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Cyclosporin H, Boc-MLF and Boc-FLFLF are Antagonists that Preferentially Inhibit Activity Triggered Through the Formyl Peptide Receptor

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Abstract—In order to properly interpret receptor inhibition experiments, the precise receptor specificities of the employed antagonists are of crucial importance. Lately, a great number of agonists for various formyl peptide receptors have been identified using a selection of antagonists. However, some confusion exists as to the precise receptor specificities of many of these antagonists. We have investigated the effects of formyl peptide receptor family antagonists on the neutrophil response induced by agonists for the formyl peptide receptor (FPR) and the formyl peptide receptor like 1 (FPRL1). To determine FPR- and FPRL1-specific interactions, these antagonists should not be used at used at concentrations above 10 μM. Signaling through FPR was inhibited by low concentrations of the antagonists cyclosporin H, Boc-MLF (also termed Boc-1), and Boc-FLFLFL (also termed Boc-2), while higher concentrations also partly inhibited the signaling through FPRL1. The antagonist WRWWWW (WRW_4) specifically inhibited the signaling through FPRL1 at low concentrations but at high concentrations also partly the signaling through FPR. Based on the difference in potency of cyclosporin H and the two Boc-peptides, we suggest using cyclosporin H as a specific inhibitor for FPR. To specifically inhibit the FPRL1 response the antagonist WRW_4 should be used.

KEY WORDS: formyl peptide receptors; inhibitors; Cyclosporin H; Boc-MLF; Boc-FLFLF.

INTRODUCTION

Neutrophil granulocytes, professional phagocytes of the innate immune system, are activated by soluble chemoattractants serving as "danger signals", and following the discovery of bacteria-derived, formylated peptides as potent neutrophil activators [1], the list of structurally well-characterized leukocyte chemoattractants and their receptors has steadily grown. The receptors all belonging to a pertussis toxin-sensitive subfamily within the G protein-coupled receptor (GPCR) superfamily [2–5]. The formyl peptide receptor (FPR) was the first neutrophil GPCR to be cloned and sequenced [6]. Soon after the FPR sequence was published, two orphan FPR-like receptors, FPRL1 and FPRL2, were cloned [6, 7]. The FPR is activated by formylated peptides [1, 8, 9], and already 25 years ago it was shown that replacement of the formyl group in one such peptide, formylmethionyl-leucyl-phenylala-

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nine (fMLF), by a tert-butyloxycarbonyl (Boc) group generates a receptor-specific antagonist [10], but the basic characterization of this antagonist was performed before the existence of FPRL1 was known. This receptor possesses large functional similarities with the FPR [11–14]. During the last decade, the knowledge about the FPR family in terms of structure, expression patterns, signaling processes, biological roles and regulation of signaling has increased enormously [13, 14], but the results presented with these Boc-peptide receptor antagonists suggest that the specificity of these may require a reevaluation [15–18]. This is illustrated by the fact that one of the receptors mediating the down-regulatory/anti-inflammatory signal induced by lipoxinA_4 (LXA_4) was identified as FPRL1 [18], an identification that relied on the effects of the antagonistic peptide FLFLF (also called Boc-2) containing a tert-butyloxylcarbonyl (Boc) group at the N-terminus [18]. This peptide has, however, been claimed by other researchers to be a nonselective antagonist for both FPRL1 and FPR [16, 17, 19, 20]. Moreover, the Boc-MLF peptide, regarded for a long time as an FPR specific antagonist, was recently shown to antagonize also the FPRL1-agonist serum amyloid A (SAA) [15]. The precise specificity of the different FPR/FPRL1 antagonist thus needs to be determined.

MATERIALS AND METHODS

Isolation of Human Neutrophils

Blood neutrophils were isolated from buffy coats obtained from healthy blood donors, using dextran sedimentation and Ficoll-Paque gradient centrifugation [21]. All cells were washed and resuspended (1×10^7/ml) in Krebs-Ringer phosphate buffer containing 10 mM glucose, 1 mM Ca^{2+}, and 1.5 mM Mg^{2+} (KRG, pH 7.3).

Peptides and Peptide Receptor Antagonists

The hexapeptides Trp-Lys-Tyr-Met-Val-L/DMet-NH_2 (WKYMVM/m) were synthesized and HPLC purified by Alta Bioscience (University of Birmingham, UK). The formylated peptide *N*-formyl-Met-Leu-Phe (fMLF) and the Boc-peptides *N*-formyl-Met-Leu-Phe (Boc-MLF) and *N*-tert-butoxycarbonyl-L-Phe-D-Leu L-Phe-D-Leu-L-Phe (Boc-FLFLFL) were from Sigma Chemical Co. (St. Louis, MO). The Arg-Trp-Trp-Trp-Trp-$CONH_2$ (WRW_4) peptide was from GenScript Corp (Piscataway, NJ) and cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland) but is also commercially available at Axxora platforms, Eton Bioscience Inc. and Hanzhou Onicon Chemical Co, Ltd. The peptides/receptor antagonists were dissolved in dimethyl sulfoxide to 10^{-2} M and stored at −70°C until use. Further dilutions were made in KRG.

Neutrophil NADPH-oxidase Activity

The NADPH-oxidase activity was determined using an isoluminol-enhanced chemiluminescence (CL) system [22]. The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Co. Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 900 µl reaction mixture containing $1–2 \times 10^5$ neutrophils, horseradish peroxidase (HRP; 4U) and isoluminol (2×10^{-5} M) [23]. The tubes were equilibrated in the Biolumat for 5 min at 37°C, after which the stimulus (0.1 ml) was added. By a direct comparison of the superoxide dismutase (SOD) inhibitable reduction of cytochrome *C* and SOD inhibitable CL, 7.2×10^7 cpm were found to correspond to a production of 1 nmol of superoxide (a millimolar extinction coefficient for cytochrome *C* of 21.1 was used. Details about the CL technique is given in [22].

RESULTS AND DISCUSSION

The Antagonists Boc-MLF and Boc-FLFLF is Specific for Receptors with in the Formyl Peptide Receptor Family only if the Concentration is Kept Lower than 10 µM

According to the assumed receptor specificity of the two Boc-antagonists (Boc-MLF and Boc-FLFLF) as well as of other FPR/FPRL1 specific antagonists [13], the peptides should not block neutrophil activity induced by the chemotactic split product from complement component 5 (C5a). This triggers a neutrophil response through the C5a receptor (C5aR), a GPCR distinct from the FPR receptor family. The antagonists, cyclosporin H, Boc-MLF, Boc-FLFLF and WRWWWW (WRW_4), did not block the NADPH-oxidase activity induced by C5a, provided that the concentration of the antagonists was equal to or lower than 10 µM, (shown for the Boc-peptides in Fig. 1). Higher concentrations of the antagonists reduced the C5a-induced cellular response,

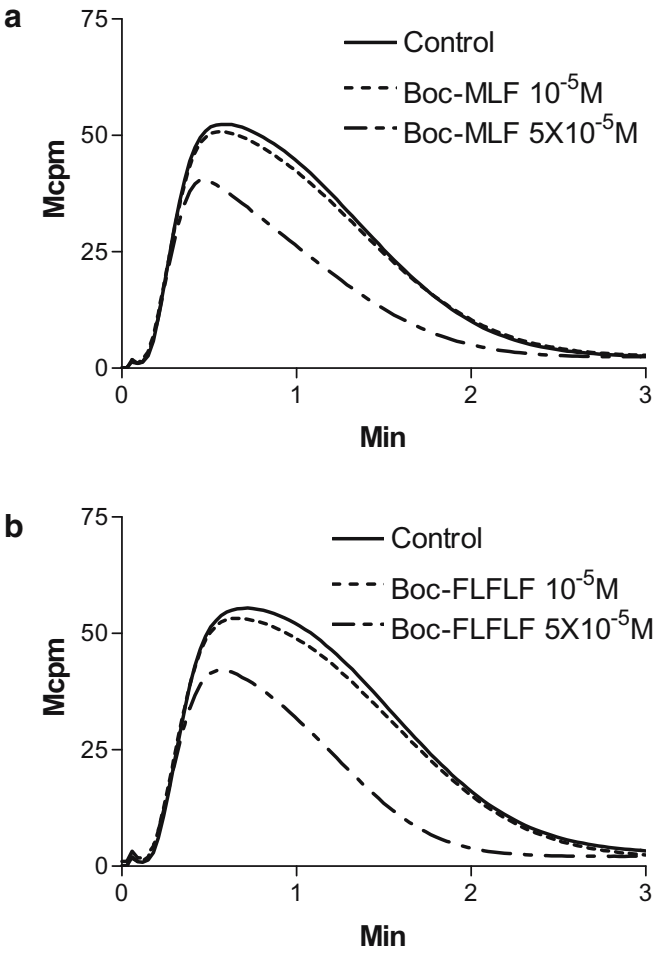

Fig. 1. The superoxide production in neutrophils (2×10^5) was measured with an isoluminol-amplified chemiluminescence technique using a chemiluminometer. In short, a reaction mixture containing neutrophils, isoluminol, and horse-radish peroxidase were pre-warmed for 5 min at 37°C before the addition of stimuli (see [23] for details about the technique). Neutrophil superoxide production induced by C5a (100 ng/ml final concentration) was determined in the absence (*solid line*) or presence of the FPR antagonists **a** Boc-MLF (*broken lines*) or **b** Boc-FLFLF (*broken lines*). The figures **a** and **b**, respectively, show one representative experiment out of three. Mcpm, 10^6 counts per minute.

suggesting that 10 μM is the highest antagonist concentration that can be used with retained receptor specificity.

Boc-MLF and Boc-FLFLF were also Fairly Specific FPR Antagonists at Concentrations up to 5 μM

The peptides fMLF and WKYMVM are both potent neutrophil activators that use different receptors to trigger a cell response. The fMLF peptide preferentially activates FPR whereas WKYMVM preferentially activates FPRL1 [13], therefore, these peptides were used to determine the specificity of the antagonists. The activity induced by fMLF was blocked by the two Boc-peptides with an inhibition efficiency approaching 100% at increasing concentrations (Fig. 2a,b). These antagonists only marginally reduced the response induced by WKYMVM at concentrations up to 5 μM (Fig. 2a,b). The cyclic undecapeptide, cyclosporin H (CsH), has been suggested to be a fairly specific FPR antagonist [24, 25], and accordingly, this peptide blocked the activity induced by fMLF with an EC_{50} value of around 0.1 μM and with an inhibition efficiency of 100% at increasing concentrations (Fig. 2c). This inhibitor did not reduce the response induced by the FPRL1 specific agonist WKYMVM up to concentrations of around 2.5×10^{-6}M and the inhibition did not reach 50% even in the presence of highest concentration (10^{-5}M) of the antagonist (Fig. 2c). The antagonist WRW_4 blocked the activity induced by WKYMVM with an EC_{50} value of 0.2 μM with an inhibition efficiency of 100% at increasing concentrations. The antagonist reduced the response induced by fMLF very much less (Fig. 2d). Depending

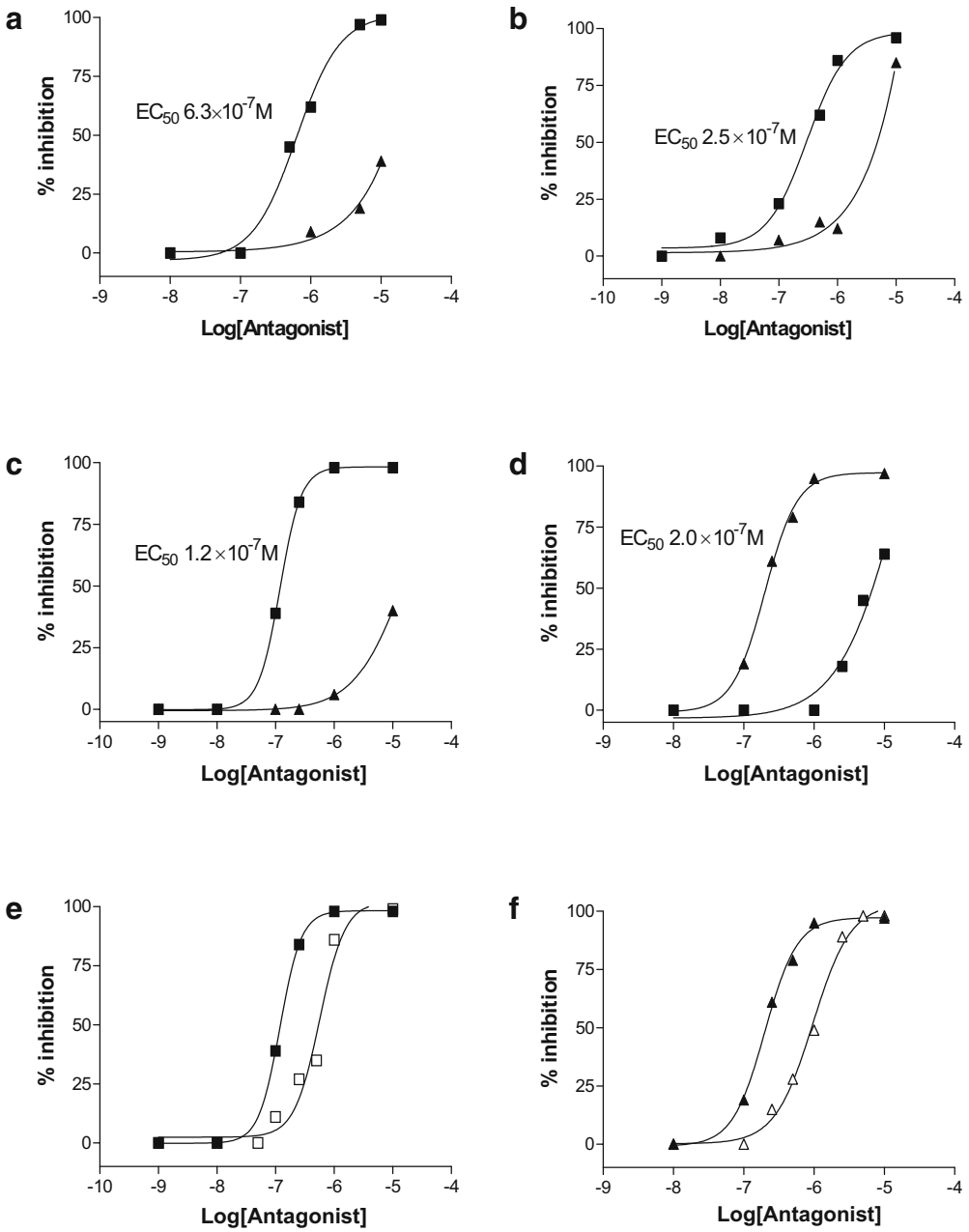

Fig. 2. The neutrophil (2×10^5) superoxide production was determined by chemiluminescence. Neutrophils and antagonists **a** Boc-MLF, **b** Boc-FLFLF, **c** cyclosporin H, **d** WRW_4, **e** cyclosporin H and **f** WRW_4 at different concentrations were incubated at 37°C for 5 min and the superoxide production was induced by the FPR agonist fMLF, final concentration (*filled squares*) 2×10^{-8} M or (*empty squares*) 10^{-7} M, and the FPRL1 agonist WKYMVM, final concentration (*filled triangles*) 2×10^{-8} M or (*empty triangles*) 10^{-7} M. The reduction in superoxide production was calculated comparing the peak values of the response with or without any antagonist, a dose response curve was drawn and the EC_{50} value was calculated using the software Graph Pad Prism 2.0 (San Diego, CA). The figures, respectively, show one representative experiment out of three.

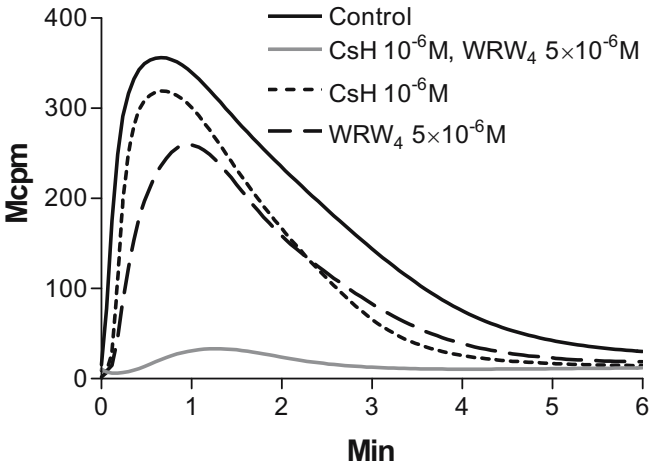

Fig. 3. Neutrophils (10^6) superoxide production induced by WKYMVm, was not inhibited by the FPR antagonist CsH or the FPRL1 antagonist WRW_4 (*broken lines*). When combined the two antagonists potently reduced the activity (*solid line*).

on the agonist concentration, the inhibition curves shifted. At a low concentration of the specific agonist the antagonists receptor preference was more marked (Fig. 2e,f). We conclude that WRW_4 is a fairly good FPRL1 antagonist whereas cyclosporin H was found to be the most potent and selective FPR antagonist. Some receptor overlap was seen at high concentrations of all antagonists. Boc-MLF and Boc-FLFLF peptides were also fairly specific FPR antagonists and could therefore be regarded as specific, rather then nonselective for receptors within the family of FPRs [16, 17, 19, 20] or being specific for FPRL1 [18].

Activity Induced through Two (or more) Different Types of Receptors

When exposing neutrophils to WKYMVm (the m denotes that the L-Met has been replaced by a D-Met at the carboxyl end), the response was largely insensitive to cyclosporin H and WRW_4 (Fig. 3). We have earlier shown that WKYMVm binds both FPR and FPRL1 [26], and accordingly the neutrophil activity was potently reduced when cyclosporin H and WRW_4 were combined (Fig 3). It should always be kept in mind, that when an agonist mediates its effect through two (or more) different types of receptors with equal/similar potency, the involvement of one of those receptors might be masked because, when an antagonist specifically inhibits one receptor type, the agonist switches to another receptor.

In the earlier mentioned publications, in which the Boc-peptides were regarded as nonselective within the family of FPR's or even as being specific for FPRL1 [15–18], very high concentrations of antagonists were used. The identification of FPRL1 as a signaling LXA_4-receptor was based on effects of 400 μM of the antagonist [18]. The SAA induced calcium response was inhibited with 25 μM of the antagonist Boc-MLF [15]. No conclusions about receptor involvement can be drawn from these results since the selectivity of the antagonists are lost at these concentrations. The concentration of each antagonist should be kept below 10 μM to keep the specificity with in the FPR family. The well known fact that not only the antagonist, but also the agonist concentration is of importance for the inhibition profile with receptor specific antagonists, suggests that in order to properly determine the involvement of FPR and FPRL1 in a cellular response the concentration of the agonist under investigation is kept as low as possible and that the effects of WRW_4 and cyclosporin H are determined in parallel.

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