Introduction

Screening for the discovery of potential antitumor active seed compounds from natural sources is still important today. Macarpine, an antitumor-active O₆-benzo[c]phenanthridine alkaloid, is one of the attractive compounds. The synthetic pathway of macarpine was established by Ishikawa et al. (1). Additionally they found that one of the key synthetic precursor of macarpine, 6-(6-methoxybenzo[1,3]dioxol-5-yl)naphtho[2,3-d][1,3]dioxole-5,8-dione 5-(O-methyloxime) (abbreviated QO-1), also showed strong cytotoxic activity (< 0.1 μM) against the Hela S3 cell line. QO-1 is one of the 2-aryl-1,4-naphthoquinone-1-oxime methyl ether derivatives. The chemical name and structure of QO-1 along with those of macarpine are shown in Fig. 1A. It has been suggested in a preliminary experiment on the structure–activity relationship (SAR) that the 6,7-methylenedioxy-1,4-naphthoquinone-1-oxime methyl ether skeleton, a common core structure in the 2-aryl-1,4-naphthoquinone-1-oxime methyl ether derivatives, could play an important role for the activity of these compounds (2, 3).

As a target of drug development for cancer therapy, microtubules are an effective target for seed compounds of antitumor drugs derived from natural products. There are examples of successful cancer drugs that target microtubules, and to date, microtubules may be considered one of the most validated therapeutic targets identified.

New 2-Aryl-1,4-naphthoquinone-1-oxime Methyl Ether Compound Induces Microtubule Depolymerization and Subsequent Apoptosis

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Abstract. In this study, we describe the antitumor activity of QO-1, one of the new 2-aryl-1,4-naphthoquinone-1-oxime methyl ether derivatives. QO-1 is a derivative of macarpine, a natural occurring product from Rutaceae plant. It could potently inhibit cell growth when tested on 19 cancer cell lines. To investigate its mechanism, two cell lines (HeLa and MCF-7) sensitive to QO-1 were selected. Based on flow cytometry, it was found to induce G2/M-phase arrest. Moreover, it could cause microtubule depolymerization both in vitro and in vivo. On the other hand, QO-1 activated spindle assembly checkpoint (SAC) proteins. Expression of Bub1, one of the SAC, was gradually increased, reaching a peak after 16 – 20 h, and then gradually decreased. Instead, QO-1 increased the sub-G1 population, which suggested a cell death population. Actually, expression of Bcl-2 family proteins and activation of caspase-3/7 were evidences of apoptosis. Consistent with these results, cells with DNA fragmentation and multinucleated cells were increased time-dependently after QO-1 exposure. In conclusion, QO-1 has promising antitumor effects via microtubule depolymerization.

Keywords: naphthoquinone-1-oxime, microtubule depolymerization, cytotoxicity, spindle assembly checkpoint, cell cycle
For cancer therapy. For example, vinblastine, discovered in 1958, is used to treat Hodgkin lymphoma (4), and paclitaxel, isolated in 1971, is used to treat breast cancer and non–small-cell lung cancer (5). These drugs induce apoptosis by damaging DNA, which is recognized at the G1 checkpoint or by directly attacking microtubules during the M phase (mitotic catastrophe). These drugs are very useful in cancer therapy but can cause serious side effects such as myelosuppression. Additionally, the basic skeletons of these drugs can cause physical problems in drug formulation and limit selection of therapies. How- ever, epothilone, a new tubulin-binding agent, is reported to have a different basic skeleton than existing drugs and fewer side effects (6). Moreover, the novel microtubule inhibitor eribulin (E7389) was approved in April 2011 for the treatment of inoperable recurrent breast cancer. It is a synthetic analog of the marine product halicondrin B. It caused significant and clinically meaningful improve-
proteins are important for inducing apoptosis caused by tubulin-binding drugs.

In this study, we analyzed the mechanism of antitumor activity of QO-1 in cancer cell lines to determine if the compound exerts the activity via cell cycle regulation and SAC proteins and also if it could directly attack microtubules.

**Materials and Methods**

**Reagents**

All cultures and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Macarpine and QO-1 were chemically synthesized by Ishikawa et al. (1, 2). Macarpine, QO-1, paclitaxel (TAX), vinblastine (VBL), and vinorelbine (VNR) (Wako Pure Chemical Industry, Osaka) were dissolved in and diluted with dimethyl sulfoxide (DMSO) and stored at −20°C at a concentration of 5 mM. Antibodies used in this study were given in the sections on western blotting and immunofluorescence.

**Cell lines and culture conditions**

The Mes13 cell line (derived from mouse kidney cell) was kindly provided by Dr. I. Ishii (Chiba University). All other cell lines were purchased from ATCC (Manassas, VA, USA), unless otherwise indicated. As cancer cell lines, we used 15 cell lines. Caki-1 (human kidney cancer cell line) was grown in McCoy's 5A (Wako). HeLa (human cervical cancer cell line), obtained from RIKEN (Tsukuba); A498 and 786-O (human kidney cancer); A549, PCP, and PC14 (human lung cancer) were grown in DMEM (Wako). MCF-7 and MDA-MB-423 (human breast cancer); KLM-1, Paca2, and PANC-1 (human pancreas cancer); DU-145 and PC3 (human prostate cancer); and HCT-15 (human colon cancer) were grown in RPMI1640 (Wako). As non–tumor cell lines, we used Mes-13 and Met-5A (human mesothelial cell line). Mes-13 was grown in DMEM supplemented with 0.01 M HEPES buffer solution and Met-5A was grown in RPMI1640-Glutamax (Gibco BRL, Langley, OK, USA). All cells were cultured with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA), 1.0 units/ml penicillin (Gibco BRL), and 2.0 mg/ml streptomycin (Gibco BRL) at 37°C in atmosphere with 5% CO₂.

**Cytotoxicity assay (MTT assay)**

To compare QO-1 activity with the parental compound macarpine, 3.0 × 10³ MCF-7 cells were seeded in each well of a 96-well plate. After 24-h incubation, an optimum concentration gradient of macarpine and QO-1 were added to each well, followed by culturing for 48 h. Finally, to assess the sensitivity of the cells to these compounds, cell viability was assessed using the proliferation reagent 3-(4,5-dimethyl thiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Dojindo, Kumamoto), according to the manufacturer’s instructions. Control cells were treated with 0.1% DMSO, which is the vehicle for these compounds. On the other hand, QO-1 activity was also assayed against other cell lines, totally 22 (20 cancer cell lines and 2 non-cancerous cell lines) cell lines. IC₅₀ values were calculated by GraphPad Prism 5J software (GraphPad Software, Inc., La Jolla, CA, USA)

**Cell cycle analysis**

A total of 2.0 × 10⁶ cells were seeded in 60-mm dishes and cultured for 24 h. The cells were then cultured with QO-1 (at IC₅₀ value for each cell line). After incubation for each indicated period, the cells were fixed in 80% ethanol (Wako) and incubated for 30 min at room temperature in PBS containing 50 μg/ml propidium iodide (Wako), 2.8% FBS, 0.01% sodium azide (Wako), and 200 μg/ml RNase A. Finally the cell suspension was filtered with a nylon mesh filter, and the filtrate was analyzed using a MoFlo cell sorter (Dako, Tokyo).

**Western blotting analysis**

A total of 1.0 × 10⁶ HeLa or MCF-7 cells were seeded in 60-mm dishes and cultured for 24 h. Cells were collected by scraping and then dissolved in ice-cold lysis buffer [50 mM Tris-HCl (pH 6.5), 10% glycerol, 10% β-mercaptoethanol (Wako), 0.5 mM phenylmethane sulfonyl fluoride (PMSF) solution, 2% sodium dodecyl sulfate (SDS) (Wako), 1 mM sodium orthovanadate, and 1% protease inhibitor cocktail]. Each sample, including 20 μg of protein, was electrophoresed through a 5% or 10% SDS-polyacrylamide (Wako) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Atto Corp., Tokyo). The membranes were blocked with Tris-buffered saline – 0.1% PBS-T [13.7 mM NaCl (Wako), 2.5 mM Tris, 0.05% Tween20 (Wako)] containing 0.3% skim milk (Yukijirushi, Tokyo). The membranes were then subjected to immunoblotting with each polyclonal antibody for Bub1 (1:1000; Abcam, Cambridge, UK), Mad2 (1:1000, Abcam), Bax (1:1000; Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (1:200, N-19: sc-492; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin B1 (1:200, Santa Cruz Biotechnology), β-actin (1:2000), goat anti-mouse IgG-HRP, and donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology). These antibodies were diluted with Immuno Enhancer (Wako) or PBS-T containing 0.1% skim milk. Detection was accomplished using Immobilon Western (Millipore, Tokyo) and was detected by LAS-1000 Plus (Fuji Film, Tokyo). Protein bands were measured by Scion Image
In vivo tubulin polymerization assay

According to previous reports (31, 32), microtubule sensitizer, TAX (25 nM) for another 24 h. Cells were washed in pre-warmed PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed, pre-incubated with 3% BSA in 0.1% Saponin/PBS(−) as a blocking step, and then incubated with each primary antibody, followed by a secondary antibody, if needed. Rat Anti Tubulin α (MCA78G) (Serotec, Oxford, UK) (1:1000) was used as the primary antibody to identify microtubules, followed by the secondary antibody Alexa Fluor® 488 Anti-rat IgG (goat) (A21208) (Invitrogen, Carlsbad, CA, USA) antibody (1:1000). Finally, to detect the nucleus, coverslips were treated with RNaseA (100 μg/ml), PI (50 μg/ml) in PBS(−) for 30 min. After washing, coverslips were mounted on glass slides and both fluorescence and Nomarski photomicrographs were taken using a confocal microscope (FV500; Olympus, Tokyo).

Statistical analysis

The data were analyzed with one-way analysis of variance, followed by Student’s t-test, Dunnett’s test, or Tukey-Kramer test. A P-value of less than 0.05 was considered significant.

Results

Anti-proliferation effect of QO-1 compared to macarpine

QO-1, which are the compounds derived from macarpine (see structures in Fig. 1A), inhibited cell proliferation in a concentration-dependent manner in MCF-7 cells. The effect of QO-1 was significantly more potent than macarpine at the respective concentrations (Fig. 1B). Moreover, QO-1 also caused concentration-dependent growth inhibition against 19 of 20 cancer cell lines including MCF-7 (Table 1). In only MIA Paca2, one of the pancreatic cancer cell lines, QO-1 showed no cytotoxic effect. On the other hand, QO-1 was also tested in non-cancerous cells. Met-5A was resistant to QO-1 even at high concentrations, although the Mes-13growth was inhibited by QO-1 in concentration-dependent manner (Table 1).

In particular, these compounds effectively and reproducibly attacked HeLa (IC50: QO-1, 0.10 μM) and MCF-7 (IC50: QO-1, 0.20 μM) cells. As shown in Table 1, there...
were also other cell lines that seemed to be quite sensitive to QO-1. However, on the other hand, we previously checked if QO-1 might affect the cell cycle in HeLa and MCF-7 cells (unpublished data). So we chose these two cell lines to investigate the functional mechanism of QO-1.

**QO-1 induced G2/M phase arrest**

To investigate the mechanism of QO-1-induced growth inhibition, we next determined if QO-1 affected the progression of the cell cycle using flow cytometry. The distribution of the whole cell population after exposure to QO-1 is shown in Fig. 2. Also we looked at the sub-G1 phase and G2/M phase population (Tables 2 and 3), which were the phases affected by QO-1. In QO-1-treated cells, the G1 population gradually shrunk and then slightly grew from 24 to 48 h exposure to QO-1 (Fig. 2). In contrast, the G2/M population immediately grew and then shrunk sharply from 24 to 48 h exposure to QO-1 both in HeLa (Table 2, upper line) and MCF-7 cells (Table 3, upper line). It suggests that G2/M arrest had occurred, but some mitotically exited cells continued progression and entered a second round of DNA replication (adaptation). On the other hand, the sub-G1 population, which is a marker of apoptosis, started to increase with a disappearance of the G2/M population both in HeLa (Table 2, lower line) and MCF-7 cells (Table 3, lower line).

**The SAC proteins induced by QO-1**

Compared with control cells (Fig. 3A, left), it was observed that expression of Bub1, a member of the SAC protein family, synchronously increased with the growth of the G2/M population (Fig. 2) by QO-1 exposure (Fig. 3A, right). It reached the peak around 8 to 20 h after exposure in both cell lines (Fig. 3B, left), but on the other hand, Mad2, which is complexed with p55Cdc, was almost stable in this study (Fig. 3: A and B, right). To examine the progression from the G2 to M phase in detail, we also checked cyclin B1 expression. It is ubiquitinated via APC/C^Cdc20 during progression from M to the next G1 phase, so protecting cyclin B1 from degradation is important to maintain the mitotic state. In both HeLa and MCF-7 cells, expression of cyclin B1 was gradually upregulated around 8 to 24 h after QO-1 exposure (Fig. 3A, right). Additionally, cyclin B1 expression disappeared after 48 h exposure to QO-1 in both cells (Fig. 3A, right). This result indicates the possibility of mitotic exit, which agrees with the flow cytometry results of 48 h where the G2/M phase population was decreased compared to 24 h.

**Effect of QO-1 on microtubule polymerization**

Because QO-1 induced M-phase arrest, we determined if QO-1 could directly affect microtubule polymerization. QO-1 did not increase microtubule polymerization, in contrast to paclitaxel that promotes microtubule polymerization (Fig. 4A). On the other hand, a microtubule-depolymerizing agent, vinorelbine, inhibited microtubule polymerization at 37°C where the polymerization was considered to increase automatically. QO-1 also exhibited half the inhibitory effect of vinorelbine against microtubule polymerization (Fig. 4B). Moreover, the in vivo polymerization study revealed that QO-1 also increases the ratio of soluble tubulin (non-polymerized tubulin) as vinorelbine did in the cellular environment, although paclitaxel increased the ratio of polymerized tubulin as expected (Fig. 4: C, D).
Apoptosis pathways induced by QO-1

The Bcl-2 family is known as a crucial regulator of apoptosis. Phosphorylation of Bcl-2 is induced on serine residues in tumor cells arrested by microtubule-targeting drugs (33), which is associated with inactivation of its anti-apoptotic function. In both HeLa and MCF-7 cells, phospho-Bcl-2 expression was observed by 24-h exposure to QO-1 (Fig. 5A, upper bands of Bcl-2). On the other hand, Bax, known as a pro-apoptotic protein belonging to the Bcl-2 family, seemed to increase during 48-h exposure to QO-1 in MCF-7 cells (Fig. 5A, right). Bax induces the release of downstream factors such as cytochrome c that leads to activation of caspase-3, the key executioner of apoptosis. We investigated if cell

Table 2. Transition of G2/M and sub-G1 population in HeLa cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>48</th>
</tr>
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<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2/M (%)</td>
<td>22.9</td>
<td>28.1</td>
<td>26.7</td>
<td>20.0</td>
<td>18.6</td>
<td>18.4</td>
<td>20.2</td>
<td>15.9</td>
</tr>
<tr>
<td>S.D.</td>
<td>3.1</td>
<td>5.2</td>
<td>4.4</td>
<td>3.5</td>
<td>0.6</td>
<td>0.4</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>QO-1 G2/M (%)</td>
<td>-</td>
<td>35.1</td>
<td>46.7</td>
<td>61.0***</td>
<td>62.9****</td>
<td>61.8**</td>
<td>58.7*</td>
<td>28.8*</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.5</td>
<td>5.0</td>
<td>6.7</td>
<td>10.5</td>
<td>14.8</td>
<td>29.5</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>Control sub-G1 (%)</td>
<td>8.0</td>
<td>6.3*</td>
<td>4.3***</td>
<td>4.5***</td>
<td>4.0***</td>
<td>4.1***</td>
<td>1.6***</td>
<td>3.1***</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.4</td>
<td>0.9</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td>0.9</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>QO-1 sub-G1 (%)</td>
<td>-</td>
<td>6.5</td>
<td>5.7</td>
<td>5.7</td>
<td>10.3</td>
<td>12.0</td>
<td>8.3*</td>
<td>43.0***</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.3</td>
<td>1.6</td>
<td>1.0</td>
<td>2.6</td>
<td>3.1</td>
<td>0.4</td>
<td>17.1</td>
<td></td>
</tr>
</tbody>
</table>

Data show the mean of three independent experiments. *P < 0.05, ***P < 0.001 vs. each “Control 0 h”, Dunnett’s test. †P < 0.05, ††P < 0.01 vs. each “Control” at the same time point, Student’s t-test.
Naphthoquinone-1-oxime Induces Apostosis

Table 3. Transition of G2/M and sub-G1 population in MCF-7 cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control G2/M (%)</th>
<th>S.D.</th>
<th>Control sub-G1 (%)</th>
<th>S.D.</th>
<th>QO-1 G2/M (%)</th>
<th>S.D.</th>
<th>QO-1 sub-G1 (%)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23.5</td>
<td>1.1</td>
<td>0.9</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>30.4</td>
<td>5.9</td>
<td>1.6</td>
<td>1.0</td>
<td>40.8*</td>
<td>-</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>29.3</td>
<td>3.2</td>
<td>7.1</td>
<td>5.8</td>
<td>57.6***</td>
<td>1.7</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>19.8</td>
<td>3.1</td>
<td>3.0</td>
<td>3.0</td>
<td>70.6***</td>
<td>4.0</td>
<td>4.6</td>
<td>3.5</td>
</tr>
<tr>
<td>16</td>
<td>24.3</td>
<td>1.9</td>
<td>2.5</td>
<td>1.1</td>
<td>68.8***</td>
<td>9.5</td>
<td>12</td>
<td>1.2</td>
</tr>
<tr>
<td>20</td>
<td>25.5</td>
<td>7.6</td>
<td>1.7</td>
<td>1.1</td>
<td>75.0***</td>
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<td>2.6</td>
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<td>1.4</td>
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</tr>
<tr>
<td>48</td>
<td>21.9</td>
<td>4.2</td>
<td>1.7</td>
<td>0.7</td>
<td>49.7***</td>
<td>8.5</td>
<td>23.4***</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Data show the mean of three independent experiments. *P < 0.05, **P < 0.01 vs. each “Control 0 h”, Dunnett’s test. ‘P < 0.05, ‘’P < 0.01, ‘’’P < 0.01 vs. each “Control” at the same time point, Student’s t-test.

dearth involved the caspase pathway. Similar to paclitaxel, QO-1 was found to activate caspase-3/7 in HeLa cells (Fig. 5B), although it did not in MCF-7 cells, which are known to have mutant caspase-3 (data not shown).

**Abnormal mitosis and apoptotic-like shape of cells induced by QO-1**

After exposure to QO-1, the cell morphology showed abnormal mitosis. To confirm the effect of QO-1 on microtubules in cells, we finally the visualized cellular spindle apparatus, which is composed of microtubules (Fig. 5C). Microtubules were stained green (Alexa Fluor® 488 conjugate) and DNA was stained red using PI.

Confocal micrographs of control cells showed normal radial arrays of microtubules in interphase cells (Fig. 5C: a, b) and typical mitotic process (Fig. 5C: c). On the other hand, QO-1-treated cells had abnormal multipolar spindles after 16-h exposure (Fig. 5C: e). There were also multinucleated cells treated with QO-1 for 16 h (Fig. 5C: f) and 48 h (Fig. 5C: l), similar to those treated with vinorelbine (Fig. 5C: m), which might result from an abnormal multipolar mitosis. Moreover, abnormal chromosomes that failed to make chromosomal pairs and several fragmented DNA pieces and clusters were observed at 16 h (Fig. 5C: d), 24 h (Fig. 5C: g, h), and 48 h (Fig. 5C: j) after QO-1 exposure, similar to those treated with vinorelbine (Fig. 5C: m, n).

**Discussion**

It has been known that organic structures with a naphthoquinone 1-oxime core, which is found in QO-1, exert antiviral activity. In this study, QO-1 showed cytotoxic activity in a wide variety of cancer cell lines. It also attacked normal cells, like Mes-13 cells, but the effect was mild on Met-5A cells. The significant difference between these two types of was the growth rate; Mes-13 cells grew fast and Met-5A cell grew slowly. Because it attacks tubulin, like vinca alkaloids do, QO-1 is expected to affect multiply proliferating cells. Thus the above difference is derived from the growth rate of each cell line.

QO-1 increased the number of cells in the G2/M population with a peak at 8 to 20 h (Fig. 3A). Thus QO-1 displayed the pharmacological activity of a microtubule-depolymerization agent. This suggests that the cytotoxic activity of QO-1 might derive from mitotic arrest. Duration of mitosis does not dictate subsequent cell fate like cell death, but activation of SAC that leads to mitotic arrest is required whether a cell dies in mitosis or dies in the subsequent interphase (34). To clarify the SAC involvement in QO-1-induced cell death, we examined the change of SAC proteins, Bub1 and Mad2, over time.

After QO-1 exposure, expression of Bub1 greatly increased from 8 to 20 h and then disappeared or became very weak at 48 h (Fig. 3), concomitantly with G2/M-phase arrest as analyzed by flow cytometry (Fig. 2 and Tables 2 and 3). However, the expression of the other SAC protein, Mad2, did not change. To explain the above results, the following reason was suggested: Mad2 makes a complex with p55Cdc, which is a direct activator of APC/C. Even if QO-1 did not affect Mad2 expression, there would still be a possibility to affect its complex, so further study to detect the factors that make the complex like p55Cdc or activation of APC/C is needed. On the other hand, SAC activation correlates with a significant increase in the concentration of SAC proteins at kinetochores (11). So detecting the localization of SAC expression at kinetochores will help to assess how long SAC activation is maintained by QO-1. Actually, a specific marker of mitotic arrest (cyclin B1 accumulation) was observed after QO-1 exposure, like that observed with the tubulin-binding agent paclitaxel (Fig. 3A). Cyclin B1 is a necessary factor to maintain mitosis, and also it is considered that delaying cyclin B1 degradation allows...
Fig. 3. Effect of QO-1 on SAC proteins Bub1 and Mad2 and cell cycle regulator protein cyclin B1 in HeLa and MCF-7 cells. Cells were treated with the vehicle or with QO-1 (HeLa, 0.10 μM; MCF-7, 0.20 μM) for the indicated time. Paclitaxel (TAX) at 50 nM was used as a positive control. After exposure to QO-1 for the indicated periods, protein expressions of Bub1, Mad2, cyclin B1, and β-actin were analyzed by western blotting as described in Materials and Methods. β-Actin was used as an internal standard. A: Representative photos of Bub1, Mad2, and cyclin B1 in HeLa and MCF-7 cells. B: Transitional change of Bub1 and Mad2 in HeLa and MCF-7 cells. Data shown are each the mean ± S.D. of three independent experiments.
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more time for the death signal to accumulate (34). Combining the results of cyclin B1 and Bub1 expressions with the flow cytometry data, it was clear that mitotic arrest was maintained until 24-h exposure, although mitotic exit already happened at the 48-h exposure point. Microscopic morphology of QO-1-treated cells also exhibited typical mitotic arrest (Fig. 5C). If cell death occurs, derived from mitotic arrest, what is the mechanism? There are abundant reports concerning mitotic cell death, although the pathways are not completely merged. Microtubule-active drugs inhibit spindle functions during mitosis and cause M-phase arrest. During M-phase arrest induced by these drugs, p53 is gradually stabilized. It is accompanied by induction of p21, which promotes postmitotic G1 arrest (35). Therefore prolonged time in mitosis induces both cell death in mitosis and mitotic exit known as mitotic slippage. In the latter case, slipped-out cells display multinucleated morphology (aneuploidy cells). It is suggested that an excessively huge DNA content eventually accumulates genotoxic stress in the post-mitotic G1 phase (36), and then p53 responds to DNA damage, triggering apoptosis. Additionally, such slippage cells do not necessarily undergo cell death and some cells may still be alive and remain in interphase or enter a second mitosis (34). On the other hand, high concentrations of vinflunine, one of the vinca alkaloids, characteristically induce a G2/M block and Bcl-2 phosphorylation, while low concentrations of vinflunine suppress microtubule dynamics and slow down mitotic progression but fail to block cells in the G2/M, following exit mitosis, a p53-dependent post-mitotic G1 arrest (37).

We observed both mitotic arrested cells and some aneuploidy cells after QO-1 exposure (Fig. 5C: i, l). Addition-

![Graph A](image)

**Microtubule polymerization (difficult condition for polymerization)**

- Control
- Paclitaxel 10 μM
- QO-1 10 μM

**Microtubule polymerization (promoting condition for polymerization)**

- Control
- Vinorelbine 5 μM
- QO-1 5 μM

**Fig. 4.** Effect of QO-1 on tubulin assembly. A: Promoting effect on in vitro microtubule polymerization. Paclitaxel was used as a positive control. MAP-rich tubulin (1.0 mg/ml) was incubated at room temperature for 3,600 s, and then the indicated compounds were added at the indicated concentrations. $A_{340}$ values were recorded once per 60 s. The results are presented as the difference of absorbance at each time point. B: Inhibitory effect on in vitro microtubule polymerization. Vinorelbine was used as a positive control. MAP-rich tubulin (1.0 mg/ml) was incubated at 37°C for 3,600 s, and then the indicated compounds were added at the indicated concentrations. $A_{340}$ values were recorded once every 300 s. The results are presented as the difference of absorbance at each time point. C and D: In vivo microtubule polymerization in HeLa cell. Cells were prepared as described in Materials and Methods. 0 h, cells not treated with any drug; Control 24 h, cells treated with 0.1% DMSO for 24 h; QO-1 24 h, cells treated with QO-1 (0.10 μM) for 24 h; VNR 24 h, cells treated with vinorelbine (25 nM) for 24 h; TAX 24 h, cells treated with paclitaxel (25 nM) for 24 h. C: Representative photos of α-tubulin (component of microtubule). S, soluble (non-polymerized) tubulin; P, polymerized tubulin. D: Ratio of soluble tubulin and polymerized tubulin. Data shown are each the mean ± S.D. of three independent experiments. ***P < 0.001 (Student’s t-test).
ally, we observed that the sub-G₁ population grew sharply after QO-1 exposure for 48 h in HeLa cells and also the M-phase population decreased at the same time (Fig. 2 and Tables 2 and 3). At that time, the G₁ population was not so changed compared with that at 24 h (Fig. 2). This indicates the progression from M to the next G₁ phase might occur at some level, but most of the sub-G₁ population might be derived from cells that directly underwent mitotic cell death under these experimental concentrations. We need further study to confirm which pathway is major.

Mitochondrial Bcl-2 family members have a crucial
role in vinca alkaloids or paclitaxel-induced apoptosis. Although the molecular mechanisms that link mitotic arrest and apoptosis are poorly understood, mitochondria is considered as the point of convergence for the apoptotic signals induced by the vinca alkaloids (37). We examined if Bax and Bcl-2 proteins contribute to apoptosis induced by QO-1. As a result, expression of Bax relatively increased in MCF-7 after exposure to QO-1, and the inactive form of Bcl-2 expression was also increased in both cell lines. So QO-1 might shift the Bcl-2 family balance toward apoptosis.

Finally we determined if the caspase family is involved in QO-1 induced apoptosis. Tubulin-binding agents can induce apoptosis both via a caspase-dependent pathway and caspase-independent pathway (38). For instance, nocodazole induces caspase-dependent apoptosis in some cell lines (39, 40). Caspase-3 activation can lead to cleavage of Bub1 before nocodazole-induced apoptosis (41). Vice versa, the cleavage of Bub1 is necessary for apoptosis via a caspase pathway in HeLa and MCF-7 cells (42). In this study, QO-1 induced similar microtubule-depolymerization effects as nocodazole does. Hence, QO-1 was expected to induce apoptosis after degradation of SAC proteins like nocodazole does. As mentioned above, the sub-G1 population grew after the G2/M population shrunk (Fig. 2), and Bub1 expression decreased or disappeared at the time point of sub-G1 increase (Fig. 3). These results support the above assumption about apoptosis being induced by QO-1. Like paclitaxel, QO-1 activated caspase-3/7 in HeLa cells but not in MCF-7 cells. This suggested that QO-1-induced apoptosis occurs via different pathways in each cell line. Although caspase-3 is commonly activated by numerous death signals, it is not expressed in the MCF-7 cell line. This cell line is known to undergo cell death in response to stimulation by TNF-α, staurosporine, and other agents. Those previous findings suggested that caspase-3 is necessary for apoptosis-inducendo nuclear events and DNA fragmentation, but may not be essential for cell death itself. Kagawa et al. showed Bax overexpression induced apoptosis in MCF-7 cells, while restoration of caspase-3 did not affect cell death induced by Bax (43). Although the precise mechanism is not yet clear, it is certain that some apoptotic events occurred in MCF-7 cells in the absence of caspase-3. The mechanism that compensates for the absence of caspase-3 function in MCF-7 cells needs further clarification. Moreover, previously it was found that a caspase inhibitor allowed mitotic cells more time for slippage and reduced the number of cell deaths in mitosis (34). Gascoigne et al. speculated that cell fate is dictated by two competing but independent mechanisms, one involving caspase activation and the other is protecting cyclin B1 from degradation (34). Therefore our results of cyclin B1 accumulation and activation of caspase 3 (in HeLa cells) are consistent with the previous reports.

Above all, we found that QO-1 exerted growth inhibition and cytotoxicity, which partly derived from its inhibiting activity on microtubule polymerization and following activation of SAC. Despite these findings, the precise mechanism remains unclear. A possibility was suggested that SAC activation by QO-1 might induce apoptotic factors. Activation of SAC proteins and following events via the Bcl-2 family or caspase-3/7 might result in potent apoptosis. Further studies are needed, but QO-1 activity revealed not only its promising effect but also proves the common core structure of such compounds to be a valuable innovation for applicable antitumor active seed compounds.

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