Selective in Vivo Accumulation of N-Acetyl-4-S-cysteaminylphenol in B16F10 Murine Melanoma and Enhancement of Its in Vitro and in Vivo Antimelanoma Effect by Combination of Buthionine Sulfoximine

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ABSTRACT

In order to develop a new chemotherapeutic agent based on exploitation of the specific metabolic pathway of malignant melanoma, a phenolic thioether, N-acetyl-4-S-cysteaminylphenol (NA-CAP), the substrate of melanin-forming enzyme, tyrosinase was developed. Our previous in vivo studies have clearly shown that this compound has a significant and selective melanocytoxicity and antimelanoma effect. This study further examined the specificity of the antimelanoma effect of NA-CAP through the study of biodistribution and accumulation of NA-CAP in B16F10 melanoma-bearing mice. We also tested the antimelanoma effect of NA-CAP by combination treatment with buthionine sulfoximine on the growth of in vitro culture cells and in vivo B16F10 melanoma lung colonies. We found a selective accumulation of 14C-labeled NA-CAP into s.c. transplants and lung colonies of melanoma grown in C57BL mice. This accumulation was mediated by selective covalent binding of NA-CAP to the melanoma tissues. The combination of NA-CAP and buthionine sulfoximine significantly increased the chemosensitivity of B16F10 melanoma cells in vitro and reduced the number of in vivo melanoma lung colonies. We conclude that NA-CAP acts as an alkylating agent to melanoma tissue and that the combination of buthionine sulfoximine enhances the therapeutic index of this potent melanoma-specific drug through the depletion of tissue glutathione.

INTRODUCTION

Melanin synthesis is a biological property unique to pigment cells and highly expressed in malignant melanoma. This metabolic pathway is mediated by tyrosinase (EC 1.14.18.1) and its related enzymes (1). In our attempt to develop a melanoma-specific chemotherapeutic agent with agents through the exploitation of this melanogenic pathway, an N-acetyl homologue of CAP,3 NA-CAP, was synthesized. We have shown that NA-CAP is a tyrosinase substrate (2). A single i.p. administration of NA-CAP results in a selective destruction (necrosis) of pigment cells (melanocytes) in hair follicles of black mice, and multiple exposure to this drug causes a significant reduction in the size of s.c.-inoculated B16F10 melanoma tissues (3) as well as the number and size of B16F10 melanoma colonies in lungs (4). This in vivo antitumor effect also appears to be selective in human tissues inasmuch as the s.c. transplantation experiment using athymic nude mice has clearly shown a significant growth retardation of human melanoma but not control of human ovarian carcinoma (5).

Recently, a number of studies have indicated that GSH is an important limiting factor for the therapeutic efficacy of drugs to cancer cells (6). GSH is an intracellular non-protein sulfhydryl compound which acts as a major component of the cellular defense against toxic challenges such as ionizing radiation, hyperthermia, and cytotoxic drugs. Thus, the depletion of GSH in cells and tissues can increase the susceptibility of cancer cells to both radiation and/or chemotherapy (7).

The role of GSH in the synthesis of melanin pigment by normal melanocytes has been fairly extensively studied, but the major function of GSH in the neoplastic counterpart of melanocytes, melanoma cells, has not yet been clarified (8, 9). This is partly related to the fact that GSH is directly involved in the synthesis of melanin pigment in melanoma cells where the two types of melanin pigment, brown-black eumelanin and yellow-red pheomelanin, are synthesized simultaneously to form a "mixed-type melanin" (10). The synthesis of the latter pigment requires GSH or other sulfhydryl compounds. Therefore, GSH can be involved in both melanin synthesis and cellular defense against cytotoxic drugs in melanoma cells. Previous studies have demonstrated that administration of BSO, a specific inhibitor of y-glutamlycysteine synthetase, the rate-limiting enzyme of the GSH synthesis, is a useful method in elucidating the role of GSH in cells (11). Kable et al. (12) and Karg et al. (13) were the first investigators who reported a significant sensitivity of melanoma cells growing in vitro to BSO as well as an increased cytotoxicity of redox agents to these cells in combination with BSO. Recently, Prezioso et al. (14) and Thrall and Meadows (15) confirmed the importance of GSH for melanoma cells by presenting an increased antimelanoma effect of synthetic catechols by BSO.

In order to clarify the mechanism of cytotoxicity of NA-CAP to melanoma cells, we first examined the in vivo biodistribution/accumulation of NA-CAP in melanoma and normal tissues in mice. Next, we studied the involvement of GSH in the in vitro and in vivo chemosensitivity of melanoma cells against NA-CAP using BSO. We now report a selective accumulation of NA-CAP in melanoma tissues and a marked enhancement of the antimelanoma effect of NA-CAP in combination with BSO.

MATERIALS AND METHODS

Chemicals. NA-CAP and its 14C-labeled homologue were synthesized in our laboratory by a conjugation of appropriately substituted thiophenols with 2-methyl-2-oxazolines using the Wehmeister reaction (16, 17). The purity of the drugs was confirmed to 99.9% by high-performance liquid chromatography measurements. BSO was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Melanoma Cell Line. The origin and growth characteristics of a murine B16F10 melanoma cell line with a high affinity to form lung melanoma colonies were described previously (3). The cells were grown as monolayers in T-75 flasks in minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated in 37°C in a humidified atmosphere of 5% CO₂ in air.

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3 The abbreviations used are: CAP, cysteaminylphenol; NA-CAP, N-acetyl-4-S-cysteaminylphenol; BSO, buthionine sulfoximine; GSH, glutathione; MT, 3,4,5-tri methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ANOVA, analysis of variance; IC₅₀, 50% inhibitory concentration.

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In Vivo Antimelanoma Assay. Cell growth in the presence or absence of experimental drugs was determined using a modified MTT-microculture tetrazolium assay described by Mosmann (18). Briefly, exponentially growing B16F10 cells were harvested, counted for cell viability, and inoculated at the concentration of 5 x 10^4 cells/well (100-μl volume) into 96-well microtiter plates (Costar, Cambridge, MA) using a multichannel pipet. After 24 h, water solutions of drugs were added (100-μl volume) to triplicate culture wells. Cells were cultured for the next 5 days. MTT stock solution was prepared at 5 mg/ml concentration in Dulbecco’s PBS (GIBCO) and stored at 4°C. On day 6, 50 μl of MTT solution, diluted to 1 mg/ml using minimal essential medium without serum, were added to each well. After a 4-h incubation, the supernatant from wells was removed, 150 μl of 100% dimethyl sulfoxide (spectrophotometric grade) were added to dissolve MTT-formazan product, and absorbance at 540 nm was measured with a microplate reader (EAR 400 AT).

The percentage of survival fraction of cells was calculated using:

\[
\% \text{ survival fraction} = \frac{\text{Absorbance in wells with drug}}{\text{Absorbance in control wells without drug}} \times 100%
\]

In Vivo Covalent Binding. Mice having both B16F10 melanoma lung colonies and s.c. melanoma tumors were given a single i.p. injection of [14C]NA-CAP (2.0 mmol/kg; 5.0 μCi). The control animal was chosen for the group without having any B16F10 melanoma transplant. These animals were sacrificed 48 h postinjection by cervical dislocation and used for measurements of the covalent incorporation of NA-CAP into tissues as described previously (19). Briefly, lungs with and without B16F10 melanoma colonies, s.c. melanoma tumors, kidneys, and livers were excised, rinsed in saline solution, blotted dry, immediately frozen with dry ice, and stored at −90°C. Tissues were homogenized with 0.05 M neutral phosphate buffer (1:4, w/v) and precipitated with 20% trichloroacetic acid. All precipitates were extensively washed with methanol-water solution (eight times volume). When the radioactivity of supernatants reached the background levels (about six washes), the tissue precipitate was dissolved in 1 N NaOH and aliquoted in Aquasol-2 (NEN Research Products, Boston, MA); the amount of the bound radiolabeled metabolite was determined using a Beckman LS 3801 liquid scintillation counter (Beckman). Protein concentrations were measured using the Bradford assay (Bio-Rad). Covalently bound radioactivity was expressed as nmol of radioactivity per mg of tissue protein.

Whole-body Autoradiography. Female C57BL/6J mice (aged 5 weeks; The Jackson Laboratory, Bar Harbor, ME) having both B16F10 melanoma lung colonies and s.c. melanoma nodules received a single i.p. injection of [14C]NA-CAP (2.0 mmol/kg; 5.0 μCi) and were sacrificed at 48 h postinjection by inhalation of carbon dioxide. The method of whole-body autoradiography was described previously (20). Briefly, the animals were mounted in a gel of carboxymethyl cellulose and frozen at −90°C; sections were cut 20 μm thick. The sections were freeze dried and processed to whole-body autoradiography by their opposition against X-ray film for 4 weeks.

In Vivo Antimelanoma Bioassay Experimental Protocols. Murine B16F10 melanoma cells were cultured to subconfluent state and harvested. Cell viability was determined by trypsin blue exclusion test. On day 0, 5 x 10^4 cells in 0.2 ml PBS were given i.v. to C57BL mice through the lateral tail vein. On day 5, the mice were randomized in four treatment groups, five animals each, and subjected to therapeutic regimens. The test drugs were dissolved in normal saline, sterilized by membrane filtration, and administered i.p. daily for 15 days. Control mice (group 1) were given normal saline solution; group 2 mice were given saline with NA-CAP 2.0 mmol/kg; group 3 mice received saline with BSO 2.0 mmol/kg; and group 4 mice received saline with a combination of NA-CAP 2.0 mmol/kg and BSO 2.0 mmol/kg. In addition, the groups 3 and 4 were supplemented with 30 mM BSO in drinking water. On day 21, mice were sacrificed by cervical dislocation, and their lungs were removed. The number of melanoma lung colonies was counted, and the size of the colonies was measured under a dissecting microscope. The lungs were weighed immediately after removal. The results for the lung colony formation assay were expressed as a percentage of the control group, b/a x 100%, where a is a value in the control group and b is a value in the experimental group.

Glutathione Assay. Mice were given a single s.c. or i.v. injection of 5 x 10^6 B16F10 melanoma cells in 0.2 ml of PBS and then were randomly distributed into four groups, each group having five animals. On day 15 after the cell inoculation, each animal received a single i.p. injection of drug solution with the following specifications: group 1 mice were given normal saline solution; group 2 were given saline with NA-CAP 2.0 mmol/kg; group 3 received saline with BSO 2.0 mmol/kg; and group 4 mice received saline with NA-CAP 2.0 mmol/kg plus BSO 2.0 mmol/kg. Two h after the i.p. injection, animals were sacrificed by cervical dislocation, and their s.c. melanoma tumors, lungs, and livers were excised, immediately frozen in liquid nitrogen solution, and processed for GSH determination. The tissue content of GSH was measured by the method of Tietze (21) and Griffith (22). Briefly, frozen tissues were weighed and homogenized in five times volume (w/v) of 1% (w/v) picric acid on ice using a glass-ball manual homogenizer. Homogenates were centrifuged, supernatants were removed, and the total GSH content in tissues was determined by glutathione (disulfide) reductase assay.

Histopathological Procedures. Aseptically removed lungs from each group of animals were placed in 10% formalin solution. Tissues were then embedded into paraffin, sectioned, and stained with hematoxylin and eosin.

Statistics. Statistical analysis in each of experimental groups was made using a computerized statistical program, SPSS/PC+. Multiple comparisons among groups were performed by ANOVA followed by multiple range tests using the method of least significant differences. The evaluation of synergy for the in vitro cytotoxicity studies was done using isobolographic methods (23).

RESULTS

Biodistribution of NA-CAP

In Vivo Covalent Binding. Five different tissues were examined for covalent binding of radio-labeled NA-CAP intermediate(s) (Table 1). The maximum binding of NA-CAP was detected in the s.c. melanoma tumor and melanoma colony-bearing lung tissues. ANOVA and range test analysis showed that radioactivity incorporated into the melanoma-bearing tissues was much greater than in nonmelanoma-bearing tissue, the difference being highly statistically significant (P < 0.001). In contrast, the nonmelanoma-bearing organs (i.e., lung, kidney, and liver) did not reveal any significant accumulation of radio-labels (P > 0.05).

In Vivo Biodistribution by Whole-body Autoradiography. The whole-body autoradiography study confirmed the above covalent binding study. The [14C]NA-CAP was seen only in melanoma-bearing organ and tissue, i.e., the lung with melanoma colonies and the s.c. melanoma tissue. At 48 h after a single i.p. injection of [14C]NA-CAP to the mouse, radioactive material was cleared from the body and was not detectable in any normal organs except the lumen of the large intestine. This finding indicates that a significant detoxication of NA-CAP occurs in the liver and is followed by the excretion of NA-CAP metabolites into the bile. The s.c. melanoma tumor and the lung with melanoma colonies were the only organs displaying a significant accumulation of the radioactivity (Fig. 1).

Cytotoxicity and Antimelanoma Effect of NA-CAP

In Vitro Cytotoxicity of NA-CAP with and without BSO. Fig. 2 shows the in vitro growth inhibition of melanoma cells by a single

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Covalent binding (nmol covalently bound/mg of protein)</th>
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<tbody>
<tr>
<td>Lungs with B16F10 melanoma colonies</td>
<td>0.330 ± 0.118</td>
</tr>
<tr>
<td>Lungs without B16F10 melanoma colonies</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>B16F10 melanoma s.c. tumors</td>
<td>0.521 ± 0.076</td>
</tr>
<tr>
<td>Liver</td>
<td>0.013 ± 0.003</td>
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<tr>
<td>Kidney</td>
<td>0.008 ± 0.001</td>
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* Female C57BL/6J mice received a single i.p. injection of 2.0 mmol/kg of [14C]NA-CAP. The animals were sacrificed at 48 h postinjection, and the covalent binding was determined as described in “Materials and Methods.”

| The data are presented as the means ± SD (n = 3). |
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more effective because the IC₅₀ was 61.0 ± 4.4 µg/ml. To study the in vitro growth inhibition of the two drug combinations in detail, we used an NA-CAP concentration scale from 10.0 to 100.0 µg/ml and BSO concentrations of either 25.0 or 50.0 µg/ml per well. The combination treatment resulted in a further decrease of IC₅₀ with the IC₅₀ for NA-CAP plus BSO 25.0 µg/ml and NA-CAP plus BSO 50.0 µg/ml being 29.2 ± 3.1 and 4.5 ± 0.9 µg/ml, respectively.

The results indicate a high susceptibility of B16F10 melanoma cells to BSO and suggest the importance of the intracellular GSH level for the proliferation and survival of melanoma cells. Isobolographic analysis of the interaction relationship between BSO and NA-CAP when used together is shown in Fig. 3. The analysis reveals that at IC₅₀ concentrations, the combination of the two drugs act synergistically, and the combination effect is more than simply additive. Using the data plotted in Fig. 3 for a BSO concentration of 25 µg/ml the interaction index between BSO and NA-CAP is 0.6071 (23). An interaction index less than unity represents a synergistic relationship with a lower index indicating a greater amount of synergy. This would indicate that BSO and NA-CAP interacted in a highly synergistic way.

In Vivo Antimelanoma Effect of NA-CAP in Combination with BSO on Lung Melanoma Colonies. ANOVA and range test analysis showed that the number of B16F10 melanoma colonies in lungs, the size of the melanoma colonies, and the weight of fresh lung tissue with melanoma colonies were significantly lower in groups treated with either NA-CAP or BSO alone compared to controls (P < 0.05; Figs. 4 and 5). Moreover, the antimelanoma effect of the combination treatment using NA-CAP and BSO together was significantly greater than using either treatment alone for reducing the number of colonies and weight of melanoma-containing lungs (P < 0.05). This combination was also significantly more effective in reducing the size of melanoma colonies than BSO alone (P < 0.05), but compared to NA-CAP alone, the improvement with the combination did not reach any statistical significance.

Importantly, the combination of NA-CAP with BSO did not enhance the systemic toxicity of NA-CAP as evaluated by general observations of the animals and changes in weight. The only observed side effect was a brief hypoactivity of animals which occurred shortly after i.p. injection of NA-CAP and resolved spontaneously in 2 h. The average animal body weight on the last day of experiments was the same as on day zero.

administration of NA-CAP or BSO as well as a combined administration of the two drugs against highly pigmented B16F10 melanoma cells. Using the MTT assay, we measured a dose-dependency of cell survival at day 5 of the continuous drug exposure. The IC₅₀ values for each group of drug treatments were obtained from dose-response curves. The control values for calculation of survival fraction were the same for the single drug as well as combination treatment. The IC₅₀ for NACAP was about 148.0 ± 12.2 µg/ml, which indicated a high chemoresistance of cells to NA-CAP. The treatment with BSO was

![Fig. 1. In vivo whole-body autoradiograph showing the biodistribution of [14C]NA-CAP in a B16F10 melanoma-containing C57BL/6J mouse. a, the unstained tissue slice of the whole body of a C57BL mouse. b, an autoradiogram of the same tissue slice of a.](image-url)

![Fig. 2. In vitro cytotoxicity of NA-CAP with and without BSO. Dose-response curves for NA-CAP, BSO, combination of NA-CAP plus BSO 25 µg/ml, and NA-CAP plus BSO 50 µg/ml tested against B16F10 melanoma cells by the MTT dye reduction assay. An additional data point not shown on this graph is for 200 µg/ml of NA-CAP alone showing a 40.4% survival fraction. The means of IC₅₀ derived from three independent tests, each run in triplicates, were 148.0 ± 12.2 µg/ml for NA-CAP alone; 61.0 ± 4.4 µg/ml for BSO alone; 29.2 ± 3.1 µg/ml for NA-CAP plus BSO 25 µg/ml; and 4.5 ± 0.9 µg/ml for NA-CAP plus BSO 50 µg/ml.](image-url)

![Fig. 3. Isobolographic analysis of BSO versus NA-CAP derived from dose-response curves. The zero interaction isobole represents a purely additive relationship between BSO and NA-CAP which is ineffective to give a 50% survival fraction of cells in vitro (i.e., IC₅₀). However, the points derived from experimental data points for the combined use of BSO and NA-CAP are well below this zero interaction isobole, yielding a concave-up curve which represents synergy between these two agents.](image-url)
Histopathological Changes of Melanoma Colonies in Lungs after NA-CAP Treatment. B16F10 melanoma colonies were in general located around or close to blood vessels in the lung. In the control group, the lung tissue revealed numerous large foci of darkly pigmented cells without significant infiltration with mononuclear cells. The lung melanoma colonies from drug-treated groups revealed histopathological findings basically similar to those of controls, but they were clearly less numerous and smaller. There was no evidence for an increased number of amelanotic colonies in lungs after the drug therapy.

Tissue GSH Content after NA-CAP Treatment with or without BSO. Table 2 presents the effect of various drug regimens on total GSH content. In the lung with melanoma colonies, a single dose of 2.0 mmol/kg of NA-CAP alone or BSO alone caused some depletion of GSH, i.e., 92 or 76% of controls, respectively, at 2 h after an i.p. injection. With a single i.p. injection of NA-CAP plus BSO, the total GSH content in the lung was reduced as much as 48% of the control.

In order to clarify the contribution of melanoma tissue itself in the total depletion of GSH in the melanoma colony-bearing lung, we also measured the GSH content in the s.c. melanoma tumor and the liver of the same animals as well as in the lung of control animals without B16F10 melanoma colonies before and after treatment. NA-CAP alone without BSO treatment depleted GSH content in the lung (without B16F10 melanoma colonies), normal liver, and the s.c. melanoma tissue to 84, 79, and 90% of controls without any treatment, respectively. The treatment with BSO alone decreased the GSH content as much as 59, 42, and 84% of controls in the normal lung (without melanoma colonies), the normal liver, and the s.c. melanoma tissue, respectively. The combination treatment with NA-CAP plus BSO resulted in GSH depletion to 44% in the normal lung (without melanoma colonies), 21% in the normal liver, and 45% in the s.c. melanoma tissue.

By ANOVA and range test analysis, the combination of BSO with NA-CAP showed a significantly higher depletion of GSH in all tissues tested compared to BSO or NA-CAP used alone (P < 0.05), except for one instance in normal lungs, where the added decrease of GSH with the combination compared to BSO alone approached significance (P < 0.065).

Histopathological Changes of Melanoma Colonies in Lungs after NA-CAP Treatment. B16F10 melanoma colonies were in general located around or close to blood vessels in the lung. In the control group, the lung tissue revealed numerous large foci of darkly pigmented cells without significant infiltration with mononuclear cells. The lung melanoma colonies from drug-treated groups revealed histopathological findings basically similar to those of controls, but they were clearly less numerous and smaller. There was no evidence for an increased number of amelanotic colonies in lungs after the drug therapy.
DISCUSSION

In this study, we have demonstrated a significant and specific antitumor property of NA-CAP against murine B16 melanoma. Specifically, both the whole-body autoradiography and covalent binding assays have clearly shown a selective in vivo accumulation of radiolabeled NA-CAP into melanoma tissue. Furthermore, we have found that the selective in vivo antimelanoma potency of NA-CAP was enhanced by a simultaneous administration of BSO, and this combined therapy was not associated with any significant side effect.

BSO is a potent chemosensitizer which increases the susceptibility of cancer cells to chemotherapeutic drugs through depletion of GSH (24). Since the pioneer work of Griffith and Meister in 1979 (25), a number of drugs including melphalan, bleomycin, and many other antineoplastic drugs, have been examined in combination with BSO in vitro and in vivo. Ozols et al. (26) elucidated the relationship between the GSH level and the expression of chemoresistance in human ovarian cancer cells and showed the feasibility of BSO in abolishing the chemoresistance. Currently, several clinical Phase I trial protocols in which BSO is combined with standard chemotherapeutic agents, e.g., L-phenylalanine mustard, have been conducted (27).

In contrast to ovarian carcinoma, the major cause(s) of the chemoresistance of melanoma cells have not yet been clarified. Kable et al. (12) reported a significant sensitivity of melanoma cells to BSO and increased cytotoxicity of L-DOPA in combination with BSO in vitro. Other in vitro studies demonstrated an improved cytotoxicity of 3,4-dihydroxybenzylamine and L-DOPA methylester by the addition of BSO (13, 14). Although the exact mechanism of cytotoxicity of these catecholic drugs is not fully understood, the positive role of BSO in the enhancement of their cytotoxicity is anticipated. Two mechanisms have been postulated for the cytotoxicity of catechols: (a) catechols are enzymatically converted into quinones which may react with intracellular sulfhydryl groups such as those of GSH and many proteins, with a subsequent irreversible damage to cells; and (b) the chemically unstable catechols may produce a severe oxidative stress by the process of autooxidation (28). In both cases, GSH plays a major protective role against cytotoxicity of catechols which is mediated by the action of GSH-dependent enzymes such as glutathione-S-transferases and GSH-peroxidase or by a direct binding with highly reactive chemicals. The depletion of GSH by BSO would diminish the effectiveness of the cellular defense mechanisms, which subsequently increase the cytotoxicity of catechols.

Our phenolic thioether, NA-CAP, is a chemically stable monophenol and a good substrate for mammalian tyrosinase in a cell-free system (3). Tyrosinase converts NA-CAP into catechol and then to ortho-quinone, which may then either produce semi-quinone radicals, causing a selective destruction of melanoma cells or inhibit sulfhydryl enzyme such as DNA polymerase (29) or thymidylate synthase (30). However, our measurement of GSH levels after the treatment with NA-CAP showed a significant GSH depletion in both melanoma and nonmelanoma tissues. Because of the absence of tyrosinase in nonmelanoma tissues, e.g., the liver, the reactive intermediate of NA-CAP may not be a quinone/semi-quinone produced solely by tyrosinase-NA-CAP interaction but should also include various quinone moieties formed from NA-CAP through interaction with non-tyrosinase enzymes such as peroxidase or cytochrome P-450 (31).

The chemical structure of the cytotoxic NA-CAP intermediate(s) presently remains unknown. We, however, expect that NA-CAP is an inert prodrug which is enzymatically activated into a highly reactive electrophilic intermediate(s) (2, 28, 31). The dose-dependent enhancement of the cytotoxicity of NA-CAP by BSO may support our prospective classification of NA-CAP as an alkylating agent (2). The in vivo covalent binding study indicates our premise that NA-CAP acts through alkylation and, furthermore, suggests that the target macromolecules are proteins. In fact, our preliminary study using 4-S-cysteaminylophenol, another compound similar to NA-CAP, suggests that there is such a target protein in CAP-mediated cytotoxicity (32—34). However, the pharmacokinetic process of the observed in vivo selectivity of NA-CAP could be more complex. Our biochemical studies revealed the depletion of GSH after the treatment with each of three drug regimens in melanoma as well as normal tissues. A striking finding was a small difference in the percentages of GSH changes between melanoma and normal nonmelanoma tissues after a single i.p. injection of NA-CAP with or without BSO. A limitation of the method we used to measure GSH levels as proposed by Tietze (21) and Griffith (22) is that the GSH levels are probably overestimated due to the contribution of contaminating erythrocytes within the tissues. Nonetheless, Minchinton et al. (35) similarly reported a relatively slow depletion of GSH by BSO in tumor tissues compared to that in the liver and kidney. It is possible, however, that after multiple administration of the drug, the differences in GSH concentrations may become more significant, and the degree of GSH depletion in melanoma tissue may become higher than that of normal tissues. Another explanation could include a different requirement of GSH for proper metabolic functions of normal nonmelanoma and melanoma cells. Indeed, Meister (36) suggested that BSO may significantly decrease GSH synthesis of both the normal and tumor cells without the alteration of physiological function as well as drug sensitivity of the normal cells. However, the tumor cells, due to their increased need and dependency on GSH, may exhibit abnormalities in their physiological function and sensitization to chemotherapy or radiation (36).

In any case, our in vitro studies showed synergistic cytotoxic effects of the NA-CAP plus BSO combination treatment against B16F10 melanoma cells. This may be of some practical significance since our in vivo studies may indicate that melanoma cells either have a lower critical threshold for GSH depletion to manifest cell damage or reach this critical threshold faster than normal cells. This concept would partly explain the observed selectivity of NA-CAP to melanoma tissues. It may be possible that the conditions for random alkylation of cellular proteins by NA-CAP reactive intermediate(s) occurs in vivo earlier and with a lower drug dose in melanoma cells than in normal cells. Thus, BSO may further speed up the occurrence of the conditions suitable for the alkylation of macromolecules in melanoma cells and, in this manner, enhances the in vivo therapeutic index of NA-CAP.

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