

# Melanogenesis Exploitation and Melanoma Nanomedicine: Utilization of Melanogenesis Substrate, NPrCAP for Exploiting Melanoma-Targeting Drug and its Conjugation with Magnetite Nanoparticles for Developing Melanoma Chemo-Thermo-Immunotherapy

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**Abstract:** Exploitation of a specific biological property is one of the best approaches for developing novel cancer-targeted drugs. Melanogenesis substrate, N-propionyl cysteaminyphenol (NPrCAP: amine analog of tyrosine) may provide a unique drug delivery system (DDS) because of its selective incorporation into melanoma cells. It may also act as a melanoma-targeted therapeutic drug because of its production of highly reactive free radicals (melanoma-targeted chemotherapy). Utilization of magnetite nanoparticles can also be a good platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to the alternating magnetic field (AMF). This study shows the feasibility of this approach in experimental study using *in vivo* and *in vitro* B16 melanoma cells and preliminary clinical study to a limited number of advanced melanoma patients. The therapeutic protocol against the primarily transplanted tumor with or without AMF once a day every other day for a total of three treatments not only inhibited the growth of primary transplant, but also prevented the growth of the secondary, re-challenge transplant and increased life span of the host mice. HSP70 production at the site of primary transplant and CD8<sup>+</sup>T cell infiltration at the site of the re-challenge melanoma transplant were seen. Four patients entered in the preliminary clinical trial by following the basic outline of this animal protocol and two of them showed PR and CR. We hope to establish *in situ* vaccination immunotherapy for melanoma metastases by melanogenesis-targeted chemo- and thermo-therapy.

**Keywords:** Melanoma, chemothermoimmunotherapy, chemotherapy, immunotherapy, thermo-therapy, melanogenesis, nanomedicine.

## INTRODUCTION

Management of metastatic melanoma is extremely difficult challenge for physicians and scientists. Currently only 10% with metastatic melanoma patients survive for five years because of the lack of effective therapies [1]. There is, therefore, an emerging need to develop innovative therapies for the control of advanced melanoma.

Exploitation of biological properties unique to cancer cells may provide a novel approach to overcome this difficult challenge. Melanogenesis is inherently cytotoxic and uniquely occurs in melanocytic cells; thus, tyrosine analogs that are tyrosinase substrates can be good candidates for melanoma-specific drug targeting and therapies [2]. *N*-

propionyl and *N*-acetyl derivatives of 4-*S*-cysteaminyphenol (NPr- and NAcCAP) were synthesized, and found to possess effects on *in vivo* and *in vitro* melanomas through the oxidative stress that derives from production of cytotoxic free radicals [3-7]. We now provide evidence that the unique melanogenesis cascade can be exploited for developing a novel chemo-thermo-immunologic strategy (CTI Therapy) for advanced melanoma by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/M).

Intracellular hyperthermia using magnetite nanoparticles (10-100nm-sized, Fe<sub>3</sub>O<sub>4</sub>) has been shown to be effective for treating cancers in not only primary but also metastatic lesions [8-10]. Incorporated magnetite nanoparticles generate heat within the cells after exposure to AMF due to hysteresis loss [11]. In this treatment, there is not only the heat-mediated cell death but also immune reaction due to the generation of heat shock proteins (HSPs) [12-21]. HSP expression induced by hyperthermia has been found to be

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involved in tumor immunity, providing the basis for developing a novel cancer therapy (thermo-immunotherapy).

Our approach is based upon the combination of (1) direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effect of melanogenesis-targeted drug and (2) indirect killing by immune reaction (*in situ* vaccination) after exposure to AMF. It is hoped from these rationales a strategy that a tumor-specific drug delivery system is developed and selective cell death can be achieved by exposure to AMF, which then can induce HSP expression through either necrotic or non-necrotic process or combination of the two, without damaging non-cancerous tissues and establish immune reaction targeted to other metastatic melanoma lesions, hence providing “*in situ* vaccination” strategy.

In this report we compared, by utilizing the mouse B16 melanoma system, at first, their chemotherapeutic and thermo-therapeutic effect on primary transplant of melanoma cells with and without AMF exposure (heat generation) and then examined the immunotherapeutic effect on the second, re-challenge transplant of the same melanoma cells to evaluate if the growth of distant metastatic melanomas can be inhibited. We also investigated the possible association of HSP production, CD8<sup>+</sup> T cell activation and MHC expression along with rejection of the re-challenge melanoma. Finally we will introduce the preliminary therapeutic effect of this CTI strategy which is based upon for a limited number of advanced melanoma patients.

Our final goal is the development of novel CTI therapy by establishing not only melanoma-targeted chemothermotherapy but also *in situ* vaccination immunotherapy to advanced melanoma through exploitation of melanogenesis cascade.

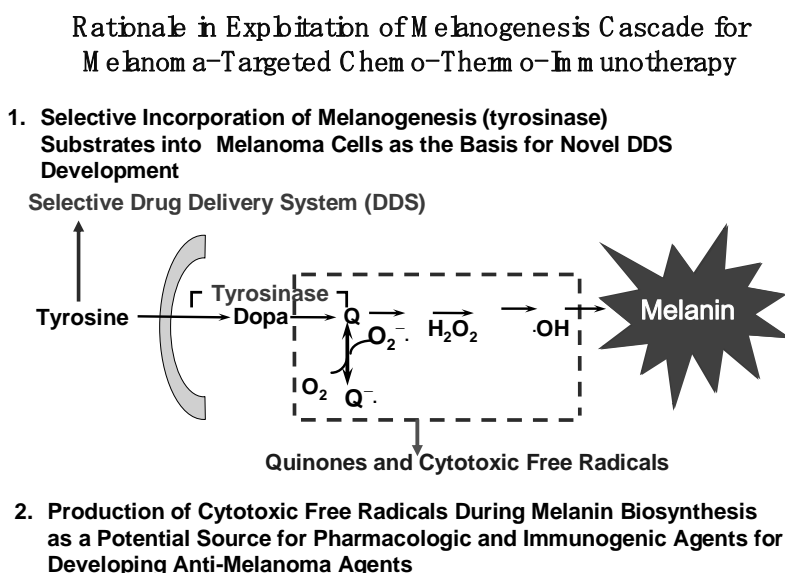
### EXPLOITATION OF MELANOGENESIS FOR POTENTIAL SOURCE IN NOVEL DRUG DEVELOPMENT TO MELANOMA

The major advance of drug discovery for targeted therapy to cancer cells may be achieved by exploiting their unique

biological property. The biological property unique to the melanocyte and melanoma cell resides the biosynthesis of melanin pigments within specific compartments, melanosomes. Melanogenesis begins with the conversion of amino acid, tyrosine to dopa and subsequently to dopa quinone in the presence of tyrosinase. This pathway is unique to all of melanocytes and melanoma cells including “amelanotic” melanoma. With the interaction of melanocyte-stimulating hormone (MSH)/melanocortin 1 receptor (MC1R), the melanogenesis cascade begins from activation of microphthalmia transcription factor (MITF) for induction of either eu- or pheomelanin biosynthesis. Tyrosinase is the major player of this cascade. It is a glycoprotein and its glycosylation process is regulated by a number of molecular chaperons, including calnexin in the endoplasmic reticulum [22,23]. Vesicular transport then occurs to carry tyrosinase and its related proteins from trans-Golgi network to melanosomal compartments. In this process a significant number of transporters, such as small GTP-binding protein, adaptor proteins and PI3kinase are involved in early melanosomal maturation, to which early and late endosomes are closely associated. Once melanin biosynthesis is completed to conduct either eu- or pheomelanogenesis within melanosomal compartments, they will move along dendritic processes and transferred to surrounding keratinocytes [24-26].

### Synthesis of Sulfur Analogs (Amine and Amide) of Tyrosine, Cysteaminylphenols

Then how can the melanogenesis cascade be exploited for better development of novel therapeutic approach to melanoma? In our approach two basic concepts are emerged toward this goal. One is that the incorporation of tyrosinase substrates, such as sulfur homologue of tyrosine (cysteinyphenol) and its amine derivative, cysteaminyphenol will be selectively incorporated into melanoma cells through active transport on the cell surface, which we believe, can be used as the basis for development of a novel drug delivery system (DDS). Another is the fact that melanin biosynthesis *per se*, if



**Fig. (1).** Two basic strategies reside in our CTI approach. One is the drug delivery system and another is the production of cytotoxic free radicals. Both are based upon tyrosinase-mediated melanogenesis.

overproduced, is toxic to melanoma cells through the production of quinone and cytotoxic free radicals, which can be used as the potential source for pharmacologic and immunologic agents for developing anti-melanoma agents (Fig. 1).

This cytotoxicity primarily derives from tyrosinase-mediated formation of dopaquinone and other quinone intermediates, which form cytotoxic free radicals. In order to utilize this unique biosynthesis pathway for cytotoxic compound in controlling melanoma growth, N-acetyl and N-propionyl derivatives of cysteaminyphenol (CAP) have been synthesized [27,28] (Fig. 2). These compounds were found to possess cytotoxic effect on *in vivo* and *in vitro* melanomas through the oxidative stress resulting from production of cytotoxic free radicals after conversion to cysteaminy catechol in the presence of tyrosinase [3-6] (Fig. 2).

### Specific Drug Delivery System and Melanocytotoxicity of Cysteaminyphenols

The specific DDS and selective cytotoxic properties were shown by a number of approaches. For example, both NPrCAP and NAcCAP can selectively disintegrate follicular melanocytes after single or multiple *ip* administration to new-born or adult C57 black mice [3, 29]. In the case of adult mice after repeated *ip* administration of NPrCAP, white follicles with 100% success can be seen at the site where hair follicles were plucked to stimulate new melanocyte growth and active tyrosinase synthesis. A single *ip* injection of NPrCAP into a new born mouse resulted in the development of silver follicles in the entire body coat. The selective disintegration of melanocytes can be seen as early as in 12 hr after a single *ip* administration. None of surrounding keratinocytes or fibroblasts showed such membrane degeneration and cell death.

The specific cytotoxicity of NPrCAP and NAcCAP was examined on various types of culture cells by MTT assay [30]. Among them, only melanocytic cells except HeLa showed the low IC50. The administration of high

concentration caused irreversible damage to melanoma cells on the colony formation assay. The cytotoxicity to these cells was dose-dependent. However, the cytotoxicity to HeLa cells on DNA synthesis was transient and reversible. The cytotoxicity on DNA synthesis inhibition was time-dependent and irreversible on melanoma cells, but was transient on HeLa cells. Molecular mechanism for cytotoxic action by NAcCAP and NPrCAP appears to involve two major target sites. One is cytostatic action which derives from the DNA synthesis inhibition through the interaction of quinone and free radicals with SH-enzymes and thymidine synthase. Another is the cytotoxic action by damage of DNA and mitochondrial ATP through oxidative stress and interaction with SH-enzyme [7] (Fig. 3). They bind protein disulphide isomerase [31].

### Selective Growth Inhibition Effect of Cysteaminyphenols to Melanoma Cells

The selectivity and specificity of our synthetic compounds to melanoma cells were evaluated by the *in vivo* and *in vitro* studies. The selective uptake of our drug by melanoma cells and tissues was shown by employing <sup>14</sup>C-labelled cysteaminyphenol. A high, specific uptake of NAcCAP was seen by melanoma cell lines, such as SKmel 23. In addition, a melanoma-bearing mouse showed, on the whole body autoradiogram, the selective uptake and covalent binding of NAcCAP in melanoma tissues of lung and skin. In another experiment, we examined to what extent one can block the melanoma growth in both *in vitro* culture and *in vivo* lung metastasis assays by administration of NAcCAP combined with BSO, buthionine sulfoxide, which blocked the effect of anti-oxidants. There was a marked growth inhibition of cultured melanoma cells in the presence of BSO, indicating that the selective cytotoxicity by our CAP is related to the quinone and free radicals. The *in vivo* lung metastasis experiment also showed the decreased number of lung melanoma colonies [3]. The problem was, however, that a fairly large number of amelanotic melanoma lesions were seen to grow in the lung. NPrCAP has been developed with the hope

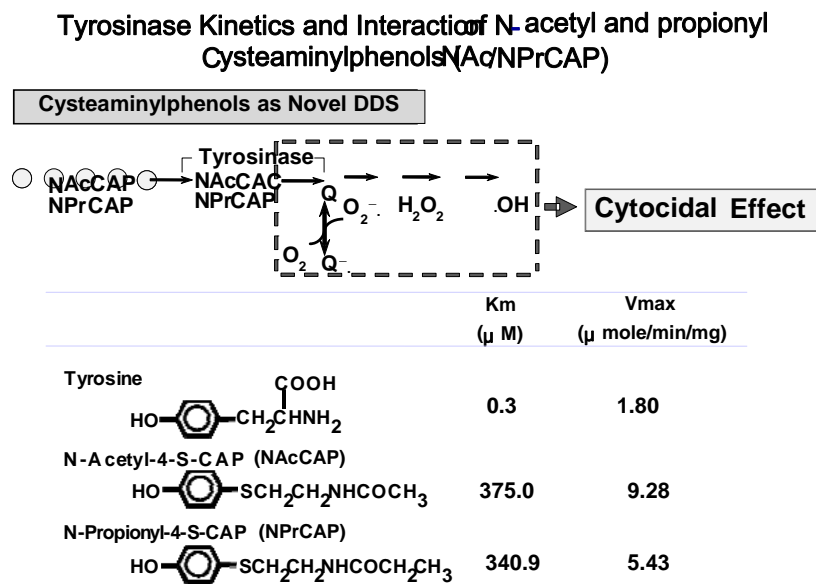
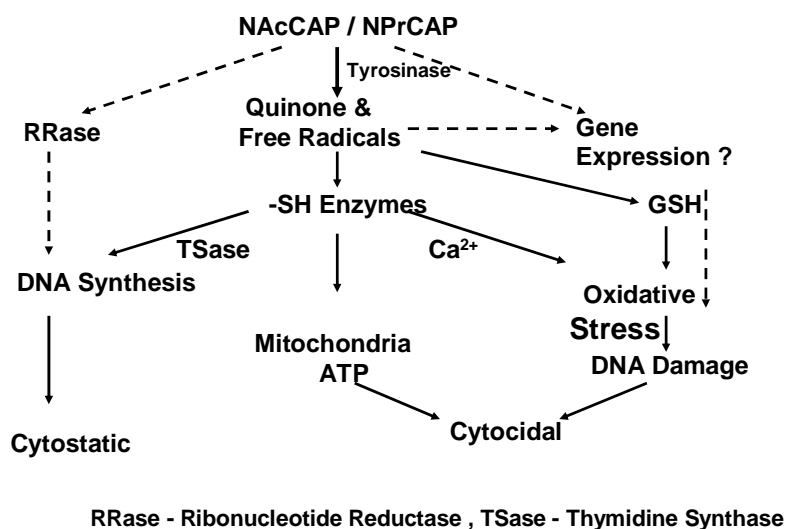


Fig. (2). Values of K<sub>m</sub> and V<sub>max</sub> were obtained by utilizing mushroom tyrosinase. The two compounds were also the substrates of B16 mouse melanoma tyrosinase.

### Molecular Targets of Cytotoxic Action by NAcCAP / NPrCAP in Melanocytes & Melanoma Cells



**Fig. (3).** Tyrosine analogs of NAcCAP and NPrCAP have two cytotoxic effects, i.e., cytotoxic and cytostatic, upon exposure to tyrosinase.

of increasing the cytotoxicity and overcoming a part of the problem.

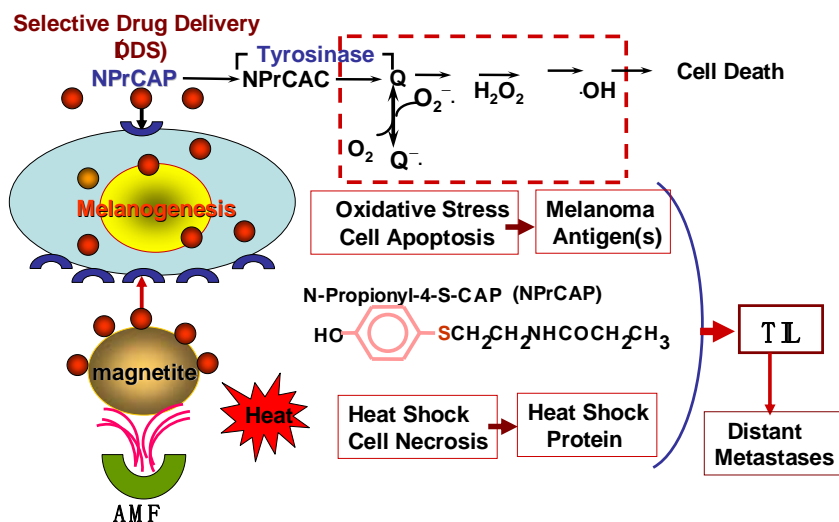
#### STRATEGY FOR DEVELOPMENT OF CHEMOTHERMO-IMMUNOTHERAPY FOR MELANOMA BY MELANOGENESIS SUBSTRATES

##### Synthesis for Conjugate of N-Propionyl Cysteaminyl-phenol and Magnetite Nanoparticles

In order to further increase the cytotoxicity to both melanotic and amelanotic cells, we conjugated NPrCAP with magnetite nanoparticles, which generate heat upon exposure to an alternating magnetic field (AMF). We expected this

combination of NPrCAP and magnetite nanoparticles to be a potential source for developing not only anti-melanoma pharmacologic but also immunogenic agent. It was expected that NPrCAP/magnetite nanoparticles complex could be selectively incorporated into melanoma cells. The degraded melanoma tissues from oxidative stress by NPrCAP and heat shock by AMF exposure would produce the synergistic effect for generating tumor-infiltrating lymphocytes, TIL that will kill melanoma cells in distant metastases (Fig. 4). Four nanoparticles were synthesized and two them, i.e., NPrCAP/M and NPrCAP/PEG/M were used for animal and human studies respectively (Fig. 5).

#### Strategy for Melanogenesis-Targeted CTI Therapy NPrCAP plus Magnetite with AMF



**Fig. (4).** NPrCAP/magnetite complex has two phases of cell destruction/death processes. One is cell apoptosis which derives from oxidative stress upon exposure to tyrosinase and another is cell necrosis that results from heat shock upon exposure to alternating magnetic field (AMF).

### Amount of NPrCAP Coupled with Cationic Magneto-Liposome, Non-Cationic Magneto-Liposome and Magnetite

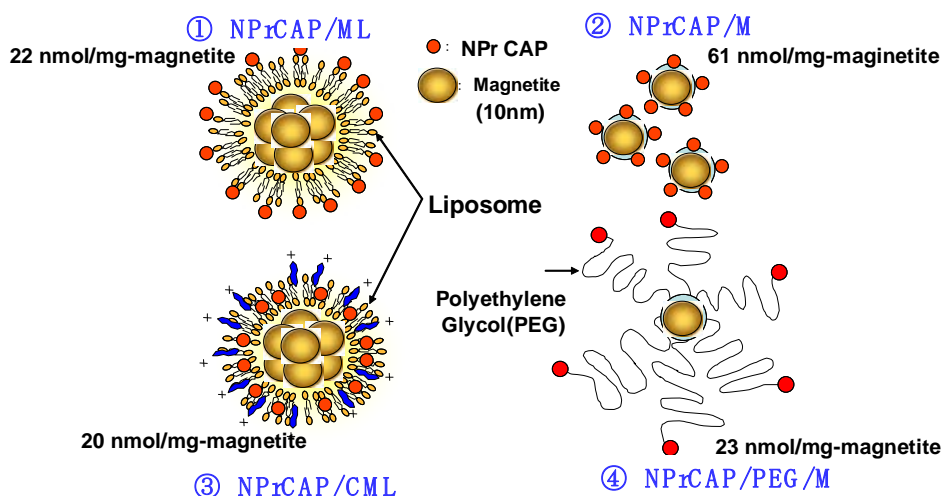


Fig. (5). ML: neutral magneto-liposome, CML: cationic magneto-liposome.

Magnetite nanoparticles have been employed for thermo-therapy in a number of cancer treatments including human gliomas and prostate cancers [32-35]. They consist of 10-100nm-sized iron oxide ( $\text{Fe}_3\text{O}_4$ ) with a surrounding polymer coating and become magnetized when placed in AMF [9]. We synthesized, in our initial study, the conjugate of NPrCAP with neutral magnetite-liposome nanoparticles (NPrCAP/ML) and 4SCAP/CML in which 4SCAP were embedded in cationic magneto-liposomes (Fig. 5). There was, however, non-specific electrostatic interaction between cationic magneto-liposomes and various non-target cells [35] and non-specific aggregations in neutral magneto-liposomes. A promising technique is the use of tumor-targeted magnetite nanoparticles, and this approach is extended by synthesizing another type of magnetite nanoparticles, NPrCAP/M and NPrCAP/PEG/M, on which NPrCAP is superficially and directly bound on the surface of magnetite nanoparticles without using liposomes [37]. Iron particles have been previously shown to be incorporated into melanocytes and melanosomes. NPrCAP/M and NPrCAP/PEG/M are chemically stable, and can be produced in large quantities and employed to effect melanoma-targeted chemotherapy (by NPrCAP) and thermo-immunotherapy (by magnetite with HSP), hence providing a basis for a novel chemo-thermo-immunotherapy (CTI therapy). Most of the experiments described below were carried out by employing NPrCAP/M except in preliminary clinical trials to which NPrCAP/PEG/M was used.

#### Development of Chemo-, Thermo- and Immunotherapy by Exploiting Melanogenesis Substrates

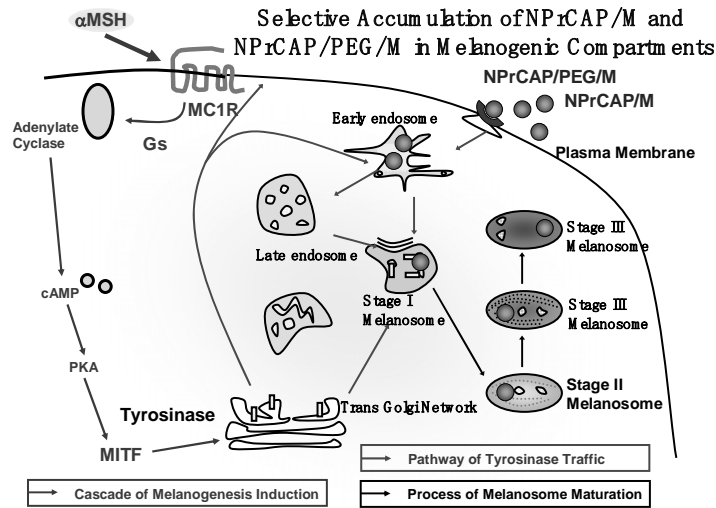
Our basic strategy in designing chemo-thermo-immunotherapy (CTI therapy) drugs is that tyrosinase substrates, NPrCAP/M, will be selectively aggregated on the melanoma cell surface by active transport through a still unknown receptor system and that they will be incorporated into early and late endosomes to which tyrosinase will also be transported from TGN to form stage I melanosomes. Once NPrCAP/M is incorporated into melanosomes, it will

be then retained and aggregated in the melanosomal compartments as there will be no melanosome transfer occurring in melanoma cells (Fig. 6). Thus we should be able to selectively destroy melanoma cells by heat generated by AMF exposure from magnetite nanoparticles which are accumulated only in melanosomal compartments. In fact, we could see NPrCAP/M nanoparticles which were selectively aggregated in melanoma cells compared to non-melanoma cells (Fig. 7). NPrCAP/M nanoparticles were found to be specifically incorporated and aggregated in melanosomal compartments at 2 weeks after *ip* administration by electron microscopy (Fig. 8). After AMF exposure, there will be selective disintegration of melanoma tissues as can be seen by Berlin Blue staining (Fig. 9) [36,37].

In hyperthermia treatment, the expression of heat shock proteins (HSPs) plays an important role in immune reactions [12-16, 38, 39]. Accumulating evidence from our group [18-20] and from others [21] implicates HSP expression induced by hyperthermia in tumor immunity and opens the door to novel cancer therapy based on hyperthermia treatment (thermo-immunotherapy). In such a strategy, a tumor-specific hyperthermia system that can induce necrotic cell death *via* HSP expression without damaging non-cancerous tissues would be highly desirable. An intracellular hyperthermia system using tumor-targeted magnetite nanoparticles facilitates tumor-specific hyperthermia; this can induce necrotic cell death *via* HSP expression, which in turn induces antitumor immunity.

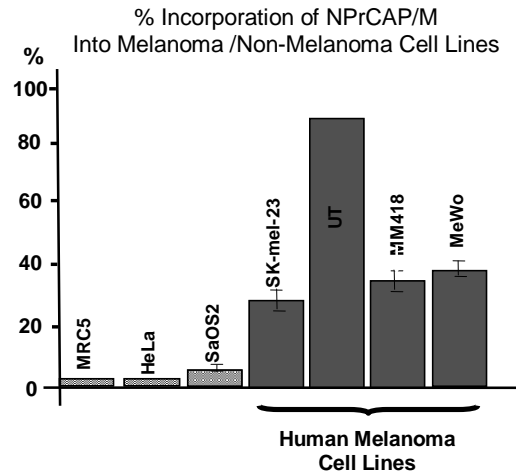
#### Protocols of Experimental Chemo-Thermo-Immunotherapy by Employing Melanogenesis Substrates

In this study, we employed three cell lines of B16 melanoma, i.e., B16F1, B16F10 and B16OVA cells and compared the thermo-therapeutic protocols in detail by evaluating the growth of the re-challenge melanoma as well as the duration and rates of survival of melanoma-bearing mice (Fig. 10). It is expected that our nanoparticles will also be selectively incorporated into human melanoma cells, (Fig. 7).



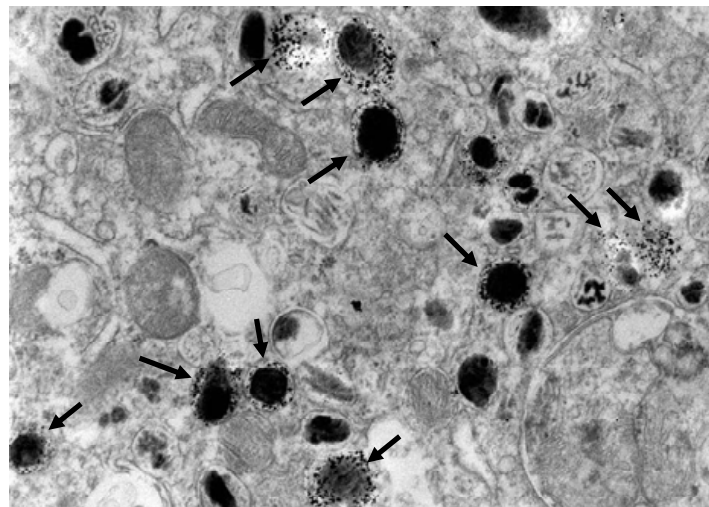
**Fig. (6).** NPrCAP/magnetite complexes (NPrCAP/M and NPrCAP/PEG:polyethylene glycol/M) are selectively incorporated into melanoma cells probably through active transport on the cell membrane and accumulate in endosomes, i.e., precursors of melanosomes.

**Selective Incorporation of NPrCAP/M into Melanoma Cells**



**Fig. (7).** NPrCAP/magnetite nanoparticles are selectively incorporated into human melanoma cells compared to non-melanocytic cells.

**Selective Accumulation of NPrCAP/M into Melanosomal Compartments at Day 15 after Administration**



**Fig. (8).** Arrows indicate magnetite particles incorporated into melanosomes.

### Selective Incorporation of NPrCAP/M into Melanoma Tissues and Their Degradation after AMF Exposure

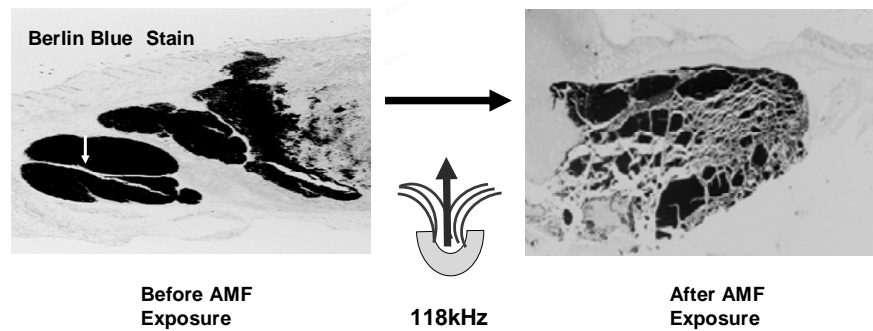


Fig. (9). NPrCAP/magnetite nanoparticles are accumulated in melanoma tissues and then degraded upon exposure to AMF.

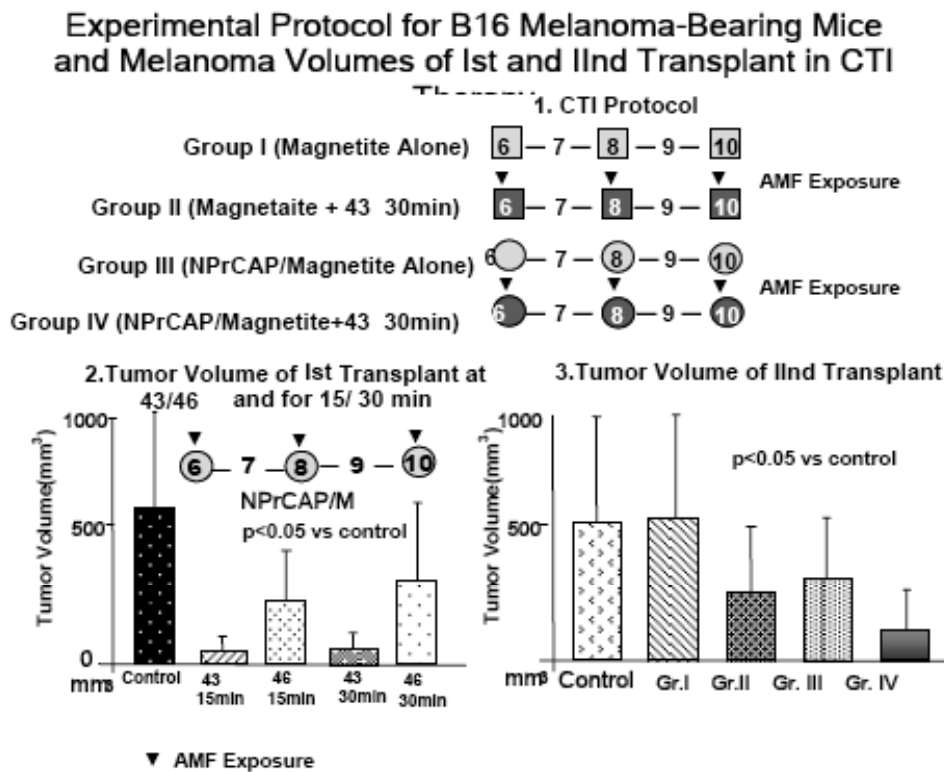


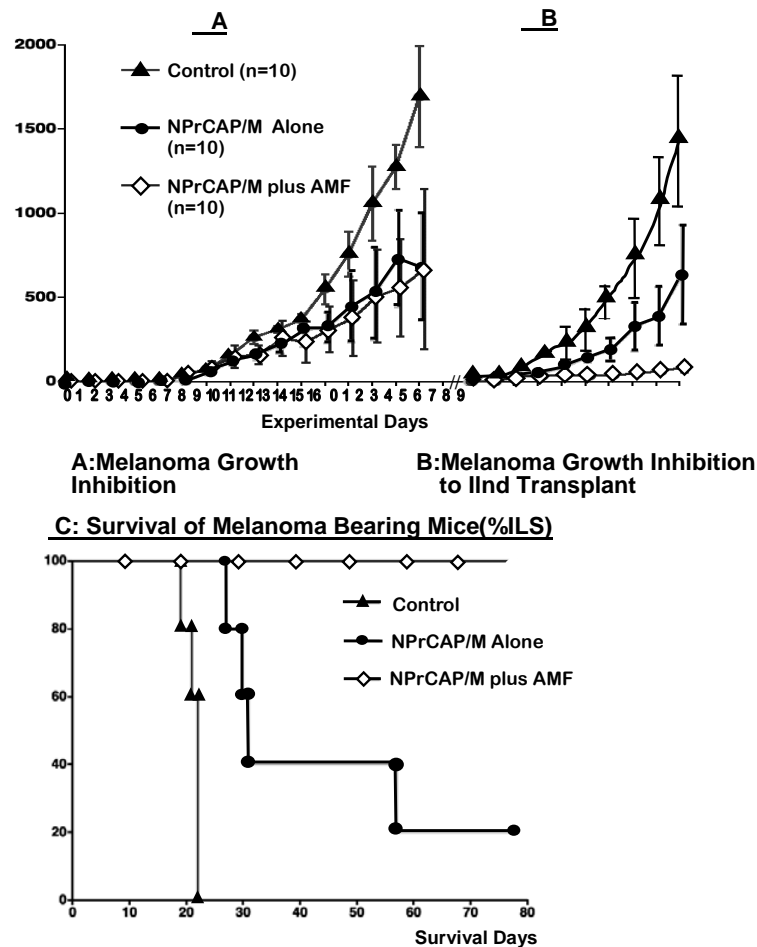
Fig. (10). The most effective thermo-immunotherapy for the growth inhibition of re-challenge melanoma transplant is achieved by the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma.

We first evaluated the chemotherapeutic effect of NPrCAP/M with or without heat. NPrCAP/M without heat inhibited growth of primary transplants to the same degree as did NPrCAP/M with heat, indicating that NPrCAP/M alone has a chemotherapeutic effect. However, there was a significant difference in the melanoma growth inhibition of re-challenge transplants between the groups of NPrCAP/M with and without heat. NPrCAP/M with AMF exposure showed the most significant growth inhibition in re-challenge melanoma and increased life span of the host animals, i.e., 30-50% complete rejection of re-challenge melanoma growth, indicating that NPrCAP/M with heat possesses a thermo-immunotherapeutic effect (Fig. 11). Specifically our study indicated that the most effective thermo-immunotherapy for re-challenge B16 melanoma can

be obtained at a temperature of 43°C for 30 min with the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma (Fig. 10). Our therapeutic conditions and their effects differ from those of magnetically mediated hyperthermia on the transplanted melanomas reported previously [40]. cationic magneto-liposomes-mediated hyperthermia for B16 melanoma showed that hyperthermia at 46°C once or twice led to regression of 40-90% of primary tumors and to 30-60% survival of mice, whereas hyperthermia at 43°C failed to induce regression of the secondary tumors with 0% survival of mice [40].

We analyzed HSP70 production in the primary tumor and CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltration into the re-challenge

## Inhibition of Melanoma Growth by NPrCAP/M



**Fig. (11).** NPrCAP/M with AMF exposure shows the most significant growth inhibition of re-challenge melanoma transplant and increased the life span of the hosts (ILS).

secondary tumor. Our study showed that NPrCAP/M-mediated hyperthermia at 43°C for 15 to 30 min and 46°C for 15 min produced a large amount of HSP70, Fig. (12). This stress protein forms a complex with intracellular peptides released from degrading tumor cells and presented by the MHC class I molecules of professional antigen-presenting cells [20]. Although hyperthermia at 46°C for 15 min could induce HSP70 as abundantly as that at 43°C for 30 min, this condition failed to suppress the re-challenge melanoma transplant as efficiently as 43°C hyperthermia Fig. (12). This suggests that immunological factors other than HSPs are at least in part responsible for rejection of the second re-challenge melanoma. Hyperthermia at 43°C for 1 hr revealed the expression of MHC class I molecules after 24 h in association with enhanced expression of HSP70 [41]. Heat treatment of tumor cells permits enhanced cross-priming, possibly *via* up-regulation of both HSPs and tumor antigen expression [21]. Thus, by inducing HSP70 and possibly MHC class I, our protocol of NPrCAP/M-mediated hyperthermia at 43°C can be an effective therapy for the treatment of advanced metastatic melanoma.

NPrCAP/M-mediated hyperthermia at a relatively low temperature (43°C) effectively inhibited the growth of second transplant, re-challenge melanoma. It may be possible that superficially bound NPrCAP possesses an important role not only in targeting nanoparticles to melanocytic cells and a chemotherapeutic effect on these cells but also in causing potentially an immunotherapeutic effect.

#### Melanocytotoxic and Immunogenic Properties of N-Propionyl Cysteaminylphenol (NPrCAP) and Magnetite Conjugates

Hyperthermia increases the expression of intracellular HSPs which is important in and necessary for the induction of antitumor immunity [42,43]. Over expression of HSPs, such as HSP 70, increases tumor immunogenicity by augmenting the chaperoning ability of antigenic peptides and presentation of antigenic peptides in MHC class I molecules [44, 45]. In this process professional antigen presenting dendritic cells play unique and important roles in taking up, processing and presenting exogenous antigens in association



### Heat Shock Protein Production

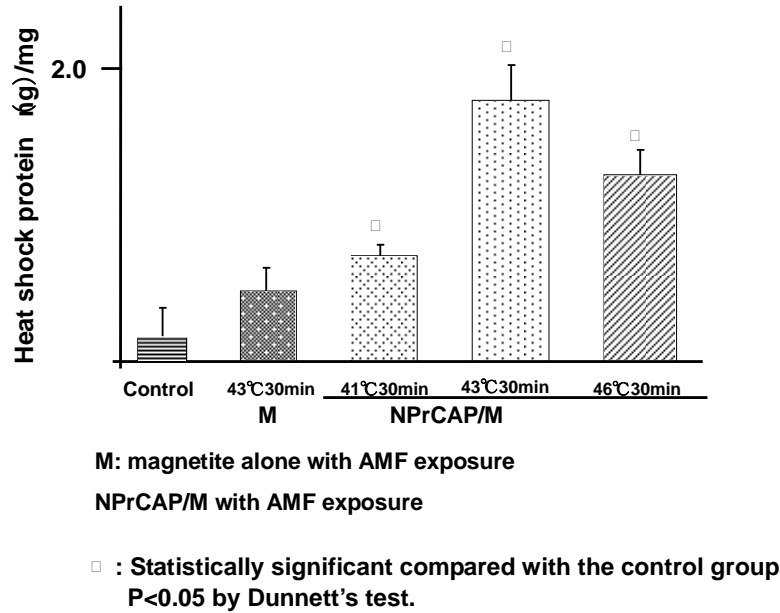


Fig. (12). NPrCAP/M with AMF exposure causes the significant production of HSP70.

with MHC class I molecules. Our working hypothesis for induction of *in situ* vaccination immunotherapy is that CTI therapy causes degradation of melanoma tissues which results in the release of HSP/melanoma antigen complex. This complex is taken up by professional antigen-presenting dendritic cells through HSP receptor. Subsequently after internalization within the dendritic cells, MHC and antigen peptide complex is presented to CD8+ T cells with the induction of acquired immunity, Fig. (13).

In our animal study it was indicated that NPrCAP/M by itself inhibits melanoma growth by not only chemotherapeutic effect but also a unique immunogenic

property [46]. Our current working hypothesis for this finding is that there is a difference in the cytotoxic mechanism and immunogenic property of NPrCAP/M between experimental groups with and without AMF exposure. The animals with NPrCAP/M plus AMF exposure resulted in non-apoptotic necrotic cell death with immune complex production of melanoma peptide as well as HSP 70 and a small amount of HSP 90. The group with NPrCAP/M plus AMF exposure showed the most significant growth inhibition of the re-challenged melanoma growth which resulted in the almost complete survival of the host animals as long as for 3 months that we have conducted our experimental protocol.

### Immune Process in Induction of *In situ* Vaccination by CTI Therapy

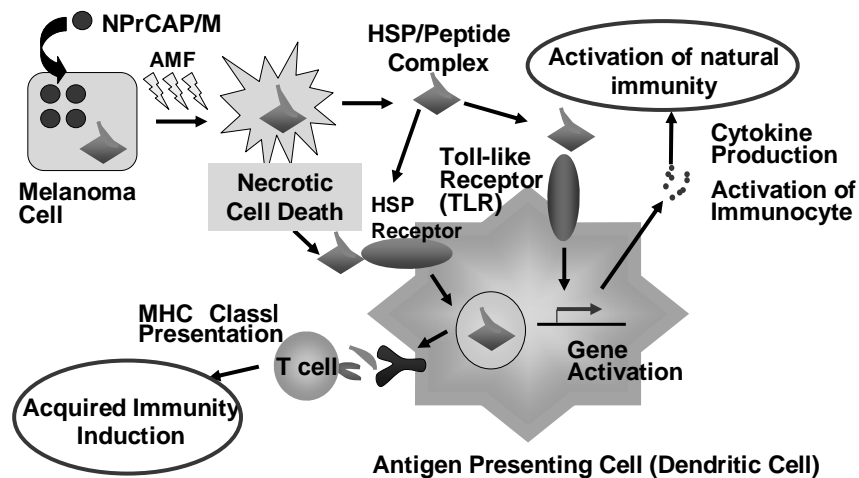


Fig. (13). CTI therapy causes the degradation of melanoma cells which results in the release of HSP/melanoma antigen complex that is taken up by antigen-presenting dendritic cells through HSP receptor.

It is, however, important to note that those animals bearing B16F1, B16F10 and B16OVA melanoma cells showed not only significant rejection of second re-challenge melanoma transplantation by administration of both NPrCAP alone and NPrCAP/M minus AMF exposure but also apoptotic or apoptotic cell death which was associated with immune complex production of HSP90 and melanoma peptide [44]. When NPrCAP was given systemically *i.p.* to black C57BL/6 mice, it caused depigmentation of black hair follicles which was found to be derived from selective apoptotic disintegration of follicular melanocytes [47]. Melanin intermediates produce reactive oxygen species such as superoxide and H<sub>2</sub>O<sub>2</sub> [5, 47, 48]. This unique biological property of melanin intermediates not only causes cell death, but also may produce immunogenic properties. The molecular interaction between NPrCAP chemo-immunotherapeutic and magnetite/AMF thermo-immunotherapeutic properties needs to be further studied.

### SUMMARY AND PERSPECTIVES

In this communication, we are able to show that:

1. NPrCAP with conjugation of magnetite nanoparticles, NPrCAP/M, with/without AMF exposure can induce cytotoxic T cells that inhibit the growth of re-challenged melanoma transplanted at the opposite site of body;
2. NPrCAP alone appears to generate both chemotherapeutic and immunotherapeutic property to B16melanoma cells through both apoptotic and non-apoptotic processes respectively;
3. Melanogenesis cascade can be utilized as the basis for developing melanoma-targeted DDS and chemo-thermo-immunotherapy agents.

Based upon these animal experiments, a preliminary human clinical trial has been carried out by employing NPrCAP/PEG/M plus AMF after we received the approval of our human clinical trials for a limited number of stage III and IV melanoma patients (Clinical Trial Research No. 18-67, Sapporo Medical University). The therapeutic protocol followed the basically identical experimental schedule as that of animal experiments. In this clinical trials, however, we utilized NPrCAP/PEG/M which was made by conjugating polyethylene glycol between NPrCAP and magnetite nanoparticles, (Fig. 5). Among four patients two of them showed complete and partial responses to our treatment and have been able to carry out normal daily activities after CTI therapy. In one patient, for example, four distant cutaneous metastasis sites were evaluated and either significant regression or shrinkage of all of these four melanoma lesions was seen. The patient was able to survive 30 months after several trials of CTI therapy. The pathological and immunological specimens revealed dense aggregation of lymphocytes and macrophages at the site of CTI therapy. Importantly there was a trend to have an almost identical distribution of CD8<sup>+</sup> T cells and MHC class I positive cells. Another patient had many lymph node metastases, but still has been surviving more than 32 months. In order to evaluate the overall therapeutic effect to advanced melanoma, it is important to have larger-scaled clinical trials and define concisely the molecular interaction between chemothera-

peutic and thermo-immunotherapeutic effect in our CTI therapy.

### ACKNOWLEDGEMENTS

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### ABBREVIATIONS

DDS	= drug delivery system
HSP	= heat shock protein
AMF	= alternating magnetic field
NPrCAP/M	= N-propionyl 4S cysteaminyphenol/magnetite nanoparticle
NPrCAP	= N-propionyl 4S cysteaminyphenol
CTI therapy	= Chemo-thermo-immunotherapy
MSH	= melanocyte stimulating hormone
MITF	= microphthalmia transcription factor
MC1R	= melanocortin 1 receptor
NACAP	= N-acetyl 4S cysteaminyphenol
BSO	= buthionine sulfoxide
PEG	= polyethylene glycol
NPrCAP/PEG/M	= N-propionyl 4-S cysteaminyphenol/polyethylene glycol/magnetite nanoparticle
ML	= non-cationic magneto-liposome
CML	= cationic magneto-liposome

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