down by centrifuge (1000 *g*, 20 min). Pellets were resuspended in cold HEPES buffer (10 mM, pH 7.9; $MgCl_2$ (1.5 mM), KCl (10 mM), DTT (0.5 mM), phenylmethylsulfonyl floride (0.5 mM), benzamidine (1 mM), leupeptin (30 $mg\,mL^{-1}$), aprotinin (5 $mg\,mL^{-1}$), and pepstatin A (5 $mg\,mL^{-1}$); all from Sigma) and incubated on ice for 5 min. Cells were lyzed by sonication. Cell lysate (25 μg) was separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham, Amersham, United Kingdom). Blots were incubated with blocking buffer (Tris-vase 11 mM, pH 7.4; NaCl (154 mM), skim milk (5%)), washed by washing buffer (Tris-vase 11 mM, pH 7.4, NaCl (154 mM), Tween-20 (0.1%)), and incubated with specific antibody to probe specific protein bcl-2. The primary bcl-2 antibody was from mouse anti-human monoclonal antibody (from Imgenex Co. San Diego). The secondary antibody (from Jackson ImmunoResearch Lab. Inc., PA) was conjugated with horseradish peroxidase at appropriate dilution by blocking buffer. The dilution factors for the primary and secondary antibodies were 1:2000 and 1:5000, respectively. The primary glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody used was a mouse monoclonal antibody to GAPDH with dilution factors 1:2000 (Biogenesis, England, UK). Immunodetection was carried out with an enhanced chemiluminescence detection system (ECL; from NEN, Boston, MA). Quantification of the amount of bcl-2 protein expression was achieved by measurement of the intensity of chemiluminescence of the second antibody with a densitometer (BioRad Gel Doc 2000, software and analysis using Gel Doc). The values in the relative bcl-2 protein expression-quantifying table represent the relative amount of bcl-2 expression in respect to GAPDH expression, divided by its control (lane 1).

Acknowledgements

This work was supported by the National Science Council (NSC 91-2113M-038-002 and NSC 91-2113M-030-011).

Keywords: antitumour agents · apoptosis · DNA damage · quinones

[1] P. J. O'Brien, *Chem. Biol. Interact.* **1991**, *80*, 1–41.
[2] A. C. Sartorelli, W. F. Hodnick, M. F. Belcourt, M. Tomasz, B. Haffty, J. J. Fisher, S. Rockwell, *Oncol. Res.* **1994**, *6*, 501–508.
[3] L. Szmigiero, K. W. Kohn, *Cancer Res.* **1984**, *44*, 4453–4457.
[4] D. Siegel, N. W. Gibson, P. C. Preush, D. Ross, *Cancer Res.* **1990**, *50*, 7293–7300.
[5] A. M. DiFrancesco, S. P. Mayalarp, S. Kim, J. Butler, M. Lee, *Anticancer Drugs* **2003**, 601–615.
[6] D. Ross, H. D. Beall, D. Siegel, R. D. Traver, D. L. Gustafson, *Br. J. Cancer* **1996**, *74*, S1–8.
[7] J. E. Moulder, S. Rockwell, *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 695–712.
[8] P. W. Vaupel, M. Hockel *in Tumor Oxygenation* (Eds.: P. W. Vaupel, D. K. Kelleher, M. Gunderoth), Gustav Fischer, Stuttgart, **1995**, pp. 219–232.
[9] R. H. Thomlinson, L. H. Gray, *Br. J. Cancer* **1955**, *9*, 539–549.
[10] L. H. Gray, A. D. Conger, M. Ebert, S. Hornsey, O. C. Scott, *Br. J. Radiol.* **1953**, *26*, 638–648.
[11] R. E. Durand, *In Vivo* (Athens) **1994**, *8*, 691–702.
[12] J. M. Brown, A. J. García, *Cancer. Res.* **1998**, *58*, 1408–1416.
[13] S. Rockwell, A. C. Sartorelli, M. Tomasz, K. A. Kennedy, *Cancer Metastasis Rev.* **1993**, *12*, 165–176.
[14] J. B. Weissberg, Y. H. Son, R. J. Papac, C. Sasaki, D. B. Fischer, R. Lawrence, S. Rockwell, A. C. Sartorelli, J. J. Fischer, *Int. J. Radiat. Oncol. Biol. Phys.* **1989**, *17*, 3–9.
[15] A. M. Rauth, R. S. Marshall, B. L. Kuehl, *Cancer Metastasis Rev.* **1993**, *12*, 153–164.
[16] E. E. Chang, H. H. Cheng, J. E. Kai, H. S. Kuo, *Chin. Pharm. J.* **1995**, *47*, 531–538.
[17] S. T. Huang, H. S. Kuo, C. L. Hsiao, L. Y. Lin, *Bioorg. Med. Chem.* **2002**, *10*, 1947–1952.
[18] S. T. Huang, H. S. Kuo, C. M. Lin, H. D. Tsai, Y. C. Peng, C. T. Chen, Y. L. Lin, *Oncology Res.* **2003**, *11*, 199–204.
[19] F. Bayer, *Chem. Abst.* **1958**, p20195f.
[20] J. M. Brown, A. J. Giaccia, *Cancer Res.* **1998**, *58*, 1408–1416.
[21] R. C. Gupta, A. Garg, K. Early, S. C. Agarwal, G. R. Lambert, S. Nesnow, *Cancer Res.* **1991**, *51*, 5198–5204.
[22] R. H. J. Hargreaves, C. C. O'Hare, J. A. Hartley, D. Ross, J. Butler, *J. Med. Chem.* **1999**, *42*, 2245–2250.
[23] J. Peter, J. Loud, E. Dimond, J. Jenkins, *Cancer Nurs.* **2001**, *24*, 446–461.
[24] A. Eastman, *Cancer Cells* **1990**, *2*, 275–280.
[25] L. F. Gibson, J. Fortney, G. Magro, S. G. Ericson, J. P. Lynch, K. S. Landreth, *Breast Cancer Res. Treat.* **1999**, 107–117.
[26] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.
[27] W. B. Mattes, J. A. Hartly, K. W. Kohn, *Biochim. Biophys. Acta* **1986**, *868*, 71–76.
[28] J. A. Hartley, M, Berardini, R. L. Souhami, *Anal. Biochem.* **1991**, *193*, 131–134.
[29] C. Dive, C. D. Gregory, D. J. Phipps, D. L. Evans, A. E. Miliner, A. L. Wyllic, *Biochim. Biophys. Acta* **1992**, *1133*, 275–281.
[30] S. S. Bacus, A. V. Gudkov, M. Lowe, L, Lyass, Y. Yung, *Oncogene* **2001**, *20*, 147–155.

Received: November 19, 2003

