Novel pharmacological MC4R agonists can efficiently activate mutated MC4R from obese patient with impaired endogenous agonist response

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Abstract

Human melanocortin 4 receptor (hMC4R) mutations with *in vitro* functional effects are responsible for 0.5–2.5% of severe obesity. Designing ligands that are able to counteract this *in vitro*-associated molecular defect is crucial to develop specific anti-obesity drugs in these genetically associated cases. We analyzed the *in vitro* effect of two novel melanocortin agonists, IRC-022493 and IRC-022511, on typical hMC4R mutations chosen based on the nature of their functional alterations, i.e. intracytoplasmic retention and/or reduced basal activity and/or reduced α -MSH potency. We assessed the *in vitro* ability of IRC-022493 and IRC-022511 to bind and activate hMC4R mutants. These mutations were found earlier in 11 obese French patients (median age (range) was 17.6 years (5.7–48.0) and body mass index (BMI)-Z-score 4.2 s.d. (1.5–5.5)). The MC4R agonists were responsible for a

significant activation of mutated hMC4R depending on the functional characteristics of the mutations. Both agonists were able to activate mutated hMC4R with decreased α -MSH potency, associated with or without decreased basal activity, to the same extent than α -MSH in wild-type MC4R. This result suggests that those mutations would be the best targets for the MC4R agonists among MC4R mutation-bearing obese patients. No specific clinical phenotype was associated with the differential response to pharmacological agonists. We identified two novel melanocortin agonists that were able *in vitro* to efficiently activate mutated hMC4R with impaired endogenous agonist functional response. These results stimulate interest in the development of these drugs for hMC4R mutations-associated obesity.

Journal of Endocrinology (2010) **207**, 177–183

Introduction

Melanocortin 4 receptor (MC4R) mutations are responsible for the most common genetic cause of human obesity (Farooqi 2008). The discovery of human MC4R (hMC4R) mutations affecting the receptor function pointed out not only the predominant role of MC4R in body weight regulation (Mutch & Clement 2006) but also reinforced the interest in the development of MC4R agonist to control obesity (Mackenzie 2006).

MC4R receptor encodes a G-protein-coupled receptor, which transduces signals by coupling to the heterotrimeric Gs protein and activating adenylate cyclase. Expressed in hypothalamic nuclei controlling food intake, MC4R

integrates an agonist (anorexigenic) signal provided by the pro-opiomelanocortin product, α -melanocyte stimulating hormone (α -MSH), and an antagonist (orexigenic) signal provided by the agouti-related peptide. The role of MC4R in energy balance was first demonstrated in mice (Huszar *et al.* 1997); MC4R invalidated mice develop severe obesity while heterozygous animals are less obese. MC4R activation both by natural and pharmacological agonists decreased food intake in rodents and improved their metabolic conditions (Fan *et al.* 1997).

In humans, the frequency of hMC4R mutations with *in vitro* functional consequences is relatively high (0.5–6%) in obese adults and children of European and North America origins (Farooqi *et al.* 2000, 2003, Vaisse *et al.* 2000,

Yeo *et al.* 2003, Hinney *et al.* 2006, Lubrano-Bertheliet *et al.* 2006, Calton *et al.* 2009), while it is significantly <1% in controls (0.1–0.6%) (Hinney *et al.* 2006, Lubrano-Bertheliet *et al.* 2006, Stutzmann *et al.* 2008). Individuals are mostly heterozygous carriers of mutated hMC4R with an autosomic dominant inheritance and variable penetrance and expressivity with age and generational influences (Stutzmann *et al.* 2008). While different classifications have been proposed (Tao & Segaloff 2003, Biebermann *et al.* 2006, Lubrano-Bertheliet *et al.* 2006, Tan *et al.* 2009), the functional consequences of hMC4R mutations can be schematically divided into the following categories: nonfunctional truncated receptor (i.e. due to missense or frameshift mutations), intracellular retention, altered basal activity, and altered α -MSH stimulation. Synthetic ligands, from classical NDP-MSH peptides to the multiple tetrapeptides and small molecule MC4R agonists, have been tested *in vitro* with distinct improvement of hMC4R functional activity depending on both agonist potency and nature of the mutation (Ujjainwalla & Sebbat 2007, Xiang *et al.* 2007). Determining which of the hMC4R mutations might be pharmacologically rescued by newly developed MC4R agonists is a promising approach to identify potential patients who may benefit the most from such drug candidates. In this study, we describe the *in vitro* effects of two novel melanocortin agonists on a set of selected mutated hMC4R, representative of previously established mutation classes (Dubern *et al.* 2001, Lubrano-Bertheliet *et al.* 2006).

Materials and Methods

hMC4R mutations

We studied the effect of IRC-022493 (Dong & Moreau 2007) and IRC-022511 (Dong *et al.* 2008), two novel MC4R agonists, on 11 hMC4R mutations described earlier in obese adults and children (Dubern *et al.* 2001, Lubrano-Bertheliet *et al.* 2006; Fig. 1B). The mutations were chosen among the various categories found in obese patients based on their known *in vitro* functional alterations such as intracytoplasmic retention (Pro299His and Ile102Ser) (class 1), reduced basal activity (Arg18Cys, Arg18His, Val50Met, and Gly231Ser) (class 2A), reduced α -MSH potency (Ser127Leu) (class 2B), or the association of both reduced basal activity and α -MSH potency (Arg305Trp, Ser58Cys, Ile102Thr, and Gly252Ser) (class 2C). Subjects were obese adults and children (median age (range) 17.6 years (5.7–48.0), and median body mass index (BMI)–Z-score (range) 4.2 s.d. (1.5–5.5)) participating in a previous genetic study performed at Hôtel-Dieu and Trousseau hospitals, Assistance Publique Hôpitaux de Paris, France. Some MC4R mutations were detected in new patients (Ser127Leu, Ileu102Thr, and Gly252Ser; Fig. 1A (Dubern *et al.* 2001, Lubrano-Bertheliet *et al.* 2006)). The subjects were phenotyped for a series of clinical and biological parameters related to obesity (anthropometric

measures, blood pressure, carbohydrate, insulin, and lipid measurements). Informed consent was obtained from all subjects and the protocol was approved by the local ethics committee.

hMC4R agonists

IRC-022493 and IRC-022511 are peptide melanocortin analogs, which specifically bind hMC4R with inhibition constants (K_i) of 2.1 and 2.4 nM respectively, being 20- and 17-fold more potent than α -MSH respectively. Activation of hMC4R by IRC-022493 and IRC-022511 stimulates intracellular cAMP production with 50% effective concentrations (EC_{50}) of 0.31 and 0.099 nM respectively, being 15- and 47-fold more potent than α -MSH respectively. By peripheral administration, IRC-022493 and IRC-022511 were shown earlier to reduce food intake and body weight gain in normal rats (Halem *et al.* 2006) and to improve obesity, hyperinsulinemia, and fatty liver disease in diet-induced obese mice (Kumar *et al.* 2009). α -MSH was purchased from Bachem (Weil am Rhein, Germany).

hMC4R cloning and expression

Wild-type and mutated hMC4R genes from genomic DNA were cloned into a pcDNA5/FRT/V5-His-TOPO expression vector. After transformation and selection of *Escherichia coli*, the transformant plasmids were extracted, and selected using the restriction enzyme EcoRI and sequenced to confirm the presence of the mutation. For each gene, one of the established transformants was amplified and purified following the manufacturer's conditions (TA expression kit, Invitrogen). CHO flip-in cells were stably cotransfected with hMC4R and pOG44 recombinase using the Flip-In system (Invitrogen) and effectene transfection reagent (Qiagen). Transfected cells were selected with hygromycin.

Binding to hMC4R

Membranes were obtained by sonication of CHO flip-in cells stably expressing wild-type or mutated hMC4R in 50 mM Tris-HCl, pH 7.4, and centrifugation at 39 000 g for 10 min at 4 °C. The pellet was resuspended in the same buffer and centrifuged at 50 000 g for 10 min at 4 °C and membranes in the resulting pellet were stored at –80 °C. Competitive inhibition of [¹²⁵I](Tyr²)-(Nle⁴-D-Phe⁷)- α -MSH ([¹²⁵I]-NDP- α -MSH; PerkinElmer, Les Ulis, France) binding was run in polypropylene 96-well plates. Cell membranes (25 µg protein/well) were incubated in 50 mM Tris-HCl, pH 7.4, containing 0.2% BSA, 5 mM MgCl₂, 1 mM CaCl₂, and 0.1 mg/ml bacitracin, with 0.4 nM [¹²⁵I]-NDP- α -MSH and increasing concentrations of unlabeled α -MSH, IRC-022493, or IRC-022511 for 2 h at 37 °C. The number of binding sites was determined by performing saturation experiments with increasing concentrations of [¹²⁵I]-NDP- α -MSH. Bound from free [¹²⁵I]-NDP- α -MSH was separated by filtration through GF/C glass fiber filter plates

Figure 1 (A) Clinical characteristic of the individuals in each mutation category. * and ° indicate mutation found in subjects previously described in Dubern *et al.* (2001) and Lubrano-Berthelie *et al.* (2006) respectively. BMI, body mass index (kg/m²); G T0, fasting glucose (mmol/l); Ins, fasting insulin (μU/ml); TG, triacylglycerol (mmol/l); TC, total cholesterol (mmol/l); the other mutations have been found recently in these French subjects. [#]Stands for subjects with noninsulin-dependant diabetes. (B) Position of the 11 chosen mutations. The mutations studied are indicated in black circles.

(Unifilter; PerkinElmer) presoaked with 0.1% (w/v) polyethylenimine, using PerkinElmer Filtermate harvester. Filters were washed three times with 50 mM Tris-HCl, pH 7.4, at 0–4 °C and assayed for radioactivity using PerkinElmer Topcount scintillation counter. Binding data were analyzed by computer-assisted nonlinear regression analysis (XL fit; IDBS, Guildford, UK).

Determination of intracellular cAMP

Intracellular cAMP levels were determined by an electrochemiluminescence (ECL) assay (Meso Scale Discovery (MSD), Gaithersburg, MD, USA). CHO flip-in cells stably expressing wild-type or mutated hMC4R were suspended in RMPI 1640 containing 0.5 mM isobutylmethylxanthine

and 0.2% BSA. They were dispensed (30 000 cells/well) in 384-well Multi-Array plates (MSD) containing integrated carbon electrodes and coated with anti-cAMP antibody. Increasing concentrations of α-MSH, IRC-022493, or IRC-022511 were added and the cells were incubated for 6 h at 37 °C. Then, a lysis buffer (HEPES-buffered saline solution with MgCl₂ and Triton X-100, pH 7.3) containing 0.2% BSA and 2.5 nM TAG ruthenium-labeled cAMP (MSD) was added and the cells were incubated for 90 min at room temperature. At the end of the incubation period, a Tris-buffered solution containing an ECL co-reactant and Triton X-100, pH 7.8, was added and cAMP levels in cell lysates were immediately determined by ECL detection with a Sector Imager 6000 reader (MSD). Data were analyzed by computer-assisted nonlinear regression analysis (XL fit; IDBS).

Table 1 Binding of α -melanocyte stimulating hormone (α -MSH), IRC-022493 and IRC-022511 to mutated human melanocortin 4 receptor (hMC4R). The inhibition constant (K_i) and number of binding sites (B_{max}) values represent the mean $\pm$ S.E.M. of three independent determinations

Mutation	K_i (nM)		B_{max} (fmol/mg protein)		Classification of mutation
	α -MSH	IRC-022493	IRC-022511	[¹²⁵ I]-NDP- α -MSH	
WT	26 $\pm$ 7.0	0.71 $\pm$ 0.27	0.71 $\pm$ 0.16	98 $\pm$ 5.7	Class 1
Ile102Ser	260 $\pm$ 89	4.7 $\pm$ 2.2	0.84 $\pm$ 0.25	23 $\pm$ 3.1	
Pro299His	—	—	—	—	
Arg18Cys	16 $\pm$ 5.7	0.37 $\pm$ 0.034	0.65 $\pm$ 0.060	40 $\pm$ 5.4	Class 2A
Arg18His	16 $\pm$ 1.2	0.49 $\pm$ 0.095	0.42 $\pm$ 0.045	61 $\pm$ 10	
Val50Met	22 $\pm$ 2.6	0.79 $\pm$ 0.17	0.63 $\pm$ 0.11	68 $\pm$ 14	
Gly231Ser	28 $\pm$ 7.3	0.58 $\pm$ 0.17	0.68 $\pm$ 0.15	104 $\pm$ 27	
Ser127Leu	160 $\pm$ 40	3.3 $\pm$ 0.13	2.6 $\pm$ 0.34	88 $\pm$ 18	Class 2B
Ser58Cys	53 $\pm$ 12	1.7 $\pm$ 4.3	0.97 $\pm$ 0.13	27 $\pm$ 5.0	Class 2C
Ile102Thr	210 $\pm$ 94	2.2 $\pm$ 0.21	0.85 $\pm$ 0.12	50 $\pm$ 2.5	
Gly252Ser	160 $\pm$ 66	2.3 $\pm$ 0.30	1.6 $\pm$ 0.29	50 $\pm$ 3.5	
Arg305Trp	130 $\pm$ 33	2.5 $\pm$ 0.57	2.2 $\pm$ 0.61	190 $\pm$ 16	

Results

We first evaluated our *in vitro* system by confirming the functional consequences of each hMC4R mutation in their ability to bind (Table 1) and be activated (Table 2) by their natural agonist α -MSH. Figure 2A illustrates α -MSH-stimulated cAMP production for one hMC4R mutation in each functional category. When compared with our previously described *in vitro* system (Vaisse *et al.* 2000, Lubrano-Berthelier *et al.* 2006), the EC₅₀ of α -MSH was generally higher but the ratio between wild-type and each mutated receptor was conserved (data not shown). When α -MSH potency was decreased (class 2B and 2C mutations and Ile102Ser), the alteration was more pronounced in the receptor activation efficacy than binding affinity with the

exception of Arg305Trp that only showed a reduced binding affinity (Tables 1 and 2). For most mutations, the number of [¹²⁵I]-NDP- α -MSH binding sites (B_{max}) was between 23 and 106% of wild-type hMC4R (Table 1). B_{max} was correlated to the maximal stimulation observed with α -MSH (E_{max}). E_{max} values were included between 38 and 111% of wild-type hMC4R (Table 2). Two exceptions were noticed: Pro299His (class 1) whose complete absence of binding and stimulation by α -MSH was observed and, intriguingly, Arg305Trp that elicited B_{max} and E_{max} of 192 and 8% of wild-type hMC4R respectively. Taken together, these results being similar to previous ones (Vaisse *et al.* 2000, Lubrano-Berthelier *et al.* 2003, 2006) confidently allowed us to use this *in vitro* system to test the pharmaceutical agonists.

Table 2 Intracellular cAMP production by α -melanocyte stimulating hormone (α -MSH), IRC-022493 and IRC-022511 on mutated human melanocortin 4 receptor (hMC4R). The 50% effective concentration (EC₅₀) and maximal stimulation (E_{max}) values represent the mean $\pm$ S.E.M. of 2–3 independent determinations

Mutation	EC ₅₀ (nM)			E_{max} (pmol/10 ⁶ cells)			Classification of mutation
	α -MSH	IRC-022493	IRC-022511	α -MSH	IRC-022493	IRC-022511	
WT	30 $\pm$ 7.5	1.5 $\pm$ 0.38	0.70 $\pm$ 0.24	1900 $\pm$ 130	2100 $\pm$ 140	2000 $\pm$ 150	Class 1
Ile102Ser	12 000 $\pm$ 820	100 $\pm$ 11	21 $\pm$ 3.0	1100 $\pm$ 51	1400 $\pm$ 57	1300 $\pm$ 59	
Pro299His	—	—	—	—	—	—	
Arg18Cys	34 $\pm$ 6.2	1.9 $\pm$ 0.55	0.92 $\pm$ 0.33	720 $\pm$ 140	770 $\pm$ 170	780 $\pm$ 160	Class 2A
Arg18His	16 $\pm$ 2.0	0.93 $\pm$ 0.18	0.43 $\pm$ 0.081	1100 $\pm$ 180	1300 $\pm$ 220	1300 $\pm$ 210	
Val50Met	37 $\pm$ 12	2.0 $\pm$ 0.80	0.96 $\pm$ 0.46	1400 $\pm$ 58	1500 $\pm$ 58	1400 $\pm$ 54	
Gly231Ser	33 $\pm$ 7.8	1.9 $\pm$ 0.52	0.79 $\pm$ 0.12	2100 $\pm$ 38	2300 $\pm$ 38	2200 $\pm$ 110	
Ser127Leu	640 $\pm$ 190	17 $\pm$ 6.0	5.2 $\pm$ 1.3	1500 $\pm$ 66	1600 $\pm$ 54	1500 $\pm$ 31	Class 2B
Ser58Cys	670 $\pm$ 90	46 $\pm$ 8.0	19 $\pm$ 4.0	800 $\pm$ 140	950 $\pm$ 150	960 $\pm$ 150	Class 2C
Ile102Thr	5800 $\pm$ 850	150 $\pm$ 57	38 $\pm$ 15	1000 $\pm$ 351	1100 $\pm$ 362	1000 $\pm$ 290	
Gly252Ser	210 $\pm$ 45	13 $\pm$ 2.0	8 $\pm$ 2.0	1400 $\pm$ 71	1500 $\pm$ 67	1500 $\pm$ 8.0	
Arg305Trp	15 $\pm$ 2.3	0.87 $\pm$ 0.15	0.61 $\pm$ 0.070	160 $\pm$ 29	170 $\pm$ 32	150 $\pm$ 32

Figure 2 (A) cAMP production by α -MSH in wild-type (black closed circle) and three hMC4R mutations with distinct functional effect: Arg18Cys (blue closed square, class 2A), Ser127Leu (red open circle, class 2B), and Ser58Cys (green open square, class 2C). Data represent the results of a typical experiment that was replicated three times. (B) Stimulation of cAMP production by IRC-022493 (red squares) and IRC-022511 (green diamonds) in comparison with α -MSH (black circles) in Gly252Ser, a class 2C hMC4R mutation. Data represent the results of a typical experiment that was replicated three times.

We evaluated the ability of the two ligands (IRC-022493 and IRC-022511) to bind and activate the hMC4R mutants. Whatever the mutation, the maximal stimulation of cAMP production ($E_{\max}$) obtained with IRC-022493 or IRC-022511 was similar to the level reached by α -MSH (Table 2). However, IRC-022493 showed a 28- to 95-fold higher affinity for MC4R (Table 1) and a 15- to 120-fold increased potency in stimulating cAMP (Table 2) than α -MSH. Comparable results were obtained with IRC-022511. IRC-022511 was 25- to 310-fold and 25- to 571-fold more potent than α -MSH in binding (Table 1) and activating (Table 2) MC4R respectively.

Both agonists revealed increased potencies as compared to α -MSH on either wild-type or the mutated hMC4R, but the

relevance in terms of beneficial activation of MC4R depended on the functional consequences of each mutation. In the case of primary major altered binding and cAMP stimulation by α -MSH, i.e. for hMC4R mutations in class 2B (decreased α -MSH potency) and class 2C (decreased basal activity and α -MSH potency), both agonists were able to reach the stimulation level of the natural ligand in wild-type hMC4R, especially for the mutations Ser127Leu, Ser58Cys, Ile102Thr, and Gly252Ser (Table 2). Figure 2B shows the comparative stimulation of cAMP production induced by α -MSH, IRC-022493, and IRC-022511 in the Gly252Ser hMC4R mutation. For hMC4R mutations associated with a decreased basal activity (class 2A), we could not predict the response even if an increased MC4R stimulation capacity was observed. In the case of a major reduced membrane expression (class 1), the agonists may lead to an enhanced hMC4R activation when part of the receptors remains reachable to the ligands (Ile102Ser). Otherwise, no benefit can be provided by the agonists (Pro299His).

We examined whether a specific clinical phenotype would be associated with the presence of hMC4R mutations that might be preferentially targeted by the agonists. No significant difference for each clinical and metabolic phenotype (age, sex, BMI-Z-score, blood pressure, and metabolic parameters) was found between the subjects separated according to the *in vitro* response of hMC4R mutations to the agonists (data not shown).

Discussion

This work shows that novel melanocortin agonists, IRC-022493 and IRC-022511, are able to offset *in vitro* the impaired endogenous agonist response of class 2B and 2C mutated hMC4R to the level of α -MSH in wild-type receptor. In a recent study (Xiang *et al.* 2007), distinct relative potencies versus α -MSH were observed for synthetic agonists depending on the mutated receptor as compared with wild-type hMC4R. In agreement with that work, differences in IRC-022493 and IRC-022511 potency depended on the mutation type, although in this study, the potency ratio of IRC-022493 or IRC-022511 versus α -MSH was unchanged in mutated hMC4R as compared to wild-type hMC4R. In case of primary intracellular retention (class 1), the pharmacological agonists can improve hMC4R stimulation only if the receptor remains accessible to the ligands (Ile102Ser). When the hMC4R mutation induces a reduced basal activity with normal responsiveness to α -MSH (class 2A), the physiological response to the agonists cannot be anticipated but undesired overstimulation of the receptor might not be excluded. The beneficial effects of IRC-022493 and IRC-022511 appear to be of marked interest for hMC4R mutations with decreased α -MSH response (class 2B and 2C). Mutations that are rescued by the pharmacological agonists (Ser58Cys, Ile102Thr, Ile102Ser, Ser127Leu, and Gly252Ser) are located in key domains involved in interactions between

hMC4R and the ligands. Except for Ser127Leu, they are positioned closer to the cytoplasmic side of the lipid bilayer and are likely to be involved in ligand-induced conformational changes important for G-protein-induced signal transduction events (Xiang *et al.* 2007). Ser127Leu is located in the third transmembrane domain and was described to be an important residue for interacting with the endogenous melanocortin conserved His-Phe-Arg-Trp residues. Ser127 residue might be important for receptor local hydrogen bonding interactions that might shift the hMC4R from the inactive to the active conformation state on ligand binding (Xiang *et al.* 2007).

Our results reinforce the importance of the functional characterization of hMC4R mutations to categorize the different classes of MC4R mutations, and thus identify mutation-bearing patients who might benefit the most from MC4R agonist therapy. Mutations with decreased endogenous α -MSH response appear to be the best candidate responders for IRC-022493 and IRC-022511. Moreover, no specific clinical phenotype permits the selection of patients, who could benefit from such pharmacological treatment. Most mutations are found at the heterozygous state and little is known about MC4R dimerization *in vivo*. In addition, previous studies showed no dominant negative effect for intracellular retained receptors (Ho & MacKenzie 1999, Yeo *et al.* 2003) and only one hMC4R mutation was described to result in a dominant-negative effect (Biebermann *et al.* 2003). Whether the pharmacological agonists could stimulate the mutated hMC4R without affecting the wild-type hMC4R protein remains to be explored.

No significant differences in clinical and metabolic phenotype were found between the subjects separated according to the *in vitro* response of hMC4R mutations to the two novel agonists. However, the spread variability of plasma triacylglycerol (TG) observed in obese patients may be in accordance with studies showing the direct role of the central melanocortin system on peripheral lipid metabolism (Nogueiras *et al.* 2007). Indeed, blockade of MC4R directly increases lipid uptake and TG synthesis in the periphery most likely through effects on autonomic outflow, inducing a decrease in plasma TG. Furthermore, it was demonstrated very recently that IRC-022493 improves hepatic steatosis reducing liver lipid and TG content in diet-induced obese mice (Kumar *et al.* 2009). Those data suggest that MC4R agonists may have a protective effect on lipid metabolism and especially TG synthesis.

In both preclinical and clinical studies, the role of the melanocortin system in penile erection, in modulation of the cardiovascular system, in inflammatory responses, and in pain processing has been described suggesting the risk of side effects when using melanocortin agonists (Adan *et al.* 2006, Shadiack *et al.* 2007). However, a very recent clinical study showed the influence of a pharmacological agonist on blood pressure and underlined the interest of MC4R agonist administration to MC4R mutation carriers since they

had lower blood pressure than MC4R wild-type carriers (Greenfield *et al.* 2009).

In conclusion, we have shown that *in vitro* functional classification of hMC4R mutations using pharmacological agonists is an attractive approach for identifying the most promising responders among the obese population of MC4R mutation-bearing patients. Using this strategy, we demonstrated that mutated hMC4R with impaired endogenous agonist functional response can be activated by IRC-022493 and IRC-022511, and might represent a valuable therapeutic target for these novel melanocortin agonists. This observation suggests an interest in the development of specific drugs in hMC4R mutations-associated obesity.

Declaration of interest

P R, P P, F A, D B D, J Z D, and C T are employed by IPSEN. B D, C L-B, R A, A B, and K C declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by the APHP (PHRC 1996), the GIP-ANR (National Agency of Research), the Benjamin Délessert Institute, the Guigoz Company, and the IPSEN group.

Author contribution statement

P R, B D, and C L-B designed the study. K C, A B, and B D recruited and performed the phenotyping of the patients. P R, P P, R A, F A, D B D, J Z D, and C T developed the agonists and experiments. P R, B D, P P, and K C analyzed the data and wrote the paper.

Acknowledgements

We thank the patients and their families for their participation in the genetic study. We also thank R Delille and J G Marin for help in receptor cloning and expression, and E Ferrandis for his helpful suggestions.

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Received in final form 29 July 2010

Accepted 9 August 2010

Made available online as an Accepted Preprint
9 August 2010