厚生労働科学研究費補助金（医療機器開発推進研究事業）
分担研究報告書

新規薬剤（NPrCAP/PEG/DNM）を用いたCTI療法 の メラノーマ転移巣に対する 効果の解析

米田　明弘　札幌医科大学皮膚科学講座講師

研究の要旨

我々は、これまで悪性黒色腫に特異な形質であるメラノジェネシスを分子標的とした薬剤NPrCAPに鉄ナノ微粒子およびポリエチレングリコールを結合させた製剤（NPrCAP/PEG/M）を合成し、磁場照射による化学温熱免疫療法を開発し、その臨床的有用性を確認した。しかし、NPrCAP/PEG/Mは低拡散性で化学的生物学的免疫誘導体として不安定であり、高拡散性新規製剤の開発が必要とされた。我々は、より高拡散性のある新規薬剤（NPrCAP/PEG/DNM）を開発し、マウス背部皮下に移植された腫瘍内における高拡散性をすでに確認した。我々は同薬剤を全身投与した際の薬剤分布を確認した。転移性肺腫瘍への特異的取り込みは認めなかったが、局所療法後の肺転移拒絶モデルにおいて、非移植コントロールマウスに比較し、腫瘍細胞の肺への拒絶を示した。新規薬剤を用いた本治療が腫瘍局所への抗腫瘍効果のみならず遠隔転移巣への治療効果を発揮する可能性を示唆した。

A．研究目的

我々は新規メラノーマ治療法を開発すべく平成17年度より３年間厚生労働省科学研究・医療機器開発推進研究事業（ナノメディシン）の補助下、医・工・化学連携によりナノ微粒子薬剤開発と磁場発生機器・治療施設の改良と化学・温熱・免疫「CTI（chemo-thermo-immuno）」療法の開発を行っている。基礎的動物実験の結果を基にメラノーマ腫瘍局所内投与に基づくCTI療法（第Ｉ世代）を、倫理委員会の許可を受け、臨床試験（学内限定第I,II相）を平成19年３月より開始した。現在まで４症例がエントリーされ、うち２例はCTI療法後、全身皮膚・リンパ節転移巣が完全・部分消失した。

我々はCTI療法の理念そのものの有用性を確認したが、新規薬剤と継続的維持療法の更なる開発が必要とされた。本申請は次世代型メラノーマ療法を確立するために必要な①全身投与が可能な新規薬剤と②次世代型化学・温熱・免疫（CTI）療法を開発する。

具体的には（1）メラノジェネシスを分子標的とした新規DDSとして我々が開発したNPrCAP（N-propionyl cysteaminylphenol）とデキストラン被覆マグネタイト・ナノ微粒子（DNM）結合体（NPrCAP/PEG/DNM）を基礎とし全身投与が可能な新規製剤を開発し、安定したGMP製剤の大量合成法を確立する。（2）NPrCAP/PEG/DNM製剤の安定性・抗腫瘍効果の解明とCTI療法分子標的・免疫機構の解析を行う。（3）CTI 療法により局所病変のみならず、遠隔転移巣への抗腫瘍効果を解析する。（4）新規薬剤の高拡散性を利用し、従来の局所療法においても、高い腫瘍特異的取り込みを達成する。腫瘍局所に対する高い抗腫瘍効果のみならず、メラノジェネシスへの選択的

取り込みに際し発生する酸化ストレスによる熱ショック蛋白とメラノソーム蛋白結合体の産生による化学・免疫効果を発現させる。これにより、肺等への遠隔転移巣に対する腫瘍拒絶効果を達成する。

B．研究方法

(1) 新規NPrCAP/PEG/DNMとメラノジェネシス標的との関連の化学療法、免疫療法効果至的治療効果の検討

新規製剤がメラノジェネシス酵素（チロシン酸化還元酵素）の基質であり、メラノーマ細胞に親和性を持つことが必須条件であるが、DDSとしてのメラノジェネシス標的機序を解明する。

(2) 新規NPrCAP/PEG/DNMの腫瘍治療効果・安全性の検討

新規製剤のin vivo 抗腫瘍効果を検討する。薬剤細胞殺効果及び直接の免疫誘導効果を検討する。我々の過去の動物を用いた研究方法はすでに確立されているので、既存製剤（NPrCAP/PEG/M、NPrCAP/M）新規開発製剤（NPrCAP/PEG/DNM）と比較した有効性について検討する。具体的には、深部臓器メラノーマ担癌マウスにCTI療法を行い、治療後に再度メラノーマを移植し、これら移植腫瘍への拒絶反応と宿主延命の効果をみる。また、同様に新規製剤の安全性についても平行して検討する。

(3) 新規NPrCAP/PEG/DNMを用いたCTI療法による遠隔転移巣腫瘍拒絶効果の検討

背部メラノーマ担癌マウスに対し、CTI療法を施行後、尾静脈よりメラノーマ細胞を注入した際の、腫瘍生着を検討する。

C．研究結果

1．新規薬剤(NPrCAP/PEG/DNM)の全身投与における薬剤分布を検討した。

メラノーマ細胞B16F010をマウス尾静脈より静注し、肺転移を生じさせ、本薬剤を投与した。薬剤投与４日後に各臓器への薬剤の分布を検討した。肺転移巣への選択的取り込みを期待したが、肺への取り込みは十分ではなく、肝、脾にほとんどは取り込まれた（図１）。

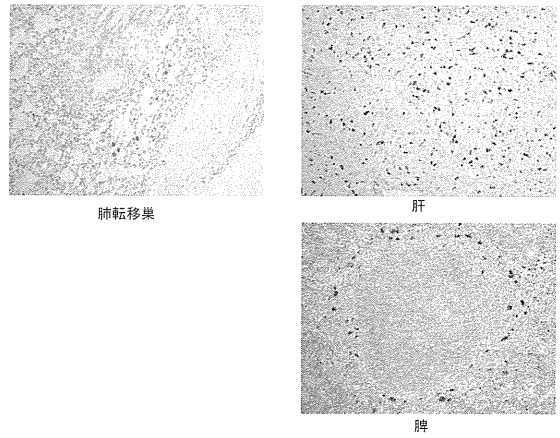


図１

2．新規薬剤(NPrCAP/PEG/DNM)の局所腫瘍内分散性の検討

新規薬剤を使用し、マウス背部に移植したメラノーマ細胞（B16F0）に対し、CTI治療を施行した。３日後の薬剤分布を検討した。従来の薬剤に比較し、高い拡散性を示し、（図２）磁場照射により、十分な温度上昇も得られた（図３）。

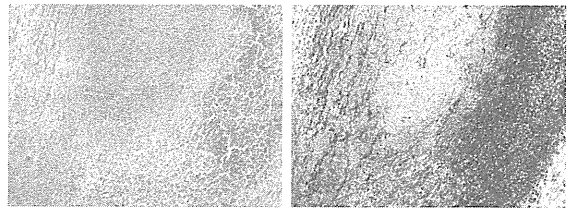


図２　　H&E　　ベルリンブルー染色体

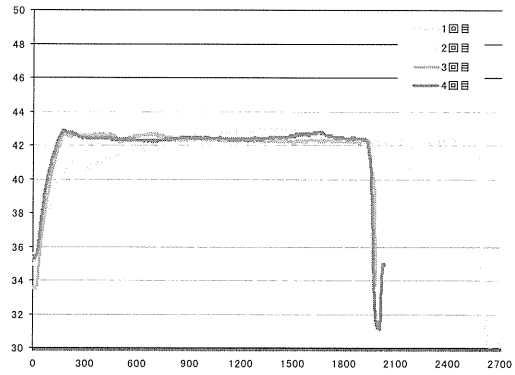


図３

2. 2　新規薬剤を用いたCTI療法による肺転移拒絶を検討した。

マウス背部皮下にメラノーマ細胞（B16F0）を移植後、CTI療法を施行し、肺転移を生じさせた。非移植コントロールマウスに比較し、肺転移は拒絶された（図４）。

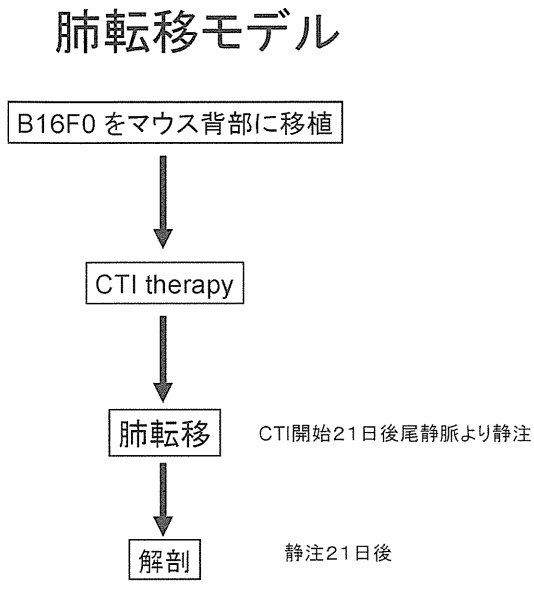


図４

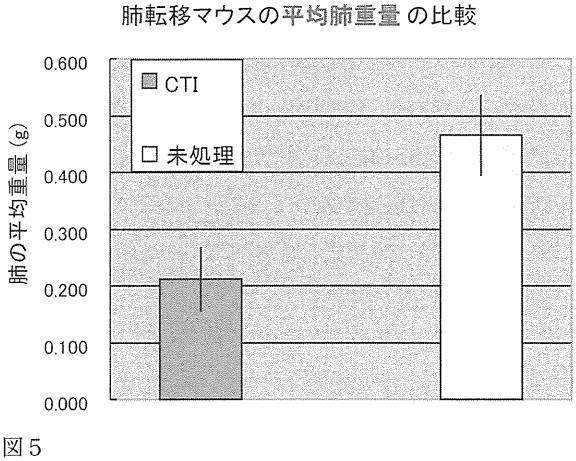


図５

CTI療法施行21日目に尾静脈よりメラノーマ細胞を静注した。21日後の肺重量を比較した。非移植群に比較し、CTI療法群では、肺重量の減少を示し、肺移植の拒絶を認めた（図５）。

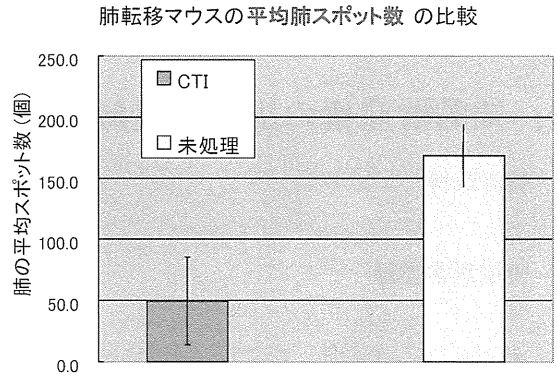


図５

同様に肺表面の腫瘍数を計測した。非移植群に比較し、CTI療法群では、平均肺スポットの減少を認め、肺移植の拒絶を認めた。

D．健康危険情報

E．研究発表

1．論文発表

Yoneta A, Horimoto K, Nakahashi K, Mori S, Maeda K, Yamashita T. A case of cystic basal cell carcinoma which shows a homogenous blue/black area under dermatoscopy. J Skin Cancer 2011; 450472: 1-4.

2．学会発表

Yoneta A, Nishizaka T, Hida T, Goto S, Ozawa M, Yamashita T: Two cases of advanced melanoma treated with dendritic cell vaccination therapy. 22nd World Congress of Dermatology. 2011 May 24-29. Seoul, Korea.

Yoneta A, Tamura Y, Nohara S, Ito A, Honda H, Wakamatsu K, Ito S, Yamashita T, Jimbow K: Development and evaluation of antitumor effect of novel NPrCAP-magnetite nanoparticles for chemo-thermo-immunotherapy in malignant melanoma. 21st International Pigment Cell

Conference. 2011 Sep 21-24: Bordeaux, France.

F．知的財産権の出願・登録状況

1．特許取得

2．実用新案登録

3．その他

厚生労働科学研究費補助金（医療機器開発推進研究事業）
総合研究報告書

「新規CTI治療薬剤の合成とGMP製剤化」に関する研究

野原　　聡　名糖産業株式会社 業務管理職

研究要旨

メラノジェネシスを分子標的とした新規DDS製剤として、NPrCAP（N-propionyl cysteaminyl phenol）と磁性ナノ粒子（DNM）との結合体:NPrCAP/PEG/DNM（図１参照）が提案/開発されてきた。しかし、従来の製剤は凝集しており製薬として注射投与するには不適であったため、最初の取り組みとして、H21年度に分散性の向上を目指しその改善に至った。翌22年度は改良製剤の大量合成-GMP化に着手し、その結果、合計14ロット（800mL相当）の合成が行われた。合成された新規製剤サンプルはH23年度より加温試験および急性/慢性毒性試験（前臨床試験の一部）に供され、将来の臨床試験に向けたデータ確保に貢献した。また、臨床で実用化されている加温機器：サーモトロンRF-8を利用した加温にも成功した。

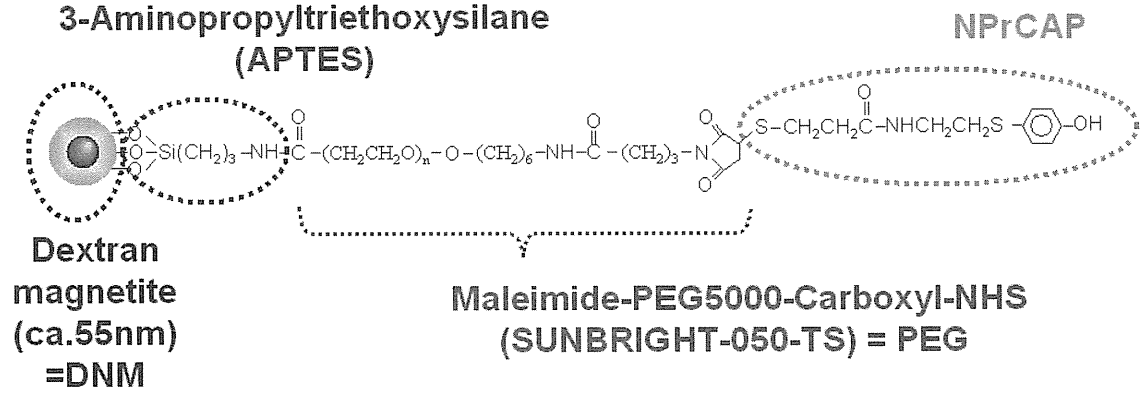


図１

A．研究目的

３ヵ年の研究計画としては、H21年度：分散性の改善（以降、新製剤と呼ぶ）、H22年度：新製剤の大量化とGMP化、H23年度：大量化した製剤を使用した前臨床試験の開始及び加温試験のデータ蓄積、を大まかな目的とした。各年度のさらに詳細については次に示す。

【H21年度：分散性改善＝新規製剤の創出】

旧製剤の凝集の原因について、合成の観点から検討し、改善することを目的とする。また、翌22年度からは量産化をGMP基準下で合成する計画としているため、名糖産業㈱名古屋研究所内に本資金にて専用クリーンルームを設置することを第２の目的とする。

【H22年度：新製剤の大量化とGMP化】

H21年度に創出した新製剤を、GMP基準に則った大量量産化の確立を目的とする。

【H23年度：前臨床試験と加温試験】

新規製剤を臨床試験に進めるため、第一ステップとなる前臨床試験に着手することを目的とする。また、本製剤の加温については、一般に誘導加温型装置を用いるが、該機器の医療分野への進出が遅々としている状況である。そこで、実現可能な代替機器の確保も今年度中の目的の一つとする。

B．研究方法

各課題（目的）に対する研究取り組み方法を項目毎に列記する。

【分散性の改善】

旧製剤の凝集原因は、NP r CAPとマグネタイトを連結させるスペーサーのAPTESによるランダムな粒子間反応に起因している可能性が高いため、その反応条件を検討/改良することによる。また、その他試薬の反応についても詳細に調査し、最適な反応条件を初年度一年以内に見出す。

【大量合成化-GMP】

上記分散性の改良および合成法の改良が達成された際には、次にGMP準拠マニュアル（Standard Operating Procedure）を作成し、それに則り反応の繰り返し再現性を確認する。（凡そ２年目の計画）

【前臨床試験の着手】

上記の再現性確認で得られるサンプルを使用し、ラット急性毒性、及び慢性毒性試験を㈱化合物安全性研究所に委託実施する。

【加温試験方法】

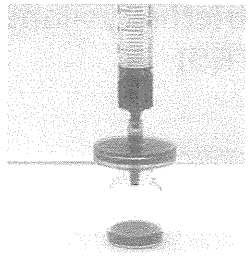
現在、本邦で唯一医療機器認可され、国内で約100台が普及しているハイパーサーミア装置：サーモトロンRF-8（山本ビニター株式会社）を利用することとする。本機器は所謂、誘電加温型であり、磁性ナノ粒子の発熱については一般概念化されていないが、本研究協力者の小林（中部大学教授）らの研究によると磁性ナノ粒子を効率良く発熱する報告がある（Jpn. J. Cancer Res., 90, 699, 1999）。よって、本研究でもこの研究を基に確証的なデータの取得を進める。

C．研究結果・考察

【分散性の改善】

反応条件を検討した結果、APTES反応時の鉄濃度を旧来の10mg/mLから１mg/mLに減じることにより、反応後も粒子径が殆ど変化しないことが判明した。これにより、新製剤は最終的に0.2umのフィルターが通過可能となり、薬剤を製造する上で極めて重要である滅菌充填が可能となった（写真１）。

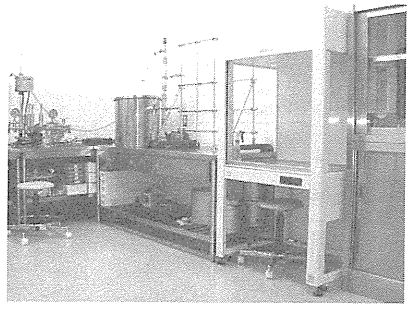
（写真１）



【GMP製剤化エリアの設置】

名糖産業㈱名古屋研究所内には写真２のとおり、専用クリーンルームが設置され（H21年12月）、翌年からのGMP準拠・新製剤量産化を行うエリアが基盤整備された。

（写真２、クリーンルーム）



【大量合成化】

最適化した条件を手本化したSOPを忠実に遵守し繰り返し合成を行った結果、計14ロット（800mL相当）を合成し、得られたサンプルは再現性が確認できた。これにより、製剤の規格を暫定的に決定した。

【前臨床試験】

㈱化合物安全性研究所に委託したラット急性毒性、慢性毒性の試験結果は、毒性に問題ないとのことであった。（詳細については別途記載）

【加温試験】

サーモトロンRF-8による加温試験は、分担研究者の井藤（九州大・准教授）及び山本ビニター㈱のご協力により本年度中に二度実施され、当社はその試験用サンプルを供給した。実験結果については別途井藤らの報告にある通り、高い発熱効果が確認できたため、今後は最適な薬剤濃度、投与量、加温時間（薬剤としての処方）を決定するための系統的データの取得が必要となる。

【その他】

当初の研究計画にはなかったが、本製剤の核である抗メラノーマ物質NP r CAPの機能解明の一助とすべく、また新たな応用可能性も目指し副題として検討した。

即ち、NP r CAPは水に溶けにくいという性質であることから、水溶性NPrCAPを開発すべく、水に対する溶解性が著しく高く、かつ粘性の低い多糖類であるデキストラン（グルコースのα1-6結合型高分子）との複合体を試作した。本試作品の評価は分担研究者の中山（川崎医大・教授）らにより行われ、その報告によると、サイトカイン、インターロイキン類の誘導作用を示すことが明らかになった。この効果はアポトーシス誘導剤としての応用可能性が考えられるため、現在、構造活性相関データの取得に着手し、より高い活性の開発と、メカニズムの解明を進めている。

D．健康危険情報

特になし

E．研究発表

1．論文発表　なし
2．学会発表　なし

F．知的財産権の出願・登録状況

1．特許取得　なし
2．実用新案登録　なし
3．その他　なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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 and Jimbow K	N-propionyl-cysteaminylphenol-magnetite conjugate (NPrCAP/M) is a nanoparticle for the targeted growth suppression of melanoma cells	J Invest Dermatol	129	2233-2241	2009
Jimbow K, Kamiya T and Hida T	Epidermal melanin unit and aging of skin; biological and molecular significance of pheomelanin in constitutive photo-aging process	加齢皮膚医学セミナー	4	53-64	2009
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Ⅳ．研究成果の刊行物・別刷

N-Propionyl-Cysteaminylphenol-Magnetite Conjugate (NPrCAP/M) Is a Nanoparticle for the Targeted Growth Suppression of Melanoma Cells

Makito Sato[1], Toshiharu Yamashita[1], Masae Ohkura[1], Yasue Osai[1], Akiko Sato[1], Tomoaki Takada[1], Hidenobu Matsusaka[1], Ichiro Ono[1], Yasuaki Tamura[2], Noriyuki Sato[2], Yasushi Sasaki[3], Akira Ito[4], Hiroyuki Honda[5], Kazumasa Wakamatsu[6], Shosuke Ito[6] and Kowichi Jimbow[1]

A magnetite nanoparticle, NPrCAP/M, was produced for intracellular hyperthermia treatment of melanoma by conjugating *N*-propionyl-cysteaminylphenol (NPrCAP) with magnetite and used for the study of selective targeting and degradation of melanoma cells. NPrCAP/M, like NPrCAP, was integrated as a substrate in the oxidative reaction by mushroom tyrosinase. Melanoma, but not non-melanoma, cells incorporated larger amounts of iron than magnetite from NPrCAP/M. When mice bearing a B16F1 melanoma and a lymphoma on opposite flanks were given NPrCAP/M, iron was observed only in B16F1 melanoma cells and iron particles (NPrCAP/M) were identified within late-stage melanosomes by electron microscopy. When cells were treated with NPrCAP/M or magnetite and heated to 43°C by an external alternating magnetic field (AMF), melanoma cells were degraded 1.7- to 5.4-fold more significantly by NPrCAP/M than by magnetite. Growth of transplanted B16 melanoma was suppressed effectively by NPrCAP/M-mediated hyperthermia, suggesting a clinical application of NPrCAP/M to lesional therapy for melanoma. Finally, melanoma cells treated with NPrCAP/M plus AMF showed little sub-G1 fraction and no caspase 3 activation, suggesting that the NPrCAP/M-mediated hyperthermia induced non-apoptotic cell death. These results suggest that NPrCAP/M may be useful in targeted therapy for melanoma by inducing non-apoptotic cell death after appropriate heating by the AMF.

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INTRODUCTION

Although early lesions of primary melanoma are curable by excision, successful treatment of metastatic melanoma has been elusive thus far. The current systemic therapies have little effect on the overall survival period or rate of advanced melanoma (Balch *et al.*, 2001). Because melanogenesis is inherently toxic and uniquely expressed in melanocytic cells, tyrosine analogs can be good candidates for melanoma-specific targeting and therapy (Jimbow *et al.*, 1993). To develop melanocytotoxic compounds for rational chemotherapy for melanoma, *N*-acetyl-cysteaminylphenol and *N*-propionyl-cysteaminylphenol (NPrCAP) were synthesized. These compounds showed selective cytotoxicity against melanoma cells *in vivo* and *in vitro* (Jimbow *et al.*, 1984; Miura *et al.*, 1990; Alena *et al.*, 1994; Tandon *et al.*, 1998). They have both cytostatic and cytocidal effects on melanoma cells (Thomas *et al.*, 1999), and induce apoptosis in follicular melanocytes of mice (Minamitsuji *et al.*, 1999). Thus, these synthetic compounds would provide the basis for the development of novel anti-melanoma agents.

Iron oxide and magnetite nanoparticles are becoming versatile tools for medical imaging of lymph nodes and are excellent candidates for hyperthermia induced by an external alternating magnetic field (AMF) due to the loss of hysteresis (Leary *et al.*, 2006; van Vlerken and Amiji, 2006). Local hyperthermia is induced in tumors by injecting magnetite nanoparticles into the core of the solid tumor and AMF irradiation results in shrinkage of animal tumors (Luderer *et al.*, 1983; Minamimura *et al.*, 2000). Magnetite cationic liposomes (MCL) have been generated for the selective accumulation of magnetite nanoparticles in tumor tissues,

[1]*Department of Dermatology, Sapporo Medical University, Sapporo, Japan;* [2]*Department of 1st Pathology, Sapporo Medical University, Sapporo, Japan;* [3]*Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University, Sapporo, Japan;* [4]*Department of Chemical Engineering, Faculty of Engineering, Kyushu University, Fukuoka, Japan;* [5]*Department of Biotechnology, Nagoya University, Nagoya, Japan and* [6]*Department of Chemistry, Fujita Health University, Aichi, Japan*

Correspondence: Dr Toshiharu Yamashita, Department of Dermatology, Sapporo Medical University School of Medicine, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan. E-mail: yamasita@sapmed.ac.jp

Abbreviations: 4-S-CAP, 4-S-cysteaminylphenol; AMF, alternating magnetic field; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MCL, magnetite cationic liposome; NPrCAP, N-propionyl-(4-S-) cysteaminylphenol; PBS, phosphate-buffered saline

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and MCL-mediated hyperthermia has inhibited growth or induced complete regression of various tumors in the transplanted animals (Yanase *et al.*, 1998; Ito *et al.*, 2003; Kawai *et al.*, 2005). If the magnetite nanoparticle technology is taken a step farther to achieve a selective delivery system to tumors, guided hyperthermia could be achieved for treatment of metastatic tumors.

Recently, we synthesized an MCL in which NPrCAP was encapsulated within the liposomes, resulting in both intracellular hyperthermia and cytotoxicity when injected into animal melanoma (Ito *et al.*, 2007). Here, we introduce another magnetite nanoparticle, NPrCAP/M, to which NPrCAP was superficially bound to enhance its targeting activity to melanoma cells. The possible mechanisms of NPrCAP/M-mediated hyperthermia against melanoma are discussed.

RESULTS

Incorporation of *N*-(1-mercaptopropionyl)-4-*S*-cysteaminylphenol (NPrCAP-SH) with magnetite

The degree of incorporation of NPrCAP-SH with magnetite was determined by HCl hydrolysis of NPrCAP/M followed by HPLC analysis of the 4-*S*-cysteaminylphenol (4-*S*-CAP) produced. We measured 4-*S*-CAP as an index of the degree of NPrCAP-SH incorporation as they share the same structural units. The results indicated that the degree of incorporation of NPrCAP-SH with magnetite was 405 nmol mg^{-1} magnetite. When B16F1 cells were cultured in NPrCAP/M-containing medium, collected, and exposed to the AMF generator, the temperature rose sharply from 30 to 50°C within 10 minutes and decreased immediately after the machine was switched off (Figure 1).

NPrCAP/M as substrate for tyrosinase

We examined whether NPrCAP/M could act as a substrate for tyrosinase. 4-*S*-CAP itself was found to be a good substrate for mushroom tyrosinase; tyrosinase oxidation of 4-*S*-CAP (100 μM) in the presence of cysteine yielded 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol through *ortho*-quinone within 10 minutes. HPLC showed that the reaction was almost completed within 10 minutes with half of the 4-*S*-CAP remaining after 4.2 minutes. At the same time, the expected catechol derivative was produced at 85 μM (85% yield) at 10 minutes. As NPrCAP/M has the same structural units as 4-*S*-CAP, it was expected to be a substrate for tyrosinase. If this were the case, 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol would be obtained by HCl hydrolysis of the cysteinylcatechol derivative of NPrCAP/M produced after tyrosinase oxidation of NPrCAP/M in the presence of cysteine. NPrCAP/M fell to half of the initial concentration after 69 minutes, and the concentration of 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol produced after 3 hours was 80 μM (80% yield) (Figure 2). Thus, the ratio of 4-*S*-CAP to NPrCAP/M in the reaction velocity on tyrosinase oxidation was 16. These results indicate that NPrCAP/M served as a substrate for mushroom tyrosinase.

Measurement of the magnetite incorporated into cells treated with NPrCAP/M

To examine whether NPrCAP/M could be incorporated into melanoma cells more preferentially than magnetite alone, we compared amounts of iron molecules in cells after culture in the NPrCAP/M- or magnetite-containing medium. To prevent non-specific adsorption of the particles to the cells, culture flasks were filled with NPrCAP/M-containing medium and rotated. After cells were collected and lysed, the amount of iron was measured. As shown in Figure 3, MM418, 70W, B16F1, SK-mel-23, TXM18, AK-1, and 96E melanoma cells incorporated large amounts of iron derived from NPrCAP/M compared with that from magnetite alone. Primary human melanocytes and non-pigmented SK-mel-24 and SK-mel-118 cells captured a relatively large amount of NPrCAP/M; however, the amount was not significantly different from that from magnetite treatment or almost the same as for magnetite (Figure 3).

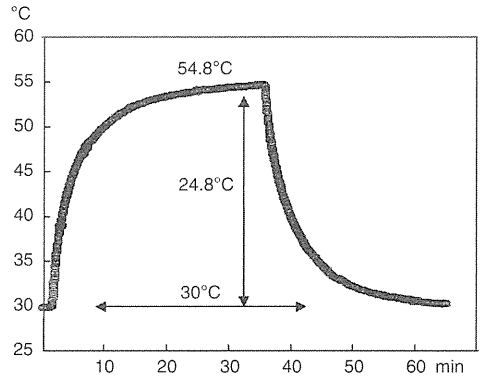

Figure 1. Heat generation in cells treated with NPrCAP/M and irradiated by AMF. 2×10^6 B16F1 cells were cultured in NPrCAP/M (5.0 mg magnetite equivalent)-containing medium for 30 minutes, collected, and exposed to the center of the coil of the AMF generator. The temperature at the center of the cell pellets was measured using an optical fiber probe. A rapid increase and decrease in temperature were observed in the cell pellet during AMF irradiation.

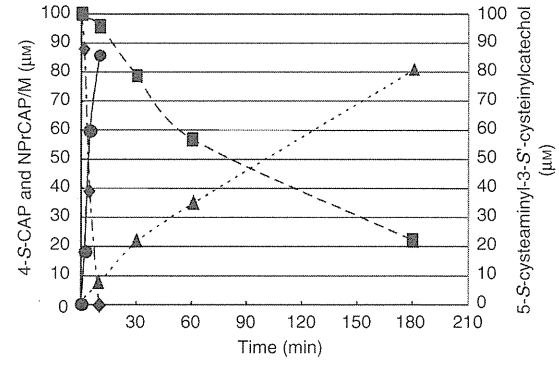

Figure 2. NPrCAP/M is incorporated into the tyrosinase oxidative reaction *in vitro*. The concentrations of the substrate remaining as 4-*S*-CAP and the 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol produced were measured by HPLC analysis after hydrolysis with HCl. ◆: 4-*S*-CAP, ■: NPrCAP/M, ●: 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol from 4-*S*-CAP, ▲: 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol from NPrCAP/M.

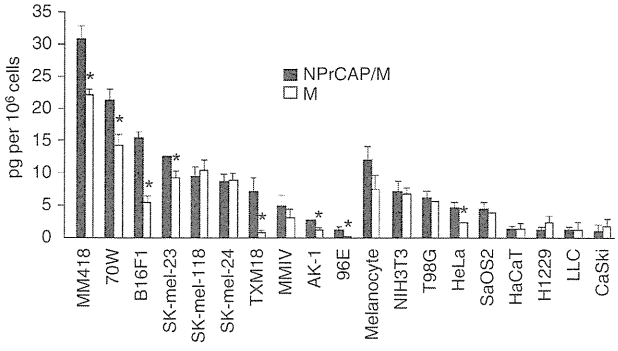

Figure 3. **Uptake of magnetite nanoparticles into melanoma and non-melanoma cells.** 75 cm^2 flasks containing growing cells were filled with NPrCAP/M- or magnetite-containing medium and fixed on a slanted disc, which was rotated slowly for 30 minutes. Incorporated iron was measured by the potassium thiocyanate method. Melanoma cell line names (MM418 to 96E) are written in gothic. Data and bars are mean ± SD of three independent experiments (*$P<0.05$).

NPrCAP/M is delivered to transplanted B16F1 melanomas

We then tested whether NPrCAP/M could be delivered to B16F1 melanoma tumors transplanted into syngeneic C57BL/6 mice. In five sets of experiments, each of which consisted of three to five mice, we transplanted a B16F1 melanoma onto the left flank and an EG7 or RMA lymphoma onto the right flank, and we injected NPrCAP/M or magnetite into the intraperitoneal cavity. After being allowed to grow for 2 weeks, tumors were excised and examined for the presence of iron (NPrCAP/M) by Berlin blue staining. Blue-stained cells were detected in 11 of the 14 melanomas, but in none of the 14 lymphomas (Figure 4a and b, Table 1). Meanwhile, in the B16F1- and EG7-bearing mice given magnetite, blue-stained tumor cells were not detected in either the melanoma or lymphoma tissues.

B16F1 melanomas were removed and examined for the subcellular localization of iron particles by electron microscopy. B16F1 cells in the NPrCAP/M-injected mice contained iron particles within dense ellipsoidal organelles, corresponding to late-stage melanosomes (data not shown). This suggested that NPrCAP/M was finally delivered to the melanogenesis system of the melanocytic cells.

Cytotoxic effects of magnetically mediated hyperthermia on melanoma cells

Because melanoma cells preferentially take up NPrCAP/M, it was expected that NPrCAP/M-treated melanoma cells would be selectively degraded by the AMF irradiation. MM418, SK-mel-23, B16F1, and TXM18 melanoma and H1229, HaCaT, HeLa, and SaOS2 non-melanoma cells were cultured in the NPrCAP/M- or magnetite-containing medium, collected, and irradiated by AMF at 43°C for 30 minutes. Figure 5 shows the results for NPrCAP/M- or magnetite-treated cells with or without hyperthermia induced by AMF. All the melanoma cells tested were degraded more significantly by NPrCAP/M with AMF than by magnetite with AMF, with differences ranging from 1.7-fold in SK-mel-23 to 5.4-fold in B16F1 cells (Figure 5a), whereas non-melanoma cells were degraded

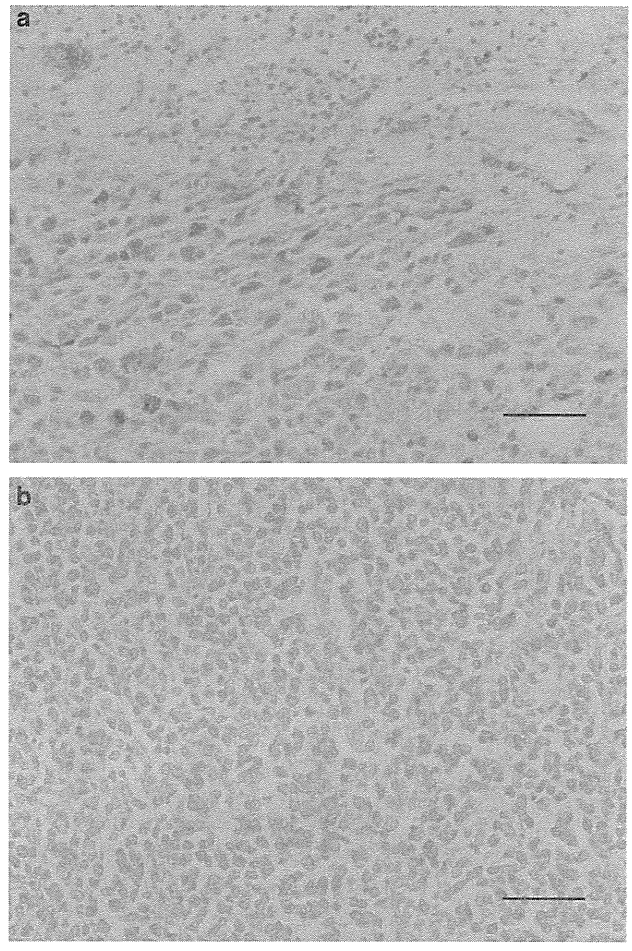

Figure 4. **Intraperitoneal NPrCAP/M nanoparticles were delivered to the subcutaneously transplanted melanoma tumors.** Mice bearing B16F1 and lymphoma tumors received i.p. administration of NPrCAP/M and were maintained for 14 days. Tumors were then removed and processed for hematoxylin-eosin and Berlin blue staining. Iron-containing blue-stained tumor cells were detected in the B16F1 tissues (**a**), but not in the EG7 (**b**) or RMA lymphoma tissues. Data are for five or four independent mice, each. Bars represent 50 μm (**a**, **b**).

almost equally by NPrCAP/M and magnetite (Figure 5b). These results suggested that NPrCAP/M could induce the death of melanoma cells more selectively and significantly than that of non-melanoma cells at the relatively low temperature of 43°C.

Hyperthermia mediated by NPrCAP/M effectively suppresses growth of mouse melanoma

To evaluate whether NPrCAP/M-mediated hyperthermia could suppress melanoma in the mouse model, we treated the subcutaneously transplanted B16F1 melanoma and measured the volumes of tumors (Figure 6). As shown in Figure 6a, B16F1 melanoma in mice treated by magnetite injection followed by AMF irradiation and NPrCAP/M injection followed by AMF irradiation resulted in statistically significant suppression of tumor growth compared with the untreated melanoma (Figure 6a and b). NPrCAP/M-mediated hyperthermia seemed to suppress growth of the melanoma

more than hyperthermia mediated by magnetite alone; however, differences between the two groups were not statistically significant.

Table 1. Presence of iron-containing tumor cells in mice injected with NPrCAP/M or magnetite

	Number of mice bearing a Berlin-blue positive tumor/number of total mice tested		
	B16F1	EG7	RMA
Exp I NPrCAP/M	5/5	0/5	NT
Exp II NPrCAP/M	3/4	NT	0/4
Exp III NPrCAP/M	3/5	0/5	NT
Exp IV magnetite	0/3	0/3	NT
Exp V magnetite	0/3	NT	0/3

Mice bearing B16F1 melanoma and EG7 or RMA lymphoma on the left and right flanks, respectively, were intraperitoneally given NPrCAP/M or magnetite. Tumors were excised and the presence of iron was examined by Berlin-blue staining.

Non-apoptotic cell death is induced by NPrCAP-mediated intracellular hyperthermia

Cellular DNA was prepared after cells had been cultured in the NPrCAP/M-containing medium followed by AMF at 43°C and subjected to analysis by flow cytometry. B16F1 and SK-mel-23 cells infected with a recombinant adenovirus expressing Ad-p63′ showed evident sub-G1 fractions, whereas cells subjected to NPrCAP/M-mediated hyperthermia contained little sub-G1 DNA (Figure 7). Levels of caspases 3, 8, and 9 in B16F1 and SK-mel-23 cells after NPrCAP/M-mediated hyperthermia were as low as those without NPrCAP/M or after NPrCAP/M treatment without hyperthermia (Figure 8). These results suggested that NPrCAP/M-mediated hyperthermia induced non-apoptotic cell death or necrosis.

DISCUSSION

The temperature at the center of the pellet of NPrCAP/M-treated B16F1 cells rose to over 50°C within 10 minutes; thus, the NPrCAP/M was a good heat generator, comparable to MCL or 4-S-CAP-loaded magnetite (Shinkai *et al.*, 1996;

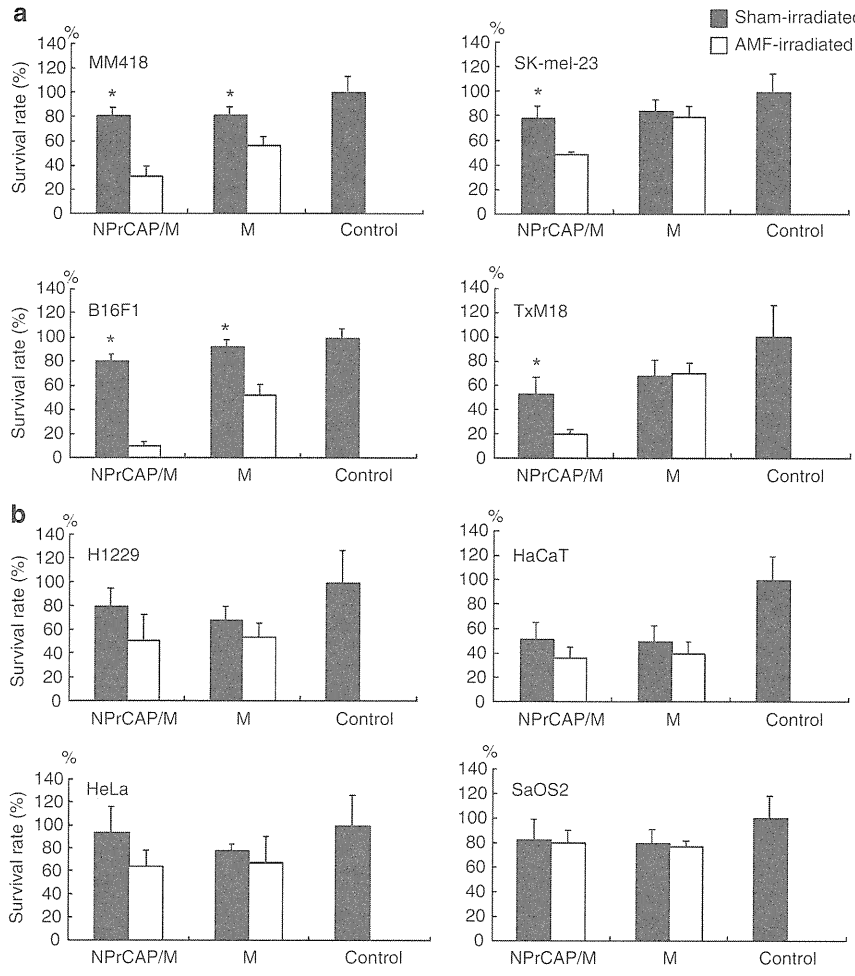

Figure 5. NPrCAP/M plus AMF treatment degraded melanoma cells more significantly than NPrCAP/M without the AMF. After cells were cultured in the NPrCAP/M-containing medium and collected, cell pellets were exposed to sham (■) or AMF (□) irradiation. Treated cells were collected and the number of viable cells not stained by trypan blue was counted. Data and bars are mean ± SD of three independent experiments (*$P<0.05$). (**a** and **b**) show results for melanoma and non-melanoma cell lines, respectively.

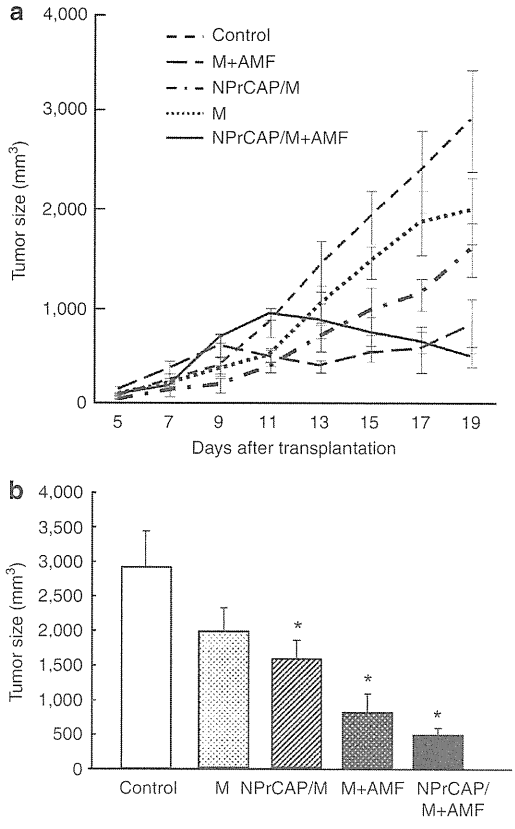

Figure 6. Tumor volumes of the B16F1-bearing mice treated by magnetite- and NPrCAP/M-mediated hyperthermia. (**a**) Comparison of groups in the first 19 days after tumor transplantation. Magnetite or NPrCAP/M (4 mg of magnetite or its equivalent) was injected directly into subcutaneous B16F1 tumors, which were then irradiated with an AMF at 46 °C for 30 minutes. Each point represents the mean ± SD of five mice. All data are presented as mean ± SD. (**b**) Comparison of tumor volumes in each group on the 19th day. *$P<0.005$, tumors treated by NPrCAP/M with the AMF, magnetite (M) with the AMF, and NPrCAP/M without the AMF were significantly different from those of control mice.

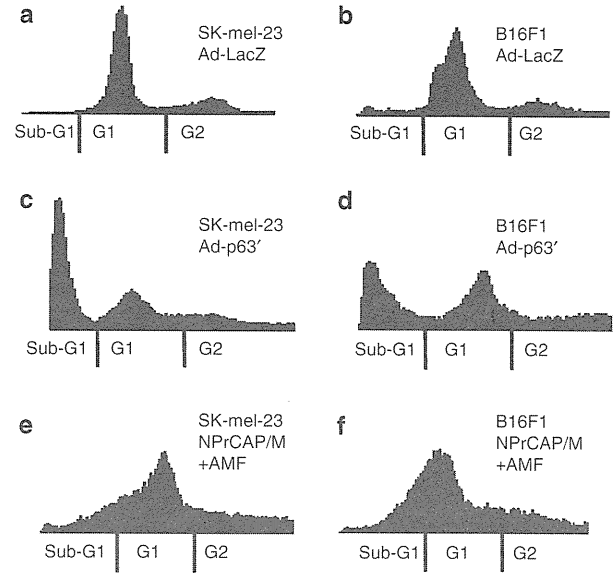

Figure 7. The sub-G1 fraction was not observed in NPrCAP/M-treated and AMF exposed cells. After cells were treated with NPrCAP/M followed by AMF irradiation and culture for 24 or 48 hours, adherent and floating cells were collected and sub-G1, G1, S, and G2/M populations were quantified with a FACScan Cell Sorter. (**a**): SK-mel-23 with Ad-LacZ, (**b**): B16F1 with LacZ, (**c**): SK-mel-23 with Ad-p63′, (**d**): B16F1 with Ad-p63′, (**e**): SK-mel-23 with NPrCAP/M + AMF, (**f**): B16F1 with NPrCAP/M + AMF.

Yanase *et al.*, 1997; Ito *et al.*, 2007). To examine NPrCAP/M as a tyrosinase substrate, we could not use the spectrophotometric assay owing to the brown suspension of the substrate. Thus, we used a method based on the fact that *ortho*-quinone obtained from tyrosinase oxidation of the substrate can be trapped with cysteine, and we monitored the cysteine adduct with HPLC. Tandon *et al.* (1998) have reported that NPrCAP is a very good substrate for tyrosinase and the enzyme's kinetic parameters (K_m and V_{max}) were found to be similar to those of the homolog *N*-acetyl-4-*S*-CAP. They also reported that the K_m values for 4-*S*-CAP and NPrCAP were 117 and 340 μM, whereas the V_{max} values were 39.0 and 5.4 μmol min^{-1} mg^{-1} of protein, respectively. In this study, the ratio of 4-*S*-CAP to NPrCAP/M in the reaction velocity on tyrosine oxidation was 16. The reaction velocity was not as good as for 4-*S*-CAP (Figure 2). However, it should be efficient enough, if we consider the bulky structure of NPrCAP/M.

Pigmented melanoma cells, such as B16F1, MM418, 70W, and SK-mel-23, captured larger amounts of iron from NPrCAP/M than from magnetite (Figure 3). These pigmented melanoma cells also captured magnetite particles without NPrCAP, although the amount of iron from the magnetite was lower than that from NPrCAP/M. It is unclear why non-pigmented AK-1 and 96E and non-melanoma HeLa cells incorporated NPrCAP/M more efficiently than magnetite. It is possible that an unidentified receptor for cysteaminyl-phenols might be present on the surface or in the cytoplasm of the cells. When mice bearing B16F1 melanoma were intraperitoneally injected with NPrCAP/M, a total of 11 of 14 melanoma tissues on 14 mice contained B16F1 cells showing Berlin blue iron staining. As NPrCAP/M was injected into the peritoneal cavity in the mice, the nanoparticles were delivered to the B16 melanoma in the subcutis through the bloodstream. In the B16F1 tumors in the mice injected with magnetite, blue-stained cells were detected in the encapsulating fibroblast-like cells, but not in the tumor cells, suggesting that NPrCAP/M, but not magnetite, was preferentially delivered to the B16F1 cells. However, a large part of NPrCAP/M given i.p. was captured in reticuloendothelial cell systems such as the liver and spleen in the mice (data not shown). Clinical trials using the present magnetite-NPrCAP nanoparticles might be limited to lesional therapy against melanoma. We have proceeded to a phase I/II study of the effect of NPrCAP/M-mediated hyperthermia not only on treated tumors but also on non-treated metastatic tumors.

Hyperthermia reduces cell viability and proliferation in a time- and temperature-dependent manner in melanoma