Resolution Pharmacology therapeutic innovation in inflammation.ACTH: The Forgotten Therapy

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Abstract

Although anti-inflammatory drugs are among the most common class of marketed drugs, chronic inflammatory conditions such as rheumatoid arthritis, multiple sclerosis or inflammatory bowel disease still represent unmet needs. New first-in-class drugs might be discovered in the future but the repurpose and further development of old drugs also offers promise for these conditions. This is the case of the melanocortin adrenocorticotropin hormone, ACTH, used in patients since 1952 but regarded as the last therapeutic option when other medications, such as glucocorticoids, cannot be used. Better understanding on its physiological and pharmacological mechanisms of actions and new insights on melanocortin receptors biology have revived the interest on rescuing this old and effective drug. ACTH does not only induce cortisol production, as previously assumed, but it also exerts anti-inflammatory actions by targeting melanocortin receptors present on immune cells. The endogenous agonists for these receptors (ACTH, α-, β-, and γ-melanocyte stimulating hormones), are also produced locally by immune cells, indicating the existence of an endogenous anti-inflammatory tissue-protective circuit involving the melanocortin system. These findings suggested that new ACTH-like melanocortin drugs devoid of steroidogenic actions, and hence side effects, could be developed. This review summarizes the actions of ACTH and melanocortin drugs, their role as endogenous pro-resolving mediators, their current clinical use and provides an overview on how recent advances on GPCR functioning may lead to a novel class of drugs.

Keywords

ACTH, drug discovery, GPCR, inflammation, melanocortin, pro-resolving mediator.

Abbreviations

ACTH, adrenocorticotropin hormone; GC, glucocorticoids; GPCR, G-protein coupled receptor; HPA, hypothalamic-pituitary-adrenal axis; MC, melanocortin; MSH, melanocyte stimulating hormone; POMC, pro-opiomelanocortin hormone; RA, rheumatoid arthritis;

1. Introduction

Do we need new drugs to treat human diseases? Do we need novel therapeutic targets? Despite the over 2,000 drugs currently approved for the use in humans [1], the need for novel treatments is obvious for treating chronic, orphan, incurable and life-threatening diseases, cancer, difficult to manage conditions such as diabetes or sepsis, emergent infections, etc. These will undoubtedly require the discovery of new molecules or interventions to be successfully managed. However, another option is to apply old drugs to new diseases, a strategy known as drug repositioning [2]. The importance of such strategy is significant as the addition of a new indication to an already developed drug dramatically reduces the high cost and time of pharmaceutical R&D required to market a drug, factors of particular relevance for neglected, orphan or rare diseases.

One might also wonder if most of pharmaceutically relevant targets have already been discovered. It has been estimated that current approved therapies are directed to less than 400 targets in addition to another <500 undergoing clinical trials [1]. Although limited by the number of potential molecular targets actually involved in a particular disease and the "only" 30 000 genes contained in the human genome, undoubtedly new targets will emerge. However, yet again, innovative approaches suggest alternative ways to activate known targets by the use, for instance, of biased agonists or allosteric modulators, as discussed later.

This review focuses on the adrenocorticotropin hormone, ACTH, an old forgotten melanocortin peptide that might potentially be rescued and repurposed for new indications. ACTH was approved by the FDA for use in humans in 1952, only three years after it was first tested in rheumatoid arthritis (RA) [3]. By that time, ACTH -acting by stimulating the adrenal cortex to produce cortisol- was used for several conditions such as RA, gout, lupus, rheumatic fever, psoriasis or ulcerative colitis [4]. Philip S. Hench, Edward C. Kendall and Tadeus Reichstein were awarded in 1950 the Nobel Prize in Physiology or Medicine for these discoveries on ACTH and adrenal hormones. However, highly efficient methods for glucocorticoid (GC) synthesis were developed some years later causing a drop in price and oral forms also became available making GC the treatment of choice to the detriment of ACTH.

Interestingly, exactly 50 years after its approval, a novel mechanism of action was envisaged by Getting *et al*, who discovered that the anti-inflammatory actions of ACTH were retained in adrenalectomized rats using a model of knee gout [5]. This cortisol-independent effect was mediated by the melanocortin (MC) receptor MC_3, which is

expressed in immune cells and in the brain, presenting this receptor as a novel therapeutic target for ACTH-like drugs devoid of cortisol-related side effects. These findings have pioneered a two-fold revived interest in ACTH therapy, first by reconsidering the use of ACTH for new indications, particularly in cases where GC are not recommended, and second by proposing innovative therapeutic targets, i.e. the melanocortin (MC) system for the development of endogenous-based anti-inflammatory therapies.

The MC system, quite unknown outside its field, is responsible for common features and processes within our body such as the colour of our eyes, our skin tanning ability upon sun exposure or whether we are lean or obese. This system can modulate blood pressure, exert anti-microbial actions or even predict anesthetics requirements [6, 7]. These wide-ranging actions make MC receptors very attractive for drug development. This review focuses on the anti-inflammatory actions of ACTH and other MC drugs and the current clinical use and future directions on drug development.

2. Melanocortins and their receptors

2.1 Melanocortin ligands

Melanocortin receptors (MC_{1-5}) belong to the class A (rhodopsin-like) family of G protein-coupled receptors (GPCRs). The broad and tissue-selective distribution of these receptors and their ligands accounts for their multiple and disparate actions of the MC system in the body. ACTH and α-,β-, and γ-melanocyte stimulating hormones (MSH) are the four endogenous melanocortin agonists, all derived from the common larger precursor pro-opiomelanocortin (POMC) protein. Cleavage of POMC is tissue specific and is performed by prohormone convertase (PC) 1 alone, leading to ACTH production, or by both PC1 and PC2, producing α,β, and γMSH (Figure 1) [8]. All MC agonists contain the common amino-acid motif HFRW, this being the minimum sequence required for receptor binding and activation. However, activation of MC_2 also requires the sequence KKRRP, only present in ACTH (and the synthetic $ACTH_{1-24}$) [9]. Hence, ACTH, α,β, and γMSH can activate $MC_{1,3,4,5}$ with varying affinities, with MC_1 and MC_3 exhibiting the highest affinity for α-MSH and γ-MSH respectively [8] while MC_2 can only be activated by ACTH. Interestingly the MC system is the only GPCR family for which naturally occurring antagonists have been identified [10, 11]. Agouti signaling (ASIP) and agouti-related (AGRP) proteins both act as inverse agonists by decreasing basal levels of constitutive agonist-independent activity of MCRs.

However, if the complexity of the MC system was not sufficient, the antagonist AGRP was recently associated with $G_{i/o}$ protein signalling activation and consequently it should be referred now as a biased agonist [12].

2.2 Melanocortin receptors

The five MCRs present high degree of homology (Figure 2) with around 70 and 80% similarity between human MC_1-MC_3 and MC_3-MC_4 respectively, explaining the low selectivity that natural, and also synthetic, peptides present. MCRs are the smallest GPCRs known with an unusually small second extracellular domain and several potential N-glycosylation sites in the amino-terminus and potential phosphorylation sites particularly in the second cytosolic loop. Of importance is also the conserved Cys in the C-terminus, suggested to be involved in anchoring the receptor within the plasma membrane [13]. Despite this resemblance, their actions are very distinct, possibly, in part, due to their specific tissue distribution. MC_1, with a extremely short C-terminus, is mainly expressed in melanocytes and regulates the conversion of pheomelanin (yellow/red) into eumelanin (dark), determining skin pigmentation. It is encoded by a highly polymorphic gene, which associates with skin cancer susceptibility [14]. MC_2 is the responsible for the steroidogenic actions of ACTH. It is expressed in the adrenal cortex, where upon activation, causes the up-regulation of the enzymes responsible for the synthesis of cortisol [15]. Mutations in MC_2 cause familial glucocorticoid deficiency type 1. MC_3 possess the longest N-terminus of the five MCRs, and it is expressed in the brain and in cells of the immune system, where it regulates energy homeostasis and inflammatory responses, respectively. Along with MC_3, MC_4 plays a mayor role in regulating energy homeostasis and variants in *MC4R* gene represent the most common cause of monogenic obesity [16]. The last receptor of the family, MC_5, was identified in 1994. Singular experiments using shampooed mice and swim tests demonstrated a role for this receptor in sebum production and thermoregulation [17] as well as their expression in other exocrine glands. Interestingly, MC_5 is also expressed in immune cells where it seems to exert protective anti-inflammatory actions [18]. A more comprehensive review on these receptors can be found in [6, 8, 19].

2.3 Signal transduction and melanocortin receptor regulation.

GPCRs are associated with a very complex pharmacology, which is essential to understand to design and develop new drugs. They are generally very promiscuous receptors, able to bind multiple ligands of very different nature including peptides, lipids, aminoacids, hormones, nucleotides or even light [20]. It is also accepted now that GPCR signaling is not as simple as a linear sequence of events (ligand-receptor-pathway-biological effect) as previously believed, but they rather act via a sort of organized network of pathways [21]. MCRs are coupled to $G_{\alpha s}$ proteins leading to the conversion of cytoplasmic ATP into cAMP by adenylyl cyclase. cAMP acts as a second messenger and activates protein kinase A (PKA). Besides this canonical pathway, other signaling cascades, dependent or not on cAMP, have also been described. Phosphorylation of extracellular-signal-regulated protein kinases ERK1/2 have been described for all MCRs and intracellular Ca^{2+} mobilization have been reported so far for $MC_{3,4,5}$ [22-28]. ACTH can also induce phosphorylation of p38 kinase in keratinocytes via MC_1 and MC_2 [29]. In addition, activation of c-Jun N-terminal kinase (JNK) and Jak/STAT pathways have been observed in MC_4 transfected HEK293 cells and Ba/F3 B lymphocytes expressing MC_5, respectively [30, 31].

The signaling studies summarized above have been conducted using a mixture of conditions: primary or transfected cells, human or mouse receptors, natural or synthetic drugs and presumably a variety of culture conditions and experimental designs. Although generalizations are usually very tempting in terms of associating a receptor with a signaling pathway, caution should be taken when making these assumptions, considering the complexity of GPCRs biology. For example, the elevations in intracellular Ca^{2+} observed in MC_3 transfected Hepa cells was only observed in the presence of the PKA inhibitor H-89 and not following αMSH treatment alone, indicating that MCR signaling can be context dependent [26]. In addition, MC_3 could induce ERK1/2 phosphorylation when activated by the peptide NDP-αMSH [24] but not by melanotan II (MT-II) [32] suggesting ligand-specific conformational states. Interestingly, single nucleotide polymorphisms (SNPs) can differentially affect MC_1 signaling by selectively reducing cAMP but not ERK1/2 signaling [33]. Taken together, deep analysis and consideration of differential signaling is of paramount importance for example when characterizing new drugs.

Ligand-independent constitutive activity is a common feature of GPCRs and it has been demonstrated for $MC_{1,3,4}$ and $_5$ [34, 35]. In particular, the intrinsic activity of MC_1 and MC_4 is key for their physiological actions and of relevance for drug discovery as inverse agonists may be developed to modulate their activity. MC_4 constitutive activity is required for maintaining energy homeostasis and it has been shown to be driven by

the N-terminal domain of the receptor that acts as a "self-ligand" [35]. The relevance of the basal activity of MC_1 however differs between human and mouse. In the mouse it is crucial in determining coat colour: *Pomc*-/- mice are still black despite the absence of any endogenous agonist because MC_1 constitutive cAMP level is sufficient to trigger full eumelanogenic activity [36]. On the other hand, POMC deficiency in humans leads to red hair pigmentation [37].

Dimerization and desensitization are also important aspects on MCRs regulation. Homodimerization of $MC_{1,3}$ and $_4$, as well as heterodimerization of MC_1/MC_3 have been reported and suggested to exist constitutively [38, 39]. Furthermore, heterodimers with non-melanocortin receptor have been found with MC_3 and the growth hormone secretagogue receptor, and MC_4 with GPR7 [40]. Homologous desensitization via ligand-induced internalization have been described for $MC_{1,2,3}$ and $_4$ and some reports suggest that internalized receptors are likely degraded rather than recycled [41].

3. MC as pro-resolving molecules

3.1 The resolution of inflammation

Recent advances in drug discovery are questioning the classical and reductionist 'one-drug one-target' approach to propose a new strategy called polypharmacology. This emerging approach in rational drug design proposes the use of drugs that can modulate several targets at a time for maximal efficacy, in contrast to the classical target-based rationale aiming for drugs with maximal selectivity and minimal side effects. This new vision is based on systems biology studies that understand biological functions as networks of events produced by the interaction of several components within the cells rather than by one single molecule [42]. Growing evidence suggests that affecting one single target is often insufficient because compensatory pathways usually counter-balance the inhibition of that given target. It is not a coincidence that this "new" concept called polypharmacology actually resemblances the way that Nature wisely designed its own defensive mechanisms. Our body's anti-inflammatory/ pro-resolving repertoire includes a number of receptors and their endogenous ligands, that acting in coordination lead to the restoration of homeostasis after the inflammatory insult. It is not one single molecule or receptor that leads to resolution but rather an integrated and synchronized network of signals and events. It is not exceptional then to find most of the endogenous pro-resolving mediators -resolvins, lipoxins, galectins, protectins, maresins, melanocortins, annexin A1 [43, 44]- in, for example, peritoneal

fluid from the zymosan-induced peritonitis, a mouse model commonly used to study resolution mechanisms [45].

The resolution of inflammation as a field was formally established in 2007 during a meeting of the British Pharmacological Society, where a panel of experts defined a framework for the study of resolution mechanisms and their therapeutic exploitation [46]. A consensus report defined resolution as an *active* rather than a passive process, that counter-regulate pro-inflammatory signals for tissue protection, leading to the restoration of homeostasis, after tissue insult. Multiple endogenous mediators have been identified and characterized since then and extensively reviewed [43, 47, 48]. Likewise remarkable is the appreciation that inflammatory diseases could derive from excessive pro-inflammatory signals as much as from defective counter-regulatory, i.e. pro-resolving, signals, shifting the perception and potential therapeutic strategies for several conditions [49].

Endogenous pro-resolving resources, besides acting in an orchestrated manner as explained earlier, also present an interesting and unique feature: they exhibit mild to moderate effects in a wide variety of actions, rather than causing dramatic inhibition on one specific mediator or target (Figure 3). With exceptions, we have learnt that maximal selectivity does not necessarily equates to maximal efficacy. The pro-resolving mode of action helps to bypass potential compensatory mechanisms that might explain, for example, why any targeted anti-chemokine therapy have been successfully developed yet to treat inflammatory conditions [50]. Another example includes strategies to inhibit the IL-6 pathway: it was found that mice lacking IL-6R on B cells develop exacerbated arthritis, as this cytokine is important for the development of protective IL-10-producing regulatory B cells [51]. This indicates that what we call 'pro'-inflammatory mediators have also a protective role. The pro-resolving strategy is not about 'bad' mechanisms that need to be shut down, it is about modulation and reaching a balance between the different 'pro' and 'anti' mechanisms. What resolution-based pharmacology proposes is to develop novel drugs that act by mimicking the way our body naturally resolves inflammation, by promoting the existing protective mechanisms using analogs of the natural mediators or small molecules targeting their receptors [43, 44].

3.2 Anti-inflammatory and pro-resolving actions of ACTH and MC drugs.

Melanocortins are molecules produced during inflammation with a role in controlling and balancing the inflammatory process, i.e. they are natural pro-resolving mediators. In general terms, they exert anti-inflammatory actions via two independent mechanisms. The first of them to be described consists on the induction of cortisol production by the adrenal cortex. This mechanism is restricted to the MC peptide ACTH, as it is the only natural peptide that can activate MC_2 (Figure 4). On the other hand, this mechanism is also responsible for the major side effects of long-term ACTH therapy, which are similar to those produced by GC therapy: Cushing's syndrome, fluid retention, glaucoma, cardiovascular disorders, etc. It is worth noting that GC can be synthesized locally in organs such as the skin [52], where they modulate local inflammation although the role or contribution of the MC system in this process is still not known. Melanocortins extra-adrenal actions, first pointed out by Ferrari *et al* in 1955 [53] when ACTH was already used in clinics, are exerted directly on cells of the immune system and play an important role in the anti-inflammatory actions of MC drugs. MC can be synthesized by immune cells [54] and hence be produced at sites of inflammation, such as the synovial fluid in RA patients [55], suggesting the existence of localized and finely regulated anti-inflammatory circuits independent of the hypothalamic-pituitary-adrenal (HPA) axis. The MC receptors are expressed in macrophages, mast cells, neutrophils and lymphocytes and of relevance for rheumatic diseases, they are also operative in osteoclasts, osteoblasts and chondrocytes and fibroblasts [56-60].

The protective actions are related to inhibition of leukocyte transmigration, reduction on cytokines and production of anti-inflammatory signals. Both receptors MC_1 and MC_3 are associated with reduction on leukocyte trafficking, as demonstrated by the use of mutant mice lacking either of these receptors in a model of vascular inflammation using intravital microscopy [61, 62]. In addition, αMSH reduced the expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1 induced by LPS in endothelial cells [63]. ACTH was able to reduce neutrophil infiltration in a model of crystal inflammation and to reduce the production of the chemoattractant cytokine CXCL-1, effects prevented when the $MC_{3/4}$ antagonist SHU9119 was used [64]. Similarly, the synthetic αMSH analog AP214, reduced neutrophil influx in the zymosan peritonitis model and the release of IL-1β, IL-6 and TNFα by peritoneal macrophages [65]. Interestingly, αMSH stimulation of monocytes resulted in the production of the anti-inflammatory cytokine IL-10 [66]. Melanocortins also promote strictly pro-resolving actions such as the clearance of apoptotic cells (efferocytosis) [65] and wound healing [67]. Protective actions on the joints are also associated with a reduction in osteoclast

formation [68], reduced production of metalloproteases by chondrocytes [69] and increase in osteoblast differentiation, this latter effect driven by ACTH acting via MC_2 expressed in these cells [56]. At the molecular level, the anti-inflammatory actions of MC drugs have been associated with the inhibition of the nuclear transcription factor NF-κB [70].

4. Current use and clinical evidence of ACTH and MC drugs

4.1. ACTH formulations

The current clinical use of ACTH is paradoxically not based on the current knowledge on ACTH actions summarized in the previous section. In the practice, ACTH use is still supported merely by their ability to induce GC release. It is worth noting that when ACTH was first commercialized, none of the extra-adrenal actions of ACTH had been discovered yet, and it is only now when the relevance of these effects is emerging [71-73]. There are currently two ACTH formulations available in the U.S.A. One is known as H.P. Acthar® Gel [74], an injectable formulation consisting of porcine ACTH purified from pituitary extracts. It is mainly used for the treatment of infantile spasms and acute exacerbations of multiple sclerosis although it is also indicated for rheumatic, dermatologic, allergic or respiratory diseases, among others. The second formulation, marketed as Cortrosyn™ [75], is a synthetic form of ACTH consisting on the first 24 amino acids, also referred as cosyntropin (see Figure 1 for nomenclature), which fully retains the steroidogenic activity of the full-length protein. This product, however, is only intended as a diagnostic agent for the screening of adrenal insufficiency. To this end, plasma cortisol concentration is measured generally 30 minutes after $ACTH_{1-24}$ administration. Synacthen® Depot [76] is the ACTH product available in the U.K. As Cortrosyn™, its structure corresponds to $ACTH_{1-24}$ although in this case it is indicated for both therapeutic and diagnostic use. As stated in its label, Synacthen Depot® can be used for short-term therapy in conditions for which GC are indicated in principle, in patients unable to tolerate GC therapy or when GC have been ineffective.

4.2 Clinical uses and clinical trials

ACTH may then be used for numerous conditions such as ulcerative colitis, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, uveitis, etc, but the most common uses seem to be infantile spasms, multiple sclerosis, nephrotic syndrome and gout. Infantile spasms, also known as West syndrome, is a medical condition usually diagnosed within the first year of life consisting on seizures and mental retardation with poor prognosis. H.P. Acthar® Gel is the only treatment available in the U.S.A. for this condition although as stated in the leaflet , the mechanism of action is unknown. A review on clinical trials on ACTH for this condition can be found in [77]. The evidence for the efficacy of ACTH in the treatment of multiple sclerosis relapses dates from the 60s, when Miller *et al* conducted a controlled study in 40 patients [78]. ACTH was considered at that time the gold standard therapy for multiple sclerosis relapses but its use decreased with the advent of GCs. The efficacy of ACTH in the treatment of nephrotic syndrome is well documented. This MC peptide is able to improve proteinuria, reduce kidney inflammation and correct dyslipidemia, actions not fully explained by induction of GCs [71]. The American College of Rheumatology also included ACTH in their guidelines for the management of gout [79], considering subcutaneous injections of 25-40 IU ACTH in patients unable to take oral anti-inflammatory medications. A recent retrospective study on 181 gout patients reported positive response in 77.9% of patients within one day after ACTH injection [80]. Efficacy has been shown too for other crystal-induced joint inflammation such as pyrophosphate crystal arthritis [81].

For all the indications described above, there is now evidence that the alternative mechanism of action of ACTH discovered by Getting *et al*, involving the activation of MC_3, might contribute to the efficacy of ACTH. This has been discussed in several reviews on infantile spasms [82], gout [83], proteinuric nephropaties [71], multiple sclerosis [84] and also lupus [85], suggesting a renaissance of ACTH therapy for several pathologies. However, most of these conditions can be treated with GCs. Given that the side effects of ACTH are similar to those produced by GCs, and possibly more importantly the elevated cost of some of the ACTH formulations, the use of this melanocortin peptide is very limited, usually regarded as a second choice when GC therapy is not possible. Hence, cheaper non-steroidogenic melanocortin drugs are needed. The pressure faced by pharmaceutical industry in developing the most innovative drugs might have sounded incompatible with the rescue or further development of an old drug that has been used for more than 60 years. As Cronstein and Terkeltaub wrote in their article "*It is surprising that so few systematic studies on the best treatment [ACTH] of acute gouty arthritis have been carried out.*" [83]. This

trend is sensibly changing and MC drug discovery is slowly progressing into industry, evidenced by the large number of companies and active clinical trials testing ACTH and other MC drugs. Table 1 summarizes the clinical trials currently compiled in the World Health Organization International Clinical Trials Registry Platform (http://apps.who.int/trialsearch/default.aspx). ACTH is further being tested for the indications described before such as gout or infantile spasms in addition to novel conditions such as rheumatoid arthritis, psoriatic arthritis, atopic dermatitis or diabetic nephropathy.

Regarding other MC drugs, afamelanotide (NDP-αMSH), a non-steroidogenic melanocortin peptide analog of the endogenous αMSH (see Figure 1) is awaiting marketing authorization from the European Medicines Agency for the treatment of the orphan disease erythropoietic protoporphyria and it is currently approved in Italy and Switzerland [86]. This highly potent analog presents improved stability due to the substitution of methionine at position 4 with norleucin (Nle), as biological activity decreases when this amino acid is oxidized. The modification at position 7 (D-Phe instead of Phe) also protects against proteolytic enzymes [87]. Marketed as Scenesse®, the product consists of a small subcutaneous implant, containing 16 mg of slowly released active ingredient. Erythropoietic protoporphyria is a rare disease characterized by severe phototoxicity resulting in intolerable pain and skin blistering shortly after the skin is exposed to sunlight. Afamelanotide confers UV protection in this disease by promoting melanin production [86]. This drug is also under clinical development for other skin conditions such as vitiligo, solar urticaria or acne vulgaris (Table 1). The anti-inflammatory peptide AP214 (ABT-719) is currently being developed by Abbvie, after being acquired from the former Action Pharma. Phase II studies for the treatment of acute kidney injury have been recently completed (NCT01777165).

5. Therapeutic perspectives for MC drug discovery

5.1 Understanding the needs

Overall, MCRs are very attractive targets for drug development, covering an extensive repertoire of plausible indications including obesity, cachexia, melanoma, acne vulgaris, vitiligo, or cardiovascular disease, all of them in addition to the already established efficacy of the melanocortin ACTH in joint diseases, nephrotic syndrome, multiple sclerosis or lupus. Then, why these seductive targets for the pharmaceutical

industry are still underused? Researchers have been focused on developing selective MC drugs for several decades, but achieving selectivity, in order to avoid off-targets side effects, is proven to be difficult. Amino acid substitution or other strategies based on modifications of the endogenous peptides led to new molecules with increased stability or potency, such as NDP-αMSH [87] or some degree of selectivity, such as DTrp^{8}-γMSH [88], with preferential binding to MC_3 over MC_1. These two drugs have been invaluable for the characterization of the anti-inflammatory actions of melanocortins, as discussed earlier, although unsatisfactory receptor selectivity prevented their clinical use to treat inflammation. Small molecules selective for MC_1 and MC_4 have also been discovered but thus far no MC_3 selective drugs have been identified. We might, however, have been pursuing the wrong aim. Is it *receptor* selectivity what is needed or *pathway* selectivity, as the emerging field of ligand bias suggests? To treat obesity, can we assume that a drug that activates MC_4 will also activate the mutated form of the receptor causing the disease? Shouldn't mutated variants of the receptors be included in the drug screenings? Similarly for drugs targeting MC_1, will molecules active at the wild type receptor behave in the same manner in the more than 60 natural variants identified for the *MC1R* gene? If the aim is to treat inflammation, molecules that activate MC_3 unable to cross the blood brain barrier would be ideal. But, would a promiscuous dual MC_1/MC_3 molecule be more advantageous, as the concept of polypharmacology suggests? In addition, considering that these two receptors are usually co-expressed in immune and joint cells (Figure 4) and they constitutively heterodimerize [38], this approach seems reasonable.

Integration of all the new knowledge on MCRs structure and sequence homology, genetics, signal transduction, receptor regulation and biological outcomes described in this review is fundamental to understand what type of molecule is needed in each particular therapeutic indication and to undergo the conceptual innovation needed for successful development of MC drugs.

5.2 Understanding MCRs pharmacology

GPCRs represent a privileged class of membrane receptors as targets for drug discovery, accounting for approximately 50% of all marketed drugs [89] but these strikingly represent only the 7% of the 365 non-olfactory known GPCRs. Although many of them may not have clinical usefulness, the opportunities to develop new GPCR-based therapies are still enormous. There is however a more important reason why new drugs targeting GPCRs are expected to be developed. New concepts on

GPCR biology such as ligand bias or allostery can be exploited pharmacologically to design molecules with improved therapeutic or safety profile. These concepts are slowly being introduced too into MC drug discovery and will be discussed next.

An allosteric modulator is a molecule that, binding to a site distinct from that of the orthosteric/endogenous agonist, is able to potentiate (positive allosteric modulator, PAM) or to decrease (negative allosteric modulator, NAM) the activity of the endogenous ligand. In addition, allosteric modulators are inactive in the absence of the endogenous ligand [90]. Among the advantages of PAMs for melanocortin drug discovery is that selective drugs could potentially be identified, as the allosteric sites are less conserved than orthosteric sites (Figure 5). In addition, drugs are expected to have fewer side effects as they potentiate the effects of the natural ligand while being inactive on their own. The development of PAMs at MC_4 are of interest for the treatment of severe obesity caused by MC_4 haploinsufficiency, i.e. when one single copy of the gene is functional [91]. The rationale is that a PAM will return the levels of MC_4 receptor activity to normal by making the healthy copy of the receptor more active. The first high throughput screening carried out, identified several molecules with PAM activity at MC_4, although they did not display receptor selectivity [91, 92]. NAMs have also been identified at the receptor MC_5. The physiological roles of this receptor are however not fully known and further research would be necessary to determine the therapeutic potential of these drugs [93].

GPCRs can be seen as switches, maintained in an inactive state, and activated to elicit an intracellular response when bound to an agonist. It is now known however that multiple active states can exist for a given receptor, and that each of those active conformations could be able to activate a different signalling cascade. An agonist with the ability to stabilize only a subset of the possible active conformations is what is known as a biased agonist [94]. The term biased is always relative to the endogenous ligand. For example, if the endogenous melanocortin αMSH activates cAMP, intracellular Ca^{2+} influx and ERK1/2 phosphorylation, a drug that only induces ERK1/2 phosphorylation when acting on the same receptor would be considered a biased agonist. The relevance of this new kind of agonists relates to the possibility of developing drugs that selectively activate a particular pathway to achieve functional selectivity. To this end, it is crucial to understand what is the functional outcome of all these pathways, and very importantly, if the pathways leading to side effects are distinct from the therapeutically relevant ones. This has been shown for opioids analgesics. The side effects of respiratory depression and constipation, could be eliminated with drugs that do not induce β-arrestin 2 recruitment, while preserving the

analgesic properties [95]. Ligand bias has been identified for MC drugs, including some endogenous ligands. As mentioned earlier in section 2.1, the natural antagonist AGRP is now considered a biased agonist because in addition to the antagonism on the cAMP pathway at MC_4, it can also activate $G_{i/o}$ protein-induced signaling [12, 96]. The impact of this finding is enormous. Can we conclude if a novel molecule is an agonist or an antagonist by measuring only one signalling pathway? How many of the drugs currently characterized as antagonists could actually be acting as biased agonists? Drug screening programmes should be updated to incorporate the therapeutically relevant signalling cascades instead of focusing on the most easily automated screening method. In MC drug discovery, cAMP is almost the only pathway used for screening although the identification of ligand-specific conformational states for synthetic molecules at MC_4 alerted about the importance of ligand bias in MC pharmacology. It was found that non-peptide and peptide ligands signal differently: while peptide agonists such as αMSH induce cAMP and internalization, several non-peptide small molecules induce cAMP but do not cause internalization of the receptor [97, 98]. The finding that non-conserved amino acids seem to be responsible for the non-peptide ligand bias might help to rationally design novel biased ligands at MC_4 although the advantage or potential value of biased agonists at MC_4 still needs to be elucidated.

The relevance of biased agonists at MC_1 might be more patent. The pigmentary side effects derived from MC_1 activation (although whether or not this is an unwanted effect is another debate), are known to be dependent on cAMP and could be prevented with a biased agonist. Some reports associate cAMP pathway with the anti-inflammatory actions of MC drugs [70]. However, Doyle *et al* found that the MC_1 selective molecule BMS-470539 retained its anti-inflammatory activity on *MC1R* variants in which ligand-induced canonical cAMP signalling is compromised [33] suggesting that other pathways are involved. In addition, we recently found that the drug AP1189 exerts anti-inflammatory actions independently of cAMP. This drug acts as a biased agonist by inducing ERK1/2 phosphorilation and Ca^{2+} mobilization via MC_1 and MC_3, while not activating the cAMP pathway, leading to anti-inflammatory actions without promoting melanogenesis (*Montero-Melendez et al, 2015 The Journal of Immunology, in press*).

MCRs genetic variants represent another fundamental aspect to be considered for MC drug discovery. Many of the *MC1R* gene variants (reviewed in [13]) are associated with decreased cAMP response to αMSH or reduced constitutive activity, measured as cAMP too. However, mutations of this receptor can impact differently on the signalling pathways. For example, the variant D294H, associated with red hair, was

unable to induce cAMP synthesis upon ligand binding (NDP-αMSH) but was fully responsive in terms of ERK1/2 phosphorylation [99]. Mutations can then create biased receptors with different signalling preferences compared with the wild type form. Furthermore, mutations can also lead to receptors that signal differently depending on the ligand. For example, the *MC1R* variant R163Q showed reduced ERK1/2 phosphorylation when treated with αMSH but normal response when stimulated with BMS-470539 [33]. Besides the complexity, the key message of these findings is that the clinical efficacy of any drug candidate should be addressed in receptors variants as found in the pathology of interest as they could affect clinical outcome. In fact, this has been addressed for the drug afamelanotide, under clinical development for vitiligo (see section 4.2) for its tanning ability. It was found that *MC1R* gene polymorphisms associated with red hair did not interfere with the ability of afamelanotide to induce eumelanin synthesis [100]. Variants at *MC4R* gene are also of relevance for the development of anti-obesity drugs. The importance of considering genetic variants in drug discovery programmes was recently recognized by Haslach *et al*. In their study they identified a number of peptides that can restore the defective response of *MC4R* variants carrying loss-of-function mutations associated with obesity [101].

Other aspects of MCRs pharmacology such as dimerization events or receptor desensitization might also have an impact on drug actions although further investigation is necessary to understand the functional consequences and relevance of these processes for drug discovery programmes.

Conclusions

GPCRs are very sophisticated components of the cell. The features that make them unique targets for drug discovery -ligand bias, allostery, oligomerization, ligand promiscuity, genetic variants, endogenous agonists/antagonists, etc- need to be fully understood and incorporated into the drug discovery process. ACTH therapy was once abandoned due to the unawareness of its real potential at that time and the limited knowledge of its extra-adrenal actions. The advances achieved during the last few decades on the understanding of MCRs biology and pharmacology and the recognition of melanocortins as part of the tightly coordinated repertoire of pro-resolving mediators, have revived the interest on ACTH and melanocortin drugs for inflammatory conditions. The current interest of industry in developing MC drugs reinforces this view. We have a better perception of the relevance of identifying the needs: should we target MC_1 or MC_3? The wild type form or the mutated one? We have a different perspective on how new drugs should be screened and characterized by including more than one signalling pathway or by identifying the therapeutically relevant ones. We now know strategies to avoid side effects with biased agonists or PAMs. In summary, we are now in a better position to develop translational melanocortin drugs.

Clearly, the once *forgotten therapy* is now the starting point for a novel promising class of drugs.

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Tables

Table 1. Clinical trials on melanocortin drugs according to the WHO International Clinical Trials Registry Platform.

Figure Legends

Figure 1. Schematic representation and nomenclature of the natural melanocortin peptides and some of the synthetic derivatives developed. Melanocortin (MC) peptides (ACTH, αMSH, βMSH and γMSH) derive from the common precursor protein pro-opiomelanocortin (POMC), which is cleaved and processed by several enzymes to produce the different melanocortin peptides. These enzymes include prohormone convertases 1 and 2 (PC1 and PC2), peptidyl α-amidating monooxygenase (PAM), carboxypeptidase E (CPE) and N-acetyltransferase (NAT). Other non-melanocortin but biologically active peptides are also generated from POMC processing such as β-endorphin. All MC peptides contain the common sequence HFRW, necessary for binding to all MC receptors. Only steroidogenic MC peptides (i.e. those able to activate MC_2 in the adrenal cortex to induce cortisol production) also contain the sequence KKRRP. ACTH, usually referred as corticotropin, is the major component of the H.P. Acthar® Gel. The synthetic derivative $ACTH_{1-24}$, also known as cosyntropin or tetracosactide (and marketed as Synacthen® Depot in U.K. and Cortrosyn™ in the U.S.A.) retains full MC_2-derived steroidogenic activity. αMSH derivatives (non-steroidogenic) under clinical development include afamelanotide, with the structure [Nle^4,D-Phe^7]-αMSH (usually referred as NDP or NDP-αMSH) and marketed as Scenesse®, and AP214, also known as ABT-719, with the structure $(Lys)_6$-αMSH. DTrp^8-γMSH is a synthetic analog of γMSH in which the amino-acid tryptophan at position 8 was substituted by its D-isomer. This peptide has been used extensively to study the anti-inflammatory actions of melanocortins.

Figure 2. Melanocortin receptors structure and sequence comparison. Amino acid sequence alignments of the human MCRs were generated with the software T-Coffee version 9.03.r1318. The highest variable regions are coloured in green and low variable ones are uncoloured. Highly variable regions include the extracellular (EC) N-terminus and first EC loop, and the intracellular (IC) C-terminus and third IC loop. Transmembrane domains (orthosteric sites) are the most well conserved regions.

Figure 3. The concept of pro-resolving based pharmacology. Target-based approach consists on the development of a drug that affects a specific target with a specific mode of action. Anakinra, Infliximab and Natalizumab are examples of biologic

therapies targeting IL-1β, TNFα and α4 intergin respectively. Biologics against the monocyte chemoattractant chemokine CCL-2 are also under investigation. Neutrophil elastase (NE) inhibitors are undergoing clinical trials for cardiovascular disease. RGD-AnxAV is a synthetic variant of annexin AV that enhances engulfment of apoptotic cells. On the other hand, pro-resolving mediators such as αMSH exert moderate actions but targeting, with one single drug, multiple aspects of the inflammatory response simultaneously.

Figure 4. Anti-inflammatory mechanisms of action of melanocortin drugs. ACTH is a major component of the hypothalamic-pituitary-adrenal (HPA) axis. It is released by the anterior pituitary gland upon stimulation with the hypothalamic corticotropin releasing hormone (CRH), produced by the paraventricular nucleus in response to biological stress. ACTH activates the receptor MC_2 on the adrenal cortex, leading to the production of corticosteroids. This mechanism explains the glucocorticoid (GC) dependent anti-inflammatory actions of ACTH. Cortisol binds to the GC receptor and via genomic and rapid non-genomic mechanisms reduce the inflammatory response. This potent anti-inflammatory mechanism is only employed by steroidogenic melanocortins (ACTH and $ACTH_{1-24}$) although it is also responsible for significant and limiting side effects. However, ACTH and non-steroidogenic melanocortins also deliver anti-inflammation by activating other MCRs expressed in immune cells, in particular MC_1, MC_3 and MC_5. Macrophages, neutrophils, lymphocytes and cells of the endothelium express these receptors, which upon activation lead to the reduction in leukocyte infiltration, inhibition of cytokines production and increased phagocytosis, among other actions. MCRs are highly expressed in bone, cartilage and other cells of the joints (chondrocytes, osteoclasts, osteoblasts, fibroblasts) bringing in actions of relevance for rheumatoid arthritis and joint inflammatory conditions. Another GC-independent mechanism, less explored therapeutically, is afforded by MC_3 and MC_4 expressed in the brain via efferent anti-inflammatory signals (cholinergic and sympathetic neurons). This central control of peripheral inflammation leads to the activation of adrenergic and nicotinic receptors in immune cells causing inhibition of NF-κB. Drugs with the ability to cross the blood-brain barrier may also act via this mechanism although side effects related to alterations in the blood pressure may occur.

Figure 5. Opportunities for MC drug discovery. The orthosteric sites (i.e. the regions were the endogenous peptides bind) present very low variability among the

five receptors (MC_1-MC_5). Asterisks indicate the regions of highest variability. However, the allosteric sites of MC receptors present very high variability providing opportunities for the development of new drugs selective for the receptor of interest, such as positive allosteric modulators (PAMs). The high variability of intracellular regions may be associated with differential signal transduction and hence different biological outcomes, suggesting the development of biased agonists for maximal therapeutic effect and minimal toxicity.

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Table 1

Acc. No.	Intervention	Health condition	Primary Sponsor	Country
ACTH and ACTH$_{1-24}$ (steroidogenic melanocortins)				
EUCTR2006-000788-27-GB	Synacthen Depot®	Infantile spasms	Royal United Hospital Bath NHS Trust	UK, Germany
EUCTR2011-000069-11-ES	Tetracosactide	Acute gout	Fernando Perez Ruiz	Spain
ISRCTN70791258	Synacthen Depot®	Idiopathic membranous nephropathy	University Hospital in Lund	Sweden
ISRCTN78654111	ACTH	Infantile spasms	Beijing Children's Hospital	China
JPRN-UMIN000012511	ACTH	Atopic dermatitis and aged skin	Tsurumai Kouen Clinic	Japan
NCT00694863	Tetracosactide	Idiopathic membranous nephropathy	Radboud University	Netherlands
NCT00805753	H.P. Acthar® Gel	Idiopathic membranous nephropathy	Mayo Clinic	US
NCT00947895	H.P. Acthar® Gel	Multiple sclerosis	Neurologique Foundation, Inc	US
NCT00986960	H.P. Acthar® Gel	Multiple sclerosis	University at Buffalo	US
NCT01021540	H.P. Acthar® Gel	Nephrotic syndrome	Arizona Kidney Disease and Hypertension Center	US
NCT01028287	H.P. Acthar® Gel	Diabetic nephropathy, nephrotic syndrome	Southeast Renal Research Institute	US
NCT01049451	ACTH	Multiple sclerosis	University of Southern California	US
NCT01093157	H.P. Acthar® Gel	Glomerulonephritis	University Health Network, Toronto	Canada
NCT01129284	H.P. Acthar® Gel	Resistant nephrotic syndrome	Columbia University	US
NCT01155141	H.P. Acthar® Gel	Idiopathic glomerulosclerosis	Stanford University	US
NCT01367964	ACTH	Infantile spasms	Ann & Robert H Lurie Children's Hospital of Chicago	US
NCT01386554	H.P. Acthar® Gel	Idiopathic membranous nephropathy	Questcor Pharmaceuticals, Inc	Canada
NCT01601236	H.P. Acthar® Gel	Diabetic nephropathy	Questcor Pharmaceuticals, Inc	US
NCT01764711	Cosyntropin	Postural orthostatic tachycardia syndrome	Vanderbilt University	US
NCT01769937	H.P. Acthar® Gel	Systemic lupus erythematosus	Fiechtner, Justus J	US
NCT01838174	H.P. Acthar® Gel	Acute optic neuritis	Elliot Frohman	US
NCT01888354	H.P. Acthar® Gel	Multiple sclerosis	The University of Texas Health Science Center, Houston	US
NCT01906372	H.P. Acthar® Gel	Refractory dermatomyositis or polymyositis	University of Pittsburgh	US
NCT01906658	H.P. Acthar® Gel	Amyotrophic lateral sclerosis	Questcor Pharmaceuticals, Inc	US
NCT01939132	H.P. Acthar® Gel	Psoriatic arthritis	Fiechtner, Justus J	US
NCT01950234	H.P. Acthar® Gel	Multiple sclerosis	University of Minnesota	US
NCT01966718	H.P. Acthar® Gel	Rheumatoid arthritis	Arthritis Treatment Center, Maryland	US
NCT02006849	H.P. Acthar® Gel	Kidney disease	Wake Forest School of Medicine	US
NCT02030028	H.P. Acthar® Gel	Rheumatoid arthritis	University of Pittsburgh	US
NCT02092883	ACTH	Infantile spasms	Wayne State University	US
NCT02113735	H.P. Acthar® Gel	Acute respiratory distress syndrome	Questcor Pharmaceuticals, Inc	US
NCT02132195	H.P. Acthar® Gel	Nephrotic syndrome	Emory University	US
SLCTR/2010/010	ACTH	Infantile spasms	Faculty of Medicine, Colombo	Sri Lanka
Other melanocortin drugs (non-steroidogenic)				
EUCTR2008-002143-16-GB	Afamelanotide	Solar urticaria	Clinuvel Pharmaceuticals Ltd	UK
EUCTR2009-017359-92-DE	Afamelanotide	Polymorphic light eruption	Clinuvel Pharmaceuticals Ltd	Belgium, Germany, Netherlands
EUCTR2009-018024-15-DE	Afamelanotide	Acne vulgaris	Clinuvel Pharmaceuticals Ltd	Germany
EUCTR2010-022630-92-DK	AP214	Prevention of postsurgical kidney injury after cardiac surgery	Action Pharma A/S	Denmark
NCT00004496	αMSH	Acute renal failure	FDA Office of Orphan Products Development	US
NCT00829192	Afamelanotide	Actinic keratoses, carcinoma, squamous cell	Clinuvel Pharmaceuticals Ltd	Australia, Europe
NCT01430195	Afamelanotide	Vitiligo	Clinuvel Pharmaceuticals Ltd	US
NCT01605136	Afamelanotide	Erythropoietic protoporphyria	Clinuvel Pharmaceuticals Ltd	US
NCT01777165	ABT-719 (AP214)	Acute kidney injury	AbbVie	Denmark, US
NCT01897519	ABT-719 (AP214)	Cardiothoracic or vascular surgery	AbbVie	Denmark, US

NCT02041195	RM-493	Obesity	Rhythm Metabolic, Inc	US

Figure 1

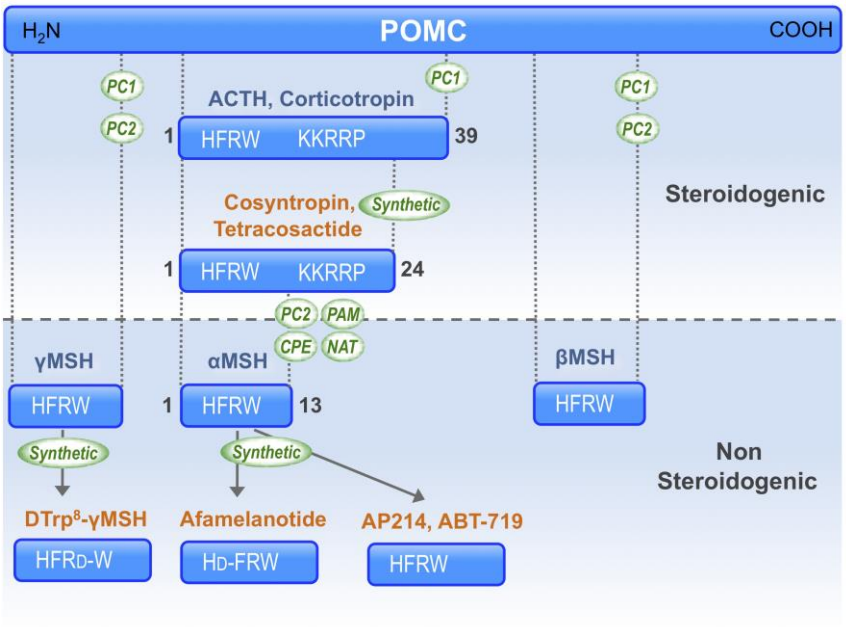

H2N
POMC
COOH
PC1
PC2
ACTH, Corticotropin
1
HFRW KKRRP
39
Cosyntropin, Tetracosactide
Synthetic
1
HFRW KKRRP
24
Steroidogenic
PC2
PAM
CPE
NAT
γMSH
αMSH
βMSH
HFRW
1
HFRW
13
HFRW
Synthetic
Synthetic
Non Steroidogenic
DTrp8-γMSH
HFRD-W
Afamelanotide
HD-FRW
AP214, ABT-719
HFRW

Figure 2

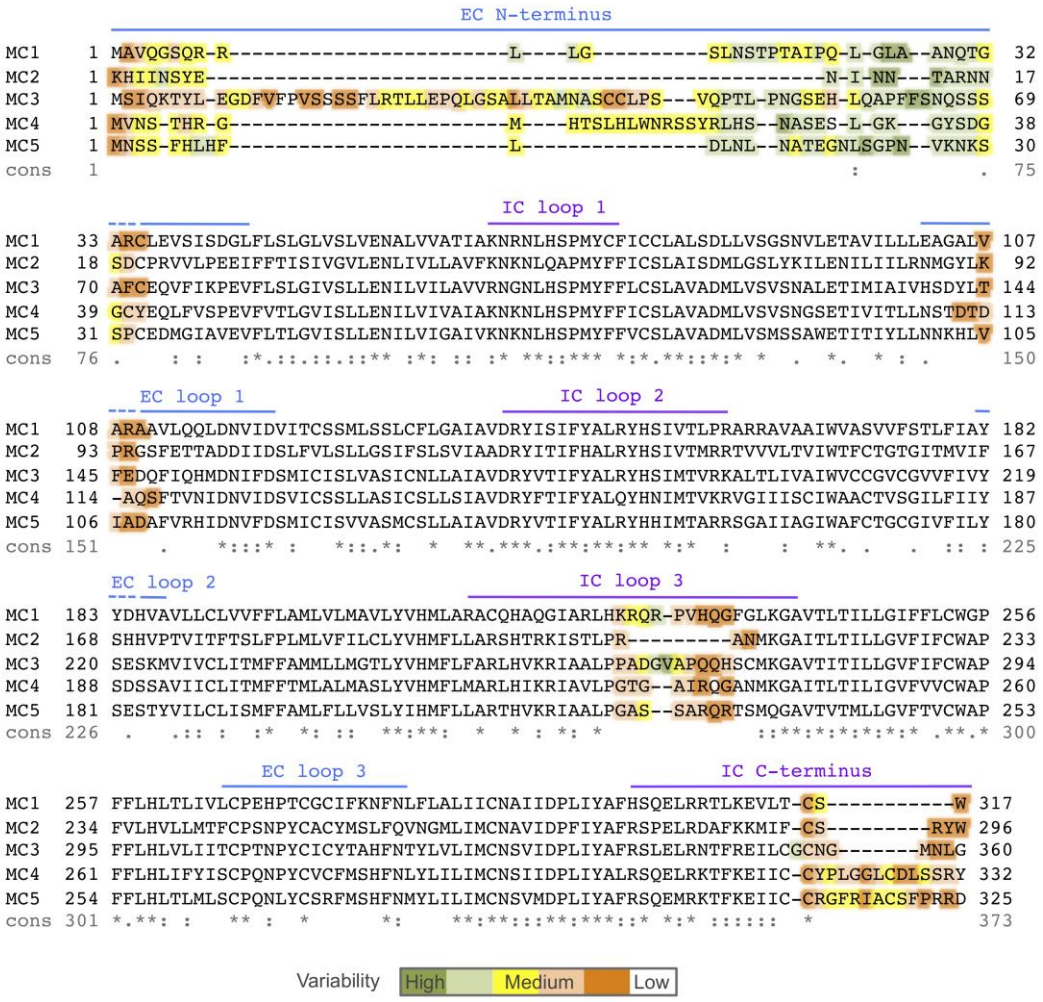

EC N-terminus
IC loop 1
EC loop 1
IC loop 2
EC loop 2
IC loop 3
EC loop 3
IC C-terminus
Variability
High
Medium
Low

Figure 3

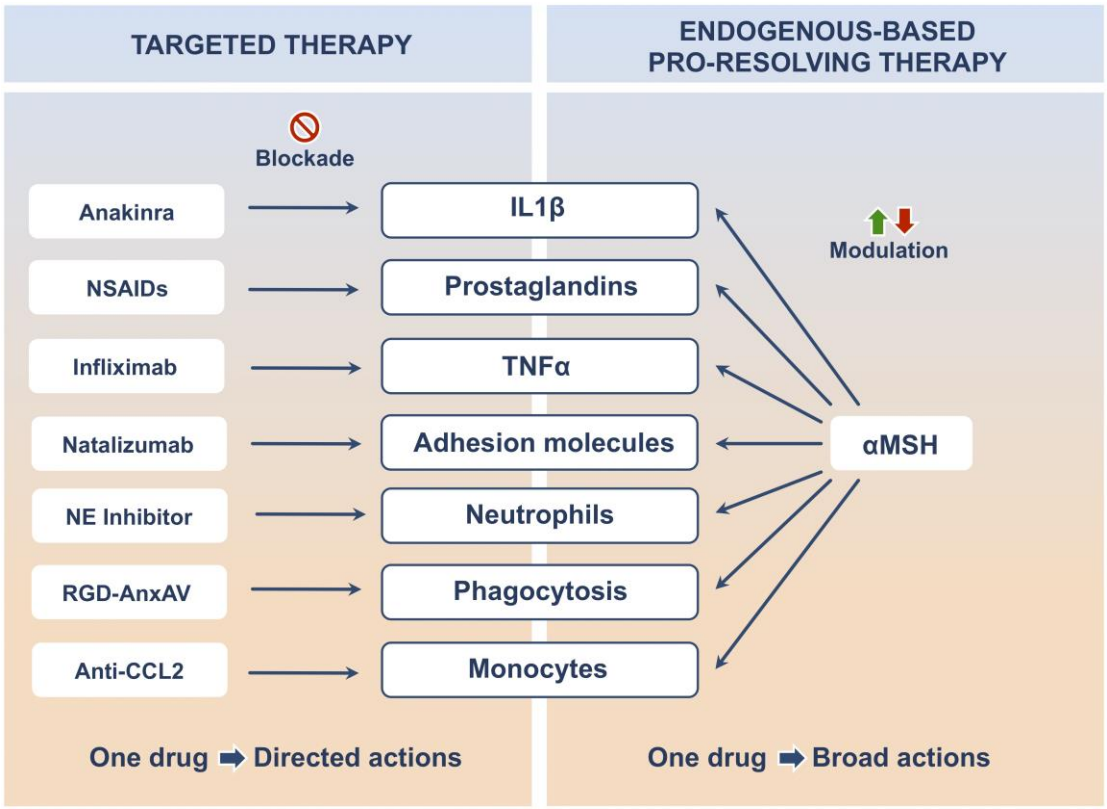

TARGETED THERAPY
ENDOGENOUS-BASED PRO-RESOLVING THERAPY
Blockade
Anakinra
NSAIDs
Infliximab
Natalizumab
NE Inhibitor
RGD-AnxAV
Anti-CCL2
IL1β
Prostaglandins
TNFα
Adhesion molecules
Neutrophils
Phagocytosis
Monocytes
Modulation
αMSH
One drug ➡ Directed actions
One drug ➡ Broad actions

Figure 4

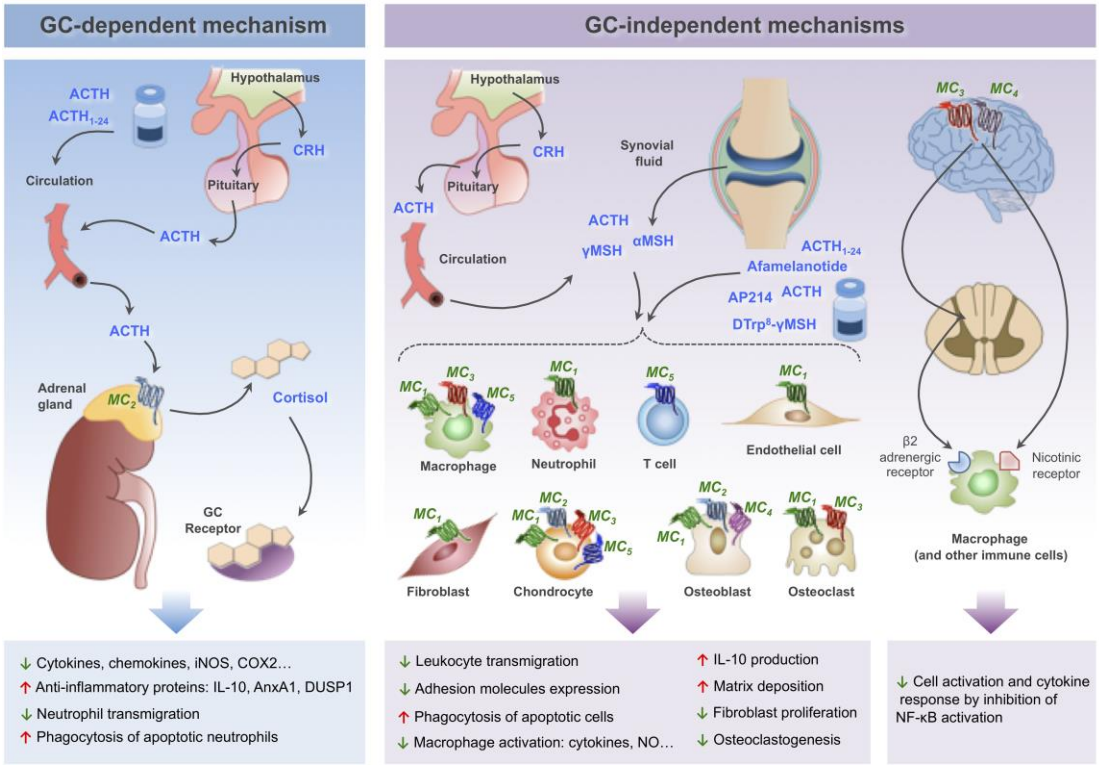
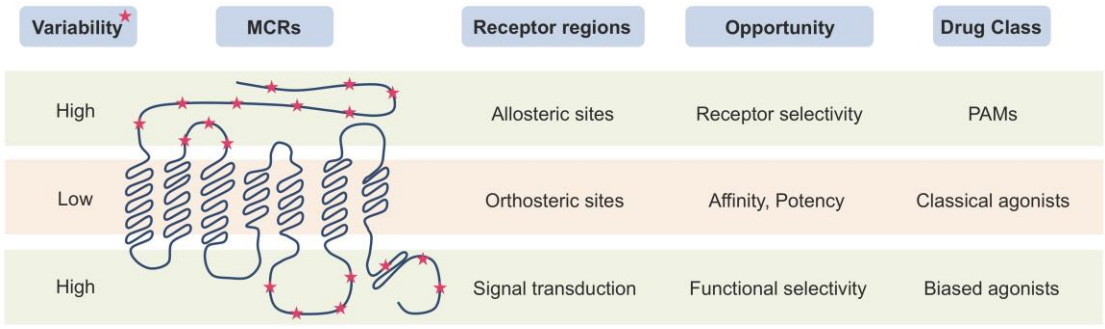

GC-dependent mechanism
GC-independent mechanisms
Hypothalamus
CRH
Pituitary
ACTH
ACTH1-24
Circulation
Adrenal gland
MC2
Cortisol
GC Receptor
Synovial fluid
αMSH
γMSH
ACTH1-24
Afamelanotide
AP214
DTrp8-γMSH
MC1
MC3
MC5
Macrophage
Neutrophil
T cell
Endothelial cell
MC4
β2 adrenergic receptor
Nicotinic receptor
Macrophage (and other immune cells)
Fibroblast
Chondrocyte
Osteoblast
Osteoclast
↓ Cytokines, chemokines, iNOS, COX2…
↑ Anti-inflammatory proteins: IL-10, AnxA1, DUSP1
↓ Neutrophil transmigration
↑ Phagocytosis of apoptotic neutrophils
↓ Leukocyte transmigration
↓ Adhesion molecules expression
↑ Phagocytosis of apoptotic cells
↓ Macrophage activation: cytokines, NO…
↑ IL-10 production
↑ Matrix deposition
↓ Fibroblast proliferation
↓ Osteoclastogenesis
↓ Cell activation and cytokine response by inhibition of NF-κB activation

Figure 5


Variability
MCRs
Receptor regions
Opportunity
Drug Class
High
Allosteric sites
Receptor selectivity
PAMs
Low
Orthosteric sites
Affinity, Potency
Classical agonists
High
Signal transduction
Functional selectivity
Biased agonists