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Dihydro-1,4-benzothiazine-6,7-dione, the Ultimate Toxic Metabolite of 4-S-Cysteaminylphenol and 4-S-Cysteaminylcatechol*

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Abstract. 4-S-Cysteaminylphenol (4-S-CAP) and the corresponding catechol 4-S-cysteaminylcatechol (4-S-CAC) have been evaluated for melanocytotoxicity. It was shown recently that tyrosinase oxidation of these substrates produces a violet pigment, dihydro-1,4-benzothiazine-6,7-dione (BQ). In this study we examined whether BQ is the ultimate toxic metabolite produced in melanoma cells from 4-S-CAP/4-S-CAC. Biochemical experiments showed that (1) BQ was formed by autoxidation of 4-S-CAC as well as by tyrosinase oxidation of 4-S-CAP/4-S-CAC, (2) BQ reacted rapidly with thiols such as reduced glutathione (GSH), and (3) BQ inhibited the activity of alcohol dehydrogenase, an SH enzyme. *In vitro* experiments showed that (1) the cytotoxicity of 4-S-CAC was mostly prevented by catalase and superoxide dismutase, (2) BQ was highly cytotoxic to B16 melanoma cells (IC_{50} being 3.9 μM as compared with 507 μM for 4-S-CAP), (3) BQ was metabolized rapidly to a GSH adduct in melanoma cells, and (4) the same GSH adduct was also formed upon incubation of melanoma cells with 4-S-CAP, the reaction being tyrosinase dependent. *In vivo* experiments showed that intratumoral administration of BQ (0.5 μmol) inhibited the subcutaneous growth of B16 melanoma nearly as effectively as 4-S-CAP/4-S-CAC (20 μmol). These results indicate that BQ is the ultimate toxic metabolite produced by tyrosinase oxidation of 4-S-CAP/4-S-CAC. BQ deprives melanoma cells of GSH and may inactivate SH enzymes essential for DNA synthesis and cell proliferation by covalent binding through their cysteine residues, thereby exerting melanocytotoxicity. Cytotoxicity of 4-S-CAC depends mostly on autoxidation producing BQ and active oxygens. BIOCHEM PHARMACOL 53;10:1435–1444, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. phenol; catechol; tyrosinase; melanocyte; melanoma; cytotoxicity

In normal melanocytes and malignant melanoma cells, the specific enzyme tyrosinase catalyzes the oxidative conversion of the common amino acid L-tyrosine to melanin pigments [1]. Exploitation of melanogenesis in developing antitumor agents specific for malignant melanoma has been a challenging subject (for a review, see Ref. 2). 4-Methoxyphenol (4-hydroxyanisole) was introduced in 1969 as a possible melanocytotoxic agent [3, 4]. In recent years, more attention has been paid to another phenolic compound, 4-S-CAP,‖ that appeared to be the most promising antimelanoma agent (Fig. 1). 4-S-CAP is a good substrate for tyrosinase, and an oxidized form binds to SH enzymes [6]. *In vitro* experiments have shown that 4-S-CAP is incorporated into melanoma cells and becomes cytotoxic by inhibiting DNA synthesis; these phenomena are correlated to the degree of pigmentation [7]. Several *in vivo* studies have also shown that 4-S-CAP inhibits growth of B16 mouse melanoma and increases the life span of B16 melanoma-bearing mice [8, 9]. The phenol also destroys melanocytes in hair follicles of black mice and reduces the number of metastatic B16-F10 colonies in the lung [10].

However, 4-S-CAP has a certain limitation: its strong hypotensive effect as a substrate for dopamine-β-hydroxylase [11], leading to a rather low LD_{50} value of 400 mg/kg [9]. 4-S-CAP is also converted rapidly to the cytotoxic aldehyde form by the action of plasma monoamine oxidase in culture medium [12], although this deamination seems to play a relatively minor role in the *in vivo* catabolism of this compound [11]. In an attempt to increase the efficacy of 4-S-CAP, we recently synthesized the corresponding catechol 4-S-CAC and evaluated it as an antimelanoma agent [13]. We found that 4-S-CAC is a good substrate for melanoma tyrosinase and is highly cytotoxic to melanoma cells both *in vitro* and *in vivo*.

With respect to the mechanism of cytotoxicity of 4-S-CAP (and 4-S-CAC), we previously reported that upon

* This paper is dedicated to the late Professor Keisuke Fujita who died on June 11, 1995.

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‖ *Abbreviations:* 4-S-CAP, 4-S-cysteaminylphenol; 4-S-CAC, 4-S-cysteaminylcatechol; ADH, alcohol dehydrogenase; 4-S-CAQ, 4-S-cysteaminyl-1,2-benzoquinone; BQ, dihydro-1,4-benzothiazine-6,7-dione; GSH, reduced glutathione; SOD, superoxide dismutase; NEM, N-ethylmaleimide; and BSO, buthionine sulfoximine.

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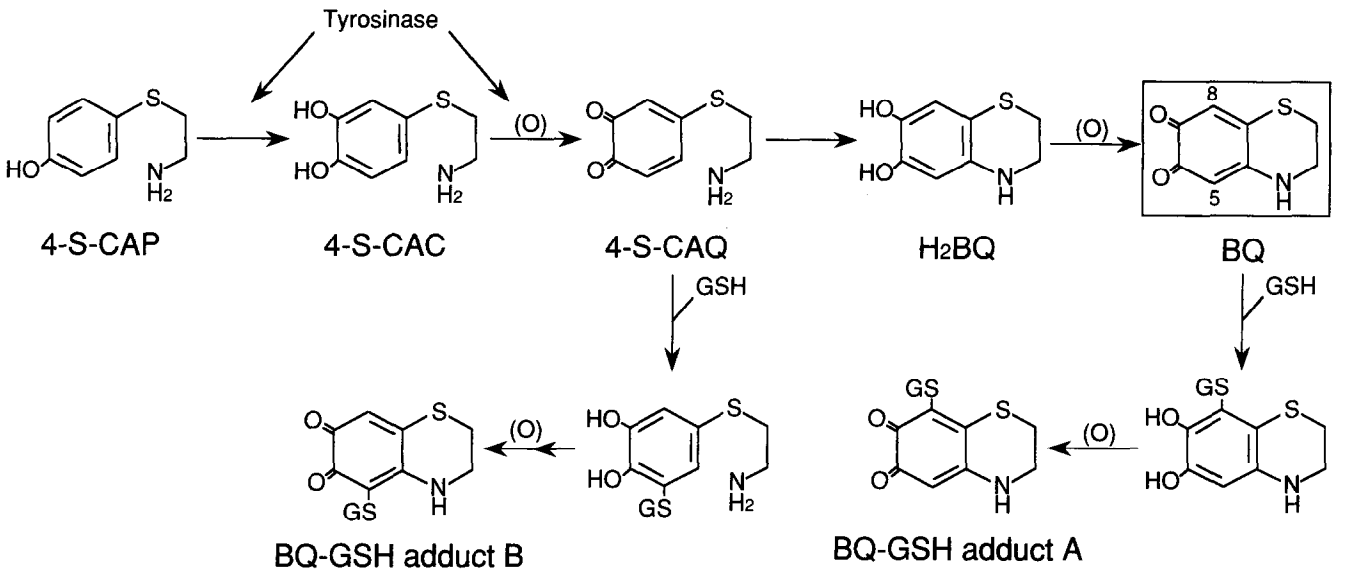

FIG. 1. Proposed metabolic pathway for 4-*S*-CAP and 4-*S*-CAC. Tyrosinase oxidation of 4-*S*-CAP or 4-*S*-CAC produces 4-*S*-CAQ, which is converted rapidly to the cyclized form (H_2BQ) through an intramolecular addition reaction of the amino group. H_2BQ is oxidized to BQ through a redox reaction with 4-*S*-CAQ [5]. The present study showed that BQ reacted with GSH to produce an unstable BQ–GSH adduct in a reduced form, which is oxidized rapidly to a more stable BQ–GSH adduct A. 4-*S*-CAQ is able to bind GSH to form an adduct [6] which is converted to the isomeric BQ–GSH adduct B through oxidation, cyclization, and re-oxidation.

tyrosinase oxidation, 4-*S*-CAP was able to bind to cysteine quantitatively and bound to an SH enzyme, ADH, to a lesser extent [6]. We suggested that 4-*S*-CAP/4-*S*-CAC is oxidized by tyrosinase present in melanoma cells to the corresponding *o*-quinone 4-*S*-CAQ (Fig. 1), which would conjugate with SH enzymes through their cysteine residues, thereby exerting cytotoxic effects [6]. 4-*S*-CAQ, being extremely reactive, is present only as a transient intermediate. A more recent chemical study by Mascagna *et al.* [5] has demonstrated that a violet pigment is produced from 4-*S*-CAP and 4-*S*-CAC upon tyrosinase oxidation, its structure being BQ (Fig. 1). In the present study, we examined the mechanism of melanocytotoxicity of 4-*S*-CAP and 4-*S*-CAC and demonstrated that the ultimate toxic metabolite is the stable quinone BQ rather than the transient, unstable 4-*S*-CAQ.

MATERIALS AND METHODS

Chemicals

4-*S*-CAP · HCl was prepared by the method of Padgette *et al.* [14], and 4-*S*-CAC · HCl was synthesized by our previously reported method [13]. BQ was prepared by the method of Mascagna *et al.* [5] and extracted with 1-butanol. The molar extinction coefficient of BQ was determined to be 16,500 $mol^{-1} \cdot L \cdot cm^{-1}$ by tyrosinase oxidation of 4-*S*-CAP. GSH, phenylthiourea, mushroom tyrosinase (4200 U/mg), catalase (5800 U/mg), SOD (3500 U/mg), and ADH (from baker's yeast; 340 U/mg) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3,5-Di-*tert*-butyl-1,2-benzoquinone was from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other chemicals were of analytical grade.

Spectrophotometry, HPLC, and Biochemical Methods

Spectra were measured using a JASCO Ubest V-520SR spectrophotometer equipped with a STR-458 constant temperature cell holder (JASCO, Tokyo, Japan). Oxidation of 4-*S*-CAP and 4-*S*-CAC was carried out in 50 mM sodium phosphate buffer, pH 6.8, with or without tyrosinase added.

The HPLC system consisted of a JASCO PU-880 HPLC pump, a JASCO 851-AS sampler, a JASCO 807-IT integrator, and either a JASCO 875-UV UV/VIS detector or a JASCO 840-EC electrochemical detector. 4-*S*-CAP, BQ, and BQ metabolites were separated on a JASCO Crestpack C18S column (C_{18}-reversed phase; 4.6 × 150 mm; particle size 5 μm). The mobile phase was methanol:water:1 M perchloric acid (10:90:1.5, by vol.). The column was maintained at 40°, and the flow rate was 0.7 mL/min. 4-*S*-CAP was detected at 250 nm, and BQ and its metabolites were detected at 379 nm. GSH was determined by the method of Imai *et al.* [15]. The 3,5-di-*tert*-butyl-1,2-benzoquinone-GSH adduct was separated on a JASCO Crestpack (see above) at 40° with a mobile phase of methanol:water: 1 M perchloric acid (65:35:1.5, by vol.) and detected using the electrochemical detector at 1200 mV versus mercury/mercury chloride electrode.

ADH activities were measured by following the formation of NADH in the presence of ethanol and NAD^+. Briefly, the method was based on that reported by Roth [16] with minor modifications. Thirty microliters of a reaction mixture was added to a 3-mL solution of 0.7 M ethanol and 0.5 mM NAD^+ in 60 mM sodium pyrophosphate, and the increase of absorbance at 340 nm was followed at 25°.

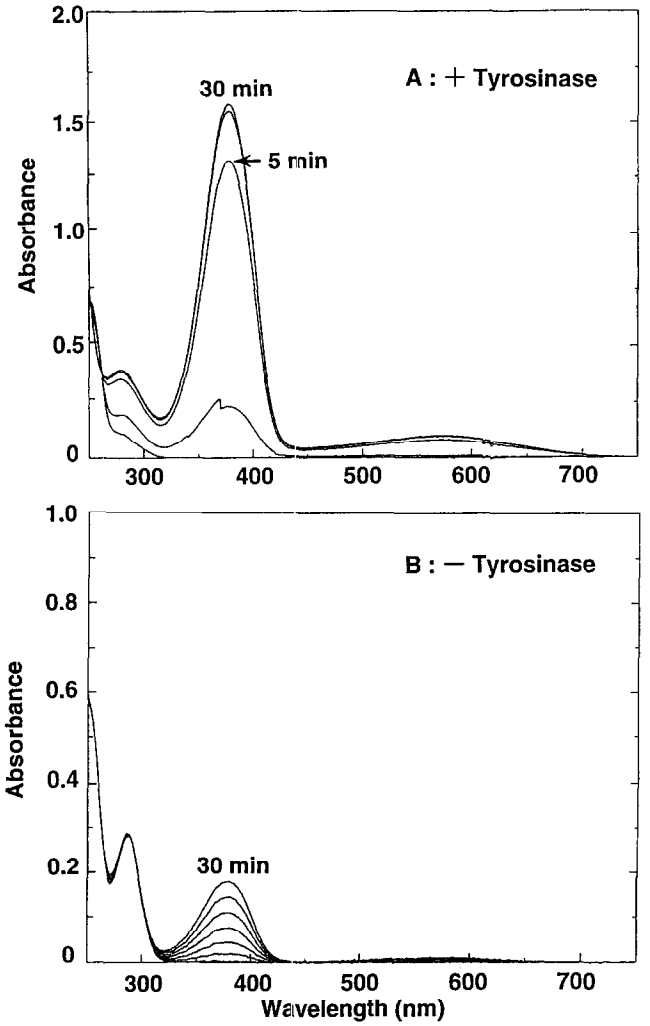

FIG. 2. Changes in absorption spectra during oxidation of 4-S-CAC. Spectra were taken at intervals of 5 min at a scan speed of 20 nm/sec. (A) Tyrosinase oxidation of 4-S-CAC (100 μM) at pH 6.8 and 37°. Spectra were also taken before and immediately after the addition of tyrosinase (5 μg/mL). Tyrosinase oxidation of 4-S-CAP (100 μM) gave similar results (not shown). (B) Autoxidation of 4-S-CAC (100 μM) at pH 6.8 and 37°.

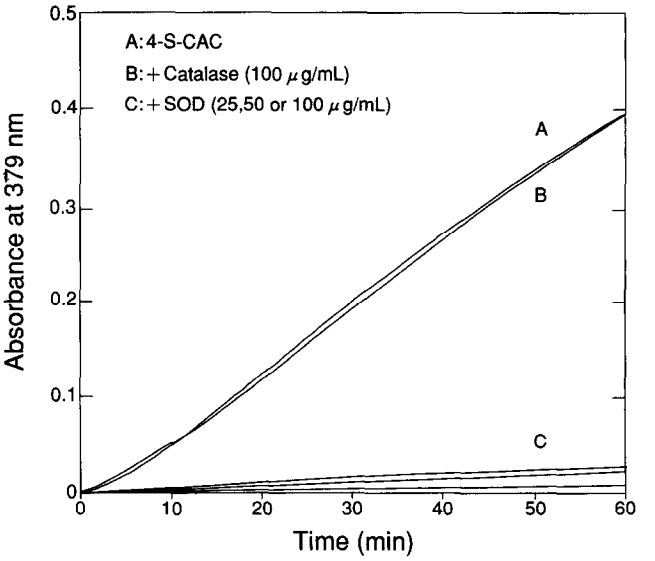

FIG. 3. Effects of catalase and SOD on autoxidation of 4-S-CAC. 4-S-CAC (100 μM) was oxidized by air at pH 6.8 and 37° in the absence or presence of catalase (100 μg/mL) or SOD (25, 50, or 100 μg/mL), and changes in the absorbance at 379 nm were followed continuously. Inhibition of BQ formation was the greatest at the 25 μg/mL concentration of SOD, followed by the 50 μg/mL concentration. A repeated experiment gave a similar result.

In Vitro *Studies*

B16-F1 mouse melanoma cells were cultured as monolayers at 37° in RPMI 1640 medium (GIBCO BRL, Life Technologies, Inc., Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Flow Laboratories, North Ryde, Australia), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified 5% CO_2 atmosphere. Exponentially growing cells were harvested by trypsinization with 0.25% trypsin-EDTA, counted in a Sysmex F-610 Micro Cell Counter (Kobe, Japan), and inoculated at a concentration of 2 or 5 × 10^5 cells/dish in 60-mm Falcon petri dishes. Twenty-four hours later, the cells were used for experiments.

In Vivo *Studies*

B16 melanoma cells (1 × 10^6 cells) were inoculated s.c. in the axillary regions of 25 C57BL/6 mice (5-week-old mice; 18–20 g). When the tumors reached an estimated volume of approximately 100 mm^3 (on day 8–9), the 25 mice were divided into groups of 5 mice each and received a single intratumoral injection of vehicle only (control group) or vehicle containing either 4-S-CAP, 4-S-CAC, or BQ. Tumor diameters were measured with calipers at intervals of 2–3 days, and tumor volumes were calculated by the formula: long axis · (short axis)^2 · 1/2. The measurement was terminated when the mean tumor volume of the control group exceeded 1000 mm^3.

RESULTS

Biochemical Studies

As reported by Mascagna *et al.* [5] tyrosinase oxidation of 4-S-CAP and 4-S-CAC yielded a violet pigment, BQ, having absorption maxima at 379 and 573 nm (Fig. 2A). Autoxidation of 4-S-CAC slowly produced the same violet pigment in a yield of *ca.* 11% in the first 30 min (Fig. 2B). We previously showed using HPLC, that autoxidation of 4-S-CAC proceeded to an extent of *ca.* 15% in 30 min at pH 6.8 [13].

The effects of catalase and SOD on the formation of BQ upon autoxidation were examined next. Catalase had no effect in preventing BQ formation, while SOD almost completely (*ca.* 95%) prevented it (Fig. 3).

We previously proposed that the cytotoxicity of 4-S-

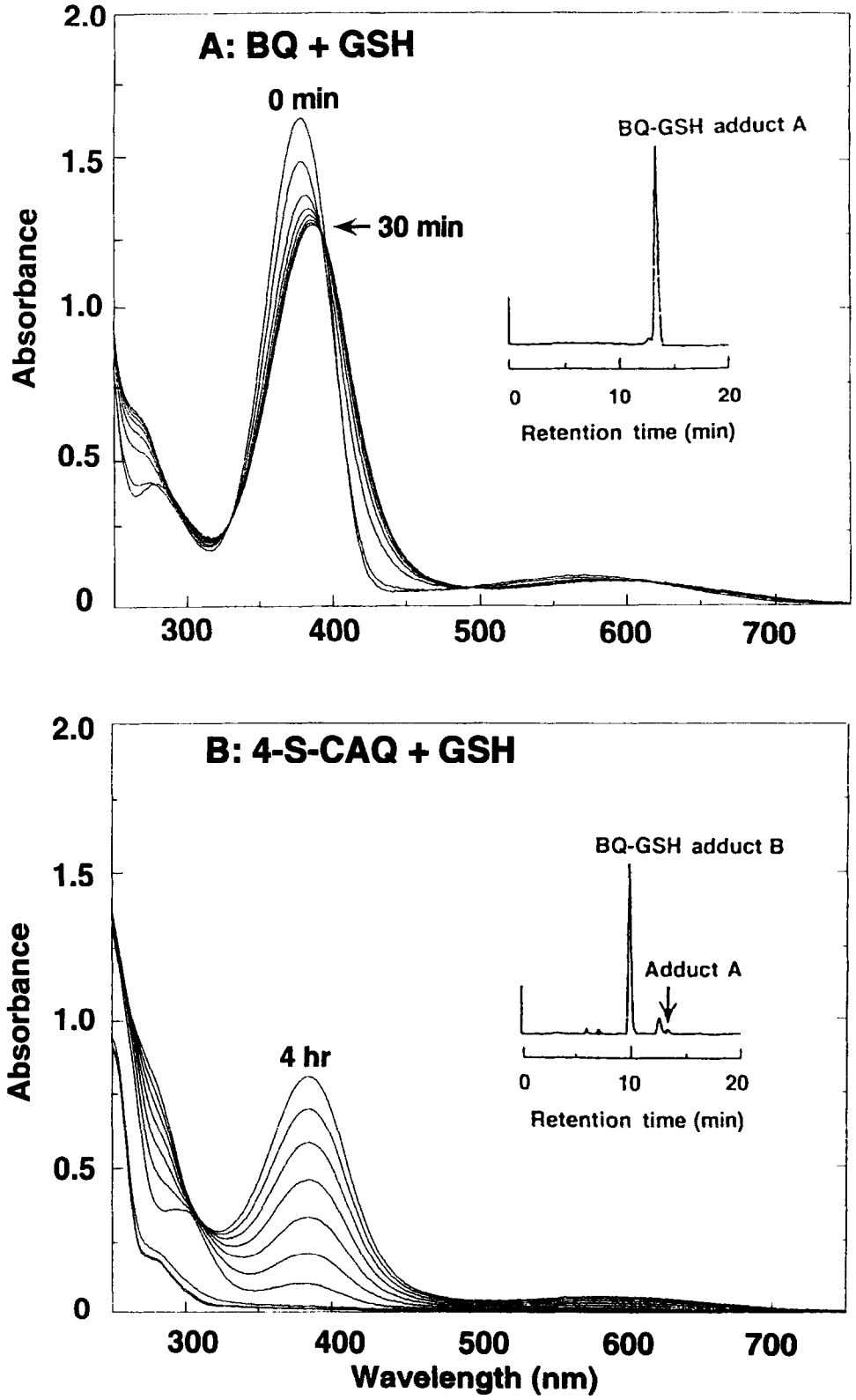

FIG. 4. Reaction of BQ and 4-S-CAQ with GSH. (A) A BQ solution (100 μM in pH 6.8 buffer) was prepared by oxidation of 4-S-CAP with tyrosinase (5 μg/mL). To this, GSH (200 μM) was added, and spectra were taken immediately thereafter and at intervals of 5 min at 37°. (B) A 4-S-CAP solution (100 μM in pH 6.8 buffer) was oxidized with tyrosinase (20 μg/mL) in the presence of GSH (200 μM). Spectra were taken at intervals of 30 min at 37°. The insets show HPLC chromatograms of the reaction mixture at 30 min for BQ and at 4 hr for 4-S-CAP. Retention times for BQ, the adduct A, and the adduct B were 10.4, 13.4, and 9.9 min, respectively.

CAP (and 4-S-CAC) is due to the inhibition of SH enzymes essential for cell survival through covalent binding to 4-S-CAQ (Fig. 1) [6]. We therefore investigated whether BQ is able to bind to SH enzymes through a similar mechanism. Figure 4A shows that BQ rapidly (more than 50% reaction in 5 min) reacted with GSH, thereby forming another violet pigment with slight bathochromic shifts of absorption maxima to 386 and 585 nm. The reaction probably proceeds via the addition of GSH through the SH group followed by a rapid oxidation to the quinone form (as shown in Fig. 1), based on the following evidence. HPLC analysis of the reaction products showed that two isomeric

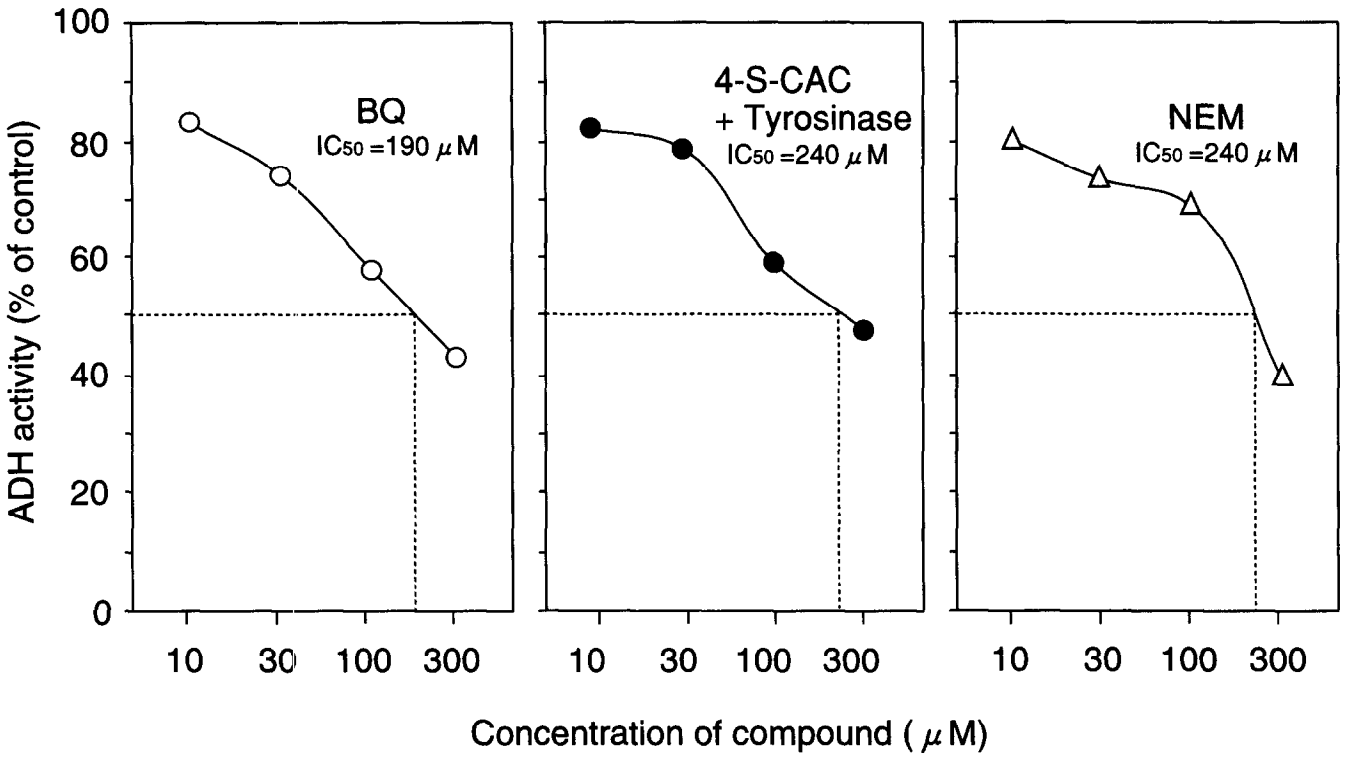

FIG. 5. Comparison of inhibition of ADH activity by BQ, 4-S-CAQ, and NEM. ADH (1 μM as the monomer; 35 μg/mL) was incubated in buffer (pH 6.8) for 30 min at 37° with the indicated concentration of preformed BQ (prepared by 5 μg/mL tyrosinase), 4-S-CAC in the presence of 5 μg/mL tyrosinase, or NEM. Residual ADH activities were measured as described in Materials and Methods. Results are averages for two separate experiments, which gave reproducible results. Control ADH activity was 92 ± 1.1 nmol ethanol oxidized/min/mg protein.

adducts were formed in a ratio of 83:1 (Fig. 4A inset). We assigned the 8-S-substituted structure, adduct A, to the major isomer because the 5 position is less reactive due to the electron-donating effect of the NH group. Cysteine and thioglycolic acid were also found to react rapidly with BQ (data not shown). The following amino acids having functional groups were found not to react with BQ: cystine, methionine, lysine, arginine, histidine, tryptophan, and aspartic acid.

Reaction of 4-S-CAQ, a transient intermediate, with GSH was examined next. Tyrosinase oxidation of 4-S-CAP in the presence of GSH proceeded rather slowly, giving a violet pigment with an absorption spectrum similar to BQ–GSH adduct A (Fig. 4B). HPLC analysis of the reaction products showed that BQ–GSH adduct A was a minor product (Fig. 4B inset). We assigned the 5-S-substituted structure, adduct B, to the major isomer because we previously showed that the addition of cysteine to 4-S-CAQ occurs mostly at the 6 position [6]. The ratio of BQ–GSH adduct A:adduct B was approximately 1:34.

We next studied the inhibitory effect of BQ on the activity of the SH enzyme ADH. An IC_{50} value of 190 μM was obtained for preformed BQ against 1 μM ADH, which was close to an IC_{50} value of 240 μM for 4-S-CAC in the presence of tyrosinase (Fig. 5). This suggests that BQ rather than 4-S-CAQ is the ultimate inhibitor of SH enzymes because the *N*-acetyl derivative of 4-S-CAQ, *N*-acetyl-4-S-CAQ, is known to react extremely rapidly with thiols [17]. Although a rather high concentration of BQ was required to inhibit the ADH activity, this was not surprising because NEM, a potent SH reagent [18], also had a similar IC_{50} value of 240 μM.

In Vitro *Studies*

We compared the cytotoxicity of preformed BQ against B16-F1 melanoma cells with that of its precursors. As shown in Fig. 6, BQ was much more cytotoxic, with an IC_{50} value of 3.9 μM, as compared with 4-S-CAP (IC_{50} of 507 μM) and 4-S-CAC (IC_{50} of 15.8 μM). 4-S-CAC became significantly less cytotoxic in the presence of catalase and SOD, with an IC_{50} value of 353 μM.

The effects of catalase and SOD on the cytotoxicity of 4-S-CAC were examined next. A 1-hr exposure of B16 melanoma cells to 15 μM 4-S-CAC resulted in a 50% inhibition of growth rate, as expected (Fig. 7). Catalase partly protected B16 cells from the cytotoxicity of 4-S-CAC, since the cell number was recovered to 72% of the control in the presence of 100 μg/mL catalase. SOD was much more effective in preventing the cytotoxicity of 4-S-CAC; cell number was increased to 90% of the control in the presence of 100 μg/mL SOD. These results, taken together with those reported in Fig. 3, do not necessarily mean that the superoxide radical exerts a direct cytotoxic

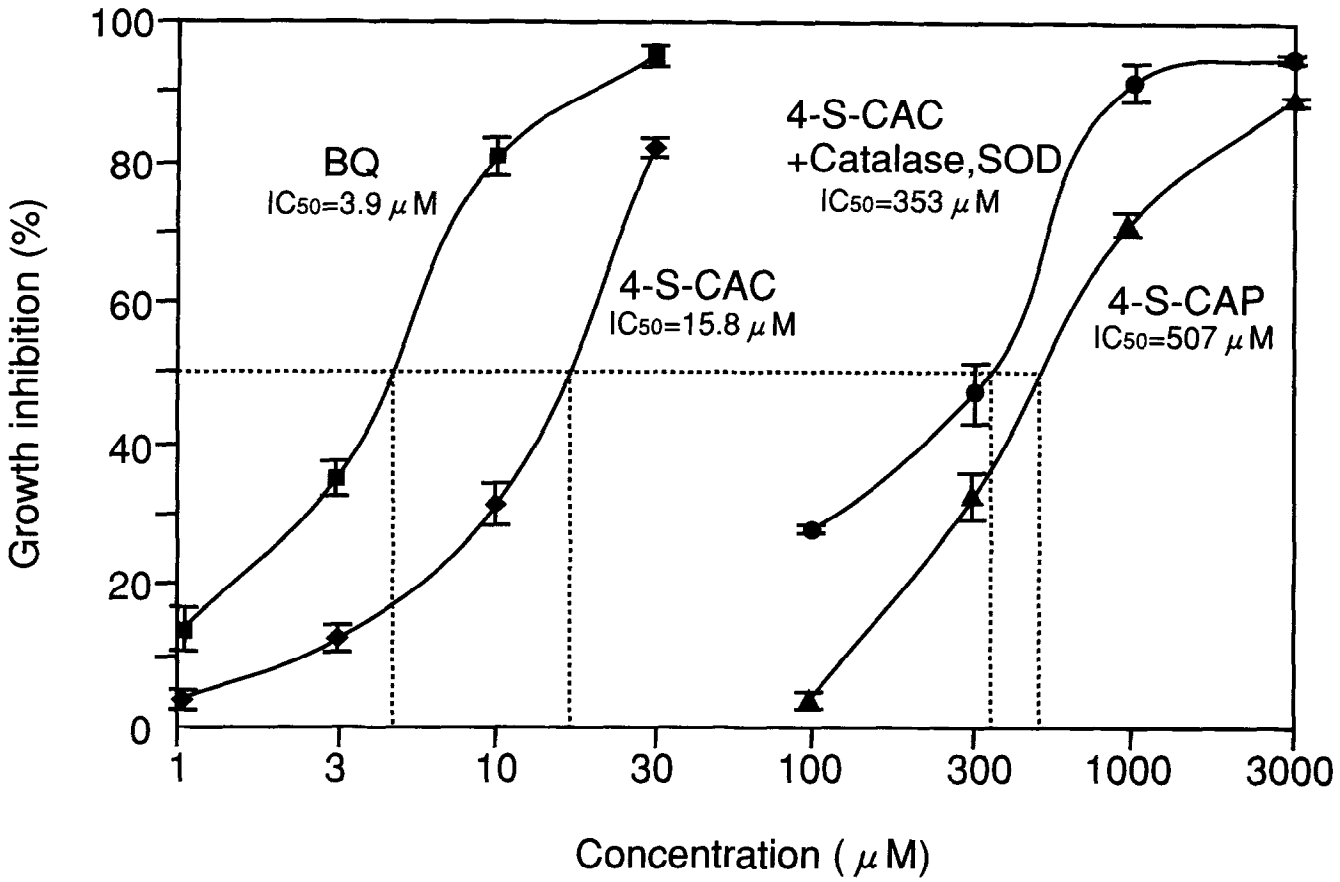

FIG. 6. Comparison of cytotoxicities of BQ, 4-*S*-CAP, and 4-*S*-CAC toward B16-F1 melanoma cells *in vitro*. Two hundred thousand B16-F1 melanoma cells were plated in 60-mm Falcon petri dishes in RPMI 1640–10% fetal bovine serum. Twenty-four hours after inoculation, the medium was replaced with serum-free RPMI 1640 medium, and the cells were exposed for 60 min to preformed BQ, 4-*S*-CAP, 4-*S*-CAC, or 4-*S*-CAC in the presence of catalase and SOD (each 100 μg/mL) at the 4 concentrations indicated. The solution of preformed BQ (100 μM) was prepared by oxidation of 4-*S*-CAP with tyrosinase (5 μg/mL) for 30 min at 37°. After 60 min of exposure, the medium was replaced with RPMI 1640–10% fetal bovine serum and incubated for 48 hr. Then cells were harvested by trypsinization and counted. Cell numbers relative to the control were calculated on the basis of three separate experiments, with each experiment being performed in duplicate. Data are expressed as means ± SEM. Control cell number was 17 ± 0.4 × 10^5/dish.

effect. Rather, they can be explained by assuming that the superoxide radical formed upon autoxidation of 4-*S*-CAC mediates the production of BQ and hydrogen peroxide outside of the cells, both of which would exert the cytotoxic effects of 4-*S*-CAC. Cytotoxicity of preformed BQ (10 μM) was not prevented by the addition of catalase and SOD (each 100 μg/mL), indicating that active oxygens, if produced outside of cells, do not play a significant role in BQ cytotoxicity.

Uptake and metabolism of BQ and 4-*S*-CAP were examined next. When B16 melanoma cells were incubated with BQ (10 μM), BQ was not detected in the cells, but the BQ–GSH adduct A was produced rapidly, with a maximum level of 0.74 nmol/10^6 cells at 15 min (Fig. 8A). The fate of the BQ–GSH adduct thereafter is not clear at present. The intracellular GSH level decreased rapidly in the first 5 min and decreased gradually thereafter to 20% of the original level in 60 min. When incubated with 4-*S*-CAP (1 mM), B16 melanoma cells rapidly took up 4-*S*-CAP, with a maximum level of 23 nmol/10^6 cells at 5 min (Fig. 8B). The 4-*S*-CAP content decreased slightly thereafter, indicating saturation within 5 min. HPLC analysis of the 4-*S*-CAP metabolites showed that BQ–GSH adduct A, but not adduct B, was produced gradually with a maximum level of 0.34 nmol/10^6 cells at 60 min. BQ again was not detected at any incubation time. The intracellular GSH level decreased gradually and almost linearly to 30% of the original level in 60 min. Addition of phenylthiourea (100 μM), a potent tyrosinase inhibitor [19], to the 4-*S*-CAP-containing medium completely inhibited the formation of adduct A (<0.01 nmol/10^6 cells at 60 min) and prevented the decrease in GSH level (data not shown). These results unambiguously indicated that the formation of BQ–GSH adduct A and the decrease in GSH level were tyrosinase dependent. When melanoma cells were incubated with 4-*S*-CAC (500 μM) in the presence of catalase and SOD (each 100 μg/mL), BQ–GSH adduct A was produced slowly with a maximum level of 0.16 nmol/10^6 cells at 60 min.

In both experiments with BQ and 4-*S*-CAP, the rates of decrease in GSH levels were faster than the rates of

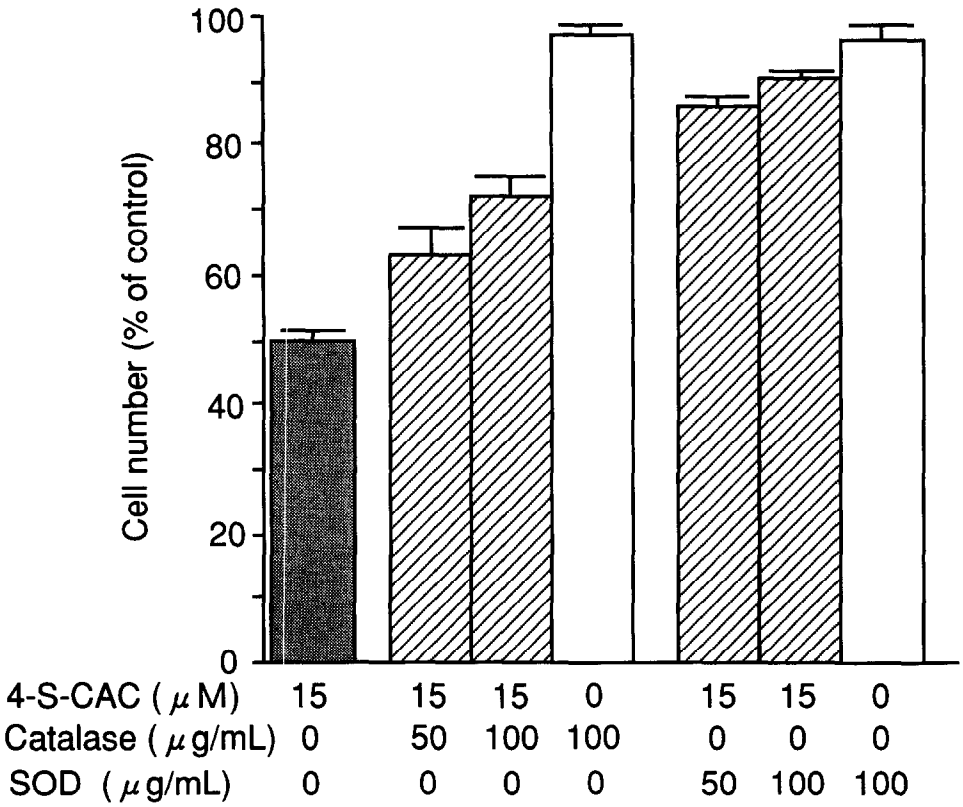

FIG. 7. Effects of catalase and SOD on the cytotoxicity of 4-S-CAC. Twenty-four hours after the inoculation of 2×10^5 B16-F1 melanoma cells as detailed for Fig. 6, the medium was replaced with serum-free medium containing the indicated concentrations of catalase or SOD. Ten minutes later, an aqueous solution of 4-S-CAC · HCl was added to a final concentration of 15 μM. After a 60-min incubation, the medium was replaced, the cells were allowed to grow for 48 hr, and then were counted, as detailed for Fig. 6. Control cell number was $16 \pm 0.7 \times 10^5$/dish.

BQ–GSH adduct formation. This could be explained by assuming that formation of one molecule of BQ–GSH adduct A produces one molecule of hydrogen peroxide by autoxidation of the reduced form of the adduct. Hydrogen peroxide thus formed may be decomposed by the action of glutathione peroxidase with a concomitant decrease in GSH level.

In Vivo *Studies*

We examined the growth inhibition of B16 melanoma tumors *in vivo* by 4-S-CAP, 4-S-CAC, and BQ by directly injecting the compounds into s.c. tumor masses. As shown in Fig. 9A, 25 μmol of 4-S-CAP or 4-S-CAC was equally effective in inhibiting the growth of B16 melanoma, with 73 and 77% inhibition, respectively. The effects were dose-dependent; 10 μmol of 4-S-CAP or 4-S-CAC inhibited tumor growth by 48 and 16%, respectively. BQ at a dose of 0.5 μmol inhibited tumor growth of B16 melanoma (80%) nearly as effectively as 20 μmol of 4-S-CAP (93%) or 4-S-CAC (85%, Fig. 9B). Higher doses of BQ could not be tested because of its low solubility. 4-S-CAP was less active than 4-S-CAC in the *in vitro* system (Fig. 6), but the *in vivo* antitumor effect of 4-S-CAP was comparable to or better than 4-S-CAC. This discrepancy, however, became negligible in the cytotoxicity of 4-S-CAC in the presence of catalase and SOD (Fig. 6). In the *in vivo* system, the lower efficacy of 4-S-CAP as a substrate for tyrosinase compared with 4-S-CAC [13] might be compensated for by a higher uptake into melanoma cells because of a higher lipophilicity.

DISCUSSION

Mascagna *et al.* [5] have shown that enzymic (tyrosinase) or chemical oxidation of 4-S-CAP or 4-S-CAC leads to the formation of the hitherto unknown quinone BQ. The present study has shown clearly that BQ is produced in melanoma cells when incubated with its precursor 4-S-CAP. BQ is 10–100 times more toxic to melanoma cells *in vitro* and *in vivo* than its precursors 4-S-CAP and 4-S-CAC, indicating that BQ is the ultimate cytotoxic metabolite derived from 4-S-CAP and 4-S-CAC. It is very likely that the oxidation of 4-S-CAP to BQ in melanoma cells is catalyzed by tyrosinase, as 4-S-CAP is a good substrate for mammalian tyrosinase [13]. BQ reacts rapidly with cellular GSH to form the BQ–GSH adduct A, thereby depriving melanoma cells of GSH. BQ that escapes from GSH detoxification may react with SH enzymes essential for cell proliferation and survival. In the *in vitro* experiments, serum-free medium was used to avoid the oxidative deamination of 4-S-CAP and production of hydrogen peroxide catalyzed by monoamine oxidase that is present in fetal bovine serum [12].

Cooksey *et al.* [20] have proposed that the tyrosinase-dependent cytotoxicity of 4-substituted phenols originates, at least in part, in the reactivities of the derived *o*-quinones with crucial SH groups within cells. In the present study, we have shown that an *o*-quinone, BQ, conjugates with a number of highly reactive thiols and binds to ADH, an SH enzyme, thereby inhibiting the enzyme activity. Previously, Prezioso *et al.* [21] reported that "oxidized 4-S-CAP" (most

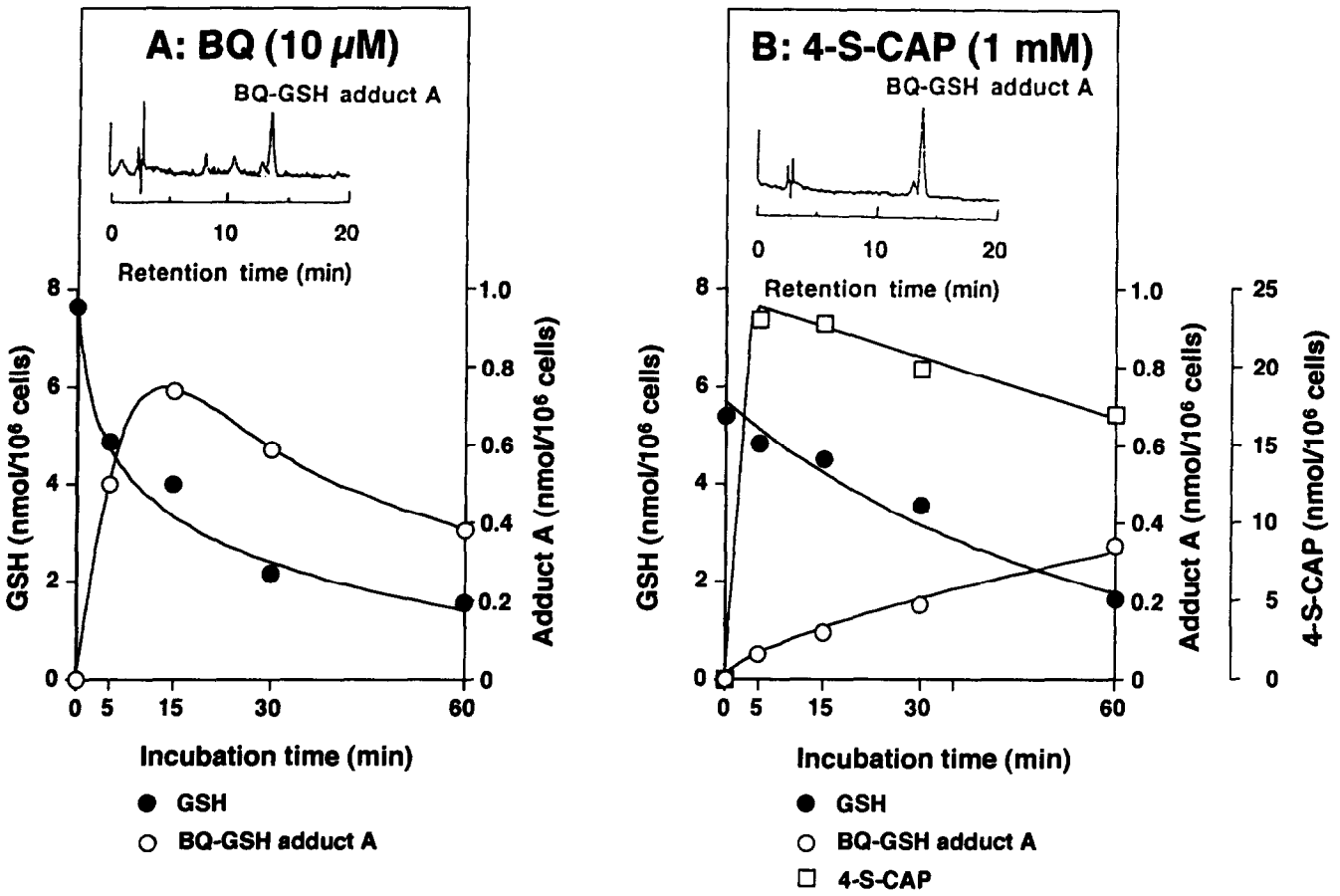

FIG. 8. Uptake and metabolism of BQ and 4-*S*-CAP in B16 melanoma cells. Five hundred thousand B16-F1 melanoma cells were plated in 60-mm Falcon petri dishes in RPMI 1640–10% fetal bovine serum. Twenty-four hours later, the medium was replaced with serum-free medium containing BQ (10 μM) or 4-*S*-CAP (1 mM) and incubated for 5, 15, 30, or 60 min. The cells were then washed two times with phosphate buffered saline, pH 7.2, and disrupted in 0.4 M perchloric acid (1 mL) with a cell scraper. After a further disruption with an ultrasonic cell disrupter, supernatants were analyzed for the 4-*S*-CAP taken up into cells (for experiment B) and for the BQ–GSH adducts produced in the cells. GSH contents in the cells were also analyzed. (A) Uptake and metabolism of BQ (10 μM). (B) Uptake and metabolism of 4-*S*-CAP (1 mM). The insets show HPLC chromatograms at 15 min for BQ and at 60 min for 4-*S*-CAP, indicating the formation of BQ–GSH adduct A. Note that BQ–GSH adduct A is the major isomer not only from BQ but also from 4-*S*-CAP. BQ itself was not detected. The results reported are from a single experiment performed in duplicate; independent experiments gave similar results. GSH contents at 0 min were 7.6 and 5.4 nmol/10^6 cells for (A) and (B), respectively. The corresponding cysteine contents at 0 min were 0.16 and 0.14 nmol/10^6 cells, respectively.

likely BQ) inhibited thymidylate synthase in cell-free extracts with an IC_{50} value of <10 μM, while 5 mM GSH significantly blocked the inhibition with an IC_{50} value of 175 μM. Taken together, these results strongly suggest that BQ exerts its cytotoxicity through covalent binding to SH enzymes, such as thymidylate synthase, that are essential for DNA synthesis and cell proliferation.

GSH depletion itself would augment cytotoxicity of drugs to melanoma cells. Alena *et al.* [22] have shown that BSO is strongly cytotoxic to B16-F10 melanoma cells, with an IC_{50} value of 61 μM. BSO is a specific inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzymes of GSH synthesis [23]. Melanoma cells become more vulnerable to the melanocytotoxic action of *N*-acetyl-4-*S*-CAP [24] and to oxidative stress after GSH depletion [25]. Therefore, enhancement of the antimelanoma effects of 4-*S*-CAP and 4-*S*-CAC (or BQ) would be expected by combined administration of BSO.

The cytotoxicity of catechols has been ascribed to two mechanisms: production of active oxygen species upon autoxidation [26] versus covalent binding of quinone oxidation products [27]. Graham *et al.* [27] and Tiffany-Castiglioni *et al.* [28] showed that 6-hydroxydopamine, a potent neurotoxin, kills neuroblastoma cells through the production of active oxygen species, especially hydrogen peroxide, while for dopamine the reaction of quinone oxidation products with SH enzymes also contributes to the cytotoxicity.

The present study has demonstrated that autoxidation of 4-*S*-CAC resulted in the production of BQ and hydrogen peroxide as final products and that both of these reactive species contributed to the cytotoxicity of 4-*S*-CAC. SOD

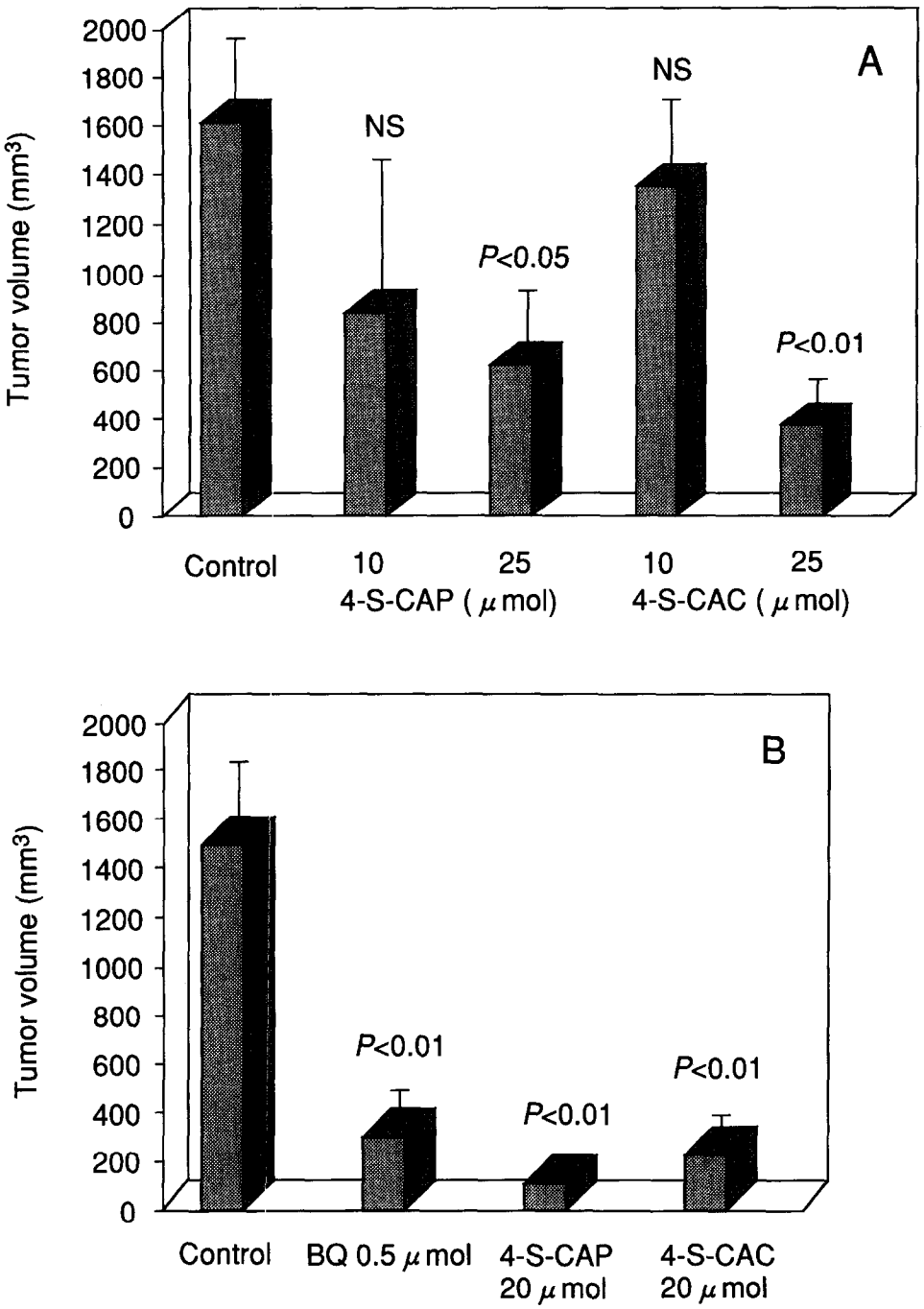

FIG. 9. Comparison of growth inhibition of B16 melanoma by BQ, 4-S-CAP, and 4-S-CAC *in vivo*. Compounds were injected s.c. into B16 melanoma tumors grown to a volume of approximately 100 mm^3 (9 days after tumor inoculation). (A) Control mice received a single injection of 100 μL vehicle (0.9% NaCl), and treated mice received a single injection of 100 μL vehicle containing the indicated amount of 4-S-CAP · HCl or 4-S-CAC · HCl. (B) Control mice received a single injection of 100 μL vehicle (0.9% NaCl-dimethyl sulfoxide, 8:2, by vol.), and treated mice received a single injection of 100 μL vehicle containing the indicated amount of BQ, 4-S-CAP · HCl or 4-S-CAC · HCl. Dimethyl sulfoxide was used to dissolve BQ, which is virtually insoluble in water. In the treated mice, some tumors became necrotic and did not grow during the observation periods; the tumor volume for those tumors was considered to be 100 mm^3. The number of tumors that became necrotic were 3/5 for mice treated with BQ, 5/5 for mice treated with 4-S-CAP, and 3/5 for mice treated with 4-S-CAC. NS; not significant.

almost completely inhibited BQ formation and hence cytotoxicity. The generation of BQ and hydrogen peroxide upon autoxidation of 4-S-CAC may proceed through several steps starting with reaction (1), similar to those seen in the autoxidation of 6-hydroxydopamine [28, 29]:

$$4\text{-S-CAC} + O_2 \rightarrow 4\text{-S-CAQ}^{\cdot} + O_2^{\cdot-} + 2H^+ \qquad (1)$$

SOD appears to terminate reactions producing not only 4-S-CAQ (and hence BQ) but also hydrogen peroxide (except for a trace amount of hydrogen peroxide that was formed by dismutation of superoxide radicals). This is why SOD is much more effective than catalase in preventing the cytotoxicity of 4-S-CAC. Autoxidation of 4-S-CAC produces hydrogen peroxide concomitant with BQ, and thus it appears likely that in studies using cultured cells, both the covalent binding of BQ and the toxic effect of hydrogen peroxide play comparable roles in exerting the cytotoxicity of 4-S-CAC.

In a previous study, we suggested that tyrosinase oxidation of 4-S-CAP produces a highly reactive 4-S-CAQ, which conjugates to some extent with SH enzymes, thus exerting cytotoxic effects [6]. This proposal was based on the observation that acid hydrolysis of reaction products

between 4-S-CAQ and ADH yielded a product of covalent binding through cysteine residues. Although this binding might actually take place, its extent should not be high because the intramolecular cyclization to form dihydroBQ proceeds very fast. The present study has clearly shown that BQ but not 4-S-CAQ conjugates with GSH when produced intracellularly in melanoma cells. It will be interesting to determine how 4-S-CAQ escapes from reacting with GSH and undergoes intramolecular cyclization to form BQ. BQ, once formed, is a rather stable quinone, yet it has a reasonably good reactivity toward SH enzymes.

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