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Review

# Utilize conjugated melanotropins for the earlier diagnosis and treatment of melanoma

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ABSTRACT

Peptides serve as effective drugs and contrast agents in the clinic today. However the inherent drawbacks of peptide structures can limit their efficacy as drugs. To overcome this we have been developing new methods to create 'tailor-made' peptides and peptide mimetics with improved pharmacological and physical properties. In this work we introduce novel peptide and small molecule conjugated molecules for earlier diagnosis and treatment of melanoma.

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**Contents**



## 1. Introduction

Multifunctional nanoparticles (MNPs) have received much increasing interest due to their envisioned advanced technological and biomedical applications (Pankhurst et al., 2003; Tartaj et al., 2005). For example, by incorporating magnetic properties and luminescent properties into the same nanoparticle, it is possible to manipulate these materials within tailored magnetic fields, and to achieve sensitive read-out by making use of the non-photobleaching properties of the luminescent particle. Through the incorporation of highly specific targeting agents and other functional ligands, such as fluorophores and permeation enhancers, the applicability and efficacy of these MNPs have greatly increased. The magnetic core is capable of generating enhanced contrast in magnetic resonance imaging (MRI) and the heat generated due to its movement in an alternating magnetic field can cause thermal death (magnetic fluid hyperthermia or MFH) of tumor cells (Corot et al., 2004, 2006). Our latest work of developing peptides and small conjugated molecules provides an

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overview of the experimental methods for peptide and small molecule biomarker conjugation with an emphasis on specific applications for therapeutics and biomedical research.

The human melanocortin 1 receptor ($hMC_1R$) is widely distributed both in the peripheral and central nervous systems in humans regulating skin pigmentation, immune response etc. (Hadley, 1988). The alpha-melanocyte-stimulating hormone (alpha-MSH: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Lys-Pro-Val-$NH_2$) is the endogenous nonselective agonist towards all subtypes of melanocortin receptors (Hruby, 1982; Hruby et al., 1987) except for the human melanocortin 2 receptor. Alpha-MSH and its analogs binding to the melanoma melanocortin 1 receptor is of interest with regard to its potential use in targeting cytotoxic drugs or imaging of melanoma (Hruby et al., 1992, 1998; Hruby, 2001, 2002; Lunec et al., 1992). Tools such as iodinated, photoaffinity-labeled, biotinylated and fluorescent melanocortins are widely used to study the fate of the ligand during its interaction with the receptor (Sharma et al., 1996; Tatro et al., 1992; Libert et al., 1989; Al-Obeidi et al., 1990; Niles and Makarski, 1978; Mourino et al., 1991). We demonstrate the feasibility of integrated nano-particle conjugated to α-MSH analogs, both peptides MTII: Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-$NH_2$ (Al-Obeidi et al., 1989a, 1989b) and small molecules (THIQ: N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1ylmethyl) piperidin-1-yl]-2-oxoethylamine) (Sebhat et al., 2002) that effectively image melanocortin receptors overexpressed on HEK293 cells.

## 2. Material and methods

### 2.1. Peptide synthesis

The super potent agonists of human melanocortin 1 receptor, MTII and the Rho-MTII, are synthesized following the standard procedure from previous work (Fig. 1a) (Cai et al., 2004a, 2004b). All peptides in this project will be synthesized manually by the $N^{\alpha}$-Fmoc solid phase methodology,(Cai et al., 2005a, 2005b) using Bromophenol Blue pH indicator to monitor the extent of coupling reactions as described by Krchnak et al. (1988) Rink amide AM resin (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl) phenoxy resin, 0.5 g, 0.637 mmol/g) was placed into a 50 ml polypropylene syringe with the frit on the bottom and swollen in DMF (20 ml) for 1 h. The Fmoc protecting group on the Rink linker was removed by 25% piperidine in DMF (1×5 min and 1×15 min). The resin is washed with DMF (4×15 ml), then washed with 0.02 M HOBt solution in DMF, stained with 0.05 mM solution of Bromophenol Blue in 0.02 M HOBt/DMF solution, and wash with 0.02 M HOBt/DMF solution (4×15 ml). The first $N^{\alpha}$-Fmoc amino acid ml coupled using preactivated ester (3 eq. of $N^{\alpha}$-Fmoc amino acid, 3 eq. of HOBt, and 3 eq. of DIC) in DMF. The coupling mixture was transferred into the syringe with the resin and shaken for 60 min, at which point the blue color of the resin changed to yellow, indicating complete coupling. The resin was washed with DMF (3×15 ml), and with DCM (3×15 ml), and the unreacted amino groups were capped using acetic anhydride (2 ml) and pyridine (2 ml) in DCM (15 ml) for 30 min, and then the resin was once again washed with DMF (6×15 ml). The peptide sequences are completed by consecutively coupling the appropriate amino acids and then the dicarboxylic acid linkers using the procedure described above. Pyrazinedicarboxylic and succinic acids were converted into their corresponding monoallyl esters prior to appending to the peptides to minimize competing formation of cyclic imides, as previously described (Mayorov et al., 2008). The other dicarboxylic acids were used as commercially available. The orthogonal allylic protection for the side chain of $Lys^{11}$ and the linker (if applicable) was removed with 0.1 eq. $Pd(PPh_3)_4$/20 eq. $PhSiH_3$ in DCM (2×30 min) prior to the peptide cyclization (Mayorov et al., 2006a, 2006b). The deprotected resin-bound peptide was washed with DCM (6×5 ml), and DMF (3×5 ml). The peptide cyclizations were accomplished as described previously (Mayorov et al., 2008), with 6 eq. DIC, 6 eq. Cl-HOBt in THF (36 h), and were monitored by Kaiser ninhydrin test (Kaiser et al., 1970). The DIC/Cl-HOBt treatment was repeated until a negative Kaiser test was obtained. Upon completion of cyclization the resin was treated with 5% solution of sodium diethyldithiocarbamate trihydrate in DMF (20 min) to remove any remaining traces of the Pd catalyst (Mayorov et al., 2006a, 2006b, 2008), then washed with DMF (5×15 ml), DCM (3×15 ml), methanol (5×15 ml), and diethyl ether (5×15 ml), and dried under reduced pressure (16 h). The cyclized peptides were cleaved off the solid support with 82.5% v/v TFA, 5% water, 5% thioanisol, 2.5% 1,2-ethanedithiol, and 5% phenol (5 ml, 3 h), and the crude peptides were precipitated out by the addition of a chilled 3:1 mixture of diethyl ether and petroleum ether (50 ml) to give white precipitates. The resulting peptide suspensions were centrifuged for 10 min at 6500 rpm, and the liquid was decanted. The crude peptides were washed with diethyl ether (4×50 ml), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues were dissolved in 2 M acetic acid, and the insoluble impurities were removed by passing the solutions through Gelman Laboratory Acrodisc 13 mm syringe filters with 0.45 μM PTFE membranes (Pall Corporation, East Hills, NY). The clear filtrates were lyophilized, the obtained white powders (50–80 mg) are redissolved in glacial acetic acid (1 ml), the resulting solutions were diluted with water (4 ml) to a peptide concentration of about 10–15 mg/ml, and passed through a Sephadex G-15 column (520×30 mm) using 1 M aqueous acetic acid as the eluent. Fractions containing the target peptides, as determined by TLC, were combined and lyophilized. Final purification was accomplished by preparative RP-HPLC on a $C_{18}$-bonded silica column (Vydac 218TP152022, 250×22 mm, 15–20 μm, 300 Å) using a Shimadzu SCL-10A HPLC system. The peptides are eluted with a linear gradient of 20–80% acetonitrile in 0.1% aqueous TFA solution over 50 min with 10 ml/min flow rate. The purified peptides were isolated in 25–30% overall yield. The structures of the pure peptides were confirmed by $^1$H NMR in DMSO-$d_6$ and by high resolution electrospray ionization (ESI) mass-spectrometry using an IonSpec Fourier transform mass spectrometer with a HiRes ESI source.

### 2.2. Peptide labeling

Dissolve 5.0 mg of H-Aca-Nle-c[Asp-His-*D*-Phe-Arg-Trp-Lys-$NH_2$ (H-Aca-MTII) in 200 μl of N,N-dimethylformamide (DMF). In a 10 ml flask, dissolve 2 mg of the integrated nano-particles (its carboxylic acid form) in 300 μl DMF. Add 20–30 mg of the resin-bound carbodiimide (EDC). Allow to stir at room temperature for 5 min. Add the peptide solution, and then 50 μl diisopropylethylamine (DIPEA). Leave stirring in the dark for 4–5 h. Put a small piece of glass wool into a glass pipette and filter the reaction mixture through the glass wool plug. Rinse with DMF (the resin will be highly colored with excess dye and should remain so after washes).

Collect the filtrate into a round-bottom flask and rotovap DMF off. Dissolve the dark syrupy residue in 1 M acetic acid and lyophilize. Purify by a gradual stepwise 50 min gradient (10% acetonitrile/0.1% TFA-water—80% acetonitrile/0.1% TFA-water) (Cai et al., 2004a, 2004b).

The schematic synthesis of Rho-THIQ are shown in Fig. 1b.

### 2.3. Biological activity assays. Receptor binding assays

Competition binding assay are carried out using commercial available melanoma cells A375 (ATCC) as described previously (Cai et al., 2005a, 2005b, 2004a) using [$^{125}$I]-[$Nle^4$,D-$Phe^7$]-α-MSH (Perkin Elmer Life Science) as the radioligand.

### 2.4. Data analysis

$IC_{50}$ and $EC_{50}$ values represent the mean of two experiments performed in triplicate. $IC_{50}$ and $EC_{50}$ estimates and their associated

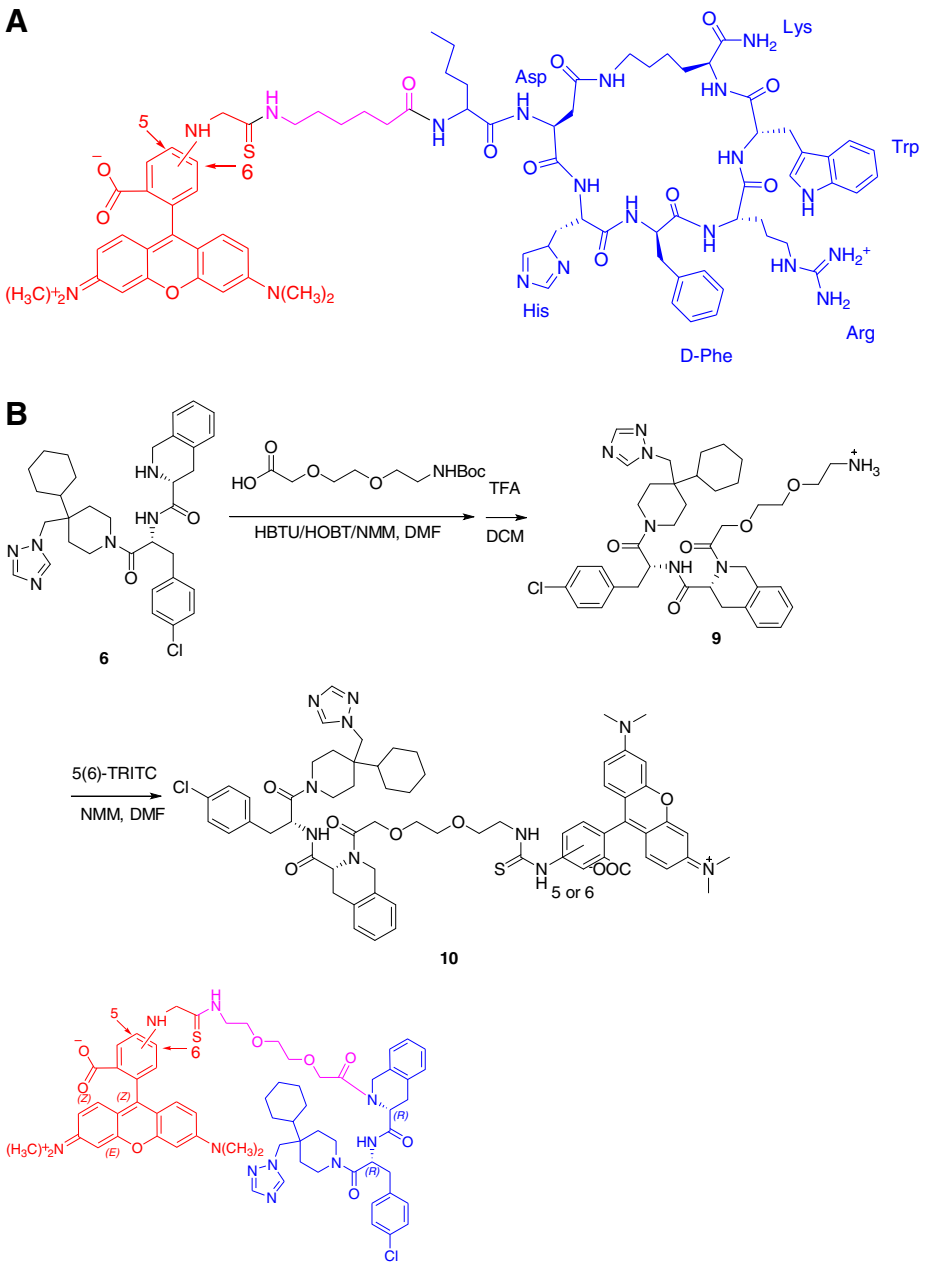



**Fig. 1.** (A) Rho-MTII. (B) Schematic synthesis of Rho-THIQ.

standard errors were determined by fitting the data using a nonlinear least squares analysis, with the help of GraphPad Prism 5 (GraphPad Software, San Diego, CA).

### 2.5. Confocal fluorescence laser scanning microscopy

To directly visualize agonist mediated receptor internalization and β-arrestins re-localization, human melanoma cells (A375) were grown on 35 mm Petri-dishes to 50% confluency, and transiently co-transfected with cDNAs encoding the β-arrestin2-GFP fusion proteins. 48 h later, the cells were washed with MEM medium without serum, and Rho-MTII with the binding buffer were added to a final concentration of 10 nM. Confocal fluorescent microscopy was performed on a BioRad laser scanning microscope using a 63×1.4 numerical aperture water immersion lens with dual line-switching excitation—(488 nm for GFP, and 500 nm for Rhodamine) and emission (515–540 nm GFP, and 570 nm for Rhodamine) filter sets.

### 2.6. Animal studies

The human melanoma A375 cell line is obtained from the American Type Culture Collection (ATCC). Using the Experimental Mouse Shared Service (EMSS) at the Arizona Cancer Center (AZCC), the cells will be grown in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin streptomycin and maintained in a humidified atmosphere of air containing 5% $CO_2$ at 37 °C. When the cell cultures are grown to 95%–100% confluence, the cells will be harvested by incubation with 1 ml trypsin solution/5 ml of Hanks balanced salt solution. The cells (90–100% viability) will be counted and resuspended in sterile saline. A total volume of 200 μl with $10^7$ cells will be injected subcutaneously into the right shoulder area of athymic nude mice (CD1 nu/nu mice) from Charles River Laboratories (Wilmington, MA). The mice are housed under pathogen-free conditions in micro-isolator cages with laboratory chow and water available. The volume of tumor is monitored every other day. The mice are imaged when tumors

reach a size of 300–500 $mm^3$ using a multiple modality low-light imaging system, Arizona Box. The Arizona Box was built to provide a versatile projection imaging system for low-light modalities such as chemiluminescence, phosphorescence, fluorescence, and even gamma ray imaging. The imaging system utilizes a back-illuminated CCD (VersArray 1300B; Roper Scientific) that provides a large field of view, high quantum efficiency, and very low noise. The CCD has a 1340×1300 imaging array and 20×20-micron pixels that provide a large imaging area with high spatial resolution. The CCD is cooled to −100 °C, at which temperature its dark current is essentially negligible (4 electrons/h). The mice with A375 xenografts are anesthesized with 1%–2% isoflurane. Anesthetized mice are placed on a heated 8-inch square specimen stage. The animal is so positioned that the tumor localizes in the center of field of view. Fluorescent Rhodamine labeled peptide is injected via the tail vein. Five minutes postinjection, the mice are imaged with excitation at 561 nm and emission at 609 nm using readout speed of 50 kHz, in which the readout noise level is reduced to 4 electrons per pixel. The fluorescence emission is captured using Chroma Technology optical filters mounted in standard camera lens filter rings. The images are repeatedly acquired at 1, 3, 6, and 24 h post-injection.

## 3. Results and discussion

Herein, we have designed a practical method for generating fluorescence labeled melanotropin and the necessary chemistry for covalently coupling the integrated nanostructured with G-protein couple receptor (GPCR) targeted ligands. G-protein coupled receptors constitute a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals. Since 50% of pharmaceuticals target G-protein couple receptors, new methods for imaging G-protein couple receptors at cell surfaces are of much interest. As proof of principle, we choose to target the conjugated melanotropin to the human melanocortin 1 receptor. We demonstrated the feasibility of integrated nano-particle conjugated to α-MSH analogs, both peptides (MTII: Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-$NH_2$ and small molecules (THIQ: N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4- (1H-1,2,4-triazol-1ylmethyl) piperidin-1-yl]-2-oxoethylamine) that effectively image melanocortin receptors overexpressed on HEK293 cells as well as on melanoma cells. In our earlier work, we have set up the techniques to track the real time cellular response via the agonist activation such as MTII and THIQ (Fig. 1) in the melanocortin system (Cai et al., 2004a, 2004b). Basically, we used Rhodamine labeled agonists (peptide and small molecule) activate HEK cells stable express hMC receptors. We found that the agonist induced endocytosis occurred within few minutes. Both of peptide agonist (MTII) and small molecule agonist (THIQ) are endocytosized. The fluorescence intensity normally relies on the binding constant rate. In this case, Rho-MTII bind at melanocortin $MC_1$ receptor in a potent manner (nano-molar range), whereas THIQ is a weaker binder at the melanocortin $MC_1$ receptor. Fig. 2**.** shows that HEK 293 cells co-expressed melanocortin $MC_1$ receptor and GFP-β-Arrestin2 were activated by Rho-MTII and Rho-THIQ. The top images show that HEK 293 cells, highly expressed both of melanocortin $MC_1$ receptor and GFP-β-Arrestin2, are activated by potent agonist of melanocortin $MC_1$ receptor Rho-MTII. Rho-MTII is internalized via β-Arrestin2 mediated endocytosis. Rho-THIQ is a weaker binder at melanocortin $MC_1$ receptor, however, the Rho-THIQ internalized inside of the cell very quickly. This is likely attributed to its smaller size or higher permeation capability.

Further experiments have shown that both MTII and THIQ can also bind at human melanoma cells (A375: ATCC CRL 1619). The binding affinities are 10 nM and 100 nM respectively (Table 1). Therefore, we used human melanoma cells (A375) as our target cells for this study. To avoid non-specific binding, we also incorporate melanocortin $MC_1$-receptor selective ligands, such as melanocortin 1 receptor agonist Ac-c[Asp(Me)-His(Me)-D-Phe-Arg(Me)-Trp(Me)-Lys(Me)]-$NH_2$, (Doedens et al., 2010) and Ac-Nle-c[Asp-His-*D*-Nal(2′)-Arg-Trp-Lys-$NH_2$ (SHU9119) (Hruby et al., 1995) to target melanoma cells (A375). The bio-conjugate procedure is same as MTII shown above.

Animal model experiments showed that the mice with A375 xenografts are able to image after tail injection with Rho-MTII. Fig. 3 shown the most significant image of A375 xenografts after inject with Rho-MTII in 5 min (Upper left). After 1 h injection, the image became smaller and some of the Rhodamine dye comes out from the urine. 24 h later the fluorescence imaging is getting dimmer but it is quite clear compared to the background (lower right). It is interesting to note that two weeks after injection the tumor has significantly shrunk (Fig. 4). This observation gives further evidence that MTII is able to target the melanoma cells (Sharma et al., 1996; Tatro et al., 1992; Libert et al., 1989; Al-Obeidi et al., 1990; Niles and Makarski, 1978; Mourino et al., 1991) and shrink the tumor at earlier stage. After two weeks the tumor started growing again. The reason for this

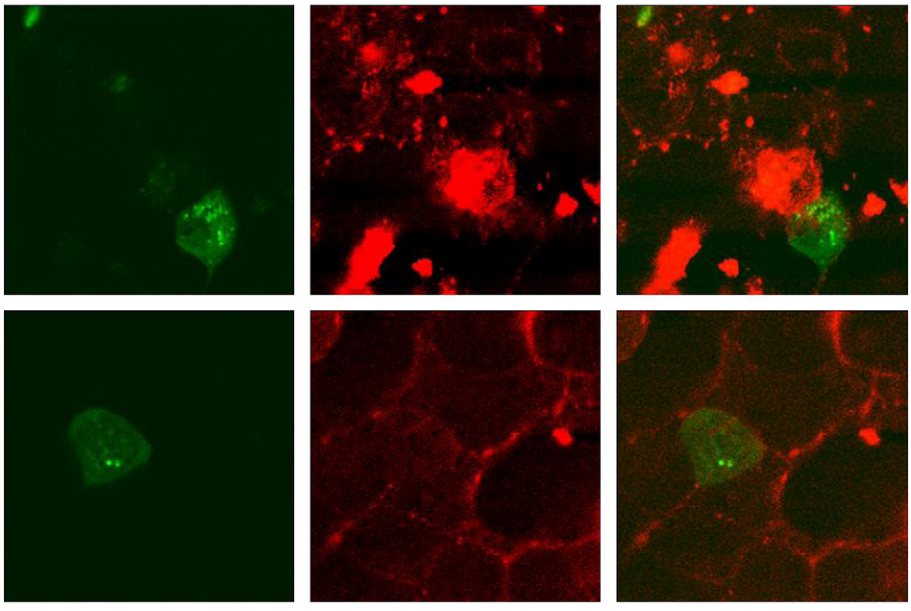
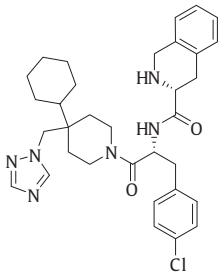


**Fig. 2.** HEK 293 cells highly expressed melanocortin 1 receptor and GFP-β-Arrestin 2 are treated with Rho-MTII (Upper) and Rho-THIQ (Down).

**Table 1**
Binding and cAMP assays of melanocortin $MC_1$ receptor selective ligands to melanoma cells (A375).

| Name | Sequence | $IC_{50}$ (nM) | % BE | $EC_{50}$ (nM) | Act% |
|---|---|---|---|---|---|
| THIQ |  | 93 ± 6 | 60 ± 4 | 950 ± 25 | 49 ± 4 |
| MT-II | Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-$NH_2$ | 6.7 ± 2 | 100 ± 10 | 111.8 ± 0.6 | 100 |
| SHU911 | Ac-Nle-c[Asp-His-D-Nal-Arg-Trp-Lys]-$NH_2$ | 102.7 ± 9 | 79.5 ± 3 | 3.3 ± 2.6 | 54.9 |

$IC_{50}$ = concentration of peptide at 50% specific binding ($N = 4$). % BE = maximal % of $^{125}$I-NDP-α-MSH displacement observed at 10 μM. $EC_{50}$ = Effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$). Act% = % of cAMP produced at 10 μM ligand concentration, in relation to MT-II. The peptides were tested at a range of concentration from $10^{-10}$ to $10^{-5}$ M.

observation might be contributed by the anti-inflammation function of the melanoctropins. The detail mechanism behind this observation is still not clear. Combined with magnetic particle on these peptides and applied with MRI on melanoma, chances to kill the melanoma cells will be much higher.

## 4. Conclusions and future perspective

High-resolution imaging of molecules intrinsically involved in malignancy and metastasis would be of great value for clinical detection and staging of tumors. Magnetic nanoparticles possess unique magnetic properties and the ability to function at the cellular and molecular level of biological interactions, making them an attractive platform as contrast agents for magnetic resonance imaging (MRI) and as carriers for drug delivery (Dobson, 2006; Ferrari, 2005). Recent advances in nanotechnology have improved the ability to specifically tailor the features and properties of magnetic nanoparticles for these biomedical applications. To better address specific clinical needs, multifunctional magnetic nanoparticles with improved magnetic properties and non-fouling and modifiable surfaces are now being developed for early detection, accurate diagnosis, and effective treatment of malignant tumors, cardiovascular disease, and neurological disease. Through the incorporation of highly specific targeting agents and other functional ligands, such as fluorophores and permeation enhancers, the applicability and efficacy of these magnetic nanoparticles could be greatly enhanced. One promising approach to increasing the local accumulation of integrated nanoparticles in diseased tissue, termed active targeting or specific targeting, is to couple the nanoparticles with targeting molecules that display high affinity toward unique molecular signatures found on malignant cells. These receptor-ligand or antigen–antibody interactions are expected to effectively prolong the agent's residence time in malignant tissues. To be investigated targeting ligands include proteins, peptides, aptamers, and small molecules, the anticipated guided delivery of nanoparticles and binding events will be monitored by using MRI and luminescence techniques. Subsequent treatment of diseased cells including tumors will be executed by the heating effect generated by the magnetic nanoparticles in an oscillating magnetic field.

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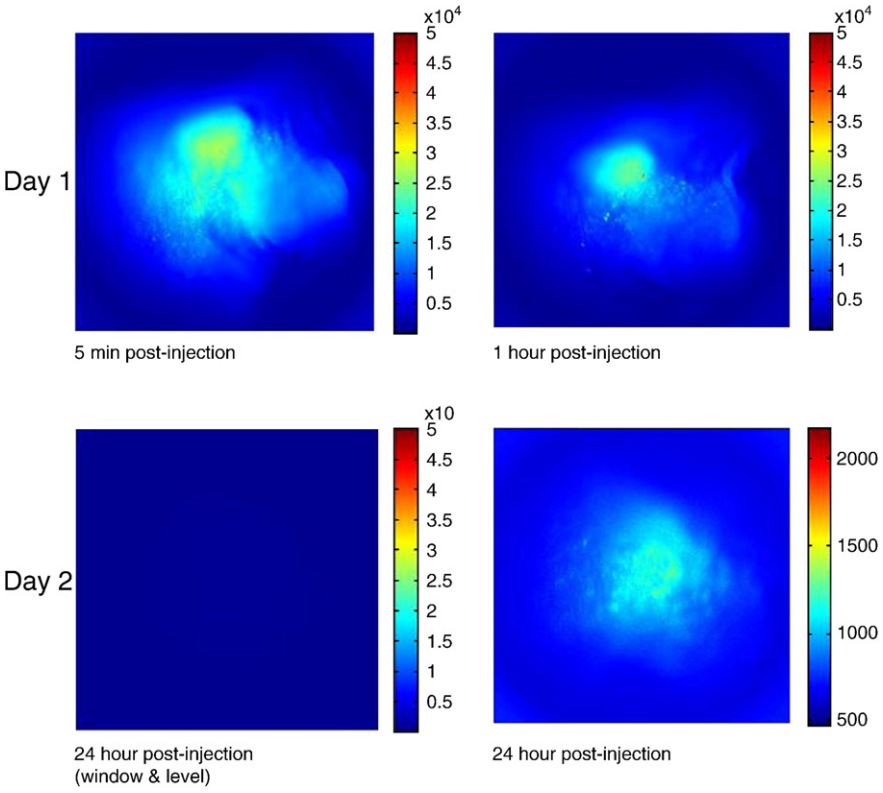


**Fig. 3.** Mouse treated with Rho-MT-II analogs targeting melanoma tumor.

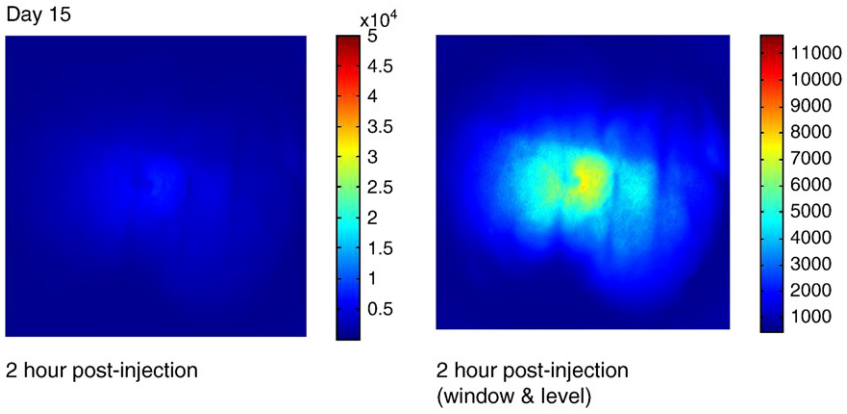


**Fig. 4.** Two weeks after first injection of the Rho-MTII. The second treatment of Rho-MTII on the same mouse the tumor size is significant reduced compared to day 2.

## References


Al-Obeidi, F., Hadley, M.E., Pettitt, B.M., Hruby, V.J., 1989a. Design of a new class of superpotent cyclic α-melanotropins based on quenched dynamic simulations. J. Am. Chem. Soc. 111, 3413–3416.

Al-Obeidi, F., de Lauro Castrucci, A.M., Hadley, M.E., Hruby, V.J., 1989b. Potent and prolonged acting cyclic lactam analogues of α-melanotropin: design based on molecular dynamics. J. Med. Chem. 32, 2555–2561.

Al-Obeidi, F., Mulcahy, M., Pitt, V.S., Begay, V., Hadley, M.E., Hruby, V.J., 1990. Synthesis and actions of a melanotropin conjugate, Ac-[Nle$^4$, Glu(gamma-4′-hydroxyanilide)$^5$, D-Phe$^7$] Alpha-MSH4-10-NH2, on melanocytes and melanoma cells in vitro. J. Pharm. Sci. 79 (6), 500–504.

Cai, M., Cai, C., Mayorov, A.V., Xiong, C., Cabello, C.M., Soloshonok, V.A., Swift, J.R., Trivedi, D., Hruby, V.J., 2004a. Biological and conformational study of β-substituted prolines in MT-II template: steric effects leading to human MC5 receptor selectivity. J. Peptide Res. 63, 116–131.

Cai, M., Stankova, M., Pond, S.J.K., Mayorov, A.V., Perry, J.W., Yamamura, H.I., Trivedi, D., Hruby, V.J., 2004b. Real time differentiation of g-protein coupled receptor (GPCR) agonist and antagonist by two photon fluorescence laser microscopy. J. Am. Chem. Soc. 126, 7160–7161.

Cai, M., Mayorov, A.V., Cabello, C., Stankova, M., Trivedi, D., Hruby, V.J., 2005a. Novel 3D pharmacophore of α-MSH/γ-MSH hybrids leads to selective human $MC_1R$ and MC3R analogues. J. Med. Chem. 48, 1839–1848.

Cai, M., Mayorov, A.V., Ying, J., Stankova, M., Trivedi, D., Cabello, C., Hruby, V.J., 2005b. Design of novel melanotropin agonists and antagonists with high potency and selectivity for human melanocortin receptors. Peptides 26, 1481–1485.

Corot, C., Petry, K.G., Trivedi, R., Saleh, A., Jonkmanns, C., Le Bas, J.F., Blezer, E., M.R., Brochet, Foster-Gareau, P., Baleriaux, D., Gaillard, S., Dousset, V., 2004. Macrophage imaging in central nervous system and in carotid atherosclerotic plaque using ultrasmall superparamagnetic iron oxide in magnetic resonance imaging. Investig. Radiol. 39, 619–625.

Corot, C., Robert, P., Idee, J.M., Port, M., 2006. Recent advances in iron oxide nanocrystal technology for medical imaging. Adv. Drug Deliv. Rev. 58, 1471–1504.

Dobson, J., 2006. Magnetic nanoparticles for drug delivery. Drug Dev. Res. 67, 55–60.

Doedens, L., Opperer, F., Cai, M., Beck, J.G., Dedek, M., Palmer, E., Hruby, V.J., Kessler, H., 2010. Multiple N-methylation of MT-II backbone amide bonds leads to Melanocortin receptor subtype h $MC_1R$ selectivity: pharmacological and conformational studies. J. Am. Chem. Soc. 132 (23), 8115–8128.

Ferrari, M., 2005. Cancer nanotechnology: opportunities and challenges. Nat. Rev. Cancer 161–171.

Hadley, M.E. (Ed.), 1988. The Melanotropic Peptides, vols. I–III. CRC Press, Boca Raton, Florida.

Hruby, V.J., 1982. Conformational restrictions of biologically active peptides via amino acid side chain groups. Life Sci. 31, 189–199.

Hruby, V.J., 2001. Design in topographical space of peptide and peptidomimetic ligands that affect behavior. A chemist's glimpse at the mind-body problem. Acc. Chem. Res. 34, 389–397.

Hruby, V.J., 2002. Designing peptide receptor agonists and antagonists. Nat. Rev. Drug Discov. 1, 847–858.

Hruby, V.J., Wilkes, B.C., Hadley, M.E., Al-Obeidi, F., Sawyer, T.K., Staples, D.J., de Vaux, A.E., Dym, O., de Lauro Castrucci, A.-M., Hintz, M.F., Riehm, J.R., Rao, R.R., 1987. α-melanotropin: the minimum active sequence in the frog skin bioassay. J. Med. Chem. 30, 2126–2130.

Hruby, V.J., Sharma, S.D., Collins, N., Matsunaga, T.O., Russell, K.C., Ed. Grant, G.A., 1992. Applications of synthetic peptides to biological and medical research. Synthetic Peptides: A User's Guide. W. H. Freeman and Company, New York, pp. 259–345.

Hruby, V.J., Lu, D., Sharma, S.D., de L. Castrucci, A., Kesterson, R.A., Al-Obeidi, F.A., Hadley, M.E., Cone, R.D., 1995. Cyclic lactam α-melanotropin analogues of Ac-Nle$^4$-cyclo[Asp$^5$, D-Phe$^7$, Lys$^{10}$] α-melanocyte-stimulating hormone-(4-10)-$NH_2$ with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors. J. Med. Chem. 38, 3454–3461.

Hruby, V.J., Meyer, J.P., Hecht, S.M., 1998. Chemical Synthesis of Peptides Bioorganic Chemistry. Peptides and Proteins. Oxford Ed. University Press, NY, pp. 27–64.

Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I., 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. 34, 595–598.

Krchnak, V., Vagner, J., Safar, P., Lebl, M., 1988. Amino-acids and peptides. part CCVI. Noninvasive continuous monitoring of solid-phase peptide-synthesis by acid-base indicator. Collect. Czech. Chem. Commun. 53, 2542–2548.

Libert, A., Ghanem, G., Arnould, R., Lejeune, F.J., 1989. Use of an alpha-melanocyte-stimulating hormone analog to improve alpha-melanocyte-stimulating hormone receptor binding assay in human melanoma. Pigment Cell Res. 2 (6), 510–518.

Lunec, J., Pieron, C., Thody, A.J., 1992. MSH receptor expression and the relationship to melanogenesis and metastatic activity in B16 melanoma. Melanoma Res. 2 (1), 5–12.

Mayorov, A.V., Han, So-Y, Cai, M., Hammer, M.R., Trivedi, D., Hruby, V.J., 2006a. Effects of macrocycle size and rigidity on melanocortin receptor-1 and -5 selectivity in cyclic actammelanocyte-stimulating hormone analogs. Chem. Biol. Drug Des. 67 (5), 329–335.

Mayorov, A.V., Cai, M., Chandler, K.B., Petrov, R.R., Van Scoy, A.R., Yu, Z., Tanaka, D.K., Trivedi, D., Hruby, V.J., 2006b. Development of cyclic gamma-MSH analogs with selective hMC3R agonist and hMC3R/hMC5R antagonist activities. J. Med. Chem. 49 (6), 1946–1952.

Mayorov, A.V., Cai, M., Palmer, E.S., Dedek, M.M., Cain, J.P., Van Scoy, A.R., Tan, B., Vagner, J., Trivedi, D., Hruby, V.J., 2008. Structure-activity relationships of cyclic lactam analogues of Alpha-Melanocyte-Stimulating Hormone (Alpha-MSH) targeting the human melanocortin-3 receptor. J. Med. Chem. 51 (2), 187–195.

Niles, R.M., Makarski, J.S., 1978. Control of melanogenesis in mouse melanoma cells of varying metastatic potential. J. Nat. Cancer. Inst. (1940-1978) 61 (2), 523–526.

Pankhurst, Q.A., Connolly, J., Jones, S.K., Dobson, J., 2003. Applications of magnetic nanoparticles in biomedicine. J. Phys. D Appl. Phys. 36, R167–R181.

Sebhat, I.K., Martin, W.J., Ye, Z., Barakat, K., Mosley, R.T., Johnston, D.B.R., Bakshi, Raman, Palucki, Brenda, Weinberg, D.H., MacNeil, T., Kalyani, R.N., Tang, R., Stearns, Ralph A., Miller, R.R., Tamvakopoulos, C., Strack, A.M., McGowan, E., Cashen, D.E., Drisko, J.E., Hom, G.J., Howard, A.D., MacIntyre, D.E., van der Ploeg, L.H.T., Patchett, A.A., Nargund, R.P., Nargund, R.P., 2002. Design and pharmacology of N-[(3R)-1,2,3,4-tetrahydroisoquinolinium- 3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-xoethylamine (1), a potent, selective, melanocortin subtype-4 receptor agonist. J. Med. Chem. 45 (21), 4589–4593.

Sharma, S.D., Jiang, J., Hadley, M.E., Bentley, D.L., Hruby, V.J., 1996. Melanotropic peptide-conjugated beads for microscopic visualization and characterization of melanoma melanotropin receptors. Proc. Natl. Acad. Sci. USA 93, 13715–13720.

Tartaj, P., Morales, M.D., Veintemillas-Verdaguer, S., Gonzalez-Carreno, T., Serna, C.J., 2005. The preparation of magnetic nanoparticles for applications in biomedicine. Biomaterials 26, 3995–4021.

Tatro, J.B., Wen, Z., Entwistle, M.L., Atkins, M.B., Smith, T.J., Reichlin, S., Murphy, J.R., 1992. Interaction of an Alpha-melanocyte-stimulating hormone-diphtheria toxin fusion protein with melanotropin receptors in human melanoma metastases. Cancer Res. 52 (9), 2545–2548.