Structure-based Search for Peptide Ligands that Cross-react with Melanocortin Receptors

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Purpose. To define sequence motifs that can be used to identify peptide ligands of the melanocortin receptor (MCR).

Methods. Screening of combinatorial libraries has led to identification of D-Trp-Nle-NH_2 (Nle, norleucine) and D-Trp-Arg-NH_2 as the smallest structures known to antagonize the amphibian MCR (1). As the basis of a search paradigm, peptide-ligands containing these or similar motifs within their larger primary structure were examined for ability to antagonize amphibian and recombinant human MCRs. Compounds examined include analogs of substance *P*, leutinizing-hormone releasing-hormone, endothelin, neurotensin, and opioid-somatostatin.

Results. Of seven compounds tested containing the predetermined search motif D-Trp-AA_x (where AA_x is Arg, Leu, Nle, or Ile), six were found to have previously unrecognized antagonist activity at the amphibian MCR (K_d 30 to 5000 nM). In contrast, of 14 similar control peptides lacking the D-Trp-AA_x search motif, only somatostatin displayed measurable antagonist potency. The anticancer peptide, [Arg^8, D-$Trp^{7,9}$, N-methyl-Phe^8]-substance *P*, was the most potent of the motif-containing peptides with a K_d of 31 nM. The μ-opioid antagonist D-Phe-cyclic[Cys-Tyr-D-*Trp*-*Arg*-Thr-Pen]-Thr-NH_2 (CTAP) also blocked the amphibian MCR (K_d 1 μM), but the related μ-antagonist CTOP, different only by only by substitution of Arg with ornithine within the search motif, was found to agonize the amphibian MCR (EC_{50} 67 nM). CTAP and the anticancer peptide were also tested on human MCRs (hMCRs); while CTAP competed with α-MSH at the hMC1 receptor, the anticancer peptide had no effect or was slightly stimulatory.

Conclusions. We have identified dipeptide motifs that help distinguish antagonist ligands of the amphibian MCR from ligands known to interact with other G-protein coupled receptors. This approach might be generally applicable if motifs can identified for other receptors and their subtypes. In studies employing CTAP and CTOP, analogs previously considered highly selective for the μ-opioid receptor, cross-reaction with MCRs must be considered.

KEY WORDS: database search design; structure-activity relationships; drug cross-reactivity; melanocortin receptors; opioid peptides; substance *P* analogs; anticancer peptide; leutinizing-hormone releasing-hormone (LHRH) peptides.

INTRODUCTION

Models to predict ligand-receptor interactions rely on the known structure of at least one component of the pair. For peptide interactions with seven transmembrane G-protein coupled receptors (GPCRs), adequate information about receptor structure is still unavailable, and ligand flexibility makes it difficult to establish which tertiary structure fits the receptor binding pocket. Prediction of peptide-receptor interactions based on amino acid sequence has faired no better; few if any sequence motifs have been identified that reliably forecast binding of peptide ligands to target receptors. The need to fully resolve peptide tertiary structure in molecular modeling, however, might be circumvented by utilizing sequence information contained within short peptidic elements capable of binding the receptor. Di- or tripeptide sequence motifs are restricted in the conformational space they can occupy, and we predict that larger peptides containing such motifs will be flexible enough to allow receptor interaction. In this report we define dipeptide motifs that confer on larger peptides the ability to antagonize a target amphibian MCR. Our results indicate that such motifs can serve to identify cross-reactivities to the MCR for ligands known to bind other GPCRs.

Receptor binding motifs can be identified form combinatorial libraries, as demonstrated for the amphibian MCR using cultured melanophore cells (1,2). Derived from *Xenopus laevis* embryos, melanophores are capable of rapid color change by translocating cytoplasmic pigment vesicles. Translocation in turn, is triggered by activation of GPCRs that cause pigment vesicles to aggregate towards the perinuclear region of the cell (lightened state) or disperse throughout the cytoplasm (darkened state). Pigment aggregation can be induced with melatonin, whereas α-melanocyte-stimulating hormone (α-MSH), VIP/PACAP (3), vasotocin (2), or histamine (this report) cause dispersion. Melanophore cells were used recently in development of a high-throughput combinatorial assay for screening ligands to GPCRs, and to identify antagonists of the MCR (1–3).

α-MSH has effects on pigmentation, cell growth, thermal regulation, learning, and immune function (4–10). In melanophores, pigment translocation evoked by the action of α-MSH (a tridecapeptide) can be antagonized by D-Trp-Arg-Leu-NH_2 and a series of related short peptides identified from random screens of a tripeptide combinatorial library using *Xenopus* melanophores. D-Trp-Arg-Leu-NH_2 has also been shown to block the human MC1 receptor (1,2). Combinatorial extensions to either end of one tripeptide antagonist, D-Trp-Arg-Nle-NH_2, had only limited effects on antagonist activity (unpublished observation) suggesting that activity is not easily perturbed or stericly hindered by nearby amino acids. Further tests also revealed that the even shorter dipeptide fragments D-Trp-Nle-NH_2 and D-Trp-Arg-NH_2 retain antagonist potency at the amphibian MCR (1). Therefore, D-Trp-Arg and D-Trp-Leu may represent minimal structural motifs for binding and antagonist activity at the MCR.

In this report we search for peptide ligands containing one of a predetermined set of motif structures and test for interactions with the MCR. Our survey consists of peptides known to interact with other closely related GPCRs including the substance *P*, neurotensin, endothelin, LHRH, and opioid receptors. Each peptide was tested for effects on pigment translocation in frog melanophores. Results indicate that short dipeptide motifs can convey cross-reactivity to the MCR when embedded in larger peptides developed as ligands to other GPCRs.

MATERIALS AND METHODS

Cell Cultures

Xenopus laevis fibroblast and melanophore cell cultures were prepared using methods described elsewhere (11–16).

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Briefly, *Xenopus* embryos were produced by inducing adult female frogs to lay eggs with injections of human chorionic gonadotropin (Sigma) and fertilizing the eggs *in vitro*, or were obtained with kind assistance from Roslyn McKendry of the University of California at Berkeley. Stage 22 to 35 (17) embryos were rinsed, dissected and plated, and colonies of melanophores grown in conditioned medium containing 50 nM α-MSH (12, 13, 15). Melanophores were separated from other cells by isolating colonies mechanically and by passing cells over a Percoll (Sigma) density gradient (11, 15). Pure melanophores were grown in conditioned medium supplemented with 50 nM α-MSH (13). After 3–4 months α-MSH was withdrawn and conditioned medium gradually replaced with non-conditioned culture medium (5 parts L-15, 2 parts fetal bovine serum, 3 parts deionized-distilled H_2O, 100 U/mL penicillin, and 100 μg/mL streptomycin).

Transformations

cDNA containing inserts of the murine μ-opiate receptor (pRc/CMV·mμOR; from Lei Yu, Indiana University, Indianapolis, IN; 18), the human MC1 receptor (pcDNAI/NEO·hMC1; from Roger Cone, Vollum Institute, Portland, OR; 19), or the human MC3 or MC4 receptors (pcCMV·hMC3 and pcCMV·hMC4; from Ira Gantz, University of Michigan), were used for fibroblast and melanophore transformations. Approximately 8×10^{-6} cells per 600 mL in 70% (Ca^{2+}-Mg^{2+})-free-phosphate-buffered saline (0.14 g/L KH_2PO_4, 1.51 g/L $Na_2HPO_4\cdot7H_2O$, 0.14 g/L KCl, 5.6 g/L NaCl, pH 7.2) and 20 μg of test cDNA were mixed, placed into 0.4 cm cuvettes, and transfected with a Gene Pulser electorporation apparatus (450 V, 960 mF; BioRad, Hercules, CA). Electroporated cells were then plated to confluency in 96- or 12-well tissue culture plates (Falcon) and, once attached, rinsed with fresh medium and incubated 48 hr before use.

Concentration-Response Measurements

Melanophore concentration-response curves were obtained from 96-well plates by measuring absorbance (630 nm) through a monolayer of cells using a BT2000 Microplate Reader (FisherBiotech, Pittsburgh PA), and fit using a logistic equation (20) as described elsewhere (1,2). Initial absorbance (A_i) was measured upon addition of test peptides after a 90 min preincubation with carrier (7 parts L-15 medium, 3 parts distilled-deionized H_2O containing 0.5% (w/w) bovine serum albumin). Preincubations were performed in the presence or absence of 1 nM melatonin (Sigma) to initiate tests from either a dispersed (high absorbance) or aggregated (low absorbance) state. Test peptides were applied for 90 min, unless otherwise specified, in the absence or presence of varying concentrations of α-MSH, and a second or final absorbance reading (A_f) taken. The percent of initial absorbance ($\%A_i$) was then calculated using the equation $\%A_i = 100(A_f/A_i)$. Test drugs include: CTAP and CTOP (from Victor Hruby, University of Arizona, Tuson); LHRH, [D-Trp^6]-LHRH, [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-LHRH, substance *P* (SP), [D-Arg^1, D-*Trp*^{7,9}, *Leu*^{11}]-*SP (spantide), [Arg*^{6}, D-Trp^{7,9}, N-Me-Phe^8]-SP-(6-11) (anticancer peptide), SP-(fragment-6-11), [Tyr^6, D-Phe^7, D-His^9]-SP-(fragment-6-11), [D-Trp^{11}]-neurotensin and N-Acetyl-[D-Trp^{16}]-endothelin-1-(fragment-16-21), [D-Arg^2]-kyotorphin, [Arg^8]-vasotocin, histamine, and PACAP-27 (from Sigma); DADLE (Tyr-D-Ala-Gly-Phe-D-Leu), DAMGO (Tyr-D-Ala-Gly-Phe(N-Me)-Gly-ol; MPS), dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), DPDPE (Tyr-cyclic[D-Pen-Gly-Phe-D-Pen]), DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr), and β-endorphin (from NIH/Multiple Peptide Systems, San Diego, CA); and D-Trp-Arg-Nle-NH_2 and D-Trp-Arg-Leu-NH_2 (from the UCSF Biomolecular Resource Center).

Cyclic-AMP Measurements

Intracellular cyclic-adenosine-3′, 5′-monophosphate (cAMP) was quantitated in *Xenopus* fibroblasts transfected with pcDNAI/NEO·hMC1 or vector alone (pcDNAI/NEO), by measuring displacement of [8-^{3}H]-cAMP from a cAMP-binding protein (21) with a [8-^{3}H]-cAMP kit from Amersham. 48 hr after transfection with test plasmid, fibroblasts were plated to confluency in 12-well tissue culture plates, rinsed for 1 hr with 70% (vol/vol) L-15 medium (Sigma) containing 0.5% bovine serum albumin (Sigma) and rinsed again for 5 min with added 0.5 mM 3-isobutyl-1-methlylxanthine (Sigma). After a 30 min exposure to test ligands in presence of 3-isobutyl-1-methlylxanthine, the cells were rinsed twice with ice-cold 70% phosphate-buffered saline (0.07 g/L $CaCl_2$, 0.07 g/L $MgCl_2\cdot6H_2O$, 0.14 g/L KH_2PO_4, 1.51 g/L $Na_2HPO_4\cdot7H_2O$, 0.14 g/L KCl, 5.6 g/L NaCl, pH 7.4), and intracellular cAMP extracted with 1 mL of 60% (vol/vol) ethanol. Cellular debris was removed by benchtop centrifugation (10,000 rpm for 1 min) and taking 400 μL aliquots of supernatant. Aliquots were then lyophilized and cAMP content quantified.

Sequence Searches

Database searches were performed by examining known GPCR ligands for the ordered tri-amino acid sequences D-Trp[or D-Phe](1)-Arg[or Ile](2)-Leu[or Nle](3), or the ordered di-amino acid sequences D-Trp(1)-Arg(2), or D-Trp(1)-Leu[or Nle](2). Subsequently, analogs of motif-containing peptides, not containing motifs themselves, were selected as controls. These control peptides were selected on the basis of similarity in structure or function to motif-bearing peptides.

RESULTS

Initial database searches for ligands with MCR binding potential were conducted using the motif D-Trp(1)-Arg(2)-Leu(3) since the peptide D-Trp-Arg-Leu-NH_2 is sufficient to antagonize the amphibian MCR (1,2). When no peptide with this tripeptide motif was found however, the search was broadened to include D-Phe at position 1, Ile at position 2, or Nle at position 3, as peptides with these sequences were also found to active (1,2). A single match, [D-Trp^{11}]-neurotensin, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Pro-D-*Trp-Ile-Leu*, was found containing the substructure motif D-Trp-Ile-Leu. [D-Trp^{11}]-neurotensin had an IC_{50} of 4.5 ± 2 μM against 600 pM α-MSH at the frog MCR.

CTAP Antagonizes the Amphibian MC Receptor

Searches based on two amino acids produced more numerous matches then three amino acid motifs (Fig. 1). Motifs D-Trp(1)-Arg(2) and D-Trp(1)-Leu(2) were used because D-Trp-Arg-NH_2 and D-Trp-Nle-NH_2 were found to actively block the

Motif Containing Peptides

Anticancer Peptide	Arg-D-Trp-Phe(N-Me)-**D-Trp-Leu**-Met-NH_2
Spantide	D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-**D-Trp-Leu**-Leu-NH_2
CTAP	Cyclic-D-Phe-Cys-Tyr-**D-Trp-Arg**-Thr-Pen-Thr-NH_2
N-Ac-[D-Trp^{16}]-ET-1-(16-21)	Ac-**D-Trp-Leu**-Asp-Ile-Ile-Trp
[D-Trp^6]-LHRH	pGlu-His-Trp-Ser-Tyr-**D-Trp-Leu**-Arg-Pro-Gly-NH_2
[D-$pGlu^1$,D-Phe^2,D-$Trp^{3,6}$]-LHRH	D-pGlu-D-Phe-D-Trp-Ser-Tyr-**D-Trp-Leu**-Arg-Pro-Gly-NH_2
[D-Trp^{11}]-NT	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Pro-**D-Trp-Ile**-Leu

Control Peptides With Pseudo-motif Structures

[Tyr^6, D-Phe^7,D-His^9]-SP	Tyr-D-Phe-Phe-**D-His-Leu**-Met-NH_2
CTOP	Cyclic-D-Phe-Cys-Tyr-**D-Trp-Orn**-Thr-Pen-Thr-NH_2

Fig. 1. Motif structures in seven test peptides are aligned and appear to be randomly located throughout the larger peptides (top). Two of 23 control peptides selected also contained pseudo-motif structures, but were ineffective as MCR antagonists (bottom).

amphibian MCR (1). Although lower in potency than the parent tripeptide D-Trp-Arg-Nle-NH_2, dipeptide motifs have less conformational flexibility and may be more reliable indicators of receptor affinity when embedded in larger peptides. One search motif, D-Trp(1)-Arg(2), produced three matches of which only CTAP was tested. CTAP was found to inhibit responses to α-MSH with a K_d of 2.9 ± 0.3 μM (Fig. 2A and Table I). Without α-MSH, in the presence or absence of 1 nM melatonin, CTAP evoked no response in wild-type, non-transfected melanophores. When melanophore cells, which lack endogenous μ-receptors, are transfected with cDNA containing a murine μ-opiate receptor (CMV·mμOR), opiate responses can be measured. In transfected cells both CTAP and naloxone block morphine (1 μM) induced pigment aggregation (IC_{50} of 42 and 300 nM, respectively). However, in non-transfected cells, neither morphine nor naloxone, at concentrations more than 100-fold higher than the IC_{50} of CTAP, had any effect in blocking responses to α-MSH. Therefore, the effects of CTAP were not due to any endogenous μ-opioid-like effects.

The selectivity of CTAP for the MCR was examined by comparing the ability of CTAP to block other GPCRs endogenous to melanophores. When CTAP was titrated against near EC_{50} concentrations of histamine (Fig. 3), [Arg^8]-vasotocin (1 nM; $EC_{50} \approx 1$ nM; data not shown), or PACAP-27 (500 pM; $EC_{50} \approx 300$ pM; data not shown), no significant inhibition of receptor mediated pigment dispersion was observed, suggesting that the inhibitory effects of CTAP are specific to the MCR.

CTOP Activates the Amphibian MC Receptor

The μ-antagonist CTOP, which differs from CTAP by only a single amino acid in the search motif domain, was selected as a control because its of structural and functional similarity to CTAP. When tested on the amphibian MCR, CTOP produced a dose dependent dispersion of melanophore pigment granules with an EC_{50} of 67 ± 19 nM (3.4 ± 0.1 μM in the presence of 1 nM melatonin). This agonist-like effect was blocked by the MSH antagonist D-Trp-Arg-Leu-NH_2 suggesting that CTOP was acting by direct stimulation of the MCR. Other opioid peptides, used as controls because they did not contain search motifs, showed no significant inhibitory effects; these include DADLE, DAMGO, dermorphin, DPDPE, DSLET, and [D-Arg^2]-kyotorphin (Table I). β-Endorphin was weakly inhibitory, with an IC_{50} of approximately 10 μM against 600 pM α-MSH, the IC_{50} used as an arbitrary cutoff for significant interactions.

Anticancer Peptide and Spantide Antagonize the Amphibian MC Receptor

The D-Trp(1)-Leu(2) search motif produced the largest number of matches including [D-Arg^1, D-$Trp^{7,9}$, Leu^{11}]-SP (spantide) and [Arg^6, D-$Trp^{7,9}$, N-Me-Phe^8]-SP-(fragment-6-11) (anticancer peptide), both substance *P* antagonists. The anticancer peptide was the most potent inhibitor with a K_d of 31 ± 4 nM, and the slightly larger spantide molecule had a K_d of 354 ± 75 nM (Table I). Both compounds were much less effective in blocking responses elicited by EC_{50} concentrations of [Arg^8]-vasotocin or PACAP-27, indicating selectivity toward the MCR (Fig. 4). The anticancer peptide did produce some inhibition when applied in combination with [Arg^8]-vasotocin ($IC_{50} \approx 10$ μM), but this likely represent a general inhibition not specific to any of the receptors tested.

Similar compounds lacking the search motif showed little or no antagonism of α-MSH. Substance *P* and SP-(fragment 6-11) had IC_{50} values > 100 μM against 600 pM α-MSH, and [Tyr^6, D-Phe^7, D-His^9]-SP-(fragment 6-11), which is similar to the anticancer peptide, and contains D-His-Leu instead of D-Trp-Leu at positions 9 and 10, was only marginally effective with an IC_{50} in excess of 10 μM (Tab. 1).

LHRH Analogs and Other Peptides

Among other peptides containing the D-Trp(1)-Leu(2) search motif three were tested. LHRH antagonist [D-$pGlu^1$, D-Phe^2, D-$Trp^{3,6}$]-LHRH inhibited α-MSH with a K_d of 285 ± 40 nM and the LHRH agonist [D-Trp^6]-LHRH had an IC_{50} of 1.1 ± 0.1 μM against 600 pM α-MSH. These first two ligands have opposite actions at the LHRH receptor, yet both motif-bearing peptides inhibit the amphibian MCR (Fig. 5). The mild inhibitory action of LHRH itself on responses to α-MSH was also seen when α-MSH was replaced with [Arg^8]-vasotocin suggesting a non-specific, generalized inhibition of melanophore responses at high concentrations. This non-specific effect was also seen at high concentrations of both motif-bearing LHRH analogs when [Arg^8]-vasotocin was used in place of α-MSH. The endothelin analog N-acetyl-[D-Trp^{16}]-endothelin-1-(fragment-16-21) stimulated pigment dispersion in melano-

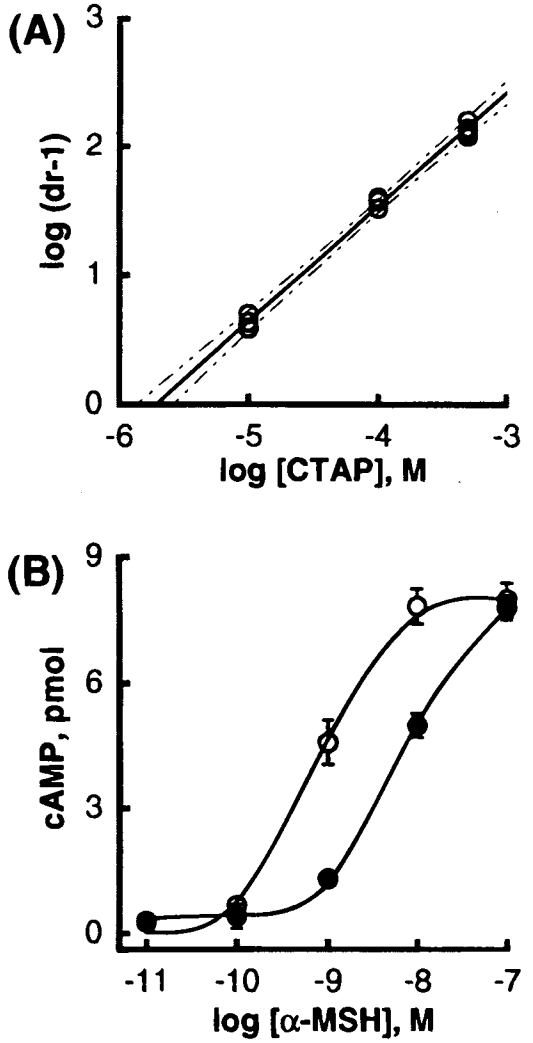

Fig. 2. Antagonist K_d's were calculated using Schild regression analysis (A). The negative logarithm of the equilibrium K_d for CTAP, shown as example, is 2.9 ± 0.3 μM, and the slope of the regression is 0.9 ± 0.1. Broken lines indicate the 99% confidence level. Each point represents the dose ratio (dr) of EC_{50} values for α-MSH measured in the absence and presence of 10, 100, and 500 μM CTAP. Functional antagonism of a human MC1 receptor by CTAP (B) was demonstrated in transfected *Xenopus* fibroblasts by measuring α-MSH stimulated cAMP accumulation in the absence (○; EC_{50} = 0.75 ± 0.04 nM) and presence (●; EC_{50} = 7.4 ± 0.9 nM) of 50 μM CTAP. Each point represents the mean and SD of 3 independent measurements 2 hrs after addition of peptides.

phore cells in the absence of α-MSH, perhaps by activating an endogenous endothelin receptor (22). This prevented direct measurement of activity specific to the amphibian MCR, and testing of this compound remains inconclusive.

Testing Compounds on Human MC Receptors

To test whether the action of motif-bearing peptides in blocking a-MSH was restricted to the amphibian receptor, CTAP and the anticancer peptide were examined on subtypes of the human MCR. In *Xenopus* fibroblasts transfected with the hMC1 receptor, CTAP (50 μM) inhibited α-MSH stimulated increases in cytosolic cAMP levels (Fig. 2B), indicating antagonist affinity for the hMC1 receptor. α-MSH did not change cAMP levels in non-transfected cells, or cells transfected with the vector alone, nor did CTAP inhibit forskolin stimulated cAMP levels in transfected cells (data not shown). Although the anticancer peptide was very effective in blocking responses to α-MSH in melanophores, it did not appear to block responses at the human MC1, MC3, or MC4 receptors. In transfected fibroblasts, the anticancer peptide (1 μM) had a slight stimulatory effect on the hMC1 receptor and appeared to have no effect, agonist or otherwise, on the hMC3 and hMC4 receptors (data not shown).

DISCUSSION

Motif Structure

In this report we have defined dipeptide motifs that when present within a larger peptide structure increase the likelyhood of antagonist interaction with a target amphibian MCR. Of the seven non-MCR peptide analogs tested containing a predetermined motif D-Trp-AA_x (where AA_x is Arg, Leu, Nle, or Ile), six had significant antagonist potencies with K_d values between 30 and 5000 nM when applied to melanophore cells (Table I). The test of one test compound was inconclusive because of secondary effects at a different site of action. Of 14 control peptides, selected on the basis of similarity to motif containing peptides, only somatostatin displayed antagonist activity. Two control peptides, CTOP and [Tyr^6, D-Phe^7, D-His^9]-SP-(fragment-6-11), contain "pseudo-motif" structures with partial resemblance to motifs found in CTAP and the anticancer peptide, respectively (Fig. 1). Despite structural and functional similarity between real- and pseudo-motif peptides, there was a dramatic divergence in antagonistic activity, an further indication that motif integrity is important for receptor interaction with the melanophore MCR.

The paucity of motif-containing peptides identified in database searches was largely due to the presence of a D-amino acid in the motif structure, as most peptide databases are comprised entirely of natural L-amino acids. Although the all L-isomer peptide Trp-Arg-Nle-NH_2 did have some antagonist potency when tested on melanophores (1), it was 100-fold less potent than with D-Trp at position 1, and was not used as a search candidate. No matches were found using the D-Trp(1)-Arg(2)-Leu(3) search motif, however, and broadening the search to include "permissible" substitutions, i.e. substitutions that can be made without seriously degrading potency of the parent tripeptide molecule (1), yielded only one match, [D-Trp^{11}]-neurotensin, a moderate to weak antagonist of the amphibian MCR.

Decreasing the length of the search motif to two amino acids was more effective at increasing the number of matches than was increasing the variability of the larger tripeptide motifs. Small motif structures have dual advantages for use in search paradigms: First, the probability of finding positive matches increases as search motifs become smaller. And second, if the probability that a particular motif accurately predicts receptor interaction depends on its ability to mimic some structure the binding pocket can recognize, then small motifs are less susceptible to distortion when embedded within a larger peptide molecule. However, small motif structures alone cannot, for example, account for the high activity observed with the anticancer pep-

Table I. Inhibition of α-MSH Mediatated Pigment Dispersion in Melanophores[a]

Motif Peptides	IC_{50}	K_d	EC_{50}
Anticancer Peptide	35 ± 14	31 ± 4	≈10 000
Spantide	483 ± 189	354 ± 75	>10 000
[pD-Glu[1], D-Phe[2], D-Trp[3,4]]-LHRH	525 ± 20	285 ± 40	>10 000
[D-Trp[6]]-LHRH	1 100 ± 100		>10 000
CTAP	2 800 ± 700	2 900 ± 300	>10 000
[D-Trp[11]]-NT	4 500 ± 200		>10 000
N-Ac-[D-Trp[16]]-ET-1 (16–21)	undetermined		undetermined
Control Peptides[b]	IC_{50}		EC_{50}
Somatostatin	3 500 ± 3 100		>10 000
β-Endorphin	≥10 000		>10 000
CTOP	>10 000		67 ± 19

[a] All values are in nM. IC_{50} measurements were made in the presence of 600 pM α-MSH and EC_{50} measurements in the absence of α-MSH. [b] Other control peptides with IC_{50} and EC_{50} values >10 μM include: Substance *P*, SP-(fragment-6-11), [Tyr[6], D-Phe[7], D-His[9]]-SP-(fragment-6-11), LHRH, naloxone, morphine, dermorphin, DAMGO, DADLE, DPDPE, DSLET, and [D-Arg[2]]-kyotorphin.

tide; additional interactions with other portions of the ligand likely contribute to receptor affinity.

The hypothesis that small motif structures can predict receptor interactions is further supported by earlier studies examining structure-activity relationships in other species (23, 24). Amino acid substitutions in the growth hormone-releasing peptide analog His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2, used because of its similarity to α-MSH-(fragment-6-11), revealed MCR antagonism by two peptides containing the D-Trp-Arg search motif, namely, [D-Trp[7], Arg[8], D-Phe[10]]-α-MSH-(fragment-6-11) and acetyl-[D-Trp[7], Arg[8], D-Phe[10]]-α-MSH-(fragment-7-10). These results strengthen the relevance of D-Trp-Arg as a MCR search motif.

Interactions with Human MC Receptors

Motif structures defined by screening combinatorial libraries using amphibian maelanophores are less predictive of interactions with human MCRs. Such species and subtype differences are also illustrated with the MSH-antagonist Met-Pro-D-Phe-Arg-D-Trp-Phe-Lys-Pro-Val-NH_2 (1,3), which is a potent antagonist of α-MSH at the amphibian receptor (IC_{50} ≈ 20 nM) but served as an agonist of a recombinant hMC1 receptor transfected into *Xenopus* fibroblasts (unpublished observation). In this report, the anticancer peptide, containing a D-Trp(1)-Leu(2) search motif, did not block the human MC1, MC3, and

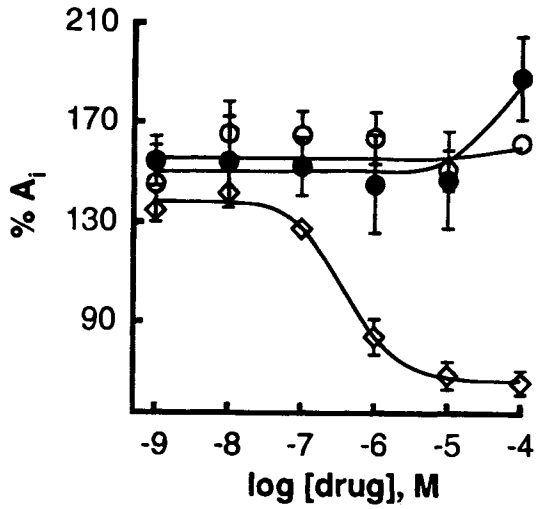

Fig. 3. Antagonist specificity of motif-containing peptides for the MCR was tested by comparison to non-MCR agonists. CTAP, which inhibits α-MSH, did not block the histamine receptor endogenous to melanophore cells. Activation of pigment dispersion by 100 nM histamine (EC_{50} ≈ 25 nM) was antagonized by the ranitidine (◇) but not by triprolidine (●) or CTAP (○). Each point represents the mean and SD of four independent measurements taken in the presence of 1 nM melatonin.

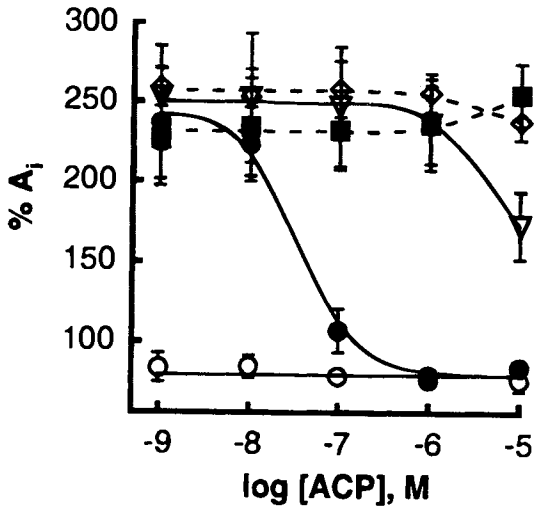

Fig. 4. Inhibition of melanophore pigment dispersion by the anticancer peptide (ACP) and related compounds on are compared. Concentration-response curves were measured in the absence (○) and presence (●) of 600 pM α-MSH, or in the presence (▽) of 1 nM [Arg[8]]-vasotocin (EC_{50} 1 nM). The IC_{50} for ACP was 35 ± 14 nM with α-MSH and >10 μM when [Arg[8]]-vasotocin was used. When ACP was replaced (broken lines) with substance-*P*-(fragment-6-11) (■) or [Tyr[6], D-Phe[7], D-His[9]]-SP-(fragment-6-11) (◇), little or no inhibition of α-MSH was observed. Each point represents the mean and SD of four independent measurements taken in the presence of 1 nM melatonin.

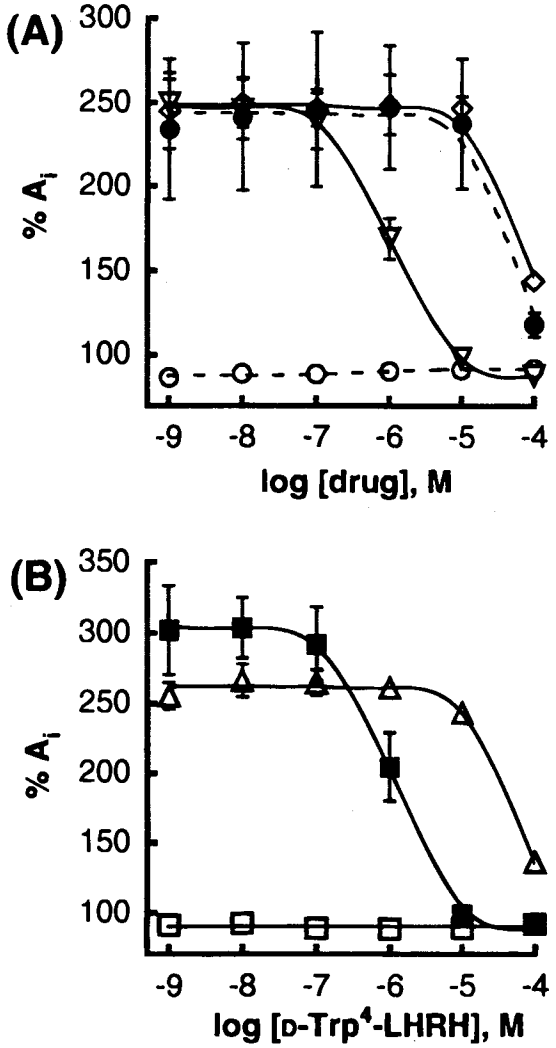

Fig. 5. Inhibition of α-MSH by LHRH analogs are tested in melanophore cells and compared to LHRH. In (A), [D-pGlu[1], D-Phe[2], D-Trp[3,6]]-LHRH (solid lines) inhibited responses to 600 pM α-MSH (▽; IC_{50} = 525 ± 20 nM), but was less effective inhibiting responses to 1 nM [Arg[8]]-vasotocin (◇; IC_{50} > 10 μM). The response to LHRH (broken lines) is shown in the absence (○) and presence (●) of 600 pM α-MSH. In (B), inhibition of MSH by [D-Trp[6]]-LHRH was tested in the absence (□) and presence (■; IC_{50} = 1.1 ± 20 μM) of 600 pM α-MSH. When α-MSH was replaced with 1 nM [Arg[8]]-vasotocin (△), the inhibition produced by [D-Trp[6]]-LHRH was >10 μM, similar to that produced by LHRH itself. Each point represents the mean and SD of four independent measurements taken in the presence of 1 nM melatonin.

MC4 receptors, whereas it did block the amphibian receptor. On the other hand, CTAP, which contains a D-Trp(1)-Arg(2) search motif, appears to be active at both the amphibian MC and human MC1 receptors. Therefore, it will be necessary to carefully define structural motifs for distinct but closely related receptor subtypes in order to increase the chance that positive correlations will be found.

The Opposing Actions of CTAP and CTOP

The finding that CTAP and CTOP interact with MCRs could change the interpretation of experimental results attributed to their capacity as μ-opiate antagonists. For example, cross-reaction between opiate and MC systems could be of consequence to the study of temperature regulation and homeostasis. Administration of opiates has long been known to induce alterations in body temperature, but these studies have been fraught with contradictory findings (25, 26). On the other hand, centrally applied α-MSH is a potent antipyretic, and the MSH analog [Nle[4], D-Phe[7]]-α-MSH is among the most potent antipyretic agents known (6, 27), actions that might be antagonized or mimicked by CTAP or CTOP.

The finding that somatostatin antagonizes the MCR was somewhat unexpected. It was selected as a control because CTAP and CTOP were derived from analogs of somatostatin, but neither analog is thought to have somatostatin-like activity (28, 29). Despite the lack of somatostatin-like activity, it is interesting that CTAP and CTOP share with somatostatin the ability to interact with the amphibian MSH receptor. Interesting also because melanocortin, somatostatin, and opioid receptors all share considerable sequence similarity and, because the melanocortin analog adrenocorticotropin (ACTH) has been shown to have partial antagonistic-like actions on central opioid receptors (29, 30). Indeed ACTH and C-terminal fragments corresponding to the sequence of α-MSH, were approximately equipotent in counteracting the effects of morphine-induced analgesia. Perhaps central opioid and melanocortin receptors are more closely related, and commonalties between the two systems broader, than previously recognized. These cross-relationships were made conspicuous with the use of a dipeptide motif indicative of MCR activity. Whether the D-Trp-Orn motif in CTOP has broader significance remains to be seen.

In conclusion, the presence of a di-amino acid motif D-Trp-AA_x (where AA_x is Arg, Leu, Nle, or Ile) is highly correlated with antagonist activity of peptide ligands at the amphibian MCR. These motifs have led to identification of previously unrecognized interactions between non-MC GPCR peptide-ligands and the amphibian MCR. However, the motifs have only limited predictive value for the human MC1 receptor; therefore, to be broadly applicable, short motif elements may have to be defined separately for each GPCR and their subtypes. This approach may simplify the search for cross-reacting peptide ligands and help us identify unexpected drug interactions.

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