Melanogenesis Exploitation and Melanoma Nanomedecine: 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 cysteaminylphenol (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 $CD8^+$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 thermotherapy.

Keywords: Melanoma, chemothermoimmunotherapy, chemotherapy, immunotherapy, thermotherapy, 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*-cysteaminylphenol (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_3O_4) 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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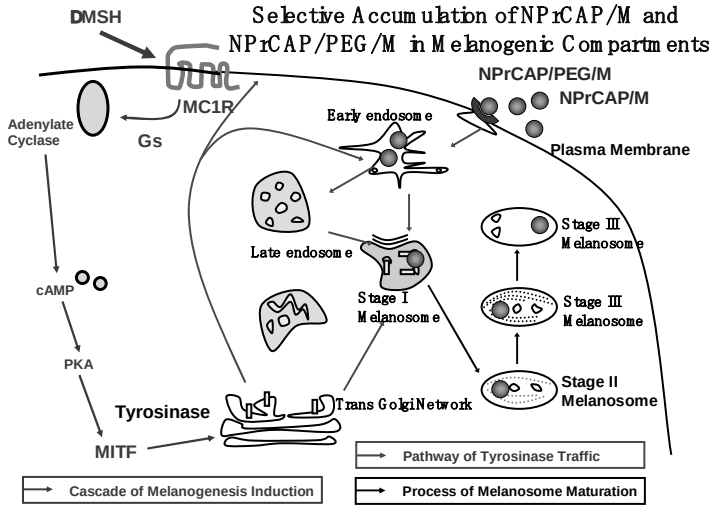

Fig. (6). NPrCAP/magnetite complexes (NPrCAP/M and NPrCAP/PEG:polyethylene glycol/M are selectively incorporated into melanoma cells probably through active transport on the cell membrane and accumulate in endosomes, i.e., precursors of melanosomes.

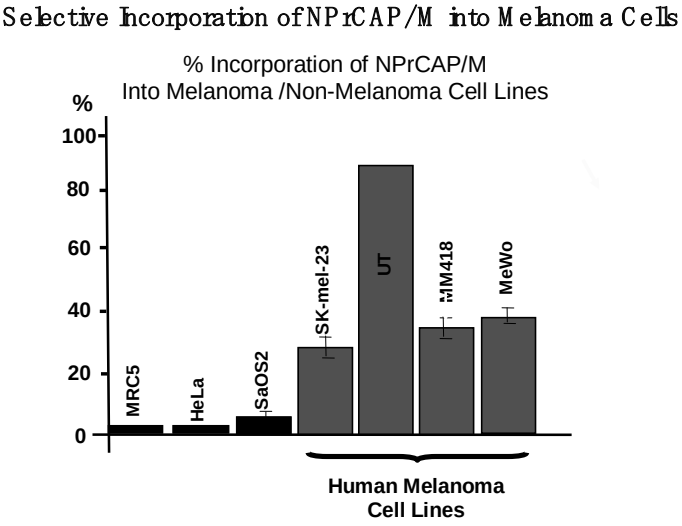

Fig. (7). NPrCAP/magnetite nanoparticles are selectively incorporated into human melanoma cells compared to non-melanocytic cells.

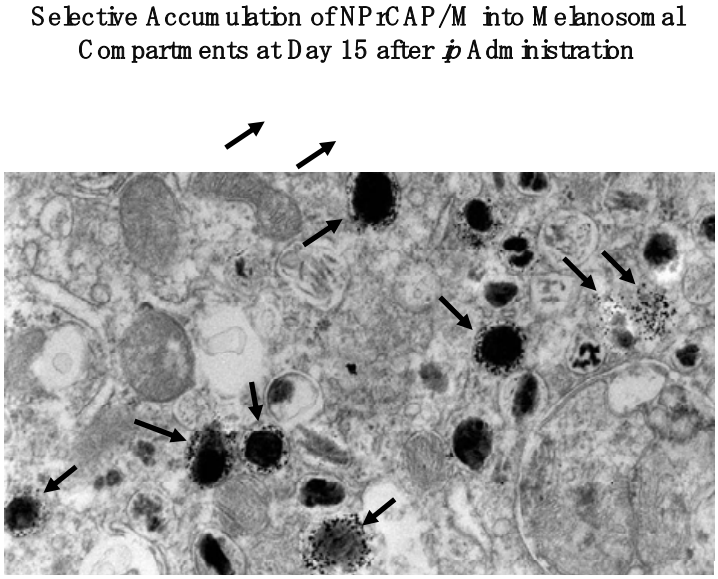

Fig. (8). Arrows indicate magnetite particles incorporated into melanosomes.

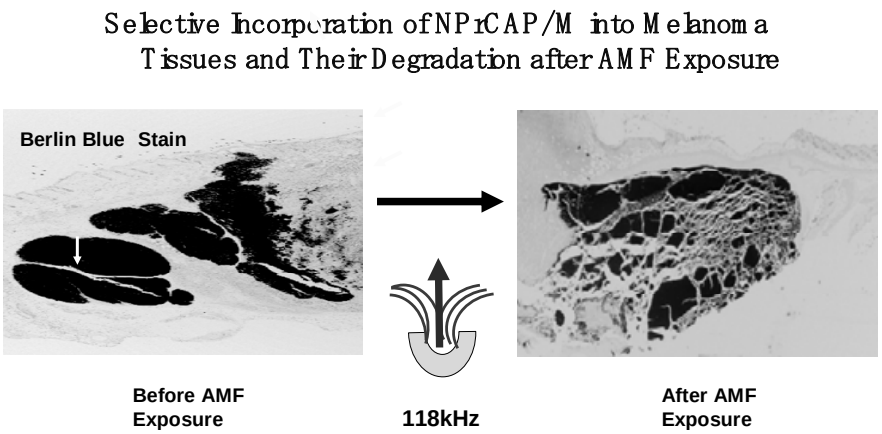

Fig. (9). NPrCAP/magnetite nanoparticles are accumulated in melanoma tissues and then degraded upon exposure to AMF.

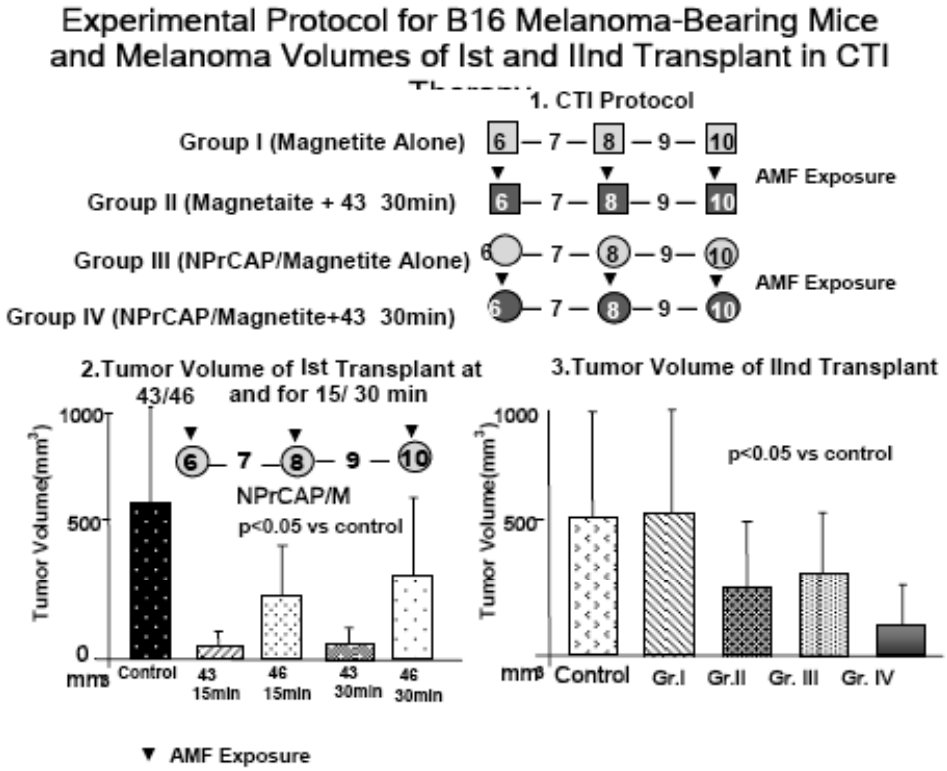

Fig. (10). The most effective thermo-immunotherapy for the growth inhibition of re-challenge melanoma transplant is achieved by the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma.

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 $CD4^+$ and $CD8^+$ T cell infiltration into the re-challenge

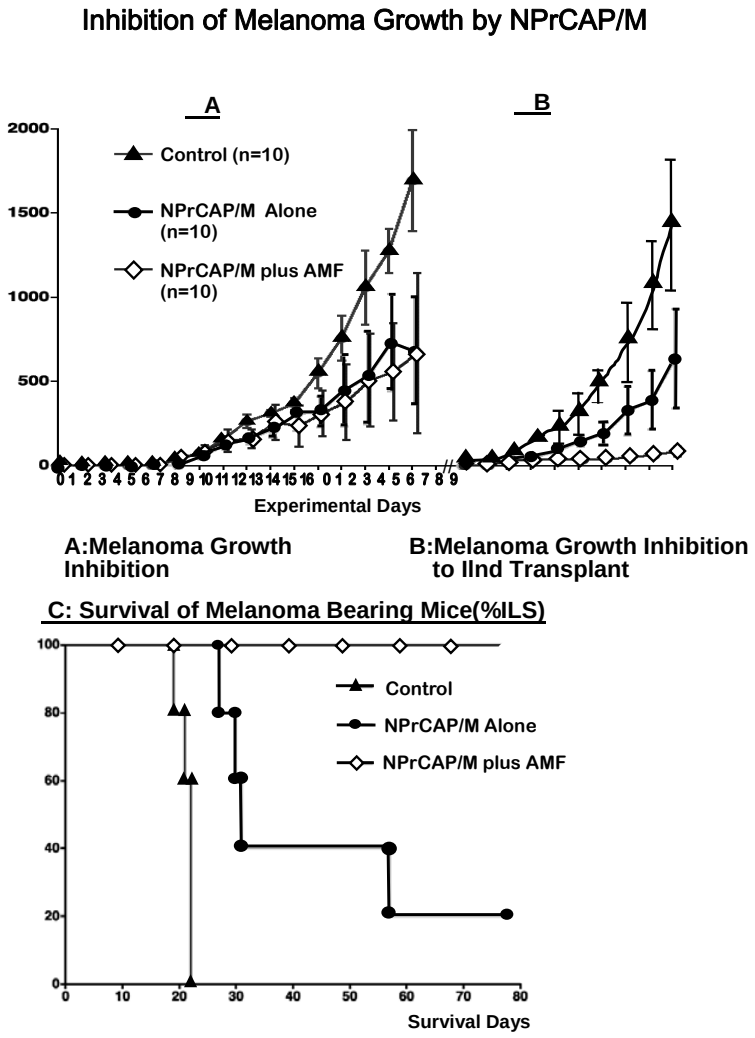

Fig. (11). NPrCAP/M with AMF exposure shows the most significant growth inhibition of re-challenge melanoma transplant and increased the life span of the hosts (ILS).

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 thermotherapy 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 thermotherapy 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° 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

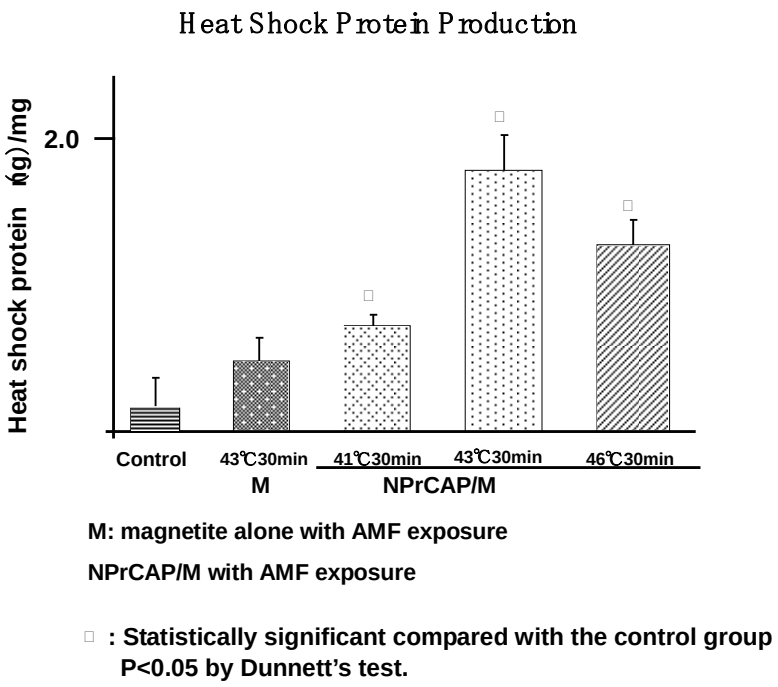

Fig. (12). NPrCAP/M with AMF exposure causes the significant production of HSP70.

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 cyototoxic 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.

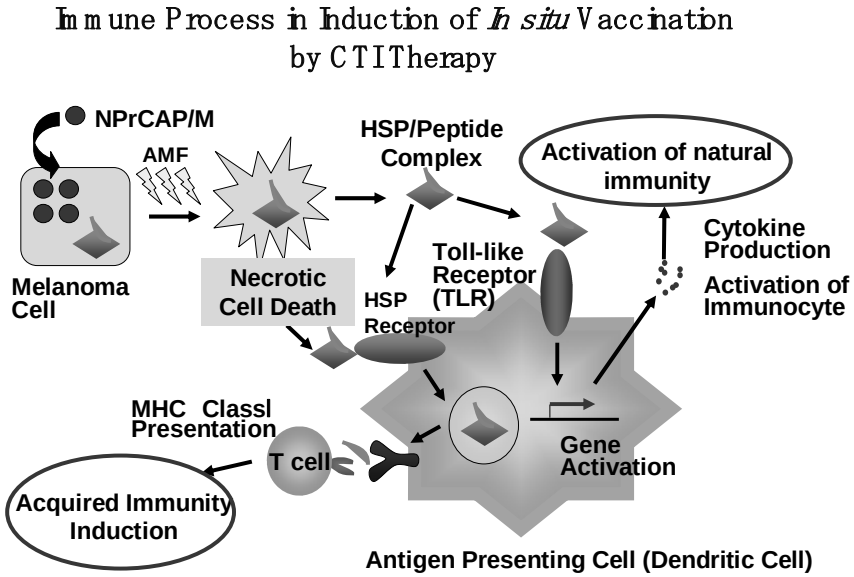

Fig. (13). CTI therapy causes the degradation of melanoma cells which results in the release of HSP/melanoma antigen complex that is taken up by antigen-presenting dendritic cells through HSP receptor.

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_2O_2 [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 B 16melanoma 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 $CD8^+$ T cells and MHC class 1 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 chemotherapeutic and thermo-immunotherapeutic effect in our CTI therapy.

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ABBREVIATIONS

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

REFERENCES

[1] Balch, C.M.; Buzaid, A.C.; Soong, S.J.; Atkins, M.B.; Cascinelli, N.; Coit, D.G.; Fleming, I.D.; Gershenwald, J.E.; Houghton, A. Jr.; bKirkwood, J.M.; McMasters, K.M.; Mihm, M.F.; Morton, D.L.; Reintgen, D.S.; Ross, M.I.; Sober, A.; Thompson, J.A.; Thompson, J.F. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J. Clin. Oncol.* **2001**, *19*, 3635-48.

[2] Jimbow, K.; Iwashina, T.; Alena, F.; Yamada, K.; Pankovich, J.; Umemura, T. Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma. *J. Invest. Dermatol., **1993**, 100*(Suppl. 2), pp、231S-8S.

[3] Alena, F.; Ishikawa, T.; Gili, A.; Jimbow, K. Selective in vivo accumulation of N-acetyl-4-S-cysteaminylphenol in B16F10 murine melanoma and enhancement of its *in vitro* and *in vivo* antimelanoma effect by combination of buthionine sulfoximine. *Cancer Res.*, **1994**, *54*, 2661-6.

[4] Pankovich, J.M.; Jimbow, K. Tyrosine transport in a human melanoma cell line as a basis for selective transport of cytotoxic analogues. *Biochemistry*, **1991**, *15*(28), 721-5.

[5] Reszka, K.; Jimbow, K. In: *Electron donor and acceptor properties of melanin pigments in the skin*. Fuchs J, Packer L. Oxidative Stress in Dermatology. Marcel Dekker, Inc. New York, 1993; pp.287-320.

[6] Tandon, M.; Thomas, P.D.; Shokravi, M.; Singh, S.; Samra, S.; Chang, D.; Jimbow, K. Synthesis and antitumour effect of the melanogenesis-based antimelanoma agent N-propionyl-4-S-cysteaminylphenol. *Biochem. Pharmacol.*, **1998**, *15*, 2023-9.

[7] Thomas P.D.; Kishi, H.; Cao, H.; Ota, M.; Yamashita, T.; Singh, S.; Jimbow, K. Selective incorporation and specific cytocidal effect as the cellular basis for the antimelanoma action of sulphur containing tyrosine analogs. *J. Invest. Dermatol.*, **1999**, *113*, 928-34.

[8] Ito, A.; Shinkai, M.; Honda, H.; Kobayashi, T. Medical application of functionalized magnetic nanoparticles. *J. Biosci. Bioeng*., **2005**, *100*, 1-11.

[9] Kawai, N.; Ito, A.; Nakahara, Y.; Futakuchi, M.; Shirai, T.; Honda, H.; Kobayashi, T.; Kohri, K. Anticancer effect of hyperthermia on prostate cancer mediated by magnetite cationic liposomes and immune-response induction in transplanted syngeneic rats. *Prostate* **2005**, *64*,373-81.

[10] Yanase, M.; Shinkai, M.; Honda, H.; Wakabayashi, T.; Yoshida, J.; Kobayashi, T. Antitumor immunity induction by intracellular hyperthermia using magnetite cationic liposomes. *Jpn. J. Cancer Res.*, **1998**, *89*, 775-82.

[11] Shinkai, M.; Yanase, M.; Honda, H.; Wakabayashi, T.; Yoshida, J.; Kobayashi, T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: in vitro study. *Jpn. J. Cancer Res.*, **1996**, *87*, 1179-83.

[12] Ménoret, A.; Chandawarkar, R. Heat-shock protein-based anticancer immunotherapy: an idea whose time has come. *Semin. Oncol.*, **1998**, *25*, 654-60

[13] Srivastava, P.K.; Ménoret, A.; Basu, S.; Binder, R.; Quade K. Heat shock proteins come of age: primitive functions acquired new roles in an adaptive world. *Immunity* **1998**, *8*, 657–65.

[14] Tamura, Y.; Tsuboi, N.; Sato, N.; Kikuchi, K. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J. Immunol.*, **1993**, *51*, 5516-24.

[15] Tamura, Y.; Peng, P.; Liu, K.; Daou, M.; Srivastava, P.K. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* **1997**, *278*, 117-20.

[16] Tamura, Y.; Sato, N. Heat shock proteins: chaperoning of innate and adaptive immunities. *Jpn. J. Hyperthermic Oncol.*, **2003**, *19*, 131-9.

[17] Srivastava, P.K. Immunotherapy for human cancer using heat shock protein-peptide complexes. *Curr. Oncol. Rep.*, **2005**, *7*, 104–8.

[18] Tamura, Y.; Takashima, S.; Cho, J.M.; Qi, W.; Kamiguchi, K.; Torigoe, T.; Takahashi, S.; Hirai, I.; Sato, N.; Kikuchi, K. Inhibition of natural killer cell cytotoxicity by cell growth-related molecules. *Jpn. J. Cancer Res.*, **1996**, *87*, 623-30.

[19] Ueda, G.; Tamura, Y.; Hirai, I.; Kamiguchi, K.; Ichimiya, S.; Torigoe, T.; Hiratsuka, H.; Sunakawa, H.; Sato, N. Tumor-derived heat shock protein 70-pulsed dendritic cells elicit tumor-specific cytotoxic T lymphocytes (CTLs) and tumor immunity. *Cancer Sci.*, **2004**, *9*, 248-53.

[20] Ito, A.; Honda, H.; Kobayashi, T. Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novel concept of "heat-controlled necrosis" with heat shock protein expression. *Cancer Immunol. Immunother.*, **2006**, *55*, 320-8.

[21] Shi, H.; Cao, T.; Connolly, J.E.; Monnet, L.; Bennett, L.; Chapel, S.; Bagnis, C.; Mannoni, P.; Davoust, J.; Palucka, A.K.; Banchereau, J. Hyperthermia enhances CTL cross-priming. *J. Immunol.*, **2006**, *176*, 2134-41.

[22] Dakour, J.; Vinayagamoorthy, M.; Chen, H.; Luo, D.; Dixon, W.; Jimbow, K. Identification of A cDNA for Ca^{++}-binding Calnexin-like phosphoprotein (p90) on melanosomes in normal and malignant human melanocytes. *Exp. Cell Res.*, **1993**, *209*, 288-300.

[23] Toyofuku, K.; Wada, I.; Hirosaki, K.; Park, J.S.; Hori, Y.; Jimbow, K. Involvement of calnexin in maturation of tyrosinase as a molecular chaperone. *J. Biochem.*, **1999**, *125*, 82-9.

[24] Jimbow, K.; Gomez, P.F.; Toyofuku, K.; Chang, D.; Miura, S.; Tsujiya, H.; Park, J.S. Biological role of tyrosinase related protein and its biosynthesis and transport from TGN to stage I melanosome, late endosome, through gene transfection study. *Pigment Cell Res.*, **1997**, *10*, 206-13.

[25] Jimbow, K.; Park, J.S.; Kato, F.; Hirosaki, K.; Toyofuku, K.; Hua, C.; Yamashita, T. Assembly, target signal and intracellular transport of tyrosinase gene family protein in the initial stage of melanosome biogenesis. *Pigment Cell Res.*, **2000**, *13*, 222-9.

[26] Jimbow, K.; Hua, C.; Gomez, P.F.; Hirosaki, K.; Shinoda, K.; Salopek, T.G.; Matsusaka, H.; Jin, H.Y.; Yamashita, T. Intracellular vesicular trafficking of tyrosinase gene family protein in eu- and pheomelanosome biogenesis. *Pigment Cell Res.*, **2000**, *13* (Suppl. 8), 110-7.

[27] Miura, T.; Jimbow, K.; Ito, S. The *in vivo* antimelanoma effect of 4-S-cysteaminylphenol and its n-acetyl derivative. *Int. J. Cancer* **1990**, *46*, 931-4.

[28] Tandon, M.; Thomas, P.D.; Shokravi, M.; Singh, S.; Samra, S.; Chang, D.; Jimbow, K. Synthesis of the melanogenesis-based antimelanoma agent, N-propionyl-4-S-cysteaminylphenol, and screening of depigmenting and anti-tumour effects. *Biol. Pharmacol.*, **1998**, *55*, 2023-9.

[29] Ito, S.; Kato, T.; Ishikawa, K.; Kasuga, T.; Jimbow, K. Mechanism of selective toxicity of 4-S-cysteinylphenol and 4-S-cysteaminylphenol to melanocytes. *Biochem. Pharmacol.*, **1987**, *36*, 2007-11.

[30] Gili, A.; Thomas, P.D.; Ota, M.; Jimbow, K. Comparison of in vitro cytotoxicity of N-acetyl and N-propionyl derivatives fo phenolic thioether amines in melanoma and neuroblastoma cells and the relationship to tyrosinase and tyrosine hydroxylase enzyme activity. *Melanoma Res.*, **2000**, *10*, 9-15.

[31] Parsons, P.G.; Favier, F.; McEwan, M.; Takahashi, T.; Jimbow, K.; Ito, S. Action of cysteaminylphenols on human melanoma cells *in vivo* and *in vitro*: 4-S-cysteaminylphenol binds protein disulphide isomerase. *Melanoma Res.*, **1992**, *1*, 97-104.

[32] van Landeghem, F.K.; Maier-Hauff, K.; Jordan, A.; Hoffmann, K.T.; Gneveckow, U.; Scholz, R.; Thiesen, B.; Brück, W.; von Deimling, A. Post-mortem studies in glioblastoma patients treated with thermotherapy using magnetic nanoparticles. *Biomaterials* **2009**, *30*, 52-7.

[33] Thiesen, B.; Jordan, A. Clinical applications of magnetic nanoparticles for hyperthermia. *Int. J. Hyperthermia* **2008**, *24*, 467-74.

[34] Johannsen, M.; Gneveckow, U.; Eckelt, L.; Feussner, A.; Waldöfner, N.; Scholz, R.; Deger, S.; Wust, P.; Loening, S.A.; Jordan, A. Clinical hyperthermia of prostate cancer using magnetic nanoparticles: presentation of a new interstitial technique. *Int J. Hyperthermia*, **2005**, *21*, 637-47.

[35] Ito, A.; Fujioka, M.; Yoshida, T.; Wakamatsu, K.; Ito, S.; Yamashita, T.; Jimbow, K.; Honda, H. 4-S-cysteaminylphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma. *Cancer Sci*, **2007**, *98*, 424-30.

[36] Takada, T.; Yamashita, T.; Sato, M.; Sato, A.; Ono, I.; Tamura, Y.; Sato, N.; Miyamoto, A.; Ito, A.; Honda, H.; Wakamatsu, K.; Ito, S.; Jimbow, K. Growth inhibition of re-challenge B16 melanoma transplant by conjugates of melanogenesis substrate and magnetite nanoparticles as the basis for developing melanoma-targeted chemo-thermo-immunotherapy. *J. Biomed. Biotechnol.*, **2010**, *2009*, 457936.

[37] Sato, M.; Yamashita, T.; Ohkura, M.; Osai, Y.; Sato, A.; Takada, T.; Matsusaka, H.; Ono, I.; Tamura, Y.; Sato, N.; Sasaki, Y.; Ito, A.; Honda, H.; Wakamatsu, K.; Ito, S. Jimbow, K. N-Propionyl-Cysteaminylphenol-Magnetite Conjugate (NPrCAP/M) Is a Nanoparticle for the Targeted Growth Suppression of Melanoma Cells. *J. Invest. Dermatol.*, **2009**, *129*, 2233-41.

[38] Lindquist, S. The heat-shock response. *Ann. Rev. Biochem.*, **1986**, *55*, 1151-91.

[39] Konno, A.; Sato, N.; Yagihashi, A.; Torigoe, T.; Cho, J.M.; Torimoto, K.; Hara, I.; Wada, Y.; Okubo, M.; Takahashi, N.; Kikuchi, K. Heat- or stress-inducible transformation-associated cell surface antigen on the activated H-ras oncogene-transfected rat fibroblast. *Cancer Res*, **1989**, *49*, 6578-82.

[40] Suzuki, M.; Shinkai, M.; Honda, H.; Kobayashi, T. Anticancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes. *Melanoma Res.*, **2003**, *13*, 129-35.

[41] Ito, A.; Shinkai, M.; Honda, H.; Wakabayashi, T.; Yoshida, J.; Kobayashi, T. Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia. *Cancer Immunol. Immunother.*, **2001**, *50*, 515-22.

[42] Ito, A.; Shinkai, M.; Honda, H.; Yoshikawa, K.; Saga, S.; Wakabayashi, T.; Yoshida, J.; Kobayashi, T. Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles. *Cancer Immunol Immunother*, **2003**, *52*, 80-8.

[43] Mise, K.; Kan, N.; Okino, T.; Nakanishi, M.; Satoh, K.; Teramura, Y.; Yamasaki, S.; Ohgaki, K.; Tobe, T. Effect of heat treatment on tumor cells and antitumor effector cells. *Cancer Res.*, **1990**, *50*, 6199-202.

[44] Ito, A.; Matsuoka, F.; Honda, H.; Kobayashi, T. Heat shock protein 70 gene therapy combined with hyperthermia using magnetic nanoparticles. *Cancer Gene Ther.*, **2003**, *10*, 918-25.

[45] Yanase, M.; Shinkai, M.; Honda, H.; Wakabayashi, T.; Yoshida, J.; Kobayashi, T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: an in vivo study. *Jpn J. Cancer Res.*, **1998**, *89*, 463-9.

[46] Osai, Y.; Ohkura, M.; Tamura, Y.; Sato, N.; Ito, A.; Honda, H.; Wakamatsu, K.; Ito, S.; Yamashita, T.; Jimbow, K. Intratumoral administration of melanoma targeting N-propionyl cysteaminylphenol induces in vivo anti-melanoma effect and tumor specific immunity. *Pigment Cell Melanoma Res.*, **2008**, *21*, 329 (Abstract).

[47] Minamitsuji, Y.; Toyofuku, K.; Sugiyama, S.; Jimbow, K. Sulphur containing tyrosinase analogs can cause selective melanocytoxicity involving tyrosinase-mediated apoptosis. *J. Invest. Dermatol.*, **1999**, *4*, 130S-6S.

[48] Jimbow, K.; Miyake, Y.; Homma, K.; Yasuda, K.; Izumi, Y.; Tsutsumi, A.; Ito, S. Characterization of melanogenesis and morphogenesis of melanosomes by physicochemical properties of melanin and melanosomes in malignant melanoma. *Cancer Res.*, **1984**, *44*, 1128-34.

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