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Analogs of sub-nanomolar hMC1R agonist LK-184 [$Ph(CH_2)_3CO$-His-D-Phe-Arg-Trp-NH_2]. An additional binding site within the human melanocortin receptor 1?

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Abstract—Twenty nine analogs of a superpotent MC1R agonist LK-184 (**1**) were tested at human melanocortin receptors (hMC1, hMC3, and hMC4Rs). All derivatives with the spacer between the N-terminus and the aromatic ring longer or shorter than C_3 were much less potent at hMC1R than **1**. Only LK-312 $PhCO(CH_2)_3CO$-His-D-Phe-Arg-Trp-NH_2 (**3**), partially mimicking the π-system of **1**, had an EC_{50} of 0.05 nM at hMC1R, which confirms the localization of the π-binding zone of the receptor. Truncation of **1** to $Ph(CH_2)_3CO$-His-D-Phe-Arg-NH_2 gave a full MC1 agonist, LK-394 (**30**), with an EC_{50} of 5 nM and a weak partial agonism at MC3/4Rs. This suggests the existence of an additional binding site within hMC1R next to that for the core sequence His-D-Phe-Arg-Trp-NH_2.

Melanocortin receptors (MCRs) are present in different tissues, perform a plethora of physiological functions and are a target of intensive pharmacological research.[1,2] Five MCRs (MC1R–MC5R) have been cloned. The natural agonists for the MC1, MC3, MC4 and MC5Rs—α-, β-, and γ-melanocyte stimulating hormones (MSH) and many of their synthetic analogs (MT-I, MT-II) are very potent but nonselective.[1]

Recently, using a series of end-capped analogs of the melanocortin core fragment His^6-D-Phe^7-Arg^8-Trp^9 (HfRW), we have identified a MC1R agonist, LK-184, $Ph(CH_2)_3CO$-HfRW-NH_2 (**1**) with an EC_{50} of 0.01 nM and ca. 500-fold selectivity for MC1R compared to MC3 and MC4Rs (Table 1). Based on the SAR obtained in this series, a model of the region of human melanocortin receptors (hMC1, hMC3, and hMC4R) adjacent to His^6 was proposed. It contained a large open hydrophobic area common for MC1, MC3, and MC4Rs and a putative aromatic π-binding zone about three carbons apart from the N-terminal binding site for hMC1R.[3] In order to explore the receptor requirements further and confirm the existence of the π-binding zone, we made a series of analogs of LK-184 (**1**) end-capped with arylalkanoic acids (**2**–**6**, **12**–**21**, **25**–**29**) and their isosteres (**7**–**11**, **22**–**24**) (Table 1).

Compounds **2**–**29** with purity >95% (>98% for **16**, >99% for **17**, **18**, **20**, **21**) were obtained, analyzed, and assayed as previously described.[3] (*S*)-(−)-Tropic acid was obtained by the acid hydrolysis of (−)-scopolamine·HCl (Sigma) and had $[\alpha]_D^{20}$ −79 (*c* 0.02, water) [lit.[4] −76 (*c* 2)]. Urea **7** and carbamates **8** and **11** were obtained by end-capping of the resin bound NH_2-tetrapeptide with the corresponding isocyanate or chloroformates followed by the same cleavage–deprotection as for the amides. The EC_{50} values for compounds **1**–**30** (all of them are full agonists, except for **30**) reported in Table 1 are the average of at least two separate experiments in duplicate.

The *p*-hydroxymethylphenyl precursors **A** and **B**, synthesized from commercially available acids 4-$HOCH_2C_6H_4O(CH_2)_nCOOH$ ($n = 1$ HMPA, $n = 2$ HMPBA, Advanced ChemTech) were reduced to the corresponding *p*-tolyl derivatives **9** and **10** during cleavage from the Rink resin with TFA in the presence of triisopropylsilane (TIS).

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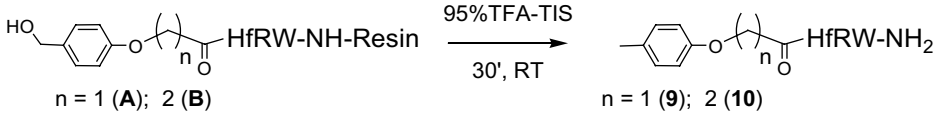
Table 1. Functional activity of melanocortins at human MC1, MC3, and MC4Rs (mean ± SEM)

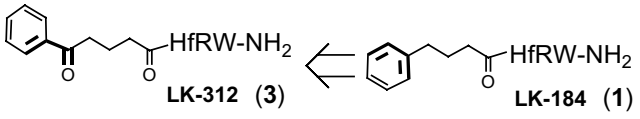
	R′CO-HfRWNH$_2$	EC_{50} (nM)		
	R′	hMC1R	hMC3R	hMC4R
	MT-I	0.07 ± 0.01	0.36 ± 0.14	0.51 ± 0.07
1[3]	Ph(CH$_2$)$_3$ (LK-184)	0.009 ± 0.004	4.7 ± 1.2	4.6 ± 2.8
2	Ph(CH$_2$)$_4$	0.27 ± 0.11	4.0 ± 2.0	6.5 ± 0.5
3	PhCO(CH$_2$)$_3$ (LK-312)	0.05 ± 0.01	3.0 ± 1.0	5.0 ± 0
4	3,5-F$_2$C$_6$H$_3$CO(CH$_2$)$_2$	0.2 ± 0.1	7.5 ± 0.5	1.5 ± 0.5
5	4-FC$_6$H$_4$(CH$_2$)$_2$	0.95 ± 0.05	13.0 ± 7.0	10.0 ± 5.0
6	4-ClC$_6$H$_4$(CH$_2$)$_2$	0.7 ± 0.1	8.0 ± 3.0	11.5 ± 7.5
7	4-FC$_6$H$_4$CH$_2$NH	0.6 ± 0.4	2.0 ± 1.0	2.0 ± 1.0
8	4-ClC$_6$H$_4$CH$_2$O	0.4 ± 0.1	6.5 ± 1.5	7.5 ± 1.5
9	4-CH$_3$C$_6$H$_4$OCH$_2$	1.0 ± 0	45.0 ± 6.0	20.5 ± 6.5
10	4-CH$_3$C$_6$H$_4$O(CH$_2$)$_2$	7.0 ± 2.6	121 ± 9.0	57.3 ± 22.4
11	4-NO$_2$C$_6$H$_4$CH$_2$	0.4 ± 0.1	4.7 ± 1.3	1.5 ± 0.5
12	4-HOC$_6$H$_4$CH$_2$	3.0 ± 0	55.0 ± 11.0	5.0 ± 1.0
13	3,4-(HO)$_2$C$_6$H$_3$CH$_2$	5.0 ± 3.0	34.5 ± 8.5	12.0 ± 8.0
14	3,4-(CH$_2$)O$_2$C$_6$H$_3$CH$_2$	1.8 ± 0.7	6.5 ± 3.5	2.5 ± 0.5
15	3-CF$_3$C$_6$H$_4$O	292 ± 177	1950 ± 580	390 ± 280
16	(*R*)-(+)-PhC(OCF$_3$)CH$_3$ (MTPA)	60.5 ± 13.5	67.5 ± 6.7	81.0 ± 6.0
17	(*S*)-(−)-PhC(OCF$_3$)CH$_3$ (MTPA)	32.5 ± 15.5	38.0 ± 18.0	33.5 ± 6.5
18	(*S*)-(−)-PhCH(CH$_2$OH) (L-tropic)	1.1 ± 0.7	3.0 ± 0	19.5 ± 9.9
19	(*R*)-(+)-PhCH(CH$_2$OH) (D-tropic)	5.5 ± 1.5	27.5 ± 8.5	9.0 ± 2.6
20	4-*i*-BuC$_6$H$_4$CH(Me)	2.0 ± 0	70.0 ± 7.0	32.5 ± 3.5
21	(*S*)-(+)-4-*i*-BuC$_6$H$_4$CH(Me)	0.41 ± 0.34	17.5 ± 2.5	10.5 ± 1.5
22	4-ClC$_6$H$_4$OC(Me)$_2$	8.3 ± 3.8	22.5 ± 9.5	13.0 ± 3.0
23	2,5-Me$_2$C$_6$H$_3$O(CH$_2$)$_3$C(Me)$_2$	3.5 ± 0.5	3.5 ± 1.5	2.5 ± 0.5
24	4-ClC$_6$H$_4$CONH(CH$_2$)$_2$C$_6$H$_4$OC(Me)$_2$	3.0 ± 1.0	15.0 ± 3.0	17.0 ± 7.0
25	1-Naphthoic	3.5 ± 1.0	38.0 ± 3.0	9.5 ± 5.5
26	6-AcO-2-Naphthoic	6.0 ± 1.0	13.5 ± 5.5	8.5 ± 5.5
27	1-Naphtylacetic	3.5 ± 1.5	23.5 ± 10.5	11.0 ± 5.0
28	3-Ph-2-(PhCOO)propionic	0.5 ± 0.1	3.5 ± 1.5	9.0 ± 3.0
29	(Ph)$_2$C(OH)	3.5 ± 1.5	7.5 ± 0.5	17.0 ± 0
30	Ph(CH$_2$)$_3$CO-HfR-NH$_2$ (LK-394)[a]	5.0 ± 0.6	25,000 ± 0[b]	61 ± 26[b]
31[3]	*p*-ClC$_6$H$_4$OCH$_2$	2.0 ± 0.6	6.0 ± 4.0	11.5 ± 1.5
32[3]	*p*-ClC$_6$H$_4$CH$_2$	9.5 ± 5.1	9.3 ± 0.7	7.5 ± 0.5
33[3]	PhCH$_2$	2.0 ± 1.0	28.0 ± 13.7	13.0 ± 6.0
34[3]	Ph(CH$_2$)$_2$	0.5 ± 0.1	17.0 ± 2.0	20.0 ± 9.0

[a] K_i MC1/3/4: 47.0 ± 14.1, MC 7625 ± 1525, MC 11,680 ± 820.
[b] Efficacy at hMC3R 11%, hMC4R 24%—a partial agonist.



According to LC–MS, in both cases the reduction occurs before cleavage. NMR spectra (300 MHz, CD_3OD) unequivocally confirm the structure of **9**. The proton spectrum shows a singlet of CH_3 at 2.26 ppm (3H) and, two doublets (16 Hz, 2×1H) of OC*H*$_2$CONH at 4.47 and 4.55 ppm. The signals of the CH_3 (19.34 ppm) and OCH_2 (67.04 ppm) groups are well separated in the ^{13}C spectrum also. On the contrary, the signals of both CH_2 groups of the starting acid HO*CH*$_2$C$_6$H$_4$O*CH*$_2$COOH have very close chemical shifts in both ^1H (4.67 and 4.55 ppm, 2×2H) and ^{13}C (64.76 and 63.66 ppm) NMR spectra.

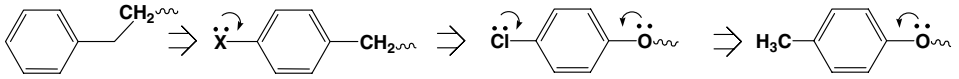
The SAR for the compounds **1**–**29** discussed here (Table 1) confirms our previous hypothesis about the existence and localization of the π-binding zone in hMC1R.[3] The extension of the carbon chain in **2** by just one CH_2 group relative to **1** leads to a 30-fold loss in potency at MC1R, since the Ph ring is offset from the 'optimal' position. At the same time LK-312 (**3**), the 5-oxo-analog of **2** partially mimicking π-system of **1**, had an EC_{50} of 0.05 nM at hMC1R comparable with an EC_{50} of 0.01 nM for **1**.



Since **3**, having the Ph ring in the same place as **2** is practically equipotent to **1**, the drop in potency for **2** relative to **1** is not caused by the fact that MC1R cannot

accommodate this ring. The potency of **1**–**3** at hMC3/4R is the same within experimental error, which shows that as in the case with hMC1R their hydrophobic area extends beyond that previously mapped but within this new range the hMC3/4Rs have no π-binding region.

Compound **4**, the 4-oxo-analog of **1**, with an extended conjugated π-system due to the 3,5-difluoro substitution, has potency at MC1R lower than **1** and the same as **1** within MC3/4Rs. It seems that the π-binding region at hMC1 is very compact, since our attempts to mimic the π-system of the Ph ring of LK-184 with a range of substituted phenyls (**5**–**15**) attached through shorter spacers were not successful.



1 X=F (**5**), Cl (**6**) **31** **9**

The *p*-F- and *p*-Cl-Ph-propionamides **5** and **6** show practically the same potency as the unsubstituted Ph-propionamide[3] **34** reported earlier (Table 1). Thus, extension of the conjugated π-system in **5** and **6** into the π-binding site of **1** does not compensate for the shorter distance between the Ph and N-terminus of HfRW in **5**, **6**, and **34**. The same applies to urea **7** isosteric to **5** and urethane **8** isosteric to **6**, though **7** has slightly better potency compared to **5** at hMC4R. Positioning of the oxygen in the chain (**8**, **31**) as well as an increase in the length of π-conjugation by substitution of CH_2 next to the aromatic ring in **6** for O (**8**) has no effect on activity. At the same time, substitution of *p*-Cl in **31** for isosteric nonconjugated *p*-Me in **9** lead to a drop in MC3 potency without affecting MC1 and MC4Rs. A homolog of **9** with a longer spacer (**10**) is less active but it shows a similar selectivity profile with a drop in potency at MC3R.

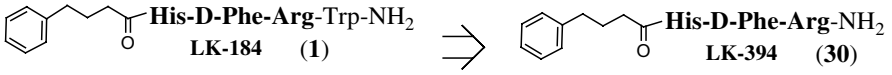
Except for *p*-NO_2 compound **11**, ring substituted phenylacetic derivatives **11**–**14** have no advantages over the unsubstituted compound **33**. Introduction of polar hydrogen bonding OH groups in the 4- or 3,4-positions (**12**, **13**) decreases the potency at MC3R, while blocking them with a methylene bridge (**14**) increases it compared to **33**. Urethane analog **15** of the above phenylacetic derivatives has low activity at hMC1/3/4Rs.

To map the area next to the HfRW N-terminus of hMCRs in detail, we used a series of chiral α-substituted phenylacetic compounds **16**–**20** and a racemic Ibuprofen derivative **21**. The Mosher's acid (MTPA) derivatives **16** and **17** are less potent than the less hindered α,α-unsubstituted compounds discussed above, though the drop in activity is not dramatic. One can see that (*R*)-(+)-derivative **16** is about two times less potent at MC1/3/4Rs relative to its (*S*)-(−)-isomer **17**. The analysis of charge distribution (AM1 and PM3) and hydrophobicity for this pair with Chem3D (CambridgeSoft) showed that the distribution of hydrophobic/hydrophilic properties of the solvent accessible surface for **16**/**17** is rather similar. This was confirmed experimentally by the fact that their retention times under reverse-phase conditions, which are proportional to the exposed hydrophobic surface, coincide.

Opposite to that, end-capping of the tetrapeptide by racemic tropic acid gave two peaks with excellent resolution (retention time 5.7 and 6.1 min, 1 mL/min, MetaChem Technologies Polaris 3μ C-18-A column 4.6×250 mm, 20 min gradient from 20% to 100% MeCN in water with 0.1% H_3PO_4) belonging to diastereomers **18** and **19** with different solvent accessible surfaces, that is, with different conformations in solution (most likely due to hydrogen bonding). Their absolute configuration was determined using (*S*)-(−)-tropic acid, which gave only isomer **18**. The latter was more potent that **19** at MC1 and MC3Rs but the difference was not as significant as expected from the HPLC data. Ibuprofen, lacking the polar hydrogen bonding OH group was used as another chiral probe for MCRs. Though (*R*)-(−)-ibuprofen was not available, comparison of (*S*)-(+)-derivative **21** with racemate **20** demonstrated that the (*S*)-(+) configuration provides better interaction with MC1 and MC3Rs but not with MC4R.

Achiral α,α-dimethylsubstituted derivatives of the fibrate family (Clofibrate **22**, Gemfibroil **23**, Benzafibrate **24**) show close potencies at MC1/3/4Rs, with **23** being the most potent and nonselective. The fact, that all three MCR subtypes have a weak response to chirality of the α-carbon (**16**–**21**), tolerate both α,α-disubstitution with hydrophobic groups (**16**, **17**, **22**–**24**) or isosteric substitution of α-carbon with polar NH (**7**) or O (**8**), as well as can accommodate such big molecules as **24**–**29** is in complete agreement with our idea of an 'open' hydrophobic area near the HfRW N-terminus of hMCRs.[3]

There are two published putative 3D models of the hMC1R with different localization of the ligand binding site. The first model places the binding site between transmembrane (TM) segments TM4/5/6/7 and emphasizes the role of extracellular loops,[5] while the work of the Swedish group[2,6] places the binding site between TM1/2/3/6/7 and questions the crucial role of extracellular loops in binding and receptor functioning.[7,8] Taking into account the diversity in size and physical properties of acceptable R′ in this and our previous publication,[3] the most logical conclusion is that the binding of the end-capping group occurs at a TM adjacent to the binding site of the core sequence HfRW-NH_2 or, rather, at an extracellular loop.



LK-184 (**1**) **LK-394** (**30**)

To confirm the existence of this additional binding site, we truncated the most potent MC1R tetrapeptide agonist LK-184 (**1**) to tripeptide LK-394 (**30**). It is well documented, that truncation of the core tetrapeptide His^6-D-Phe^7-Arg^8-Trp^9-NH_2 to His^6-D-Phe^7-Arg^8-NH_2 [9,10] or mutation of Trp^9 [11] leads to the loss of melanocortin activity at MC1R, as well as at MC3R.[12] Recently published Ala and Pro scans of a cyclic melanocortin agonist MT-II at hMC3/4/5Rs[13,14] and Ala scan of the core tetrapeptide at mouse MC1/3/4/5Rs[15] also confirmed the crucial role of Trp^9 in ligand binding at MCRs and their functioning. On the contrary, the truncation of **1** with the Ph group presumably involved in π-binding at additional site, leads to a full hMC1R agonist **30** with EC_{50} 5 nM comparable to 13 nM[3] for Ac-His-D-Phe-Arg-Trp-NH_2 and with only weak partial agonism at hMC3 and hMC4Rs (Table 1). Since tripeptide **30**, lacking Trp^9, retains potency and function of the melanocortin core tetrapeptide and the binding of the remaining His-D-Phe-Arg-NH_2 fragment most likely occurs at the same site as of the full sequence His-D-Phe-Arg-Trp-NH_2, we can assume that N-terminal $Ph(CH_2)_3$ group of LK-394 binds at the recently identified by us[3] π-binding site of hMC1R.

Thus, using a series of end-capped tetrapeptides **2**–**30** we have confirmed the existence and localization of the π-binding site at hMC1R, proved the concept of an 'open' hydrophobic area next to the N-terminal amide binding sites for the core sequence His^6-D-Phe^7-Arg^8-Trp^9-NH_2 and discovered a tripeptide full hMC1R agonist LK-394 (**30**) with high MC1R versus MC3/MC4Rs selectivity. The body of data obtained by the end-capping of HfRW-NH_2 strongly suggests that the binding of the end-capping group occurs at an alternative transmembrane region compared to the binding site of the core sequence HfRW-NH_2 or, rather, at an extracellular loop. The position of this putative alternative binding site is to be determined.

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