Novel MC2R Antagonists Decrease Cortisol in Primary Human Adrenal Cortical Cells and Corticosterone in an *in vivo* Model of Hypercortisolism

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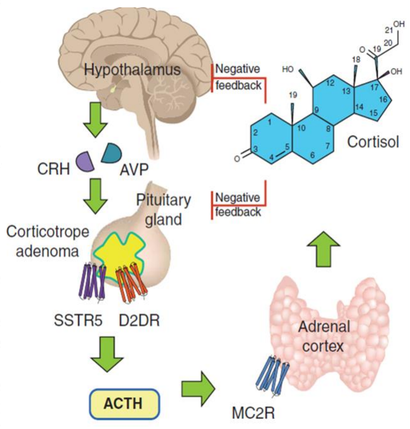
INTRODUCTION

Adrenocorticotropic hormone (ACTH), is a peptide hormone that regulates glucocorticoid (GC) production by the adrenal gland through interaction with the melanocortin 2 receptor (MC2R). ACTH production by corticotropic cells in the anterior pituitary is regulated by corticotropic releasing hormone (CRH) and arginine vasopressin (AVP). The secretion of ACTH is negatively regulated by the glucocorticoid cortisol. Chronic elevation of ACTH is associated with Cushing's disease (CD) and Congenital Adrenal Hyperplasia (CAH).



CD is characterized by the hypersecretion of ACTH by pituitary adenomas and loss of negative feedback control leading to chronic overproduction of cortisol which is ultimately responsible for the disease morbidity and mortality. The first line therapy, surgical removal of the pituitary tumor, suffers from high recurrence rates while pharmacotherapies are limited due to insufficient efficacy or side effects. Therefore, there is a clear unmet need for new treatments (1-8).

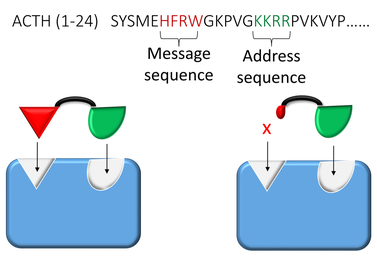
In CAH, a loss of cortisol thus, reduced negative regulation of ACTH, results in ACTH dependent overproduction of adrenal androgens. The standard of care for CAH is treatment with GC. However, supraphysiological levels of GC are needed to efficiently suppress androgens, leading to side effects (1,8).

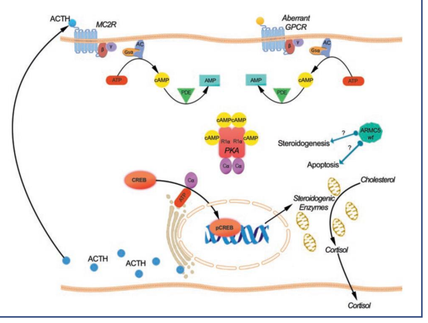
Of the 5 melanocortin receptor subtypes, MC2R specifically interacts with ACTH and is selectively expressed in the adrenal gland. The hormone, which is a 39-residue peptide consisting of a common message sequence and unique address sequence, is the only known endogenous ligand for the MC2R (9,10). The receptor itself is a GPCR that stimulates cAMP production through coupling to Gαs leading to expression of steroidogenic enzymes.

The interaction of ACTH and MC2R is proposed to occur through a multi-step mechanism whereby the address portion of the peptide engages the receptor to promote a second interaction with the message sequence (10). We hypothesized that driving the potency and selectivity of the address-receptor interaction while disrupting the message-receptor interaction would lead to selective MC2R antagonists.

Such MC2R antagonists have the potential to efficiently regulate ACTH driven pathophysiology while avoiding the side effects of the current therapies in CD and CAH.

We report the discovery and pharmacological characterization of novel peptide MC2R antagonists in an optimized in vivo model of ACTH over secretion. Screening of rationally designed peptide libraries in functional assays allowed the identification of potent and selective MC2R peptide antagonists. These compounds suppressed ACTH induced MC2R signaling and displayed a significant reduction of cortisol levels in primary human adrenal cortical cells. The optimized animal model of ACTH induced GC secretion was used to demonstrate the efficacy of our novel MC2R antagonists. These results demonstrate the potential of MC2R antagonists to address unmet needs in the treatment of CD and CAH patients.





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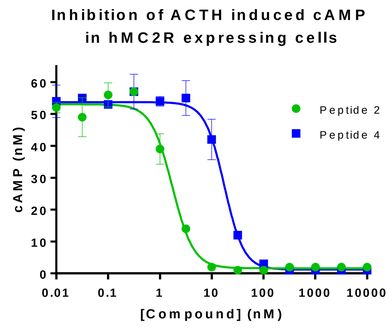
In vitro functional screening and selectivity assays

In vitro screening and functional cell based assays

We generated more than 100 rationally designed peptides based on the structural and conformational features of ACTH(1-39). Peptides were screened *in vitro* in cell based functional assays for agonist and antagonist activity at the MC2R and were counter-screened for agonist and antagonist activity against all other human melanocortin receptors. Functional reduction of ACTH-induced cortisol production in primary human adrenal cortical was utilized for screening compounds with high potency and specificity. Data from MC2R and primary cell assays were utilized as starting points for peptide optimization.

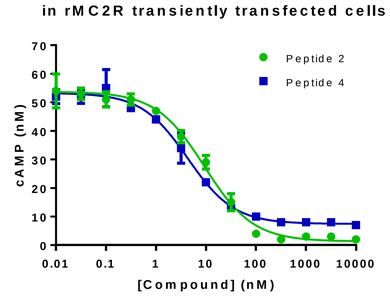


In vitro screening assay in hMC2R cell line

GeneBLAzer® MC2R-CRE-bla-CHO-K1 (ThermoFisher Scientific) are a stable cell lines expressing human melanocortin 2 receptor and the human melanocortin 2 receptor accessory protein (MRAP) that is required for receptor trafficking and functional activity (10). These cells were utilized for compound screening *in vitro* via inhibition of ACTH-induced cAMP measured by homogenous time resolved fluorescence (HTRF, cisbio). Cells were exposed to varying concentration of compound in the presence of the EC80 of ACTH(1-39).

Increasing concentrations of MC2R antagonist compounds up to 10μM are incubated with cells for 20 minutes followed by stimulation with ACTH(1-39) at EC80 for 30 minutes at room temperature. Cells were also tested for agonist activity by HTRF in these cells in the absence of ACTH(1-39).

All peptides were also measured by HTRF for cAMP agonist and antagonist activity using CHO GeneBLAzer® stable cell lines expressing MC1R, MC3R, MC4R and MC5R.

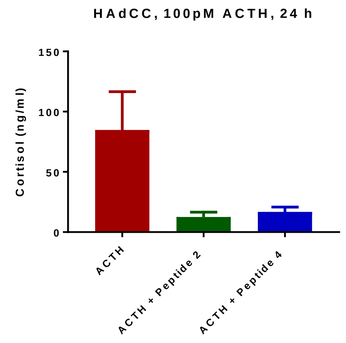


In Vitro screening assay in transiently transfected rMC2R cells:

Prior to *in vivo* testing, antagonist activity was confirmed *in vitro* at the rat MC2R. CHO-K1 cells were transiently transfected with rat-specific MC2R and MRAP. Cells were incubated for 24 hours prior to the cAMP HTRF assay. Concentration response curves of MC2R antagonist peptides up to 10μM were incubated with cells at 37°C for 20 minutes, followed by exposure to the EC80 of ACTH(1-39) for 30 minutes at 37°C. Peptides were also tested up to 10μM in the absence of ACTH(1-39) to check for agonist activity.

For peptides 2 and 4, the IC50 values ranged from 2 to 19nM at human MC2R in vitro and from 2 to 10nM at rat MC2R. Representative graphs are shown.

These data demonstrate that we identified peptides with antagonist activity at the human and rat MC2 receptor. These peptides also showed selectivity over human MC1R, MC3R, MC4R and MC5R and an absence of agonist activity at any of the melanocortin receptors.



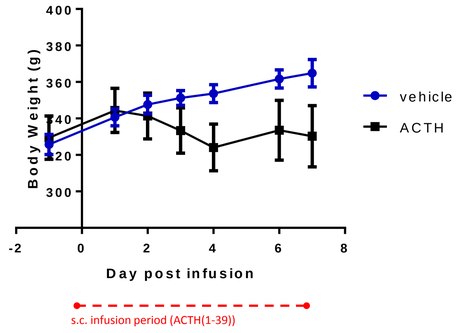
In vitro reduction of ACTH-induced cortisol production in primary human adrenal cortical cells:

Adrenal cortical cells are capable of producing cortisol in response to stimulation with ACTH(1-39) (11). Functional activity of peptide compounds was measured by inhibition of ACTH-induced cortisol production in human adrenal cortical cells. Primary human adrenal cortical cells (HAdCC, Sciencell) were cultured as previously described (11). Cells were treated for 24 hours with 100pM ACTH(1-39) (Tocris Bioscience) in the presence or absence of 1.2 μM MC2R antagonist peptides at 37°C. Following this incubation, conditioned media was collected and assayed for cortisol concentration by HTRF assay kit (cisbio). In this single experiment, at the concentration used, both peptide 2 and peptide 4 reduced ACTH-stimulated cortisol production in primary human cells. Data are represented as mean +/- SD with 2 replicates per group.

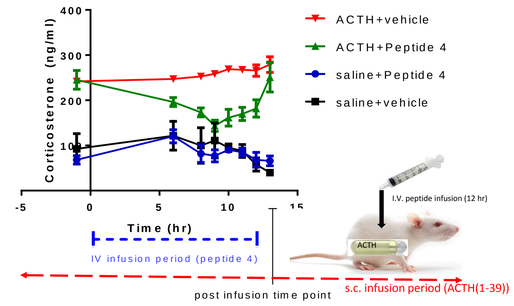
In vivo reduction of corticosterone in a hypercortisolism model

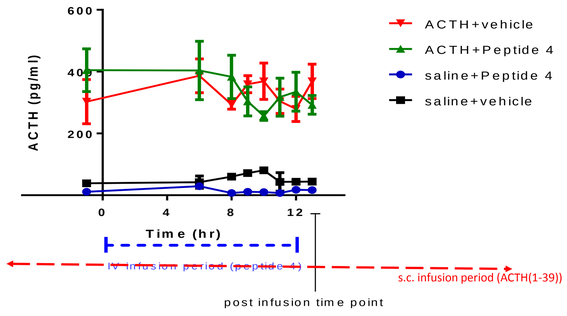
In vivo pharmacodynamic model of hypercortisolism

To evaluate the *in vivo* efficacy of MC2R peptide antagonists, we developed a rat model of hypercortisolism by exogenously increasing plasma ACTH levels via continuous administration of ACTH(1-39) through a subcutaneous pump. ACTH levels were increased similar to the levels found in Cushing's Disease patients (12).



Dual (jugular vein and carotid artery) catheterized male Sprague-Dawley rats were implanted subcutaneously with Alzet® osmotic pumps administering 0.05 mg/kg/day ACTH(1-39) (Tocris) or vehicle for 7 or 14 days. Repeated exposure to ACTH leads to a reduction in body weight, primarily through reduced food intake (13). As expected, continuous infusion of ACTH(1-39) s.c. led to reduced body weight over 7 days and was utilized as a biomarker of elevated ACTH prior to inclusion in pharmacodynamics studies.





Peptide 4 reduces corticosterone level in hypercortisolism model
Two days following pump implantation, animals were attached to automated blood samplers (ABS2, Instech Laboratories) and allowed to acclimated for several hours to allow recovery of normal corticosterone levels following transfer and handling. Following acclimation, a baseline sample is collected and animals were then remotely IV infused with peptide 4 or vehicle at 2.2 mg/kg/day for 12 hours. Samples were collected every hour from 6 to 12 hours of the infusion period. One hour after the completion of IV infusion, an additional post-infusion sample was collected. Peptide 4 reduced corticosterone levels over the infusion period without affecting plasma ACTH.

Plasma concentrations of ACTH and corticosterone were determined using the Milliplex Rat Stress Hormone Magnetic Bead Panel (EMD Millipore) read with a Luminex MAGPIX imager (EMD Millipore). Data are represented as mean +/- SEM, n=4 rats/group.

CONCLUSION

We have identified selective and efficacious peptide MC2R antagonists in the low nM range by rational design and screening in functional cell based assays. In human primary adrenal cortical cells, MC2R peptide antagonists decreased ACTH-induced cortisol production. Utilizing an *in vivo* rodent model of hypercortisolism, we also demonstrated that MC2R peptide antagonists decreased elevated plasma corticosterone without affecting increased plasma ACTH concentration.

These selective MC2R antagonists have the potential to generate lead structures towards the discovery of novel treatments for CAH and CD.

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