# Studies on the Cytotoxicity of Asterriquinone Derivatives

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The antitumor agent, asterriquinone (ARQ) is a known metabolite isolated from the mycelium of *Aspergillus terreus* IFO 6123[1]. YAMAMOTO *et al.* reported that ARQ analogues having a 2,5-dimethoxy-*p*-benzoquinone moiety (ARQ dimethyl ether: ARQDMe)[2,3] and the 2,5-diamino-*p*-benzoquinone moiety (diamino ARQ: ARQDA)[4] did not show any cytotoxicity. As reported in our previous communication[5], a new metabolite, asterridinone, which has a furo[3,2-*b*]furan moiety instead of the *p*-benzoquinone moiety of ARQ, did not show cytotoxic activity towards P388 mouse leukemia cells, while isoasterriquinone and neoasterriquinone which have the *tert*- or *iso*-pentenyl groups at the different position of the indole ring, were active as was ARQ. These results indicate that the 2,5-dihydroxy-*p*-benzoquinone structure is more important for cytotoxic activity than the kind of prenyl groups and its position. In this study, to assess the contribution of the hydroxy group in the *p*-benzoquinone moiety to the cytotoxic activity of ARQ, we synthesized several ARQ derivatives, of which the one or two hydroxy group(s) were substituted with the acetyl, methoxy, and/or amino groups, and examined their cytotoxicity towards P388 cells.

## Materials and Methods

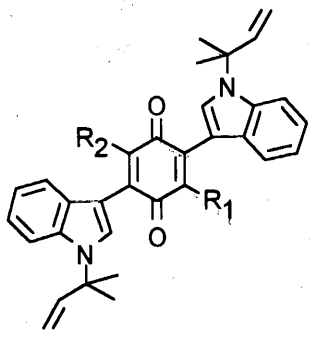
### Test Compounds

ARQ and ARQ monoacetate (ARQAc) were isolated as the metabolic products from mycelium of *Aspergillus terreus* IFO 6123[5]. ARQ diacetate (ARQDAc)[6] and ARQDMe[1] were prepared from ARQ by the usual method. ARQ monomethyl ether acetate (ARQMeAc)[6] was prepared by methylation of ARQAc and ARQ monomethyl ether (ARQMe)[6] was prepared from ARQMeAc by alkaline hydrolysis. ARQDA was prepared from ARQDMe as described previously[4]. Monoamino ARQ (ARQA) was prepared from ARQMe in the same manner of ARQDA. Monoamino ARQ monomethyl ether (ARQAMe) was also obtained by methylation of ARQA. The chemical structures of the compounds used in this study are shown in Figure 1.

### 2-Amino-3,6-bis[1-(1,1-dimethyl-2-propenyl)-1*H*-indol-3-yl]-5-hydroxy-2,5-cyclohexadiene-1,4-dione (ARQA)

ARQMe (30 mg, 0.058 mmol) was treated with 5% (v/v) aq $NH_3$ (5 ml) in EtOH (50 ml) and allowed to stand overnight at room temperature. The resulting precipitate was collected, dissolved in diethyl ether and washed with 5% (w/v) aq $NaHCO_3$. The organic layer was washed with water, dried over anhydrous $Na_2SO_4$, evaporated under reduced pressure and the residue was purified by column chromatography ($SiO_2$, $CH_2Cl_2$). ARQA (23 mg, 0.045 mmol) was obtained in 78% yield. Dark reddish green prisms (from EtOH) of mp 217~218°C (dec.). EI-MS *m/z*: 505 ($M^+$). HREI-MS *m/z*: 505.2368 (Calcd for $C_{32}H_{31}N_3O_3$: 505.2365). IR (KBr) $cm^{-1}$: 3488, 3350, 3320, 1656. UV $\lambda_{max}$ nm (log $\varepsilon$) in EtOH: 224 (4.67), 280 (4.36), 294 (4.40), 460 (3.36). $^1$H-NMR ($CDCl_3$) $\delta$: 1.83 (12H, s, $2C(CH_3)_2CH=CH_2$), 3.52 (2H, br s, $NH_2$), 5.26 (1H, d, $J$=17.6 Hz, $C(CH_3)_2CH=CH_2$), 5.27 (1H, d, $J$=17.6 Hz,

Fig. 1. Chemical structures of ARQ and its derivatives.




| | $R_1$ | $R_2$ |
|---|---|---|
| asterriquinone (ARQ) | OH | OH |
| ARQ monoacetate (ARQAc) | OAc | OH |
| ARQ diacetate (ARQDAc) | OAc | OAc |
| ARQ monomethyl ether (ARQMe) | OH | OMe |
| ARQMe acetate (ARQMeAc) | OAc | OMe |
| ARQ dimethyl ether (ARQDMe) | OMe | OMe |
| monoamino ARQ (ARQA) | $NH_2$ | OH |
| ARQA monomethyl ether (ARQAMe) | $NH_2$ | OMe |
| diamino ARQ (ARQDA) | $NH_2$ | $NH_2$ |

$C(CH_3)_2CH=CH_2$), 5.28 (2H, d, $J=10.4$ Hz, $2C(CH_3)_2CH=CH_2$), 6.22 (2H, dd, $J=17.6$, 10.4 Hz, $2C(CH_3)_2CH=CH_2$), 7.11~7.20 (4H, m, Ar-H), 7.40~7.42 (1H, m, Ar-H), 7.53 (1H, s, Ar-H), 7.56~7.63 (3H, m, Ar-H), 7.68 (1H, s, Ar-H), 8.47 (1H, s, OH).

2-Amino-3,6-bis[1-(1,1-dimethyl-2-propenyl)-1*H*-indol-3-yl]-5-methoxy-2,5-cyclohexadiene-1,4-dione (ARQAMe)

ARQA (10 mg, 0.020 mmol) was treated with ethereal diazomethane at room temperature for 30 minutes. After evaporation, the residue was recrystallized from *n*-hexane to give ARQAMe (10 mg, 0.019 mmol) in 94% yield. Dark red needles of mp 147~149°C (dec.). EI-MS *m/z*: 519 ($M^+$). HREI-MS *m/z*: 519.2522 (Calcd for $C_{33}H_{33}N_3O_3$: 519.2522). IR (KBr) $cm^{-1}$: 3508, 3372, 1650. UV $\lambda_{max}$ nm ($\log\varepsilon$) in EtOH: 223 (4.70), 294 (4.44), 448 (3.56). $^1$H-NMR ($CDCl_3$) $\delta$: 1.83 (12H, s, $2C(CH_3)_2CH=CH_2$), 3.85 (3H, s, $OCH_3$), 5.24 (2H, d, $J=17.6$ Hz, $2C(CH_3)_2CH=CH_2$), 5.27 (2H, d, $J=10.4$ Hz, $2C(CH_3)_2CH=CH_2$), 5.30 (2H, s, $NH_2$), 6.23 (2H, dd, $J=17.6$, 10.4 Hz, $C(CH_3)_2CH=CH_2$), 7.12~7.18 (4H, m, Ar-H), 7.40~7.42 (1H, m, Ar-H), 7.52~7.54 (1H, m, Ar-H), 7.56 (1H, s, Ar-H), 7.58~7.58 (1H, m, Ar-H), 7.60 (1H, s, Ar-H), 7.62~7.62 (1H, m, Ar-H).

Assay for Cytotoxic Activity *In Vitro*

P388 mouse leukemia cells were kindly supplied by Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Cells were passaged weekly through female BALB/c × DBA/2 (CD2F1) mice (Nippon SLC, Hamamatsu, Japan) and harvested from tumor-bearing mice 6~7 days after transplantation. Cells were suspended in PRMI 1640 supplemented with 10% fetal bovine serum, 20 $\mu$M 2-mercaptoethanol, and 100 $\mu$g/ml kanamycin at a density of $1.5\times10^4$ cells/ml and cultured with or without a compound at 37°C for 72 hours in a 5% $CO_2$ incubator. The compounds were dissolved in DMSO and final concentration of DMSO was adjusted so as to be 0.5% of culture medium. The survival of the cells was measured by MTT assay[7], and the 50% growth-inhibitory concentration ($IC_{50}$) of the compound was determined.

## Results and Discussion

As shown in Table 1, ARQDA and ARQDMe were inactive, as reported[2~4]. But, ARQA, ARQMe and ARQAc, which have one hydroxy group in the *p*-benzoquinone moiety, showed same or more cytotoxic activity against P388 cells than ARQ. ARQA had been defeated the cytotoxic activity by methylation of hydroxy group (ARQAMe, $IC_{50}$: >100 $\mu$M). Therefore, methylated ARQAc (ARQMeAc) which have no free hydroxy group in the molecule, showed more potent cytotoxicity than ARQMe. These results indicated that one free hydroxy group substituted to the *p*-benzoquinone moiety is important in order for the ARQ derivatives to exhibit cytotoxicity, and suggested that the acetylated ARQ derivatives were converted into corresponding free hydroxy derivatives by the hydrolysis of the ester group(s) in the cells.

Table 1. Cytotoxicities of ARQ and its derivatives against P388 cells.

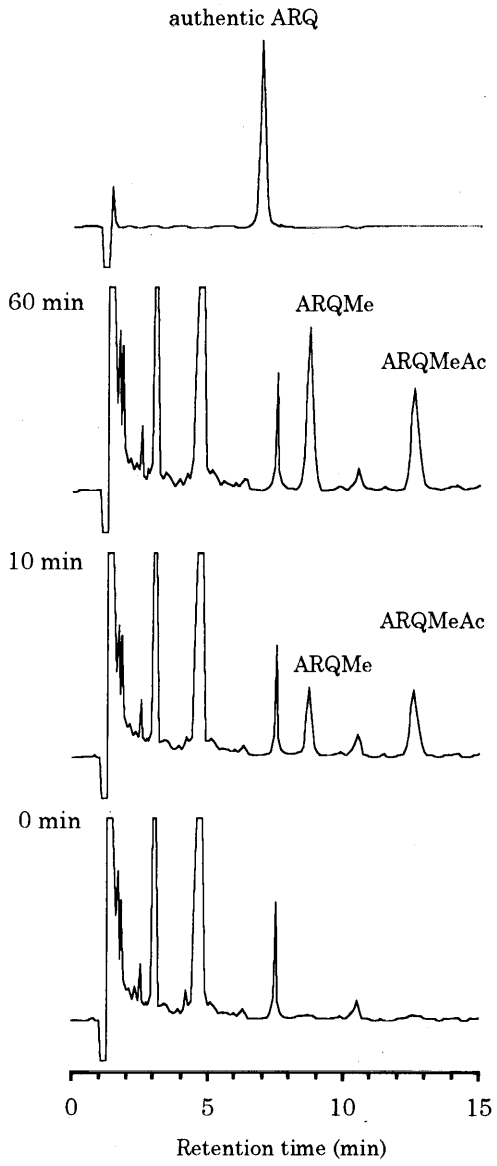
| Compound | $IC_{50}$ ($\mu$M) |
|---|---|
| ARQ | 1.28 |
| ARQAc | 1.25 |
| ARQDAc | 1.07 |
| ARQMe | 0.74 |
| ARQMeAc | 0.56 |
| ARQDMe | >100 |
| ARQA | 0.75 |
| ARQAMe | >100 |
| ARQDA | >100 |

When P388 cells were incubated with ARQMeAc for 60 minutes at 37°C, time course of intracellular content of the compound and its metabolic change in the cells were determined.

Figure 2 shows HPLC profiles of authentic ARQ and of the cell extract. Two peaks (Fig. 2; 10 minutes and 60 minutes) except for cell origin (Fig. 2; 0 minute) were observed in the cells after incubation, and they were assigned to ARQMe (retention time: 8.8 minutes) and ARQMeAc (retention time: 12.6 minutes) by direct comparison with authentic samples. During incubation for 60 minutes, ARQ (Fig. 2; ARQ, retention time: 6.9 minutes) was never detected in the cells. We have preliminarily confirmed that ARQMeAc was stable in the culture medium without cells (data not shown).

Figure 3 shows the changes in the intracellular amounts of ARQMeAc and ARQMe. Intracellular ARQMeAc was gradually increased, and ARQMe also appeared in the cells 10 minutes after addition of ARQMeAc and increased with the incubation time. After 60 minutes, the cells were washed and further incubated for 30 minutes in fresh medium without compound, and the fate of the compounds in the cells was observed. Although total

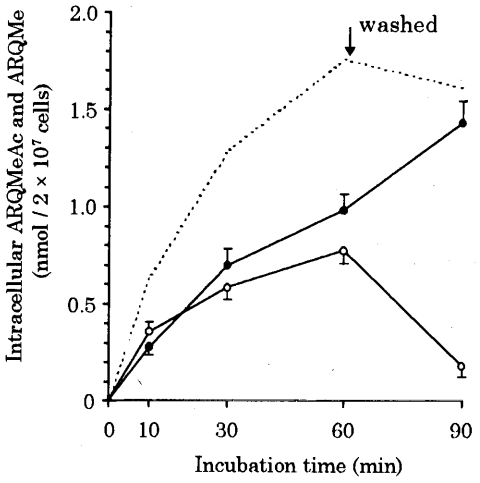
Fig. 2. HPLC profiles of the cell extract of P388 cells after incubation with ARQMeAc.




P388 cells ($2 \times 10^6$ cells/ml) were suspended in Hanks' solution (pH 7.4) and incubated with 3 $\mu$M ARQAcMe in a volume of 10 ml for the designated period in a 5% $CO_2$ incubator at 37°C. Cells were collected by centrifugation at 2°C, 2000 rpm for 5 minutes, washed twice with chilled phosphate-buffered saline (pH 7.4) and extracted with acetone in a sonicator for 10 minutes. The extract was evaporated under a nitrogen stream at room temperature. The residue was dissolved in 200 $\mu$l of MeOH and used for HPLC analysis. HPLC analysis was done using $\mu$BONDASHERE 5 $\mu$ C18-100Å (3.9 × 150 mm, Nihon Millipore Ltd., Tokyo, Japan), elution with 1 mM oxalic acid - MeOH (20 : 80, v/v), 20 $\mu$l injection, at a flow rate of 1.0 ml/minute and monitored at 254 nm.

Fig. 3. Intracellular accumulation of ARQMeAc and ARQMe.

Symbols: (○) ARQMeAc, (●) ARQMe, and doted line showed total amounts of ARQMeAc plus ARQMe.




Cells ($2 \times 10^7$ cells/10 ml) were cultured in Hanks' solution (pH 7.4) containing 3 $\mu$M ARQMeAc for the indicated times (10, 30, and 60 minutes) at 37°C in a 5% $CO_2$ incubator. After 60 minutes, washing out of extracellular compound, cells were further incubated in fresh medium for 30 minutes. The content of the compound was determined by reference to standard curve for each compound. Data are the means ± S.E. of at least three independent experiments done in triplicate.

amounts of ARQMeAc plus ARQMe was decreased only a little, intracellular ARQMe was increased in the contrast with the rapid decrease of ARQMeAc. These results indicated that the acetoxy group in the ARQMeAc molecule is biologically hydrolyzed to the hydroxy group (ARQMe) (Fig. 2). However, because the biotransformation of ARQMe to ARQ was never observed in the cells, the ether bond appears to be insensitive to the biological ether bond cleavage. Consequently, even if ARQDAc and ARQMeAc are fully substituted derivatives of the two hydroxy groups of the *p*-benzoquinone moiety, they showed cytotoxic activity after hydrolysis of the ester bond in the cell. While, ARQDMe could not show cytotoxicity because having two ether bond.

In conclusion, this study indicates that the existence of at least one hydroxy group or hydrolyzable ester group in the *p*-benzoquinone moiety of ARQ derivatives is sufficient to elicit these biological actions, and that the 2,5-dihydroxy-*p*-benzoquinone structure is not essential.

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