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# Receptor Binding Affinities and Biological Activities of Linear and Cyclic Melanocortins in B16 Murine Melanoma Cells Expressing the Native MC1 Receptor

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## Abstract

Cyclic α-melanocyte-stimulating hormone (α-MSH) analogues produced by disulphide bridging (e.g. [Cys$^4$,Cys$^{10}$]α-MSH) are known to be almost equipotent to the native hormone in amphibian skin bioassays and as a consequence have been proposed as a paradigm for the active conformation of native MSH at the pigment cell MC1 receptor. However this proposal has been somewhat speculative as there is no published data comparing biological activity of cyclic MSH analogues with data on receptor binding. This study addresses this problem by comparing tyrosinase stimulatory activity with their receptor binding affinity in B16 murine melanoma cells expressing the native MC1 melanocortin receptor.

Cyclic [Cys$^4$,Cys$^{10}$]α-MSH showed almost the same affinity for the MC1 receptor as α-MSH, but the linear analogue [Cys$^4$,Cys$^{10}$]α-MSH bound less strongly. Both had biological activities similar to that of the natural ligand. Introduction of D-Phe into the ring in position 7 increased both affinity and activity of the cyclic compound.

The study suggests that the intrinsic efficacy of cyclic [Cys$^4$,Cys$^{10}$]α-MSH analogues is similar to native α-MSH. Our studies support the proposal that the cyclic structure serves as a good model for the active conformation of linear α-MSH.

Melanocortins have several functions both the periphery and the CNS where MSH peptides participate in temperature control (Murphy et al 1982; Raible & Knicherbocker 1993) and inflammation (Ceriani et al 1994), interact with neurotransmitters (deWied & Jolles 1982; O'Donohue & Dorsa 1982) and show behavioural effects (deWied & Jolles 1982). At least three different receptors for MSH peptides are expressed in various regions of the brain (Chhajlani et al 1993; Gantz et al 1993a, b, 1994; Roselli-Rehfuss et al 1993). The involvement of α-MSH in the pigmentation of amphibia has long been known (Eberle 1988) and structure-activity relationships have been studied extensively in amphibian pigment cells and mammalian melanoma cells. The amino-acid sequence of the pigment cell receptor, known as the MC1 receptor, is now available (Chhajlani & Wikberg 1992; Mountjoy et al 1992) and this has evoked renewed interest.

Cyclic peptide derivatives are a valuable tool in conformational studies of biological molecules. As they adopt a more defined range of conformations than their linear counterparts, their active conformation is more predictable, and they are amenable to conformational analysis by means of NMR spectroscopy, circular dichroism, IR spectroscopy and molecular modelling. Also, they may have an energetic advantage compared with linear ligands as they do not necessarily need to adopt an active conformation prior to receptor binding. For MSH, two approaches have been taken to introduce a ring structure into the molecule: either formation of a disulphide bridge or lactam cyclisation. The first cyclic melanotropic peptide synthesised was [Cys$^4$,Cys$^{10}$]α-MSH (Fig. 1), a peptide with slightly higher melanotropic activity than α-MSH in amphibian bioassays (Sawyer et al 1982). [Cys$^4$,Cys$^{10}$]α-MSH with its derivatives can be regarded as a model for the active conformation of the α-MSH molecule as these compounds have similar activity to α-MSH in mammalian cells. Lactam analogues of α-MSH have also been synthesized and appear to have a significant amount of biological activity (Sugg et al 1988; Hadley et al 1989). Although biological data is available for all these compounds, mainly from reptilian bioassays, receptor binding data has not been reported previously for any of the cyclized analogues.

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## Materials and Methods

*Peptide synthesis and radioiodination*

α-MSH, α-MSH$_{(4-13)}$ and [Nle$^4$,D-Phe$^7$]α-MSH were synthesized by solid-phase techniques employing Fmoc strategy (Atherton & Sheppard 1989) on a MilliGen 9050 automatic synthesizer as described previously (Ahmed et al 1992; Sahm et al 1994) [Cys$^4$,Cys$^{10}$]α-MSH was synthesized using similar methods. Deprotection and cleavage from the resin was carried out using a mixture of 95% trifluoroacetic acid, 2·5% ethanedithiol and 2·5% anisole. The crude peptide (100 mg) was dissolved in 3 g glacial acetic acid and the solution degassed with $N_2$. Degassed water (250 mL) was then added and the mixture titrated to pH 7·5 with $NH_4OH$. The flask was filled to the neck with degassed water and the

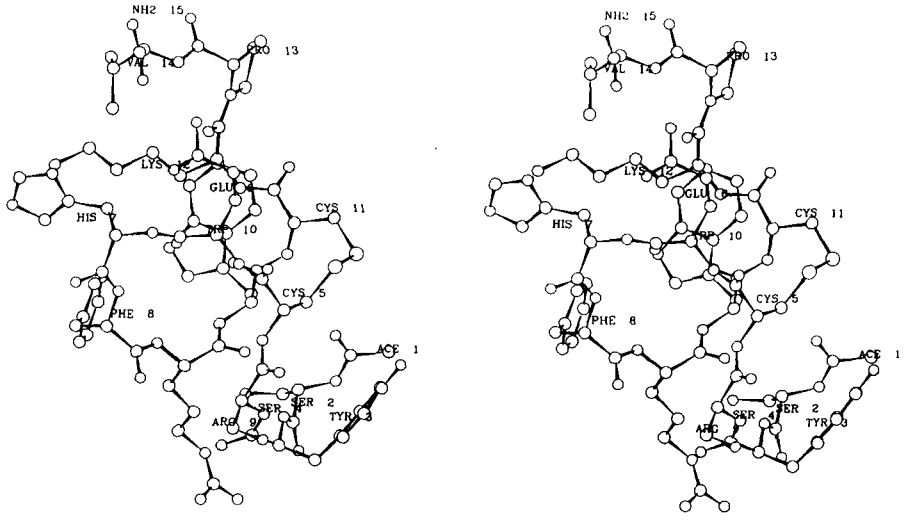

FIG. 1. Stereoview of [Cys$^4$,Cys$^{10}$]α-MSH. A low energy conformation was drawn using INSIGHT II. Note that the program requires the acetyl group at the *N*-terminus to be numbered so that numbering of the amino acid residues in this figure is raised by one.

solution was exposed to air until the cyclization was complete. The cyclized peptide was separated from the buffer using a C18 Bond Elut column packed with Spherisorb ODS. The adsorbed peptide was washed off using acetonitrile-water, lyophilized and purified by HPLC. Identity was confirmed by FABMS: M + H calc. 1681·74, found 1682. Ac-[Cys$^4$,D-Phe$^7$,Cys$^{10}$]α-MSH$_{(4\text{-}13)}$-NH$_2$ was obtained from Bachem, Switzerland. [Nle$^4$,D-Phe$^7$]α-MSH was radioiodinated at the Tyr$^2$ following a literature method (Eberle 1988).

*Cell culture*

B16 murine melanoma cells (donated by L. R. Kelland, Institute of Cancer Research, Sutton, UK) were grown in RPMI 1640 medium (Gibco) supplemented with 10% foetal calf serum (Gibco), 1% non-essential amino acids, 50 int. units mL$^{-1}$ penicillin and 50 mg mL$^{-1}$ streptomycin (Gibco) at 37°C in a humidified atmosphere of 95% air-5% CO2.

*Receptor-binding assay*

The dissociation constant ($K_d$) of [$^{125}$I-Tyr$^2$,Nle$^4$,D-Phe$^7$]α-MSH was established in replicate binding isotherms to be 0·478 ± 0·08 nM (Sahm et al 1994). For competitive-binding experiments, cells were incubated in 24-well plates for 8 h at 0–4°C with the serum-free RPMI 1640 medium containing 25 mM HEPES, 2% BSA, 0·1 nM [$^{125}$I-Tyr$^2$,Nle$^4$,D-Phe$^7$]α-MSH and various concentrations of the non-labelled peptides. The binding medium was then washed off, the cells lysed in 1M NaOH and the radioactivity counted on a γ-counter.

*Tyrosinase assay*

Cells were incubated in 24-well plates for 48 h with RPMI 1640 medium in the presence of 0·2 mCi mL$^{-1}$ 3′,5′-[$^3$H]-L-tyrosine and various concentrations of the peptides following the method of Pomerantz (1966). After the incubation, the medium was removed, treated with 0·2 mL charcoal suspension (10%), centrifuged, and the radioactivity of the supernatant was counted on a liquid scintillation counter. The level of tritium activity in the supernatant is indicative of the presence of tritiated water, and hence tyrosinase activity.

*Melanin assay*

Cells were incubated in 96-well plates for 72 h in the presence of the peptides; the absorbance of the wells was then measured at 405 nm on a microplate reader. The amount of melanin produced is directly proportional to the concentration of peptide in the medium (Siegrist & Eberle 1986).

Table 1. Dissociation constants and EC50 values of linear and cyclic α-MSH analogues. Each value is the mean of three or more experiments.

| | Binding affinity ($K_d \times 10^8$) (n = 3) | Tyrosinase assay (EC50 × $10^{10}$) (n = 3) | Melanin assay (EC50 × $10^{10}$) |
|---|---|---|---|
| α-MSH | 2·06 ± 0·0531 | 6·10 ± 7·73 | 16·2 ± 8·89 (n = 5) |
| [Cys$^4$, Cys$^{10}$]α-MSH | 35·2 ± 21·7 | 9·60 ± 5·02 | 56·1 ± 34·6 (n = 3) |
| [Cys$^4$, Cys$^{10}$]α-MSH | 8·32 ± 0·899 | 9·51 ± 6·53 | 48·3 ± 43·8 (n = 4) |
| Ac-[Cys$^4$,D-Phe$^7$,Cys$^{10}$]α-MSH$_{(4\text{-}13)}$-NH$_2$ | 0·666 ± 0·202 | 0·58 ± 0·309 | n.d. |

n.d. = not determined

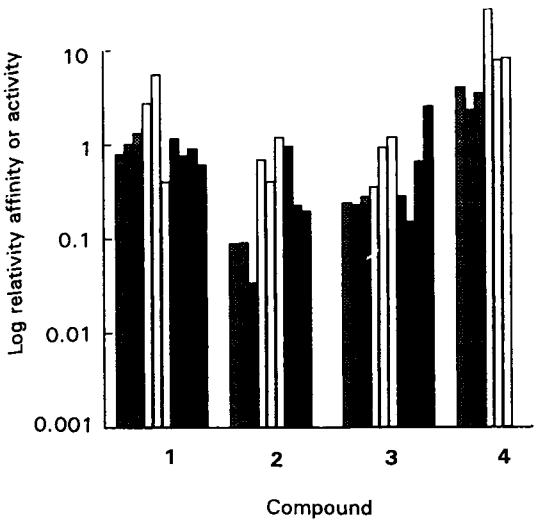


FIG 2. Binding and biological activity of α-MSH (1), [Cys⁴,Cys¹⁰]α-MSH (2); [Cys⁴,Cys¹⁰]α-MSH (3); Ac-[Cys⁴,D-Phe⁷, Cys¹⁰]α-$MSH_{(4-13)}$-$NH_2$ (4) relative to α-MSH. Cross-hatched, binding assay; white, tyrosinase assay; black, melanin assay.

*Statistical data analysis*

Data was analysed by one-way analysis of variance following Fisher's multiple comparison procedure. The coefficient of variance associated with the four replicates used in each of the assays was generally less than 10%.

## Results and Discussion

Results of the competitive binding assay and the biological assays are shown in Table 1 and Fig. 2; data relative to α-MSH are shown in Table 2. [Nle⁴,D-Phe⁷]α-MSH had approximately 10 times the affinity for the receptor than α-MSH itself. Due to its ease of radioiodination compared with α-MSH and its increased stability it has been used as an internal standard in all assays. The affinity of the radiolabelled derivative was previously found to be 4·2 times that of non-labelled [Nle⁴,D-Phe⁷]α-MSH (Sahm et al 1994).

Although [Cys⁴,Cys¹⁰]α-MSH was reported to be more active than α-MSH in amphibian bioassays (Sawyer et al 1982; Knittel et al 1983), its binding affinity was slightly lower than that of the natural ligand. It had a significantly higher affinity than its linear counterpart which showed only six percent of the affinity of α-MSH. The tyrosinase assay, however, suggested that the biological activities of the two forms were identical. This was consistent with results from the melanin biosynthesis assay.

Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]α-$MSH_{(4-13)}$-$NH_2$ was slightly more active than α-MSH, but less so than [Nle⁴,D-Phe⁷]α-MSH. Since the affinity and activity of Ac-α-$MSH_{(4-13)}$-$NH_2$ are not significantly different from α-MSH, this indicates that the increased potency of Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]α-$MSH_{(4-13)}$-$NH_2$ is likely to be due to the D-Phe residue in position 7.

These results indicate that the cyclic analogues retain a significant amount of affinity and activity. They can therefore be considered as models for the active conformation of the native peptide, α-MSH. Deletion of the three *N*-terminal amino acids of native α-MSH, Ser-Tyr-Ser, did not have a marked effect on the activity of the analogues studied here; this is in agreement with previous studies (Cody et al 1984; Sahm et al 1994;). The [Cys⁴,Cys¹⁰]α-MSH analogues are designed to leave both putative active regions of the peptide, residues 6–9 and 11–13, unchanged. An additional factor to consider is the observation that the ring size of the molecule favours binding to the receptor, since it has been reported that a reduced ring size achieved by introduction of mercaptoacetic acid in position 4 significantly reduced the activity of the peptide, whereas substitution with mercaptopropionic acid, leaving the ring size unchanged, did not affect activity in amphibian bioassays (Lebl et al 1984).

It has been proposed that the structure of the cyclic derivative stabilises a β-turn of the peptide backbone within the active site between positions 6 and 9 (Sawyer et al 1982), a conformation that would also be favoured by the introduction of the D-Phe residue in [Nle⁴,D-Phe⁷]α-MSH. To date the presence of a β-turn has not been proven by analytical means although there have been some NMR studies on cyclic lactam analogues of α-MSH (Sugg et al 1988). The similarity of the biological activity of the linear and the cyclic form of [Cys⁴,Cys¹⁰]α-MSH raises the question of whether the disulphide bridge was reduced under the conditions of the tyrosinase assay and the melanin assay, or whether the observed similarity in activity might simply be due to the greater variation generally associated with this kind of biological assay compared to the binding assay. Introduction of the D-Phe residue into the cyclic compounds appeared favourable for both binding and biological activity in B16 melanoma cells, whereas in cyclic lactam analogues the reverse was observed with the L-Phe analogues of [D-Orn⁵, Glu⁸]α-MSH. These were more potent than their D-Phe counterparts in amphibian bioassays (Sugg et al 1988).

The work reported here indicates that the biological activity of the Cys⁴,Cys¹⁰ cyclic derivatives is closely related to their receptor binding affinity, suggesting that the intrinsic efficacy of the cyclic derivatives is similar to native

Table 2. Affinities and activities of cyclic α-MSH analogues relative to α-MSH.

| | Binding affinity | Tyrosinase assay | Melanin assay |
|---|---|---|---|
| α-MSH | 1·00 | 1·00 | 1·00 |
| [Cys⁴, Cys¹⁰]α-MSH | 0·06 | 0·63 | 0·28 |
| [Cys⁴, Cys¹⁰]α-MSH | 0·24 | 0·64 | 0·33 |
| Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]α-$MSH_{(4-13)}$-$NH_2$ | 3·09 | 10·05 | n.d. |

n.d. = not determined

melanocortins. This observation supports the hypothesis that the likely binding conformation of melanocortins can be modelled using such cyclic compounds. Thus further NMR and molecular modelling studies on these compounds could aid the understanding of the molecular interaction of melanocortins with their receptors.

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