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Review

Immunomodulation of melanoma by chemo-thermo-immuno-therapy using conjugates of melanogenesis substrate NPrCAP and magnetite nanoparticles

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Abstract: A major advance of drug discovery and targeted therapy directed to cancer cells may be achieved by exploitation and immunomodulation of their unique biological property. This review summarizes our efforts to develop novel chemo-thermo-immuno-therapy (CTI therapy) by conjugating a melanogenesis substrate, N-propionyl cysteaminylphenol (NPrCAP: amine analog of tyrosine), with magnetite nanoparticles (MNP). In our approach, NPrCAP provides a unique drug delivery system (DDS) because of its selective incorporation into melanoma cells. It also functions as a melanoma-targeted therapeutic drug because of its production of highly reactive free radicals (melanoma-targeted chemotherapy). Moreover, utilization of MNP is a platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to an alternating magnetic field (AMF). The feasibility of our approach was successfully shown in experimental *in vivo* and *in vitro* mouse melanoma models and in preliminary clinical trials to a limited number of advanced melanoma patients.

Keywords: Melanoma; Chemo-thermo-immuno-therapy; Melanogenesis; Magnetite nanoparticle; Drug delivery system; Heat shock protein; *In situ* vaccine therapy; Immune checkpoint inhibitor

1. Introduction

Management of advanced metastatic melanoma is an extremely difficult challenge for physicians and scientists because of the limited availability of effective therapies. There is, therefore, a critical need to develop innovative therapies for the control of

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advanced melanoma. Exploitation of biological properties unique to cancer cells may provide a novel approach to overcome this difficult challenge.

The biological property unique to melanoma cells and their precursor cells, melanocytes, is the biosynthesis of melanin pigments, melanogenesis, within specific cellular compartments called 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 melanocytes and melanoma cells, including "amelanotic" melanoma [1]. With the interaction of melanocyte-stimulating hormone (MSH) and the melanocortin 1 receptor (MC1R) [2], the melanogenesis cascade proceeds with 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. Vesicular transport then carries tyrosinase and its related proteins (TRPs) from the trans-Golgi network to melanosomal compartments. A significant number of transporters in this process are involved in early melanosomal maturation, to which early and late endosomes are closely associated [3]. Once melanin biosynthesis is completed, melanosomes move along dendritic processes on melanocytes and are transferred to surrounding keratinocytes in skin. However, melanoma cells do not develop many dendrites and retain melanosomes within their cytoplasm, hence forming a "black mole" [4].

Tyrosine analogs that are the substrates of the melanin-forming enzyme tyrosinase can be some of the best candidates for developing specific melanoma targeting drugs and therapies. *N*-acetyl and *N*-propionyl derivatives of 4-*S*-cysteaminylphenol (NAc- and NPr-CAP) were synthesized and found to be much better substrates of tyrosinase than tyrosine (Figure 1) [5].

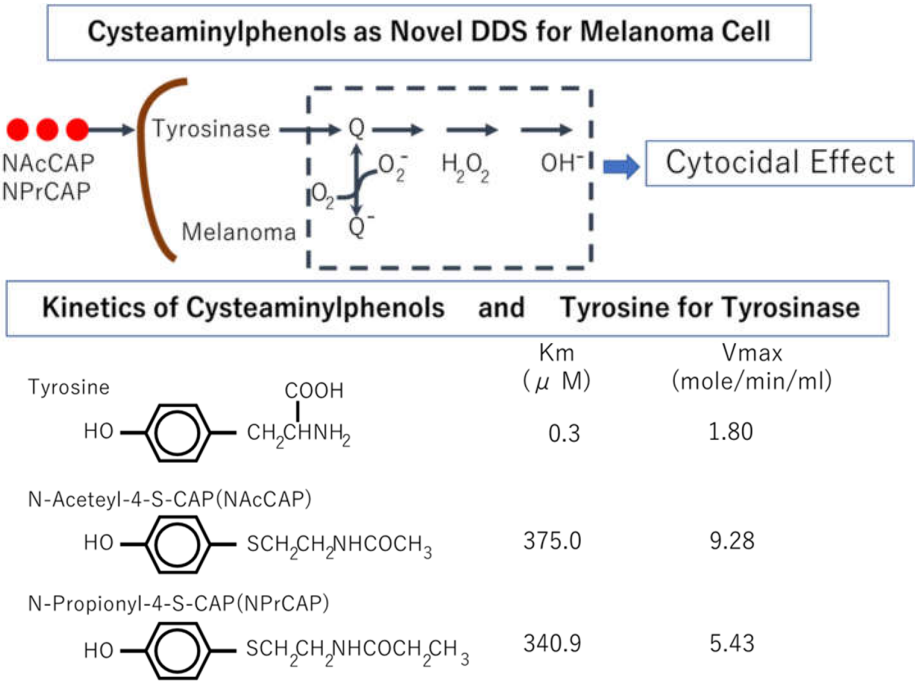

Figure 1. Comparison of kinetics of tyrosine, NAcCAP and NPrCAP for mushroom tyrosinase. NAcCAP and NPrCAP produce a significant amount of cytocidal free radicals through the interaction with tyrosinase.

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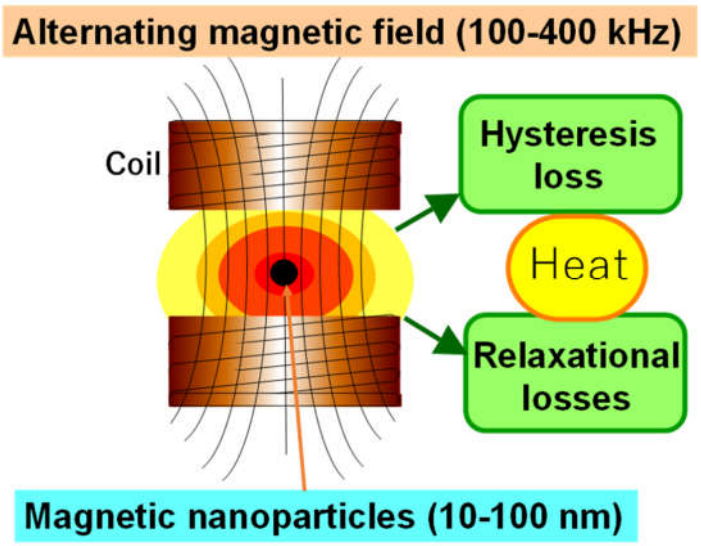

Figure 2. Mechanism of heat generation of magnetic nanoparticles under an alternating magnetic field.

Intracellular hyperthermia using magnetite nanoparticles (MNP) (10-100nm-sized, Fe_3O_4) has been shown to be effective for treating cancers [6,7]. Incorporated MNP generate heat within the cells after exposure to an alternating magnetic field (AMF), due to hysteresis loss [8-11] (Figure 2). In this treatment, there is not only heat-mediated cell death but also an immune reaction due to the generation of heat shock proteins (HSPs) [12]. HSP expression induced by hyperthermia has been found to be involved in cancer immunomodulation, providing the basis for developing a novel cancer thermo-immunotherapy.

In this report we compared chemo-therapeutic and thermo-therapeutic effects on primary transplanted B16 mouse melanoma cells on the flank with and without AMF exposure (heat generation) and then examined the immune-therapeutic effect on a second, re-challenge transplant of the same melanoma cells on the opposite side flank to evaluate if the growth of distant metastatic melanomas can be inhibited. We also investigated the possible association of HSP production, $CD8^+$ T cell activation and MHC expression with rejection of the re-challenge melanoma transplants. We also introduce our preliminary therapeutic effort of this CTI strategy for a limited number of advanced melanoma patients.

2. Synthesis of novel conjugates of NPrCAP and magnetite nanoparticles for developing melanoma-targeted chemo-thermo-immuno-therapy (CTI therapy)

2.1. Preparation of 4-S-CAP-loaded magnetite cationic liposomes and measurement of in vitro/in vivo antimelanoma effects; Starting rational basis for developing CTI therapy

CTI therapy is based on the combination of chemotherapy using melanogenesis substrates and hyperthermia using magnetic nanoparticles, and the resulting antitumor immune response induced by *in situ* vaccination through dying tumor cells. Analogs of the melanogenesis substrate tyrosine are good candidates, such as the sulfur homolog of tyrosine 4-*S*-cysteaminylphenol (4-*S*-CAP), which causes cytotoxicity of melanoma [13,14] and can be used for melanoma-targeted chemotherapy. In addition, intracellular hyperthermia can be generated by loading magnetic nanoparticles into tumor cells followed by inductive heating of the nanoparticles under an alternating magnetic field

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(AMF). A promising approach of the initial step to improve uptake of nanoparticles into tumor cells was to use cationic liposomes. We developed magnetite cationic liposomes (MCLs) that have 10-fold higher affinity for tumor cells than neutrally charged magnetite liposomes [15]. To test the combined effects of chemotherapy using 4-*S*-CAP and hyperthermia using MNP on melanoma, we prepared 4-*S*-CAP-loaded magnetite cationic liposomes (4-*S*-CAP/MCLs) (Figure 3) [16].

An *in vitro* experiment showed that 4-*S*-CAP in 4-*S*-CAP/MCLs had a dose-de-

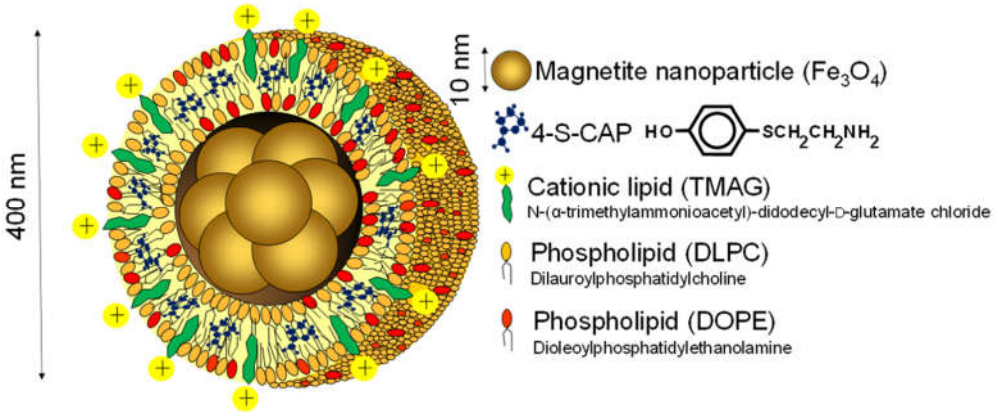

Figure 3. Illustration of 4-S-cysteaminylphenol (4-S-CAP)/magnetite cationic liposome (MCL).

pendent antiproliferative effect on B16 melanoma cells, and the combination treatment of 4-*S*-CAP with hyperthermia was determined to have an additive effect [16]. As a mechanism, the cytotoxicity of 4-*S*-CAP in melanoma cells depends mostly on its production of reactive oxygen species (ROS) [17]. Hyperthermia also induces ROS in various cells [18], and ROS may play an important role in the additive effect of the combined treatment of 4-*S*-CAP and hyperthermia on melanoma cells. Moreover, 4-*S*-CAP/MCLs were injected into melanoma nodules in mice and the mice were irradiated with an AMF for 30 min. During AMF irradiation, the temperature of the melanoma nodules increased to 45°C and tumor growth was strongly suppressed for 12 days, including complete regression of 17% (1/6) of melanoma nodules [16]. These results suggest that melanogenesis substrate-conjugated magnetic nanoparticles are a potent tool for melanoma therapy.

2.2. *New synthetic method of NPrCAP-SH for CTI therapy*

A synthetic route for *N*-(1-mercaptopropionyl)-4-*S*-cysteaminyl phenol (NPrCAP-SH) has been reported already [8]. However, there was room for improvement in the synthetic process of NPrCAP-SH, because (1) the reagent *N*-succinimidyl-3-[2-pyridyldithio] propionate, used in the process described in the previous paper [8], was

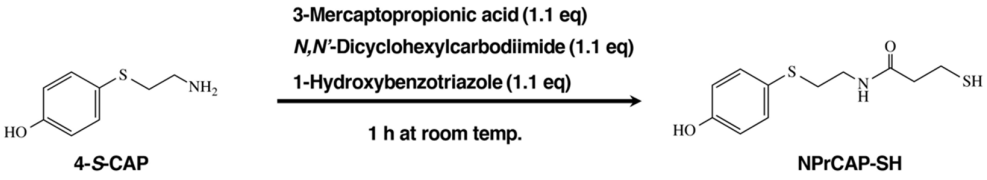

Figure 4. One-pot synthesis of NPrCAP-SH.

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very expensive, and (2) it was not easy to separate NPrCAP-SH from the by-products yielded in that synthetic process. Thus, it was necessary to develop a new synthetic method that shortens the reaction time, uses less expensive reagents and can generate NPrCAP-SH with larger quantities and higher purity.

4-*S*-cysteaminylphenol (4-*S*-CAP), obtained by hydrolyzing *N*-acetyl-4-*S*-CAP (NAcCAP) with 6M HCl by the method of Padgette et al. [19], was reacted with 3-mercaptopropionic acid, *N*,*N*′-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in *N*,*N*-dimethylformamide for 1 h at room temperature. The resultant oily compound was purified by silica gel column chromatography to give NPrCAP-SH (90%) as a colorless crystal after recrystallization. Thus, we have established an efficient and reproducible one-pot method for synthesis of NPrCAP-SH (Figure 4).

We synthesized four NPrCAP derivatives bound to MNP as shown in Figure 5 for CTI therapy, and we have already reported the synthetic methods for three of these derivatives (NPrCAP/MNP, NPrCAP/PEG/MNP and NPCMD) [8, 20-22]. In this review, we report a new method for the synthesis of NPrCAP/PEG/APTES/DNM, which is described below.

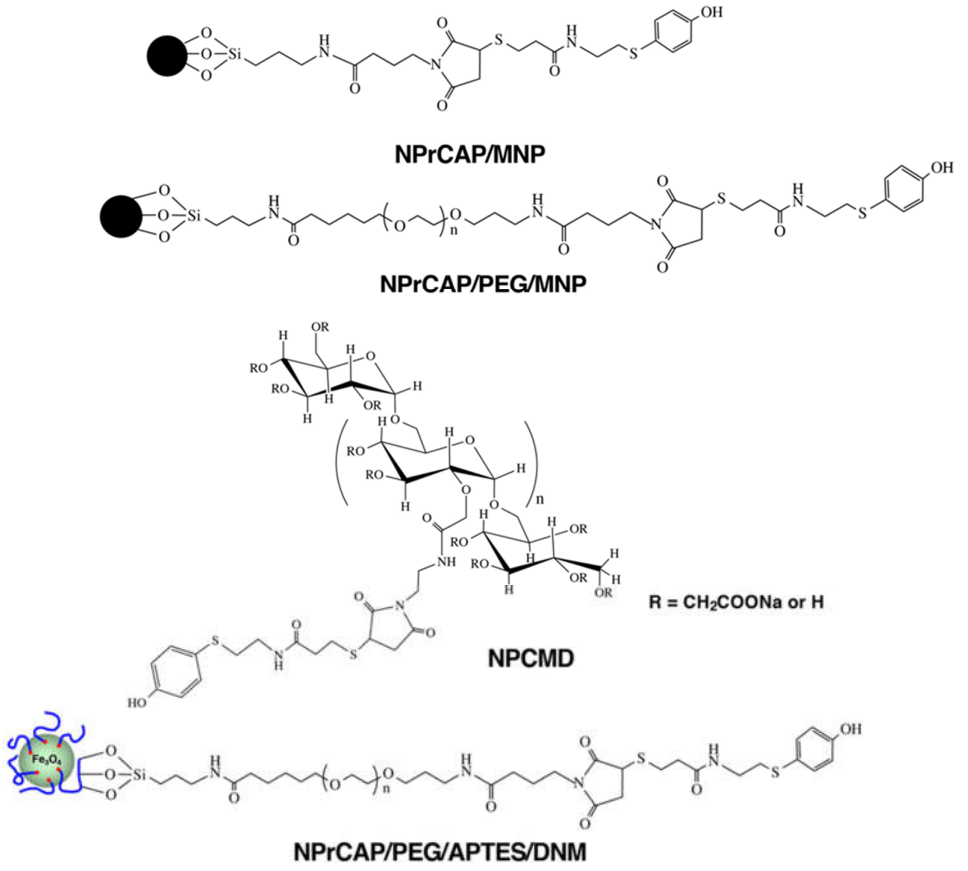

Figure 5. Various kinds of NPrCAP derivatives bound to MNP. NPrCAP/MNP: *N*-propionyl-4-*S*-cysteaminylphenol/magnetite nanoparticle. NPrCAP/PEG/MNP: *N*-propionyl-4-*S*-cysteaminylphenol/polyethylene glycol/magnetite nanoparticle. NPCMD: *N*-propionyl-4-*S*-cysteaminylphenol/maleimide-dextran. NPrCAP/PEG/APTES/DNM: *N*-propionyl-4-*S*-cysteaminylphenol/polyethylene glycol/3-aminopropyltriethoxysilane/dextran nanomagnetite.

2.3. *Synthesis of NPrCAP/PEG/APTES/DNM bound to dextran nanomagnetite*

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Dextran nanomagnetite (DNM) was prepared by adding dextran in water to a magnetite suspension. DNM thus prepared was first reacted with 3-aminopropyltriethoxysilane (APTES) to form APTES/DNM, and then with PEG-NPrCAP obtained by reacting NPrCAP-SH and PEG for 1 h at room temperature. The mixture was kept for 4 to 6 h at room temperature, and then kept in a refrigerator overnight to synthesize NPrCAP/PEG/APTES/DNM (Figure 6).

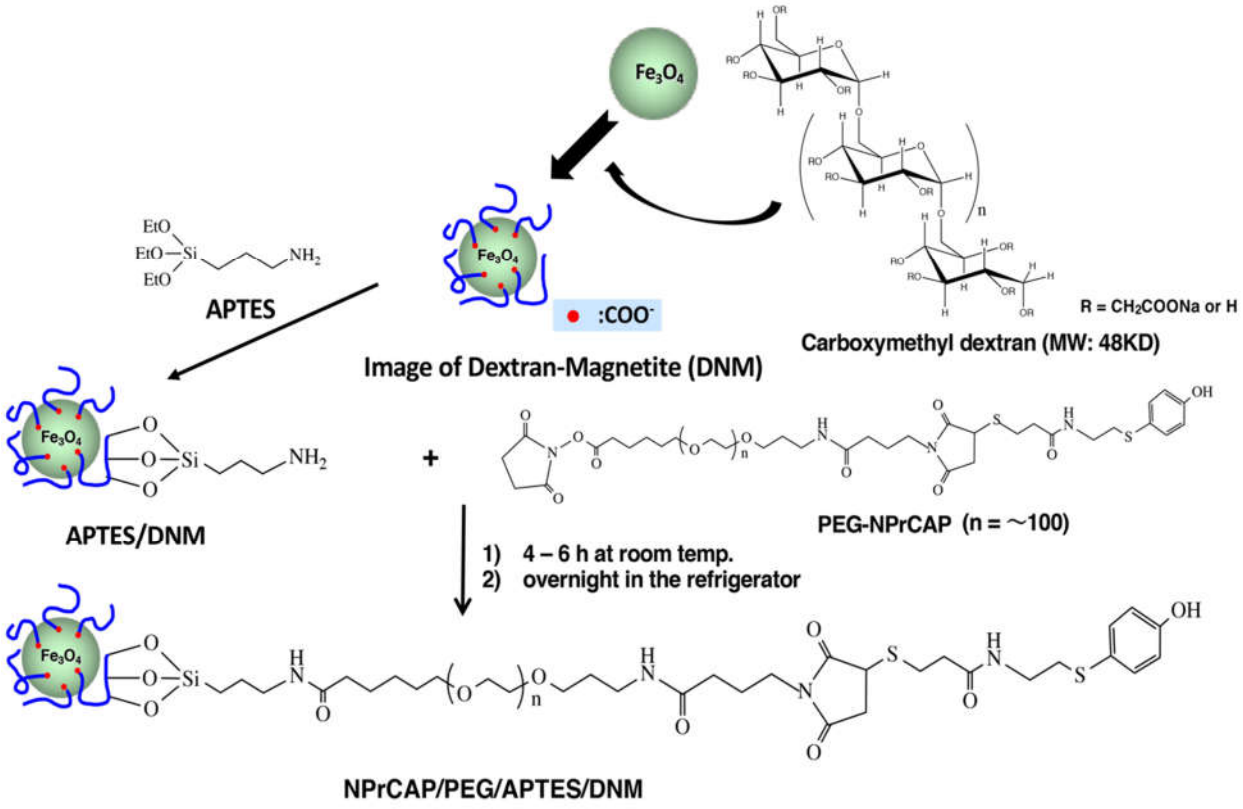

Figure 6. Image of DNM and synthesis of NPrCAP/PEG/APTES/DNM.

2.4. *Quantification of NPrCAP bound to DNM*

In order to olysis. On the other hand, the inclusion of 1% phenol in 6M HCl suppressed the decomposition of 4-*S*-CAP, which did not decompose even with 2 h reaction time. Based on the above results, the following experimental conditions to check the amount of NPrCAP bound to NPrCAP/PEG/APTES/DNM were established: 1) the NPrCAP/PEG/APTES/DNM suspension is reacted under 6M HCl containing 1% phenol for 1 h, and 2) the reaction mixture is diluted 10-fold with 0.1 M HCl and then 4-*S*-CAP is quantified by high-performance liquid chromatography (HPLC) analysis (Figure 7).

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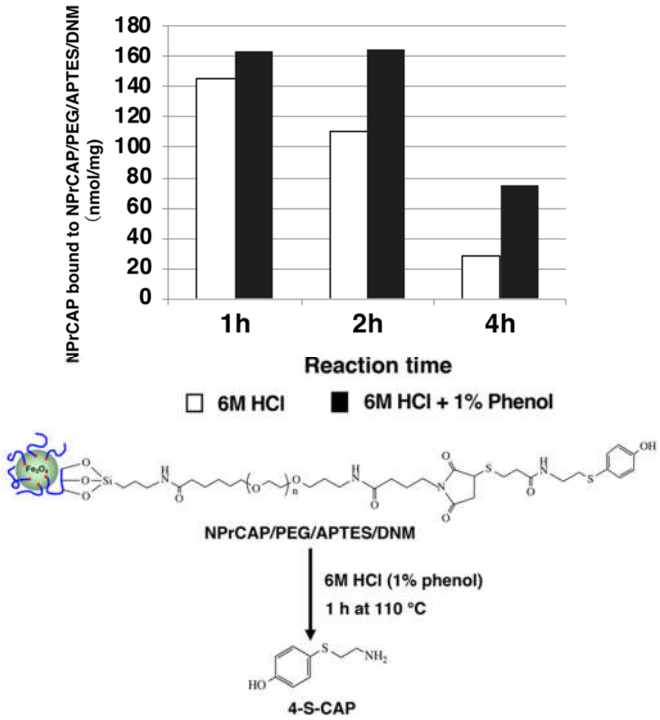

Figure 7. Quantification of NPrCAP bound to NPrCAP/PEG/APTES/DNM, and production of 4-*S*-CAP from NPrCAP/PEG/APTES/DNM by 6M HCl containing 1% phenol.

2.5. *The different reactivities of NPrCAP/MNP and NPrCAP/PEG/APTES/DNM as substrates for tyrosinase*

We examined whether NPrCAP/MNP and NPrCAP/PEG/APTES/DNM could act as substrates for tyrosinase. 4-*S*-CAP itself was found to be a good substrate for tyrosinase because tyrosinase oxidation of 4-*S*-CAP in the presence of cysteine yielded 5-*S*-cysteaminyl-3-*S*-cysteinylcatechol (CA-CysC) through *ortho*-quinone within 10 min (Figure 8a) [8,23,24]. HPLC analysis showed that the reaction was almost completed within 10 min, with half of the 4-*S*-CAP remaining after 4.2 min [8,23]. HPLC analysis showed that CA-CysC derived from 4-*S*-CAP was produced at 85 μM (85% yield) at 10 min. The reaction rate constant (*k*) of 4-*S*-CAP was 0.17 min^{-1}. As NPrCAP/MNP has the same structural units as 4-*S*-CAP, it was expected to be a substrate for tyrosinase. If this were the case, CA-CysC would be obtained by HCl hydrolysis, in the presence of 1% phenol, of the cysteinylcatechol derivative of NPrCAP/MNP produced after tyrosinase oxidation of NPrCAP/MNP in the presence of cysteine. NPrCAP/MNP fell to half of the initial concentration after 82 min, and CA-CysC produced after 180 min was 80 μM (80% yield) (Figure 8a). Thus, the ratio of 4-*S*-CAP to NPrCAP/MNP in the reaction velocity on tyrosinase oxidation was 19.5, and the reaction rate constant (*k*) of NPrCAP/MNP was 8.5 x 10^{-3} min^{-1}. These results indicate that NPrCAP/MNP served as a substrate for tyrosinase. On the other hand, in the case of NPrCAP/PEG/APTES/DNM, the time for reduction to half of the initial concentration was 17 min, and the concentration of CA-CysC produced after 180 min was 98 μM (98% yield) (Figure 8a,b). The ratio of 4-*S*-CAP to NPrCAP/PEG/APTES/DNM in the reaction velocity on tyrosinase oxidation was 4.0, and

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the reaction rate constant (k) of NPrCAP/PEG/APTES/DNM was 4.1 x 10^{-2} min^{-1}. Thus, the tyrosinase oxidation of NPrCAP/PEG/APTES/DNM was about 5 times faster than that of NPrCAP/MNP. This was predicted because 1) the dispersibility of NPrCAP/PEG/APTES/DNM is greater and 2) in NPrCAP/PEG/APTES/DNM, the side chain is longer, and the steric hindrance of the aromatic ring site was alleviated.

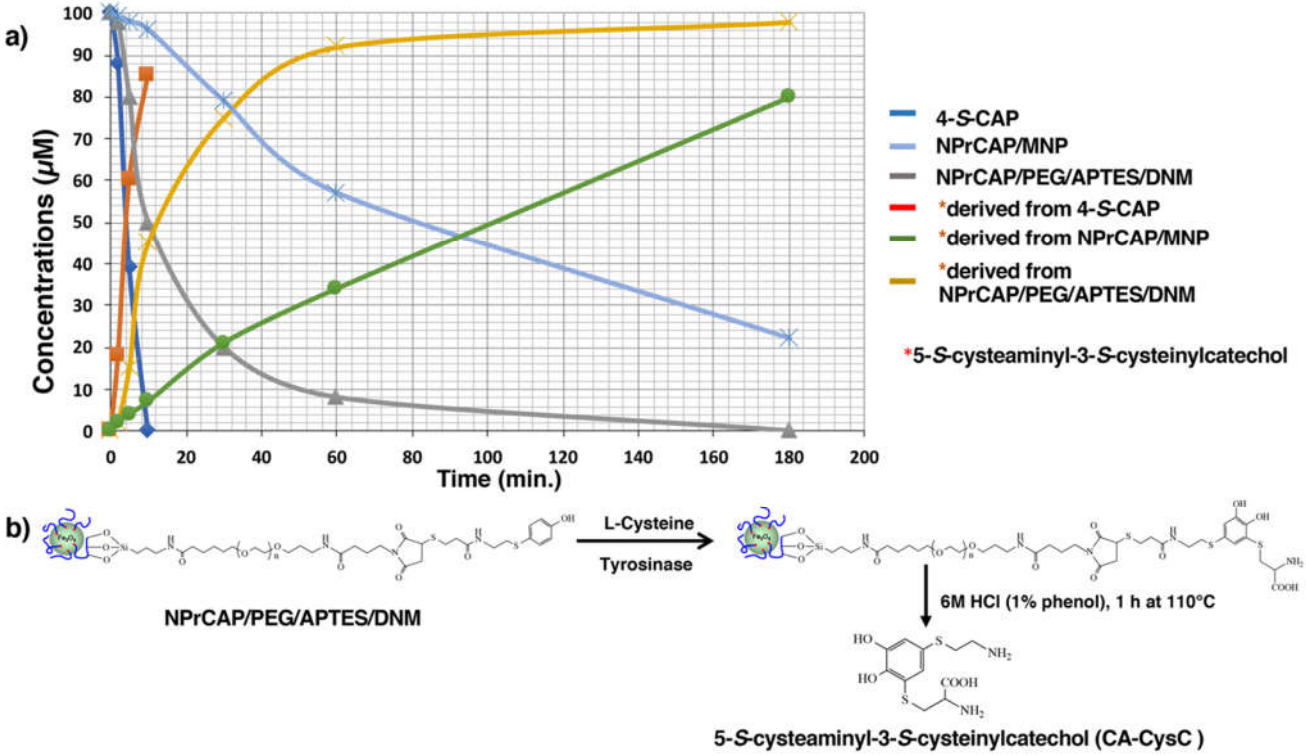

Figure 8. a) Tyrosinase reaction of 4-*S*-CAP, NPrCAP/PEG/APTES/DNM and NPrCAP/MNP, and yield of 5-*S*-cysteaminyl-3-*S*-cysteinylcatechol (CA-CysC). NPrCAP/MNP and NPrCAP/PEG/APTES/DNM are incorporated into the tyrosinase oxidative reaction *in vitro*. The concentrations of the substrate remaining as 4-*S*-CAP and the CA-CysC produced were measured by HPLC analysis after hydrolysis with 6M HCl. * is CA-CysC (derived from 4-*S*-CAP, NPrCAP/MNP and NPrCAP/PEG/APTES/DNM). B) Tyrosinase oxidation of NPrCAP/PEG/APTES/DNM in the presence of cysteine followed by 6M HCl containing 1% phenol yielded CA-CysC as well as 4-*S*-CAP and NPrCAP/MNP.

3. Selective inhibition of melanoma growth by NPrCAP/MNP conjugates in a mouse melanoma model

3.1. Melanoma-Targeting Drug Delivery and Growth Inhibition

Two basic findings emerged in our studies to exploit the melanogenesis cascade to develop a better, novel therapeutic approach to melanoma treatment. One is that modified tyrosinase substrates such as the sulfur homologue of tyrosine, cysteinylphenol (CP), and its amine analogue cysteaminylphenol (CAP) will be selectively incorporated

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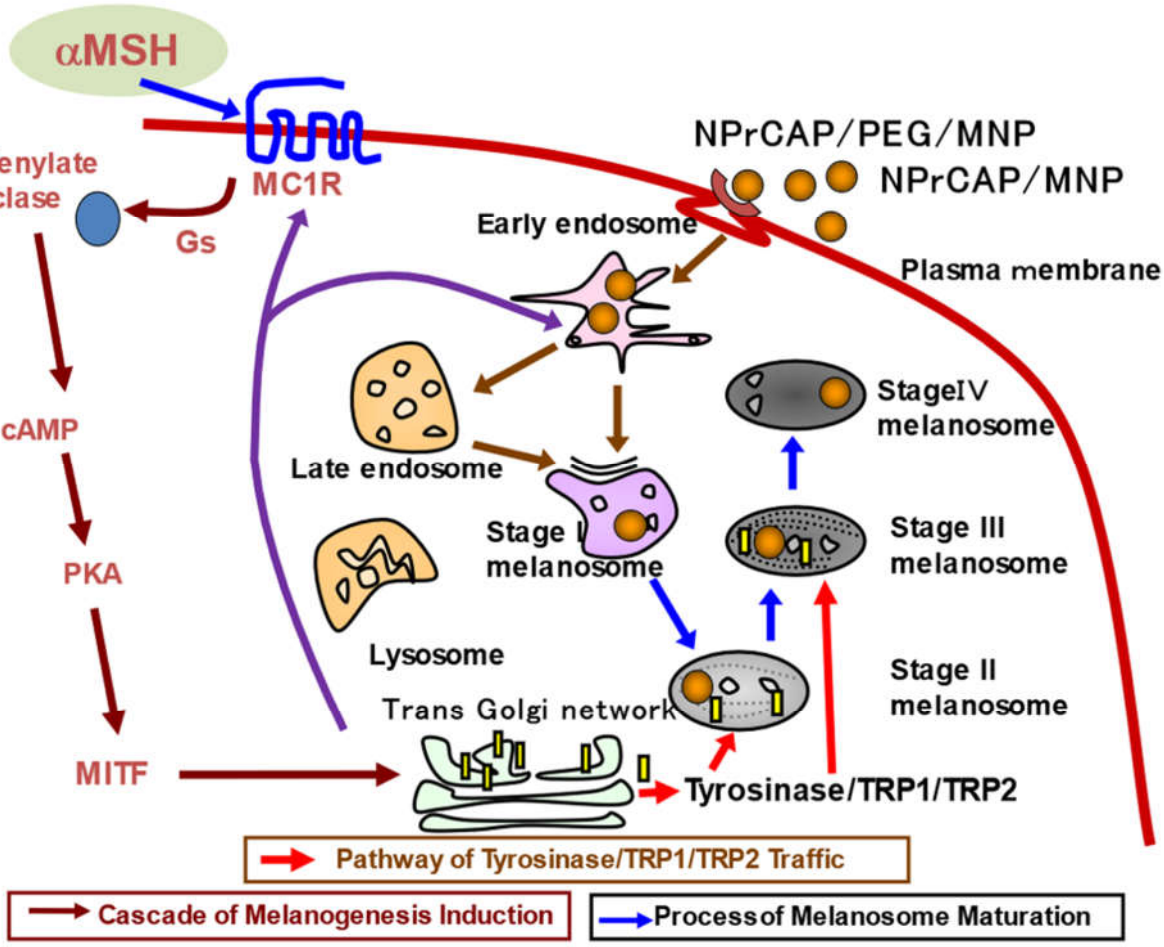

Figure 9. Selective accumulation of NPrCAP/MNP and NPrCAP/PEG/MNP. (KC: keratinocyte; MC: melanocyte; DMC: degenerating melanocyte; BL: basal lamina; PA: hair papilla).

into melanoma cells through active transport on the cell surface, which we believe can be used as the basis for developing a novel drug delivery system (DDS) (Figure 9) [25-28].

Another finding is that melanin biosynthesis *per se*, if overproduced, is toxic to melanoma cells through the production of quinone and cytotoxic free radicals, which can be used as a potential source for development of pharmacologic and immunologic anti-melanoma agents [29].

The melanoma-targeting 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 C57BL/6 black mice. At a site on adult mice where hair follicles were plucked to stimulate new melanocyte growth and activate tyrosinase biosynthesis, repeated *ip* administration of NPrCAP yielded white follicles with 100% success. A single *ip* injection of NPrCAP into new born mice resulted in the development of silver follicles in the entire body coat. The selective disintegration of melanocytes can be seen by electron microscopic examination as early as 12 h after a single *ip* administration. None of

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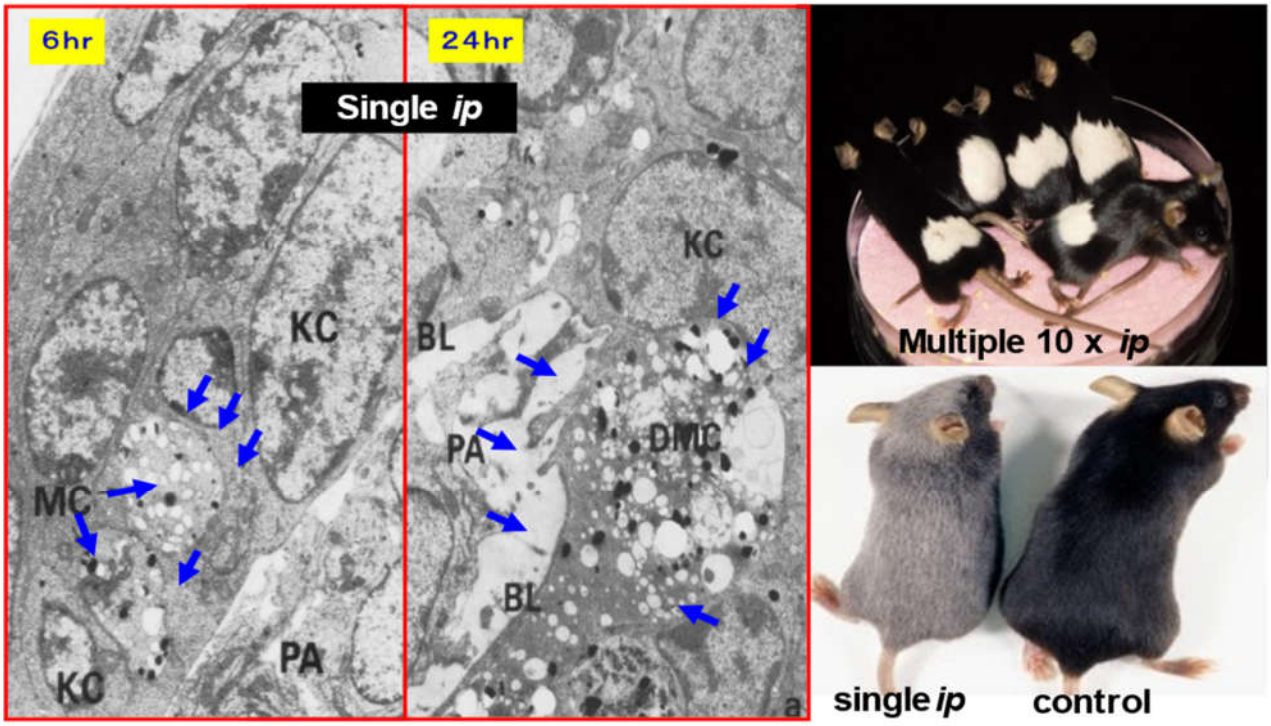

Figure 10. Selective cytotoxicities of NAcCAP and NPrCAP to black hair follicles of new born and adult C57BL/6 mice by *ip* administration

surrounding keratinocytes or fibroblasts showed membrane degeneration or cell death (Figure 10) [30-31].

In experiments using melanoma-bearing mice, NAcCAP and NPrCAP were found to be selectively incorporated into melanoma transplants and retained within melanoma cells, exerting a cytotoxic effect through oxidative stress that may derive from tyrosinase-catalyzed production of cytotoxic free radicals from the tyrosine analogs (chemotherapeutic effect) [32].

The specific cytotoxicities of NPrCAP and NAcCAP were further examined in various types of cultured cells by MTT assay [33,34]. Among them, only melanocytic cells and HeLa cells showed low IC50 values. The administration of high concentrations caused irreversible damage to melanoma cells in colony formation assays and the cytotoxicity to these cells was dose-dependent. However, the cytotoxicity to HeLa cells on DNA synthesis was transient and reversible. The molecular mechanism for the cytotoxic action by NAcCAP and NPrCAP appears to involve two major biological processes. One is cytostatic action that derives from DNA synthesis inhibition through the interaction of quinone and free radicals with the SH-enzyme protein disulphide isomerase. Another is cytocidal action by damage of DNA and mitochondrial ATP through oxidative stress. Combining NAcCAP with buthionine sulfoxide (BSO), which blocks the effect of anti-oxidants, revealed a marked growth inhibition of cultured melanoma cells, indicating again that the selective cytotoxicity of our CAP is related to quinone and free radicals (Figure 11) [34].

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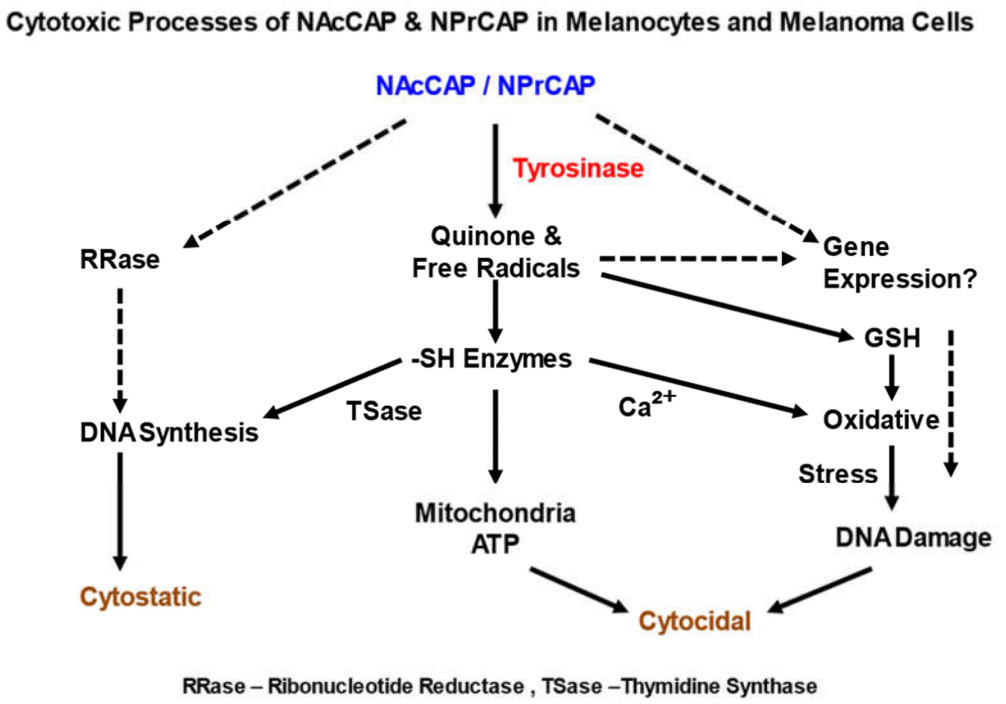

Figure 11. Molecular cascade of cytotoxic action of NAcCAP and NPrCAP in melanocytes and melanoma cells.

In B16F10 melanoma-bearing mice, administration of NAcCAP, when combined with BSO, yielded marked growth inhibition of melanoma cells, indicating that the selective cytotoxicity of our CAP is related to the production of quinones and free radicals. *In vivo* lung metastasis assays in these mice showed a decreased number of lung melanoma colonies in the presence of BSO [30]. However, the problem with NAcCAP administration was that a fairly large number of amelanotic melanoma lesions was seen to grow in the lung. Administration of NPrCAP, however, has improved this "amelanosis" problem, showing fewer amelanotic lesions and increased cytotoxic growth inhibition.

Subsequent experiments using NPrCAP conjugated with MNP revealed selective disintegration of melanoma tissues upon exposure to AMF [9]. Thus, our approach of exploitation of melanogenesis substrates and synthesis of NPrCAP has provided a firm basis to develop melanoma-targeting rational therapy. In addition, NAcCAP may be an ideal choice for development of a novel depigmenting agent for skin diseases such as melasma [5].

3.2. Growth Inhibition of Re-challenge Melanoma Transplant

In this study, we first evaluated the chemotherapeutic effect of NPrCAP/MNP on primary transplants of B16 F1melanoma with (group 1) and without (group 2) heat

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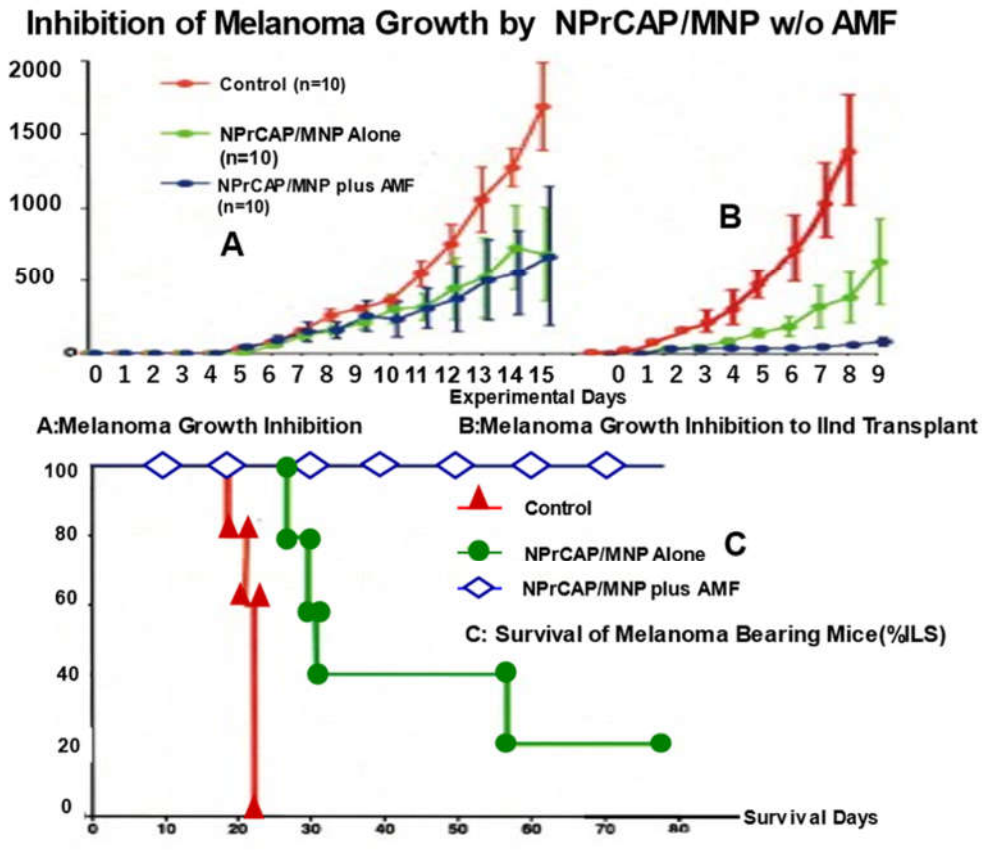

Figure 12. Melanoma growth inhibition by NPrCAP/MNP with/without AMF (A, B) and host survival curves, % ILS(C).

exposure. Significant growth inhibition of the primary transplants was observed in both groups of mice(Figure 12A). However only the goup of mice treated with NPrCAP/MNP plus AMF showed alomost complete growth inhibition of rechallenge IInd melanoma transplant (Figure 12B), indicating that NPrCAP/MNP alone has a certain chemotherapeutic effect, and that the group treated with both NPrCAP and AMF heat revealed much greater melanoma growth inhibition of rechallenge IInd transplant as well as %ILF of melanoma-bearing host mice (Figure 12C).

Importantly, when mice were re-challenged with a second B16F1 transplant at day 53 after treatment from day 6 to day 10 and subsequent excision of the primary transplant at day 13 (Figure 13a), there was a marked difference in growth inhibition of the re-challenge transplants between the groups of NPrCAP/M treatment with vs. without heat. NPrCAP/M with AMF exposure yielded significant growth inhibition of re-challenge melanoma transplants and complete tumor rejection in 30% of the host animals (Figure 13b,c), indicating that NPrCAP/MNP with heat exerts a thermo-immunotherapeutic effect. [8,9]. Moreover, our experiments indicated that the most effective primary tumor thermo-immunotherapy for inhibiting the growth of re-challenge B16F1 melanoma transplants was obtained when the treatment was repeated three times, once every other day, with AMF exposure at a temperature of 43°C for 30

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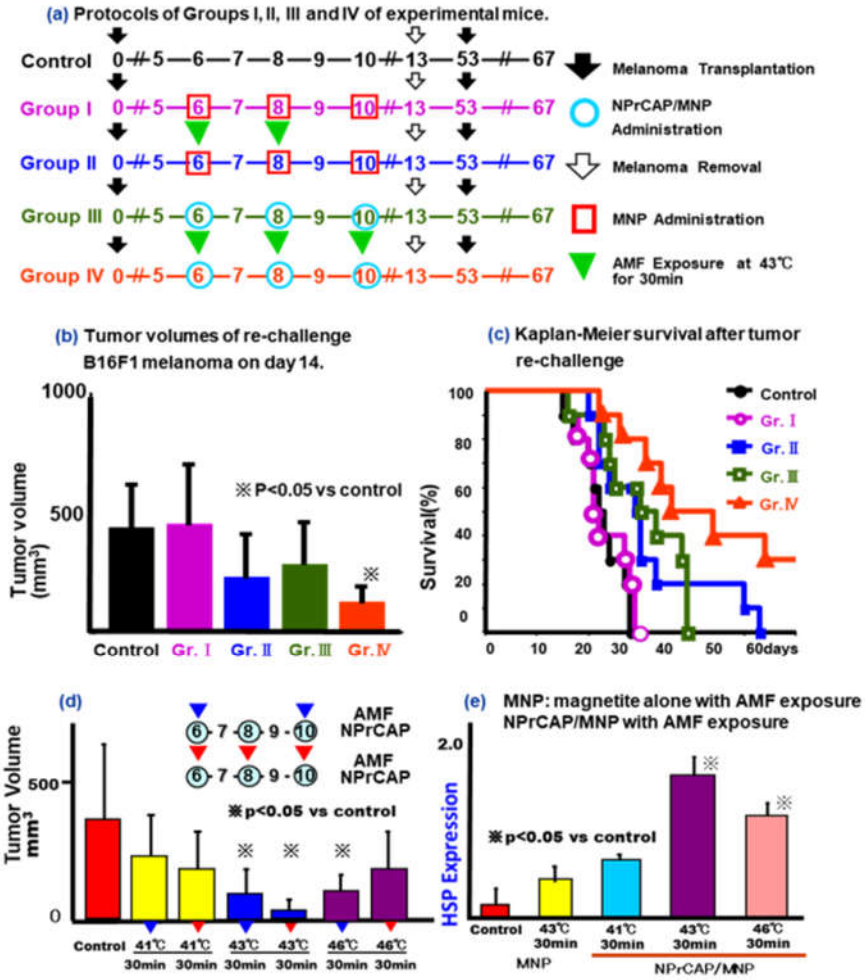

Figure 13. Tumor growth, heat-shock protein and survival of mice re-challenged with melanoma transplants after treatment of initial transplants. a) Experimental protocols for the five groups of mice, all of which underwent initial B16F1 melanoma transplants on day 0, removal of the tumors on day 13, and re-challenge with B16F1 on the opposite flank on day 53. The Control group received no treatment, Group I received MNP without AMF on the days indicated, Group II received MNP + AMF. Group III received NPrCAP/MNP without AMF, and Group IV received NPrCAP/MNP + AMF. b) Volumes of re-challenge B16F1 tumors 14 days after transplantation (67 days after primary transplanation). c) Kaplan-Meier survival curves after primary and re-challenge melanoma transplants. d) Effects of AMF frequency and temperature on re-challenge B16F1 tumor volumes in mice treated with NPrCAP/MNP on days 6, 8, and 10 after primary tumor transplantation plus AMF on days 6 and 10 (blue inverted triangles) or on days 6,8 and 10 (red inverted triangles). Tumor volumes were measured on day 67, 14 days after re-challenge transplantation. e) Heat shock protein (HSP) expression in primary B16F1 tumors treated with MNP or NPrCAP/MNP together with exposure to AMF at the indicated temperatures.

min (Figure 13d). A previous study using cationic magneto-liposomes-mediated

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hyperthermia with 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 [16].

3.3. *Induction of NPrCAP-mediated melanoma apoptosis*

The chemical agent NPrCAP has been shown to be a good substrate for tyrosinase and to be selectively incorporated into melanoma cells, causing cytotoxicity [23,34]. We examined the molecular mechanism of NPrCAP-mediated cytotoxicity to melanoma cells by focusing on intracellular reactive oxygen species (ROS).

When melanocytic and non-melanocytic cells were exposed to NPrCAP (0.5-3.0 mM) for one hour and cultured for 24 more hours, growth of pigmented B16F1, 70W and M1 melanoma cells was inhibited in a concentration dependent manner, while growth of NIH3T3 fibroblast cells was not affected. An inactive form, N-propionyl-2-*S*-cysteaminylphenol, showed no growth inhibitory effect on B16F1 or NIH3T3 cells.

To examine the mechanism of the cell death induced by NPrCAP, cellular DNA and caspase activation were analyzed by flow cytometry and caspase 3 assay. After pigmented B16F1, non-pigmented TXM18 melanoma, NIH3T3 fibroblast and RMA mouse lymphoma cells were exposed to 1 mM NPrCAP for one hour and cultured for 24 more hours, adherent and floating cells were collected and processed for flow cytometry. The results showed that the sub-G1 fraction was increased in the NPrCAP-treated B16F1 cells, comparable to the increase in TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L-exposed B16F1, but was not elevated in the NPrCAP-treated NIH3T3, TXM18, or RMA cells [10]. For analysis of caspase 3 activation, cells were cultured in medium containing NPrCAP for one hour and then caspase 3/7 activity was measured using a Caspase-Glo3/7 Assay kit (Promega, Madison, WI) and a luminescence microplate reader. The assay detected caspase 3/7 activity that was remarkably increased (35.8-fold) in the NPrCAP-treated B16F1 cells, comparable to TRAIL-exposed cells. On the other hand, NIH3T3, RMA and TXM18 cells treated with NPrCAP showed increases of only 4.1-, 1.4- and 1.4-fold, respectively [10]. These findings suggested that NPrCAP induces apoptotic cell death selectively in pigmented melanoma cells in association with increased caspase 3 activity.

To analyze the relationship between NPrCAP-mediated apoptosis and ROS production in melanocytic cells, we examined ROS generation using flow cytometry. Pigmented and non-pigmented melanoma cells were cultured with the general ROS indicator CM-H2DCFDA (5 μM) for 30 min and then with NPrCAP (NPr-4-*S*-CAP) or the inactive NPr-2-*S*-CAP (0.5-6 mM) for one hour. The cells were then collected and processed for flow cytometry to quantify the ROS-containing cell fraction M2 [10]. Pigmented B16F1, 70W, G361 and M-1 melanoma cells, but not non-pigmented TXM18 and SK-mel-24 melanoma cells, produced significant amounts of ROS in the presence of NPr-4-*S*-CAP (Figure 14). The results suggested that NPrCAP selectively produced ROS in pigmented melanoma cells and that melanin biosynthesis was essential for NPrCAP to produce ROS.

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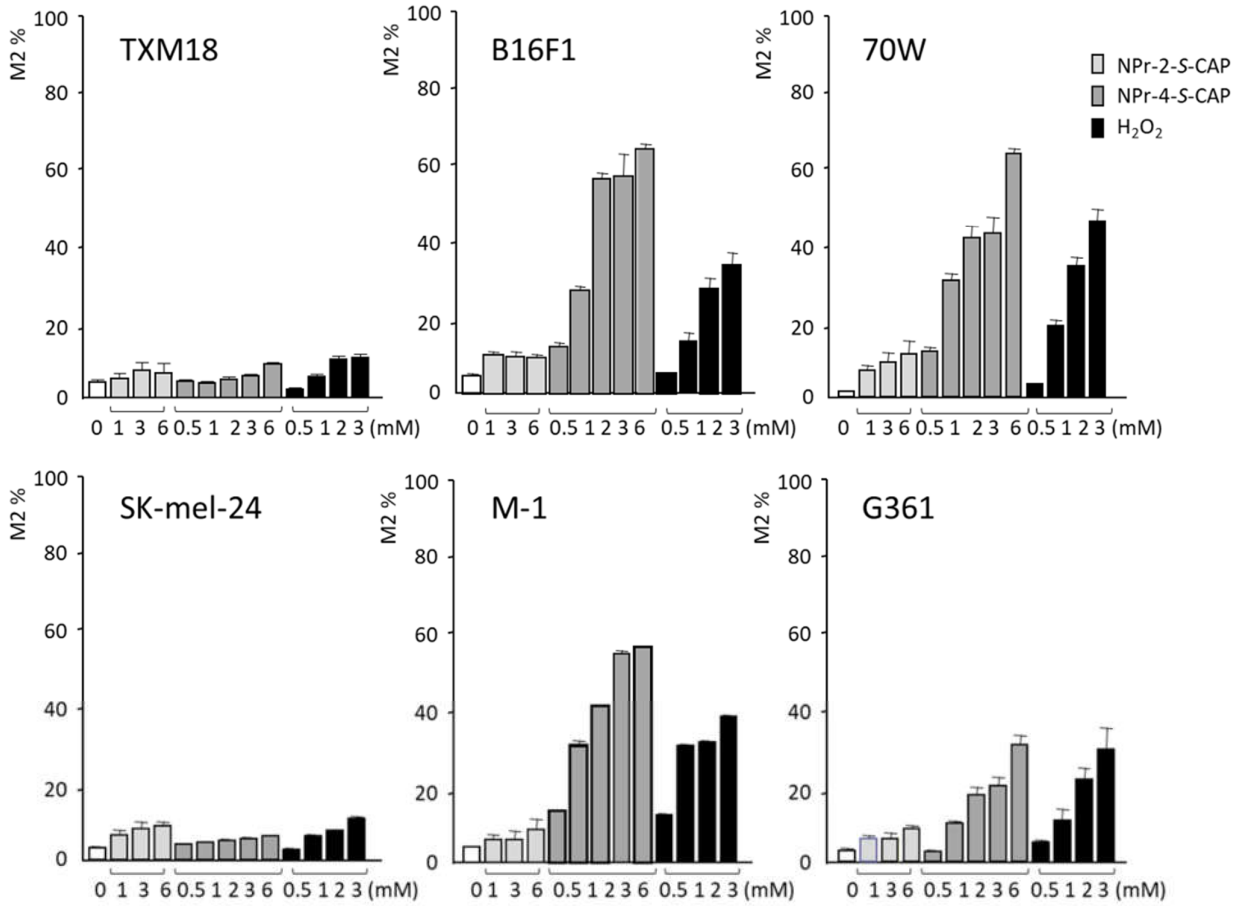

Figure 14. Pigmented but not non-pigmented melanoma cells produce ROS in the presence of NPr-4-*S*-CAP.

4. Specificity and Mechanism of Immunomodulation by CTI therapy in Melanoma

4.1. NPrCAP as neo-antigen producer

NPrCAP is a good substrate for tyrosinase [33] and is selectively incorporated into melanoma cells, which causes cytotoxicity *in vitro* and *in vivo* [33, 34]. To clarify the molecular mechanism of NPrCAP-mediated cytotoxicity to melanoma cells, Ishi-Osai reported that mice treated with intratumoral injections of NPrCAP to suppress the growth of primary B16F1 melanoma transplants also rejected secondary, re-challenge tumors [10]. The participation of $CD8^+$ T cells was suggested for the NPrCAP-mediated anti-B16F1 melanoma immunity.

Phenolic substrates as prohaptens are oxidized by tyrosinase to produce *ortho*-quinones, which act as haptens that covalently bind to tyrosinase or other melanosomal proteins to generate potential neo-antigens [35-37]. These neo-antigens trigger an immunological response cascade that results in a melanocyte-specific, delayed-type hypersensitivity reaction leading to melanocyte elimination or melanoma rejection.

Based on the haptenation theory, Ito et al. examined the oxidation of NPrCAP and

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its subsequent binding to sulfhydryl compounds (thiols) [24]. They demonstrated that NPrCAP is oxidized by tyrosinase to form a highly reactive *ortho*-quinone (*N*-propionyl-4-*S*-cysteaminyl-1,2-benzoquinone, NPrCAQ; Figure 15), which binds covalently to biologically relevant thiols, including proteins through cysteine residues. The production and release of NPrCAQ-protein adducts was verified in B16F1 melanoma cells *in vitro* and in B16F1 melanoma-bearing mice *in vivo* through the detection of CA-CysC after acid hydrolysis of the protein fraction (Figure 15). These results suggested that the phenol NPrCAP, acting as a prohapten, can be oxidized in melanoma cells by tyrosinase to the active quinone-hapten NPrCAQ, which binds to melanosomal proteins through their cysteine residues to form possible neo-antigens, thus triggering the immunological response.

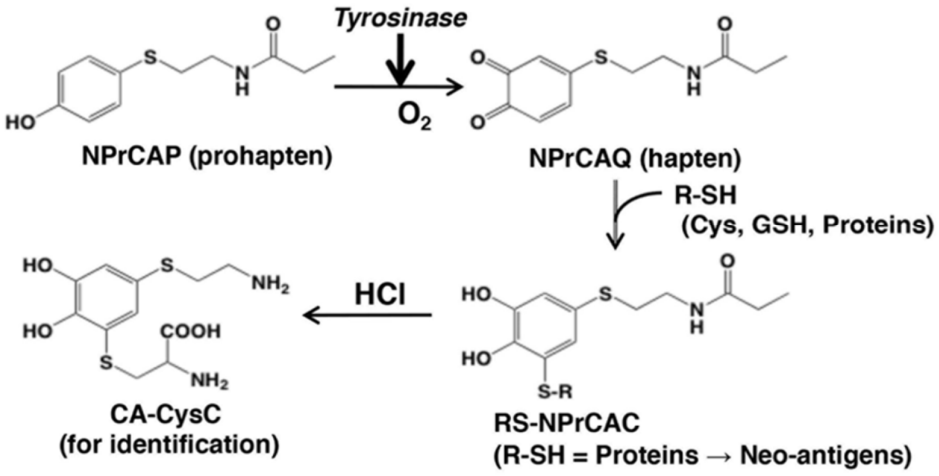

Figure 15. Tyrosinase activation of NPrCAP (prohapten) and binding of the quinone-hapten NPrCAQ to proteins through cysteine residues [24]. Oxidation of NPrCAP with tyrosinase produces the quinone NPrCAQ, which binds to thiols (cysteine, reduced glutathione, melanosomal proteins). The production of NPrCAQ-thiol adducts can be confirmed by the detection of CA-CysC after acid hydrolysis.

4.2. *T-cell Receptor Repertoires of Tumor-Infiltrating Lymphocytes*

Cytotoxic T lymphocytes (CTLs) play a significant role in antitumor immunity, and the presence of tumor-infiltrating lymphocytes (TILs) has been considered to be a favorable clinical prognostic indicator [38]. To further understand the T-cell response to melanoma in CTI therapy and to develop a more effective strategy based on immunomodulation, we investigated the diversity of TILs after CTI therapy [12] (Figure 16). The immune response of CTLs is mediated via T-cell receptors (TCRs) consisting of

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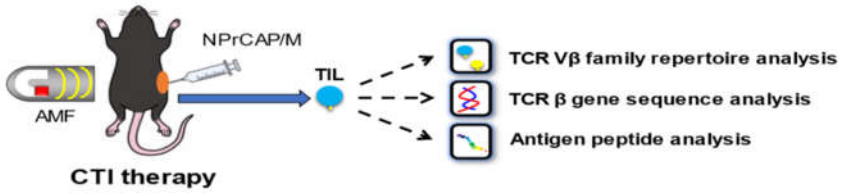

Figure 16. Schematic illustration of T-cell receptor (TCR) repertoire analysis of tumor-infiltrating lymphocytes (TILs) after CTI therapy for B16 melanoma.

α and β chains. In the variable (V) regions, the gene sequence encoding the third complementarity-determining region (CDR3), which is called the hypervariable region, is considered to play the most important role in antigen recognition [39]. We analyzed the diversity of the TCR Vβ family to investigate the qualitative changes of TILs after CTI therapy. Almost all TCR Vβ families (in total 21 TCR Vβ families were analyzed) were detected in untreated B16 melanoma in C57BL/6 mice, whereas the TCR repertoire was restricted to a few TCR Vβ families in TILs after CTI therapy. Among them, expression of the Vβ11 gene was confirmed with good reproducibility, suggesting that T cells expressing the TCR Vβ11 were activated by CTI therapy in B16 melanoma. In addition, we succeeded in analysis of the CDR3 gene sequence of TCR Vβ11 in TILs after CTI therapy [24]. Consistent with our result, it was reported that a B16 melanoma-specific CD8+ T cell line, AB1, expressed TCR Vβ11 [40], suggesting that clonal expansion of Vβ11+ TILs can be a useful biomarker for the T-cell response to B16 melanoma in mice. Furthermore, the same group reported that the AB1 cells recognized a melanoma antigen, tyrosinase related protein-2 (TRP-2) peptide, which was consistent with a report by Singh et al. showing that a TRP-2 peptide-specific CD8+ T cell clone expressed Vβ11 [41]. In order to identify the antigen specificity of TILs after CTI therapy of B16 melanoma, we investigated the interferon (IFN)-γ production ability using melanoma antigen peptides such as TRP-1$_{222\text{-}229}$, TRP-2$_{180\text{-}188}$ and gp100$_{25\text{-}33}$. When stimulated with the TRP-2 peptide, T cells were activated to secrete IFN-γ, indicating that TILs induced by CTI therapy of B16 melanoma responded to the TRP-2 peptide. Taken together, these findings show that tumor-specific TILs were produced after CTI therapy, and suggeste that TCR Vβ11+ T cells are particularly important for immunity against B16 melanoma. Moreover, the melanoma antigen peptides selected by TIL analysis (e.g., TRP-2 peptide for B16 melanoma) may be used to boost antitumor immunity induced by CTI therapy.

4.3. *CTI Therapy as in situ Peptide Vaccine Immunotherapy*

By comparing the antitumor effect of NPrCAP/MNP with and without AMF exposure, we observed that NPrCAP/MNP with AMF exposure had a superior antitumor effect compared with that of NPrCAP/MNP alone. Furthermore, mice bearing primary melanoma tumors treated with NPrCAP/MNP plus AMF showed significant suppression of re-challenge, second transplant melanoma growth (Figure.12 B) and

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increased life spans, *i.e.*, almost complete rejection of re-challenge melanoma (Figure12C), whereas NPrCAP/MNP without AMF was much less effective, with 30-50% rejection of re-challenge melanoma. These results indicate that NPrCAP/MNP with AMF exposure has a strong immunotherapeutic effect [8,9]. Therefore, we investigated the underlying mechanisms for the induction of antitumor immunity induced by NPrCAP/MNP with AMF exposure. Incorporated MNP exposed to an AMF generate heat within cells due to hysteresis loss or relaxational loss [15]. It has been demonstrated that intracellular hyperthermia using MNP is effective for the treatment of certain types of cancer, in not only primary but also metastatic lesions [42-45]. Hyperthermic treatment using cationic magnetite liposomes containing 10 nm MNP induced antitumor immunity by enhancement of HSP expression [44]. It has been demonstrated that various types of HSPs bind antigenic peptides, and these antigen peptides are cross-presented to specific cytotoxic T cells (CTLs) by professional antigen presenting cells including dendritic cells (DCs). This exogenous pathway is called cross-presentation and is important for the development of $CD8^+$ T cell responses against tumors and infectious pathogens that do not have access to the classical MHC class I pathway [46,47]. In our study using B16-OVA melanoma cells, treatment with NPrCAP/MNP with AMF exposure resulted in increased expression of HSPs, including Hsp72, Hsp90 and ER-resident stress proteins such as gp96, in melanoma cells [11]. Moreover, these HSPs (Hsp72, Hsp90 and gp96) were secreted in extracellular milieu and were taken-up by DCs. These DCs presented melanoma-associated antigen peptides (OVA peptide and TRP2 peptide) through cross-presentation

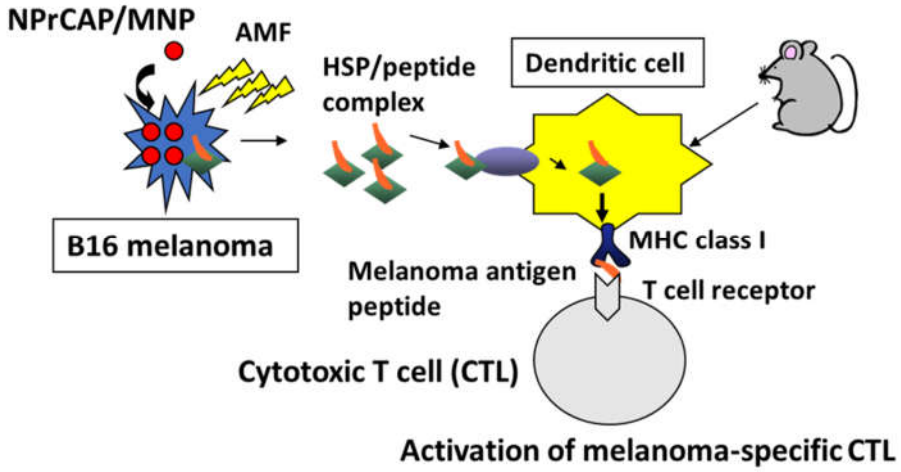

Figure 17. Induction of cytotoxic T cells specific for melanoma-associated antigen peptide by intracellular hyperthermia generated by NPrCAP with AMF. (1) Treatment of melanoma using NPrCAP/MNP with AMF induces various types of HSPs that bind melanoma-associated antigen peptides, and necrotic melanoma cells release HSP-antigen peptide complexes in the tumor microenvironment. (2) Infiltrated dendritic cells (DCs), which sense the inflammation induced by hyperthermia, take-up HSP-peptide complexes and cross-present HSP-chaperoned antigen peptides to antigen specific cytotoxic T lymphocytes (CTLs).

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of HSP-bound peptide(s) to specific $CD8^+$ T cells. Among HSPs, Hsp72 was shown to be largely responsible for the augmented antigen presentation to $CD8^+$ T cells. As Hsp72 is known to be most highly upregulated among several HSPs in response to heat shock, newly synthesized Hsp72 has more chances to bind melanoma-associated antigen peptides.

Thus, our hyperthermia using NPrCAP/MNP with AMF exposure induced an anti-melanoma cytotoxic T lymphocyte (CTL) response through cross-presentation of melanoma-specific antigen peptides bound to hyperthermia-induced HSPs by DCs. More importantly, intracellular hyperthermia using NPrCAP/MNP can be a promising treatment for the prevention of recurrence and/or distant metastasis of melanoma, because systemic antimelanoma immunity is induced by this therapy (Figure 17).

If the treatment with NPrCAP/MNP plus AMF could prevent distant melanoma metastasis such as lung and distant cutaneous metastases, it would be a great gospel for patients with advanced melanoma. Therefore, we examined whether treatment of primary cutaneous B16 melanoma with intracellular hyperthermia using NPrCAP/MNP with AMF can inhibit lung colonization of intravenously injected secondary challenge B16 melanoma cells. We observed that NPrCAP/MNP plus AMF clearly inhibited lung metastasis compared with NPrCAP/MNP alone. These results indicated that intracellular hyperthermia using NPrCAP/MNP with AMF elicited systemic antimelanoma immunity and prevented lung metastasis and recurrence of melanoma.

Thus, CTI therapy using NPrCAP/MNP with AMF against advanced melanoma is a promising strategy not only for the treatment of primary melanoma but also for prevention of the recurrence of melanoma.

5. Approach to human advanced melanoma patients

5.1. Scale-up production of NPrCAP/PEG/APTES/DNM for clinical application

Based on NPrCAP/PEG/APTES/DNM, which is a PEG-mediated conjugate of NPrCAP and APTES with DNM, we tested further improvements of the synthesis conditions for development of a good manufacturing practice (GMP)-based production process. Water dispersibility is important for injectable drugs. We found that aggregation of NPrCAP/PEG/APTES/DNM was caused by particle-to-particle interactions due to APTES. By reducing the iron concentration from 10 mg/mL to 1 mg/mL during the APTES reaction, we found that the particle size of NPrCAP/PEG/APTES/DNM did not increase even after the reaction. Thus, this new formulation of NPrCAP/PEG/APTES/DNM can pass through a 0.2 μm filter, enabling sterilization, which is extremely important in the manufacture of drugs. Furthermore, equipment for the production of NPrCAP/PEG/APTES/DNM compliant with GMP was installed in the labolatory of Meito Sangyo Co., Ltd. (Nagoya, Japan). Meito Sangyo has manufactured ferucarbotran, the drug substance of Resovist [48], which is sold by Bayer Schering Pharma (Berlin, Germany) as a clinically available MRI contrast agent, in compliance with GMP. As a result of repeated synthesis while complying with the standard operating procedure (SOP), a total of 14 lots (800 mL) of NPrCAP/PEG/APTES/DNM

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were synthesized, and the reproducibility was comfirmed. Taken together, the standard of the formulation of NPrCAP/PEG/APTES/DNM was determined.

5.2. *Preliminary human clinical trial of CTI therapy for advanced melanoma patients*

Based on our animal experiments and sucessful production of GMP grade NPrCAP/PEG/APTES/DNM, a preliminary human clinical trial (Phase I/II) has been carried out with a limited number of stage III and IV melanoma patients, after receiving informed consents from the patients and institutional approval of our human clinical trial protocol (Clinical Trial Research No. 18-67, Sapporo Medical University).

The therapeutic protocol followed the basically identical experimental schedule as that of the animal experiments (Figures 18 and 19). Among four patients treated with NPrCAP/PEG/APTES/DNM plus AMF exposure, two of them showed complete and partial responses, respectively, and have been able to carry out normal daily activities after the CTI therapy. In one of those two responding patients, four distant cutaneous metastasis sites were evaluated and either significant regression or shrinkage of all four lesions was seen. That patient was able to survive 36 months after several cycles of CTI therapy. The pathological and immunological specimens revealed dense aggregations of lymphocytes

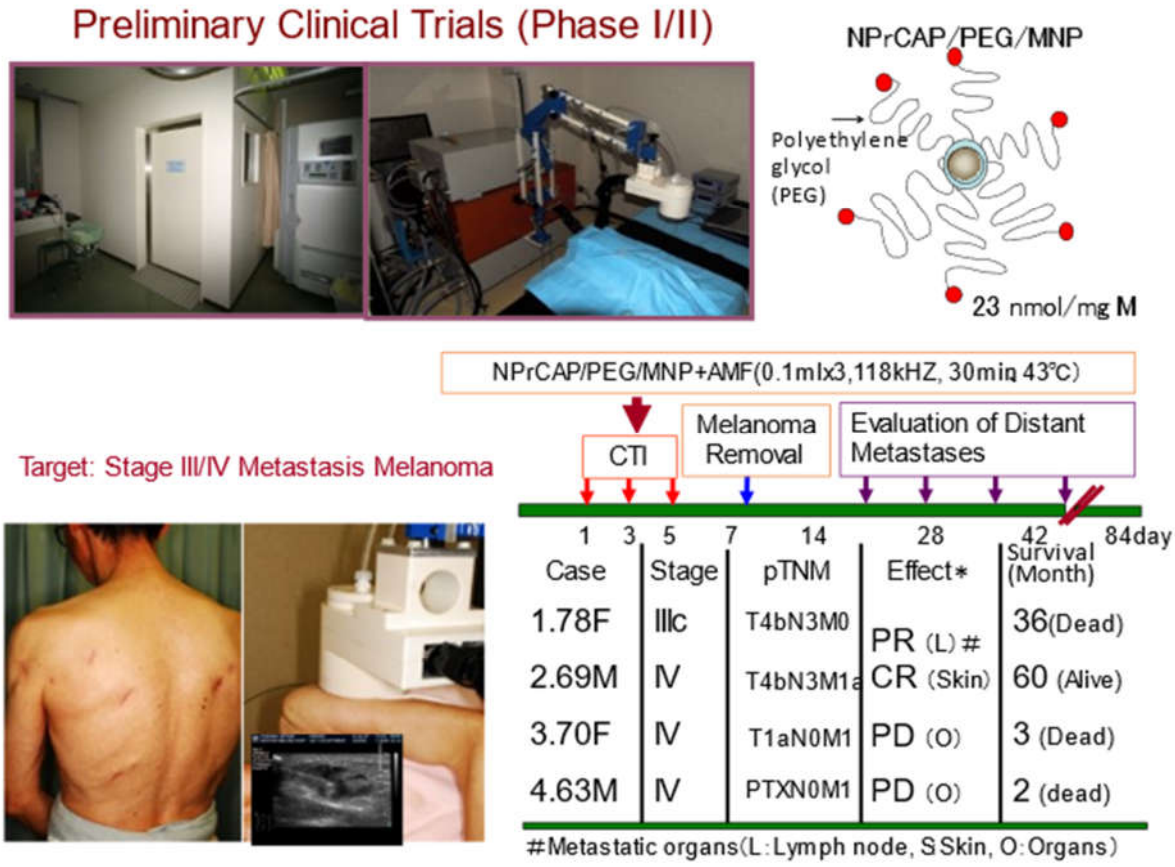

Figure 18. Preliminary clinical trials of CTI therapy for advanced metastatic melanoma patients in stages 3 and 4.

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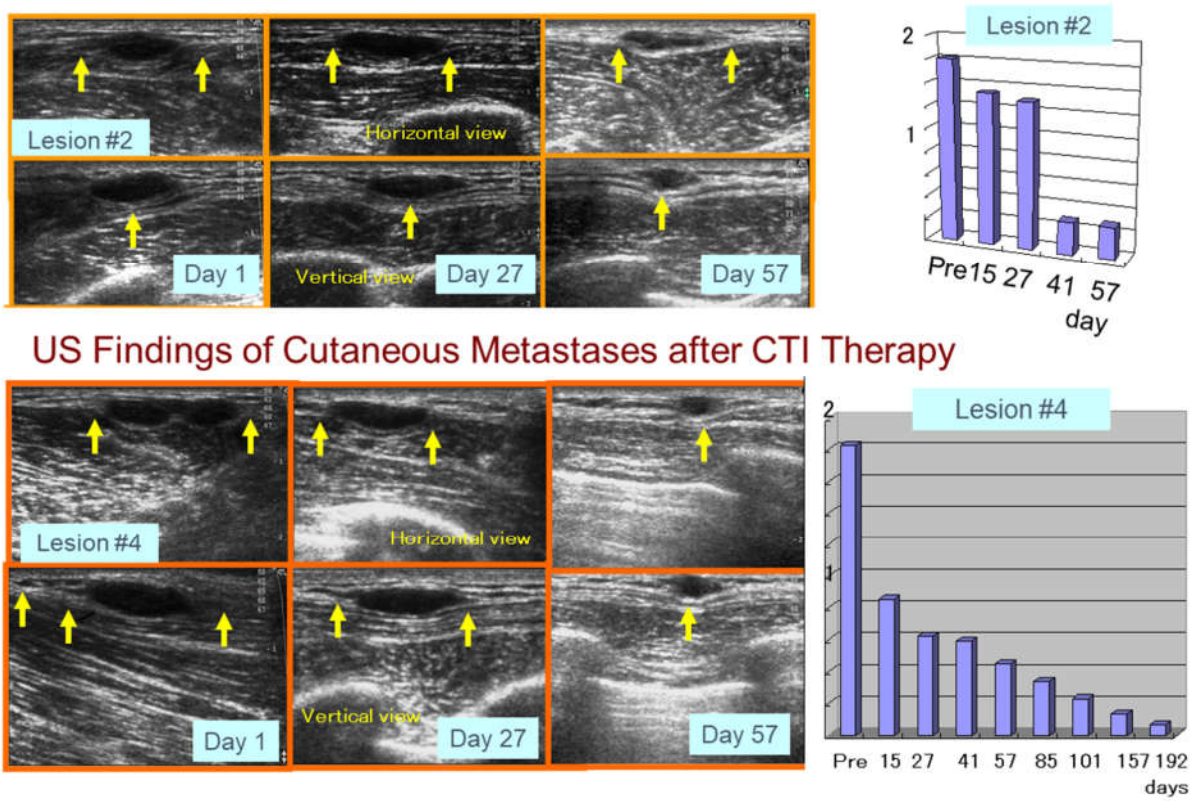

Figure 19. Ultrasound findings of distant melanoma metastases after CTI therapy.

and macrophages at the site of CTI therapy. Importantly, there was a trend toward an almost identical distribution of $CD8^+$ T cells and MHC class 1 positive cells. The other responding patient had many lymph node metastases, but has survived more than 32 months so far. In order to evaluate the overall therapeutic value for advanced melanoma, it is important to have larger-scaled clinical trials and define concisely the molecular interactions between the chemotherapeutic and thermo-immunotherapeutic effects in our CTI therapy.

6. Conclusion and perspective

While there is a significant advance in early detection, malignant melanoma continues to be a large contributor to cutaneous cancer-related mortality. In melanoma patients with metastases, the clinical effect of cytotoxic anticancer drugs including decarbonize has been limited. Over the last decade, however, an influx of novel systemic-targeted therapeutics using immune check inhibitors (ICIs) and BRAF/MEK inhibitors has been introduced and improved both quality of life and survival for advanced stage patients.

The approved ICIs for melanoma are the antibodies to programmed cell death protein 1 (PD-1) (nivolumab, pembrolizumab), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (ipilimumab) and Lymphocyte-activation gene 3 (LAG-3) (relatlimab). They are used for the management of unresectable or metastatic melanoma or adjuvant setting regardless of BRAF-mutation status [49]. They contribute to

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extending overall survival. However, the clinical effect is not fully satisfactory, particularly in acral and mucosal melanoma [50].

Targetted therapies are antoher approach to inhibit uncontrolled cellular proliferation and subsequent resistance. They include BRAF/mitogen -activated protein kinase extracellular receptor kinase (MEK) inhibition, which is seen as first -line management in those patients suffering from stage III or metastatic disease with a proven mutation. Others include talimogene laherparepvec (T-VEC), interleukin (IL), interferon (IFN) that are used as well-tolerable therapeutics. Overall effects of these alternatives are, however, limited. It is, therefore, necessary to have further effort for developing melanoma-targeted immunomodulative systemic therapies.

Here in this communication, we have introduced a novel approach that provides chemotherapeutic, thermotherapeutic and immunotherapeutic effects. This approach is based upon;

(1) melanogenesis substrate, NPrCAP with conjugate of MNPs, NPrCAP/MNP, with AMF exposure can generate cytotoxic T cells that inhibit the growth of re-challenged melanomas which are transplanted at the opposite distant site of body,
(2) NPrCAP alone appears to generate some chemotherapeutic and immunotherapeutic properties through both apoptotic and non-apoptotic processes,
(3) unique melanogenesis cascade can be employed for developing a novel chemo-thermo-immunologic strategy (CTI Therapy) to advanced melanoma patients. It is achieved by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/MNP), and
(4) together with AMF exposure, NPrCAP/MNP can induce cytotoxic T cells that inhibit the growth of re-challenge melanoma transplants at the opposite distant site of the body from the treated primary melanoma.

It may, therefore, be considered that we have provided evidence that the unique melanogenesis cascade can be employed for developing a novel chemo-thermo-immunologic strategy (CTI therapy) for advanced melanoma patients.

Our approach is based on the combination of direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effects of NPrCAP/MNP with exposure to AMF and indirect killing by immune reaction (*in situ* vaccination immuno-therapy). This rationale also provides a strategy for producing a tumor-specific drug delivery system (DDS) that achieves selective melanoma cell death. This can then induce HSP production through either necrotic or non-necrotic process or a combination of the two, without damaging non-cancerous tissues. This cascade can further generate an immune reaction targeted to other metastatic melanoma lesions, hence providing an "*in situ* vaccination" strategy" (Figure 20).

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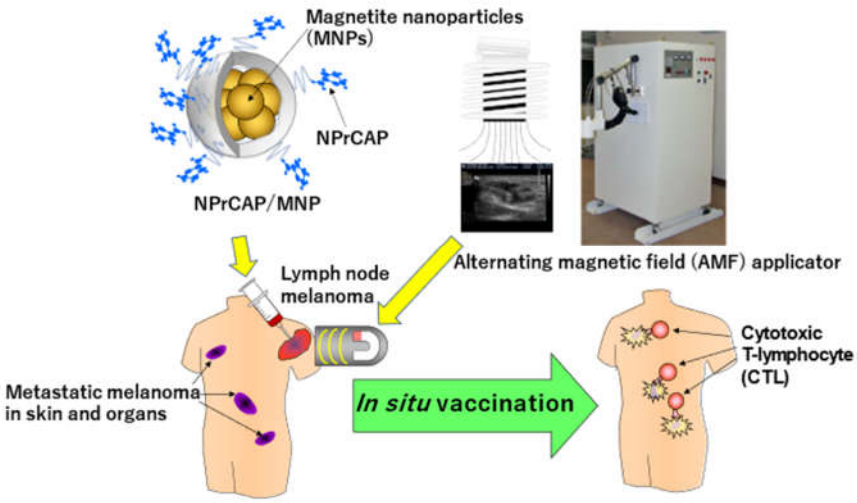

Figure 20. Flow of CTI therapy using conjugates of NPrCAP and MNP with exposure to alternating magnetic field (AMF) and growth inhibition of metastatic melanoma lesions by immunomodulated *in situ* vaccination.

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Abbreviations

AMF	alternating magnetic field
APTES	3-aminopropyltriethoxysilane
CA-CysC	5-*S*-cysteaminyl-3-*S*-cysteinylcatechol
4-*S*-CAP	4-*S*-cysteaminylphenol
CDR3	third complementarity-determining region

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CP	cysteinylphenol
CTI	chemo-thermo-immuno
CTL	cytotoxic lymphocytes
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
DC	dendritic cell
DCC	*N,N'*-dicyclohexylcarbodiimide
DDS	drug delivery system
DNM	dextran nanomagnetite
HSP	heat shock protein
MCL	magnetite cationic liposome
MITF	microphthalmia transcription factor
MNP	magnetite nanoparticle(s)
MSH	melanocyte-stimulating hormone
NAcCAP	*N*-acetyl-4-*S*-CAP
NPCMD	*N*-propionyl-4-*S*-cysteaminylphenol/maleimide-dextran
NPrCAP-SH	*N*-(1-mercaptopropionyl)-4-*S*-cysteaminyl phenol
NPrCAP/MNP	*N*-propionyl-4-*S*-cysteaminylphenol/magnetite nanoparticle
NPrCAP/PEG/MNP	*N*-propionyl-4-*S*-cysteaminylphenol/polyethylene glycol/magnetite nanoparticle
NPrCAP/PEG/APTES/DNM	*N*-propionyl-4-*S*-cysteaminylphenol/polyethylene glycol/3-aminopropyltriethoxysilane/dextran nanomagnetite
NPrCAQ	*N*-propionyl-4-*S*-cysteaminyl-1,2-benzoquinone
PD-1	programmed cell death protein 1
ROS	reactive oxygen species
TCR	T-cell receptor
TIL	tumor-infiltrating lymphocyte

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