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Enhancement of gene delivery by an analogue of α-MSH in a receptor-independent fashion

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Abstract

In order to transfect melanoma specifically by receptor-mediated endocytosis we prepared dioctadecyl aminoglycylspermine (lipospermine)–DNA complexes with [Nle^4,D-Phe^7]-α-MSH(4–10), a pseudo-peptide analogue of α-melanocyte stimulating hormone (α-MSH) linked to a thiol-reactive phospholipid. With these complexes we obtained an up to 70-fold increase of transfection with B16-F1 melanoma cells. However when B16-G4F, an α-MSH receptor negative melanoma cell line was transfected, an up to 700-fold increased transfection efficiency was observed. The peptide hormone analogue was equally efficient when it was only mixed with lipospermine–DNA complexes without covalent coupling. In addition to melanoma cells we also obtained up to 30-fold increased transfection with BN cells (embryonic liver cells). Our data show that an α-MSH analogue increased transfection independently of the MSH receptor expression but reaches efficiencies approaching those obtained with peptides derived from viral fusion proteins. The absence of targeting of constructs containing [Nle^4,D-Phe^7]-α-MSH(4–10) can probably be attributed due to the relatively modest number of MSH receptors at the surface of melanoma. We suggest, however, that the peptide hormone analogue used in this study has membrane-active properties and could be of interest as helper agent to enhance non-viral gene delivery presumably by endosomal-destabilizing properties.

Keywords: Melanoma; Cationic lipid-based lipofection; Melanocyte stimulating hormone; Receptor-mediated endocytosis; Membrane-active agent

1. Introduction

One of the critical steps for gene therapy is the transfer of genes into target cells. With viral vectors high transfection efficiencies may be achieved, but these systems carry limited lengths of DNA, are potentially hazardous for patients and do not allow a convenient targeting of specific cell types. In comparison to viral vectors, synthetic transfection constructs are less efficient but they are still attractive because of the possibility to overcome most of these problems. For these reasons many efforts are concen-

Abbreviations: α-MSH, α-melanocyte stimulating hormone; [NDP]-α-MSH, [Nle^4, D-Phe^7]-α-MSH; [NDP]-α-MSH(4-10), [Nle^4, D-Phe^7]-α-MSH fragment 4–10; compound **1**, HS-CH_2-CH_2-CONH-Gly-[Nle^4, D-Phe^7]-α-MSH(4-10); compound **1**-DPPE, product obtained after reaction of **1** with DPPE-$(PEG)_3$-Mal; FCS, fetal calf serum; MEM, modified Eagle's medium; HBS, HEPES-buffered saline; lipospermine, dioctadecyl aminoglycylspermine; HPLC, high-performance liquid chromatography; RLU, relative light unit; TCEP, *tris*-(2-carboxyethyl)-phosphine; MC1, melanocortin receptor 1

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trated on increasing transfection efficiencies and tissue specificity of synthetic vectors. (Poly)cationic molecules, able to complex and condense DNA [1–4] seemed to be very attractive but no real breakthrough has occurred. Efforts on improving lipid-based gene transfer have been done by different ways: direct modification of the cationic lipid [5,6], optimization of the formulation of lipid–DNA complexes [7,8] and addition of compounds, such as amphipathic peptides [9–11] or helper lipids [5,7,8], that facilitate transfer across the endosomal membranes. On the other hand, concerning tissue specific targeting, a variety of ligands have been examined, for review see [12,13]. Recently, a small peptide ligand has been successfully used to transfect pancreatic cells [14]. In an other study, fibroblasts and HeLa cells have been targeted by an integrin binding peptide coupled to PEI [15]. Such small peptides have the advantage that they can be readily synthesized and may be less antigenic in vivo.

The incidence of melanoma in the Caucasian population is still increasing [16] and because of the difficulties to treat its disseminating forms, there is a considerable interest in specific therapies for this type of tumors. In gene therapy, several approaches seem to be promising, as for example the use of genes encoding HLA or cytokines [17,18]. Localized gene transfer will improve the treatments, as the destruction of healthy tissue could be considerably limited. In order to target specifically melanoma cells we have chosen to use a superpotent analogue of α-melanocyte stimulating hormone (α-MSH), derived from [Nle^4,D-Phe^7]-α-MSH(4–10) (compound **1**, Fig. 1) [19]. Compared to the natural hormone, this stable pseudo-peptide analogue of the fragment 4–10 of α-MSH, abbreviated here [NDP]-α-MSH(4–10), binds with a slightly lower affinity to the MSH receptor melanocortin receptor 1 (MC1) [19], a G-protein-coupled receptor which is specifically expressed on pigmented cells and in higher density on melanoma cells [20,21]. Importantly, it was shown that [NDP]-α-MSH(4–10) has a very low non-specific uptake in vivo and could be modified at both N- or C-terminal ends, for example by coupling to macromolecules or other molecular moieties, without affecting its affinity for the receptor [19,22–24]. For efficient gene delivery by ligand–receptor interaction, endocytosis of the complex is necessary. In this respect, it has been shown for B16 melanoma cells, that [NDP]-α-MSH is rapidly internalized, accompanied by a loss or down-regulation of surface MSH receptors, suggesting an internalization of the [NDP]-α-MSH–receptor complex [25].

In this study we have investigated the efficiency of gene transfer to mouse melanoma cells using [NDP]-α-MSH(4–10) coupled to an amphiphilic anchor mixed to a dioctadecyl aminoglycylspermine (lipospermine)–DNA complex. We have found that this ligand originally used for targeting purposes enhanced transfection but independently of the MSH-receptor expression. Our results suggest that [NDP]-α-MSH(4–10) might be endowed with hitherto unrecognized membrane-active properties that favor transfection.

2. Materials and methods

2.1. Materials

Compound **1**, HS-CH_2-CH_2-CONH-Gly-[Nle^4, D-Phe^7]-α-MSH(4–10) (see Fig. 1) was obtained high-performance liquid chromatography (HPLC)-purified from Neosystem (Strasbourg, France). DPPE-(polyethylene glycol (PEG))$_3$-Mal was prepared in our laboratory as described [26,27].

2.2. Cell cultures

B16-F1 and B16-G4F mouse melanoma cell lines were maintained at 37°C in a humidified air–CO_2 (5%) atmosphere using modified Eagle's medium (MEM) with Earle's salts (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 2 mM L-glutamine, 1% MEM non-essential amino acid solution, 1% MEM vitamin solution, 50 U/ml of penicillin and 50 μg/ml streptomycin. 3T3 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS. BN cells were cultured in DMEM with 0.4% glucose, supplemented with 10% FCS, 100 U/ml streptomycin, 100 IU/ml penicillin, 0.1 mg/ml kanamycin and 286 mg/ml L-glutamine.

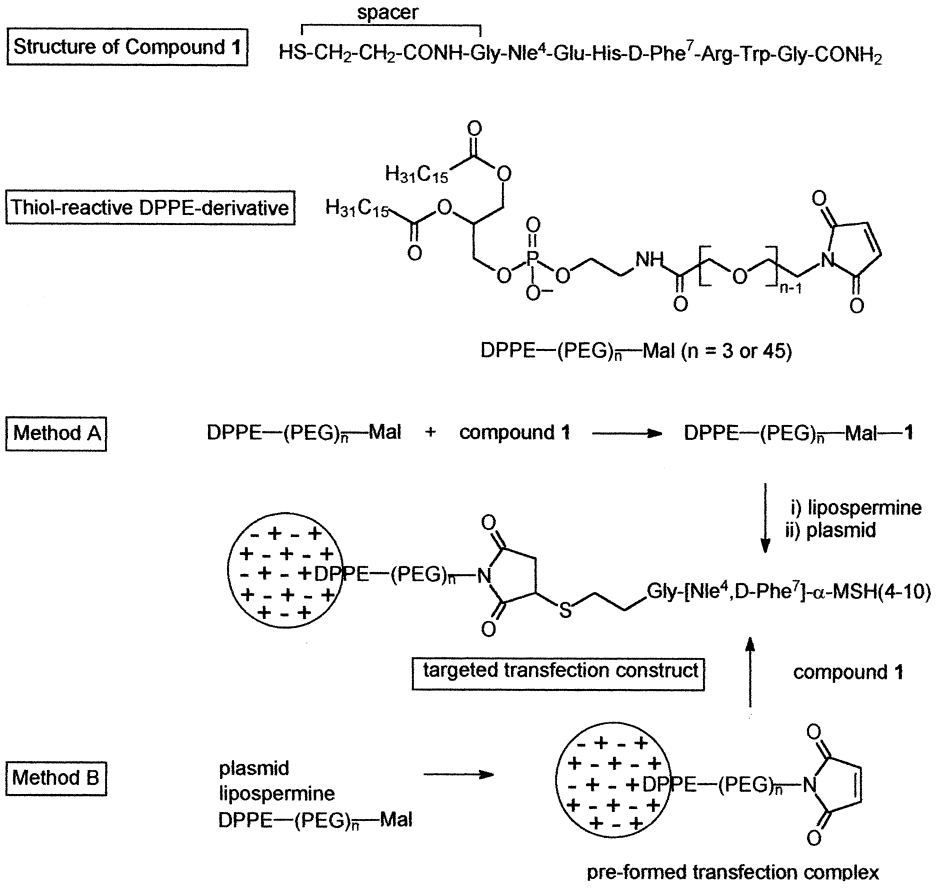

Fig. 1. Structure of the [Nle^4,D-Phe^7]-α-MSH(4–10) analogue compound **1** and of the thiol-reactive DPPE derivatives. Schematic representation of the preparation of lipoplexes for the targeted transfection of melanoma cells. Method A: compound **1** was reacted first with DPPE-(PEG)$_n$-Mal and the conjugation product was mixed with lipospermine and plasmid, at given N/P ratios, to yield the targeted transfection construct. Method B: alternatively, compound **1** was conjugated to preformed and freshly prepared transfection complexes containing DPPE-(PEG)$_n$-Mal.

2.3. Transfection protocols

Transfections were performed with 2 μg of the plasmid pCMV-Luc (Dr. M.A. Zanta, Strasbourg, France) per well in 24 well plates. For this amount of DNA, 6 nmol of lipospermine (Transfectam, Promega, gift of J.-P. Behr) are required to neutralize all the negative charges carried by the phosphates of the plasmid (i.e., molar ratio of lipospermine nitrogens to DNA phosphate N/P = 1).

Preparation of the transfection particles:

Method A (Fig. 1): Ligand **1** was first conjugated to DPPE-(PEG)$_n$-Mal, a thiol-reactive amphiphilic anchor and then added to lipospermine and plasmid to yield the transfection particles at given N/P ratios. A solution of DPPE-(PEG)$_n$-Mal (0.12–0.6 nmol) in chloroform was evaporated under vacuum. The resulting film was dispersed by bath-sonication in a mixture of 40 μl ethanol and 40 μl of HEPES-buffered saline (HBS) (pH 6.7) containing 15 mM NaCl and reacted for 2 h, at room temperature, with compound **1** previously treated with *tris*-(2-carboxyethyl)phosphine (TCEP) to reduce any disulfide bonds; finally, unreacted DPPE-(PEG)$_n$-Mal was treated with an excess (five equivalents) of 2-mercaptoethanol during 20 min. The same was done with controls which lacked **1**. After evaporation under vacuum, the resulting film was bath sonicated in the presence of 20 μl H_2O and 70 μl HBS. For three wells, lipospermine (in 10% ethanol) was then added

to the coupling reaction, the solution was vortex-mixed and 6 μg DNA (in double-distilled water) added.

Method B (Fig. 1): Alternatively, the coupling reaction of compound **1** was performed with preformed lipospermine–DNA particles of given N/P ratios containing the thiol reactive derivative DPPE-$(PEG)_n$-Mal. In this case lipospermine and DPPE-$(PEG)_n$-Mal (0.12–0.6 nmol) in chloroform were evaporated under vacuum. The lipid film was sonicated in 90 μl HBS, 6 μg DNA added and vortexed. TCEP-treated compound **1** was reacted for 2 h with the lipospermine–DNA complexes and unreacted DPPE-$(PEG)_n$-Mal was treated with an excess (five equivalents) of 2-mercaptoethanol during 20 min.

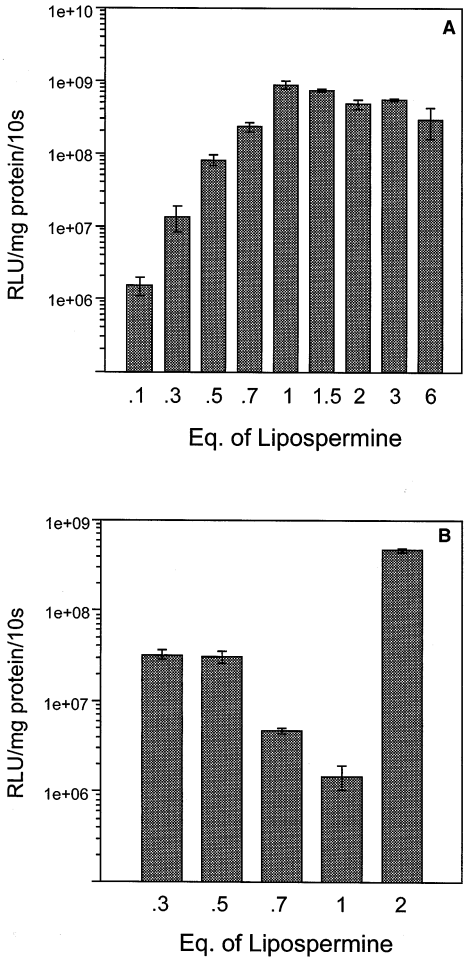

Fig. 2. Effect of N/P and buffer conditions on lipospermine–DNA complexes-mediated transfection of B16-F1 melanoma cells. Complexes were prepared with increasing charge equivalents of lipospermine in 150 mM NaCl (A) or HBS containing 15 mM NaCl (B). Cells were transfected with pCMVLuc plasmid (2 μg per well). Luciferase activities were measured 24 h after transfection and are represented as the mean values (± S.D.) of triplicates.

After the preparation of compound **1**-DPPE–lipospermine–DNA complexes 200 μl of MEM without serum was added to them and the transfection mixture was added to cells (5×10^4 cells/well) which were seeded in 24 well plates (Costar) 18 h before transfection to reach 60–70% confluence during transfection. Culture medium was replaced with 1 ml serum-free medium before transfection and 10% FCS was added 2 h after transfection. Transfections were also done in 1 ml fresh complete medium containing 10% FCS. In this case relative values for transfections with compound **1** were only slightly lower compared to transfections in serum-free medium (results not shown). The cells were cultured for an additional 24 h period and tested for reporter gene expression. Luciferase expression was quantified on cell lysates by measuring the light emission with a luminometer (Biolumat LB 9500; Berthold, Paris, France) using a commercial kit (Promega). Each transfection experiment was done in triplicate and is expressed as relative light units (RLU) integrated over 10 s, per mg of cell protein (BCA assay, Pierce, Paris, France). If not indicated otherwise experiments were performed twice or three times. For all studies representative experiments are shown because of variations of absolute values between individual experiments, depending on plasmid batches and state of the cells.

3. Results

3.1. Optimization of lipospermine–DNA lipoplexes for targeted transfection of mouse melanoma cells

To observe targeted transfection it is, in many cases, necessary to transfect cells with complexes that otherwise, in the absence of specific ligands, lead to only low transfection efficiencies. This can be observed for example with constructs that have a global charge near neutrality (N/P = 1) [8] or which are negatively charged as reported by Lee and Huang [28] for folate targeted constructs. In this latter work, it was shown that under optimal transfection conditions, when using positively charged complexes, no

further enhancement of transfection could be observed in the presence of the specific ligand; moreover this transfection was no more cell specific suggesting that the non-specific electrostatic interactions between cells and the positively charged complexes were superseding the specific ligand–receptor interactions. Similar considerations were discussed by Schaffer et al. [29] favoring the existence of a limited window where ligand-mediated transfection can be observed.

In comparison to the many other cell types transfected with the lipospermine–DNA complexes encoding luciferase reporter gene [30], B16-F1 mouse melanoma cells were the most easily transfected, even with constructs prepared at low N/P in the presence of 150 mM NaCl (Fig. 2A). In contrast, when the complexes were formed in HBS containing only 15 mM NaCl, transfection reached lowest efficiencies at N/P = 1 and increased again when the particles were globally negatively charged (analyzed down to N/P = 0.3 in Fig. 2B). Similar effects have been observed by Lee and Huang [28], which can be explained by the occurrence of small particles size for positively and negatively charged particles and large particle size for 'isoelectric' (i.e. neutral) particles.

As low N/P are sufficient to transfect efficiently B16-F1 cells, we wondered whether the transfection of these cells was still dependent on proteoglycans as reported for other cell types [31,32]. Proteoglycans are the most negative-charged components on the cell surface and they were shown to interact with lipoplexes/polyplexes carrying net positive charges. The involvement of proteoglycans in transfection could be demonstrated e.g. by inhibiting the glycosamine sulfation with sodium chlorate or by competition with free glycosaminoglycans [31,32]. To test the role of sulfated proteoglycans in the transfection of B16-F1 melanoma cells, the cells were grown in the presence of 35 mM sodium chlorate for 4 and 48 h and transfected with lipospermine–DNA complexes prepared at different N/P ratios. At 48 h, luciferase expression was less than 30% of the control even in the case of negatively charged transfection complexes (Fig. 3). To test if free exogenous glycosaminoglycans could competitively inhibit gene delivery, B16-F1 cells were transfected in the presence of heparin (40 μg/ml). Confirming the results of other groups [31,32], this resulted in a dramatic decrease of transfection efficiency to levels less than 1% of controls under all charge conditions tested (not shown). Thus, even in the case of negatively charged particles it seems that there is disruption of the complexes and liberation of DNA as predicted for positively charged particles by Szoka et al. [33,34].

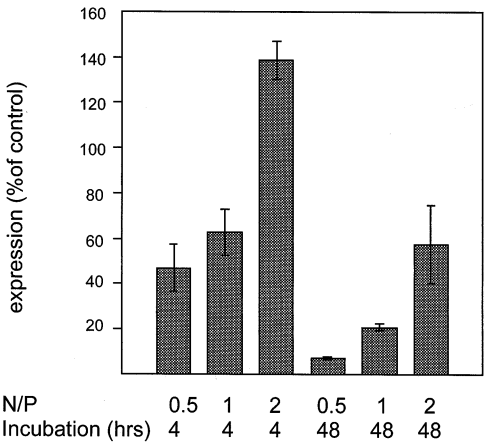

Fig. 3. Effect of sodium chlorate on transfection of B16-F1 cells by lipospermine–DNA complexes. Cells were grown for 4 or 48 h in the presence of sodium chlorate (35 mM) and transfected with complexes of different N/P. Luciferase activity was measured after 48 h in both cases. Results are presented as a percentage (±S.D.) of luciferase expression relative to control transfections obtained with untreated cells.

3.2. Preparation of targeted constructs

The preparation of the targeted constructs was achieved according to a strategy we have developed before for the coupling of e.g. triantennary galactosyl ligands to transfection constructs [5] or peptides to the surface of liposomes [27]. Thus compound **1**, which corresponds to the pseudo-peptide [Nle^4,D-Phe^7]-α-MSH(4–10) that was extended at its N-terminus with a thiol containing spacer-arm, can be easily reacted with a thiol-reactive derivative of phosphatidylethanolamine such as DPPE-$(PEG)_3$-Mal that serves as an amphipilic anchor (Fig. 1) in lipoplexes. The conjugation of **1** to the transfection constructs was realized according to two alternative methods (Fig. 1): (i) method A: **1** was first coupled to DPPE-$(PEG)_3$-Mal (2–10 mol% of lipospermine) and the reaction product was mixed with lipospermine before making the complexes with the plasmid; (ii) method B: **1** was coupled to preformed transfec-

tion complexes made by mixing lipospermine and DPPE-$(PEG)_3$-Mal (2–10 mol% of lipospermine) to the plasmid. As seen in Fig. 4B, coupling with different amounts of **1** after formation of the lipid–DNA complexes (method B) resulted in strongly decreased transfection efficiencies; however when **1** was first coupled to DPPE-$(PEG)_3$-Mal and the complexes formed after (method A), there was a 40-fold increase in efficiency starting at 5 mol% of **1** (Fig. 4A). When 10 mol% of compound **1** was used we did not observe any further increase in transfection efficiency (results not shown).

We also tested the influence on the transfection efficiencies of the spacer-arm length between the ligand and the transfection particle. We tried two different PEG-based spacers, i.e. a short spacer with three oxyethylene units and a longer spacer with 45 (average) units. We observed more than a 1000-fold decrease of transfection efficiencies when lipospermine is mixed with DPPE-$(PEG)_{45}$-Mal-compound **1** (with long PEG-spacers), independently of the coupling method, suggesting an inhibitory effect of the longer spacer (Fig. 4A,B). In fact, it has been shown that PEG alone on liposomes inhibits adhesion to cells [35]. As the long spacers did not improve transfections, all further experiments were than done with short spacers. For all following studies, complexes were prepared in HBS (pH 6.7) containing 15 mM NaCl.

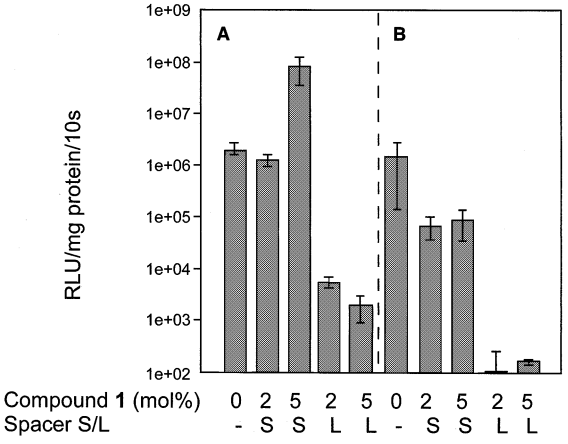

Fig. 4. Effect of the spacer-arm length and coupling method on the transfection efficiencies of B16-F1 cells by lipospermine–DNA complexes (N/P = 1). Compound **1** was conjugated before (method A, A) or after (method B, B) mixing the thiol-reactive derivative DPPE-$(PEG)_n$-Mal with lipospermine and pCMVLuc plasmid as described in Section 2. Luciferase activities were measured 24 h after transfection (2 μg plasmid per well) and are represented as the mean values (±S.D.) of triplicates. Spacers: S = $(PEG)_3$ and L = $(PEG)_{45}$. The proportions of the ligand are given as mol% of lipospermine used to prepare the lipoplexes.

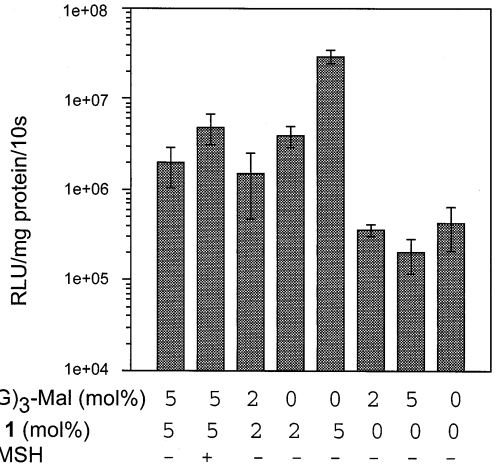

Fig. 5. Influence of compound **1** on the transfection of B16-F1 cells (MC1 receptor positive cells) by lipospermine–DNA complexes. The cells were transfected with complexes (N/P = 1) that contained compound **1** coupled to DPPE-$(PEG)_3$-Mal (method A) or complexes, lacking the thiol-reactive derivative, in the presence of free compound **1**. In competition experiments an excess of [NDP]-α-MSH (1 μg/ml) was added per well before transfection. Luciferase activity was monitored 24 h after transfection. The activities are means (±S.D.) of triplicates. The proportions of thiol-reactive DPPE derivative and of compound **1** are given as mol% of lipospermine used to prepare the lipoplexes. This experiment was performed six times with very similar results.

3.3. *Transfection of α-MSH receptor positive or negative mouse melanoma cells*

When B16-F1 cells, which have been shown to express about 10 000 MSH receptors on the cell surface [21], were transfected with targeted constructs, prepared according to method A, we observed increased efficiencies with complexes containing 5 mol% of compound **1** (Figs. 4 and 5) and a up to 70-fold increase over controls (Fig. 5) when compound **1** (TCEP- and 2-mercaptoethanol-treated) was simply mixed with lipospermine, in the absence of DPPE-$(PEG)_3$-Mal, before formation of the complexes with DNA. However when an excess of free [NDP]-α-MSH was added to the cell culture medium we never could observe any competition;

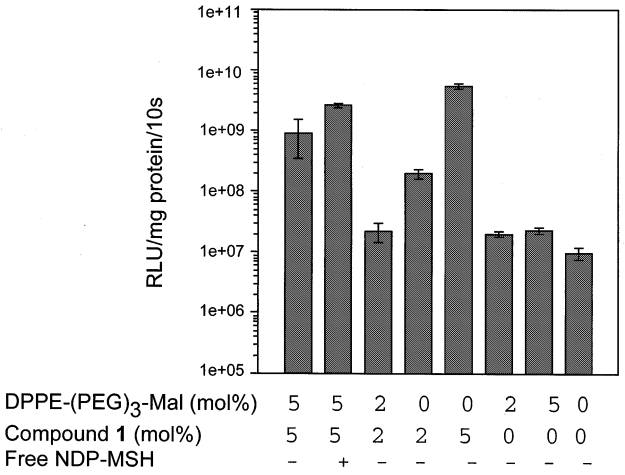

Fig. 6. Influence of compound **1** on the transfection of B16-G4F (MC1 receptor negative cells) by lipospermine–DNA complexes. The cells were transfected with complexes (N/P = 1) that contained compound **1** coupled to DPPE-$(PEG)_3$-Mal (method A). In competition experiments an excess of [NDP]-α-MSH (1 μg/ml) was added per well before transfection. Luciferase activities were monitored 24 h later and are shown as means (± S.D.) of triplicates. This experiment was performed four times with very similar results.

on the contrary, in most cases we even observed a further increase in transfection yield (Fig. 5).

As a control, the same experiments were performed with B16-G4F, i.e., a mutant line derived of B16 melanoma cells which is lacking the α-MSH receptor [36]. With this cell line we obtained up to a 700-fold increased efficiency when compound **1** was mixed with lipospermine–DNA complexes without DPPE-$(PEG)_3$-Mal. Again, we could not compete with an excess of free [NDP]-α-MSH in the culture medium (Fig. 6). To determine optimal charge ratios of lipospermine for the transfection of B16-G4F cells with compound **1**/–lipospermine–DNA complexes, constructs containing **1** coupled to DPPE-$(PEG)_3$-Mal or free were prepared with N/P ratios ranging from 0.5 to 6. Highest effects of compound **1** on transfection yields in comparison to controls (i.e. 30–90-fold, see Fig. 7), were observed with particles near neutrality, i.e. N/P = 0.5–2. Enhancement was highest (90-fold) with N/P = 2 and compound **1**–DPPE. Compound **1**, coupled or free was, up to 20 times, less effective with more positively charged complexes (N/P = 4 and 6). As observed in the former experiments (Figs. 5 and 6), when DPPE-$(PEG)_3$-Mal was mixed with lipospermine no increase of luciferase activity was observed (results not shown).

The data shown in Fig. 7 are representative for several experiments. Except for the construct DOGS–DPPE-$(PEG)_3$-Mal–compound **1** N/P = 1, the covalent coupling of **1** resulted in increased transfection efficiency. At present, we can not explain why at N/P = 1 this was not observed in this one experiment.

In order to test whether compound **1**, either covalently coupled to DPPE-$(PEG)_3$-Mal or mixed with lipospermine during the preparation of the complexes, was effective with other cell types than melanoma cells, we repeated the experiments with fibroblasts (3T3 cells), and hepatocytes (BN cells). In BN cells, compound **1** covalently linked or added to neutral particles (N/P = 1) increased the transfection yields up to 30-fold (Fig. 8). However, increased transfection efficacy was also observed with the construct containing DPPE-$(PEG)_3$-Mal in the absence of compound **1**. A similar effect of thiol-reactive derivatives on transfection yields was observed previously by Kichler et al., 1995 [37]. Nevertheless the

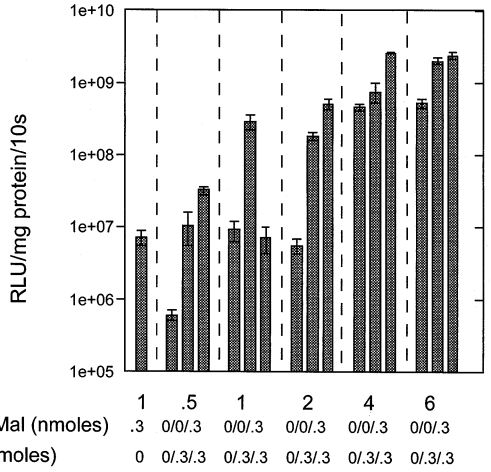

Fig. 7. Effect of N/P ratios and compound **1** on the transfection efficiencies of lipospermine–DNA complexes. B16-G4F cells were transfected with complexes containing varying proportions of lipospermine and compound **1** conjugated to DPPE-$(PEG)_3$-Mal (method A) or with complexes lacking the thiol-reactive derivative in the presence of free compound **1**. The quantities of DPPE-$(PEG)_3$-Mal and compound **1** used in these particular experiments are given in nmol; the quantities of lipospermine used to prepare the lipoplexes are indicated by the N/P ratio with N/P = 1 corresponding to 6 nmol of the cationic lipid. Luciferase activities were monitored 24 h later and are shown as means (± S.D.) of triplicates.

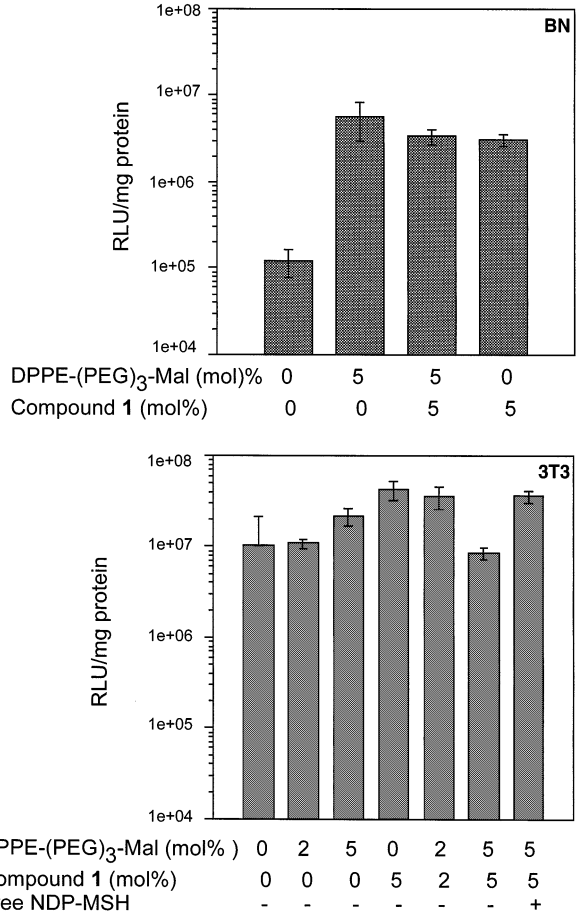

Fig. 8. Effect of compound **1** on the transfection efficiency of BN and 3T3 cells by lipospermine–DNA complexes. The cells were transfected with complexes (N/P = 1) containing increasing proportions (0–5 mol%) of compound **1** conjugated to DPPE-$(PEG)_3$-Mal (method A). In competition experiments an excess of [NDP]-α-MSH (1 μg/ml) was added per well before transfection. Luciferase activities were monitored 24 h later and are shown as means (± S.D.) of triplicates.

fact that constructs that did not contain the maleimide group were also potent in the presence of compound **1** (Fig. 8, last bar) indicates that this peptide is active per se in the transfection process.

In contrast, with 3T3 cells we could not observe any significant increase in transfection triggered by compound **1** (Fig. 8).

4. Discussion

It is now well established that mouse and human melanoma cells possess specific high-affinity receptors for α-MSH [20,21,38,39]. For that reason this peptide was utilized for the development of diagnostic tools of melanoma but also for therapeutic purposes; thus, α-MSH derivatives have been successfully used to visualize melanoma metastases in patients and malignant melanomas have been specifically targeted with MSH–toxin fusion proteins [40]. For targeting purposes α-MSH has many potential advantages: it presents a high affinity for its receptors, analogues have been synthesized which are highly stable in vivo and which have also excellent affinities for example for the MC1 receptor [19,21], because if their small size these peptides should be poorly immunogenic. Finally these analogues can be easily modified, e.g. at their N-terminus, without losing affinity for the receptor. For these reasons α-MSH, and in particular its superpotent analogue [NDP]-α-MSH(4–10), seemed to us an attractive tool for targeting melanoma cells with the aim of gene delivery.

In many cases, a condition to observe targeted gene transfer is to associate specific ligands to transfection particles that present by themselves a limited interaction with the target cells and thus generally achieve only low transfection levels. In our preliminary assays to determine optimal conditions for targeting gene transfer to melanoma cells with compound **1** we have observed that B16-F1 cells are easily transfectable with lipoplexes. In general with other cell lines tested with cationic lipid-based gene transfer complexes, e.g. with lipospermine[30], optimal transfection is observed with highly positive particles (i.e. N/P ≥ 6). This positive charge is thought to promote the binding of the complexes to the negatively charged surface of cells and their uptake via adsorptive endocytosis. In contrast, B16-F1 melanoma cells are optimally transfected with lesser positively particles (N/P ratio between 2 and 4) and are transfectable, albeit in lower yield, with neutral or even net negatively charged complexes obtained with lipospermine under standard conditions. This could be explained tentatively by the fact that melanoma cells overexpress negatively charged proteoglycans [41]. In agreement with this proposal we have shown that inhibition of the sulfation of its surface proteoglycans decreased drastically the transfection efficiency. This result, however, does not explain why cells are still transfected with complexes pre-

pared with a global negative charge. Related to these results it should be noted that relatively efficient transfection with particles that carry a net negative charge have also been observed by others [42,43]. In line with our aim, we could however decrease background transfection by preparing the complexes in HBS and the lowest levels were reached with neutral particles.

In our transfection assays we have shown that a superactive analogue of α-MSH used with lipospermine–DNA complexes increased strongly the transfection of melanoma cells. Enhancement was only observed when coupling was performed using method A. As the coupling methods described by Remy et al. [5], even decreased transfection efficiencies of B16-F1 cells (not shown) we changed the protocol and included a drying step after coupling and 2-mercaptoethanol treatment which eliminated ethanol and 2-mercaptoethanol (see Section 2.3). We suppose that particles prepared according method B inhibit transfection of melanoma cells because 2-mercaptoethanol remains in the transfection solution.

With these complexes, we could however not demonstrate any cell specific targeting, as we could not compete targeting with an excess of free [NDP]-α-MSH. In addition B16-G4F cells which lack MC1 receptors on their surface are even better transfected than B16-F1 cells. With these cells transfection is improved up to 700-fold by the presence of the ligand **1**.

Thus, even if there is some cell specific targeting, the unspecific enhancement due to compound **1** is so high that it would mask it. This seems likely, considering the relatively low number of MSH receptors expressed on the cell surface in comparison to other receptors which have been used successfully for targeting gene delivery constructs, such as for example folate receptors ($\sim 2.5 \times 10^5$ sites/cell) [24]. As compound **1** was also found to have an effect on transfection yield when it is just mixed with lipospermine when making the complexes with the plasmid, we suppose that the free ligand could interact with the constructs most probably via hydrophobic interactions (see below).

Using the same lipospermine–DNA lipoplexes, the effect of **1** on the transfection yield was also tested of other cell types such as murine fibroblasts and liver-derived cells. We could observe an enhanced transfection only with the liver cells. When compound **1** is just mixed with lipospermine, transfection increases as observed with coupled **1**. We conclude from these results that the effect of compound **1** is not restricted to cells that express the MSH receptor and therefore does not increase the transfection yields of the lipospermine-based lipoplexes by targeting this receptor.

The effect of the peptide [NDP]-α-MSH on the transfection efficiency of lipospermine–DNA complexes is therefore more reminiscent to the one described for viral fusogenic peptides when associated to polyplexes/lipoplexes [44]. These membrane-active peptides, which act by destabilizing the endosomal membranes, also lack cell specificity and for example INF6, a peptide deriving from influenza virus, which is one the most effective of these agents, was found to increase transfection up to 1000-fold with 1.5 and 2 charge eq. particles [11]. In contrast to compound **1**, no enhancement was observed with neutral particles. Under optimal transfection conditions, i.e. with the most positively charged particles, only slight enhancement could be obtained with compound **1** and this feature was also observed with viral fusogenic peptides [11]. But in contrast to fusogenic peptides compound **1** strongly increases transfection with particles which are nearly neutral, N/P between 0.5 and 2.

The mechanism for the membrane-destabilizing action of compound **1** remains to be fully elucidated. But it seems that it might be different from the known fusogenic peptides: among the peptides that Kichler et al. have tested [11], only the acidic (negatively charged) ones gave good results with lipospermine. In contrast to these viral peptides, compound **1** has a net positive charge at neutral and acidic pH. There are, however, some hints on a possible membrane activity of α-MSH and [NDP]-α-MSH. The group of Schwyzer has shown some time ago that α-MSH and other peptide hormones that bind to G-protein-coupled receptors interact directly with lipid bilayers [45,46]. More recent biophysical studies by Biaggi et al. [47,48] and Macedo et al. [49] have demonstrated that indeed α-MSH penetrates lipid bilayers. Our own studies in this area demonstrate that the analogues of α-MSH are endowed with hitherto unrecognized, and unexpected, membrane-active properties such as the promotion of membrane fu-

sion (D. Lima de Souza et al., to be published) which could well explain our present data.

Taken together, compound **1** does not seem to be a useful peptide for the targeting of cells such as melanomas. This is probably due to the relatively low expression of MC1 receptors on the surface of these cells. However, **1** seems to be a new kind of membrane-active peptide that could be used under neutral transfection conditions, in particular for melanoma cells. The other advantages, as low immunogenicity, resistance to degradation are good prerequisites for in vivo gene transfer.

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References

[1] J.-P. Behr, B. Demeneix, J.P. Loeffler, J. Perez-Mutul, Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA, Proc. Natl. Acad. Sci. USA 86 (1989) 6982–6986.

[2] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.

[3] R. Leventis, J.R. Silvius, Interaction of mammalian cells with lipid dispersion containing novel metabolizable cationic amphophiles, Biochim. Biophys. Acta 1023 (1990) 124–132.

[4] X. Gao, L. Huang, A novel cationic liposome reagent for efficient transfection of mammalian cells, Biochem. Biophys. Res. Commun. 179 (1991) 280–285.

[5] J.S. Remy, A. Kichler, V. Mordinov, F. Schuber, J.-P. Behr, Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles bearing multiantennary galactosyl ligands: a stage towards artificial viruses, Proc. Natl. Acad. Sci. USA 92 (1995) 1744–1748.

[6] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsay, M. Martin, P.L. Femgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.

[7] A.E. Thierry, P. Rabinovich, B. Peng, L.C. Mahan, J.L. Bryant, R.C. Gallo, Characterization of liposome-mediated gene delivery expression, stability and pharmacokinetics of plasmid DNA, Gene Ther. 4 (1997) 226–237.

[8] A. Kichler, W. Zauner, M. Ogris, E. Wagner, Influence of the DNA complexation medium on the transfection efficiency of lipospermine–DNA particles, Gene Ther. 5 (1998) 855–860.

[9] C. Plank, B. Oberhauser, K. Mechtler, C. Koch, E.J. Wagner, The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems, J. Biol. Chem. 269 (1994) 12918–12924.

[10] K. Mechtler, E. Wagner, Gene transfer mediated by influenza virus peptides: the role of the peptide sequences, New J. Chem. 21 (1997) 105–111.

[11] A. Kichler, K. Mechtler, J.-P. Behr, E. Wagner, Influence of membrane-active peptides on lipospermine–DNA complex-mediated gene transfer, Bioconj. Chem. 8 (1997) 213–221.

[12] F. Schuber, A. Kichler, D. Lima de Souza, B. Frisch, Ligand-mediated gene delivery, in: G. Gregoriadis and B. McCormack, Targeting of Drugs: Strategies for Gene Constructs and Delivery, NATO ASI Series, Life Sciences 323 (2000) 210–225.

[13] M.P. Deonarain, Ligand-targeted receptor-mediated vectors for gene delivery, Exp. Opin. Ther. Patents 8 (1998) 53–69.

[14] D.S. Carpenter, R.F. Minchin, Targeting of a cholecystokinin–DNA complex to pancreatic cells in vitro and in vivo, Gene Ther. 5 (1998) 848–854.

[15] P. Erbacher, J.-S. Remy, J.-P. Behr, Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway, Gene Ther. 6 (1999) 138–145.

[16] J.M. Elwood, H.K. Koh, Etiology, epidemiology, risk factors, and public health issues of melanoma, Curr. Opin. Oncol. 6 (1994) 179–187.

[17] G.J. Nabel, D. Gordon, D.K. Bishop, B.J. Nickoloff, Z.-Y. Yang, A. Aruga, M. Cameron, E. Nabel, A.E. Chang, Proc. Natl. Acad. Sci. USA 93 (1996) 15388–15393.

[18] P. Jantscheff, R. Herrmann, C. Rochlitz, Cancer gene and immunotherapy: recent developments, Med. Oncol. 16 (1999) 78–85.

[19] C. Bagutti, B. Stolz, R. Albert, C. Bruns, J. Pless, A.N. Eberle, 111In-DTPA-labelled analogues of α-melanocyte-stimulating hormone for melanoma targeting receptor binding in vitro and in vivo, Int. J. Cancer 58 (1994) 749–755.

[20] A.N. Eberle, in: The Melanotropins: Chemistry, Physiology and Mechanisms of Action, S. Karger, Basel, 1988.

[21] W. Siegrist, F. Solca, S. Stutz, L. Giuffré, S. Carrel, J. Girard, A.N. Eberle, Characterization of receptors for α-melanocyte-stimulating hormone on human melanoma cells, Cancer Res. 49 (1989) 6352–6358.

[22] D.R. Bard, C.G. Knight, D.P. Page-Thomas, Targeting of a chelating derivative of a short-chain analogue of α-melano-

cyte-stimulating hormone to Cloudman S91 melanomas, Biochem. Soc. Trans. 18 (1990) 882–883.
[23] D.R. Bard, C.G. Knight, D.P. Page-Thomas, E.P. Wraight, T.S. Maugham, The use of derivatives of α-MSH for targeting of melanoma in vivo, in: G. Gregordiadis, G. Poste, A.T. Florenz (Eds.), Targeting of Drugs: The Challenge of Peptides and Proteins, Plenum, New York, 1992.
[24] S. Sharma, M. Granberry, J. Jiang, S. Leong, M. Hadley, V. Hruby, Multivalent melanotropic peptide and fluorescent macromolecule conjugates: new reagents for characterization of melanotropin receptors, Bioconj. Chem. 5 (1994) 591–601.
[25] W. Wong, R.F. Minchin, Binding and internalization of melanocyte stimulating hormone receptor ligand [Nle4, D-Phe7] α-MSH in B16 melanoma cells, Int. J. Biochem. Cell. Biol. 28 (1996) 1223–1232.
[26] H.C. Loughrey, L.S. Choi, P.R. Cullis, M.B. Bally, Optimized procedures for the coupling of proteins to liposomes, J. Immunol. Methods 132 (1990) 25–35.
[27] B. Frisch, C. Boeckler, F. Schuber, Synthesis of short polyoxyethylene-based heterobifunctional cross-linking reagents. Application to the coupling of peptides to liposomes, Bioconj. Chem. 7 (1996) 180–186.
[28] R.J. Lee, L.J. Huang, Folate-targeted, anionic liposome-entrapped poly-lysine-condensed DNA for tumor cell-specific gene transfer, J. Biol. Chem. 271 (1996) 8481–8487.
[29] D.V. Schaffer, D.A. Lauffenburger, Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery, J. Biol. Chem. 273 (1998) 28004–28009.
[30] O. Boussif, M.A. Zanta, J.-P. Behr, Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold, Gene Ther. 3 (1996) 1074–1080.
[31] K.A. Mislik, J. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, Proc. Natl. Acad. Sci. USA 93 (1996) 12349–12354.
[32] L.C. Mounkes, W. Zhong, G. Cipres-Palacin, T. Heath, R.J. Debs, Proteoglycans mediate cationic liposome–DNA complex-based gene delivery in vitro and in vivo, J. Biol. Chem. 273 (1998) 26164–26170.
[33] Y. Xu, F.C. Szoka, Mecanism of DNA release from cationic liposome–DNA complexes used in cell transfection, Biochemistry 35 (1996) 5616–5623.
[34] O. Zelphati, F.C. Szoka, Mechanism of oligonucleotide release from cationic liposomes, Proc. Natl. Acad. Sci. USA 93 (1996) 11493–11498.
[35] H. Du, P. Chandaroy, S.W. Hui, Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion, Biochim. Biophys. Acta 1326 (1997) 236–248.
[36] F.F. Solca, J. Chluba-de Tapia, K. Iwata, A.N Eberle, B16-G4F mouse melanoma cells: an MSH receptor deficient cell clone, FEBS Lett. 322 (1993) 177–180.
[37] A. Kichler, J.S. Remy, O. Boussif, B. Frisch, C. Boeckler, J.P. Behr, F. Schuber, Efficient gene delivery with neutral complexes of lipospermine and thiol-reactive phospholipids, Biochem. Biophys. Res. Commun. 209 (1995) 444–450.
[38] G.E. Ghanem, G. Comunale, A. Liebert, G.A. Verkcammen, F.J. Lejeune, Evidence for a-melanocyte stimulating hormone (α-MSH) receptors on human malignant melanoma cells, Int. J. Cancer 41 (1988) 248–255.
[39] J.B. Tatro, M. Atkins, J.W. Mier, S. Hardason, H. Wolfe, T. Smith, M.L. Entwistle, S.J. Reichlin, Melanotropin receptors demonstrated in situ in human melanoma, Clin. Invest. 85 (1990) 1825–1832.
[40] J.B. Tatro, Z. Wen, M.L. Entwistle, M.B. Atkins, T.J. Smith, S. Reichlin, J.R. Murphy, Interaction of an α-melanocyte stimulating hormone–diphtheria toxin fusion protein with melanotropin receptors, Cancer Res. 52 (1992) 2545–2548.
[41] J. Timar, A. Ladanyi, K. Lapis, M. Moczar, Differential expression of proteoglycans on the surface of human melanoma cells characterized by altered experimental metastatic potential, Am. J. Pathol. 141 (1992) 467–474.
[42] E.R. Lee, J. Marshall, C.S. Siegel, C. Jiang, N.S. Yew, M.R. Nichols, J.B. Nietupski, R.J. Ziegler, M.B. Lane, K.X. Wang, N.C. Wan, R.K. Scheule, D.J. Harris, A.E. Smith, S.H. Cheng, Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung, Hum. Gene Ther. 14 (1996) 1701–1717.
[43] A.J. Fasbender, J. Zabner, M.J. Welsh, Optimization of cationic lipid-mediated gene transfer to airway epithelia, Am. J. Physiol. 269 (1995) L45–L51.
[44] E. Wagner, Effects of membrane-active agents in gene delivery, J. Control. Release 53 (1998) 155–158.
[45] H.U. Gremlich, U.P. Fringeli, R. Schwyzer, Conformational changes of adrenocorticotropin peptides upon interaction with lipid membranes revealed by infrared attenuated total reflection spectroscopy, Biochemistry 22 (1983) 4257–4264.
[46] D.F. Sargent, R. Schwyzer, Membrane lipid phase as catalyst for peptide receptor interactions, Proc. Natl. Acad. Sci. USA 83 (1986) 5774–5778.
[47] M.H. Biaggi, T.J.T. Pinheiro, A. Watts, M.T. Lamy-Freund, Spin label and ^{2}H-NMR studies on the interaction of melanotropic peptides with lipid bilayers, Eur. Biophys. J. 24 (1996) 251–259.
[48] M.H. Biaggi, K.A. Riske, M.T. Lamy-Freund, Melanotropic peptides lipid bilayer interaction. Comparison of the hormone α-MSH to a biologically more potent analog, Biophys. Chem. 67 (1997) 139–149.
[49] Z.S. Macedo, T.A. Furquim, A.S. Ito, Estimation of average depth of penetration of melanotropins in dimyristoylphosphatidylglycerol vesicles, Biophys. Chem. 59 (1996) 193–202.