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UNIVERSITY OF ALBERTA

EXPERIMENTAL MELANOMA-SPECIFIC CHEMOTHERAPY USING PHENOLIC THIOETHERS

BY

FRANTISEK ALENA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES

(MEDICINE)

EDMONTON, ALBERTA

FALL 1992

ISBN 0-315-77308-1

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DEGREE:	DOCTOR OF PHILOSOPHY
YEAR DEGREE GRANTED:	FALL 1992

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TO MY WIFE AND CHILDREN FOR THEIR LOVE AND SUPPORT,

TO MY MOTHER AND FATHER FOR ALL THEY'VE DONE FOR ME

ABSTRACT

Malignant melanoma is a fatal disease once metastasized in organs. This is largely related to the fact that existing chemotherapy is mostly inefficient and other modalities of treatment are only palliative.

To improve current melanoma chemotherapy, a new strategy based on the exploitation of the unique enzyme, tyrosinase, in pigment cells has been proposed. It was expected that tyrosinase would convert synthetic phenolic compounds into highly cytotoxic orthoquinones which might then selectively destroy pigment cells.

Based on this concept, a new class of synthetic tyrosinase substrates, phenolic thioethers (PTEs) was synthesized. Using a hair depigmentation and a lung B16F10 melanoma colony-forming assay *in vivo*, PTEs were tested. Of these, N-acetyl-4-S-cysteaminylphenol (NACAP), was found to possess highly selective melanocytotoxic and antimelanoma effects. However, using the MTT cytotoxicity assay, the PTEs were found to be non-selectively cytotoxic *in vitro*. Tyrosinase was shown not to be the major enzyme responsible for the cytotoxicity of PTEs *in vitro*.

PTE toxicity was further studied using an *in vivo* hair depigmentation model. Dose-dependent melanocytotoxicity of NACAP in black mice and the change of melanogenesis from the pheomelanogenic to the eumelanogenic type in yellow mice was found. The depletion of glutathione (GSH) by NACAP in pigment cells and also in non-pigment cells was found to be associated with the cytotoxicity of PTEs. Positive or negative modulations of GSH levels in tissues and cells inhibited or enhanced respectively the melanocytotoxicity or cytotoxicity of NACAP *in vitro* and *in vivo*.

It was an earlier proposal that PTEs are lipophilic prodrugs that are activated into reactive electrophilic intermediate(s) by some drug metabilizing enzyme(s) and tyrosinase. The induction of chemical injury to cells is believed to occur due to alkylation of macromolecules by these reactive intermediates of PTEs metabolism and/or oxidative stress. Melanogenesis is considered to be responsible for the selective toxicity of PTEs in

pigment cells *in vivo* indirectly by lowering a critical threshold for the induction of chemical injury by PTEs.

Tests of NACAP in combination with buthionine sulfoximine (BSO) revealed enhanced antimelanoma effects *in vivo* and *in vitro*. The drug-combination regimen showed only minor systemic toxicity. Moreover, whole-body autoradiography and *in vivo* covalent binding assays showed NACAP to be selectively accumulated in melanoma tissues. It is concluded that a combination of NACAP and BSO might be used for the development of a melanoma-specific chemotherapeutic protocol for clinical use.

ACKNOWLEDGEMENTS

I wish to thank Dr. Bill McBlain for his guidance, support and encouragement throughout my graduate program. The interest in the project and useful advice provided by Dr. Kowichi Jimbow and Committee members is also acknowledged.

I greatly appreciate the stimulating discussions and suggestions provided by Dr. Wally Dixon and my fellow graduate students.

Financial support from the National Cancer Institute of Canada and from the Alberta Cancer Board is gratefully acknowledged.

Finally, a very special thanks to my wife Jane for her love, encouragement and technical help with printing this thesis.

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I. THE ROLE OF CHEMOTHERAPY AND THE DEVELOPMENT OF NEW STRATEGIES FOR TREATMENT OF MALIGNANT MELANOMA

A. INTRODUCTION

Cancer chemotherapy has been primarily directed against tumors which are widespread from the site of origin, or have a known high propensity to metastasize (1). Melanoma is a malignancy which has a tendency to spread throughout the body in the early stage of its development when the primary skin lesion is relatively inconspicuous. For this reason, many patients are detected with an advanced or metastatic form of this disease (2). Metastatic melanoma is a frequently fatal disease because at present there is no effective modality of treatment: surgery and radiation therapy play only a palliative role and provide little long- term benefit to patients, while immunotherapy is still in an experimental stage of development (3). Chemotherapy offers some limited degree of success but exhibits a response rate less than 20-25% (3). In order to help in the treatment of melanoma patients, the improvement of existing chemotherapy is imperative.

Despite improvements to current chemotherapeutic protocols, e.g., new drug-combination regimens, or better dosing of drugs, the systemic toxicity to the host of existing antineoplastic drugs precludes the use of doses high enough to be effective against melanoma (4). One approach in reducing this undesired non-specific drug toxicity is development of drugs designed to selectively act against this specific type of cancer cell. The basis for this selective action is the exploitation of a special property of the target pigment cells.

A unique function of normal as well as neoplastic pigment cells is melanogenesis, a process which forms the pigment melanin (5). During the melanogenic process tyrosinase converts the amino acid L-tyrosine into L-dopa and dopaquinone (6). Dopaquinone is highly reactive and is potentially toxic to pigment cells.

For a long time, the melanogenic pathway has been considered exploitable for designing a rational approach for the selective chemotherapy of melanoma (7). It has also been proposed that tyrosinase may generate toxic intermediates also from synthetic substrates, tyrosine analogues, with lethal consequences to the cells (5). Hence, a range of such compounds, monophenols and catechols, has been examined for melanocytotoxic and antimelanoma properties (8). However, systemic toxicity or unfavourable pharmacokinetics have prevented these agents from use in chemotherapy in patients (9).

In previous studies, 4-S-cysteaminylphenol (4-S-CAP), a phenolic thioether (PTE) and a tyrosinase substrate, was found to possess significant melanocytotoxic and antimelanoma properties (10). However, the high systemic toxicity of this amine necessitated a modification of its chemical structure. In order to reduce the occurence of side effects, four PTEs, derivatives of 4-S-CAP, were synthesized and investigated in an attempt to find an agent with the potential for application in anti-melanoma chemotherapy.

The main objectives of this project were 1) to evaluate the PTEs use as anti-melanoma chemotherapuetic agents; 2) to examine the proposed mechanism of action of PTEs; and 3) to design an experimental drug-treatment protocol with a high efficacy against malignant melanoma using some of the PTEs as principal antimelanoma agents.

For perspective, the current state of knowledge of melanoma is reviewed and standard approaches to the treatment of this disease are outlined. Then, the details of the experimental approaches for the development of melanoma-specific chemotherapy will be described.

B. BIOLOGY OF MALIGNANT MELANOMA

Origin of Melanoma Cells. Melanoma cells are neoplastic pigment cells. Their precursors are melanocytes which originated from neural crest-derived melanoblasts (11). During early stages of gestation, melanoblasts migrate from neural crest to the skin, mucous membranes, uveal tract, and meninges, where they differentiate into melanocytes. Once in the epidermis, melanocytes secrete pigment granules, melanosomes, which are engulfed by keratinocytes, a process that continues throughout life. The melanocytes of hair follicles synthesize and secrete melanosomes to hair keratinocytes periodically, during the growing (anagen) phase of the hair cycle only. All other extracutaneous melanocytes produce measurable amounts of pigment only in embryonic and fetal periods of life and typically do not discharge their melanosomes to other cells (12).

Most melanomas occur in the skin (13) although uveal melanoma is the most common primary cancer of the eye in adults (14).

Melanocytic Nevi and Malignant Melanoma. Cutaneous melanocytic tumors can be divided into melanocytic nevi and malignant melanoma (15). Recent investigations have indicated that melanocytic nevi are a risk factor for melanoma (16). This correlation is most obvious in patients with familial dysplastic nevus syndrome (DMN), who have more than a 15-times higher risk for the development of malignant melanoma than individuals without DMN (17).

However, most malignant melanomas begin *de novo*, without evidence of a precursor, melanocytic nevus (18).

Etiology. The understanding of melanoma etiology is rudimentary. Research has focused on endogenous and exogenous factors that might contribute to the transformation of melanocytes into melanoma cells (19). Many epidemiologic studies suggest that

sunlight, in particular its ultraviolet B range, is the most important environmental factor in the pathogenesis of melanoma (20). In addition, occupational exposure to chemicals and ionizing radiation increases the likelihood of developing melanoma. Other exogenous factors which may be associated with an increased risk of melanoma include dietary factors, viruses, and trauma. A contribution of endogenous genetic factors is indicated by the occurence of melanoma in families and differences in incidence among various racial groups. Another endogenous factor seems to be an endocrine involvement, e.g., more women develop melanoma than men but women have a better prognosis than men (21).

Characteristics of Melanoma Cells. The availability of methods for both isolating and propagating melanocytic cells in culture has allowed comparisons between the characteristics of normal melanocytes and melanoma cells (22). The major differences may be summarized as follows: 1) limited life span of normal melanocytes and nevus cells versus indefinite growth of melanoma cells; 2) inability to grow without attachment to the substrate versus high colony forming efficiency in soft agar; 3) non-tumorigenicity versus tumorigenicity in athymic nude mice; 4) dependence on exogenous growth factors versus autonomous growth in protein-free medium; and 5) diploid karyotype versus non-random chromosomal abnormalities (22).

Importantly, the most prominent function of melanocytes, melanogenesis, is preserved in the majority of melanomas (23).

Melanogenesis. Melanogenesis represents a complex biosynthetic pathway with the final product being the polymeric pigment, melanin. This process occurs exclusively inside a specialized membrane-bound organelle called the melanosome (24).

The essential enzyme of melanin formation is a copper-containing enzyme named tyrosinase (monophenol monooxygenase; monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, E.C. 1.14.18.1). Tyrosinase is an bifunctional enzyme that catalyzes two

consecutive reactions of the melanin biosynthetic pathway: 1) the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-dopa); and 2) the oxidation of L-dopa to dopaquinone (25). Tyrosinase is the critical regulatory (rate-limiting) element in the melanogenic pathway and its pertuberation causes some diseases of skin, e.g., albinism (25). Some melanomas are unable to synthesize melanin.

There are two major classes of melanin pigment, eumelanin and pheomelanin (Figure I-1). Dopaquinone produced from L-tyrosine by tyrosinase is a common precursor for both. The eumelanin pathway starts with cyclization of dopaquinone into indoles, which polymerize into black-brown eumelanin. The pheomelanin pathway is initiated by conjugation of dopaquinone with L-cysteine or glutathione to yield 5-S-cysteinyldopa as a major product. The cyclization of cysteinyldopas gives rise to 1,4-benzothiazine intermediates and ultimately leads to a yellow-red macromolecular pigment, pheomelanin (26). Thus, eumelanin and pheomelanin are biogenetically related, and it is believed that the melanins in nature are mostly of mixed-type which are formed by co-polymerization of eumelanic and pheomelanic intermediates (27).

Further details concerning melanogenesis will be given in the section D of this chapter, under the description of experimental approaches to develop melanoma-specific chemotherapy.

C. CLINICAL FEATURES AND CURRENT THERAPIES OF MALIGNANT MELANOMA

Epidemiology. The incidence of cutaneous melanoma continues to rise throughout the world (18). In North America the incidence of melanoma has almost tripled during the last forty years (28). Melanoma occurs in adults of all age groups; the median age at diagnosis is 53 (19). In young adults, melanoma has the highest incidence rate of any cancer in Caucasians who are 25- to 29-years old (19).

Clinical Types of Melanoma. Primary cutaneous melanomas occur *de novo* from melanocytes located at the epidermo-dermal junction of the skin or from pre-existing melanocytic nevus cells (18). There are four major subtypes; lentigo maligna melanoma (LMM), superficial spreading melanoma (SSM), nodular melanoma (NM), and acral lentiginous melanoma (ALM) (29). Typically, LMM, SSM, and ALM demonstrate an initial radial phase of superficial peripheral growth in the epidermis followed by vertical growth through the skin and later metastatic spread. NM has a minimal radial growth and progresses directly to vertical growth. Other less common types of melanoma are mucosal and extracutaneous melanoma, e.g., eye and leptomeninges (29).

Metastatic melanoma often exhibits rapid growth accompanied by a deformation of the body. It may occur as local metastases, e.g., "satellite" lesions located within 5 cm of the primary site of melanoma, or located beyond this range but not beyond the area of regional lymph node drainage (18). If the metastatic cells escape beyond the regional lymph node drainage area, the pattern of disease may be one of diffuse involvement of many organs. Metastatic melanoma can involve almost all organs: the most commonly affected are the lung, liver, and brain. The metastases into lymph nodes are present in more than 75% of patients with advanced melanoma (18).

Clinical Staging of Melanoma. There are several staging systems for melanoma (30). The most commonly used system employs three stages: 1) stage I melanoma is a primary melanoma without apparent metastases; 2) in stage II melanoma, metastases are present in regional lymph nodes (only); and 3) in stage III melanoma with distant metastases. Clinical staging is important for prognostic and therapeutic purposes (30).

Current Therapy for Malignant Melanoma. Four major modalities of treatment are presently available: surgery, radiotherapy, immunotherapy, and chemotherapy. The role of each form of therapy depends on the stage of melanoma.

Surgery for Melanoma. Surgery is the absolute indication for early stages of melanoma (19). In stage I disease, a wide excision of a primary tumor is curative in more than 90% of cases if the thickness of the lesion is less than 1.0 mm. Surgical intervention is also indicated for patients with stage II melanoma although surgical removal of a tumor along with the radical dissection of regional lymph nodes may be curative in only 20-30% of such patients. In contrast, surgery has a limited role in the treatment of metastatic melanoma. Surgical procedures in patients with stage III melanoma are performed generally for palliative rather than curative purposes (31).

Radiation Therapy for Melanoma.. Currently, radiation therapy is used for the palliative management of patients with melanoma. It is effective for symptomatic relief in patients with painful bone metastases and metastases affecting the central nervous system (32).

Immunotherapy for Melanoma. During the last decade, several new biologic response modifiers, such as recombinant interferon alpha, have shown a significant and reproducible activity against metastatic melanoma (33). Of the investigational therapies, adoptive immunotherapy based on the *in vitro* activation of lymphokine-activated killer (LAK) cells by interleukin-2 has received the greatest attention. The majority of remissions have been, however, partial. Drawbacks of the currently used immunotherapy protocols are their considerable systemic toxicity and the relatively low response rate (15% to 25%). For this reason, improvement of immunotherapy is necessary to advance from its present investigational stage to its routine use in clinical medicine (34).

Chemotherapy for Melanoma. Chemotherapy remains the only viable and tested modality of treatment for metastatic melanoma (30). However, the efficacy of the current

standard chemotherapy is low, with reported response rates varying from 20% to 25%. Moreover, these responses are mostly partial remissions and not cures.

Drugs used to treat melanoma are administered in single-drug or drug-combination regimens (35). The principal drug for single-drug chemotherapy is dacarbazine (DTIC) (36). DTIC has produced the highest response rates against metastatic melanoma, these being about 15-25% (37). However, the responses observed with DTIC are usually short-lived (3-6 months), there is not a significant increase in survival, and the treatment is associated with nausea and vomiting. Along with DTIC, nitrosoureas have shown some antitumor activity against melanoma and are sometimes used as alternatives to DTIC (38). In addition, two other classes of drugs are used in a single-agent chemotherapy : vinca alkaloids and cisplatin. The partial response rates for these drugs are 10% to 25% (39, 40).

Based on the independent activity of DTIC and the nitrosoureas, many studies have investigated the efficacy of combining DTIC with one of nitrosoureas. The results consistantly failed to show any improvement in the response rates in comparison with those of DTIC alone (41). Other drugs that have been evaluated with DTIC include bleomycin and hydroxyurea, but these regimens also have not improved the prognosis of melanoma patients. Currently, there is an interest in combining DTIC with cisplatin and vinca alkaloids (42, 43). As compared with single agents, combination chemotherapy has not improved the rates of remissions and survival (44). Perfusion of an isolated limb, using mostly a combination of melphalan and hyperthermia, has a significant palliative effect on locally advanced melanoma of the extremity, but its impact on overall survival is controversial (45).

D. EXPERIMENTAL MELANOMA-SPECIFIC CHEMOTHERAPY

The currently available chemotherapeutic agents have failed to be effective in the treatment of metastatic malignant melanoma. The major reasons are a dose-limiting host toxicity of the antineoplastic drugs and non-selectivity of drug effects (46). Therefore, the need to develop more effective and selective antimelanoma agents is clear. The basis for such a melanoma-specific approach should be the exploitation of specific biological properties of these tumor cells.

Unique Function of Pigment Cells. As previously described, melanoma cells are neoplastic pigment cells which usually retain the most typical function of normal melanocytes, melanogenesis (23). Melanogenesis generates a number of highly reactive intermediates including orthoquinones which are potentially hazardous to pigment cells. Amplification of this cytotoxic potential may allow these cells to self-destruct.

Origin of Melanoma-Specific Strategy. Hochstein and Cohen in 1963 (7) were the first investigators who suggested this potential hazard of melanogenesis to melanocytes and the possibility of amplifying this hazard for the development of rational chemotherapy for melanoma. They proposed the manipulation of specific protective mechanisms, e.g., by inhibiting enzymes involved in the maintenance of intracellular reduced glutathione, to enhance melanocytotoxicity caused by melanogenesis (7). This concept has been further advanced mainly by other workers, e.g., Riley and Wick, who have introduced the use of synthetic tyrosinase substrates, monophenols and catechols (47, 23).

Background Findings. It has been known for a long time that many phenolic and catecholic compounds induce depigmentation in humans and animals. In 1936, Oettel

reported that black-haired cats became grey when fed with hydroquinone (48). Oliver *et al.* first reported extensive skin depigmentation in workers exposed for several months to rubber gloves containing the antioxidant monobenzylether of hydroquinone (49). Subsequent studies found that the depigmenting effect of these drugs was not due to an inhibition of tyrosinase but that other mechanisms were involved (50).

Brun surveyed a wide range of depigmenting agents and found the monomethylether of hydroquinone (4-hydroxyanisole, 4-HA) to be the most active depigmenting agent *in vivo* in that study (51). Riley showed 4-HA to be rapidly oxidized by tyrosinase into a quinone moiety while its isomer, 2-hydroxyanisole (2-HA), an agent without depigmenting potency, was found not to be a tyrosinase substrate. He concluded that an essential property for a phenolic compound to be a depigmenting agent was that it should be a substrate for tyrosinase (52). Further studies have shown that phenolic depigmenting agents cause cytoplasmic vacuolation, clumping of melanosomes, and cytolysis (53). It has been postulated that the cytotoxic action of phenols is due to some highly reactive (oxidative) intermediates, probably quinone(s), formed by tyrosinase (47).

Passi *et al.* have delineated the criteria for phenolic compounds to be substrates for tyrosinase. The capacity of phenolic substances to act as substrates requires the presence of electron donor groups as ring substituents. All of the known depigmenting phenolic compounds possess a high affinity for tyrosinase (54).

Many catechols are also good substrates for tyrosinase which is capable of oxidizing them into orthoquinone moieties. In order to exploit the potential of catechols as antimelanoma agents, Wick *et al.* initiated an extensive investigation of L-dopa and several of its derivatives, e.g., L-dopa methylester, dopamine, and 3,4-dihydroxybenzylamine (55-59). His and others' findings confirmed the significant antimelanoma properties of catechols, and indicated that the cytotoxicity of catechols is related in part to an inhibition of the sulfhydryl-dependent enzyme DNA polymerase (59).

Clinical Phase I-II Trials. The first clinical phase trials using 4HA, dopamine, and L-dopa/carbidopa were performed in the late 1970s and early 1980s.

In clinical pilot studies that used 4-HA to treat patients with advanced melanoma, administration of the drug by continuous venous infusion was ineffective while the intra-arterial administration of 4-HA was only partly effective, causing local responses in 9 out of 20 patients (60, 61). In addition, unfavourable pharmacokinetics and serious hepatotoxicity of 4-HA have prevented further clinical trials (62). Dopamine and the combination of L-dopa/carbidopa were evaluated in an attempt to treat patients with disseminated melanoma (63, 23). Although some promising initial results were achieved, the trials were discontinued because of serious dose-limiting cardiovascular side effects (63).

Current Status. The failure of the most promising catechols and monophenols to be applied to clinical chemotherapy indicated that an approach to melanoma specific chemotherapy was more complex than had been originally predicted. Moreover, recent findings have clearly shown significant non-specific cytotoxicity of phenolic compounds, this non-specific toxicity being tyrosinase-independent (64, 65). There is now substantial evidence that the cytotoxic effects of catecholic compounds are mostly due to auto-oxidation, and not tyrosinase-mediated oxidation, and that this auto-oxidation leads to the subsequent generation of reactive oxygen species (66, 67).

Problems which have been encountered during the years of intensive research were addressed at a recent international symposium (Melanogenesis - Its Chemistry as a Therapeutic Strategy in Melanoma) held in Manchester, U.K., in March 1991 (68). The participants of this symposium re-evaluated the concept of a melanoma-specific strategy and found it valid. They concluded that the melanocytotoxic and antimelanoma effects caused by phenolic compounds that are tyrosinase substrates, may indeed be exploitable

through the development of new prodrugs with minimal systemic toxicity which had more favourable pharmacokinetic properties.

Currently, newly synthesized tyrosinase substrates, the phenolic thioethers (PTEs), seem to be promising candidates in the search for clinically effective melanoma-specific agents (9).

E. AN APPROACH TO DEVELOPING MELANOMA-SPECIFIC CHEMOTHERAPY

A recent strategy in the development of chemotherapeutic agents is the attempt to design drugs with particular activities, e.g., the ability to bind to an enzyme known to be important in some biochemical pathway. Another important factor for the successful discovery of specifically acting drugs are *in vivo* and *in vitro* screening assays in which drugs can fully manifest a desired property (69). These principles were invoked in the approach to melanoma-specific chemotherapy.

Experimental Drugs. Based on the above principles for melanoma-specific strategy, Ito and Jimbow synthesized a new group of monophenolic thioether derivatives (PTEs) (70). The intended properties in these agents were: 1) to be substrates for tyrosinase; 2) to exert antimelanoma effects; and 3) to possess favourable pharmacokinetic properties. The inclusion of sulfur into the molecule of PTEs increases the lipophilicity of these compounds as was found by the comparison of partitition coefficients of PTEs to those of other monophenols (unpublished data), and thus may improve their penetration into cells. In a group of nine PTEs, 4-S-cysteaminylphenol (4-S-CAP), was found to be the most potent agent in the prolongation of the life span of melanoma-bearing mice and in the inhibition of melanoma growth (70). Moreover, 4-S-CAP showed a significant depigmentation effect in the hair follicles of black mice (10). The systemic toxicity of 4-S-CAP was moderate, but warned of a possible limitation to future clinical use. The probable basis for the observed host toxicity was the interaction of 4-S-CAP with plasma

monoamine oxidase (MAO) (71). To avoid this problem and to find even more potent melanocytotoxic and antimelanoma agents, Ito prepared four derivatives of 4-S-CAP using a synthetic method described by Wehrmeister (Figure I-2) (72).

The new PTEs were found to be tyrosinase substrates and were screened for their selective melanocytotoxicity *in vivo* and *in vitro*.

Experimental Models. A major goal of this reseach has been to develop an experimental protocol for the treatment of metastatic forms of melanoma using PTEs. The biology of metastasis includes the complex interactions between the tumor tissue and the surrounding normal tissue, which may influence the outcome of chemotherapy. In order to examine the antimelanoma potential of PTEs in conditions closely resembling those of melanoma metastases in humans, the *in vivo* lung melanoma colony forming assay developed by Fidler was used (73, 74). In this model, a suspension of murine B16F10 melanoma cells is intravenously injected into syngeneic C57BL/6J mice to embolize and subsequently produce melanoma colonies in lung (Figure I-3). Morphologically and histologically, the murine melanoma nodules show similarities to the pulmonary metastases of melanoma patients, except that heterogeneity encountered in the clinical disease is absent in the model system.

While the murine model may provide a better understanding of the mechanism of action of PTEs, however, the biologic properties and behavior of melanomas, e.g., the degree of melanization or proliferation rate, are heterogenous, which carries the inherent risk that conclusions based on observations of a limited number of melanoma cell lines may not be of general applicability. To reduce this problem, a murine hair follicle model was used that has been otherwise recognized as useful for studying the melanocytotoxicity of depigmenting agents (75). Advantages of this model are the availability of uniformly activated melanogenic follicular melanocytes, ease of experimentation, and great reproducibility of results.

In the hair follicle depigmentation model, plucking of hair promptly induces a growth phase (anagen) of new hair. During anagen, a follicle is formed by cells with an exceptionally high mitotic activity, their doubling time being 12-13 hours (76, 77). The anagen hair follicle contains two closely interactive compartments with three main types of activated cells. In the epithelial compartment, the most abundant cells are peripherally located keratinocytes while less numerous follicular melanocytes surround the tip of the dermal papilla (melanocytic zone). The mesenchymal compartment is represented by the dermal papilla which contains mostly fibroblasts (Figure I-4). The follicular melanocytes synthesize pigment-containing melanosomes which, when mature, are transfered to an adjacent follicular keratinocytes. The keratinocytes gradually move from the follicle toward the skin surface, thus forming a hair shaft. The pigmentation of the visible part of the hair shaft (the part above the skin surface) is a diary of the activity of follicular melanocytes; thus, the tip records the earliest period while the bottom part of the shaft corresponds to the most recent period (late anagen) of melanogenic activity of these cells (78). Events affecting follicular melanocytes during anagen may change their capacity for melanin production which may be evident as differences in the pigmentation of the hair shaft. Thus, the degree and type of pigmentation of newly grown hair may "record" or indicate (patho)physiologic occurencess in the activated follicular melanocytes.

For *in vitro* cytotoxicity testing of PTEs, the microculture tetrazolium (MTT) assay described by Mosmann (Figure I-5) (79) was used. The main advantages of the MTT assay are simplicity of performance using an ELISA reader and the high reproducibility of results.

F. CONCLUSIONS

The treatment of patients with metastatic malignant melanoma is frustrating since the currently available chemotherapeutic modalities are ineffective and unable to alter the poor prognosis for these patients. The low efficacy of the chemotherapy is caused by both the host dose-limiting toxicity of standard anticancer drugs and the inherent resistance of melanomas to the present array of treatment agents.

One possibility for overcoming the undesired non-specific toxicity of drugs is by a selective drug interaction with the melanogenic process which is present in the majority of melanomas. An essential property of melanoma-specific drugs is their ability to act as substrates for the tyrosinase. During melanogenesis, tyrosinase converts L-tyrosine into dopa and dopaquinone, the later being potentially toxic to the producing cell. It has been proposed that tyrosinase may also convert synthetic substrates, monophenols and catechols, into highly reactive intermediates that may exert selective antimelanoma effects.

Many drugs, mostly tyrosinase substrates, have been investigated for antimelanoma effects, however, an effective melanoma-specific chemotherapeutic protocol for the treatment of melanoma patients has not yet been developed. The main obstacles that have prevented success have been high systemic toxicity and unfavourable pharmacokinetic properties of the candidate drugs.

Based on the concept of tyrosinase-dependent activation of phenolic prodrugs into selectively melanocytotoxic and antimelanoma moieties, a new class of synthetic tyrosinase substrates, phenolic thioethers (PTEs), was synthesized. The melanocytotoxicity and antimelanoma effects, and the possible mechanisms of cytotoxicity of these new PTEs were studied in *in vivo* and *in vitro* assays, the results of which are presented in this thesis.

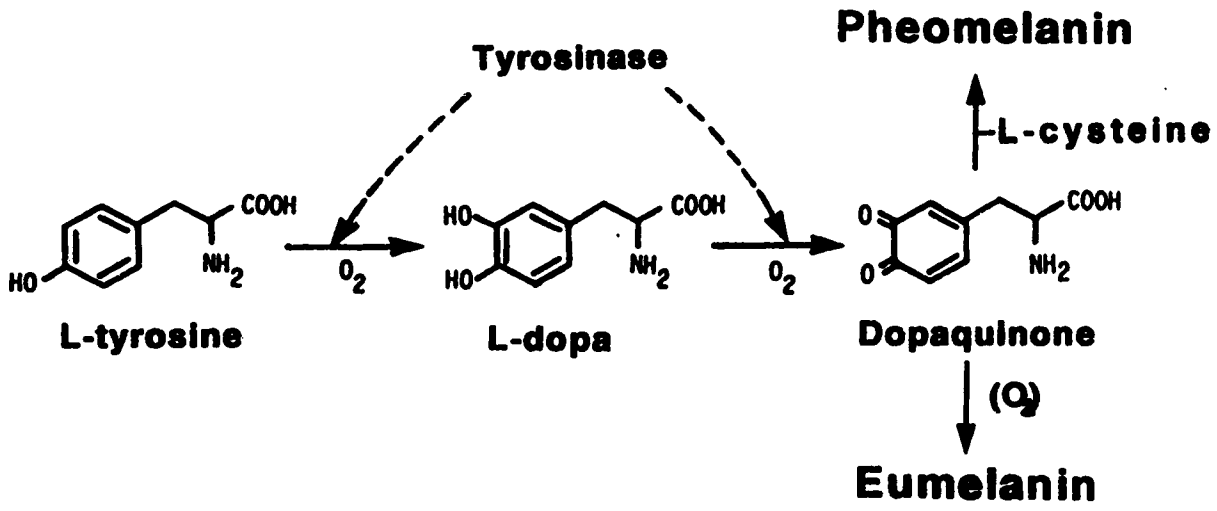


Fig. I-1. Outline of the melanin biosynthetic pathway. The enzyme, tyrosinase, converts the amino acid, L-tyrosine, into L-3,4-dihydroxyphenylalanine (L-dopa) and dopaquinone. Dopaquinone is a precursor common to both the black-brown pigment, eumelanin, and yellow-red, pheomelanin.

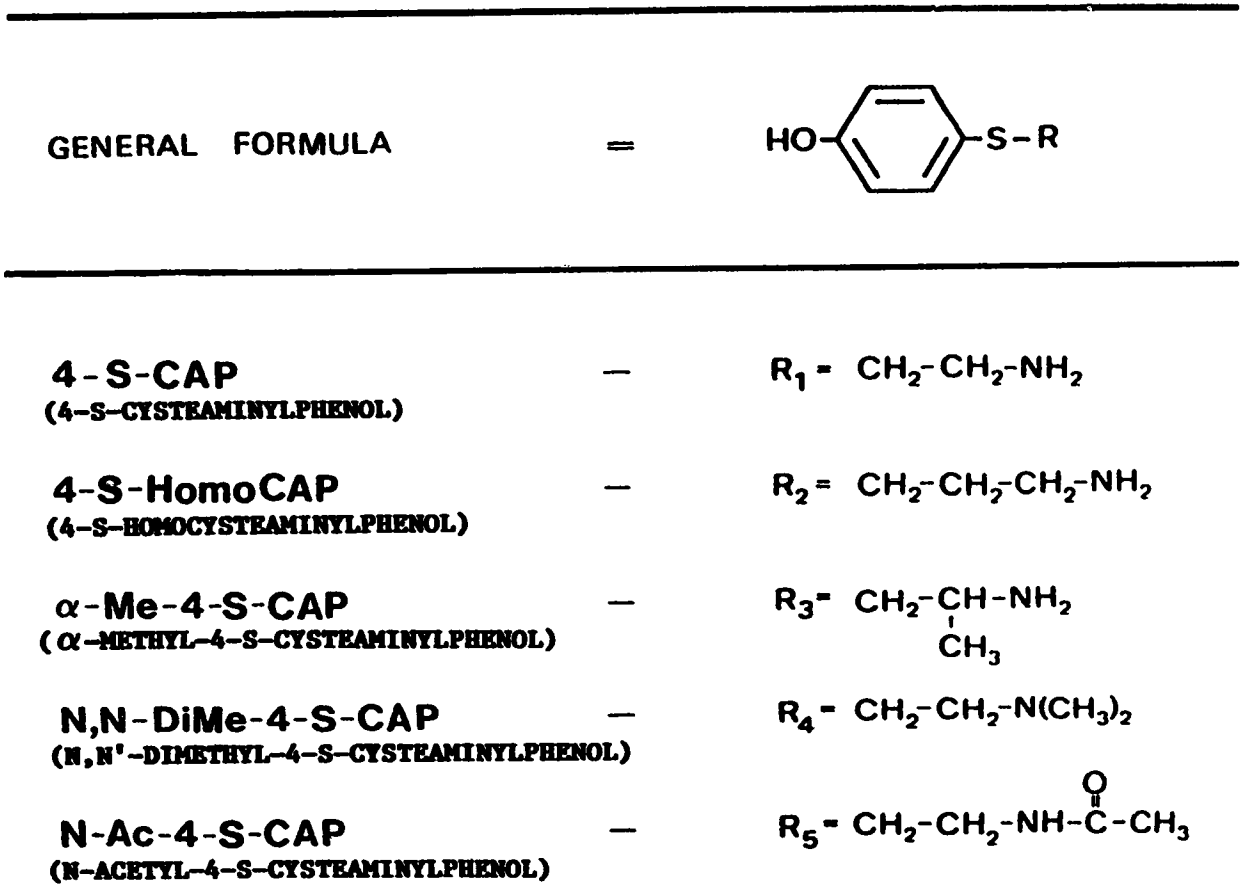


Fig. 1-2. Chemical structures of the phenolic thioethers synthesized for this study.

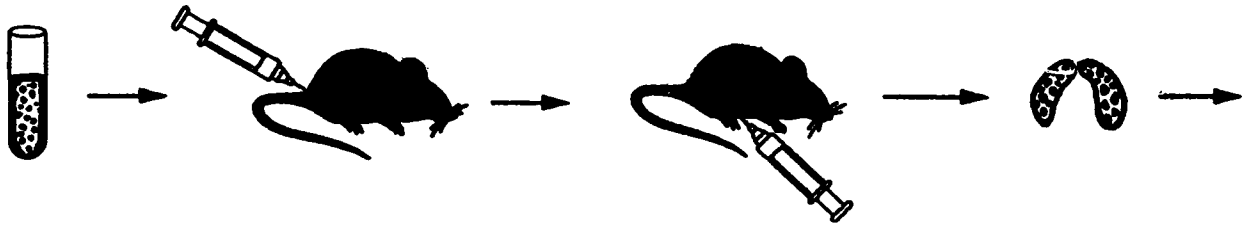
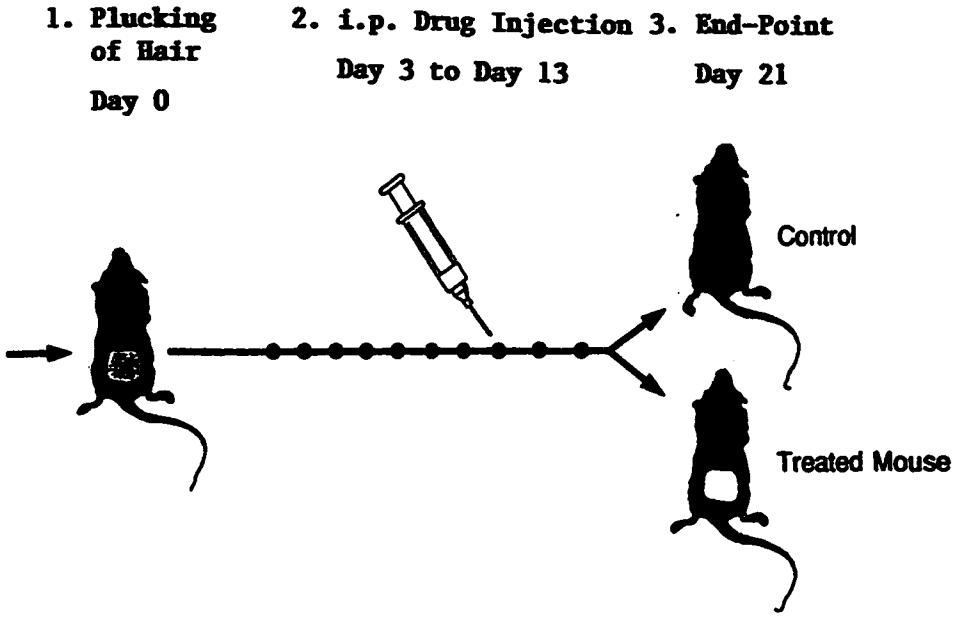


Fig. 1-3. Flow diagram of lung melanoma colony-forming assay in mice. (Based on the work of Fidler (1977)).

A. Flow Diagram of the Procedure.



B. Histologic Structure of the Anagen Hair Follicle

(Adapted from A.B. Ackerman: Histologic Diagnosis of Inflammatory Skin Diseases. Philadelphia, Lea & Febiger, 1978)

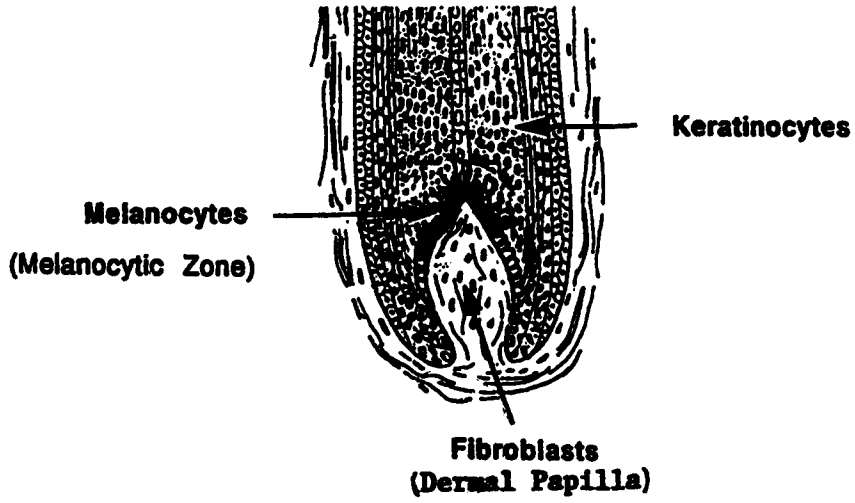


Fig. I-4. *In vivo* hair follicle depigmentation assay in mice.

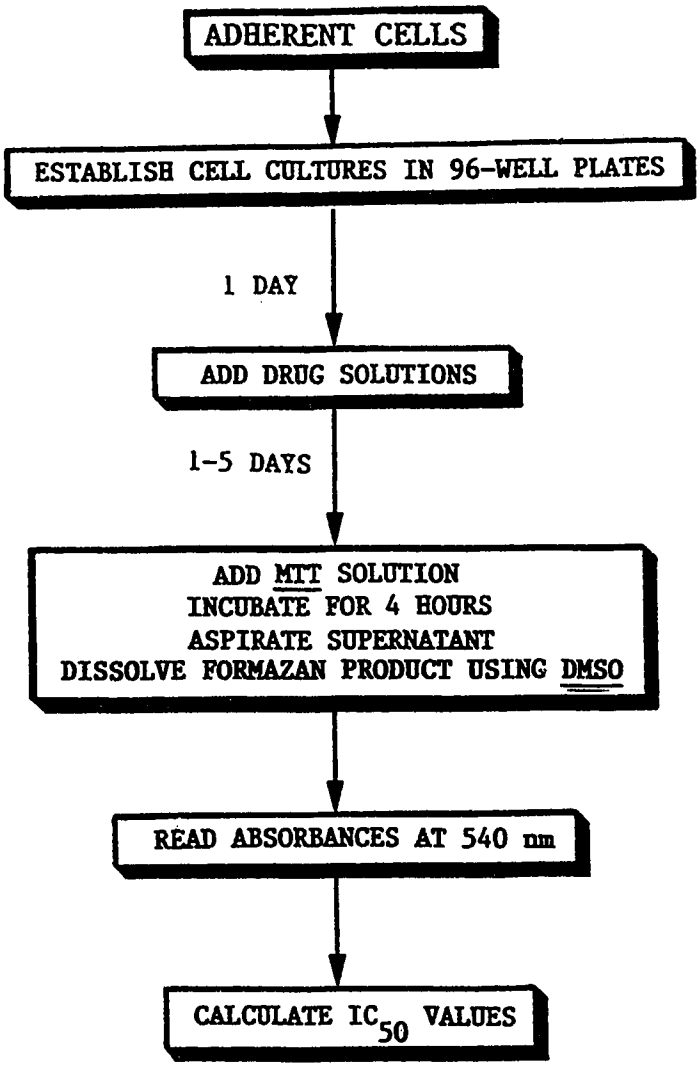


Fig. I-5. Flow diagram of *in vitro* microculture tetrazolium (MTT) assay.
(Based on the method of Mosmann (1983)).

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II. RESEARCH PLAN

A. RATIONALE

The current standard chemotherapy for the treatment of metastatic forms of cutaneous malignant melanoma is inefficient, the response rate being less than 20 to 25% in patients treated (1). For this reason, a search for new, more effective drugs to cure malignant melanoma is necessary.

In previous studies, several *p*-hydroxyphenyl derivatives, monophenols and catechols, have been shown to be selectively toxic to both melanocytes and melanoma cells *in vitro* and *in vivo* (2). A common property of these compounds is that of substrate activity for the unique enzyme of pigment cells, tyrosinase (3). During melanogenesis, tyrosinase converts L-tyrosine into L-dopa and dopaquinone (4). The highly reactive dopaquinone is potentially toxic to pigment cells. It has been proposed that tyrosinase may also convert synthetic phenolic substrates into reactive orthoquinone moieties which may exhibit selective melanocytotoxic and antimelanoma effects (5, 6).

Recently, Ito and Jimbow synthesized a new group of phenols, phenolic thioethers (PTEs), which are substrates for tyrosinase and which may be more toxic to pigment cells than the previously tested phenols due to increased lipophilicity and resulting uptake (7). It is hoped that an investigation of the biological effects of the PTEs may lead to:

a) A better understanding of the mechanism of selective melanocytotoxicity by phenols, and
b) The design of effective melanoma-specific chemotherapeutic protocols.

B. OBJECTIVES

The objectives of the research described in this thesis were:

1. To demonstrate the biological effects of PTEs *in vivo* and *in vitro*;

2. To select the most effective compound from the group of five PTEs to become the principal drug for further experimental melanoma-specific chemotherapy;

3. To test the proposed mechanism of cytotoxicity of PTEs;

4. To design an experimental drug-treatment protocol with a high efficiency against malignant melanoma using a PTE as the main chemotherapeutic agent.

C. HYPOTHESES

It is hypothesized that PTEs:

1. Are potent melanocytotoxic and antimelanoma agents *in vitro* and *in vivo*;

2. Are selectively cytotoxic to pigment cells;

3. Become cytotoxic after conversion into orthoquinone moieties by the action of tyrosinase;

4. May be cytotoxic through the mechanism of alkylation of macromolecules by reactive intermediates or by the induction of severe oxidative stress;

5. May be used in combination with other drug(s) to exhibit enhanced antimelanoma effects *in vitro* and *in vivo*.

D. CHAPTER FORMAT

The above-mentioned hypotheses were tested in a series of experiments, the results of which are here grouped as thesis chapters, and which have been either published, or are being prepared for publication as individual scientific papers.

Chapter III examines the *in vivo* melanocytotoxicity and antimelanoma effects of five PTEs (hypothesis 1). Using black mice, both the systemic toxicity (LD50) and the hair depigmentation potency of each compound was assessed. Based on these results, N-acetyl-4-S-cysteaminylphenol (N-Ac-4-S-CAP) was selected to become the principal drug for further investigation. The antimelanoma effects of N-Ac-4-S-CAP were tested against murine B16F10 melanoma cells in a lung melanoma colony forming assay. Histologic studies were performed to examine the selectivity of cytotoxic action by N-Ac-4-S-CAP (hypothesis 2). This material was published in Cancer Research, 50: 3743-3747, 1990.

In Chapter IV, the biological effects of PTEs against cancer and normal cells *in vitro* (hypothesis 1 and 2) were studied. The role of tyrosinase as a mediator of cytotoxicity by PTEs was examined in detail using a potent inhibitor of tyrosinase, phenylthiourea (hypothesis 3).

In Chapter V, the dose-dependency of the melanocytotoxic effects of N-Ac-4-S-CAP against hair follicles of black and yellow mice (hypothesis 1) was examined. Also, the glutathione (GSH) content in various tissues before and after the treatment with N-Ac-4-S-CAP was measured (hypothesis 4). Based on these findings, a novel hypothesis concerning the mechanism of cytotoxicity by PTEs is proposed.

In Chapter VI, experimental work that examined the relationship between the degree of melanocytotoxicity by N-Ac-4-S-CAP and GSH content in the skin is reported.

GSH levels in tissues were modulated either by N-acetyl-L-cysteine (NAC), or by D,L-buthionine sulfoximine (BSO) in order to increase or decrease the GSH content, respectively, and to observe the effects of GSH changes on the depigmenting potency of N-Ac-4-S-CAP against black and yellow hair follicles in mice (hypothesis 4).

In Chapter VII, a series of *in vitro* mechanistic experiments that tested hypothesis 4 are reported. A possible correlation between the morphologic changes of cells and the dose of N-Ac-4-S-CAP, the relation between dose- and time-dependent cytotoxicity by N-Ac-4-S-CAP and GSH changes in cell cultures, and the effect of GSH depletion by BSO on the cytotoxic potency of N-Ac-4-S-CAP was examined. Moreover, melanoma and non-melanoma cells were examined for the synthesis of stress proteins after a short exposure to N-Ac-4-S-CAP, and the time-course of lipid peroxidation in cells continuously exposed to N-Ac-4-S-CAP was measured.

In Chapter VIII, the antimelanoma effect of N-Ac-4-S-CAP in combination with BSO *in vitro* and *in vivo* (hypothesis 5) was assessed. Moreover, the selective incorporation of N-Ac-4-S-CAP into melanoma tissue *in vivo* was studied using the method of whole body autoradiography and a covalent binding assay (hypothesis 2).

Discussion of the melanocytotoxic and antimelanoma effects of PTEs, in particular those of N-Ac-4-S-CAP, the proposed mechanism of cytotoxicity *in vivo* and *in vitro*, and the applicability of N-Ac-4-S-CAP as the principal drug in a drug-combination protocol for the chemotherapy of human melanoma patients were summarized in Chapter IX.

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III. MELANOCYTOTOXICITY AND ANTIMELANOMA EFFECTS OF PHENOLIC AMINE COMPOUNDS IN MICE IN VIVO [1]

A. INTRODUCTION

Melanin pigment is synthesized solely within melanocytes and melanoma cells. The enzyme tyrosinase (E.C. 1.14.18.1) converts the amino acid tyrosine to L-dopa and hence to dopaquinone, which gives rise to a melanin polymer. This process occurs in a specific secretory granule, the melanosome (1, 2). There have been several attempts to use the unique melanin pathway as a basis for developing chemotherapuetic agents selectively toxic to melanoma cells, with the strategy in most cases based on L-dopa and related catechols (3-7); however, chemical instability and severe toxicity due to nonspecific oxidation have precluded success. Since phenolic compounds seem to possess fewer drawbacks and appear more promising for use in chemotherapy to combat melanoma, a new subgroup of phenolic thioethers of cysteine and cysteamine, cysteinylphenol (CP) and cysteaminylphenol (CAP), respectively, were developed (8).

Both 4-S-CP and 4-S-CAP are substrates for mammalian tyrosinase and can depigment black hair *in vivo* whereas their 2-S-isomers are not tyrosinase substrates and evidence no melanocytotoxic effects (8). Importantly, 4-S-CAP has a significant antimelanoma effect (9). Padgette *et al.* (10), however, in developing hypotensive agents, discovered that 4-S-CAP is a substrate of plasma monoamine oxidase (MAO). An earlier study from this laboratory confirmed this result *in vitro* and indicated that 4-S-CAP could produce a cytotoxic aldehyde through interaction with plasma MAO (11).

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[1] *A version of this chapter has been published. Alena, F., Jimbow, K., and Ito, S. Melanocytotoxicity and antimelanoma effects of phenolic amine compounds in mice in vivo. Cancer Res., 50: 3743-3747, 1990.*

As a potential route around these difficulties, four homologs of 4-S-CAP were synthesized: N-Ac-4-S-CAP, α-Me-4-S-CAP, 4-S-HomoCAP, and N,N-DiMe-4-S-CAP. All four compounds are substrates for tyrosinase while 4-S-HomoCAP is also a substrate for MAO (12).

The depigmentation of black follicles by test compounds given s.c. is a reliable screening method for evaluating melanocytotoxicity (13). In the present study, the four new phenolic amine compounds and the mother compound, 4-S-CAP, given both i.p. and s.c., were compared in the depigmentation assay in mice. The antimelanoma effect of 4-S-CAP and the most efficacious depigmenting compound were also assessed by assaying the formation of melanoma colonies in the lungs.

B. MATERIALS AND METHODS

Animals. Breeding pairs of C57BL/6J black mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and the female progeny were used in the study at 8 wk old and weighing 17.0 g. All procedures were approved by the University's Health Sciences Animal Welfare Committee.

Chemicals. The methods used to synthesize the phenolic amine compounds [4-S-CAP, N-Ac-4-S-CAP, α-Me-4-S-CAP, 4-S-HomoCAP, and N,N-DiMe-4-S-CAP] were reported previously (8,9,11). Briefly, the basic compound, 4-S-CAP, was prepared through refluxing a mixture of phenol and cysteamine with HBr (Figure III-1). In all experiments, the agent injected into control was normal saline solution.

LD50. The LD50 for each compound (n = 6 mice each) was established by a single i.p. injection of the test drug dissolved in normal saline solution, in a dose range of 100 to 1300 mg/kg body weight. The LD50 was 600 mg/kg for 4-S-CAP, 1200 mg/kg for

N-Ac-4-S-CAP, 500 mg/kg for α-Me-4-S-CAP, 350 mg/kg for 4-S-HomoCAP, and 300 mg/kg for N,N-DiMe-4-S-CAP.

Melanoma Cell Line. The murine B16F10 melanoma cell line with a strong metastatic property to form tumor colonies in lungs was kindly supplied by Dr. B.M. Longenecker, Department of Immunology, University of Alberta, Cells were grown in T-75 flasks in Dulbecco's MEM (GIBCO Lab. Inc., Grand Isl. NY) supplemented with 10% (v/v) fetal calf serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air.

Melanocytotoxicity In Vivo. Hairs were plucked manually from the backs of the mice, to initiate new anagen growth, a feature of which is activation of follicular melanocytes with increased expression of tyrosinase activity. Thirty mice were randomized into six groups of five (one control and one for each compound). Starting on day 1, the agents were injected i.p. or were infiltrated s.c. in an area where hair follicles had been plucked daily for 14 days, at a dose of 300 mg/kg body weight.

Melanin Content. In the same 30 mice, hair follicles were harvested on day 22 (early telogen phase) and their eumelanin content analyzed. Details of the assay, including chemical degradation of melanin and analysis by high performance liquid chromatography (HPLC), were as described before (14). Briefly, a 10-mg hair sample was homogenized in water at a concentration of 10 mg/ml; the 200-μl homogenate was transferred to a screw-capped test tube, mixed with 1M H_2SO_4 (800 μl), and oxidized with 3% $KMnO_4$. The product, pyrrole-2,3,5-tricarboxylic acid (PTCA), was analyzed by HPLC using a UV detector; samples are measured in duplicate. The eumelanin content is expressed as PTCA (ng/mg) against the PTCA content of BALB/c albino mice as control (10 ng/mg, the background value with our method, in which 1 ng corresponds to a eumelanin content of 50 mg), and the degree of depigmentation is expressed as a percentage: (PTCA content of

control pigmented sample minus PTCA content of experimental sample)/(PTCA content of control sample) x 100.

Light and Electron Microscopy. In six groups of three mice (one control and one for each compound), samples of skin and regrowing hair follicles were excised under brief general anesthesia on day 10 (late anagen phase), pre-fixed in Karnovsky's solution (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer solution), and post-fixed in 1% osmium tetroxide in cacodylate buffer solution. Tissues were stained with 1.5% uranyl acetate in 0.1 M veronal buffer for 30 min, dehydrated in graded ethanol solutions, embedded in epoxy resin, and sectioned with a Potter-Blum Ultramicrotome MT II. Sections 1.0-μm thick were stained with methylene blue for light microscopy; thin sections were stained with lead citrate for electron microscopy.

Assay of Melanoma Colony Formation in the Lungs. Murine B16F10 melanoma cells were cultured to subconfluence, harvested by applying a thin layer of 0.25% trypsin solution in EDTA, washing and resuspending in cold normal saline solution. Viable cells were identified by trypan blue dye exclusion, counted, and the cells suspension was diluted to the desired concentration. Three groups of 10 mice were studied: control, 4-S-CAP, and N-Ac-4-S-CAP. On Day 0, the mice were inoculated via the lateral tail vein with 5×10^4 cells in 0.2 ml of normal saline. The test drugs were dissolved in normal saline, sterilized by membrane filtration, and administered at a dose of 300 mg/kg (4-S-CAP) or 900 mg/kg (N-Ac-4-S-CAP); starting on day 5 after plucking, the control solution or drug was injected i.p. daily for 14 days. On day 26, the mice were killed by cervical dislocation, their lungs were removed, and the number of melanoma colonies was counted under a dissecting microscope. The results were expressed as percentage of reduction in the number of colonies: $[a\text{-}b/a] \times 100$, where a = colonies in control group and b = colonies in experimental group.

Metabolic assay. Fifteen mice randomly divided into three groups of five were given i.p. injections of a test agent (300 mg/kg) dissolved in normal saline solution. Group 1 mice were given injections of 4-S-CAP, and group 2, N-Ac-4-S-CAP; group 3 were controls. The mice were kept in metabolic cages and urine was collected at 3, 8, and 24 hr. after injection; 4-S-CAP and N-Ac-4-S-CAP content of urine samples was measured by HPLC in a system consisting of a Waters 600 E liquid chromatograph, with a Bondapak C18 column (3.9 x 300 mm; particle size, 10 μm), and a Waters 460 electrochemical detector. The mobile phase was 0.1 M potassium phosphate buffer, pH 2.1, containing 0.1 mM Na_2EDTA: methanol, 80:20 (v/v), column temperature was 60°C, and the flow rate was 1.0 ml/min. Urine samples were hydrolyzed with 0.1 M HCl before assay, and the phenols were detected at 850 mV against an Ag/AgCl reference electrode.

Statistics. All results are presented as mean ± S.D. and statistical significance assessed using the Student's *t*-test. *P* values of 0.05 or lower were considered significant.

C. RESULTS

Macroscopically Visible Depigmentation of New Growth of Black Hair Follicles (Figures III-2 & 3). This was most marked after injection of N-Ac-4-S-CAP, whether i.p. or s.c., with the new hair being almost pure white as in albino mice. The degree of depigmentation with the other phenolic amine compounds was in descending order of potency: α-Me-4-S-CAP, 4-CAP, 4-S-HomoCAP, and N,N-DiMe-4-S-CAP. Depigmentation after s.c. injection of N-Ac-4-S-CAP was similar with doses of 600 and 300 mg/kg. With 4-S-CAP and α-Me-4-S-CAP, i.p. administration induced more depigmentation than s.c. treatment, whereas 4-S-HomoCAP induced more depigmentation

when given s.c. than i.p. N,N-DiMe-4-S-CAP induced no visible changes in pigmentation in new follicles.

Melanin Content in Depigmented Follicles (Table III-1). The most potent depigmenting agent was N-Ac-4-S-CAP, which induced 98% depigmention in new growing follicles after either i.p. or s.c. administration, with little or no dose-related difference in degree. There was no statistically significant difference in melanin content between hair follicles treated with N-Ac-4-S-CAP and those of control albino mice (P = 0.1). α-Me-4-S-CAP induced 89% depigmentation after i.p. injection and 58% after s.c. administration, with the latter route causing some irritation of the skin in the plucked areas. The depigmentation potency of 4-S-CAP was similar to that of α-Me-4-S-CAP, being 87% after i.p. and 47% after s.c. administration. Interestingly, 4-S-HomoCAP evidenced greater potency when given s.c. (63.5%) than i.p. (12.1%); this was higher than with 4-S-CAP, α-Me-4-S-CAP, and N,N-DiMe-4-S-CAP given s.c.. N,N-DiMe-4-S-CAP induced no significant depigmentation when given i.p. or s.c. (7.3% and 6.1% respectively).

Light Microscopy of Depigmented Follicles (Figure III-4). In control samples (Figure III-4a), there were melanocytes in the hair bulb above the dermal papilla and both melanocytes and surrounding keratinocytes were filled with black pigment. On day 10, after N-Ac-4-S-CAP given i.p. (Figure III-4b) neither functioning melanocytes nor melanin pigments remained in the follicles but there were no vacuolar changes of cells in the hair bulb. After α-Me-4-S-CAP i.p. (Figure III-4c) there was a similar loss of functioning melanocytes and melanin pigments. For contrast, after i.p. injection of 4-S-CAP (Figure III-4d) the hair follicles contained functioning melanocytes and melanin pigments transferred into keratinocytes, although they were much sparser than in controls. After treatment with 4-S-HomoCAP (Figure III-4e) the follicles contained fairly numerous melanocytes and

melanin pigment, almost the same as seen in the controls. Few changes were evident after N,N-DiMe-4-S-CAP given i.p. (Figure III-4f).

Electron Microscopy of Depigmented Follicles after i.p. Injection for 10 days (Controls, see Figure III-5a). No melanocytes or melanosomes were visible after treatment with N-Ac-4-S-CAP (Figure III-5b), whereas the follicles contained variable numbers of functioning melanocytes and melanosomes after injection of the other compounds. Melanocytes from mice treated with α-Me-4-S-CAP (Figure III-5c) and 4-S-CAP (Figure III-5d) contained immature melanosomes at stages II and III, whereas those from controls were full of mature melanosomes at stage IV (Figure III-5a). After 4-S-HomoCAP treatment, the follicles also contained predominantly stage III melanosomes (Figure III-5e), more numerous than with α-Me-4-S-CAP or 4-S-CAP. There was little or no change from control in numbers or melanization of melanocytes after N,N-DiMe-4-S-CAP (not shown).

Antimelanoma Effects of 4-S-CAP and N-Ac-4-S-CAP. The numbers of B16F10 melanoma colonies were significantly reduced after treatment with N-Ac-4-S-CAP and 4-S-CAP (Table III-2). Furthermore, examination under a dissecting microscope revealed marked reduction in size of those remaining (diameter, 1.5-1.7 mm vs 2.5-3.1 mm in controls) and marked depigmentation or frank amelanosis of some. The only side effects noted were brief apathy and mild hypothermia immediately after i.p. injection; average body weight was the same on Day 0 and at death.

Assay of Metabolites in Urine. Urinary excretion of the unchanged compounds, without conversion or degradation, was maximal at 3 hr after i.p. injection (5.2 mg/mouse); this was 7.7% (0.399±0.018 mg) for 4-S-CAP and 13.9% (0.723±0.027) for N-Ac-4-S-CAP. Later excretion of these two compounds, respectively, was as follows: at 8 hr,

8.5% (0.433±0.023 mg) and 19.8% (1.034±0.033 mg); and at 24 hr, 8.8% (0.4458±0.021 mg) and 20.4% (1.089±0.028 mg). Most notably, 1.3% (0.066±0.003 mg) of the N-Ac-4-S-CAP i.p. dose was excreted as 4-S-CAP, indicating conversion to the mother compound of part of this homologue administered by this route.

D. DISCUSSION

Our previous study (12), *in vitro*, indicated that all of the five phenolic compounds tested were substrates of mushroom and melanoma tyrosinases but that only 4-S-HomoCAP and 4-S-CAP were substrates of tyrosinase and MAO. Interestingly, 4-S-CAP was the best substrate for mushroom tyrosinase, whereas 4-S-HomoCAP was the best for melanoma tyrosinase, a discrepancy attributed to the need for co-factors L-dopa and L-ascorbic acid in the melanoma tyrosinase reaction mixture (15). The present study, *in vivo*, indicated N-Ac-4-S-CAP and α-Me-4-S-CAP as the compounds possessing greatest activity for follicular depigmentation and 4-S-HomoCAP having the least. This indicates that the selective melanocytotoxicity of phenolic amine compounds relates to melanin synthesis and tyrosinase activity, which are high in plucked hair, and not to plasma MAO activity. The study of melanoma colonies in the lungs confirmed that N-Ac-4-S-CAP is a potent antimelanoma agent, but its effect was not absolute under the conditions and with doses used in these experiments. The marked difference in depigmentation potency of 4-S-HomoCAP given i.p. and s.c. may relate to slower degradation of the compound given s.c. and, thereby, an enhanced ability to affect melanocytes directly and induce greater depigmentation.

Previous *in vitro* studies also showed that the rate of formation of the 4-S-CAP-derived *o*-quinone, a major intermediate of 4-S-CAP melanocytotoxicity that subsequently binds to SH-containing enzymes (e.g., DNA polymerase), was very fast after the administration of 4-S-CAP and slower after α-Me-4-S-CAP (11, 12). In the present study,

however, the two compounds possessed similar ability for depigmentation *in vivo*, indicating that melanocytotoxicity related not only to the rate of *o*-quinone formation through tyrosinase but also to other mechanism(s); e.g., DNA may be damaged by free radicals generated through the interaction of tyrosinase and phenolic amine compounds. Another factor responsible for selective melanocytotoxicity might be the binding of synthetic amine compounds to melanin, which could interact later with tyrosinase. The absence of melanocytotoxicity *in vivo* in albino mice, in which tyrosinase and melanin are absent but unmelanized melanosomes are present, supports this possibility (12).

Further work is needed to establish whether phenolic amine compounds do in fact have a selective affinity for melanin. These initial findings indicate that N-Ac-4-S-CAP is an ideal candidate compound for the development of rational chemotherapy to combat melanoma although such development is hampered by the paucity of information about the compound *in vitro* as well as *in vivo*. Recently, the neurotoxicity of an amine MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was attributed to the binding of its active metabolite to neuromelanin (16). Many melanoma cells appear to be involved in pheomelanogenesis rather than eumelanogenesis (1), but it is not known whether the synthetic compounds tested here have differential high affinities for one of these pathways of melanin synthesis, how these compounds interact with gamma-glutamyl transpeptidase, which is partly responsible for pheomelanogenesis (2, 17), or whether the active compound is 4-S-CAP or N-acetyl-4-S-CAP.

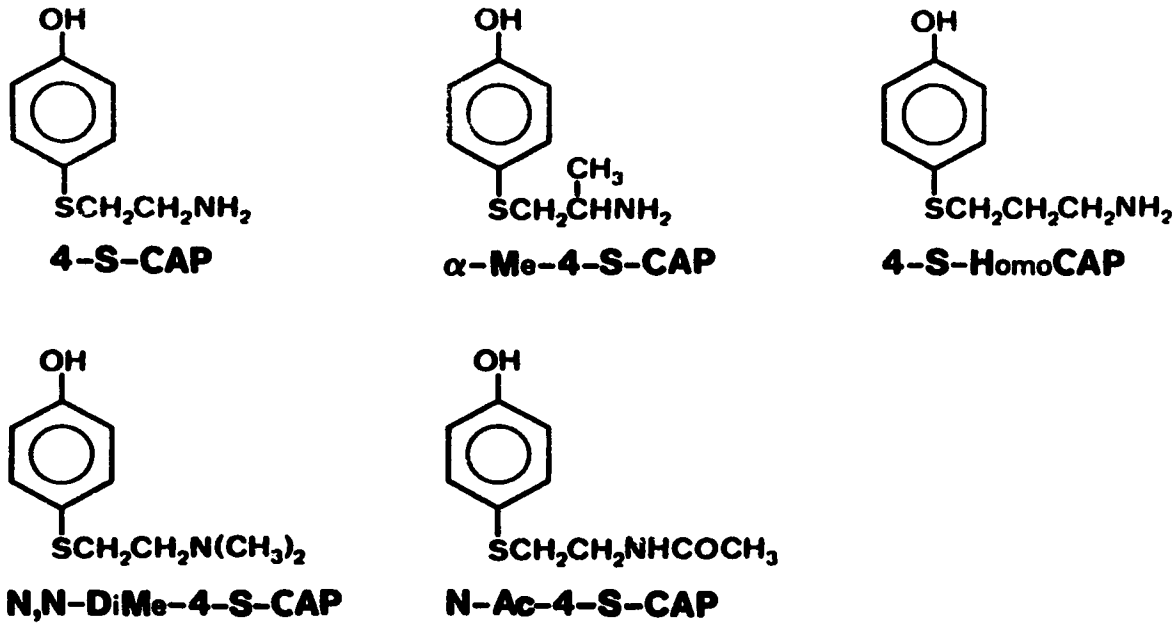

Fig. III-1. Chemical structure of phenolic amine compounds, 4-S-CAP, α-Me-4-S-CAP, 4-S-HomoCAP, N,N-DiMe-4-S-CAP, and N-Ac-4-S-CAP.

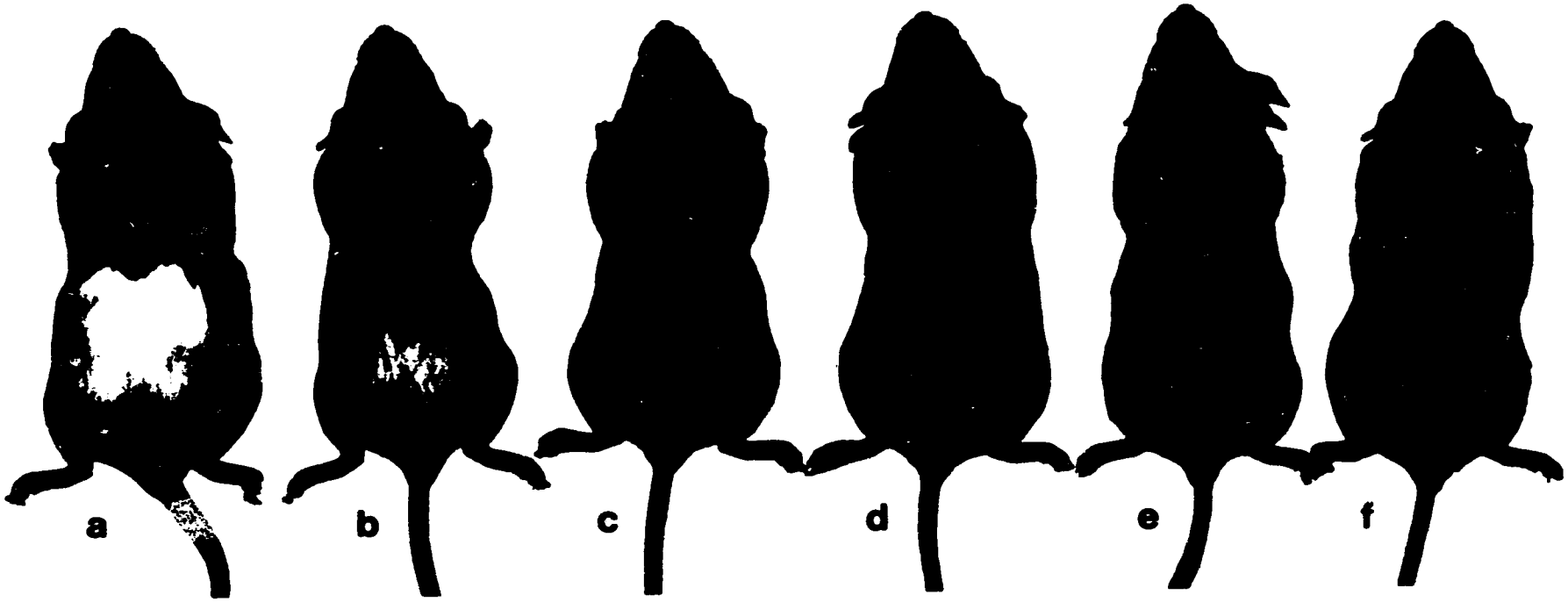

Fig. III-2. Macroscopically visible changes in black hair follicles after i.p. injection of phenolic amine compounds, 300 mg/kg daily for 14 days: a) N-Ac-4-S-CAP; b) α-Me-4-S-CAP; c) 4-S-CAP; d) 4-S-HomoCAP; and e) N,N-DiMe-4-S-CAP. f) normal saline solution (control).

Fig. III-3. Macroscopically visible changes in black hair follicles after s.c. injection of phenolic amine compounds, 300 mg/kg daily for 14 days. a) N-Ac-4-S-CAP; b) 4-S-HomoCAP; c) α -Me-4-S-CAP; d) 4-S-CAP; and e) N,N-DiMe-4-S-CAP.

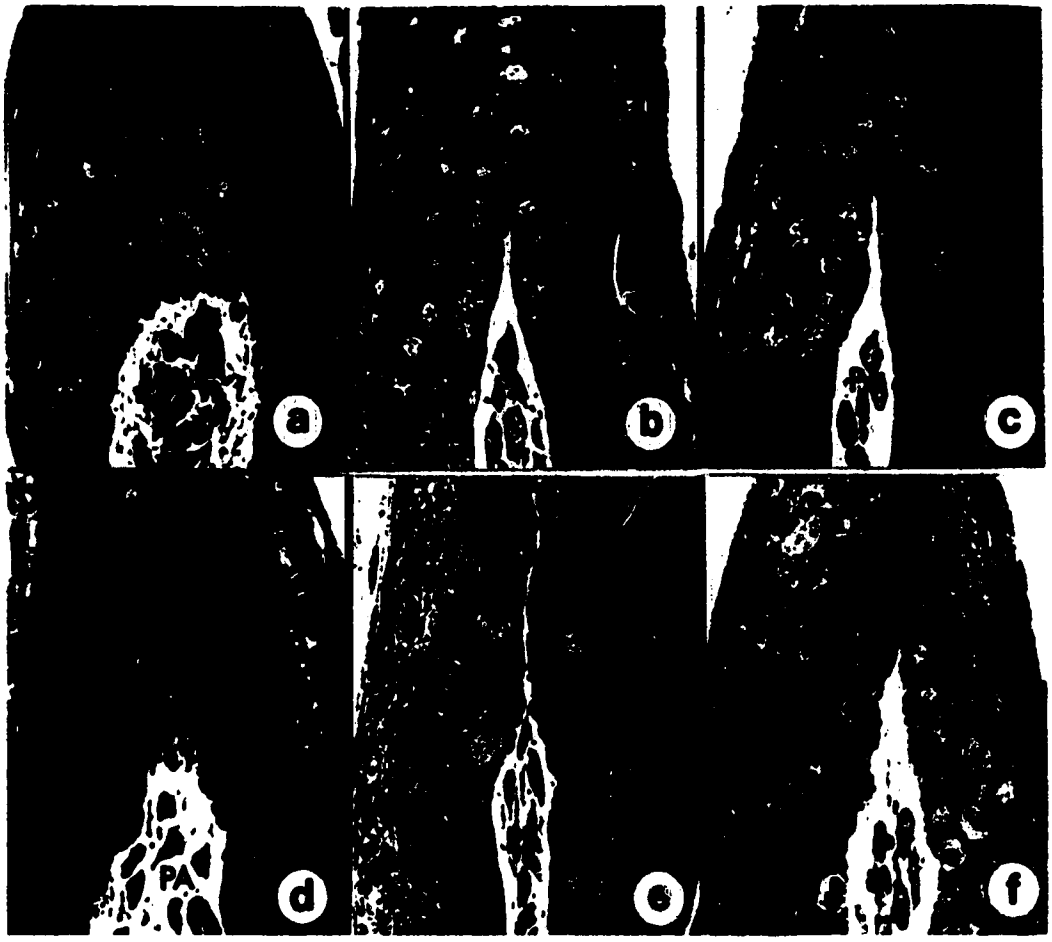

Fig. III-4. Light microscopy of hair follicles after i.p. injection of phenolic amine compounds daily for 10 days. Bar, 10 μm, PA, papilla. **a)** control. *Arrows*, normal melanocytes of hair follicle of a C57BL/6J mouse. **b)** after treatment with N-Ac-4-S-CAP; no melanocytes or melanosomes are present. **c)** after α-Me-4-S-CAP; few melanocytes are seen in the melanocytic zone above the papilla. **d)** after 4-S-CAP; the number of melanosomes in the hair bulb is greatly reduced. *Arrows*, functioning melanocytes. **e)** after 4-S-HomoCAP; melanocytes and melanosomes are present in the melanocytic zone of the bulb (*arrows*). **f)** after N,N-DiMe-4-S-CAP; the number of melanocytes and melanosomes are similar to control (a). x 1100.

Fig. III-5. Electron microscopy of hair follicles after i.p. injection with phenolic amine compounds (300 mg/kg) daily for 10 days. *Bar*, 1 μm. MC, melanocytes; MS, melanosomes; KC, keratinocytes; FB papillary fibroblasts; PA, papilla. **a)** control; no degenerative changes are seen in melanocytes, melanosomes, keratinocytes, or papillary fibroblasts. **b)** after treatment with N-Ac-4-S-CAP; no melanocytes or melanosomes are present in melanocytic zone of the hair bulb, but no degenerative changes are seen in the keratinocytes. **c)** after α-Me-4-S-CAP; individual melanosomes in the few remaining melanocytes appear sparser and more fragmented than in keratinocytes, and many are unmelanized . **d)** after 4-S-CAP ; melanosomes are reduced in size and number. **e)** after 4-S-HomoCAP; cytotoxic effects are less than with the other compounds, consisting in some reduction in number and size of melanosomes. x 5300.

Table III-1 PTCA content and depigmentation of new hair after treatment with phenolic amine compounds.

Compound injected (300 mg/kg)	PTCA[a] content (ng/mg)	Depigmentation (%)	*t* test (*P*)
Intraperitoneal			
Normal saline (control)	1089.0 ± 48.9[b]	0.0	
N-Ac-4-*S*-CAP	26.0 ± 10.3	97.6	<0.001
α-Me-4-*S*-CAP	121.3 ± 81.9	88.9	<0.001
4-*S*-CAP	146.1 ± 72.4	86.6	<0.001
4-*S*-HomoCAP	956.7 ± 77.9	12.1	>0.05
N,*N*-DiMe-4-*S*-CAP	1009.0 ± 193.5	7.3	>0.2
Subcutaneous			
Normal saline (control)	1089.0 ± 48.9	0.0	
N-AC-4-*S*-CAP	30.8 ± 9.1	97.2	<0.001
α-Me-4-*S*-CAP	458.3 ± 107.6	57.9	<0.001
4-*S*-CAP	577.9 ± 47.2	46.9	<0.001
4-*S*-HomoCAP	397.7 ± 279.3	63.5	<0.02
N,*N*-DiMe-4-*S*-CAP	1023.4 ± 110.1	6.1	>0.2

[a] PTCA = Pyrrole-2,3,5-tricarboxylic acid, a eumelanin indicator;

[b] Sample mean ± S.D. (n = 5);

Table III-2 Antimelanoma effects of 4-S-cysteaminylphenol and N-acetyl-4-S-cysteaminylphenol on the formation of B16F10 melanoma colonies in mouse lungs excised 26 days after injection of the cell suspension.

Compound	No. of mice	No. of colonies per pair of lungs	% of control	*t* test (*P*)
Normal saline (control)	10	86.5 ± 32.9[a]	100.0	
4-*S*-CAP, 300 mg/kg	10	28.0 ± 14.5	32.4	<0.001
N-Ac-4-*S*-CAP, 900 mg/kg	10	21.5 ± 10.8	24.9	<0.001

[a] Mean ± S.D.

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IV. IN VITRO CYTOTOXICITY OF PHENOLIC THIOETHERS AGAINST MELANOMA AND NON-MELANOMA CELLS: THE ROLE OF TYROSINASE AS A MEDIATOR OF CYTOTOXICITY

A. INTRODUCTION

In a previous study, five phenolic thioethers (PTEs) were shown to exert significant cytotoxicity against melanocytes and melanoma cells *in vivo* (1). The PTEs comprise a group of *p*-hydroxyphenolic derivatives, monophenols and catechols, which have been found to be selectively cytotoxic to pigment cells (2).

The common biochemical property of these melanocytotoxic phenols is an ability to act as substrates for the enzyme tyrosinase (E.C. 1.14.18.1). In a two-step reaction, tyrosinase converts phenols into catechols and catechols into potentially cytotoxic orthoquinones (3). It has been proposed that the unique presence of tyrosinase in pigment cells, and thus the production of toxic quinones exclusively in these cells, would provide a basis for the development of rational chemotherapy for malignant melanoma using synthetic tyrosinase substrates (4). Based on this proposal, several synthetic *p*-hydroxyphenolic compounds, namely 4-hydroxyanisole, dopamine, and L-dopa in combination with carbidopa, were used in clinical phase I and II trials for the treatment of human melanoma patients (5-7).

Recently, however, the role of tyrosinase in the cytotoxicity of 4-hydroxyanisole and catechols has been questioned (8-11). In contrast to previous studies, the new studies revealed a non-selective cytotoxicity of *p*-hydroxyphenolic derivatives which was independent of the tyrosinase activity.

To clarify the degree of selectivity and the role of tyrosinase in the cytotoxicity of PTEs, the effects of five PTEs were examined against melanoma and non-melanoma cells *in vitro*. Moreover, the relationship between tyrosinase and cytotoxicity of PTEs were studied

in detail, using melanoma cells with a high tyrosinase activity in the presence and/or absence of a specific inhibitor of tyrosinase, phenylthiourea (PTU).

B. MATERIALS AND METHODS

Cell Lines and Culture Conditions. The origin and biological properties of the tested cells are listed in Table IV-1. The population doubling time for each cell line was determined using daily counting of exponentially growing cells for at least three days. The tyrosinase activity of cells was measured as will be described later. Spectrophotometry was used for the measurement of melanin contents in cells; the results are expressed semiquantitatively. Cells of both stock and experimental cultures were grown in standard culture medium, MEM (GIBCO Lab. Inc., Grand Isl., NY) supplemented with 10% (v/v) fetal calf serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated at 37.0°C in a humidified atmosphere of 5% (v/v) CO_2 in air.

Drugs. The method for the synthesis of the phenolic thioethers, 4-S-cysteaminylphenol (4-S-CAP), N-acetyl-4-S-cysteaminylphenol (N-Ac-4-S-CAP), α-methyl-4-S-cysteaminylphenol (α-Me-4-S-CAP), 4-S-homocysteaminylphenol (4-S-Homo-CAP), and N,N'-dimethyl-4-S-cysteaminylphenol (N,N-DiMe-4-S-CAP) was previously reported (12). Briefly, appropriately substituted thiophenols were conjugated with 2-methyl-2-oxazolines using the Wehrmeister's reaction (12). The drugs were dissolved in distilled deionized water at the stock concentration of 2.0 mg/ml, sterilized by membrane filtration, and stored at -20.0°C. Before use, the stock solutions were thawed, dilutions made, and application to cultures accomplished within 1 hr. Phenylthiourea (PTU) was purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

Drug-Stability Bioassay at 37.0°C. This bioassay was performed in a modification of the method described by Hildebrand-Zanki (13). Briefly, five PTEs were prepared and stored in frozen aliquots for at least 10 days. The frozen drug solutions were thawed and mixed with MEM or with MEM plus 10% (v/v) fetal calf serum. The concentration of drugs in the mixture was 100 μg/ml. The mixtures were preincubated at 37°C in a 5% (v/v) CO_2 atmosphere for 5 days. Then, the appropriate dilutions of preincubated drug-MEM mixtures were made. Simultaneously, equivalent mixtures of MEM with freshly thawed drug solutions were prepared. The medium-alone mixtures were supplemented with 10% (v/v) fetal calf serum. The SK-MEL-23 melanoma cells were grown in 96-microwell plates and, after the addition of the preincubated or fresh drug-MEM mixtures, were continuosly incubated for 5 days. The evaluation procedure of the bioassay was the same as described for the MTT-microculture tetrazolium assay.

MTT-Microculture Tetrazolium Assay. Cell growth in the presence or absence of experimental drugs was determined using the modified MTT-microculture tetrazolium assay described by Mosmann (14). Briefly, exponentially growing cells were harvested, counted for cell viability, and inoculated at a concentration of 1-5 x 10^3 cells/well into 96-well microtiter plates (Costar, Cambridge, MA) using a multichannel pipet. After 24 hr, 0.9% NaCl aqueous solutions of drugs were added into triplicate culture wells. Cells were cultured for a specified number of days (1-5 days) at 37.0°C in a humidified atmosphere of 5% (v/v) CO_2 in air. The MTT stock solution was prepared at 5 mg/ml concentration in Dulbecco's PBS (GIBCO) and stored at 4.0°C. At the end point of the assay, 50 μl of MTT solution, diluted to 1 mg/ml concentration using MEM without serum, was added to each well. After a 4-hr incubation, the supernatant was removed from the wells, 150 μl of 100% DMSO (spectrophotometric grade) was added to dissolve MTT-formazan product, and the absorbance at 540 nm was measured with a microplate reader (EAR 400 AT). The survival fraction percentage of cells was calculated using the formula: **b/a x 100%** where **b** is the

mean of absorbance values in wells with drug(s), and a is the mean of absorbance values in control wells without drug(s).

Tyrosinase Assay. Tyrosine hydroxylase activity of tyrosinase was measured by Pomerantz's method (15) as modified by Townsend (16). Briefly, cells were cultured for a specified number of days (day 1-5), harvested by trypsinization, counted for viability, and pelleted by centrifugation. Cell pellets were homogenized by sonication, centrifuged, and both supernatant and particulate fractions were separately assayed for enzyme activity. The assay incubation mixture contained 0.5 μCi of (3,5-^{3}H)-L-tyrosine (46.7 Ci/mmol, NEN Research Products, Boston, MASS.), 100 μM of unlabeled L-tyrosine, 100 μM L-dopa in 0.1 M sodium-phosphate buffer, pH 6.8, and the enzyme sample. The reaction mixture was incubated at 37.0°C for 3 hr and then stopped by the addition of 0.1% charcoal on ice for 10 min. After centrifugation, the aliquots of assay samples were placed on Dowex 50W column equilibrated with 0.1 M citric acid. The ^3H_2O was washed through the column with a citrate buffer, and aliquots of the total wash mixed with Aquasol-2 were counted in a Beckman LS 3801 liquid scintillation counter. Protein concentration of the samples was measured using the Bradford assay (Bio-Rad Kit). Enzyme activity was expressed as nanomoles of tyrosine oxidized per 1 hr per mg of protein.

C. RESULTS

Drug-Stability Bioassay. The *in vitro* growth kinetics of SK-MEL-23 melanoma cells exposed to the preincubated drug-MEM mixtures were almost the same as those of the cells exposed to the freshly prepared drug-MEM mixtures (data not shown). It was found that PTEs are chemically stable compounds which did not lose their biological activity after preincubation under the culture conditions for 5 days.

Time-Course and Dose-Dependent Growth Inhibition of Melanoma and Non-Melanoma Cell Lines by N-Ac-4-S-CAP. The growth characteristics of cells in the presence and absence of drugs were studied using the MTT assay. N-Ac-4-S-CAP was chosen as the test drug for this purpose because this compound was proven to be the most potent cytotoxic PTE *in vivo*. The typical growth curves for SK-MEL-23 melanoma cells are shown in Figure IV-1. A clear dose-dependent relationship between the number of cells exposed to N-Ac-4-S-CAP (indirectly expressed by absorbance values) and the duration of cell cultivation was found. Similar findings were also observed for SK-MEL-118 (human amelanotic melanoma) and HeLa (human cervical carcinoma) cells (data not shown). Usually, at 24 to 48 hr after plating, the control cells started to grow exponentially for at least the next five days if the plating density was 1-5 x 10^3 cells/microwell. The growth curves for cells exposed to N-Ac-4-S-CAP clearly indicated that this drug is cytotoxic at higher concentrations (above 10 μg/ml) and also that the cytotoxicity is not of an immediate type but rather is a delayed type. For this reason, it was decided that day 5 of continuous drug exposure would be used as an end-point for the evaluation of drug-responses.

In Vitro **Growth Inhibition of Melanoma Cell Lines by Five Phenolic Thioethers (Table IV-2).** The relationship between the *in vitro* cytotoxicity of five PTEs and tyrosinase activity in cells was studied using one human melanotic melanoma cell line (SK-MEL-23) and three amelanotic melanoma cell lines (SK-MEL-118, MeWo, and G 361) with known tyrosinase activity *in vitro* (Table IV-1). It was found that SK-MEL-118 cells were the most susceptible to each of PTEs, their IC_{50}s being about 1.5- to 2-times lower than the IC_{50}s of the second most susceptible cells, SK-MEL-23. MeWo and G 361 cells were the least susceptible to drugs, their IC_{50}s being about 2-3 times higher than those of SK-MEL-23 cells for the majority of PTEs.

In Vitro Growth Inhibition of Melanoma and Non-melanoma Cells by 4-S-CAP and N-Ac-4-S-CAP (Table IV-3). Using melanoma and non-melanoma cell lines, the selectivity of cytotoxicity by two representative PTEs, 4-S-CAP and N-Ac-4-S-CAP, *in vitro*, were examined. It was found that the two PTEs were non-selectively cytotoxic to cells, e.g., pheochromocytoma cells were the most susceptible cells to 4-S-CAP, and lung adenocarcinoma cells were more susceptible to N-Ac-4-S-CAP than melanotic melanoma cells. No correlation was found between the susceptibility of cells to PTEs and tyrosinase activity, melanin content, and/or population doubling time of cells.

Time-Course of Tyrosinase Activity in SK-MEL-23 Melanotic Melanoma Cells in the Presence and Absence of PTU. The continuous exposure of SK-MEL-23 melanoma cells to PTU at a concentration of 100 μM for five days, efficiently inhibited the pigmentation (melanization) of these proliferating cells (Figure IV-2). This *in vitro* depigmentation effect of PTU was not associated with growth inhibition as presumed by daily counting of both exponentially growing control and PTU-treated cells (data not shown). The measurement of tyrosinase activity in SK-MEL-23 cells showed a spontaneous decrease in tyrosinase activity as a function of time and increasing cell density (Figure IV-3). The highest tyrosinase activity in control cells was observed at 24 hr after plating. At the same time, the addition of PTU resulted in the reduction of tyrosinase activity to about 22% of control. The initial increase in tyrosinase activity at 24 hr after plating was followed by a gradual decline. At day 5 after plating, the difference between the spontaneous and PTU-induced inhibition of tyrosinase activity was not significantly different.

Time-Course Correlation between Tyrosinase Activity and In Vitro Growth Inhibition of SK-MEL-23 Melanoma Cells by 4-S-CAP and N-Ac-4-S-CAP in the Presence and Absence of PTU. The cultures of SK-MEL-23 melanoma cells in 96-microwell plates

were prepared by plating the cells in the same density (5 x 10^3 cells/cm^2) as that for the studies of tyrosinase activity. The growth inhibitory effects of N-Ac-4-S-CAP and 4-S-CAP on these cells were examined in the continuous presence or absence of PTU (100 μM). These cytotoxic effects of N-Ac-4-S-CAP (25.0 μg/ml) and 4-S-CAP (5.0 μg/ml) are presented in Figure IV-4. There was no significant difference between the cytotoxicity by PTEs in the presence and absence of PTU. The same trend was observed also in cells exposed to 4-S-CAP at concentrations of 1.0 μg/ml and 10.0 μg/ml, and N-Ac-4-S-CAP at concentrations of 10.0 μg/ml and 100 μg/ml (data not shown).

D. DISCUSSION

The *in vitro* cytotoxicity of five PTEs against melanoma and non-melanoma cells were studied in order to examine the degree of selective action of PTEs against melanoma cells. Secondly, the role of tyrosinase in the process of cytotoxicity by PTEs was clarified. For the purpose of making comparisons between the susceptibilities of tested cell lines to PTEs, the parameter of IC_{50} (a drug inhibitory concentration of 50%) was used. The IC_{50} parameter in the MTT assay is defined as the drug concentration required to decrease control absorbance by 50%. Several studies have confirmed that the values of IC_{50} obtained from the MTT assay are comparable with those from assays based on counting cell numbers (17).

Many drugs exhibit their cytotoxic potential during the first two to three doublings of a cell population and short-term assays are sufficient for the evaluation of the drug cytotoxicity. However, other drugs possess a delayed or cumulative cytotoxicity and for an accurate assessment of their true cytotoxicity, mid- to long-term duration assays are neccessary (18). In order to determine the optimal duration of the MTT assay for the assessment of IC_{50}, the time-course studies of cytotoxicity by N-Ac-4-S-CAP (the principal PTE of our investigation) using melanoma and non-melanoma cells were performed. It was

found that the most reproducible results could be obtained on day 5 of continuous drug exposure. This finding indicated that N-Ac-4-S-CAP possessed a delayed cytotoxicity *in vitro* (18) and cell populations needed sufficient time for at least five to seven doublings in order to exhibit significant cytotoxic effects. Thus, the IC_{50} values for each tested drug and each cell line after 5 days of continuous drug exposure was used.

Comparisons between the growth inhibitory potency of five PTEs on a panel of four melanoma cell lines showed a significant difference between the subgroup of amine derivatives (4-S-CAP and 4-S-HomoCAP), and the subgroup consisting of the α-carbon methylated amine (α-Me-4-S-CAP), the dimethylated amine (N,N-DiMe-4-S-CAP), and the amide derivative (N-Ac-4-S-CAP). The former group was clearly more cytotoxic than the latter, e.g, the IC_{50}s of 4-S-CAP and 4-S-HomoCAP were 5- to 8-fold lower than that of N-Ac-4-S-CAP and 14- to 21-fold lower than that of α-Me-4-S-CAP, respectively, using SK-MEL-23 melanoma cells as a reference cell line. Interestingly, the most potent *in vitro* cytotoxic agent, 4-S-HomoCAP, showed the least depigmentation potency against black hair follicles and the highest systemic toxicity of all PTEs tested after i.p. administration *in vivo* (1). The depigmenting potency of 4-S-HomoCAP was improved and its systemic toxicity diminished by s.c. administration. In contrast, N-Ac-4-S-CAP and α-Me-4-S-CAP were shown to be the most potent and the least systemically toxic depigmenting agents after i.p. and s.c. administration. This observation hints at the importance of the pharmacokinetic conditions, e.g., the process of "first-pass effect" when an intraperitoneally administered drug is metabolized by the liver before it reaches systemic circulation (19), for the effectiveness of PTEs against the target tissue. Also, this finding indicates that the more suitable pharmacokinetic properties of PTE, e.g., a slower rate of detoxication and perhaps better bioavailability (in a higher concentration and for a longer time) of the drug in the target tissue are more important for the final *in vivo* effect than the actual cytototoxicity of the drug observed *in vitro* (20).

Of the four human melanoma cell lines tested, the most susceptible to each of five PTEs was SK-MEL-118 cell line. SK-MEL-118 cells are human amelanotic melanoma cells without tyrosinase activity *in vitro* (unpublished data from this laboratory). This finding suggested that *in vitro* cytotoxicity of PTEs is not mediated by tyrosinase or that there are two enzymatic systems producing the cytotoxicity of PTEs, one tyrosinase-mediated and the second tyrosinase-independent, present in pigment cells. Using a panel of melanoma and non-melanoma cell lines and two drugs, 4-S-CAP and N-Ac-4-S-CAP, as representatives for each subgroup of the studied PTEs, it was confirmed that the susceptibility of cells was not correlated to tyrosinase activity alone, e.g., human lung adenocarcinoma cells, or neuroblastoma cells, which lack tyrosinase activity, are about 1.5-times more susceptible to the PTEs than human melanotic melanoma cells with high tyrosinase activity. Similar results were reported by Inoue *et al.* (21) who studied the same PTEs *in vitro* using different melanoma and non-melanoma cell lines.

To further clarify the role of tyrosinase in the process of cytotoxicity by PTEs, melanotic melanoma cells were grown in the presence of a known selective inhibitor of tyrosinase, phenylthiourea (PTU). This drug almost completely inhibited tyrosinase activity in melanotic melanoma cells. However, this significant inhibition of tyrosinase activity did not affect the cytotoxicity of 4-S-CAP and N-Ac-4-S-CAP, and their IC_{50}s were practically the same with and without the presence of PTU. The same finding was previously reported for α-Me-4-S-CAP and N,N-DiMe-4-S-CAP (21). Moreover, the observation of Prezioso *et al.* (22) that the tyrosinase activity in melanotic melanoma cells is time- and cell density-dependent was confirmed. The highest tyrosinase activity was detected at 24 hr after plating followed by a rapid decline at 48 and 72 hr after plating to a level at 120 hr after plating which was equal to that produced by PTU inhibition. Paradoxically, the melanoma cells exposed to PTEs exhibited an insignificant reduction in their viability/survival at the time when they possessed the highest tyrosinase activity. Conversely, the highest

decrease in survival was seen at a time in which spontaneous or drug-induced inhibition of tyrosinase activity was maximal.

Interestingly, it was not possible to correlate the proliferative activity, melanin content, and susceptibility of cells to PTEs. For example, B16F10 murine melanoma cells which possessed the highest tyrosinase activity, the highest melanin content, and the shortest population doubling time, were the most resistant to the treatment with 4-S-CAP and N-Ac-4-S-CAP of all the cell lines studied. However, the previous *in vivo* study clearly indicate that the higher metabolic activity of cells is an essential predisposition of cells for susceptibility to PTE treatment. It was found that only follicular melanocytes activated by plucking exerted a chemically-induced injury by PTEs while dormant follicular melanocytes survived the same treatment without any damage. Previously, Galpine *et al.* (23) found that 4-hydroxyanisole inhibited DNA synthesis and blocked cells in the G1 phase of cell cycle. Similarly, Kable *et al.* (24) reported that catechols, L-dopa and 3,4-dihydroxybenzylamine, arrested cells in the S phase at low doses and in the G1 phase at high doses. Thus, it is possible that the cytotoxic mode of action of PTEs is also associated with whether or not the cells are in the S and/or G1 phases of the cell cycle.

In conclusion, PTEs were found to be non-selectively cytotoxic against melanoma as well as non-melanoma cells *in vitro*. This *in vitro* cytotoxicity of PTEs was tyrosinase-independent and also independent of the melanin content of the cells. These findings indicate that beside the proposed tyrosinase-mediated cytotoxicity (25, 26), there is a tyrosinase-independent enzymatic system causing the cytotoxicity of PTEs *in vitro*.

TABLE IV-1. CHARACTERISTICS OF CELL LINES USED IN IN VITRO CYTOTOXICITY ASSAY.

Cell Line	Tumor/Cell Type	Source*	Population Doubling Time (hours)	Tyrosinase Activity (nmol/h/mg prot.)	Melanin Content
	A. MELANOMAS				
1. SK-MEL-23	human melanotic melanoma	a	24.0	11.5 ± 0.3	+++
2. SK-MEL-118	human amelanotic melanoma	b	16.0	0.0	-
3. MeWo	human amelanotic melanoma	c	33.0	0.0	-
4. G 361	human amelanotic melanoma	d	16.8	0.0	-
5. B16F10	murine melanotic melanoma	e	12.6	49.2 ± 1.9	+++++
	B. NEURAL CREST DERIVED NON-MELANOMA CARCINOMAS				
6. SK-N-BE(2C)	human neuroblastoma	f	14.0	0.0	-
7. PC-12	rat pheochromocytoma	g	13.0	0.0	-
	C. NON-NEURAL CREST CARCINOMAS				
8. SK-MES-1	human lung adenocarcinoma	h	17.2	0.0	-
9. SK-OV-3	human ovarian carcinoma	i	24.0	0.0	-
10. HeLa	human cervical carcinoma	j	13.0	0.0	-
	D. NORMAL CELL STRAIN				
11. FB	human fibroblast strain	k	14.5	0.0	-

*a Dr. A.N.Houghton, Memorial Sloan-Kettering Cancer Center, NY
b Dr. A.N.Houghton, Memorial Sloan-Kettering Cancer Center, NY
c Dr. R.S.Kerbel, National Cancer Institute of Canada, Kingston,
d ATCC
e Dr. B.M.Loegensecker, Immunol., Univ.of AB, Edmonton,
f Memorial Sloan-Kettering Cancer Institute, NY,
g Dr. R.Murphy, Anatomy & Cell Biol., U of AB, Edmonton,
h ATCC
i ATCC
j ATCC
k primary culture from human neonatal foreskin

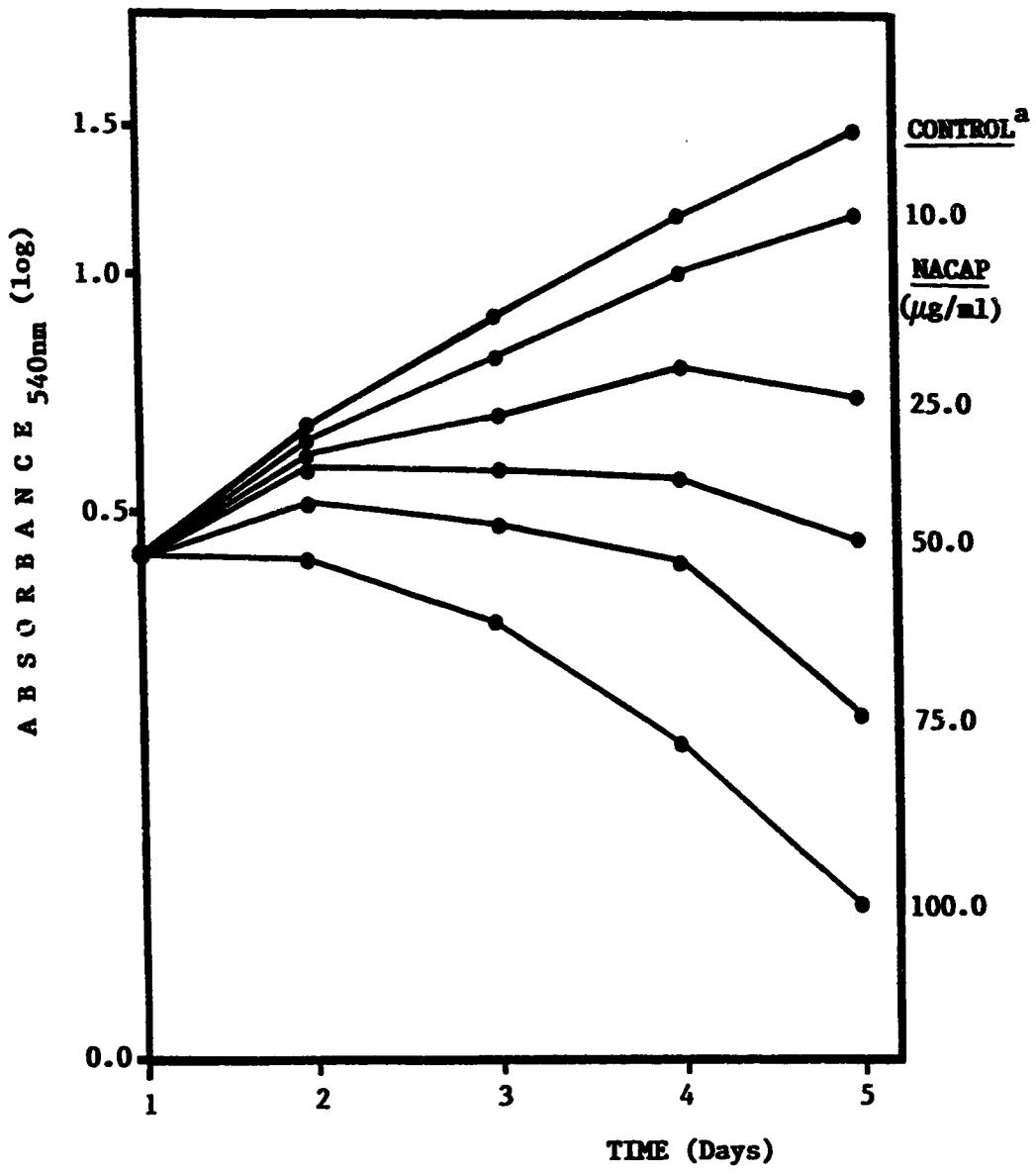

Fig. IV-1. *In Vitro* Growth Kinetics of SK-MEL-23 Melanoma Cells in the Presence or Absence of N-acetyl-4-S-cysteaminylphenol (NACAP) at Different Concentrations.

[a] Data shown as means; error bars omitted for clarity. The S.D. of three replicate wells/group were generally less than 10% of the mean value.

Table IV-2. *In Vitro* Cytotoxicity of Five Phenolic Thioethers against Melanotic and Amelanotic Melanoma Cell Lines .

	IC_{50} (μg/ml)				
CELL LINE	N-Ac-4-S-CAP	α-Me-4-S-CAP	N,N-DiMe-CAP	4-S-CAP	4-S-HomoCAP
SK-MEL-23	25.0 ± 2.4[a]	71.7 ± 6.9	56.3 ± 3.7	5.2 ± 0.2	3.0 ± 0.2
SK-MEL-118	12.9 ± 0.8	57.0 ± 5.1	35.0 ± 2.4	3.7 ± 0.3	1.6 ± 0.1
MeWo	72.3 ± 4.2	186.0 ± 6.4	125.0 ± 4.5	3.8 ± 0.1	2.6 ± 0.3
G 361	78.3 ± 6.2	88.0 ± 6.0	81.0 ± 6.6	5.9 ± 0.4	6.0 ± 0.7

[a] The mean ± S.D., (n=3);

Table IV-3. *In Vitro* Cytotoxicity of N-Acetyl-4-S-Cysteaminylphenol (N-Ac-4-S-CAP) and 4-S-Cysteaminylphenol (4-S-CAP) to Melanoma and Non-Melanoma Cells.

DRUG: 4-S-CAP			
No.	CELL LINE	TA[a]	IC_{50} (μg/ml)[b]
1.	PC-12	-	1.9 ± 0.2
2.	SK-MES-1	-	3.2 ± 0.2
3.	SK-N-BE(2C)	-	3.5 ± 0.4
4.	SK-MEL-118	-	3.7 ± 0.3
5.	MeWo	-	3.8 ± 0.1
6.	SK-MEL-23 ★	++	5.2 ± 0.2
7.	G 361	-	5.9 ± 0.4
8.	Fibroblasts	-	7.8 ± 0.3
9.	SK-OV-3	-	10.0 ± 0.6
10.	HeLa	-	12.5 ± 0.5
11.	B16 F10 ★	+++	14.2 ± 0.7

DRUG: N-Ac-4-S-CAP			
No.	CELL LINE	TA[a]	IC_{50} (μg/ml)[b]
1.	SK-MEL-118	-	12.9 ± 0.8
2.	SK-MES-1	-	14.8 ± 0.7
3.	SK-N-BE(2C)	-	16.7 ± 0.9
4.	PC-12	-	20.1 ± 0.3
5.	SK-MEL-23 ★	++	25.0 ± 2.4
6.	MeWo	-	72.3 ± 4.2
7.	HeLa	-	75.7 ± 4.1
8.	G 361	-	78.3 ± 6.2
9.	SK-OV-3	-	93.7 ± 5.4
10.	Fibroblasts	-	133.7 ±10.3
11.	B16 F10 ★	+++	149.7 ± 9.8

[a] TA = Tyrosinase activity;

[b] The sample mean ± S.D., (n=3);

★ Melanotic melanoma;

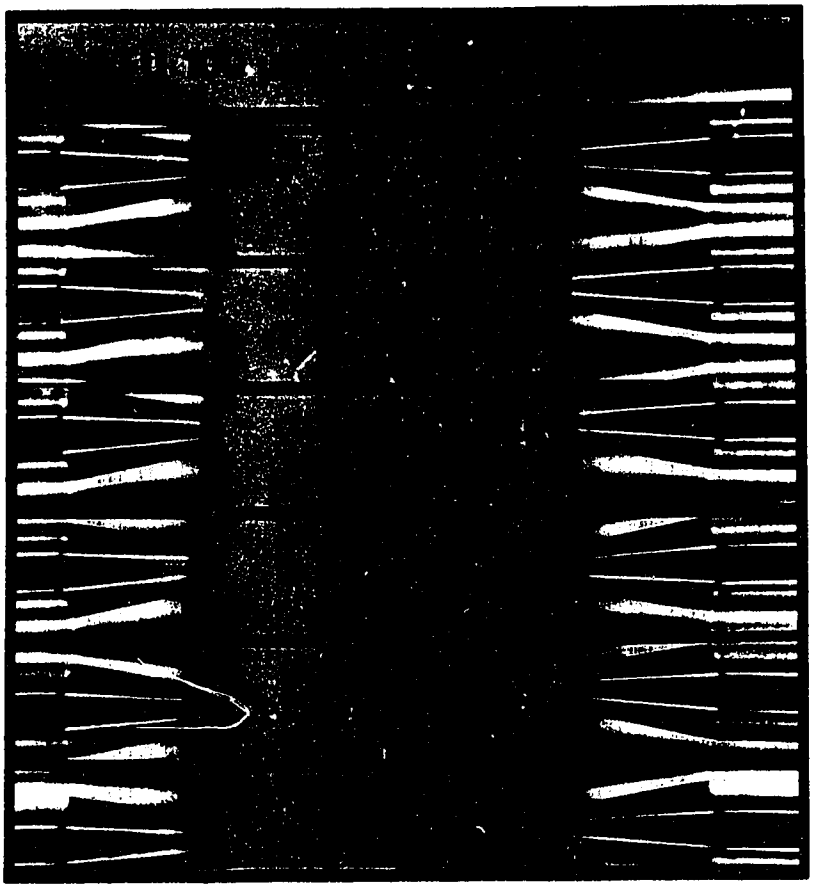

Fig. IV-2. Time-course (day 1-5) of melanization of SK-MEL-23 (human melanotic melanoma) cells grown in MEM supplemented with 10% fetal calf serum and in the presence (right side) or absence (left side) of a tyrosinase inhibitor, phenylthiourea (PTU) 100 μM. The addition of PTU almost completely inhibited the biosynthesis of melanin in melanoma cells without any inhibitory effect on cell proliferation.

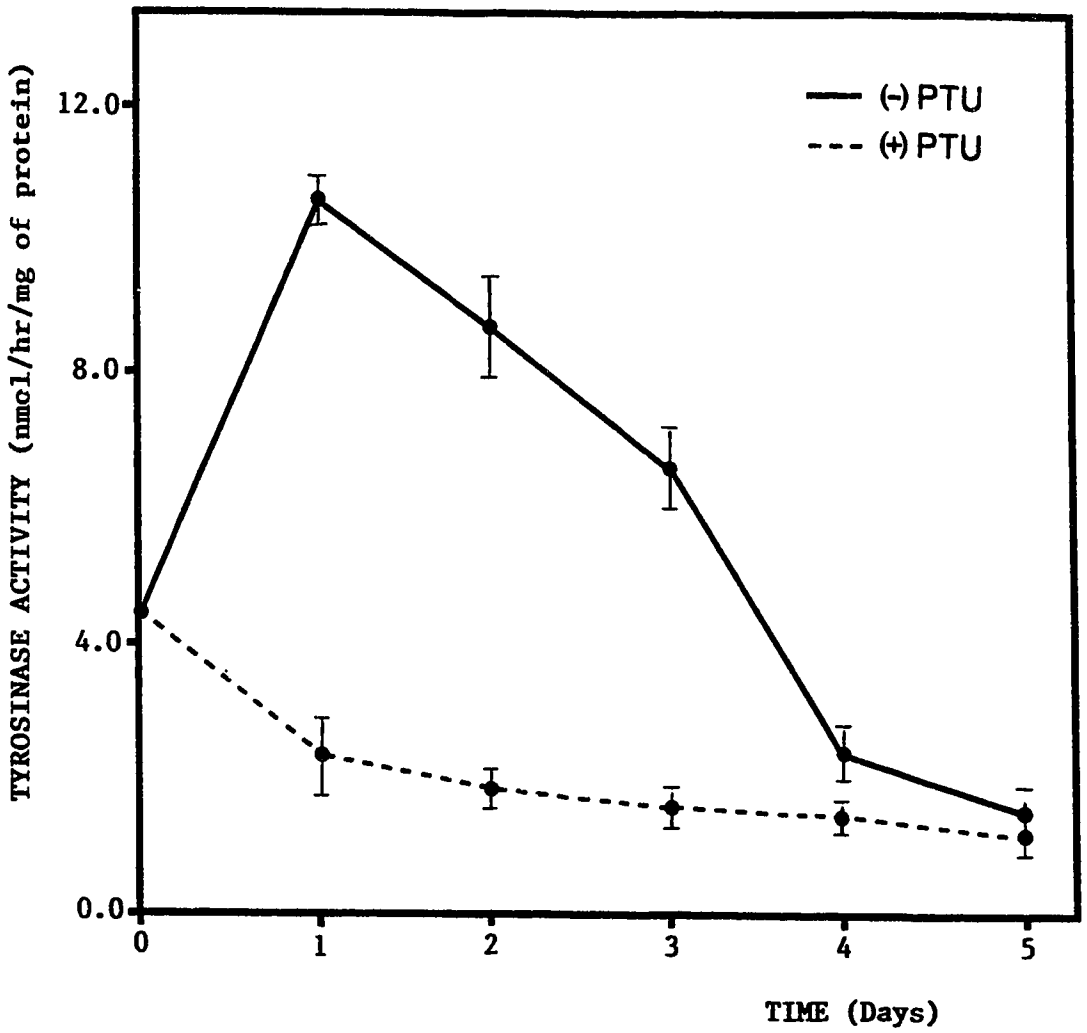

Fig. IV-3. Time-Course of Tyrosinase Activity of SK-MEL-23 Melanoma Cells in the Presence or Absence of a Tyrosinase Inhibitor, Phenylthiourea (PTU).

Exponentially growing SK-MEL-23 melanoma cells were harvested, plated into T-75 flasks in concentration of $5x10^3$ cells/cm^2 and grown in MEM with 10% fetal calf serum. At 24 hr after plating, PTU in 100 μM concentration was added into experimental flasks. Cells were harvested at 24, 48, 72, 96, and 120 hr of exposure to PTU; control cells were collected in the same sequences. The number of cells was counted using a hemocytometer and the tyrosine hydroxylase activity of tyrosinase was measured as described in Materials and Methods. Each parameter was measured in triplicate.

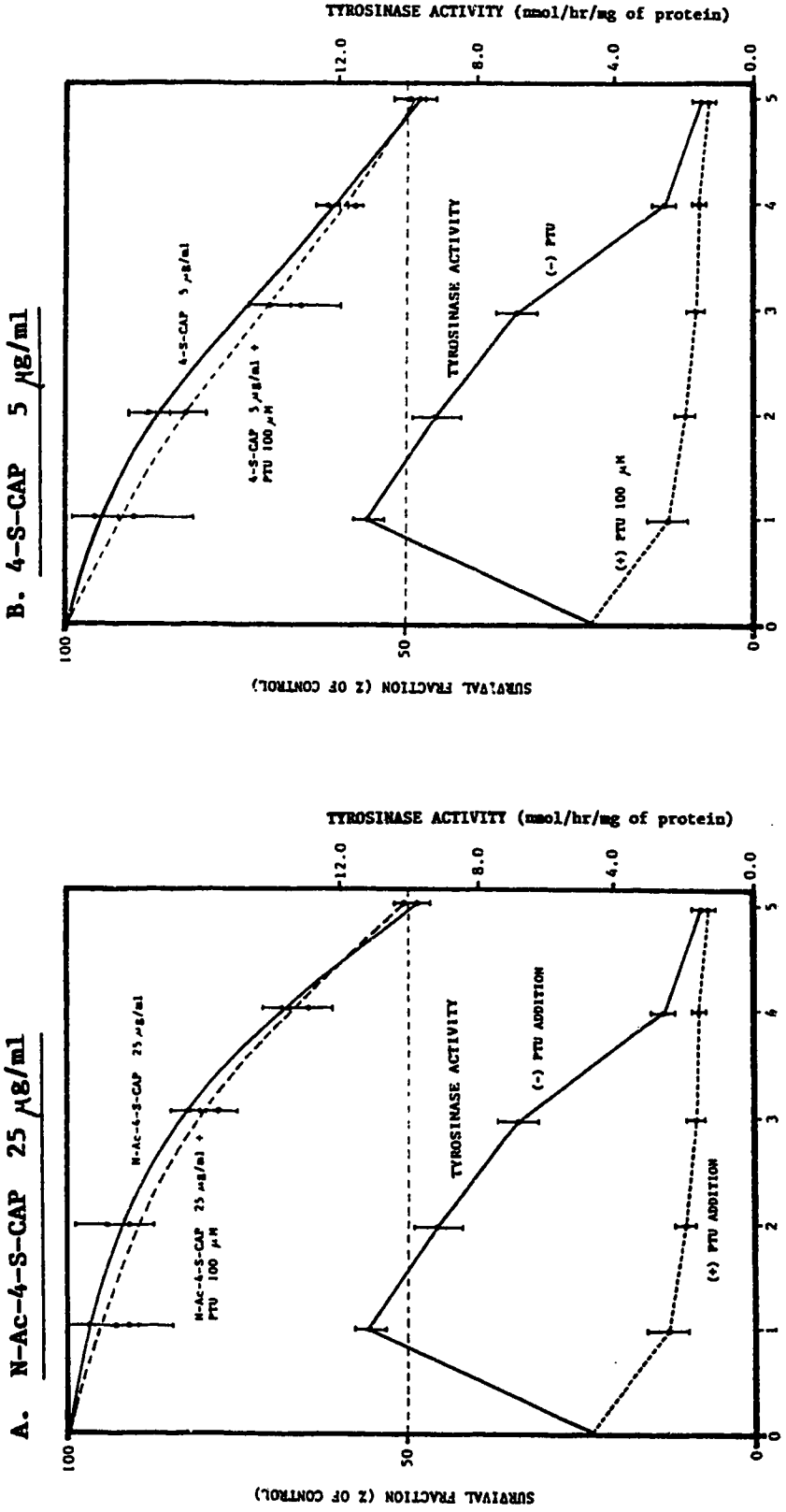

Fig. IV-4. Time-Course of *In Vitro* Cytotoxicity of N-Ac-4-S-Cysteaminylphenol (N-Ac-4-S-CAP) and 4-S-Cysteaminylphenol (4-S-CAP), and Tyrosinase Activity in Sk-MEL-23 Melanoma Cells in the Presence or Absence of Tyrosinase Inhibitor, Phenylthiourea (PTU) 100 μM.

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V. DISTINCT DEPIGMENTATION EFFECTS OF N-ACETYL-4-S-CYSTEAMINYLPHENOL IN HAIR FOLLICLES OF BLACK AND YELLOW MICE IN VIVO.

A. INTRODUCTION

Many chemicals are melanocytotoxic and cause depigmentation of the skin and hair in humans and experimental animals (1). *p*-Hydroxyphenyl derivatives, e.g., monobenzylether of hydroquinone or 4-hydroxyanisole, are the most extensively studied group of such depigmenting agents (2, 3). The common property of these depigmenting phenols is their capacity to act as substrates for the unique enzyme of pigment cells, tyrosinase (E.C. 1.14.18.1). It has been proposed that the observed selective melanocytotoxicity is mediated by toxic products of tyrosinase, the orthoquinones (4, 5). Thus, according to this theory, only pigment cells with active tyrosinase are susceptible to the cytotoxic action of *p*-hydroxyphenols.

Hair follicles of C57BL/6J mice have been recognized as a particularly useful model for studying the melanocytotoxic effects of drugs *in vivo* (6). In these mice, plucking of hairs induces the anagen phase of hair growth which is coupled with melanogenesis in activated follicular melanocytes. The C57BL/6J mice exist in several differently colored strains; the (a/a) strain is black due to the production of black pigment, eumelanin, whereas the (A^y/a) strain is yellow due to the synthesis of the yellow pigment, pheomelanin, in hair follicles (7). The plucking of hairs initiates the formation of new hair follicles with many activated follicular melanocytes and high tyrosinase activity in both black and yellow C57BL/6J mice. Therefore, one would anticipate that during the anagen phase, a potent melanocytotoxic phenol which is a substrate for tyrosinase, should cause the depigmentation of newly grown hair in both black and yellow mice. However, contrary to this expectation, researchers have succeeded only in depigmenting of hair follicles in

black mice. Attempts to depigment hair follicles in yellow mice by phenols have been unsuccessful (8). The reason for this discrepancy remains unknown.

Previously, the biological effects of a new type of *p*-hydroxyphenol, N-acetyl-4-S-cysteaminylphenol (NACAP) (9) have been presented. NACAP is a phenolic thioether (PTE) and a substrate for tyrosinase, which has been shown to possess a high depigmenting potency in the black hair follicular model as well as significant *in vivo* growth inhibitory effect against murine B16F10 melanoma. Moreover, NACAP has been shown to possess low systemic toxicity with an LD_{50} of 1200 mg/kg after a single i.p. injection. This property made it an attractive candidate compound for *in vivo* studies of the mechanism of cytotoxicity by PTEs.

In this study, the differences in the susceptibility of black and yellow follicular melanocytes to NACAP *in vivo* were examined. The details concerning the depigmentation of black hair follicles by NACAP and a demonstration of the effects of NACAP against yellow hair follicles in mice are presented. Based on the results obtained, a novel alternative hypothesis explaning the mechanism of cytotoxicity by NACAP is proposed.

B. MATERIALS AND METHODS

Animals. The black C57BL/6J (a/a) and yellow C57BL/6J-A^y (A^y/a) mice, 6 week old females, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the Animal Farm of the University of Alberta and fed *ad libitum* with water and rat/mouse chow.

Chemicals. N-acetyl-4-S-cysteaminylphenol (NACAP) was synthesized in our laboratory using a modified method previously described by Wehrmeister (10). The purity of the drug was 99.9% according to HPLC measurements. All other reagents were of analytical grade.

Melanocytotoxicity In Vivo. Mice having hair in the telogen (resting) phase of growth were briefly anesthesized by inhalation of methoxyflurane (Metophane) and hairs were manually plucked from a small area of the back. Twenty black mice and twenty yellow mice were randomly divided into four groups of five (one group was control and one group for each of three different dose-regimens of NACAP). NACAP was dissolved in normal saline solution and the solution was sterilized by membrane filtration. Starting 72 hr after plucking, the mice were given i.p. injections of 0.9% saline (0.154 M NaCl) solution (control) or solutions of NACAP in 0.154 N NaCl at concentrations of 0.5, 1.0, and 2.0 mmol/kg, respectively, once a day for 10 consecutive days. The final day of the assay was day 21 after plucking (the beginning of telogen phase).

Melanin Content. In the black and yellow mice described above, newly grown hair from previously plucked areas was harvested and its eumelanin and pheomelanin content was analyzed. Details of the melanin assays were previously reported (11). Briefly, the eumelanin content was measured by chemical degradation of eumelanin with $KMnO_4$. The product, pyrrole-2,3,5-tricarboxylic acid (PTCA) was analyzed by HPLC with a UVL detector; samples were measured in duplicate. The results were expressed as ng of PTCA per mg of wet tissue; 1 ng of PTCA corresponds to about 50 ng of eumelanin. The pheomelanin content was measured by HPLC after chemical degradation of pheomelanin by hydriodic acid into aminohydroxyphenylalanine (AHP). The results were expressed as ng of AHP per mg of tissue; 1 ng of AHP is equal to 5 ng of pheomelanin.

Electron Microscopy. On day 8 after plucking, samples of skin with regrowing hair follicles were excised under brief general anesthesia, pre-fixed in Karnovsky's solution, and post-fixed in 1% osmium tetroxide in cacodylate buffer. Tissues were stained with 1.5%

uranyl acetate, dehydrated in graded ethanol solution, embedded in epoxy resin, sectioned with a Potter-Blum Ultramicrotome MT II, and thin sections were stained with lead citrate.

Glutathione Assay. Twelve black mice and twelve yellow mice were plucked as previously described. Then, separately for each strain, mice were randomized into four groups of three animals (one control and three experimental groups treated with different doses of NACAP). On day 5 after plucking, control mice were given a single i.p. injection of normal saline solution and mice of the first, second, and third experimental groups were given a single i.p. injection of NACAP at 0.5, 1.0, and 2.0 mmol/kg concentration, respectively. Two hours after injection, animals were killed by cervical dislocation, and skin from the plucked area and samples of lung, and liver were removed and immediately frozen in liquid nitrogen. The total glutathione (GSH) content of these samples was assayed by a method that embodied those of Tietze (12) and Griffith (13). Briefly, frozen tissues were weighed and homogenized in 5.0 volumes (w/v) of 1% picric acid (w/v) on ice using a glass-glass homogenizer. Homogenates were centrifuged and the supernatant fractions (after spinning at 10,000 g for 10 min) were used to determine the total GSH content in tissues using a GSSG-reductase assay (13).

C. RESULTS

Dose-Dependent Depigmentation of Black Hair Follicules by NACAP (Figure V-1). NACAP administration during the anagen phase of hair regrowth resulted in dose-dependent effects on follicular melanocytes and resulting changes in the color of new hair. A dose of 0.5 mmol/kg of NACAP did not produce any visible depigmentation of the black follicles (Figure V-1a), whereas a dose of 1.0 mmol/kg was partially effective with the newly grown hair follicles being grey (Figure V-1b). The dose 2.0 mmol/kg of NACAP was

the most effective and the newly grown hair follicles of these mice were depigmented and pure white (Figure V-1c).

Depigmentation Effect of a Single and Multiple Injections of (NACAP 2.0 mmol/kg) on Black Hair Follicles. Ten black mice were plucked as described in Materials and Methods and distributed into two groups of five. Group 1 mice were given a single i.p. injection of NACAP (2.0 mmol/kg) on day 3 after plucking. As seen in Figure V-3a, the result of the treatment was complete (white) depigmentation of new hair. Group 2 mice were given multiple i.p. injection of NACAP (2.0 mmol/kg) daily for 150 days. The new hair after the first plucking grew white, however, after about 60 days were naturally replaced by black hair from spontaneously initiated anagen. On day 120 after the first plucking, hair on the back of mice were plucked for a second time to initiate the anagen phase. The new hair was incompletely depigmented, presenting various shades of grey (Figure V-3b). Importantly, no signs of chronic cummulative toxicity of NACAP were observed. The body weight of animals gradually increased from original 17.0 g to 28.0 g at the end of the experiment. This increase of body weight was the same as that of control (untreated) mice.

Reversible Pigmentation of New Hair after Previous Depigmentation with NACAP. In order to study whether the melanocytotoxic action of NACAP against follicular melanocytes causes reversible (temporary) or irreversible (permanent) loss of hair pigmentation in plucked area, six black mice were prepared for a depigmentation assay as described in Materials and Methods and were treated with i.p. injection of NACAP (2.0 mmol/kg), daily for 10 days. The result was pure white depigmentation of hair regrown in the plucked areas. On day 22 (early telogen phase), hairs were plucked from a small central part of the depigmented area, thus leaving a wide peripheral rim of depigmented hair. Animals were distributed into two groups of three. Group 1 mice did not receive any

additional treatment. As demonstrated in Figure V-4a, at 21 days after the second plucking, the regrown central hair was fully pigmented, its black color being of the same shade as that of the hair of control black mice. Group 2 mice were given an i.p. injection of NACAP (2.0 mmol/kg) daily for 10 days. New hair grew white and was indistinguishable from the previously depigmented hair (Figure V-4b).

Electron Microscopy of Depigmented Black Hair Follicles after i.p. Injections for Five Days. The follicles from control mice and mice treated with NACAP (0.5 mmol/kg) contained similar numbers of active melanocytes and many mature melanosomes (Figure V-5a). Melanocytes from mice treated with NACAP (1.0 mmol/kg) exhibited a reduction in number of both melanocytes and melanosomes, the latter being incompletely melanized (stage II-III), smaller, and degraded (Figure V-5b). No melanocytes or melanosomes were visible in follicles treated with NACAP (2.0 mmol/kg) (Figure V-5c). Keratinocytes and fibroblasts present in follicles did not show evidence of morphologic changes.

Effects of NACAP in Yellow Hair Follicles (Figure V-2). The effects of the NACAP treatment in yellow mice were significantly different to those seen in black mice. The doses of NACAP 0.5 and 1.0 mmol/kg did not produce any visible changes in pigmentation of new hair and the hair color remained a yellow-red of the same shade as that of controls (Figure V-2a). However, in two of five animals treated with NACAP (2.0 mmol/kg), newly grown hair was visibly dark, the color being incompletely black mixed with yellow-red (Figure V-2b). The hair color of the remaining three animals was macroscopically the same as that of controls without any dark (black) component.

Melanin Content in Chemically Treated Hair Follicles. The eumelanin and pheomelanin content of hair samples from black and yellow mice treated with different concentrations of NACAP are summarized in Table V-1. In the black mice, NACAP

treatment with 0.5, 1.0, and 2.0 mmol/kg resulted in a dose-dependent reduction in the eumelanin content of hair to 98%, 28%, and 3% of control, respectively. Only non-significant amounts of pheomelanin were detectable in the hair of black mice. In yellow mice, the treatment with NACAP with 0.5, and 1.0 mmol/kg doses did not significantly affect the pheomelanin content in hair and the AHP values were 99% and 90% of control, respectively. In these mice practically no eumelanin component was detectable (the eumelanin content was similar to that of albino mice). The effect of NACAP (2.0 mmol/kg dose) was different from that of the previous two treatments. The pheomelanin content in new hair was reduced to 56% of control, and the eumelanin content became detectable, being 28% of control black mice.

Electron Microscopy of Yellow Hair Follicle (Figure V-6). The treatment with NACAP, at 0.5 and 1.0 mmol/kg concentrations, did not produce any observable changes in the morphology of hair follicles as compared to that of controls. The treatment with NACAP 2.0 mmol/kg also did not affect the general ultrastructural morphology of follicular melanocytes. It was expected initially that a large number of melanosomes should show signs of the conversion from a typical spherical (pheomelanosomal) form to a more ellipsoidal (eumelanic) form. The ultrastructural results were, however, different from this expectation since only a very few of the melanocytes showed ellipsoidal granules similar to eumelanin-containing melanosomes.

Glutathione Contents of Various Tissues after Treatment with NACAP. The NACAP treatments resulted in a dose-dependent depletion of GSH in tissues of both black and yellow mice. As shown in Table V-2, doses of NACAP at 0.5, 1.0, and 2.0 mmol/kg reduced the GSH content in the skin to 94%, 85%, and 76% of controls in black, and to 99%, 95%, and 82% of controls in yellow mice, respectively. Importantly, the same doses of NACAP also resulted in a dose-dependent GSH depletion in the lung to 92%, 87% and

81% of controls in black and to 99%, 91%, and 86% in yellow mice, respectively, and in the liver to 99%, 86%, and 64% of controls in black and 97%, 92% and 77% of controls in yellow mice, respectively.

D. DISCUSSION

The effects of NACAP against activated follicular melanocytes of black C57BL/6J mice demonstrated the exceptional depigmenting (melanocytotoxic) potential of this compound. In black hair follicles, a clearly dose-dependent depigmentation was observed. The degree of depigmentation ranged from no visible depigmentation, through various shades of grey, to pure white depigmentation of newly grown hair.

It is widely believed that the major action of depigmenting phenolic agents against melanocytes is due to their interaction with tyrosinase (3). According to this theory, tyrosinase oxidizes phenols into highly reactive orthoquinones, and these, or their derivatives, semiquinone radicals, leak from the melanosomes into the cytosol (Figure V-7). In the cytosol, these reactants are either detoxified by conjugation with glutathione (GSH), or if unopposed, they induce a chemical injury to cells by the alkylation of macromolecules or by oxidative stress. The target organelles are thought to be the nucleus (inhibition of DNA polymerase), mitochondria (inhibition of oxidative phosphorylation), and endoplasmic reticulum with ribosomes (inhibition of protein synthesis and glycosylation). These chemical injuries may be reversible as in the pertubation of some non-essential functions, such as melanogenesis, or irreversible with resulting cell death (14).

The effects of NACAP against black hair follicles may be explained by the concept of tyrosinase-mediated cytotoxicity of phenols. It may be assumed that NACAP molecules enter the melanocyte, and thence the melanosome, and there compete with L-tyrosine, for binding to tyrosinase. Tyrosinase is a rate-limiting enzyme in melanogenesis, which implies that there is some maximum rate (limit) for the production of orthoquinone

molecules per time unit. With an increased dose of NACAP, one may also expect an increased production of orthoquinone molecules derived from NACAP, and thus the increased probability for the manifestation of its cytotoxic potential.

In Figure V-8, a model for the dose-dependent depigmentation effects of NACAP is depicted. It is postulated that the bulk of reactive intermediates derived from the NACAP dose of 0.5 mmol/kg was insufficient to induce chemical injury and consequently failed to damage the follicular melanocytes. The net result was normal black pigmentation of newly grown hair. In contrast, a dose of 1.0 mmol/kg of NACAP was capable of inducing some chemical injury to the follicular melanocytes, however, this degree of cellular damage was compatible with survival for the majority of these follicular melanocytes. The result was partial inhibition of melanogenesis and a grey depigmentation of new hair. The amount of reactive intermediates derived from a dose of 2.0 mmol/kg overcame the capacity of follicular melanocytes to survive the chemical attack and caused their total destruction. The result was complete depigmentation and new hair was white. Electron microscopic findings support this suggestion.

However, there are serious flaws in models which invoke the concept of tyrosinase-dependent cytotoxicity of phenols. For example, it remaines unexplained why orthoquinones derived from synthetic tyrosinase substrates should be more reactive than the highly reactive dopaquinone moieties derived from L-tyrosine. Also, it is known that under normal circumstances, the activated melanocytes possess several very effective protective mechanisms which diminish the hazard created by melanogenesis. The first of these is obviously that the melanogenic process occurs exclusively within a membrane-bound organelle, the melanosome, which sequesters the reactive components (15). Secondly, the abundant cytosolic nucleophiles such as glutathione, should inactivate orthoquinone molecules that leak from the melanosome. The leaking of reactants from the melanosomes is necessary for cytotoxicity, but experimental demonstration has not been reported. In fact, experiments using tyrosinase encapsulated in liposomes have

demonstrated that only minor amounts of melanogenic intermediates diffuse across the phospholipid membrane (16).

Due to the difficulties in explaining how reactants leak from the melanosome, some investigators hypothesize (17) that the tyrosinase present in the endoplasmic reticulum, Golgi apparatus, and coated vesicles, may be catalytically active and acts as a source of the extramelanosomal formation of toxic orthoquinones. Tyrosinase is a glycoprotein which is synthesized by rough endoplasmic reticulum, then it is glycosylated in Golgi complexes and packed along with "post-tyrosinase regulatory factors", e.g., dopachrome conversion factor and indole blocking factor in coated vesicles (18). The coated vesicles containing tyrosinase fuse with the membrane-delimited vacuoles of stage I melanosomes derived from smooth endoplasmic reticulum (18). Indeed, there is good evidence of a significant amount of extramelanosomal tyrosinase in pigment cells (19). Hatta *et al.* (20) reported the presence of melanin monomers within coated vesicles and premelanosomes (stage I melanosomes) in pigment cells, however, several other investigators have shown that this extramelanosomal tyrosinase is catalytically inactive (21, 22). Presently, it is accepted that in mammals, active tyrosinase is present in several subcellular organelles of the melanocyte, but, *in vivo*, melanogenic activity occurs only in melanosomes (23).

It was found that even a single i.p. injection of NACAP (2.0 mmol/kg) was capable of producing complete depigmentation of new hair when administered at the beginning of the melanogenic phase of hair growth in black mice. However, the effects of NACAP against activated follicular melanocytes in yellow mice were different from those observed in black mice. The lower doses of NACAP (0.5 and 1.0 mmol/kg) did not affect the normal yellow pigmentation of new hair. However, the dose of 2.0 mmol/kg resulted in a dark, almost black color of new hair. The black color suggested the presence of eumelanin pigment and that indication was confirmed by measurements of the eumelanin and pheomelanin content of the hair of yellow mice after treatment with NACAP.

Previously, the conversion of pheomelanogenesis to eumelanogenesis was reported by Geschwind et al. in lethal yellow mice (A^y/a) after injections with high doses of alpha-melanocyte stimulating hormone (24). Similarly, Ikejima and Takeuchi observed the production of eumelanin in the hair-bulb melanocytes of UV-irradiated A^y/a mice (25). However, a conversion of pheomelanogenesis into eumelanogenesis by synthetic drugs *in vivo* has not been described previously. Several studies had, in contrast, concluded that follicular melanocytes of yellow mice were cells which were not susceptible to depigmenting phenols (8, 26, 27). A possible explanation for this phenomenon was based on the reported low level of tyrosinase activity and resulting cytotoxic intermediates of eumelanogenesis in yellow follicular melanocytes (8).

The conversion of pheomelanogenesis into eumelanogenesis is a result of dynamic changes in melanogenic biochemistry. Both eumelanin and pheomelanin have the same natural precursor, dopaquinone. Dopaquinone is a highly reactive electrophile with a very short life span (28). In the presence of nucleophilic thiols such as cysteine or glutathione (GSH), dopaquinone forms cysteinyldopa and/or glutathionedopa conjugates. Specifically, 5-S-cysteinyldopa is a major precursor for pheomelanin synthesis. In the absence of significant amounts of thiols inside the melanosome, dopaquinone is cycled into dopachrome and further into indoles which polymerize to eumelanin (29). Thus, the critical factor which directs dopaquinone into pheomelanic or eumelanic pathway is the presence or absence of thiols at the site of melanogenesis (18).

Based on a review of melanogenic biochemistry, the possibility of thiol deficiency in follicular melanocytes after the NACAP treatment was predicted. Although it was known that follicular melanocytes represent a very small fraction of the skin activated by plucking, the GSH content in the skin of black and yellow mice was nevertheless measured. A significant, dose-dependent depletion of GSH level was observed. Since the bulk of whole skin tissue is made up of keratinocytes and fibroblasts, it was tentatively concluded that the GSH depletion was due to NACAP metabolic effects in these non-pigment cells. The

control measurements of GSH levels in the lung and liver of black and yellow mice confirmed the existence of hepatic and extrahepatic drug metabolism of NACAP which is associated with significant GSH depletion. Due to its absence in lung or liver tissues, tyrosinase could not be the sole enzyme responsible for converting an inert molecule of NACAP into a reactive intermediate(s). Thus, there is probably a common tyrosinase-independent drug-metabolizing pathway present in pigment and non-pigment cells.

In mice, the type of melanin synthesis in follicular melanocytes is determined by alleles at the agouti locus of chromosome 2 which act through the microenvironment of the hair follicle rather than through the follicular melanocytes themselves (30). Mutations at the agouti locus result in different pigmentation of hair. For example, the hair follicles of nonagouti C57BL/6J (a/a) mice synthesize a pure eumelanin while those of yellow C57BL/6J-A^y (A^y/a) mice produce a pure pheomelanin pigment due to the action of the same enzyme, tyrosinase. Granholm *et al.* (31) measured the tyrosinase activity in yellow follicular melanocytes and found it to be only about 25-35% of that in black follicular melanocytes. Other effects of the A^y mutant have also indicated that the main role of the agouti locus is the control of metabolic regulatory systems and that the site of this regulations is the smooth endoplasmic reticulum (32). The endoplasmic reticulum is known to be a major site of drug metabolism (33). Thus, it is possible that the agouti locus may negatively influence not only the expression of tyrosinase but also the expression of drug metabolizing enzymes of the endoplasmic reticulum in yellow follicular melanocytes. This hypothesis may explain, at least partially, the different susceptibility of black and yellow follicular melanocytes to depigmenting phenols.

In Figure V-9, the current hypothesis concerning the conversion of pheomelanogenesis to eumelanogenesis is demonstrated. NACAP is thought to be an inert lipophilic prodrug that enters cells and then activated into reactive, electrophilic intermediate(s), by some drug-metabolizing enzyme(s) of the endoplasmic reticulum. This reactive intermediate is rapidly conjugated with GSH. The NACAP-GSH conjugate is

probably expelled from the cell and may be detected in urine in its mercapturic acid form (34). The formation of NACAP-GSH conjugates leads to a relative depletion of GSH in the cytosol. Since GSH is a major reducing equivalent in cells, as well, as a major physiological reservoir for cysteine in many cell types, the GSH depletion may also create a deficiency of reduced cysteine in the cytoplasm and consequently inside the melanosomes (35). Cysteine is a dominant thiol in the pheomelanic melanosomes (36). The lack of soluble free cysteine in the melanosome would probably prevent the formation of 5-S-cysteinyldopa, and consequently no pheomelanin is produced. Under these circumstances, the tyrosinase product, dopaquinone, enters the eumelanic synthetic pathway. The resulting outcome is black pigmentation of newly grown hair in yellow mice.

Thus, the same dose of NACAP (2.0 mmol/kg) caused white depigmentation of hair follicles in black mice and a black pigmentation of hair follicles in yellow mice. It is proposed that the observed differences in the biological effects of NACAP in black and yellow follicular melanocytes are caused by the qualitatively identical process of drug metabolism of NACAP in the cells. However, the quantitative differences in both the metabolic activation of NACAP and consequent GSH conjugation of NACAP intermediate(s) in black and yellow mice may be a major reason for the chemically induced destruction of black melanocytes and the relatively mild functional alteration of yellow melanocytes. It may be further speculated that the significantly slower rate of activation of NACAP and/or higher reducing milieu, e.g, the actual cellular concentration and promptness to resynthesize GSH, in yellow melanocytes in contrast to the situation in black melanocytes may protect the former from the chemical damage by phenols *in vivo*. The same situation may also be true for dormant melanocytes, albino melanocytes and other unrelated non-pigment cells. This speculation may also partially explain the discrepancy between selective melanocytotoxicity of PTEs *in vivo* and their non-specific cytotoxicity *in vitro* (Chapter IV).

In summary, the cytotoxicity of PTEs appears to be mediated by tyrosinase and other drug metabolizing enzymes within the pigment cells. This tyrosinase-independent cytotoxicity of PTEs is associated with depletion of GSH and may be significantly affected by the actual intracellular level of GSH.

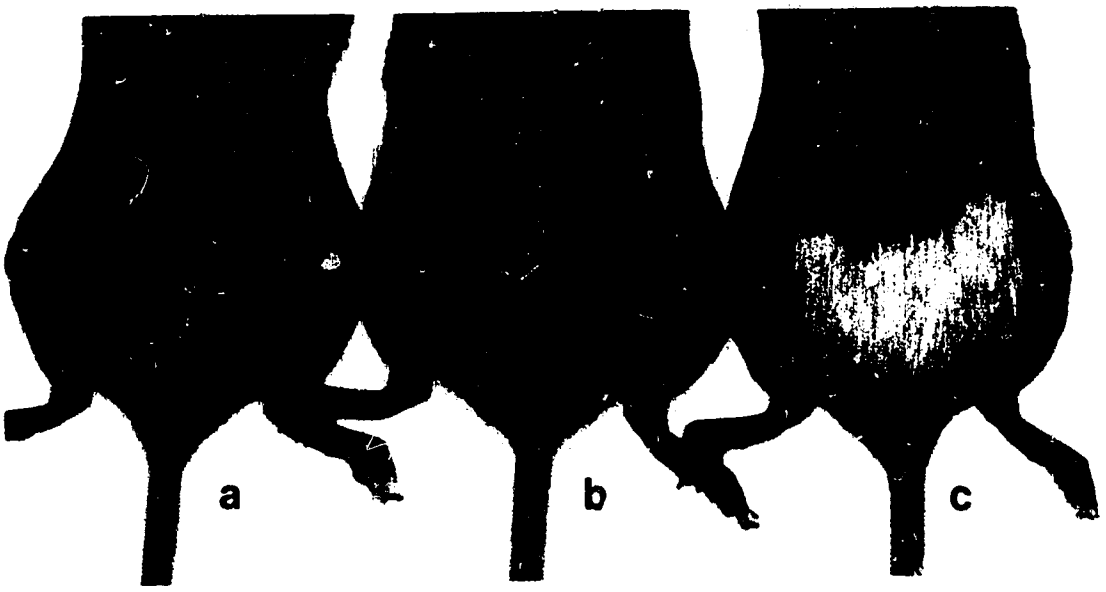

Fig. V-1. Macroscopically visible dose-dependent effects of NACAP on pigmentation of black hair follicles after i.p. injections in doses: a) 0.5 mmol/kg (black pigmentation identical to control); b) 1.0 mmol/kg (grey depigmentation), and c) 2.0 mmol/kg (white depigmentation), daily for 10 days.

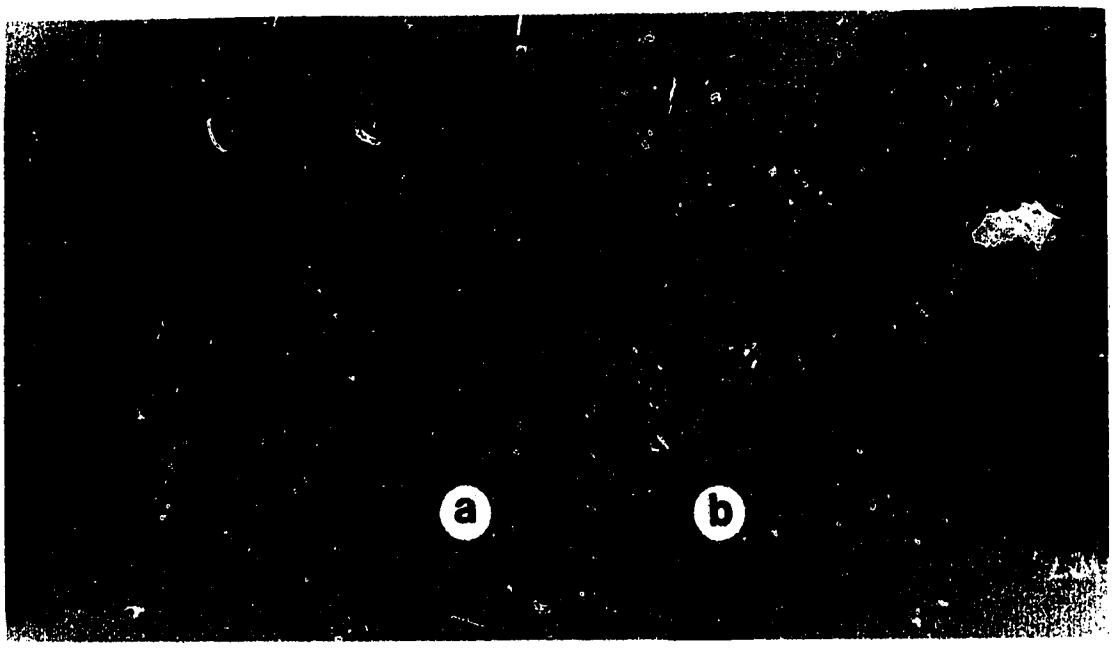

Fig. V-2. Macroscopically visible effects of NACAP on pigmentation of yellow hair follicles after i.p injection in doses: a) 0.5 and 1.0 mmol/kg (yellow pigmentation identical to control); and b) 2.0 mmol/kg (mostly black pigmentation of hair), daily for 10 days.

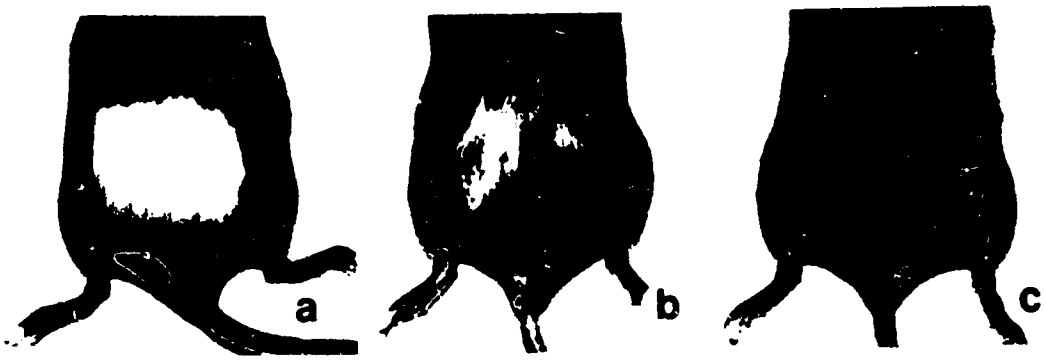

Fig. V-3. Depigmentation effect in black hair follicles after **a)** a single i.p. injection of NACAP (2.0 mmol/kg) on day 3 after plucking; and **b)** multiple injections of NACAP (2.0 mmol/kg) daily for 120 days. **c)** a control mouse treated with normal saline solution.

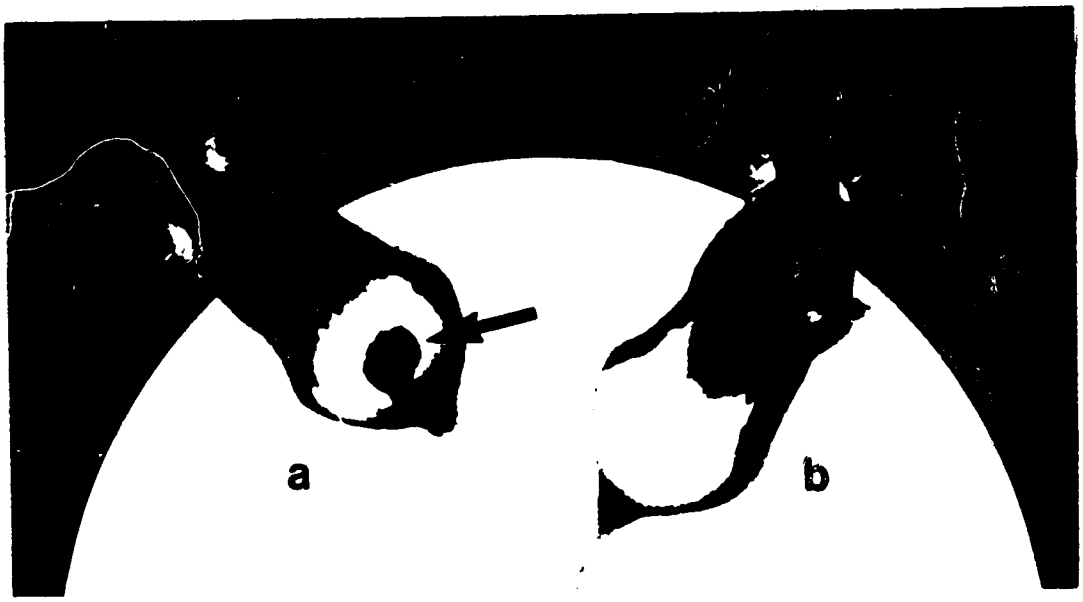

Fig. V-4. Reversible pigmentation of black hair follicles after previous chemical depigmentation. Mice were plucked and treated with i.p. injection of NACAP (2.0 mmol/kg) daily for 10 days. Twenty one days after the first plucking, the mice were plucked for the second time in a small central portion within the depigmented areas, and mouse **a** received no further treatment, whereas mouse **b** was again treated with NACAP, as previously described. New hair after the second plucking in **a** mouse was black as that of control animals, whereas hair in **b** mouse was completely depigmented into white.

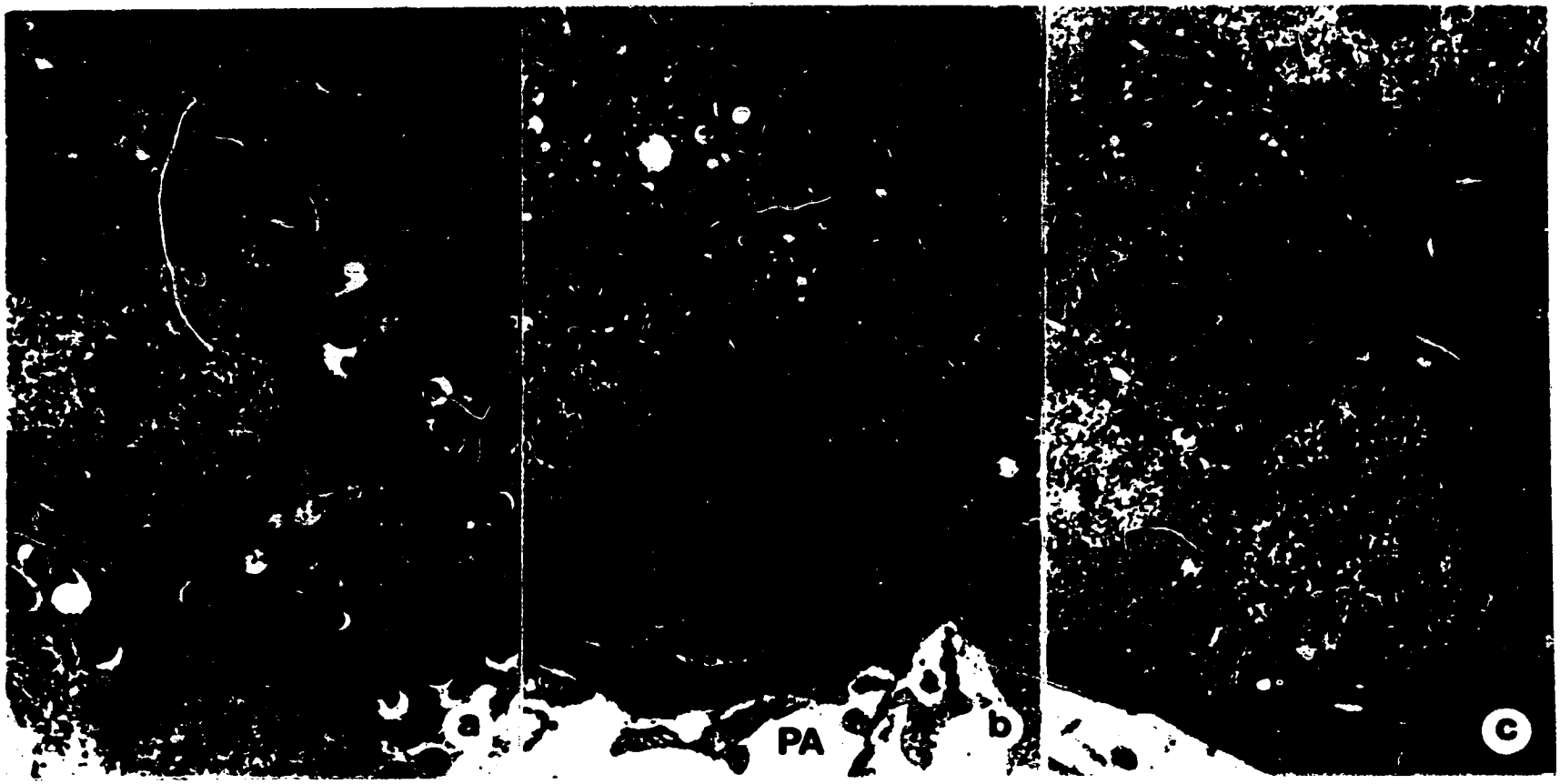

Fig. V-5. Electron micrographs of black hair follicles after i.p. injection of NACAP in various doses daily for five days. Bar, 1 μm. MC, melanocyte; KC, keratinocyte; MS, melanosome; PA, dermal papila. a) control and after treatment with 0.5 mmol/kg of NACAP; no degenerative changes are detectable and many mature melanosomes are present in the follicle. b) after treatment with 1.0 mmol/kg of NACAP ; melanosomes around few remaining melanocytes are smaller and degraded. c) after treatment with 2.0 mmol/kg of NACAP; no melanocytes or melanosomes are seen in the melanocytic zone of the hair follicle. x 7000.

Fig. V-6. Electron microscopy of yellow hair follicle A) control; B) after i.p. injection of NACAP (2.0 mmol/kg) daily for five days. MC, melanocyte; PA, dermal papilla. No significant morphologic changes are visible on melanosomes treated with NACAP. *Bar*, 1 μm.

TABLE V-1. EUMELANIN AND PHEOMELANIN CONTENTS IN HAIR OF MICE TREATED WITH N-ACETYL-4-S-CYSTEAMINYLPHENOL IN I.P. INJECTIONS DAILY FOR TEN DAYS.

MOUSE STRAIN	DOSE OF NACAP (mmol/kg)	PTCA[a] (ng/mg)	AHP[b] (ng/mg)	PTCA/AHP RATIO
Black C57BL/6J (a/a)	None (control)	1079.0±109.0[c]	55.3± 4.1	19.51
	0.5	1058.0±148.7	50.8±10.2	20.83
	1.0	298.0± 20.6	58.1±13.5	5.13
	2.0	33.3± 7.4	55.0± 9.8	0.61
Yellow C57BL/6J (A^y/a)	None (control)	16.1± 1.4	2882.1±280.2	0.0056
	0.5	15.8± 0.6	2851.9±330.4	0.0055
	1.0	15.9± 0.8	2590.9±225.7	0.0061
	2.0	304.2± 41.2	1608.4±324.9	0.1891

[a] PTCA = Pyrrole-2,3,5-tricarboxylic acid, a eumelanin indicator;

[b] AHP = Aminohydroxyphenylalanine, a pheomelanin indicator;

[c] Sample mean ± S.D. (n = 3);

TABLE V-2. GLUTATHIONE CONTENT IN THE SKIN, LUNG, AND LIVER OF BLACK AND YELLOW MICE TWO HOURS AFTER THE TREATMENT WITH A SINGLE INJECTION OF N-ACETYL-4-S-CYSTEAMINYLPHENOL IN 0.5, 1.0, AND 2.0 mmol/kg DOSES.

TREATMENT	GSH CONTENT (μmol/g of tissue)		
	SKIN	LUNG	LIVER
Black C57BL/6J mice			
None (control)	0.541±0.037[b]	2.014±0.179	5.065±0.234
NACAP 0.5[a]	0.512±0.029	1.855±0.106	5.014±0.223
NACAP 1.0	0.460±0.033	1.750±0.143	4.357±0.284
NACAP 2.0	0.372±0.021	1.633±0.138	3.205±0.380
Yellow C57BL/6J-A^y/a mice			
None (control	0.620±0.041	2.095±0.159	5.443±0.359
NACAP 0.5	0.617±0.030	2.066±0.155	5.282±0.235
NACAP 1.0	0.550±0.058	1.903±0.121	4.990±0.195
NACAP 2.0	0.499±0.054	1.787±0.112	4.166±0.315

[a] mmol/kg

[b] Mean ± S.D. (n = 3)

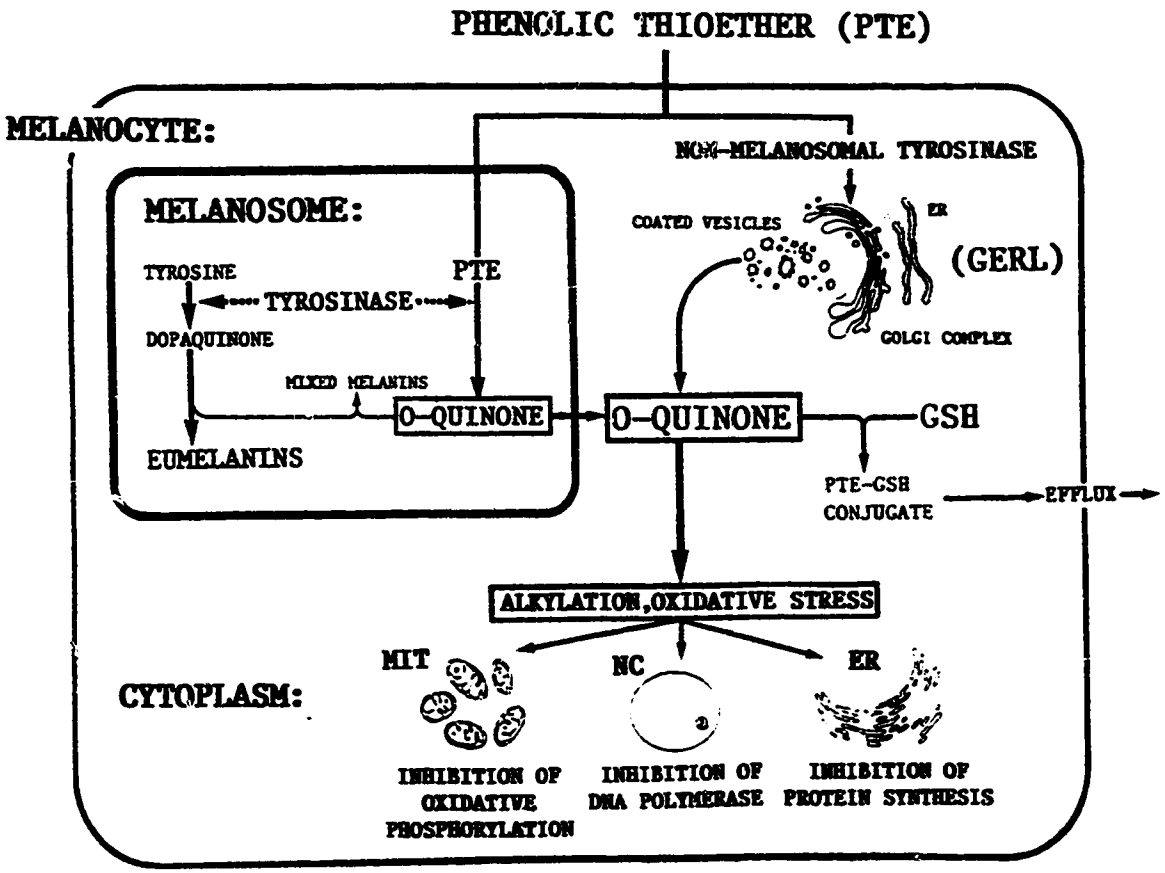

Fig. V-7. The proposed tyrosinase-mediated mechanism of melanocytotoxicity by phenolic thioethers (PTEs). After diffusion through the plasma membrane and the cytoplasm into the melanosome, a chemically inert PTE is converted by tyrosinase into a highly reactive orthoquinone moiety which damages the melanosomal membrane by the initiation of lipid peroxidation. The consequence is a leakage of orthoquionone molecules into the cytoplasm where some molecules are detoxicated by a conjugation with glutathione (GSH), while other molecules may alkylate macromolecules (proteins) or initiate oxidative stress. The final outcome is a reversible or irreversible damage to the pigment cell.

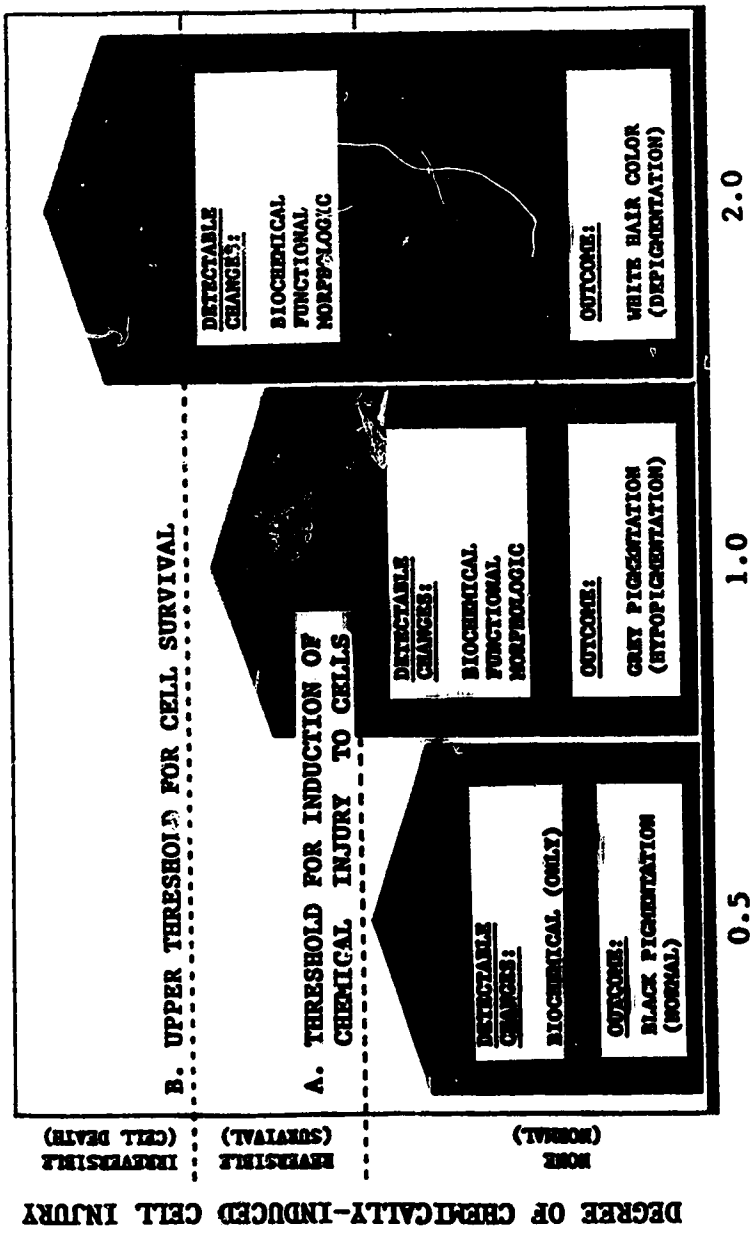

Fig. V-8. *In vivo* dose-dependent chemically induced injury to activated follicular melanocytes in black C57BL/6J mice treated with daily i.p. injection of NACAP in three different dose-regimens for 10 days.

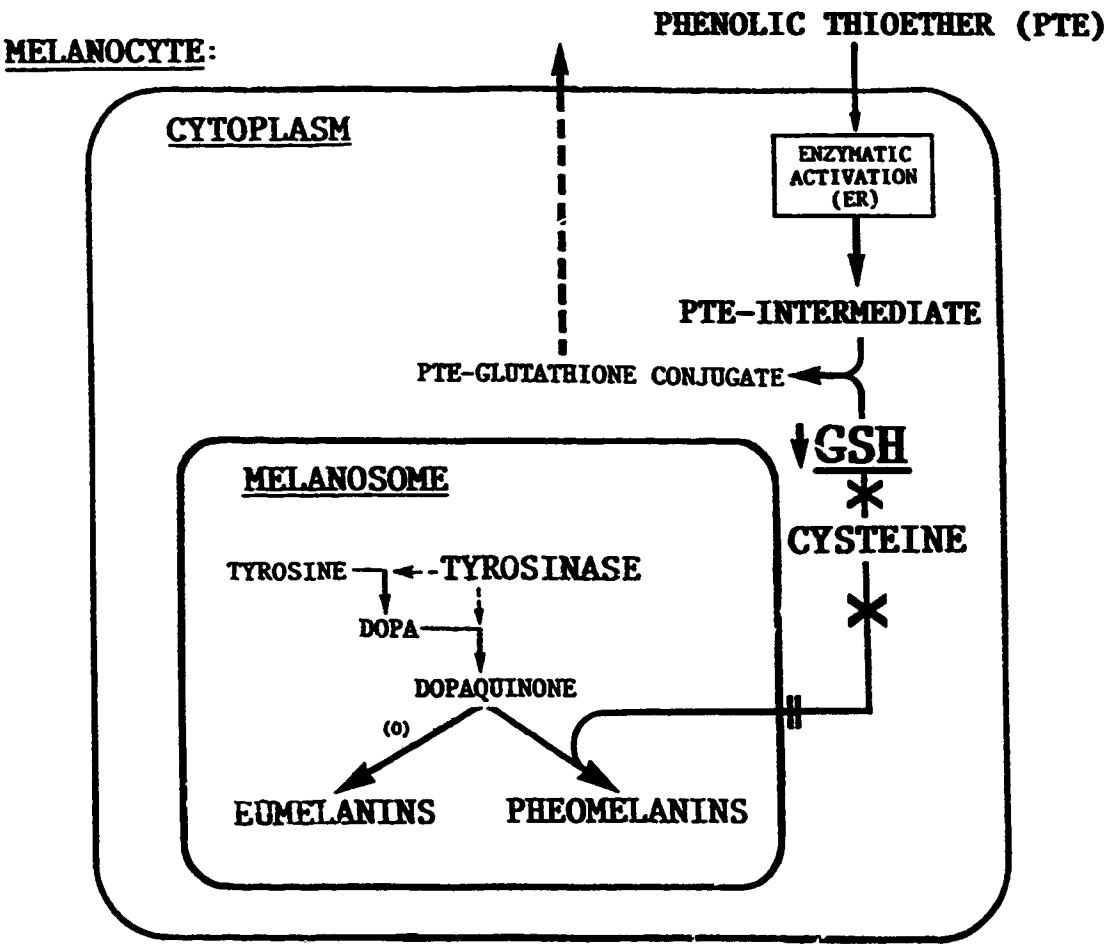

Fig. V-9. The proposed mechanism of the conversion of pheomelanogenesis to eumelanogenesis in activated follicular melanocytes of yellow C57BL/6J Ay/a mice treated with N-acetyl-4-S-cysteaminylphenol (2 mmol/kg) or other phenolic thioethers (PTE)*in vivo*. After diffusion into the cell, a chemically inert PTE is activated by some phase I drug-metabolizing enzyme (cytochrome P-450?) located in the endoplasmic reticulum into a reactive intermediate which is conjugated with glutathione (GSH). The excessive consumption of GSH leads to the depletion of cytoplasmic GSH. The consequent low level of L-cysteine in the cytoplasm causes a deficiency of L-cysteine inside the melanosome. Under this condition, the product of tyrosinase, dopaquinone, cannot bind with L-cysteine to give cysteinyldopas (precursors of pheomelanin), but enters the eumelanic biosynthetic pathway to form the pigment eumelanin.

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VI. MODULATIONS OF GLUTATHIONE AND MELANOCYTOTOXICITY OF N-ACETYL-4-S-CYSTEAMINYLPHENOL IN BLACK AND YELLOW MICE IN VIVO.

A. INTRODUCTION

We have found N-acetyl-4-S-cysteaminylphenol (NACAP) to be a selective *in vivo* melanocytotoxic agent capable of depigmenting newly grown hair in black mice to white (1). NACAP belongs to a group of phenolic compounds that are substrates for mushroom and mammalian tyrosinases (EC 1.14.18.1) and selectively toxic to pigment cells *in vivo*. Certain of these compounds, e.g., gamma-L-glutaminyl-4-hydroxybenzene (GHB), or 4-S-cysteaminylphenol (CAP) were tested for their melanocytotoxicity against follicular melanocytes in black pigmented (eumelanic) mice (2, 3). According to these reports, the tested drugs were melanocytotoxic and caused a grey depigmentation of hair in black mice. Other depigmenting phenols, e.g., the monoethylether of hydroquinone, were also tested against follicular melanocytes of yellow (pheomelanic) guinea pigs and mice (4, 5). In contrast to black follicular melanocytes, yellow follicular melanocytes were resistant to the depigmenting agents and tests failed to produce any depigmentation of yellow hair. The reason for this distinction in the susceptibility of eumelanic and pheomelanic follicular melanocytes to melanocytotoxic phenols remains poorly understood (5).

In our earlier studies of the depigmenting potency of NACAP against eumelanic and pheomelanic follicular melanocytes in mice (Chapter V). NACAP treatment at 1.0 mmol/kg caused a grey depigmentation while a dose of 2.0 mmol/kg resulted in a white depigmentation of hair. However, the same doses of NACAP had a different effect in yellow mice, the lower dose failed to affect the yellow-red color of new hair while the higher dose resulted in the change of the hair color from yellow-red to black. The reason for this phenomenon was found to be due to a conversion of pheomelanogenesis to

eumelanogenesis as a result of the depletion of tissue GSH levels caused by NACAP treatment.

In this study, we have further characterized the involvement of GSH in the melanocytotoxicity of NACAP by modulation of GSH levels in tissues of black and yellow mice using N-acetyl-L-cysteine (NAC) or buthionine sulfoximine (BSO). Evidence is here presented that the cytotoxicity of NACAP against follicular melanocytes is directly related to the GSH content in the target tissue of both black and yellow mice.

B. MATERIALS AND METHODS

Animals. The black C57BL/6J (a/a) and yellow C57BL/6J-A^y (A^y/a) mice, 6-week old females, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the Animal Farm of the University of Alberta and fed *ad libitum* with water and rat/mouse chow.

Chemicals. N-acetyl-4-S-cysteaminylphenol (NACAP) was synthetized in our laboratory using a modified method described by Wehrmeister (6). The purity of the drug was determined to be 99.9% by HPLC measurements. D,L-buthionine-(S,R)-sulfoximine (BSO), diethyl maleate (DEM), and N-acetyl-L-cysteine (NAC) were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

Melanocytotoxicity In Vivo. Mice with hair in the telogen (resting) phase of growth were briefly anesthesized by inhalation of methoxyflurane (Metophane) and hair was manually plucked from the caudal half of the back. Twenty five black mice were randomly distributed into groups of five with one group serving as control and one group for each of four different drug regimens. The same procedure was carried out with twenty five yellow mice. NACAP, BSO, and NAC were dissolved in 0.154 M NaCl solution and the solutions were sterilized by membrane filtration. DEM was diluted in sesame oil. The

experimental designs for black and yellow mice are presented in Figure VI-1. Seventy two hr after plucking, the mice were given i.p. injections of drug solutions daily for 10 consecutive days as follows: control black mice of group 1 were given 0.154 M NaCl solution and black mice of groups 2 to 4, NACAP (1.0 mmol/kg). In addition, the group 3 mice were given a s.c. injection of NAC (500 mg/kg) one hour before and 8 hours after the injection of NACAP, the group 4 mice and positive control group 5 mice received an i.p. injection of BSO (2.0 mmol/kg) one hour before the injection of NACAP. The same protocol was used also for yellow mice with the only differences being the dose of NACAP at 2.0 mmol/kg and group 4 & 5 mice receiving both an i.p. injection of BSO (2.0 mmol/kg) and a s.c. injection of DEM (0.6 ml/kg) one hour prior to NACAP injection. The end point of the assay was at day 21 after plucking (the beginning of the telogen phase).

Melanin Content. Newly grown hair from previously plucked areas on the back of black and yellow mice was harvested and the eumelanin and pheomelanin contents were determined by the method of Ito and Jimbow (7). Briefly, the eumelanin content was measured by the chemical degradation of eumelanin with $KMnO_4$. The product, pyrrole-2,3,5-tricarboxylic acid (PTCA) was measured by HPLC with a UVL detector; samples were measured in triplicates. The resuls were expressed as ng of PTCA per mg of wet tissue; 1 ng of PTCA corresponds to about 50 ng of eumelanin. The pheomelanin content was measured by HPLC after chemical degradation of pheomelanin by hydriodic acid into aminohydroxyphenylalanine (AHP). The results were expressed as ng of AHP per mg of tissue; 1 ng of AHP is equal to 5 ng of pheomelanin.

Glutathione Assay. The hair of twelve black and twelve yellow mice was plucked as previously described. For each strain, mice were randomized into four groups of three animals, consisting of one control group and three groups treated with the different drug regimens). On day 5 after plucking, control black and yellow mice were given a single i.p.

injection of 0.154 M NaCl solution and black mice from groups 2 to 4 recieved a single injection of NACAP (1.0 mmol/kg). In addition, black mice from group 3 were given a single s.c. injection of NAC (500 mg/kg) while mice from group 4 received a single injection of BSO (2.0 mmol/kg) one hour prior to NACAP. Yellow mice from groups 2 to 4 were given a single i.p. injection of NACAP (2.0 mmol/kg). In addition, the group 3 mice were given a single s.c. injection of NAC (500 mg/kg) while the group 4 mice were given a single i.p. injection of BSO (2.0 mmol/kg) and a s.c. injection of DEM (0.6 ml/kg) one hour prior to NACAP. Two hours after the injection of NACAP, animals were killed by a cervical dislocation, and their skin from the plucked areas was removed, immediately frozen in liquid nitrogen, and later used for the measurement of total glutathione (GSH) content by a method described by Tietze (8) and Griffith (9). Briefly, frozen tissues were weighed and homogenized in 5.0 volumes (w/v) of 1% picric acid (w/v) on ice using a glass-glass homogenizer. Homogenates were centrifuged, supernatants removed and used to determine the total GSH content in tissue using a GSSG-reductase assay.

C. RESULTS

Macroscopically Visible Effect of Drug Treatments on Black Hair Follicles. In Figure VI-2, the effects of the three drug regimens on the pigmentation of newly grown hair are demonstrated. A dose of 1.0 mmol/kg of NACAP reproducibly caused a partial (grey) depigmentation of hair in black mice (Figure VI-2b). The combination of NACAP (1.0 mmol/kg) with NAC (500 mg/kg) before and after the NACAP injection almost completely abolished the depigmentation effect of NACAP, the newly grown hair being black as those of controls (Figure VI-2a). The combination treatment with NACAP (1.0 mmol/kg) and BSO (2.0 mmol/kg) resulted in a significant enhancement of the NACAP depigmentation effect, the new hair being almost completely white (Figure VI-2c). A

positive control treatment of mice using BSO alone did not produce visible depigmentation of newly grown hair (data not shown).

Macroscopically Visible Effects of Drug Treatments on Pigmentation of Yellow Hair (Figure VI-3). The administration of NACAP (2.0 mmol/kg) to yellow mice resulted in predominantly black pigmentation of the upper portion of hair in one mouse and partially black (dark) pigmentation of hair in two mice of the five treated animals (Figure VI-3b). The color of new hair in all animals treated with a combination of NACAP (2.0 mmol/kg) and NAC (500 mg/kg) was the same as that of controls, that being yellowish-red (Figure VI-3a). The combination drug treatment with NACAP (2.0 mmol/kg), BSO (2.0 mmol/kg) and DEM (0.6 ml/kg) had a different effect, the new hair being mostly lightly yellowish-red and some hair being completely white. The positive control treatment of yellow mice using BSO and DEM did not result in any macroscopically visible changes in normal hair pigmentation (not shown).

Melanin Content in Chemically Treated Hair Follicles. In Table VI-1, the results of measurements of eumelanin and pheomelanin content in hair of black and yellow mice treated with three different drug regimens are presented. In black mice, the grey hair of mice treated with NACAP alone contained 29% of the eumelanin content of controls as expressed by PTCA. Black hair from mice pretreated with NAC before and after the NACAP treatment showed unremarkable reduction in eumelanin content to 96% of controls, while in the almost white hairs from mice treated with NACAP and BSO, the eumelanin content was reduced to 14% that of control. In yellow mice, the hair of control mice contained insignificant amounts of eumelanin (less than 1.4% of that of black controls). However, after the treatment with NACAP alone, the eumelanin content became clearly detectable and increased to 20% of that in black controls. At the same time, the pheomelanin content was reduced to 56% of that in yellow controls. The hair from mice treated with NAC

before and after the administration of NACAP contained negligible amounts of eumelanin and 93% of the pheomelanin content of yellow controls. The pretreatment of mice with BSO and DEM before the administration of NACAP resulted in a significant reduction in the pheomelanin content to 15% of controls.

Glutathione Contents in Skin after Various Drug Treatments (Table VI-2). The treatment with NACAP (1.0 mmol/kg in black and 2.0 mmol/kg in yellow mice) resulted in depletion of GSH in the skin of both black and yellow mice to 85.6% of black and 80.0% of yellow control mouse levels, respectively. The pretreatment of animals with NAC prevented significant GSH depletion in both murine strains, the GSH content being 98.6% of controls in black and 93.9% of controls in yellow mice. The combination treatment of black mice with NACAP and BSO, and yellow mice with NACAP, BSO and DEM, resulted in significant GSH depletion, the GSH content being 47.8% and 33.4% of controls in black and yellow mice, respectively.

D. DISCUSSION

Many phenolic compounds have been found to be selectively melanocytotoxic against black (eumelanic) follicular melanocytes, however, none of them have exhibited melanocytotoxicity against yellow (pheomelanic) hair follicles *in vivo* (4). The reason for this discrepancy has not been elucidated. However, some investigators (5) propose low levels of tyrosinase activity and consequently low levels of cytotoxic intermediates of eumelanogenesis in yellow follicular melanocytes as a possible basis for this phenomenon.

Another explanation for the different susceptibility of black and yellow melanocytes to melanocytotoxic phenols may be found in the differences between the chemistry of eu- and pheomelanogenesis. It is clear that sulfhydryl compounds such as cysteine and glutathione (GSH), are essential for the process of pheomelanogenesis by acting as

scavengers for dopaquinone to yield the pheomelanin precursors, the cysteinyldopas (10, 11). Bennedetto *et al.* (12) reported a significantly higher level of GSH in pheomelanin-producing melanocytes in comparison to that of eumelanic melanocytes. It is also known that GSH plays a major role in the protection of cells against oxidative and free radical damage as well as other types of toxicity by xenobiotics (13). Thus, it seems likely that the increased GSH level in yellow follicular melanocytes creates not only a pre-condition for pheomelanogenesis but also serves to protect these cells better against chemically induced injury.

Previously, we found that the administration of NACAP, at a dose of 1.0 mmol/kg, results in a grey depigmentation of newly grown hair in black mice while the same drug administered at a dose of 2.0 mmol/kg caused the conversion of hair pigmentation from yellow to black in yellow mice (Chapter V). These changes in hair pigmentation where associated with the depletion of GSH. Specifically, we considered the effects of the above-mentioned doses of NACAP to be intermediate (on a scale from no effect on pigmentation to maximal depigmentation effect) and to be directly associated with a moderate degree of GSH depletion in the skin.

Considerable information about the functions of GSH, especially with regard to toxicology, has come from studies in which GSH metabolism was modified, i.e., the tissue concentrations of GSH were increased or decreased using specific drugs or diet regimens (14). We adopted this approach in order to study the interrelationship between the GSH levels in tissues and biological effects of NACAP. Our ability to produce intermediate effects on hair pigmentation by NACAP, gave us the opportunity to study the complex relation between the changes in tissue GSH levels and the melanocytotoxic efficacy of NACAP in a very simple manner: the macroscopically visible changes in pigmentation of newly grown hair acted as a reliable indicator of hidden events at subcellular and cellular levels in hair follicles.

N-acetyl-L-cysteine (NAC) which is an effective agent for the stimulation of GSH synthesis by cysteine delivery (14) was used to increase GSH levels. In order to deplete GSH levels in tissues, a selective and irreversible inhibitor of gamma-glutamylcysteine synthetase, D,L-buthionine-(S,R)-sulfoximine (BSO) for both black and yellow mice was used. In addition, diethyl maleate (DEM) was used for yellow mice only (15). Recently, each of these drugs has been used for GSH modulations in pigment cells *in vitro* (16, 17). However, we have not found any report describing the use of NAC, BSO, or DEM in combination with a melanocytotoxic phenol for the purpose of hair depigmentation studies *in vivo*.

The modulations of GSH concentrations in tissues strongly influenced the effects of NACAP. The repletion of GSH by NAC effectively inhibited any melanocytoxicity in both black and yellow mice and consequently new hair was of the same pigmentation as that of controls. The enhanced GSH depletion by BSO and/or DEM converted intermediate effects of NACAP into a maximal depigmentation effect in black mice and a visible depigmentation effect in yellow mice. Our measurements indicate a direct relationship among the specific drug regimen, GSH levels in the target tissue, and changes in eumelanin/pheomelanin contents in hairs.

This report describes the first succesful attempt to depigment yellow hair follicles by a melanocytotoxic phenol *in vivo*. To achieve this depigmentation, it was necessary to combine two GSH-depleting agents, BSO and DEM, with NACAP since BSO alone at a dose of 2.0 mmol/kg was not effective in the enhancement of NACAP melanocytotoxicity necessary to produce visible depigmentation of yellow hair. It is possible that by using higher doses of BSO in combination with NACAP, visible depigmentation might be achieved. However, it is also possible that the rate of GSH depletion is more important for the induction of a severe chemical injury to yellow follicular melanocytes than the absolute level of GSH depletion seen after a relatively longer time. In this latter case, it is proposed that the administration of DEM was essential to remove the dynamic adjustment of yellow

follicular melanocytes to the conditions of low GSH levels. This was otherwise impossible to achieve using BSO alone.

Interestingly, the control treatments with BSO alone or with BSO and DEM, without the addition of NACAP, effectively depleted GSH levels in the skin, but did not affect the pigmentation of new black or yellow hair follicles. This observation suggests that the depletion of GSH to some critical threshold is only the background prerequisite necessary for reactive intermediate(s) of NACAP to exert the cytotoxicity. This may indicate that NACAP reactive intermediate(s) acts as an electrophilic alkylator of macromolecules and not as a redox cycling agent producing oxidative stress since the latter types of reactants do not require reaching of some GSH threshold to start acting (18).

A preliminary working hypothesis regarding the mechanism of cytotoxicity by NACAP and PTEs is proposed (Figure VI-4). Based on current toxicology concepts, it is hypothesized that PTEs are inert phenolic compounds which are metabolized in the liver into glucuronate and sulfate conjugates and in these forms are excreted into the urine and cleared from the body. The glucuronidation and sulfation are known to be two major detoxication pathways for phenols (19). However, we propose the existence of a minor drug-metabolizing pathway mediated by tyrosinase and some other type of oxidase which is present in hepatic as well as extrahepatic tissues and which may cause the non-selective toxicity of PTE *in vitro* (Chapter IV). The reactive intermediate(s) is conjugated with GSH directly or by the action of GSH-S-transferases (20). The rapid conjugation results in a GSH depletion with a subsequent alkylation (covalent binding) of macromolecules by intermediates, or enhanced lipid peroxidation of membranes due to the inefficient protection by GSH-peroxidase (21) The condition of GSH deficiency also alters the maintenance of cellular protein thiols with a subsequent negative impact on their many functions, e.g., the decreased activity of Ca^{2+}-dependent ATPases (22). Previously, several reports showed inhibitory effects of depigmenting phenols on mitochondrial respiration (23, 24). Thus, the inhibition of oxidative phosphorylation followed by a

deficiency in ATP production and an alteration of calcium homeostasis may be the additive mechanism of toxicity by PTEs. Ultimately, we expect that each of these components contribute in varying degrees to the induction of chemical injury to cells with the eventual outcome of cell death.

The presented *in vivo* model may explain the *in vitro* non-selective cytotoxicity by PTEs. However, using this model, it is difficult to explain the observed selective melanocytotoxicity of phenols *in vivo*. The previously accepted explanation for this phenomenon is based on the exclusive presence of tyrosinase in pigment cells with active melanogenesis (25). In contrast, the current hypothesis explains the selective melanocytotoxicity of phenols *in vivo* by dynamic quantitative differences between the drug-metabolic activity of activated pigment cells and other cell types. This concept evolved partly from the findings of Kulkarni *et al.* (26) who explain the selectivity of toxic agents mostly through differences between the metabolism of the target and nontarget tissues. The current speculation is that a fine balance between the phase I reactions (drug activation) and phase II reactions (drug conjugation) during the metabolism of PTEs may be disturbed in favour of the former which results in the overproduction of unopposed reactive intermediates. The activating enzyme is presently unknown, but we may expect its preferential specificity for the same substrates as those of tyrosinase, *p*-hydroxyphenolic compounds. This activating enzyme is commonly expressed in pigment and non-pigment cells, and under uniform culture conditions, its reactive products cause dose- and time-dependent non-selective cytotoxicity *in vitro*. However, under the complex pharmacokinetic conditions *in vivo*, which may be combined with a relative overexpression of this enzyme in melanogenic pigment cells, the production of toxic intermediates per time unit overloads a critical threshold for the induction of chemical injury which is followed by reversible and irreversible damage of activated pigment cells. The cytotoxic impact on pigment cells is then detectable at the biochemical, functional, and morphological levels as has been shown. At the same time, a process qualitatively

identical but quantitatively well below a critical threshold for induction of chemical injury occurs in other cell types of the body, e.g., non-pigment cells, but also dormant melanocytes, or even activated yellow follicular melanocytes. In these cases the same process, but at a slower rate, will cause only biochemically detectable changes without any functional and morphologic alterations. The measurements of GSH changes in various tissues in mice exposed to NACAP support this idea.

In addition to the proposed quantitative differences in drug activation, there may also be a differentially higher uptake of synthetic phenolic compounds into the activated pigment cells due to their resemblance to the natural substrate for tyrosinase, L-tyrosine. Moreover, active melanogenesis in pigment cells has been described as a significant burden to these cells, making them more susceptible to any changes in cellular redox milieu (27). For this reason, the critical threshold for induction of chemical injury in these cells may be lower than that of cells without active melanogenesis. Thus, taking into account all these factors, it is proposed that active melanogenesis is indirectly involved in the exertion of selective toxicity of PTEs to pigment cells *in vivo*.

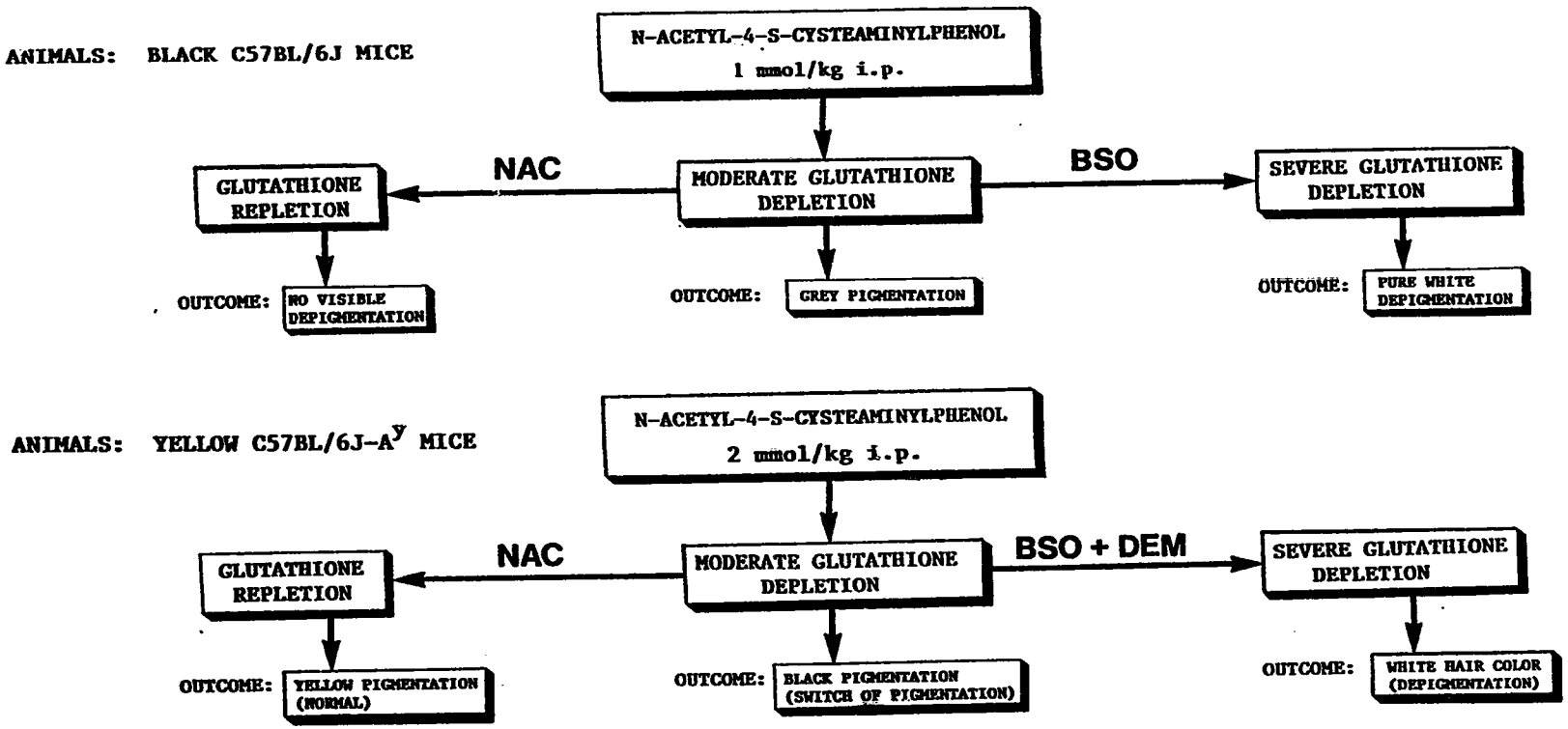

Fig. VI-1. Schemes of the experimental design for modulations of GSH levels by drugs in tissues of black and yellow C57BL/6J mice treated with N-acetyl-4-S-cysteaminylphenol (NACAP) in moderate doses, in vivo.

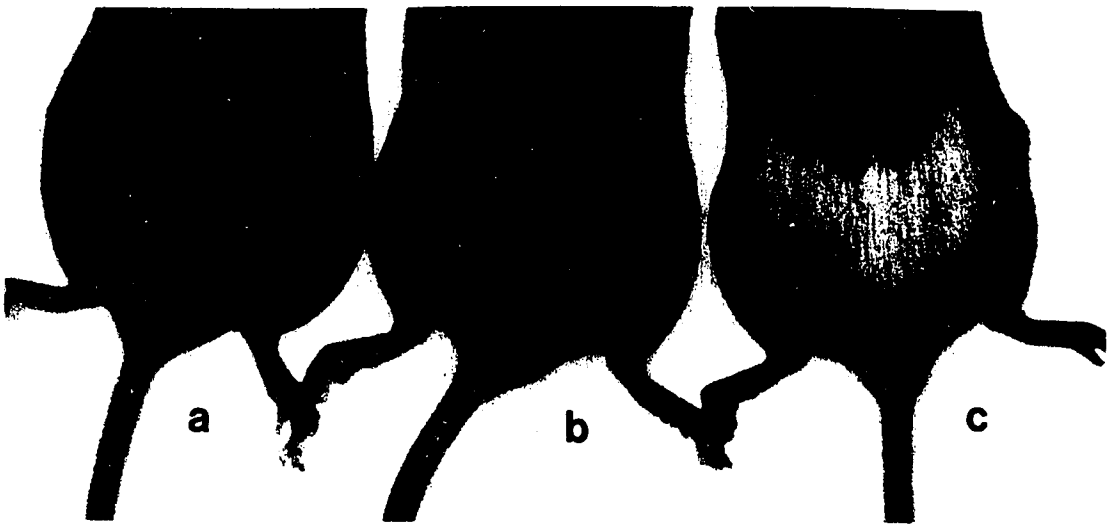

Fig. VI-2. Macroscopically visible effect of modulations of glutathione levels in skin on depigmentation potency of NACAP in black hair follicles. The effect of a) NACAP 1.0 mmol/ kg combined with N-acetyl-L-cysteine 500 mg/kg; b) NACAP 1.0 mmol/kg alone; c) NACAP 1.0 mmol/kg in combination with buthionine sulfoximine 2.0 mmol/kg.

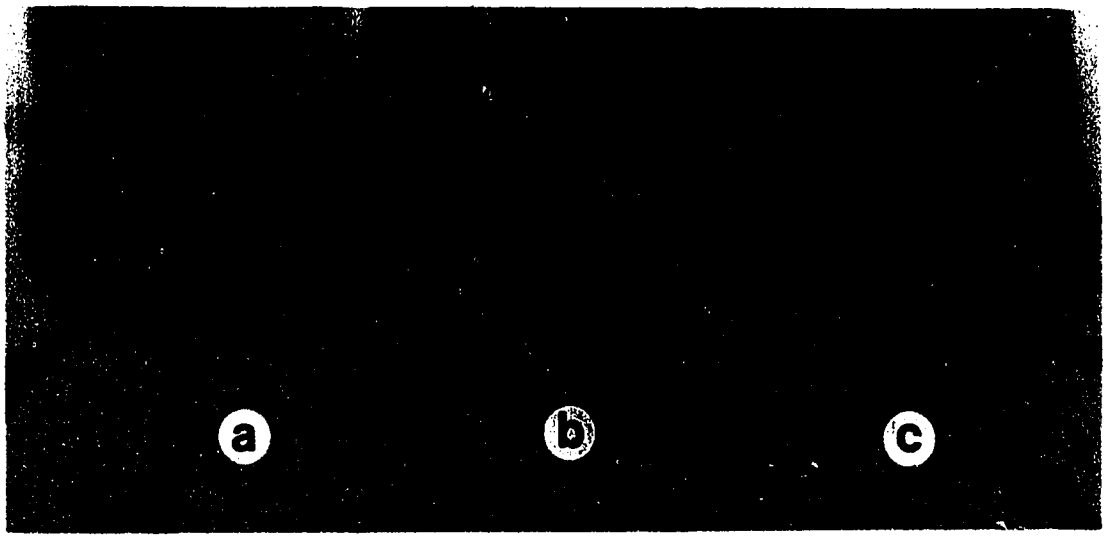

Fig. VI-3. Macroscopically visible effect of modulations of glutathione levels in skin on depigmentation potency of NACAP in yellow hair follicles. a) effect of NACAP 2.0 mmol/kg with N-acetyl-L-cysteine 500 mg/kg; b) effect of NACAP 2.0 mmol/kg alone; and c) effect of NACAP 2.0 mmol/kg in combination with buthionine sulfoximine 2.0 mmol/kg & diethyl maleate 0.6 ml/kg.

TABLE VI-1. Eumelanin and Pheomelanin Contents in Hair of Black and Yellow C57BL/6J Mice treated with N-Acetyl-4-S-Cysteaminylphenol (NACAP) and Its Combinations with N-Acetyl-Cysteine (NAC) and Buthionine Sulfoximine (BSO) *In Vivo*.

Treatments[a]:	PTCA	AHP	PTCA/AHP Ratio
	ng/mg		
A. Black C57BL/6J (a/a)			
Normal Saline (Control)	1079.0±109.0	55.3±4.1	19.51
NACAP alone	312.5± 56.8	52.1±6.2	6.01
NACAP+NAC	1037.5± 63.7	50.8±7.0	20.41
NACAP+BSO	154.2± 25.7	48.3±3.8	3.19
B. Yellow C57BL/6J(A^y/a)			
Normal Saline (Control)	16.1± 1.4	2882.1±280.2	0.006
NACAP alone	212.5±35.4	1608.4±324.9	0.132
NACAP+NAC	15.3± 0.8	2695.4±301.1	0.006
NACAP+BSO+DEM	4.3± 0.6	436.7±111.3	0.010

TABLE VI-2. Glutathione Content in the Skin of Black and Yellow Mice Treated with Different Drug-Regimens In Vivo.

Mouse Strain	Drug Treatments[a]:			
	Normal Saline (Control)	NACAP	NACAP/NAC	NACAP/BSO/DEM
Black C57BL/6J(a/a)	0.533±0.048[b]	0.456±0.020	0.526±0.031	0.255±0.041
Yellow C57BL/6J(A^y/a)	0.608±0.056	0.486±0.043	0.571±0.029	0.203±0.036

[a] Treatment of black mice: NACAP (1 mmol/kg), L-NAC (500 mg/kg), BSO (2 mmol/kg);
Treatment of yellow mice: NACAP (2 mmol/kg), L-NAC (500 mg/kg), BSO (2 mmol/kg), and DEM (0.6 ml/kg).

[b] GSH content in μmol/g of tissue; (n=3)

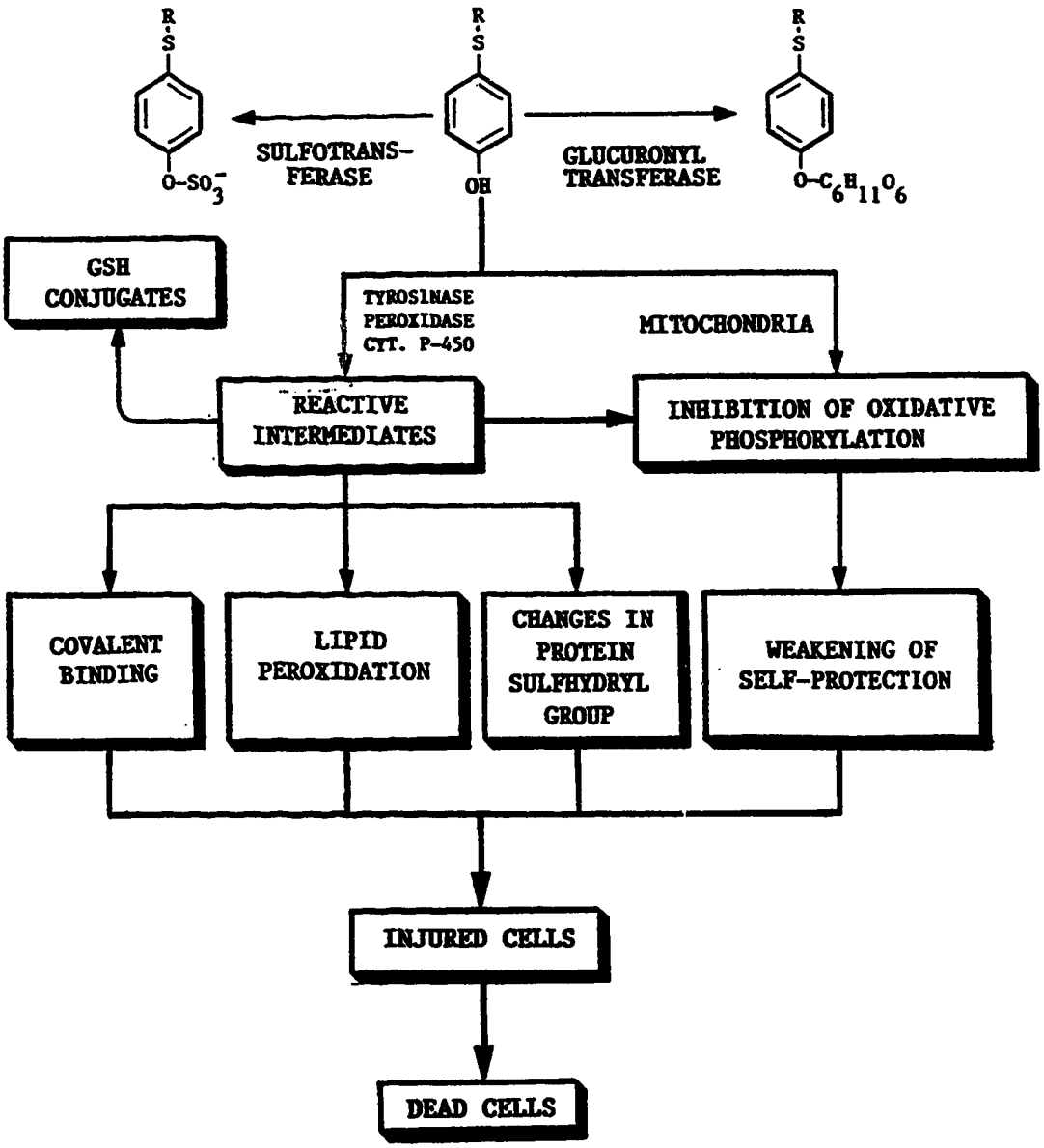

Fig. VI-4. Proposed sequence of events leading to chemically induced injury and cell death by phenolic thioethers.

(Adapted from S. Ji et al.: Biological Reactive Intermediates III., 1986)

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VII. MECHANISM OF CYTOTOXICITY OF N-ACETYL-4-S-CYSTEAMINYLPHENOL : PRELIMINARY STUDY

A. INTRODUCTION

N-acetyl-4-S-cysteaminylphenol (NACAP) is a *p*-hydroxyphenyl derivative which has been found to be selectively toxic to follicular melanocytes and melanoma cells *in vivo* (1). This drug was designed to be a substrate for tyrosinase in an attempt to develop a melanoma-specific chemotherapy by a drug interaction with the melanin biosynthetic pathway of pigment cells (2). In this approach, it was proposed that synthetic phenolic substrates may be converted inside the cells by melanosomal tyrosinase into highly reactive orthoquinone moieties which may induce chemical injury or even cause total destruction of the cells (3). Due to the exclusive presence of tyrosinase in melanocytes and melanoma cells, the cytotoxicity of synthetic tyrosinase substrates was anticipated to be selective for pigment cells only. *In vivo* findings have been in agreement with this concept (1).

However, the *in vitro* studies revealed that NACAP is non-selectively cytotoxic, some non-melanoma cells, e.g., lung adenocarcinoma or neuroblastoma cells being more susceptible to NACAP than melanotic melanoma cells, and that the cytotoxicity of NACAP against melanoma cells is tyrosinase-independent (Chapter IV). Recently, several reports have described similar contradictions between *in vivo* selectivity to pigment cells and *in vitro* non-selective cytotoxic effects of another phenolic tyrosinase substrate, 4-hydroxyanisole (4-6).

In order to clarify the discrepancy between these *in vivo* and *in vitro* findings, a possible mechanism of cytotoxicity by NACAP has been investigated. A significant depletion of glutathione (GSH) in pigment and non-pigment tissues after the treatment

with NACAP was shown (Chapter V), as well as the inhibition or enhancement of the melanocytotoxicity of NACAP by modulations of GSH levels in tissues *in vivo* (Chapter VI).

GSH is essential for a) maintenance of a reducing milieu in cells; b) reduction of ribonucleotides to form the deoxyribonucleotide precursors of DNA; and c) protection against oxidative and free radical damage, and d) detoxication of activated xenobiotics (7) The observation of GSH depletion by NACAP treatment has indicated two possible mechanisms of cytotoxicity: 1) alkylation of macromolecules by electrophilic intermediates of NACAP, or 2) induction of severe oxidative stress by free radicals derived from NACAP metabolites (8). Thus, the determination of which of these two mechanisms of cytotoxicity is the major mode of action by NACAP would indirectly characterize the nature of NACAP reactive intermediate(s).

In this study, some morphologic, functional, and biochemical correlations in cells exposed to NACAP *in vitro* have been examined. Based on these findings, a preliminary hypothesis of the major steps in the mechanism of cytotoxicity by NACAP is presented.

B. MATERIALS AND METHODS

Cell Lines. The origin and characteristics of cell lines was previously reported (Chapter IV.). Briefly, SK-MEL-23 (human melanotic melanoma) and SK-MEL-118 (human amelanotic melanoma) cell lines were kindly supplied by Dr. A.N. Houghton from the Memorial Sloan-Kettering Cancer Center, New York. HeLa (human cervical carcinoma) and SK-OV-3 (human ovarian carcinoma) cell lines were obtained from ATCC, U.S.A. Cells were grown in standard culture medium, MEM (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated at 37.0°C in a humidified atmosphere of 5% CO_2.

Chemicals. N-acetyl-4-S-cysteaminylphenol (NACAP) was synthetized in our laboratory using a method described by Wehrmeister (9). D,L-buthionine-(S,R)-sulfoximine (BSO) was purchased from Sigma Co., St. Louis, MO. All other reagents were of analytical grade.

MTT-Microculture Tetrazolium Assay. Cell growth in the presence or absence of experimental drugs was determined using a modified MTT-microculture tetrazolium assay described by Mosmann (10). Briefly, exponentially growing cells were harvested, counted for cell viability, and inoculated at the appropriate concentration (1-5 x 10^3 cells/well) into 96-well microtiter plates (Costar, Cambridge, MA) using a multichannel pipet. After 24 hr, aqueous solutions of drugs were added to culture wells in tripilicate. Cells were cultured for a specified number of hours or days at 37.0°C in a humidified atmosphere of 5% CO_2 in air. MTT stock solution was prepared at 5 mg/ml concentration in MEM (GIBCO) and stored at 4.0°C. At the end point of the assay, 50 µl of MTT solution, diluted to 1 mg/ml concentration using MEM without serum, was added to each well. After a 4-hr incubation, the supernatant from wells was removed, 150 µl of 100% DMSO (spectrophotometric grade) were added to dissolve the MTT-formazan product, and the absorbance at 540 nm was measured with a microplate reader (EAR 400 AT).

The survival fraction percentage of cells was calculated using a formula: **b/a x 100 %** were **b** is the mean of absorbance values in wells with drug (s) and **a** is the mean of absorbance values in control wells without drug(s).

Glutathione Assay. Cells were plated in the T-25 flasks (Costar) in the same densities per mm^2 ($5x10^3$ /mm^2) as those cells plated into 96-microculture plates. Cells were exposed to aqueous 0.154 M NaCl solutions of NACAP in concentrations of 10, 50, and 100 µg/ml. Each drug concentration for each cell line was tested in triplicate. Cells were harvested with trypsin/EDTA, washed with phosphate-buffered solution, and

counted. After pelleting by centrifugation, the cells were exposed to 6.5% trichloracetic acid solution at 4.0°C for 10 min to extract GSH. The total GSH content was measured using the GSSG-reductase assay described by Griffith (11). The results are expressed as nmoles of GSH per 10^6 cells and as a percentage of control.

Induction of Stress Proteins (HSP) by Heat and Toxic Chemicals. Single-cell suspensions of SK-MEL-23, SK-MEL-118, and HeLa cells ($5x10^4$ cells/ml concentration) were plated in Petri dishes with sterilized glass slides. After 72 hr, the slides with cells in the logarithmic phase of growth were washed with PBS, transfered into dishes with fresh MEM containing 10% fetal calf serum (complete culture medium), and exposed to stress (shock) inducing treatments. The negative control cells were left without any stress treatment and the positive control cells were exposed to a classic heat shock at 42.5°C for 3 hr (12). The induction of HSPs by toxic drugs was carried out by exposure of the cells to NACAP (100 μg/ml) for 1 hr or menadione (positive control) (100 μg/ml) for 30 min. After the stress treatments, cells were transfered into dishes with a drug-free complete culture medium at 37.0°C for 4 hr. After this recovery phase, cells were fixed on glass slides by ethanol:acetone (1:1) solution for 10 min at 4.0°C and stained with a monoclonal 72-kD heat shock antibody, RNP 1197 (Amersham International PLC), 1:250 dilution using the avidin-biotin method (13). 1% methylene green solution was used for counterstaining.

Determination of Lipid Peroxidation. The lipid peroxidation in both control and drug-treated SK-MEL-23 melanoma cells was determined by the measurement of the concentration of malondialdehyde (MDA) formed in cells from polyenoic fatty acids by peroxidative decomposition. The MDA concentration was measured using thiobarbituric (TBA) assay (14). Briefly, SK-MEL-23 melanotic melanoma cells were cultured in T-75 flasks in MEM supplemented with 10% fetal calf serum and NACAP (25 μg/ml) for a specified period of time (from 24 to 120 hr). Control cells and cells exposed to NACAP

were harvested by trypsin/EDTA, washed by phosphate buffered saline solution (PBS), and pelleted by centrifugation. Each cell pellet was resuspended in 2.0 ml of PBS. 1.0 ml of cell suspension was used for protein determination by the Bradford assay (Bio-Rad Kit) and 1.0 ml was used in the TBA assay. The TBA reaction mixture consisted of 2.0 ml of TCA-TBA-HCl reagent (15% w/v trichloracetic acid, TCA; 0.375% w/v thiobarbituric acid, TBA; 0.25N hydrochloric acid, HCl) and 1.0 ml of cell sample. The mixture was heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 min. The absorbance was determined at 535 nm against a blank that contained all the reagents except the biological sample. The concentration of MDA was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. The results were expressed as picomols of MDA per mg of protein.

C. RESULTS

Dose-Dependent Gross Morphologic Changes. The SK-MEL-23 melanoma cells were cultured without (control) or with the continuous presence of NACAP at various concentrations (10, 25, 50, 75, and 100 μg/ml) for 120 hr. As presented in Figure VII-1, the presence of NACAP resulted in a dose-dependent decrease in the numbers of cells, the proliferative activity of cells, and the morphology of cells. At concentrations above 25 μg/ml a visible cytotoxic effect on melanoma cells (Figure VII-1D to 1F) was seen. Typically, many cells were partly or completely detached from the bottom, became rounded, and showed blebbing on their surface. Only a few cells survived the exposure to 100 μg/ml of NACAP for 120 hr and these showed atypical dendritic morphology and no sign of proliferation (Figure VII-1F).

Time-course of Viability/Survival and Glutathione Content in Cells Continuously Exposed to NACAP (Figures VII-2 to 5). SK-MEL-23, SK-MEL-118, HeLa,

and SK-OV-3 cells were cultured in both 96-well microculture plates for assessment of their viability/survival and T-25 flasks for measurements of changes in their GSH content after exposure to NACAP at concentrations of 10, 50, and 100 μg/ml for 1, 6, 24, and 48 hr. The results were expressed as a percentage of control and showed clearly dose- and time-dependent changes in both parameters. It was found that the percentage of reduction in GSH concentration in cells for each NACAP concentration was always greater than the corresponding percentage of reduction in numbers of viable or surviving cells for each cell line and each drug concentration studied. The statistical differences in both parameters between each of four cell lines were insignificant for the first 24 hr. However, 48 hr after the continuous exposure to NACAP, SK-MEL-118 amelanotic melanoma cells showed significantly higher drug susceptibility, this being about 2-fold higher than that of SK-MEL-23 cells. This finding is in a good correlation with our observation that SK-MEL-118 cells show a visible decrease in cell density after 48 hr exposure to NACAP while SK-MEL-23, HeLa, and SK-OV-3 cells need about 72-hr exposure to exhibit microscopically visible reduction in cell densities (data not shown). Also, this finding is in an agreement with previous results in which the susceptiblity of various cells lines to NACAP using IC_{50} as a parameter after 120 hr of continuous drug exposure (Chapter IV). SK-MEL-118 was found to be the most susceptible cell line, its IC_{50} being about 2-times lower than that of SK-MEL-23 and about 6 or 7-times lower than those of HeLa and SK-OV-3, respectively.

In Vitro **Cytotoxicity of NACAP, BSO, and a Combination of NACAP and BSO (Figure VII-6).** SK-MEL-23, SK-MEL-118, and HeLa cell lines were tested for their susceptibility to NACAP alone, BSO alone, and to a combination of NACAP and BSO. SK-MEL-118 cells were found to be the most susceptible to NACAP and BSO of all three cell lines, with IC_{50}s being 12.0 and 48.0 μg/ml, respectively. In contrast, HeLa cells were the most resistant to NACAP and BSO, the IC_{50}s being 74.0 and 233.0 μg/ml, respectively. The IC_{50} values for SK-MEL-23 cells were 25.0 and 77.0 μg/ml for NACAP and BSO,

respectively. The combination treatment of cells with BSO at two different concentrations (25.0 and 50.0 μg/ml) and NACAP showed a significant additive effect on cytotoxicity of the two drugs against each of the three cell lines that have been studied. Again, the most susceptible to the combination treatment was SK-MEL-118 cell line, with the IC_{50}s being 7.0 and 5.0 μg/ml for NACAP plus BSO (25 μg/ml) and NACAP plus BSO (50 μg/ml), respectively. However, the susceptiblity of SK-MEL-23 to a combined treatment was not significantly different to those of SK-MEL-118, the IC_{50}s being 8.0 and 5.5 μg/ml, respectively. HeLa cells remained the most resistant to the drugs although the IC_{50}s for the combined drug regimens were 40.0 and 37.0 μg/ml, respectively, these being statistically significantly different to those of single-drug regimens.

Induction and Distribution of 72-kD HSP in Cells (Figure VII-7). In order to determine whether the exposure of cells to NACAP for a very short time is harmful to cells, a technique for the detection of the 72-kD HSP in cells by immunostaining was used. SK-MEL-23, SK-MEL-118, and HeLa cells were exposed to two known inducers of HSPs, heat and menadione, and the experimentally unknown NACAP. It was found that under standard culture conditions none of the tested cell lines expressed the 72-kD HSP (Figure VII-7Aa, 7Ba, 7Ca). Exposure of the cells to a classic heat-shock treatment at 42.5°C for 3 hr, resulted in positive staining for 72-kD HSP in the cytoplasm and nucleolus of cells (Figure VII-7Ab, 7Bb, 7Cb). Menadione was used as a positive control drug capable of inducing the synthesis of HSPs. Exposure of the cells to menandione at a concentration of 100 μg/ml for 30 min resulted in prompt synthesis of 72-kD HSP in cells of each cell line studied (Figure VII-7Ad, 7Bd, 7Cd). Exposure of cells to NACAP at a concentration of 100 μg/ml for 1 hr gave a similar result; the highest intensity of staining for 72-kD HSP was found in SK-MEL-118, then in SK-MEL-23, while the weakest but still clearly positive staining was detectable in HeLa cells (Figure VII-7Ac, 7Bc, 7Cc). The localization of HSP

staining in cells treated with NACAP was preferentially in the cytoplasm but some accumulation and clustering of HSP was visible also in the nucleolus of cells.

Time-course of Lipid Peroxidation in Cells Exposed to NACAP In Vitro (Figure VII-8). For preliminary testing of NACAP for its capacity to produce an oxidative stress manifested as lipid peroxidation of cellular membranes, we used SK-MEL-23 melanotic melanoma cell line. NACAP was used at a concentration of 25 μg/ml. This concentration is very close to the IC_{50} of NACAP for SK-MEL-23 cells as measured after 120 hr of continuous exposure to the drug (Chapter IV). Under these conditions, it was expected that about one half of the harvested cells will be viable at the end point of the time-course assay (day 5). During this time a steady increase in the concentration of malonic dialdehyde (MDA), an indicator of lipid peroxidation, in cells exposed to NACAP was observed. Specifically, this MDA increase was from 3-times to 7-times that of controls at 24 hr and 120 hr, respectively.

D. DISCUSSION

Cell injury may be initiated by the formation of stable (noncovalent) complexes between drugs and cellular enzymes or receptors, and through the formation of highly chemically reactive (unstable) species within the cells (8). The principal drug under investigation, NACAP, is a chemically stable and unreactive compound that requires activation by a drug metabolizing enzyme(s) to initiate lesions to cells and tissues. Drug metabolizing enzymes are classified into two categories: phase I enzymes, principally mono-oxygenases, which form a functional group in the drug molecule, and phase II enzymes which conjugate this functional group to hydrophilic molecules to produce water-soluble metabolites (15). Toxicity, which may be defined as the consequence of alterations of cellular structure and function which are essential to cell viability, may be exerted by two

major types of chemically unstable species: electrophiles and free radicals (16). The electrophiles are species deficient in an electron pair and therefore possess a propensity to covalently bind (alkylate) nucleophilic compounds, e.g., GSH or nucleophilic groups of macromolecules. Free radicals contain an odd number of electrons and may produce toxicity by alkylation or by generation of active oxygen species. Presently, the chemical nature of NACAP reactive intermediate(s) are unknown. However, in this study, several experiments were performed which may provide a preliminary insight into the mechanism of toxicity by NACAP.

NACAP was found to act as a dose-dependent cytotoxic drug. *In vitro*, SK-MEL-23 melanoma cells showed dose-dependent microscopically visible morphologic changes and a significantly reduced proliferation rate after exposure to NACAP at concentrations higher than 25 μg/ml. Typically, chemically injured cells became rounded, and blebbing on their surface was clearly identifiable. Simultaneously, the trypan blue exclusion test indicated increased permeability of cellular membranes (data not shown). Similar effects on cell morphology, e.g., formation of numerous blebs were seen in SK-MEL-23 cells exposed to menadione. Menadione is known to be metabolized by one- or two-electron reduction routes with a consequent production of oxidative stress or depletion of GSH and protein thiols (17). Recently, several studies provided sufficient evidence that the blebbing of cells exposed to menadione is caused by disturbances in thiol and calcium ion homeostasis (18, 19, 20). Thus, it may suggest that the morphologic cytoskeletal changes in cells exposed to NACAP are also linked to a severe alteration in Ca^{2+} homeostasis.

GSH plays a variety of important roles in the protection of cells against exogenous and endogenous chemical reactants (21). Also, an association between GSH depletion and increased susceptibility of cells to drugs has been well documented (22). In this context, the current comparative studies which correlated changes in cellular viability/survival and GSH content after exposure to NACAP, may not only indicate that NACAP treatment is linked with GSH depletion, but also that the depletion of GSH is a necessary predisposition

for cytotoxicity by NACAP to occur. This indication may be further supported by the finding of a significant potentiation of *in vitro* cytotoxicity by NACAP in combination with BSO. BSO depletes GSH levels by the selective inhibition of gamma-glutamylcysteine synthetase which is a rate-limiting step in the synthesis of GSH *de novo* (23). Importantly, these *in vitro* findings are in agreement with the results of previous *in vivo* studies in which the modulations of GSH in tissues inhibited (via GSH repletion) or increased (via enhanced GSH depletion) the melanocytotoxicity of NACAP against hair follicular melanocytes (Chapter VI).

Time-course studies of *in vitro* cytotoxicity by NACAP showed that significant reduction in numbers of surviving cells occured usually after more than 48 to 72 hr of a continuous exposure of cells to the drug (Chapter IV). This was interpreted to be the result of a relatively slower progression of toxic events, e.g., alkylation of macromolecules and/or lipid peroxidation of membranes, which was combined with a gradual deterioration of cellular protective mechanisms mainly due to the depletion of GSH.

However, cytotoxicity by drugs which occured after a relatively long time of continuous drug-exposure may also be due to the induction of synthesis of protective stress (heat shock) proteins (HSPs). HSPs are highly conserved families of proteins which are synthesized by cells in response to heat but also other stressors such as heavy metals, metabolic poisons, or many drugs. HSPs possess various protective functions to cells, e.g., 70-kD and 60-kD act in folding and unfolding of proteins and translocation of polypeptides under conditions of oxidative stress (24). The 72-kD gene family is the most highly conserved group of HSPs. The presence of 72-kD HSP in human melanoma and non-melanoma cell lines exposed to classic heat-shock treatment or an oxidative stress-inducing drug, menadione, was shown. Treatment of cells with NACAP at a concentration of 100 μg/ml for 1 hr, also resulted in the positive detection of 72-kD HSP. Although menadione is chemically a quinone while NACAP is a monophenol (which has to be further metabolized into a reactive quinone moiety), the finding of early synthesis of HSP

in response to NACAP treatment indicates the involvement of oxidative stress as an early event in cytotoxicity by NACAP.

In order to further study the possibility of oxidative stress as a major mode of cytotoxicity by NACAP, the time-course of lipid peroxidation in SK-MEL-23 cells exposed to this drug was examined. The results showed a gradual increase in lipid peroxidation in those cells exposed to NACAP from a 3-fold increased level at 24 hr to about 7-fold increased level compared to controls at 120 hr of continuous exposure. Although this increase is significant, it is presently impossible to estimate the physiologic importance of this observation. It seems likely that significant lipid peroxidation occurs in a later stage of the cytotoxic process and may be caused more by the inefficient repair of cell membranes due to a deficiency of GSH as a cofactor for GSH-peroxidase rather than by the direct action of a redox cycling intermediate(s) (quinone-semiquinone) of NACAP metabolism.

Perhaps more useful information about the NACAP metabolism and the chemical nature of its reactive intermediate(s) may be obtained from the analysis of urine samples from animals treated with this drug. However, this information is still not available. For this reason, the chemical nature of toxic NACAP intermediate(s) remains speculative. Previously, the major urinary excretory products of 4-hydroxyanisole (4-HA) from human patients were identified (25). 4-HA is a potent melanocytotoxic phenol, a tyrosinase substrate, which shares many chemical and biologic properties with NACAP. Pavel *et al.* (26) found the major metabolites of 4-HA to be sulphate and glucuronide conjugates of 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxyanisole. The authors concluded that the metabolism of 4-HA proceeds preferentially via ring hydroxylation. The probable anatomical site of the ring hydroxylation and conjugation was believed to be in the liver, specifically liver microsomes, although an alternative metabolic route could involve the tyrosinase-catalyzed oxidation of 4-HA (26). This finding is relevant to the present study since it clearly demonstrates the significant production of dihydroxyphenols (catechols) from phenols by enzyme(s) other than tyrosinase in pigment cells or tyrosine hydroxylase

in neuronal cells. The dihydroxyphenols are unstable compounds which may be oxidized into quinones. This tyrosinase-independent production of reactive quinonoid moieties may explain the non-selective cytotoxicity of 4-HA (27) as well as NACAP (Chapter IV) *in vitro*.

In the context of toxicology, quinones may act as oxidants or electrophiles (28). This means that both oxidative stress generated by the quinone-semiquinone redox cycling coupled with alkylation (in this case, arylation) of macromolecules by the electrophilic quinone moiety may be the mechanism of toxicity (Figure VII-9). Since the redox potential of quinones can be strongly influenced by cellular substituents as well as pH (29), it is possible to predict that both major modes of toxicity of quinones may proceed simultaneously in the same cell, and, moreover, the two processes may be present at different levels in various cell types or even in various compartments of the same cell. This variability may partially explain differences in *in vitro* and *in vivo* susceptibilities of the same cells exposed to NACAP or 4-HA.

These studies have attempted to provide some preliminary indications about the possible mechanism of toxicity by NACAP. A working hypothesis (depicted in Figure VII-10) proposes that a lipophilic drug, NACAP, enters the cell and is activated by some mono-oxygenase present in the endoplasmic reticulum and tyrosinase, into a reactive intermediate(s). The formation of reactive intermediates is linked with rapid depletion of reduced GSH either by conjugation of GSH with reactants or by increased GSH consumption due to the repair of lipid peroxidation within cells. The condition of GSH deficiency further facilitates the alkylation of macromolecules and/or severe oxidative stress with enhanced lipid peroxidation of membranes. The common outcome of these events is an uncontrolled increase in cytosolic Ca^{2+} concentration with a destructive effect on cytoskeleton, cell blebbing, and ultimately cell lysis and death.

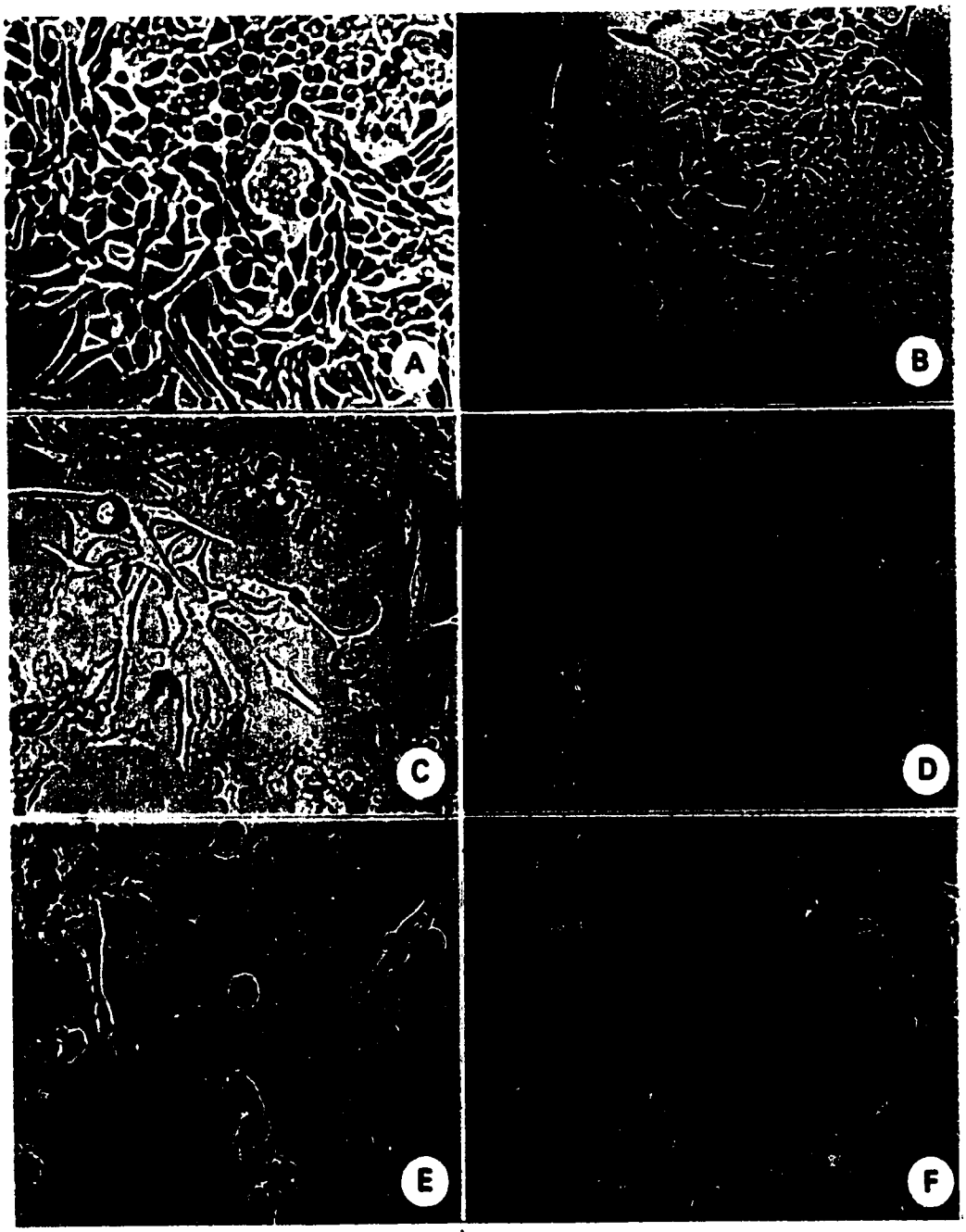

Fig. VII-1. In vitro **dose-dependent morphologic changes of SK-MEL-23 (human melanotic melanoma) cells continuously exposed to NACAP at various concentrations for 120 hr. A) control, almost confluent cell culture unexposed to the drug; B) effect of 10 µg/ml, reduced the number of proliferating cells; C) effect of 25 µg/ml, increased the number of degenerating cells; D) effect of 50 µg/ml , many degenerating cells with signs of blebbing or death; E) effect of 75 µg/ml, clusters of mostly dead cells, only few surviving cells still attached to the surface; F) effect of 100 µg/ml, only few atypically dendritic cells present, some dying cells remain attached to the bottom.** *Bar*, 20 µm.

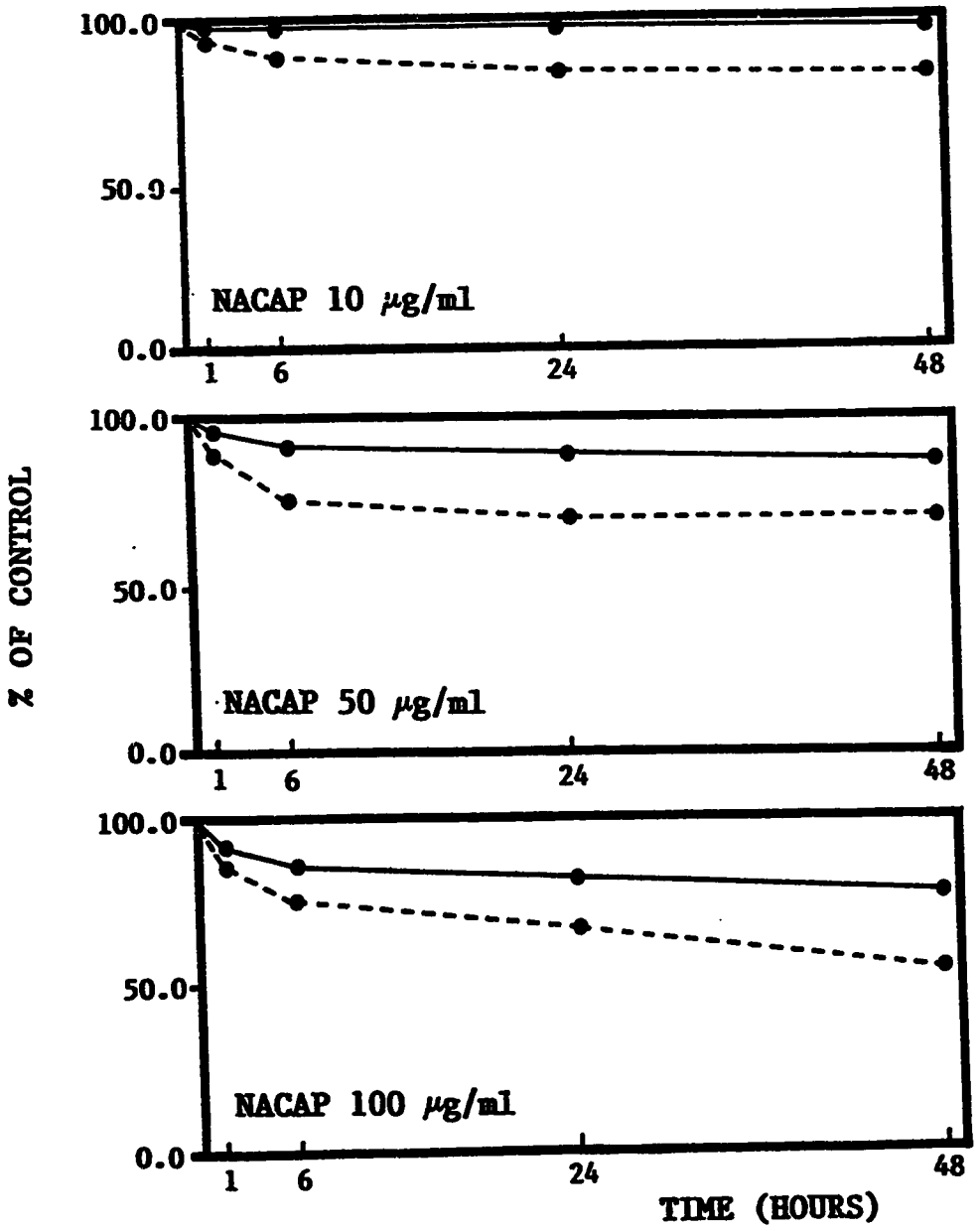

Fig. VII-2. Time-Course of Viability/Survival and Glutathione Content of SK-MEL-23 Human Melanotic Melanoma Cells Exposed to N-Acetyl-4-S-Cysteaminylphenol *In Vitro.*

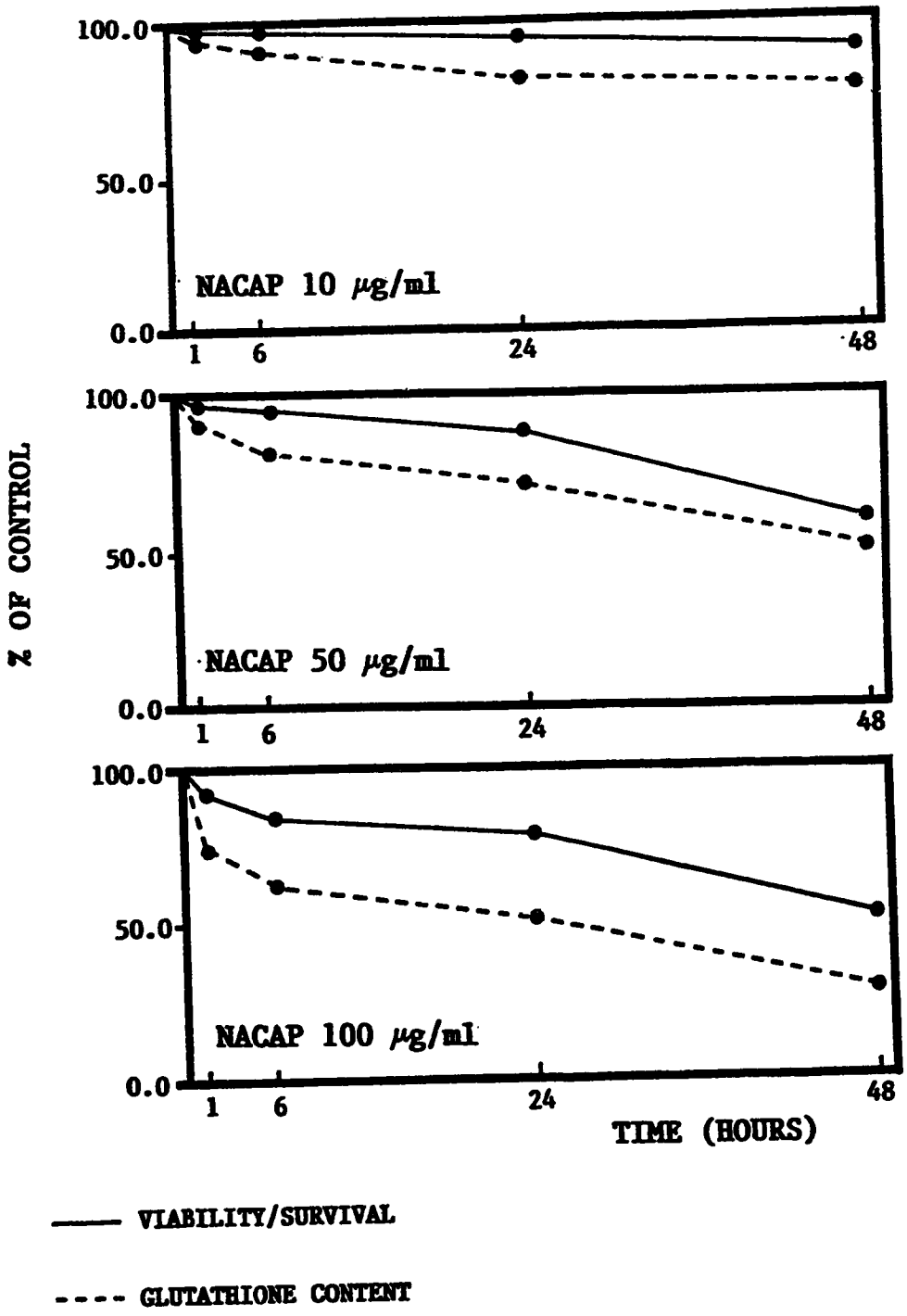

Fig. VII-3. Time-Course of Viability/Survival and Glutathione Content of SK-MEL-118 Human Amelanotic Melanoma Cells Exposed to N-Acetyl-4-S-Cysteaminylphenol *In Vitro*.

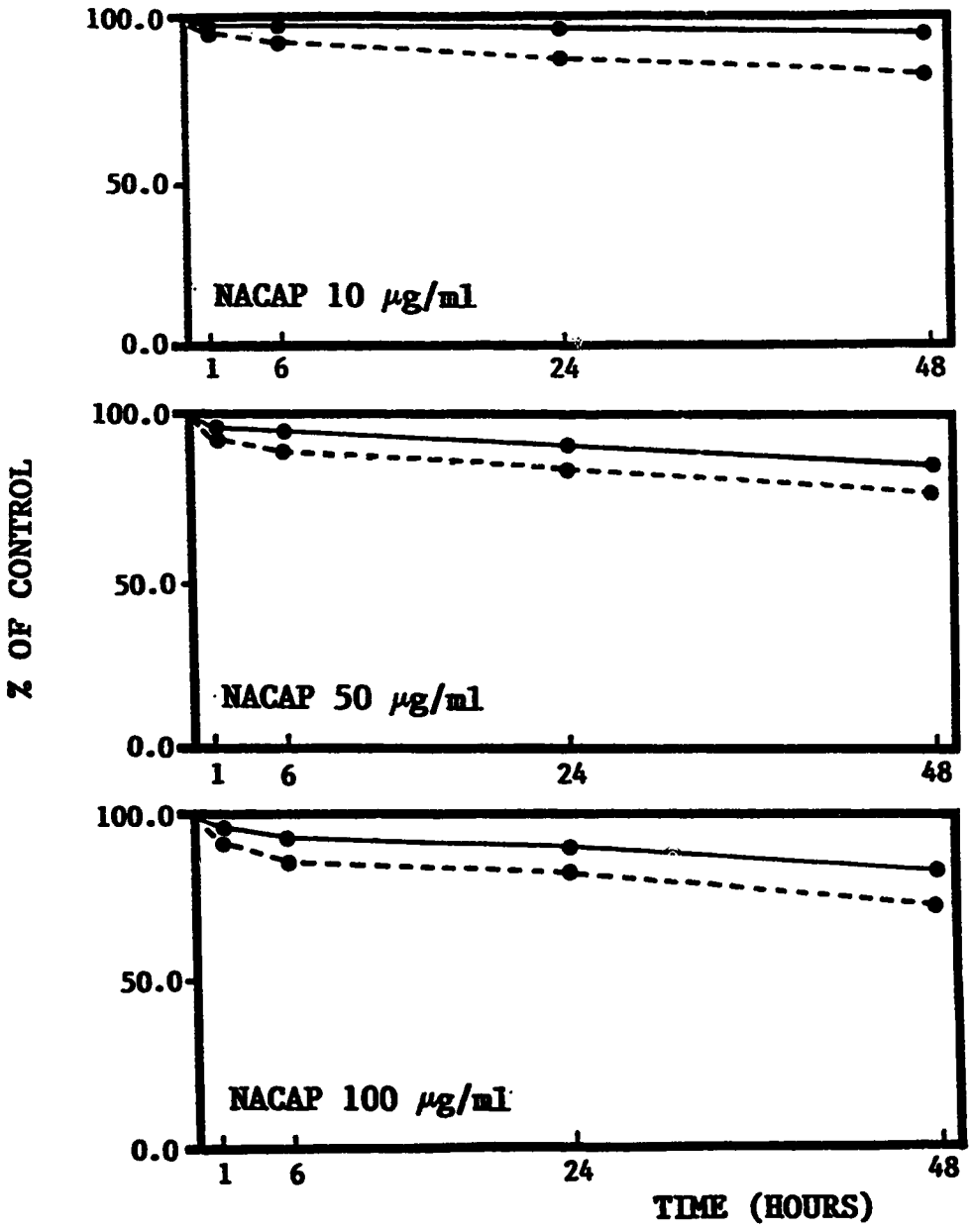

Fig. VII-4. Time-Course of Viability/Survival and Glutathione Content of HeLa Human Cervical Carcinoma Cells Exposed to N-Acetyl-4-S-Cysteaminylphenol *In Vitro.*

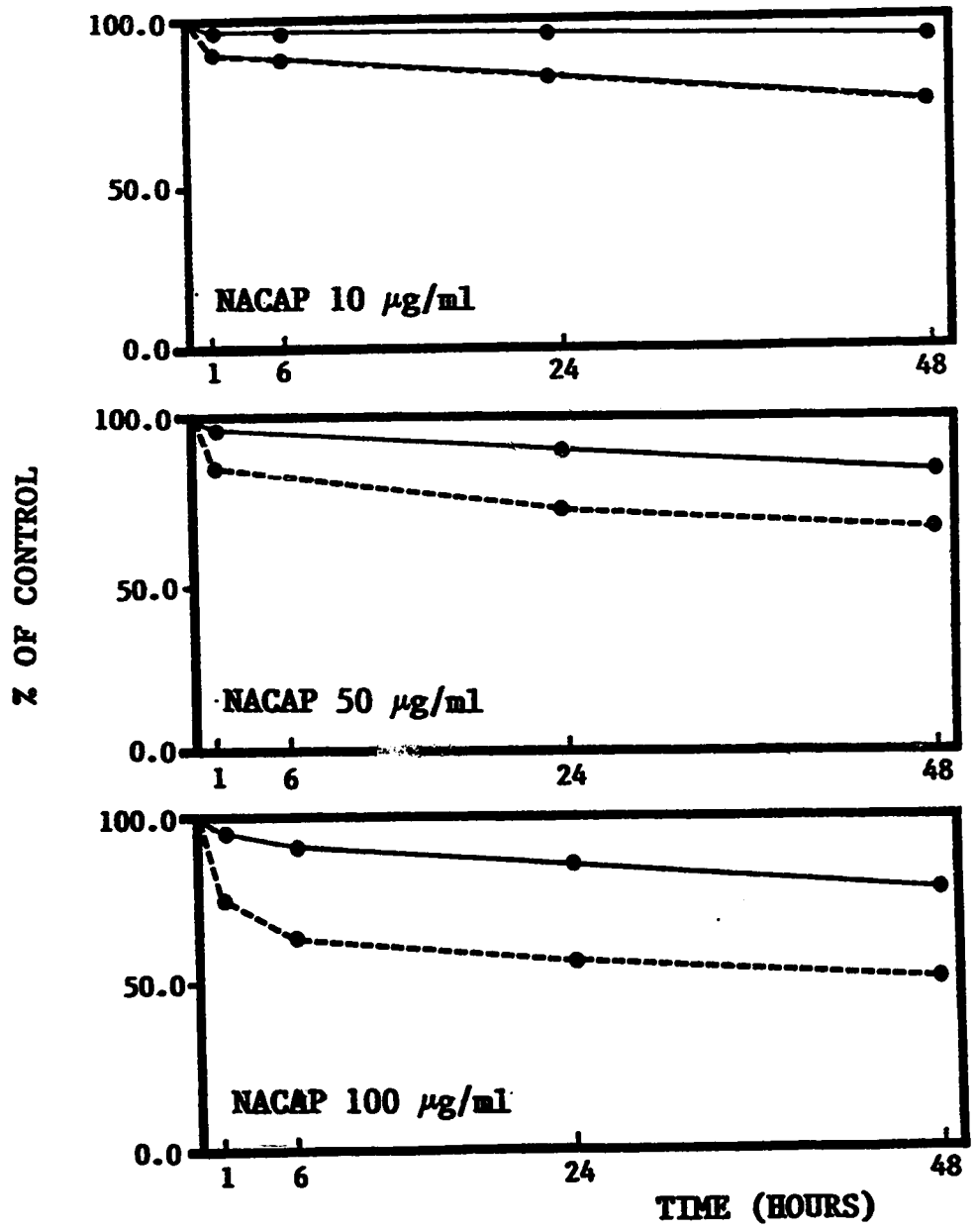

Fig. VII-5. Time-Course of Viability/Survival and Glutathione Content of SK-OV-3 Human Ovarian Carcinoma Cells Exposed to N-Acetyl-4-S-Cysteaminylphenol *In Vitro.*

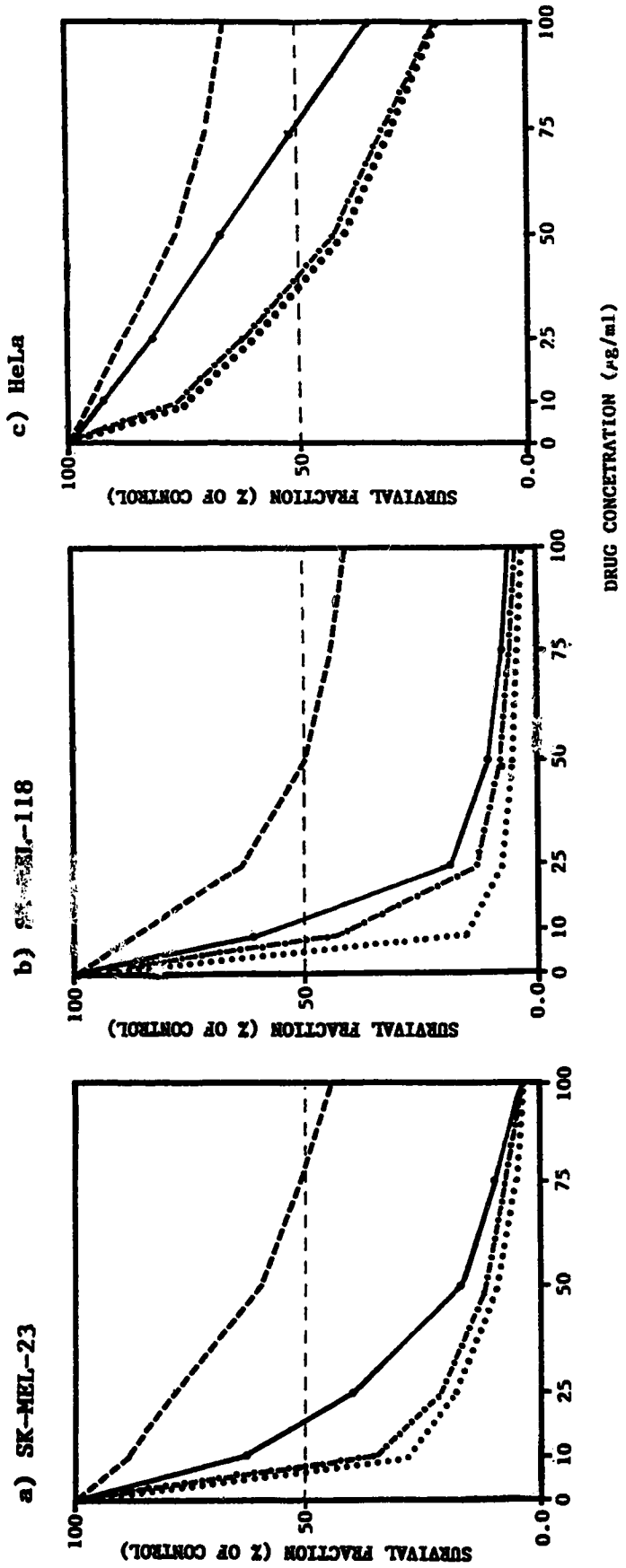

Fig. VII-6. *In Vitro* Cytotoxicity of N-Acetyl-4-S-Cysteaminylphenol (NACAP) alone, Buthionine Sulfoximine (BSO) alone, and Combination of NACAP and BSO against SK-MEL-23, SK-MEL-118, and HeLa Cells.

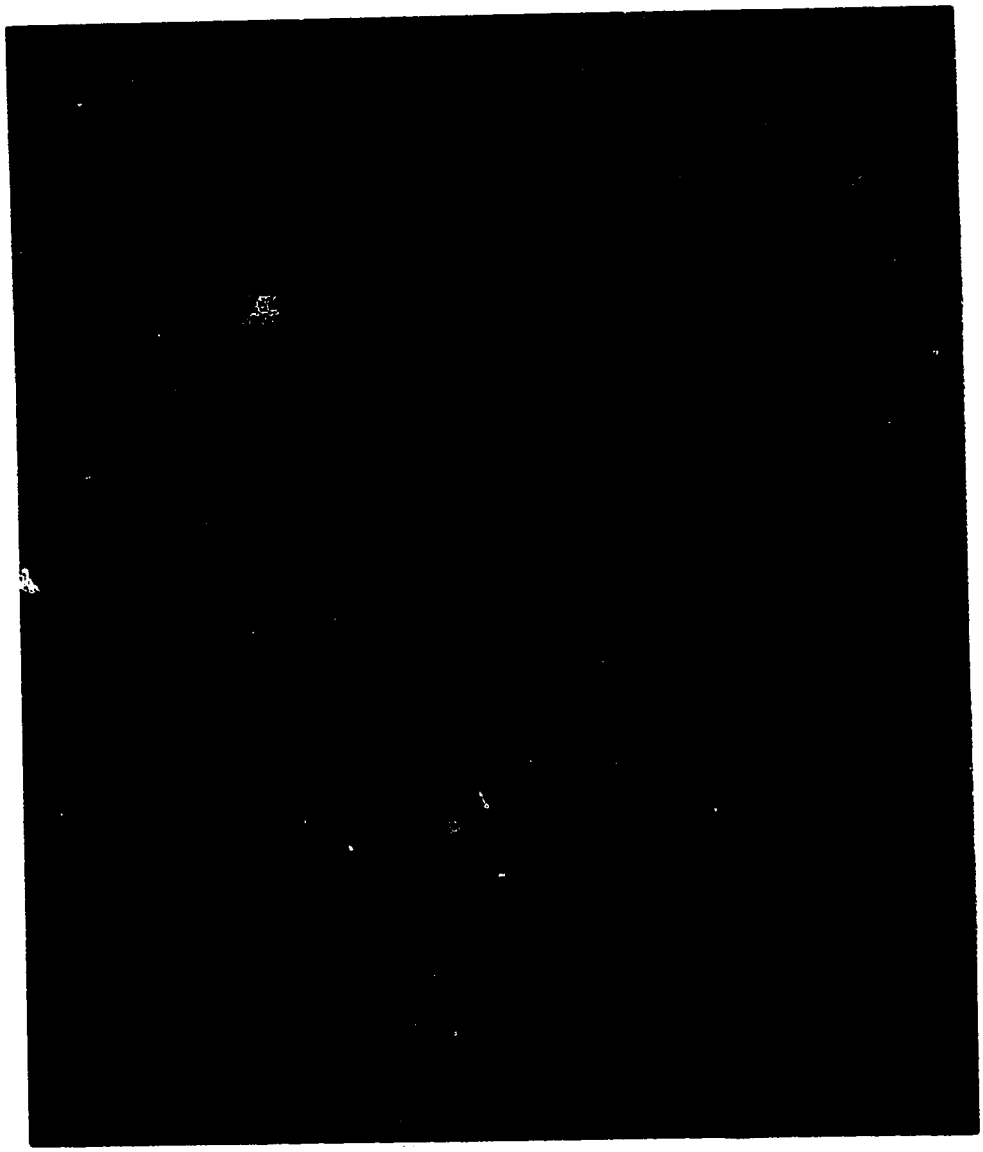

Fig. VII-7. Expression and distribution of 72-kD stress protein in cells: A) SK-MEL-23, human melanotic melanoma; B) SK-MEL-118, human amelanotic melanoma; and C) Hela, human cervical carcinoma, growing on glass slides and a) unexposed to stress (shock) stimulus (controls); b) exposed to heat-shock-treatment at 42.5 °C for 3 hr; c) exposed to NACAP (100 µg/ml) for 1 hr; and d) exposed to menadione (100 µg/ml) for 30 min. The cells exposed to stress treatments were returned to drug-free complete culture medium at 37 °C for 4 hr. After this recovery phase, cells were fixed on glass slides by ethanol:acetone (1:1) solution for 10 min at 4 °C and stained with monoclonal 72-kD heat shock antibody (RNP 1197) as described in Materials and Methods. *Bar*, 20 µm.

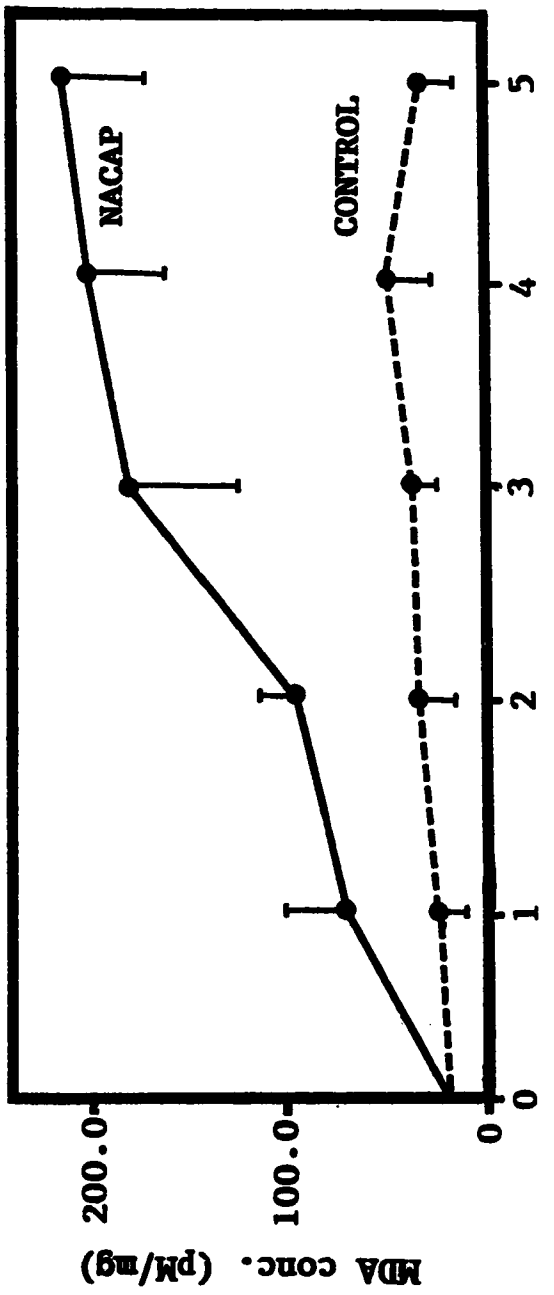

Fig. VII-8. Time-course of lipid peroxidation measured by TBA method in SK-MEL-23 (human melanotic melanoma) cells continuously exposed to N-acetyl-4-S-cysteaminylphenol in 25 μg/ml concentration in culture medium for 24 to 120 hours. The results are expressed as picomols of malonic dialdehyde (MDA) per mg of protein. Data represent the mean ± S.D. of three different experiments.

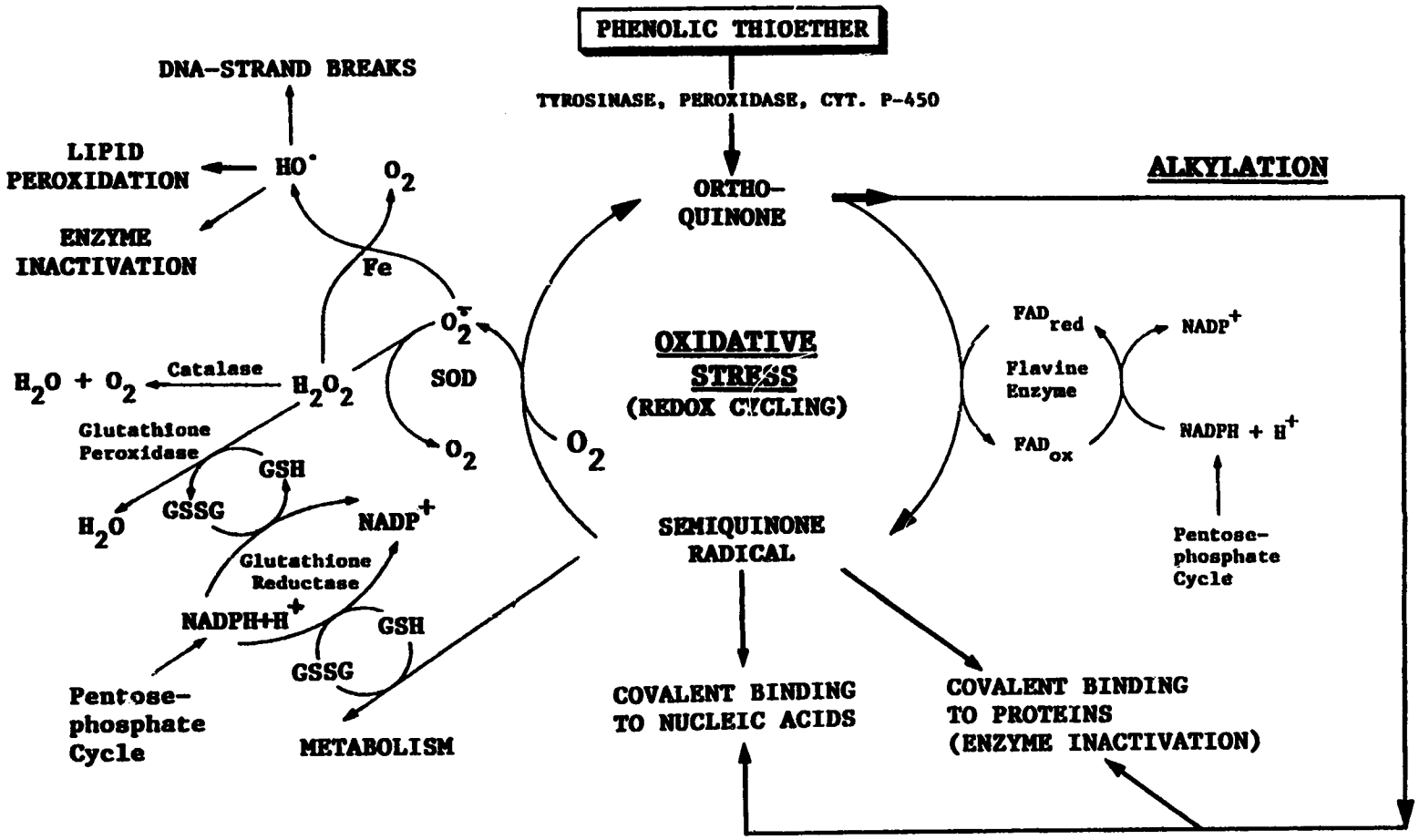

Fig. VII-9. Proposed mechanism(s) of toxicity by quinones enzymatically derived from phenolic thioethers.

(Adapted from H. Kappus: Biochem. Pharmacol. 35, 1986)

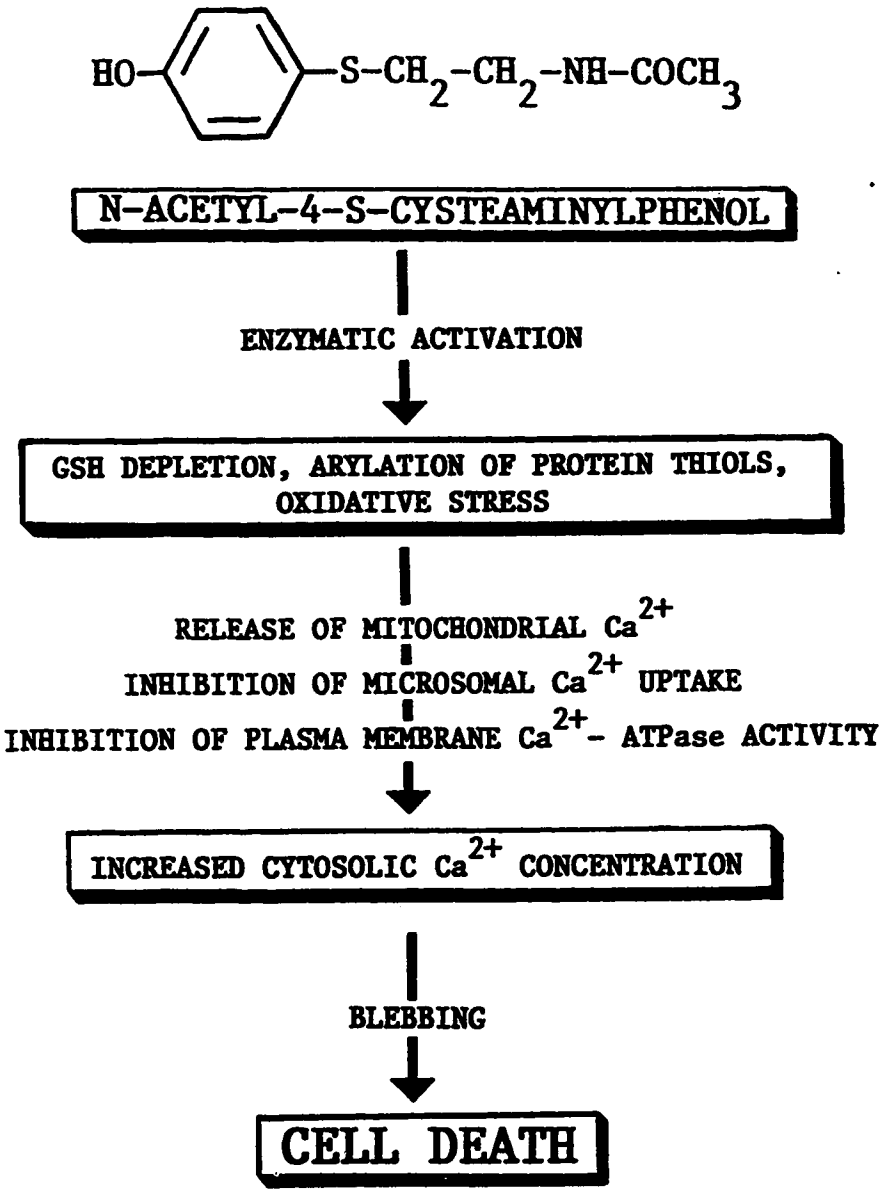

Fig. VII-10. Proposed mechanism of chemical cell injury leading to the cell death induced by exposure to N-acetyl-4-S-cysteaminylphenol.

(Adapted from P. Moldeus et al.: Pharmacol. Therap., 33, 1987)

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VIII. ENHANCEMENT OF IN VITRO AND IN VIVO ANTIMELANOMA EFFECTS OF N-ACETYL-4-S-CYSTEAMINYLPHENOL AGAINST MURINE B16F10 MELANOMA BY COMBINATION WITH BUTHIONINE SULFOXIMINE.

A. INTRODUCTION

In a previous study (1), we have shown that administration of N-acetyl-4-S-cysteaminylphenol (NACAP) to B16F10 melanoma lung colony-bearing mice results in a significant reduction in the number and size of colonies. This *in vivo* antitumor effect was melanoma-specific and had no observable toxicity to normal tissues (1). In contrast to the *in vivo* antimelanoma effects, our *in vitro* cytotoxicity studies have revealed that B16F10 melanoma cells are highly resistant to NACAP (Chapter IV). This observation is in agreement with a common finding that melanoma cells are resistant to the majority of antineoplastic drugs which have been tested (2). The mechanism of this chemoresistance is not well understood.

Many studies suggest that glutathione (GSH) may be an important factor limiting the therapeutic efficacy of cancer treatment by drugs (3). GSH is an intracellular non-protein sulfhydryl compound which is considered to be 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 may increase the susceptibility of some cancer cells to both radiation and/or chemotherapy (4).

Although the role of GSH in the synthesis of melanin by normal melanocytes has been thoroughly studied, the main functions of GSH in melanoma cells have remained unclear (5, 6). Previous studies have demonstrated that administration of buthionine sulfoximine (BSO), a specific inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis, may be a useful tool for the studying of the role of GSH in cells *in vitro* and *in vivo* (7). Kable *et al.* (8) and Karg *et al.* (9) were the first

investigators who reported a high sensitivity of melanoma cells to BSO, as well as an increased cytotoxicity of redox agents to melanoma cells in combination with BSO. Recently, Prezioso *et al.* (10) and Thrall *et al.* (11) confirmed these findings by presenting a significant enhancement of antimelanoma effect of catechols by BSO. However, these studies were performed only *in vitro*, and the efficacy of the drug combination treatment against melanoma cells using BSO *in vivo* has, hitherto, not been investigated.

In view of the above-mentioned data, the possibility for modifying the effects of NACAP with BSO against B16F10 melanoma cells was tested. This report shows enhanced antimelanoma activity of NACAP by a simultaneous treatment with BSO *in vitro* and *in vivo*. A possible mechanism of cytotoxicity is discussed and the demonstration of the selective accumulation of NACAP in melanoma tissue *in vivo* is shown.

B. MATERIALS AND METHODS

Chemicals. N-acetyl-4-S-cysteaminylphenol (NACAP) and its [^{14}C]-ring-labeled form were synthesized in our laboratory using a modified method described by Wehrmeister (12). The purity of the drugs was 99.9% according to HPLC measurements. D,L-buthionine-(S,R)-sulfoximine (BSO) was purchased from Sigma Co., St.Louis, MO. All other reagents were of analytical grade.

Melanoma Cell Line. The origin and growth characteristics of the murine B16F10 melanoma cell line which has a high affinity to form lung melanoma colonies, has been described previously (1). The cells were grown as monolayers in T-75 flasks in MEM medium (GIBCO Lab. Inc., Grand Isl. NY) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated in 37.0°C in a humidifed atmosphere of 5% CO_2 in air.

In Vitro **Antimelanoma Assay.** Cell growth in the presence or absence of experimental drugs was determined using a modified MTT-microculture tetrazolium assay described by Mosmann (13). Briefly, exponentially growing B16F10 cells were harvested, counted for cell viability, and inoculated at a concentration of $5x10^3$ cells/well (100 μl volume) in 96-well microtiter plates (Costar, Cambridge, MA) using a multichannel pipet. After 24 hr, aqueous solutions of drugs were added (100 μl volume) to culture wells in triplicate and cells were cultured for 5 days. An MTT stock solution was prepared at 5 mg/ml concentration in Dulbecco's PBS (Gibco) and stored at 4.0°C. On day 6, 50 μl of MTT solution, diluted to 1 mg/ml using MEM without serum was added to each well. After a 4-hr incubation, the supernatants from the wells were removed, 150 μl of 100% DMSO (spectrophotometric grade) was added to dissolve MTT-formazan product, and the absorbance at 540 nm was measured with a microplate reader (EAR 400 AT).

The survival fraction percentage of cells was calculated using a formula: (Absorbance in wells with drug)/(Absorbance in control wells w/o drug) x 100%.

In Vivo **Antimelanoma Assay and Experimental Protocols.** Murine B16F10 melanoma cells were cultured to a subconfluent state, harvested by trypsinization, washing, and resuspension in cold PBS. Cell viability was determined by the trypan blue exclusion test. C57BL/6J mice, 5 week old females, were purchased from The Jackson Laboratory (Bar Harbor, ME). On day 0, $5x10^4$ cells in 0.2 ml PBS were given i.v. to mice through a lateral tail vein. On day 5, the mice were randomized in four treatment groups of 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. Group 1 mice (control) were given injections of normal saline solution; group 2 mice, NACAP (2.0 mmol/kg); and group 3 mice, BSO (2.0 mmol/kg). Group 4 mice were given a combined injection of NACAP (2.0 mmol/kg) and BSO (2.0 mmol/kg). In addition, the group 3 & 4 mice were supplemented with 30 mM BSO in drinking water. On day 21,

mice were killed by cervical dislocation and their lungs were removed. The number of melanoma colonies in the lungs was counted and the size of 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 percentage of control group: b/a x 100%, where **a** is a value in control group and **b** is a value in experimental group.

Glutathione Assay. Mice were given a s.c. or i.v. injection of $5x10^4$ B16F10 melanoma cells in 0.2 ml of phosphate buffered solution (PBS, pH = 7.2) and then were randomly distributed into four groups of five animals. On day 15 after the cell inoculation, each animal was injected with a single i.p. injection of drug solution as follows: group 1 mice were given an injection of normal saline solution; group 2, NACAP (2.0 mmol/kg); group 3, BSO (2.0 mmol/kg); and group 4 mice were given injection of NACAP (2.0 mmol/kg) in combination with BSO (2.0 mmol/kg). Two hours after i.p. injection, animals were killed by cervical dislocation, and their s.c. melanoma tumors, lungs, and livers were removed and immediately frozen in liquid nitrogen to be used for GSH determination. The content of total GSH was determined using a method described by Tietze (14) and Griffith (15). Briefly, frozen tissues were weighted and homogenized in 5.0 volumes (w/v) of 1% (w/v) picric acid on ice using a glass-glass manual homogenizer. Homogenates were centrifuged, supernatants were removed and used to determine the total GSH content in tissues using a GSSG-reductase procedure.

Histopathologic Procedure. 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.

In Vivo **Covalent Binding.** Animals with both lung B16F10 melanoma colonies and s.c. growing melanoma tumors, and control animals without B16F10 melanoma

transplants were given a single i.p. injection of [^{14}C]-NACAP (2.0 mmol/kg/5.0 μCi), killed 48 hr later, and used for measurements of the covalent incorporation of NACAP into tissues as previously described (16). Briefly, lungs with and without B16F10 melanoma colonies, s.c. melanoma tumors, and livers were excised, rinsed in saline solution, blotted dry, immediately frozen with dry ice, and stored at -90.0°C. Tissues were homogenized with 0.05 M neutral phosphate buffer (1:4, w/v) and precipitated with 20% trichloracetic acid. All precipitates were repetitively washed with 8 volumes of 80% methanol : 20% water. When the radioactivity of supernatants reached the background levels (about 6-8 washes), the tissue precipitate was dissolved in 1 N NaOH, aliquoted in Aquasol-2, and the amount of bound radiolabeled metabolite was determined using a Beckman LS 3801 liquid scintillation counter. Protein concentrations were measured using the Bradford assay (Bio-Rad Kit). Covalently bound radioactivity was expressed as nmol of radioactivity per mg of tissue protein.

Whole-Body Autoradiography. A mouse with both B16F10 melanoma colonies in lungs and s.c. growing melanoma tumor was injected with a single i.p. injection of [^{14}C]-NACAP (2.0 mmol/kg, total activity 5.0 μCi/dose) and killed 48 hr later by inhalation of carbon dioxide. The method of whole-body autoradiography was previously described (17). Briefly, animal was mounted in a gel of carboxymethyl cellulose, frozen at -90.0°C, and sectioned with 20-μm thickness. The sections were freeze-dried and processed to whole-body autoradiography on X-ray film for 4 weeks.

Statistics. Statistical analysis in each of experimental groups was made using the software program SPSS/PC+. Multiple comparisons among groups were performed by analysis of variance (ANOVA) followed by a range test.

C. RESULTS

In Vitro **Cytotoxicity of NACAP and BSO.** Figure VIII-1 shows the *in vitro* growth inhibition of NACAP, BSO and combinations of the two drugs against highly pigmented B16F10 melanoma cells. Using the MTT assay, the dose-dependency of cell survival at day 5 of continuous drug exposure was measured. This end point allowed the cells 8-10 doublings with a population doubling time of about 12.6 hours. The IC_{50} values for each group of drug treatments were obtained from survival curves. The IC_{50} for NACAP was about 148.0 ± 12.2 μg/ml (7.01 ± 0.58 x 10^{-4} M) which indicated a high chemoresistance of cells to NACAP. The treatment with BSO was more effective, the IC_{50} value being about 61.0 ± 4.4 μg/ml (2.75 ± 0.20 x 10^{-4} M). To study the *in vitro* growth inhibition of the two drug combination in detail, a concentration scale of NACAP from 10.0 to 100.0 μg/ml was used, and a BSO concentration of 25.0 and 50.0 μg/ml per well. Cells were exposed to BSO two hours before the addition of NACAP. The combination treatment resulted in a significant additive decrease of IC_{50} values, the IC_{50} for NACAP plus BSO (25.0 μg/ml) and NACAP 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 suggesting the importance of the intracellular GSH for the proliferation and survival of melanoma cells. The IC_{50} values of the combination treatments, being 4.9- and 32-fold lower to the IC_{50} of NACAP alone, respectively, may indicate that the addition of BSO abolished the chemoresistance of B16F10 cells significantly and in a dose-dependent manner.

In Vivo **Antimelanoma Effect of NACAP and BSO on Melanoma Colonies in Lungs.** The numbers of B16F10 melanoma colonies were significantly reduced after treatments with NACAP alone and BSO alone as well (Figures VIII-2 & VIII-3a).

Furthermore, the size of colonies and the fresh weight of lungs with melanoma colonies treated with single-drug regimens were also significantly reduced (Figs. VIII-3b & 3c). However, the results of the combination treatment using NACAP plus BSO were still superior to those of both single-drug regimens. Except for the size of melanoma colonies treated with NACAP alone and NACAP plus BSO, all other results of the drug combination treated group of mice were significantly different to those of the control and single-drug treated groups according to the ANOVA and range tests. Importantly, the combination of NACAP with BSO did not enhance the systemic toxicity of NACAP. The only observed side effect was a brief hypoactivity of animals which occured shortly after i.p. injection of NACAP and resolved spontaneously in two hours. The average body weight of animals was the same on day 0 as that on the last day of experiments.

Histopathologic Findings. Typically, B16F10 melanoma colonies are located around or close to blood vessels in lung (Figure VIII-4a). In the control group, the lung tissue revealed large foci of darkly pigmented cells without significant infiltration with mononuclear cells. The melanoma colonies in the lungs from drug treated groups resembled those of controls but were clearly less numerous and smaller (Figure VIII-4b). There was no evidence of necrosis or increased number of amelanotic colonies in lungs of treated animals.

Effect of Drug Treatments on Glutathione Content. Table VIII-1 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 NACAP alone and BSO alone caused the depletion of GSH to 92% and 76% of controls, respectively, as measured two hours after i.p. injection. With a combined single i.p. injection of NACAP plus BSO, the total GSH content in the lung was reduced to 48% of controls. In order to examine the contribution of melanoma tissue to the total depletion of GSH in the melanoma colony-bearing lung, the GSH

content in the s.c. melanoma tumor and the liver of the same animals was determined as well as the GSH content in the lung of control animals without B16F10 melanoma colonies, before and after the treatment. NACAP alone depleted GSH in the lung without B16F10 melanoma colonies, the s.c. melanoma, and the liver, to 84%, 90%, and 79%, respectively. The treatment with BSO alone decreased the GSH concentration to 59%, 84% and 42% of controls in the lung without melanoma colonies, the s.c. melanoma, and the liver, respectively. The combination treatment with NACAP plus BSO resulted in GSH depletion to 44% in the lung without melanoma colonies, 45% in the s.c. melanoma, and 21% in the liver, respectively.

In Vivo **Covalent Binding to Tissue Protein.** Five different tissues were examined for covalent binding of radioactively labeled NACAP intermediate(s) (Table VIII-2). The maximum of binding after 48 hr post injection was detected in s.c. melanoma tumor and melanoma colony-bearing lung tissues. In contrast, the normal tissues, e.g. the lung without melanoma colonies, the kidney, as well as the liver, the organ with the highest metabolic activity against NACAP, bound insignificant amounts of radioactivity.

Whole-Body Autoradiography. 48 hr after i.p. injection of [^{14}C]-labeled NACAP in the mice, the radioactivity cleared from the body by urinary excretion, and was undetectable in any normal organ except the contents of the intestine. This finding indicates significant detoxication of NACAP in the liver which is followed by the excretion of NACAP metabolites into the bile. As presented in Figure VIII-5, the s.c. melanoma tumor and the lung with melanoma colonies were the only tissues displaying significant accumulation of the radioactivity.

D. DISCUSSION

BSO is a potent chemosensitizer which is capable of increasing the susceptibility of cancer cells to chemotherapeutic agents through depletion of GSH (18). Since the pioneering work of Griffith and Meister in 1979 (19), a number of drugs, including melphalan, bleomycin, and many other antineoplatic drugs, have been examined in combination with BSO *in vitro* and *in vivo*. Ozols and collaborators elucidated the relationship between GSH levels and the expression of chemoresistance in human ovarian cancer cells and showed the feasibility of using BSO to abolish the chemoresistance (20). Currently, several clinical trials phase I protocols in which BSO is combined with standard antineoplastics, e.g., L-phenylalanine mustard, are being conducted (21).

In contrast to ovarian carcinoma, the major cause(s) of the chemoresistance of melanomas has not yet been clarified. Kable *et al.* (8) reported a significant sensitivity of melanoma cells to BSO alone and increased cytotoxicity of L-dopa in combination with BSO *in vitro*. Other *in vitro* studies demonstrated improved cytotoxicity of 3,4-dihydroxybenzylamine and L-dopa methyl ester by the addition of BSO (10, 11). Although the mechanism of cytotoxicity by these catecholic drugs is not established, the positive role of BSO in the enhancement of their cytotoxicity has been shown. Two mechansims 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 the subsequent irreversible damage to cells, and b) the chemically unstable catechols may produce severe oxidative stress by the process of auto-oxidation (22). In both cases, GSH plays a major protective role against cytotoxicity of catechols which is mediated mostly by the GSH-dependent enzymes, glutathione-S-transferase(s), and glutathione peroxidase. The depletion of GSH by BSO diminished the effectiveness of

the cellular defence mechanisms which subsequently increases the cytotoxicity of catechols.

NACAP is a chemically stable monophenol that is a good substrate for mammalian and plant tyrosinases in a cell-free system (1). Tyrosinase converts NACAP into catechol and then to ortho-quinone which may cause the selective destruction of pigment cells. This study, however, provides the additional possibility of other metabolic pathways for NACAP which could produce cytotoxic intermediate(s) and involve cellular GSH. The measurements of GSH levels in various tissues after treatment with NACAP show significant metabolism of NACAP in both pigmented as well as non-pigmented tissues which is associated with GSH depletion. Since tyrosinase is not present in these tissues, the reactive intermediate of NACAP in the later case cannot be a quinone produced by tyrosinase. Instead, it may be a quinone moiety formed by some monophenoloxidase, e.g. peroxidase or some other metabolite produced by other xenobiotic metabolizing enzymes, e.g., cytochrome P-450 (23). Although the chemical structure of the cytotoxic intermediate(s) remains unknown, it is expected that NACAP is a prodrug which is enzymatically activated into a highly reactive electrophilic intermediate(s). The potentiation and the dose-dependent enhancement of the cytotoxicity of NACAP by BSO may support the prospective classification of NACAP as an alkylating agent. The *in vivo* covalent binding study confirms the belief that NACAP acts through alkylation, and furthermore, indicates that the target macromolecules are predominantly proteins.

In this study, the significant melanoma-specific antitumor property of NACAP has been presented. In addition, both the whole-body autoradiography and *in vivo* covalent binding assays clearly show the selective accumulation of radiolabeled NACAP into melanoma tissue. However, an explanation of the basis for the selectivity of NACAP action is complex. The biochemical studies revealed the reduction of GSH after the treatment with each of three drug regimens in melanoma as well as normal tissues. A striking finding is the small difference between the percentages of GSH change in the melanoma and

normal tissues after a single i.p. injection of NACAP as well as BSO. Similarly, Minchinton *et al.* reported a relatively slow depletion of GSH by BSO in tumor tissues compared to liver and kidney (24). It is possible, however, that after multiple injections of the drugs, the differences in GSH concentrations may become significant, the degree of GSH depletion in melanoma tissue being higher than that of normal tissues. Another explanation would include a different requirement of GSH for appropriate functioning of normal and melanoma cells. Indeed, Meister suggested that BSO may significantly decrease GSH synthesis in both normal and tumor cells, without alteration of both the physiologic functions and drug sensitivity of the normal cells. However, the tumor cells may exhibit dysfunction and sensitization to chemotherapy or to radiation under the GSH deficiency (25). The current studies suggest that, *in vivo*, melanoma cells have either a lower critical threshold for GSH depletion to manifest cell damage or reach this critical threshold faster than normal cells. This concept would explain the basis for the observed selectivity of NACAP for melanoma tissue. It is proposed that the conditions for random alkylation of cellular proteins by NACAP reactive intermediate(s) occur *in vivo* earlier and with lower doses of the drug in melanoma than in normal cells. Additionally, BSO may further speed up the occurence of the conditions suitable for the alkylation of macromolecules in melanoma tissue.

The combination treatment of B16F10 melanoma cells with NACAP and BSO has been shown to be a feasible approach for the reduction of the chemoresistance of these cells to NACAP *in vitro* and *in vivo*. The dose of both drugs (2.0 mmol/kg/day/per drug) used in *in vivo* studies produced measurable and distinct biological responses without significant side effects even after their combined administration. It is concluded that the combination regimen increased the therapeutic index of the *in vivo* selective antimelanoma drug, NACAP.

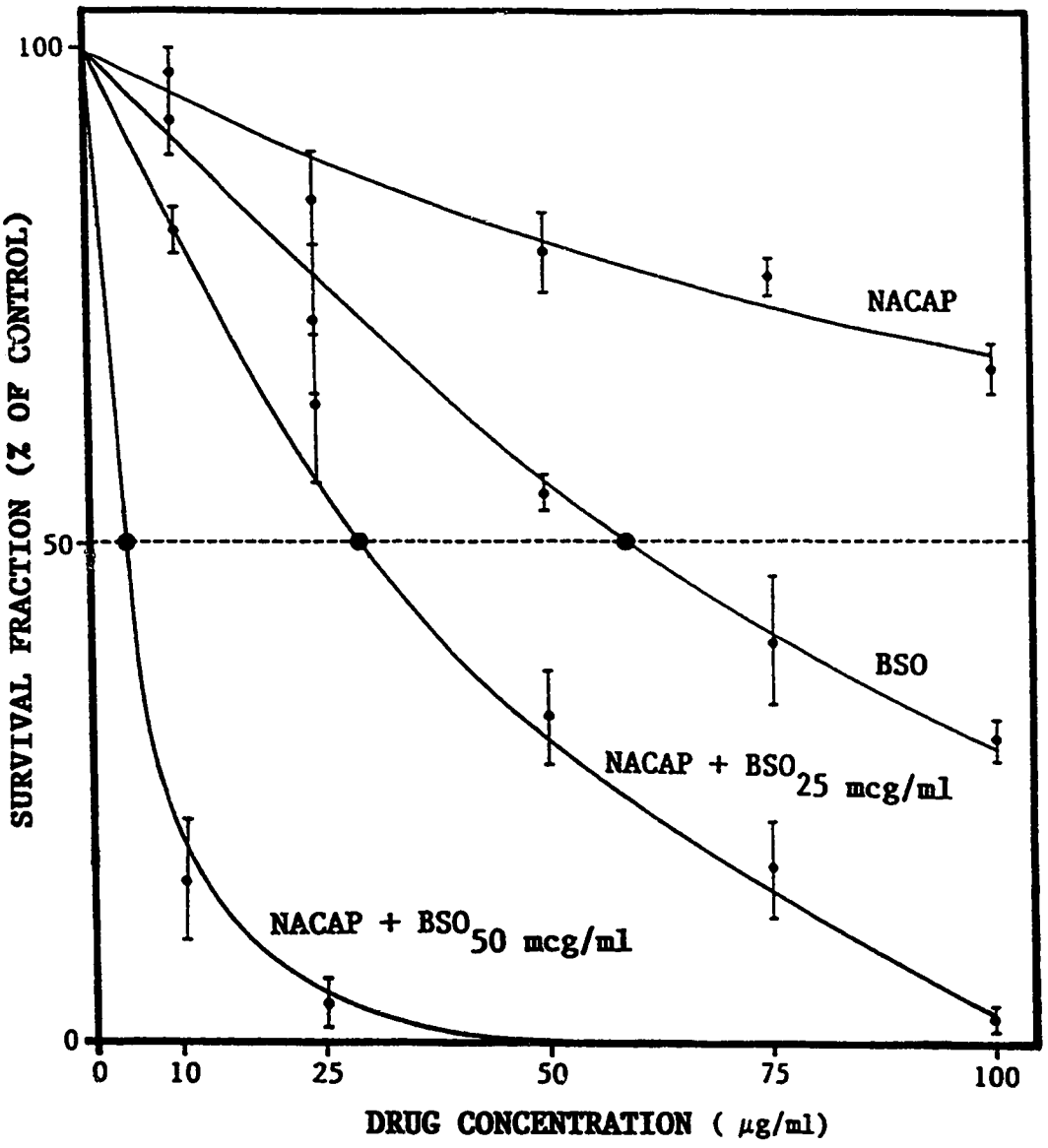


Fig. VIII-1. **Survival curves** for NACAP, BSO, combination of NACAP plus BSO 25 μg/ml, and NACAP plus BSO 50 μg/ml concentration tested against murine B16F10 melanoma cells in the MTT dye reduction assay. The means of IC 50s derived from three independent tests, each run in triplicates, were 148.0 ± 12.2 μg/ml for NACAP alone; 61.0 ± 4.4. μg/ml for BSO alone; 29.2 ± 3.1 μg/ml for NACAP plus BSO 25 μg/ml; and 4.5 ± 0.9 μg/ml for NACAP plus BSO 50 μg/ml.

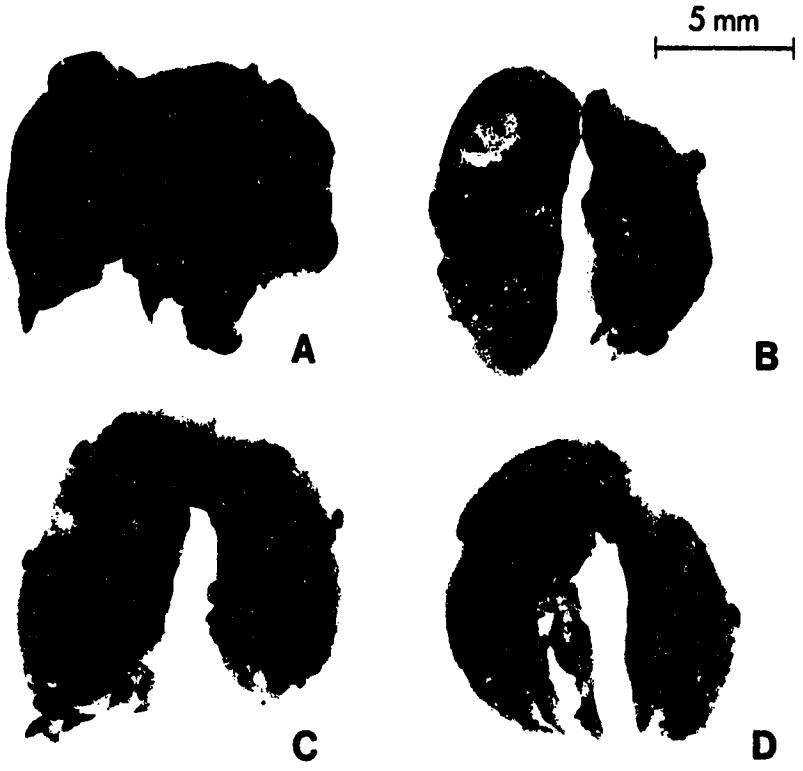
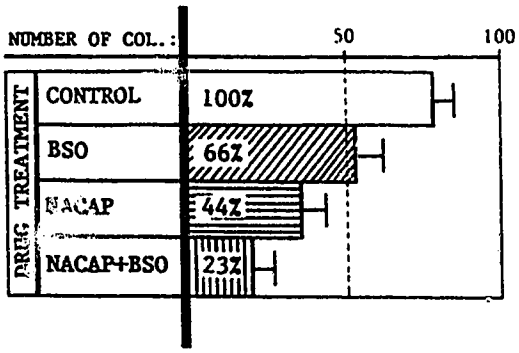
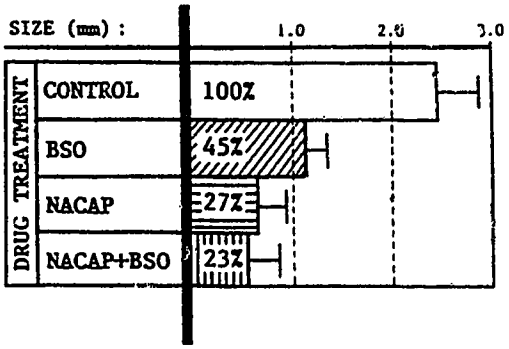


Fig. VIII-2. Representative lungs from mice 21 days after inoculations of 5x10 cells of B16F10 melanoma: **A.** from an untreated mouse injected with normal saline solution; **B.** from a mouse treated with BSO 2.0 mmol/kg; **C.** from a mouse treated with NACAP 2.0 mmol/kg; and **D.** from a mouse treated with a combination of NACAP 2.0 mmol/kg and BSO mmol/kg.

a) Number of B16F10 melanoma colonies:



b) Size of B16F10 melanoma colonies:



c) Weight of fresh B16F10 melanoma colony-bearing lungs:

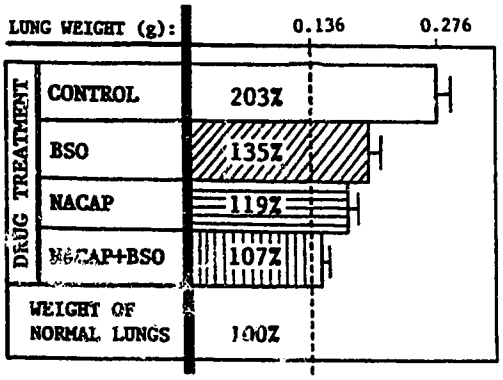


Fig. VIII-3. Effect of drug treatments by NACAP alone, BSO alone, and combination of NACAP plus BSO on the number and size of murine B16F10 melanoma colonies in lungs, and the weight of fresh B16F10 melanoma colony-bearing lungs in mice after i.p. injection of drugs daily for 15 days.

Fig. VIII-4. Light microscopy of B16F10 melanoma colonies in the lung tissue of black C57BL/6J mice. a, Alveoli; v, pulmonary blood vessel; b, bronchiole; m, melanoma colony. **a)** Control (untreated) lung. *Arrow*, a fully developed melanoma nodule surrounding a pulmonary venule. **b)** after treatment with i.p. injection of NACAP (2 mmol/kg) daily for 15 days. *Arrow*, small melanoma colonies bridging pulmonary venules with a bronchiole. *Bar*, 100 μm.

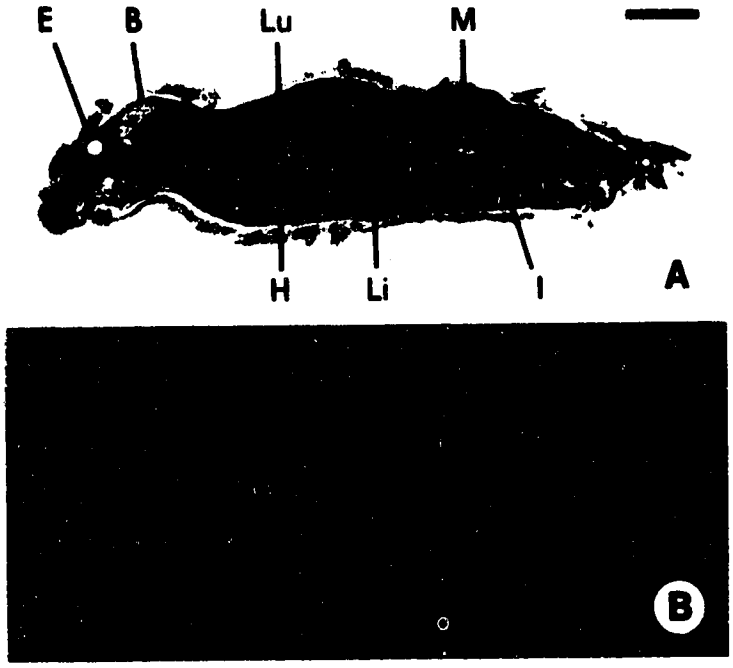


Fig. VIII-5. A whole-body autoradiogram of a mouse with both a s.c. B16F10 melanoma tumor and B16F10 lung melanoma colonies after a single i.p. injection of [^{14}C]-NACAP (5.0 μCi). 48 hr after injection, the animal was killed, frozen, and prepared for sectioning. The 20-μm sections were freeze-dried and exposed to x-ray film for whole-body autoradiography. **A)** A whole-body section without exposure to x-ray film. E, eye; B, brain; Lu, lung with B16F10 melanoma colonies; H, heart; Li, liver; Me, s.c. melanoma tumor; I, intestines. **B)** after exposure to x-ray film. Positive signal of accumulated radioactivity is clearly detectable in the melanoma colony-bearing lung and s.c. melanoma tissues, as well as in the lumen of the distal parts of intestine. *Bar*, 1 cm.

Table VIII-1. Glutathione Content in Melanoma and Non-Melanoma Tissues Two Hours after a Single i.p. Injection of NACAP, BSO, or Combination of NACAP plus BSO in Mice .

Tissue	Control	NACAP 2 mmol/kg	BSO 2 mmol/kg	NACAP + BSO ā 2 mmol/kg
Lungs with B16F10 Col.	2.005±0.190[a] 100.0±19.0%[b]	1.837±0.148 91.6± 7.4%	1.530±0.225 76.3±11.2%	0.964±0.329 48.1±16.4%
Lungs w/o B16F10 Col.	1.895±0.230 100.0±12.1%	1.583±0.174 83.5± 9.1%	1.115±0.055 58.8± 2.3%	0.830±0.085 43.8± 4.5%
B16F10 s.c. Tumor	0.822±0.068 100.0± 8.3%	0.745±0.086 90.4±10.1%	0.686±0.082 83.6±10.0%	0.366±0.099 44.5±12.0%
Liver	5.121±0.200 100.0± 3.9%	4.047±0.254 79.0± 5.0%	2.164±0.637 42.3±12.4%	1.074±0.240 21.0± 4.7%

[a] GSH content (μmol/g of tissue) expressed as a mean ± SD (n = 3);

[b] Percentage of control;

Table VIII-2. In Vivo Covalent Binding of [^{14}C]-NACAP to Lung with and without B16F10 Melanoma Colonies, B16F10 Melanoma s.c. Tumor Tissue, Liver, and Kidney.

Tissue[a]	Covalent Binding[b] (nmol covalently bound/mg of protein)
Lungs with B16F10 melanoma colonies	0.330 ± 0.118
Lungs without B16F10 melanoma colonies	0.010 ± 0.001
B16F10 melanoma s.c. tumor	0.521 ± 0.076
Liver	0.013 ± 0.003
Kidney	0.008 ± 0.001

[a] Female C57BL/6J mice received a single intraperitoneal injection of 2.0 mmol/kg [14C]-NACAP. The animals were killed 48 hr later, the tissues were removed, and the covalent binding was determined as described under Materials and Methods.

[b] The data are presented as the means ± S.D. (n=3).

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IX. SUMMARY AND DISCUSSION

The hypotheses tested in this thesis can be summarized as follows: Phenolic thioethers (PTEs) are new investigational drugs for the development of melanoma-specific chemotherapy. Certain of these compounds:

1. Are potent melanocytotoxic and antimelanoma agents *in vivo* and *in vitro*;

2. Are selectively cytotoxic to pigment cells;

3. Become cytotoxic after conversion into orthoquinone moieties by the action of tyrosinase;

4. May be cytotoxic through the mechanism of alkylation of macromolecules by reactive intermediates or by the induction of severe oxidative stress;

5. May be used in combination with other drug(s) to exhibit enhanced effect *in vitro* and *in vivo*.

These hypotheses have been verified as follows:

Hypothesis 1

Both *in vivo* and *in vitro* experiments clearly demonstrated that PTEs are potent melanocytotoxic and antimelanoma drugs (Chapter III & IV). Of five PTEs, the most *in vitro* cytotoxic agent was found to be 4-S-HomoCAP, followed by 4-S-CAP, N-Ac-4-S-CAP, N,N-DiMe-4-S-CAP, and α-Me-4-S-CAP. However, the melanocytotoxic potency of PTEs *in vivo* was different with the most powerful agent being N-Ac-4-S-CAP, followed by α-Me-4-S-

CAP, 4-S-CAP, 4-S-HomoCAP, and N,N-DiMe-4-S-CAP. The *in vivo* antimelanoma effects of both 4-S-CAP and N-Ac-4-S-CAP were found to be significant, but due to the 2-fold lower systemic toxicity, N-Ac-4-S-CAP showed better potential for use in experimental melanoma-specific chemotherapy.

Hypothesis 2

Both selective melanocytotoxic and antimelanoma effects of PTEs were observed only under *in vivo* experimental conditions (Chapter III & VIII). In contrast to expectations, the *in vitro* tests unambiguously showed PTEs to be non-selective cytotoxic drugs, since many non-pigment cells from a panel of eleven cell lines were more susceptible to PTEs than pigment cells (Chapter IV).

Hypothesis 3

In vitro tests using a specific tyrosinase inhibitor, phenylthiourea, failed to validate an essential role for tyrosinase in the mediation of cytotoxicity by PTEs (Chapter IV). It was found that inhibition of tyrosinase did not prevent or even affect the *in vitro* cytotoxicity of 4-S-CAP and N-Ac-4-S-CAP. Moreover, we found that some cells without detectable tyrosinase activity, e.g, lung adenocarcinoma, neuroblastoma, and amelanotic melanoma cells, were more susceptible to PTEs than melanotic melanoma cells expressing high levels of tyrosinase. Importantly, the cells with the highest tyrosinase activity and the highest degree of melanization, murine B16F10 melanoma cells, were the least susceptible to PTEs of all eleven cell lines which have been tested. These results also invalidate the hypothesis that the cytotoxic intermediates of PTEs are orthoquinones produced by melanosomal tyrosinase. However, on a theoretical basis, it is proposed that the cytotoxic intermediates may be quinonoid moieties formed by some enzyme(s) of the endoplasmic reticulum, e.g., mono-oxygenase(s) (Chapter V & VII).

Hypothesis 4

Both whole-body autoradiography and *in vivo* covalent binding assays clearly showed the selective incorporation of radioactively labeled N-Ac-4-S-CAP into melanoma tissues *in vivo* due to the alkylation of cellular proteins by reactive intermediates (Chapter VIII). Measurements of glutathione (GSH) levels in tissues and cells exposed to N-Ac-4-S-CAP *in vivo* or *in vitro* showed significant GSH depletion by N-Ac-4-S-CAP (Chapter V, VI, VII, VIII). Moreover, the melanocytotoxic efficacy of N-Ac-4-S-CAP *in vivo* was found to be dose-dependent, and was directly affected by GSH repletion (inhibition of melanocytotoxicity) or enhanced GSH depletion (potentiation of melanocytotoxicity) (Chapter V & VI). These findings indicated the presence of a critical GSH threshold for an induction of chemical injury to cells, and thus indirectly have supported the probability that alkylation of macromolecules by reactive intermediates is a major mode of cytotoxicity by PTEs. However, these tests also revealed increased lipid peroxidation in cells exposed to N-Ac-4-S-CAP which finding may indicate that an oxidative stress also contributes to the mechanism of cytotoxicity by PTEs (Chapter VII).

Hypothesis 5

The combination treatment with buthionine sulfoximine (BSO) resulted in significant enhancement of antimelanoma potency of N-Ac-4-S-CAP *in vitro* and *in vivo* (Chapter VII & VIII). Importantly, this drug combination did not cause significant side effects. It is concluded that BSO significantly improved the therapeutic index of N-Ac-4-S-CAP and may be useful in an experimental drug-combination protocol for melanoma-specific chemotherapy of human patients

The data presented in this thesis research confirm the previous observations of the selective melanocytotoxicity by synthetic *p*-hydroxyphenolic compounds *in vivo* (1, 2). However, the results from *in vitro* experiments clearly show the non-selective cytotoxicity

of PTEs which is not mediated by tyrosinase (Chapter IV). This finding apparently contradict the original working hypothesis which was based on tyrosinase-catalyzed oxidation of phenolic compounds to reactive intermediates (3). Previously, several investigators reported a similar discrepancy between this theoretical concept and the *in vitro* non-selective cytotoxicity of another synthetic phenol, 4-hydroxyanisole (4-HA) (4, 5, 6). Currently, it is believed by some investigators that there are two modes of cytotoxicity by *p*-hydroxyphenolic compounds *in vitro*: the first mode is mediated by tyrosinase and is exhibited exclusively in pigment cells whereas the second mode of cytotoxicity is tyrosinase-independent and exists in non-pigment cells (7). This dualistic hypothesis does not consider the significant tyrosinase-independent cytotoxicity of phenols to be the major mode of cytotoxicity for pigment cells. In addition to the complexity of this problem, it is presently unclear which enzymes are involved in the tyrosinase-independent cytotoxicity (8, 9).

In vitro tests in which pigment cells with high tyrosinase activity (SK-MEL-23 melanoma cells) were exposed to a selective inhibitor of tyrosinase, phenylthiourea, and then simulataneously to NACAP, unambiguosly showed that tyrosinase is not responsible for the mediation of cytotoxicity by PTEs even in pigment cells (Chapter IV).

In order to clarify the mechanism of cytotoxicity by PTEs, a series of *in vivo* and *in vitro* mechanistic studies using NACAP as a representative of PTEs were performed (Chapters V, VI, and VII). It was found that *in vivo* depigmentation effects (melanocytotoxicity) of NACAP in black mice were dose-dependent with a range of depigmentation from no visible depigmentation, through various shades of grey, to pure white depigmentation. However, NACAP administered in the same doses to yellow mice produced distinct effects on pigmentation of newly grown hair follicles, e.g., a dose (2.0 mmol/kg) capable of fully depigmenting a black hair follicle to white, caused the conversion of pheomelanogenesis to eumelanogenesis in yellow mice with the new hair being black. It was shown that this effect was associated with significant and dose-

dependent depletion of GSH. Moreover, it was found that GSH was also depleted in lung and liver tissues. It was proposed that NACAP is an inert lipophilic prodrug which enters cells and is then activated into reactive electrophilic intermediate(s) by some drug metabolizing enzyme(s) of the endoplasmic reticulum. This reactive intermediate is rapidly conjugated with nucleophilic GSH followed by GSH depletion. The depletion of glutathione to some modest level causes a deficiency of L-cysteine necessary for pheomelanogenesis in the melanosomes of yellow hair follicles with the consequent conversion of pheomelanogenesis to eumelanogenesis without any damage to cells. However, the increase of GSH depletion below some critical threshold is associated with reversible or irreversible chemically induced injury to cells. The modulations of GSH levels in tissues *in vivo* clearly revealed the tissue protective effect of GSH against the cytotoxicity of NACAP.

The mechanism of cytotoxicity by NACAP has been found predominantly to be alkylation (arylation) of macromolecules by reactive intermediate(s) as demonstrated by *in vivo* covalent binding assays. However, some degree of oxidative stress is also associated with the cytotoxicity of NACAP as shown by the measurement of lipid peroxidation and the positive detection of the induction of heat-shock (stress) protein(s) in cells exposed tc NACAP. These findings indicate that the reactive intermediate(s) is probably of a quinonoid nature since quinones (quinonoids) are known to act through both major modes of cytotoxicity (10). If this were true, the enzymes producing the quinonoid intermediate(s) could be tyrosinase, peroxidase and/or cytochrome P-450 (11) with the preferred substrate being a *p*-hydroxyphenolic compound.

The whole-body autoradiographic study revealed the selective accumulation of radiolabeled NACAP into melanoma tissue. It is hypothesized that the basis for the *in vivo* selective cytotoxicity of NACAP could be the differentially lower critical GSH threshold for induction of chemical injury in pigment cells than that in non-pigment cells. This diffrence could be caused by active melanogenesis in the former. It is also proposed that the fine

balance between the rate of the phase I activation of *p*-hydroxyphenols into reactive intermediates and the rate of the phase II conjugation reactions of these reactants may be altered in pigment cells with active melanogenesis *in vivo*. Thus, this new concept concerning the mechanism of cytotoxicity by PTEs (NACAP) considers melanogenesis and the tyrosinase present in activated melanocytes to be only indirectly responsible for the selective melanocytotoxicity of phenols *in vivo*.

In this study, the significant melanoma-specific antitumor property of NACAP has been presented (Chapter III). In addition, the combination treatment of B16F10 melanoma cells with NACAP and BSO has been shown to enhance this antimelanoma effect by reducing the chemoresistance of these cells to NACAP *in vitro* and *in vivo* (Chapter VIII). Importantly, this distinct antitumor effect of the combination therapy was not associated with significant systemic toxicity. Thus, it is concluded that the combination of NACAP and BSO may be a feasible approach for the development of an effective melanoma-specific chemotherapeutic protocol for human patients.

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