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The road to Insurmountability: Novel avenues to better target CC Chemokine Receptors

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Chapter 5

Synthesis and pharmacological evaluation of triazolo-pyrimidinone derivatives as noncompetitive, intracellular antagonists for CCR2/5 chemokine receptors

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ABSTRACT

Both CC Chemokines receptors 2 (CCR2) and 5 (CCR5) are involved in a variety of inflammatory and immunological diseases; however, with the exception of maraviroc, clinical trials with selective CCR2 and CCR5 antagonists have been unsuccessful. Preclinical and clinical evidence suggests that dual CCR2/CCR5 inhibition might represent a more effective strategy for the treatment of multifactorial diseases. In this regard, the high conservation of a recently discovered intracellular binding site in chemokine receptors provides a potential new avenue for the design of multitarget allosteric modulators. In this study, we synthesized and evaluated the biological activity of a series of triazolo-pyrimidinone derivatives, previously reported as CCR2 antagonists. By performing radioligand binding assays, we first confirmed that these compounds bind to the intracellular site of CCR2 with high affinity. In addition, functional assays were used to evaluate their activity on CCR5, allowing us to explore structure-affinity/activity relationships in both receptors, and thus to gain understanding of the structural requirements to modulate selectivity. Overall, triazolo-pyrimidinone derivatives were mostly selective towards CCR2; however compounds **39** and **43** were able to inhibit CCL3-induced β-arrestin recruitment in CCR5 with approximately 100 nM potency. Finally, these compounds displayed an insurmountable mechanism of inhibition in both receptors, which holds promise for improved efficacy in inflammatory diseases characterized by elevated levels of endogenous chemokines.

INTRODUCTION

CC Chemokine receptors 2 (CCR2) and 5 (CCR5) are two membrane-bound G protein-coupled receptors (GPCRs), which belong to the subfamily of chemokine receptors. Chemokine receptors are widely expressed in leukocytes, and thus, they regulate different homeostatic and inflammatory leukocyte functions upon interaction with their endogenous chemokines.[1, 2] In general, chemokine receptors interact with multiple endogenous chemokines, such as CCL2, CCL7 and CCL8 in the case of CCR2, and CCL3, CCL4 and CCL5 in the case of CCR5.[1] Furthermore, most chemokines can interact with multiple chemokine receptors, allowing for a very complex and fine-tuned system.[3, 4] Dysregulation of this system has been linked to the development of several pathophysiological conditions. For example, both CCR2 and CCR5 have been implicated in many inflammatory and immune diseases such as rheumatoid arthritis, multiple sclerosis, atherosclerosis, diabetes mellitus and psoriasis,[5, 6] rendering these proteins attractive targets for the pharmaceutical industry. As a result, many efforts have been made to bring CCR2 and CCR5 small-molecule antagonists into the clinic, although with limited success. Only maraviroc, an HIV-1 entry inhibitor selectively targeting CCR5, has been approved by the FDA and EMA,[7] while all other drug candidates have failed in clinical trials.

Recently, it has been suggested that the development of multitarget drugs—designed to interact with multiple receptors—represents a more effective approach in the treatment of complex multifactorial diseases.[8, 9] Thus, dual targeting of CCR2 and CCR5 emerges as a potentially more efficacious strategy in diseases where both receptors are involved. Indeed, combined CCR2/CCR5 inhibition has resulted in beneficial effects in several preclinical disease models and clinical studies, further supporting the use of dual antagonists.[10, 11] In this regard, several antagonists with dual CCR2/CCR5 activity have been reported in the last years, including the first dual antagonist TAK-779 and the clinical candidate cenicriviroc.[12] All of these antagonists bind to the extracellular region of CCR2 and CCR5, in a site overlapping with the chemokine's binding pocket.[13] Yet, the crystal structures of CCR2 (**Chapter 3**) and CCR9 have demonstrated that chemokine receptors can also be targeted with intracellular allosteric modulators.[14, 15] These intracellular ligands offer a number of advantages, such as noncompetitive binding and, as a consequence, insurmountable inhibition; which is particularly important due to the high local concentration of chemokines during pathological conditions (**Chapter 2**).[16, 17] In addition, the high conservation of this intracellular site allows for the design of multitarget antagonists (**Chapters 2 and 4**).[17, 18] Several high-affinity intracellular ligands have been already identified for CCR2,[19, 20] but not for CCR5; although intracellular compounds developed for CCR2 or CCR4 have been reported to bind CCR5 with much lower potency.[20, 21]

In the current study we first report that previously patented CCR2 antagonists with a triazolo-pyrimidinone scaffold, such as compound **8** (Figure 1),[22] bind to the intracellular site of the receptor with high affinity. In addition, we show that this compound is able to inhibit CCR5 with moderate activity, suggesting a potential dual CCR2/CCR5 activity for this class of compounds. Thus, a series of novel and previously reported triazolo-pyrimidinone derivatives were synthesized according to published methods[22] in order to obtain structure-affinity/activity relationships (SAR) in both CCR2 and CCR5. Radioligand binding assays and functional assays were used to evaluate their affinity towards CCR2 and activity towards CCR5. In addition, characterization of two selected compounds (**39** and **43**) in a [^{35}S]GTPγS binding assay demonstrated that these compounds inhibit both receptors in a noncompetitive, insurmountable manner. Finally, compound **43** was docked into the CCR2 crystal structure in order to shed light on the binding mode of these derivatives, in comparison to that of the crystalized CCR2-RA-[*R*] (**Chapter 3**).[14] In summary, our findings provide some insight on the CCR2/CCR5 selectivity profile of triazolo-pyrimidinone derivatives, as well as on the structural requirements for the design of multitarget or selective intracellular ligands for these receptors.

RESULTS AND DISCUSSION

Chemistry

Triazolo-pyrimidinone derivatives **6 - 43** were synthesized using a three-step synthesis approach as described by Bengtsson *et al.*[22] (Scheme 1). First, if not commercially available, the β-keto esters **1a-n** were synthesized from ethyl acetoacetate **1a** and the respective bromo or iodo alkanes **2f-h,j,k** or benzylbromide **2n**. Benzylation of the β-keto esters **1a-n** with the corresponding R^1-substituted benzylbromides (**3a-v**), at reflux, resulted in a series of benzylated β-keto esters **4aa-na, 4bb-bq, 4eq-ev** in yields between 8% and 97% (Scheme 1, Table S1). Finally a cyclisation reaction of the benzylated β-keto esters **4aa-na, 4bb-bq, 4eq-ev** with the commercially available 3,5-diamino triazole **5c** in ionic liquid BMIM-PF6 (1-butyl-3-methylimidazolium hexafluorophosphate) at 200°C under microwave irradiation resulted in final compounds **6, 9-43** in yields ranging from 4% to 83%. However final compound **7** (R^2 = H) was synthesized using H_3PO_4 in ethanol conditions and **8** (R^2 = Me) in p-toluenesulfonic acid monohydrate conditions.

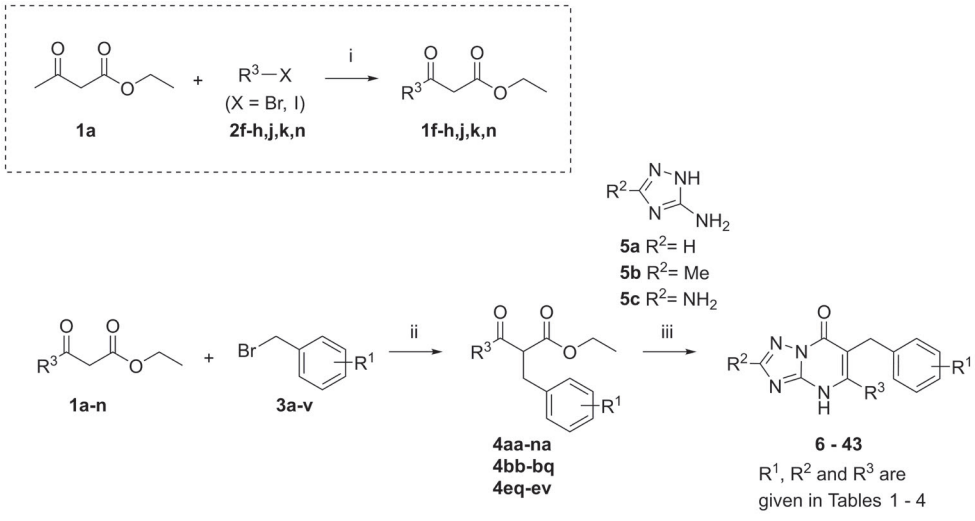
Biology

We have previously identified several CCR2 intracellular ligands belonging to different chemical scaffolds, such as CCR2-RA-[*R*], SD-24 and JNJ-27141491 (Figure 1).[19, 20] In contrast to CCR2 orthosteric ligands, these intracellular ligands lack a basic nitrogen, have lower molecular weights, unsaturated systems with haloarenes and acidic groups capable of forming hydrogen bonds.[17, 19] Other CCR2 antagonists with similar features have been described in literature, including the triazolo- or pyrazolo-pyrimidinone derivatives described in two different patents.[22, 23] To test whether they also bind to the intracellular site of the receptor, we synthesized "example 1" from the patent by Bengtsson *et al*.,[22] corresponding to the triazolo-pyrimidinone derivative **8** in our study (Figure 1). Using a [^{3}H]-CCR2-RA-[*R*] binding assay as previously described,[18] we found that compound **8** fully displaced [^{3}H]-CCR2-RA-[*R*] binding from U2OS cells stably expressing hCCR2b (U2OS-CCR2) with high affinity and a pseudo-Hill slope (n_H) close to unity, indicating a competitive interaction with [^{3}H]-CCR2-RA-[*R*] for the intracellular binding site. **8** displaced [^{3}H]-CCR2-RA-[*R*] with a pK_i of 8.90 ± 0.04 (K_i = 1.3 nM, Figure 2a and Table 1), consistent with its previously reported activity in a CCR2 calcium flux assay (IC_{50} = 16 nM).[22]

Previous studies have shown that some of these intracellular ligands are able to bind and inhibit multiple chemokine receptors, enabling the design of selective and multitarget

inhibitors.[18, 20, 21] In this regard, CCR5 is the closest homolog to CCR2, with > 90% sequence similarity of their intracellular binding pockets. From the main interactions of CCR2-RA-[*R*] to CCR2, only Val244^{6x36} is exchanged to Leu236^{6x36} in CCR5[14] (residues named according to structure-based Ballesteros—Weinstein nomenclature[24]). Thus, we investigated whether compound **8** is also able to inhibit the highly homologous CCR5. However, the much lower affinity of [^{3}H]-CCR2-RA-[*R*] for CCR5 compared to CCR2 hindered us from performing radioligand binding assays;[20] thus, we assessed the CCR5 activity of **8** with a functional β-arrestin recruitment assay after stimulation with CCL3, one of the endogenous agonists of CCR5. For this assay, we also included the intracellular ligands CCR2-RA-[*R*], SD-24 and JNJ-27141491, as well as the CCR2/CCR5 orthosteric antagonist TAK-779 as a positive control (Figure 1), since it is a potent CCR5 antagonist in a variety of functional assays.[25, 26]

Scheme 1. Synthesis scheme of the triazolo-pyrimidinone derivatives 6 – 43[a]



1a R^3= Me **1h** R^3= Pent
1b R^3= *c*Pr **1i** R^3= *c*Pent
1c R^3= Et **1j** R^3= Hex
1d R^3= Pr **1k** R^3= Hept
1e R^3= *i*Pr **1l** R^3= Ph
1f R^3= Bu **1m** R^3= 4-MePh
1g R^3= 2-EtBu **1n** R^3= CH_2CH_2Ph

3a R^1= 3-Cl **3l** R^1= 4-Me
3b R^1= H **3m** R^1= 4-F
3c R^1= 2-Me **3n** R^1= 4-Cl
3d R^1= 2-Cl **3o** R^1= 4-Br
3e R^1= 2-OMe **3p** R^1= 4-OMe
3f R^1= 3-Me **3q** R^1= 3,4-diCl
3g R^1= 3-F **3r** R^1= 2,3-diCl
3h R^1= 3-Br **3s** R^1= 2,5-diCl
3i R^1= 3-I **3t** R^1= 3,5-diCl
3j R^1= 3-OMe **3u** R^1= 3,5-diBr
3k R^1= $3\text{-}CF_3$ **3v** R^1= 3-Br. 4-Cl

4aa R^3= Me, R^1= 3-Cl
4ba R^3= *c*Pr, R^1= 3-Cl
...
Full list in Table S1

[a]Reagents and conditions: (i) NaH, n-BuLi, THF, overnight, 0°C to rt (**1a-e,i,l,m** were commercially available); (ii) DIPEA, LiCl, THF, reflux, overnight; (iii) (**8-43**, $R^2 = NH_2$) BMIM-PF6, 200°C, 1h or (**6**, R^2=H) H_3PO_4, EtOH, 170°C, 10h or (**7**, R^2 = Me) p-toluenesulfonic acid monohydrate, 180°C, 30 min.

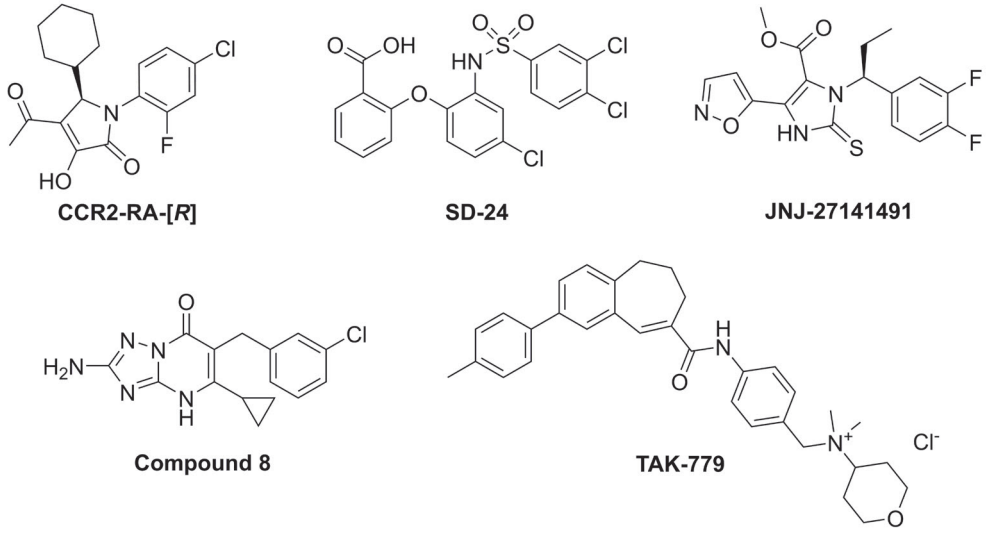


Figure 1. **Chemical structures of the orthosteric CCR2/CCR5 antagonist TAK-779 and the CCR2 intracellular ligands CCR2-RA-[R], SD-24, JNJ-27141491 and the triazolo-pyrimidinone derivative 8.** [^{3}H]-CCR2-RA-[*R*] was used in radioligand binding assays for CCR2.

In this assay, CCL3 induced β-arrestin recruitment to U2OS cells stably expressing hCCR5 (U2OS-CCR5) with a pEC_{50} of 8.3 ± 0.08 (6 nM) (Figure S1a), similar to values reported in literature.[27] As expected, TAK-779 was able to completely inhibit β-arrestin recruitment induced by an EC_{80} concentration of CCL3 (pEC_{80} = 7.9 ± 0.08), when tested at a single concentration of 1 μM (Figure S1b). In contrast, none of the intracellular ligands was able to fully inhibit CCL3-induced β-arrestin recruitment to the same level as TAK-779; in fact, only compound **8** displayed more than 70% inhibition when tested at 1 μM (Figure S1b), while CCR2-RA-[*R*], SD-24 and JNJ-27141491 led to approximately 50% inhibition or less at the same concentration of 1 μM (Figure S1b). Consistent with this low inhibition in CCR5, it was previously shown that CCR2-RA-[*R*], JNJ-27141491 and SD-24 inhibited inositol phosphate (IP) formation in CCR5 with 7 to 22-fold lower potency compared to CCR2 inhibition, respectively.[20] Preincubation of U2OS-CCR5 cells with increasing concentrations of TAK-779, before exposure to CCL3, resulted in an inhibitory potency (IC_{50}) of 6 nM, consistent with previously reported values (Table S2).[26] Also in agreement with a previous study,[20] the reference intracellular ligand CCR2-RA-[*R*] inhibited CCL3-induced β-arrestin recruitment with an IC_{50} value of 703 nM (Table S2). Moreover, while TAK-779 inhibited CCL3-induced β-arrestin recruitment with a pseudo-Hill slope close to unity (n_H = -1.1), while CCR2-RA-[*R*] inhibition showed a significantly higher Hill slope (n_H = -2.4), indicative of two different binding sites for CCL3 and CCR2-RA-[*R*] (Table S2).[28]

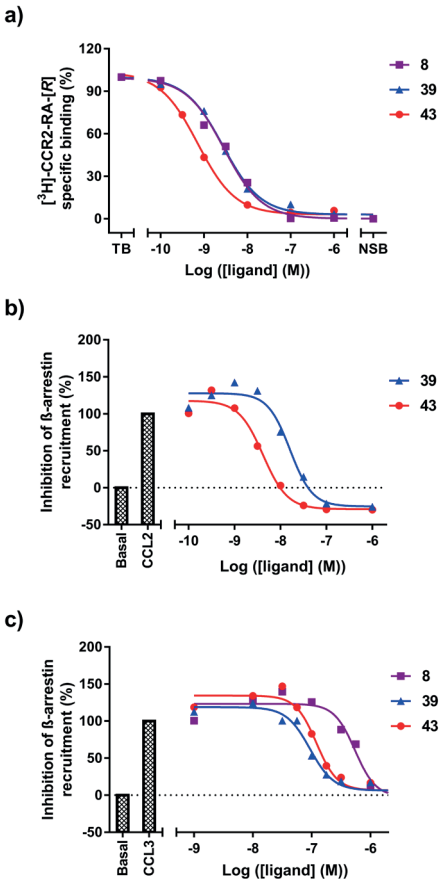


Figure 2. **Characterization of ligands in U2OS-CCR2 and U2OS-CCR5.** (a) [^{3}H]-CCR2-RA-[*R*] displacement by increasing concentrations of triazolo-pyrimidinone derivatives **8**, **39** and **43** in U2OS-CCR2 at 25°C. Data are normalized to specific binding in the absence of compound (set as 100%). (b) Inhibition of CCL2-stimulated β-arrestin recruitment in U2OS-CCR2 by increasing concentrations of compounds **39** and **43**, after stimulation with an EC_{80} concentration of CCL2 (set as 100%). (c) Inhibition of CCL3-stimulated β-arrestin recruitment in U2OS-CCR5 by increasing concentrations of compounds **8**, **39** and **43**, after stimulation with an EC_{80} concentration of CCL3 (set as 100%). All data are from single, representative experiments performed in duplicate.

As compound **8** was the best CCR5 inhibitor in this assay, displaying an IC_{50} value of 571 nM and a Hill slope of -2.2 ± 0.3 (Figure 2c and Table 1), we then synthesized several triazolo-pyrimidinone derivatives to explore their structure-affinity/activity relationships (SAR) in CCR2 and CCR5. All synthesized triazolo-pyrimidinone derivatives were evaluated in [^{3}H]-CCR2-RA-[*R*] binding assays to determine their binding affinity for CCR2, and in β-arrestin recruitment assays to determine their activity towards CCR5 (Figure 2 and Tables 1 – 3).

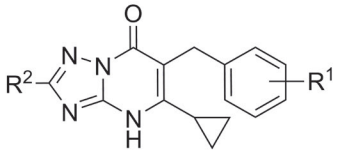
In CCR5, compounds were first screened at a concentration of 1 μM, and only those that displayed > 70% inhibition at this concentration were further evaluated in a concentration-inhibition curve to determine their potency. For better comparison, two compounds (**39** and **43**) were also tested in a CCR2 β-arrestin recruitment assay as previously described (Figure 2b).[19] Finally, we determined the mechanism of inhibition of **39** and **43** in both CCR2 and CCR5 using a [^{35}S]GTPγS binding assay (Figure 3, Table 4).

Structure-affinity/activity relationships (SAR) in CCR2 and CCR5

Analysis of the triazolo-pyrimidinone derivatives started by modifying the amino group (R^2) of the triazolo moiety (R^2, Table 1). Compared to **8**, removing the amino group (**6**) resulted in a similar affinity towards CCR2, in agreement with the similar reported IC_{50} values of approximately 20 nM for both compounds, when tested in a calcium flux assay.[22] However, in CCR5 **6** displayed a lower potency, as the inhibition of CCL3-stimulated recruitment of β-arrestin decreased to 60%, compared to 76% inhibition by **8**. The introduction of a methyl group in R^2 (**7**) was less favourable for both receptors, as both affinity for CCR2 and activity to CCR5 were reduced compared to **8**. As compound **8** displayed the highest affinity/activity for both receptors, we decided to keep the amino group in R^2 and explore different phenyl substituents (R^1, Table 1), taking **8** as the starting point.

Compared to **8**, the unsubstituted **9** showed a 5-fold decrease in affinity towards CCR2, while in CCR5 it was only able to inhibit 35% of the receptor response at 1 μM. Next, we investigated the effect of several benzyl modifications, including the influence of different substituent positions (Table 1). In the case of CCR2, meta-substituted derivatives also yielded the highest affinities in this series of compounds (**13** – **18**), whereas ortho-substituted derivatives yielded the lowest (**10** – **12**). None of the ortho-substitutions led to an improvement in affinity over **8** or the unsubstituted **9**. Introduction of a methyl (**10**) or a chloro (**11**) group in this position resulted in affinities lower than 10 nM, while the introduction of an electron-donating methoxy group further reduced the affinity to 105 nM (**12**), displaying the lowest CCR2 affinity in this series (Table 1). Moving the methyl group to meta (**13**) or para (**19**) position slightly improved the CCR2 binding affinity compared to **9**, achieving the highest affinity in meta position (**19**, 3 nM). Similarly, moving the methoxy group to meta or para position resulted in improved affinities following the meta > para > ortho order; however, the affinities remained lower than 10 nM (**17**, 13nM; **23**, 21 nM), with no improvement over **9**. This is consistent with functional data reported in the patent by Bengtsson *et al*., where similar compounds with a methoxybenzyl moiety displayed a loss of CCR2 activity compared to the unsubstituted-phenyl analogue.[22] Substitution of the meta methoxy group by an electron-withdrawing CF_3 group resulted in improved affinity over **17** (**18**, 6 nM), but no improvement over the unsubstituted **9**.

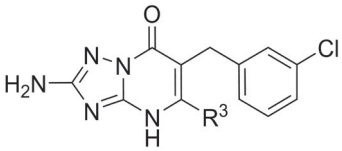
Table 1. Characterization of compounds 6 – 23 in hCCR2 and hCCR5



Compound	R^1	R^2	hCCR2 pK_i ± SEM (K_i, nM)[a]	hCCR5 pIC_{50} ± SEM (IC_{50}, nM) or inhibition at 1µM (%)[b]
6	3-Cl	H	8.76 ± 0.01 (1.7)	60%
7	3-Cl	Me	8.46 ± 0.05 (3.5)	35%
8	3-Cl	NH_2	8.90 ± 0.04 (1.3)	6.24 ± 0.004 (571)
9	H	NH_2	8.27 ± 0.10 (5.9)	35%
10	2-Me	NH_2	7.81 ± 0.05 (15.7)	28%
11	2-Cl	NH_2	7.84 ± 0.03 (14.5)	27%
12	2-OMe	NH_2	6.98 ± 0.04 (104.6)	-20%[c]
13	3-Me	NH_2	8.61 ± 0.03 (2.5)	62%
14	3-F	NH_2	8.53 ± 0.18 (3.4)	43%
15	3-Br	NH_2	9.08 ± 0.06 (0.9)	60%
16	3-I	NH_2	9.06 ± 0.02 (0.9)	66%
17	3-OMe	NH_2	7.89 ± 0.07 (13.0)	-27%[c]
18	3-CF_3	NH_2	8.26 ± 0.09 (5.9)	36%
19	4-Me	NH_2	8.46 ± 0.03 (3.5)	-57%[c]
20	4-F	NH_2	8.39 ± 0.03 (4.1)	31%
21	4-Cl	NH_2	8.74 ± 0.05 (1.8)	31%
22	4-Br	NH_2	8.84 ± 0.02 (1.5)	14%
23	4-OMe	NH_2	7.68 ± 0.05 (20.9)	-28%

Data are presented as mean pK_i/pIC_{50} ± standard error of the mean (SEM) and mean K_i/IC_{50} (nM) of at least three independent experiments performed in duplicate. [a]pK_i values from the displacement of ~6 nM [^{3}H]-CCR2-RA-[*R*] from U2OS cells stably expressing CCR2, at 25°C. [b]Percent inhibition of β-arrestin recruitment in U2OS cells stably expressing CCR5 by 1 µM compound, in presence of CCL3 (pEC_{80} = 7.9). pIC_{50} values were determined for compounds displaying more than 70% inhibition. % Inhibition values are presented as means of at least two independent experiments, performed in duplicate. [c]No inhibition was observed at the concentration of 1 µM, instead some CCL3 stimulation was measured.

Table 2. Characterization of compounds 24 – 36 in hCCR2 and hCCR5



Compound	R^3	hCCR2 $pK_i \pm SEM$ (K_i, nM)[a]	hCCR5 $pIC_{50} \pm SEM$ (IC_{50}, nM) or inhibition at 1μM (%)[b]
8	*c*Pr	8.90 ± 0.04 (1.3)	6.24 ± 0.004 (571)
24	Me	7.78 ± 0.07 (17.2)	-35%
25	Et	8.40 ± 0.07 (4.0)	29%
26	Pr	8.46 ± 0.07 (3.6)	64%
27	*i*Pr	8.72 ± 0.05 (1.9)	6.56 ± 0.05 (281)
28	Bu	8.64 ± 0.03 (2.3)	6.29 ± 0.05 (519)
29	2-EtBu	8.20 ± 0.04 (6.4)	29%
30	Pent	8.14 ± 0.03 (7.2)	38%
31	*c*Pent	8.81 ± 0.04 (1.6)	6.43 ± 0.08 (388)
32	Hex	7.66 ± 0.02 (22.0)	-63%[c]
33	Hept	6.76 ± 0.05 (178.1)	-265%[c]
34	Ph	7.64 ± 0.17 (26.7)	-41%[c]
35	4-MePh	6.81 ± 0.07 (158.8)	-13%[c]
36	CH_2CH_2Ph	7.29 ± 0.05 (52.3)	-42%[c]

Data are presented as mean pK_i/pIC_{50} ± standard error of the mean (SEM) and mean K_i/IC_{50} (nM) of at least three independent experiments performed in duplicate. [a]pK_i values from the displacement of ~6 nM [^{3}H]-CCR2-RA-[*R*] from U2OS cells stably expressing CCR2, at 25°C. [b]Percent inhibition of β-arrestin recruitment in U2OS cells stably expressing CCR5 by 1 μM compound, in presence of CCL3 (pEC_{80} = 7.9). pIC_{50} values were determined for compounds displaying more than 70% inhibition. % Inhibition values are presented as means of at least two independent experiments, performed in duplicate. [c]No inhibition was observed at the concentration of 1 μM, instead some CCL3 stimulation was measured.

The effect of introducing different halogen groups was first investigated in meta position. Overall, an increase in size and lipophilicity from fluoro to iodo resulted in improved binding affinities towards CCR2 (F, **14** < Cl, **8** < Br, **15** ≈ I, **16**). In fact, compounds **15** and **16** displayed the highest affinities in this series of derivatives (**15**, 0.8 nM; **16**, 0.9 nM). Moving the halogen substituents to the para position resulted in a similar trend in affinity (F, **20** < Cl, **21** < Br, **22**); however, their affinities were lower compared to the meta-substituted analogues. Of note, compounds with a fluorine atom in meta (**14**) or para (**20**) position displayed lower affinities than compounds with a methyl group in the equivalent position

(**13** and **19**). To gain more insight in a potential relationship between affinity and lipophilicity as observed in the halogen series, calculated log P values (cLogP) of compounds **8** – **23**, with R^1 modifications, were plotted against their pK_i values in CCR2. This analysis revealed only a slight correlation between these two parameters for this set of compounds (Fig S2a); however, this correlation was lost when all synthesized derivatives were included in this plot (Fig S2b), indicating that this is not a general trend.

In the case of CCR5, meta-substituted derivatives also outperformed their ortho- and para-substituted analogues, with some compound displaying > 60% inhibition at 1 µM; in contrast, ortho- and para-substitution resulted in compounds with low (≤ 31%) to marginal efficacy in CCR5, suggesting that substituents in ortho or para position are not tolerated in CCR5. Similarly as in CCR2, the introduction of a methoxy group was unfavourable, as it led to a complete loss of activity in CCR5 when tested at 1 µM (**12**, **17** and **23**), regardless of the position; whereas electron-withdrawing groups in meta position (**18**, $R^2 = CF_3$) did not bring any improvement over the unsubstituted **9**. Except for compound **14** bearing a meta-fluoro, which showed less than 45% inhibition, all other compounds bearing halogens in meta position led to > 60% inhibition; the same was achieved when a methyl group was placed in this position (**13**). Overall, these data indicate that meta-substituents, especially halogens, are preferred to achieve dual CCR2/CCR5 activity, while ortho- and para-substituents lead to a lower affinity but higher selectivity towards CCR2.

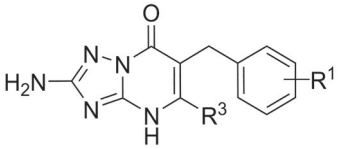
As none of the other substituents in R^2 led to a significant improvement in CCR5 activity over compound **8**, we decided to continue with this compound and investigate the effect of replacing the cyclopropyl moiety in R^3. Based on the chemical structure of **8** and CCR2-RA-[*R*] (Figure 1), we hypothesized that the cyclopropyl group in **8** interacts with $Val244^{6x36}$ in CCR2, in a similar manner as the cyclohexyl group of CCR2-RA-[*R*].[14] Thus, several triazolo-pyrimidinone derivatives were synthesized with different alkyl chains and aromatic groups in this position, in order to investigate their SAR (Table 2). Starting with the effect of alkyl substituents, we observed that increasing the size and flexibility of the alkyl chain from n = 1 (methyl) to n = 4 (butyl) resulted in a parallel increase in CCR2 affinity (17 nM for R^3 = Me (**24**); ~4 nM for R^3 = Et (**25**) and R^3 = Pr (**26**); 2 nM for R^3 = Bu (**28**)). However, further elongation of the chain length (n = 5 – 7) led to a progressive drop in affinity (7 nM for R^3 = Pent (**30**); 22 nM for R^3 = Hex (**32**); 178 nM for R^3 = Hept (**28**)), indicating that linear alkyl chains longer than five carbons might not fit in this hydrophobic pocket. The same trend was observed for CCR5 activity, as only the *n*-propyl (**26**) and *n*-butyl (**28**) substituted compound led to > 60% inhibition, albeit without improvement over **8** (**28**, 519 nM). Moreover, introduction of a hexyl or heptyl group resulted in CCL3 stimulation instead of inhibition, which was not further investigated. Increasing bulkiness via branching of alkyl groups or substitution with aliphatic rings enhanced the affinity towards CCR2, indicating that these substituents

might provide a better interaction with the receptor. For instance, the introduction of both isopropyl (**27**) and cyclopropyl (**8**) groups led to an improvement in CCR2 affinity compared to the linear analogue **26**. Moreover, compound **27** with an isopropyl substituent also yielded a 2-fold increase in CCR5 potency compared to the cyclic analogue **8**, displaying the highest potency in this series of compounds (**27**, 281 nM). In line with this trend, we observed that replacing the linear pentyl group (**30**) with a cyclopentyl group (**31**) was also beneficial for CCR2, as this derivative showed a 4.5-fold increased affinity compared to **30** (**31**, 1.6 nM). In CCR5, **31** inhibited the CCL3-induced response with a potency of 388 nM, showing a slight improvement over compound **8**. In contrast, the introduction of a 2-ethyl butyl group (**29**) resulted in reduced affinity/activity towards both CCR2 and CCR5. These data suggest that the isopropyl group is the preferred R^3 substituent when designing CCR2/CCR5 dual antagonists, as this substituent led to the highest potency in CCR5 while maintaining a high affinity for CCR2. Next, inspired by our work on CCR1/CCR2 selectivity of pyrrolone derivatives,[18] we investigated whether aromatic substituents are tolerated in this position. As expected from previous studies,[18, 29] the introduction of aromatic groups decreased 20-fold (**34**, 27 nM), 40-fold (**36**, 52 nM) and 122-fold (**35**, 159 nM) the affinity for CCR2 compared to **8**. When tested in CCR5, all derivatives showed a complete loss of activity at 1 μM, indicating that aromatic groups are not favourable for selectivity or dual activity.

With the aim of finding dual CCR2/CCR5 intracellular inhibitors, we kept the isopropyl moiety in R^3 and investigated the effect of having a di-substituted phenyl moiety in R^1, by exploring different positions and combinations of chlorine and bromine atoms (Table 3). First, and similar as **8**, we kept the cyclopropyl moiety in R^3 and combined it with di-chlorination in meta and para position (**37**). Compared to the mono-substituted analogues **8** and **21**, this compound yielded an even higher affinity to CCR2 (**37**, 0.4 nM); moreover, its ability to inhibit CCL3-induced response in CCR5 was also improved, as the potency increased to 214 nM. By replacing the cyclopropyl of **37** with an isopropyl group (**38**), we retained affinity for CCR2 (0.6 nM), but the potency for CCR5 increased by almost 2-fold (132 nM), in agreement with the higher potency observed in **27** versus **8** (Table 2). Moving one chlorine atom to ortho position, while keeping one in the adjacent meta position, yielded compound **39** with slightly lower affinity for CCR2 but even higher potency in CCR5 (**39**, 84 nM), indicating that although ortho substituents are not preferred in mono-substituted derivatives, they are still tolerated when placed in combination with halogens in other positions. However, placing the two halogens in the second and fifth position was clearly detrimental for both receptors (**40**); in CCR2, the affinity decreased by almost 40-fold, while in CCR2, the compound was only able to inhibit 20% of the CCR5 response. Placing the two halogens in the symmetrical third and fifth positions restored the affinity/activity in both receptors (**41**, 2.2 nM in CCR2 and 336 nM in CCR5). Replacing the two chlorine atoms of **41** by bromine atoms yielded derivative **42**, which retained affinity towards CCR2 but led to decrease in CCR5 activity,

as this compound was not able to inhibit > 70% of the CCL3-induced response. Finally, the combination of a bromo in meta position with a chloro in para position (**42**) improved both the affinity and activity to both receptors to similar levels as **37**, in the case of CCR2, and **38** in the case of CCR5, indicating that halogens in adjacent positions are more favourable for activity in these receptors. Of note, compounds **37**, **38** and **43** displayed the highest affinities to CCR2 in this study, while **38**, **39** and **43** displayed the highest potencies to CCR5.

Table 3. Characterization of compounds 37 – 43 in hCCR2 and hCCR5



			hCCR2	hCCR5
Compound	**R^1**	**R^3**	**pK_i ± SEM (K_i, nM)[a]**	**pIC_{50} ± SEM (IC_{50}, nM) or inhibition at 1µM (%)[b]**
37	3,4-diCl	*c*Pr	9.35 ± 0.05 (0.4)	6.67 ± 0.03 (214)
38	3,4-diCl	*i*Pr	9.22 ± 0.05 (0.6)	6.91 ± 0.09 (132)
39	2,3-diCl	*i*Pr	8.81 ± 0.07 (1.6)	7.09 ± 0.07 (84)
40	2,5-diCl	*i*Pr	7.65 ± 0.03 (22.5)	20%
41	3,5-diCl	*i*Pr	8.66 ± 0.05 (2.2)	6.49 ± 0.06 (336)
42	3,5-diBr	*i*Pr	8.68 ± 0.01 (2.1)	64%
43	3-Br, 4-Cl	*i*Pr	9.42 ± 0.02 (0.4)	6.95 ± 0.04 (115)

Data are presented as mean pK_i/pIC_{50} ± standard error of the mean (SEM) and mean K_i/IC_{50} (nM) of at least three independent experiments performed in duplicate. [a]pK_i values from the displacement of ~6 nM [^{3}H]-CCR2-RA-[*R*] from U2OS cells stably expressing CCR2, at 25°C. [b]Percent inhibition of β-arrestin recruitment in U2OS cells stably expressing CCR5 by 1 µM compound, in presence of CCL3 (pEC_{80} = 7.9). pIC_{50} values were determined for compounds displaying more than 70% inhibition. % Inhibition values are presented as means of at least two independent experiments, performed in duplicate.

It is important to note that so far we are comparing data not only between two different receptors, but also between two different assays: i) a radioligand binding assay for CCR2, in the absence of agonist, which allows the determination of true affinities (pK_i values); ii) a functional assay for CCR5 in the presence of an EC_{80} concentration of CCL3, without further correction of their IC_{50} values. To better compare the activities in both receptors, we selected compounds **39** and **43**—with the highest potency on CCR5 and the highest affinity for CCR2, respectively—and tested these in a previously described β-arrestin recruitment assay for CCR2.[19] In this assay, compound **39** inhibited CCL2-stimulated β-arrestin recruitment with a

potency of 21 nM, while compound **43** displayed a higher potency of 4 nM, consistent with their affinities. In addition, their Hill slopes (n_H = -2.5 for **39**; n_H = -3.4 for **43**) are indicative of a non-competitive form of inhibition, a further confirmation of their allosteric binding site located in the intracellular region of CCR2 (Figure 2b and Table S3). Of note, the Hill slopes in CCR5 were comparable to those in CCR2 (n_H = -3.7 for **39**; n_H = -4.4 for **43**), i.e. indicating an allosteric interaction at CCR5 as well. Comparing the IC_{50} values obtained with the functional assays in both receptors, we observe a 4-fold difference between CCR2 and CCR5 in the case of **39**, making it a potential dual-antagonist for both receptors. However, the potencies in CCR2 and CCR5 differ by 29-fold in the case of **43**, indicating that this compound is one of the more selective compounds towards CCR2.

Mechanism of inhibition of selected compounds

Selected compounds **39** and **43** were also tested in a [^{35}S]GTPγS binding assay in both CCR2 and CCR5, in order to determine their mechanism of inhibition. In the case of CCR2, we have shown that these ligands fully displace radiolabelled [^{3}H]-CCR2-RA-[*R*], indicating that triazolo-pyrimidinone derivatives bind in the same intracellular binding site. Thus, these compounds were expected to show non-competitive, insurmountable antagonism to (orthosteric) chemokine ligands, as previously demonstrated in CCR2 with CCR2-RA-[*R*][19] and JNJ-27141491[30]. To verify this, **39** and **43** were characterized in a previously described [^{35}S]GTPγS binding assay on U2OS-CCR2 membranes.[19] In this assay, CCL2-stimulation of [^{35}S]GTPγS binding in CCR2 was examined in the absence or presence of fixed concentrations of **39** and **43** (Table 4 and Figure 3a,b). In the absence of antagonist, increasing concentrations of CCL2 induced [^{35}S]GTPγS binding with an EC_{50} of 8 nM, in line with previously described parameters.[18, 19] Co-incubation of CCL2 with **39** or **43** caused a significant reduction in the maximal response of CCL2 (E_{max}) at all three antagonist concentrations tested. The lowest concentrations of antagonist did not affect the potency of CCL2, while higher concentrations significantly reduced the potency of CCL2 (Table 4 and Figures 3a,b).

To confirm our hypothesis that these two compounds also bind to an allosteric site in CCR5, i.e. the intracellular binding site, we next analysed the effect of **39** and **43** on CCL3-induced [^{35}S]GTPγS binding in U2OS-CCR5 membranes. In agreement with previous studies, CCL3 stimulated [^{35}S]GTPγS binding in CCR5 with a potency of 4 nM.[27] Similarly as in CCR2, the two compounds were able to significantly suppress the maximal response induced by CCL3 at all concentrations tested (Table 4 and Figures 3c,d). However, in contrast to CCR2, the potency of CCL3 was only significantly reduced with the highest concentration of **43** (Table 4). Such depression of the maximal response with or without a decrease of agonist potency is typical of insurmountable antagonists,[31] indicating that **39** and **43** behave as insurmountable

antagonists at both CCR2 and CCR5. Of note, insurmountable antagonism can be generally achieved by two different mechanisms: allosteric binding or slow binding kinetics, i.e. slow equilibration, of a competitive antagonist.[31] However, insurmountable inhibition due to a hemi-equilibrium is only evident in pre-incubation experiments, where the receptor is pre-incubated with the antagonist before exposure to the agonist.[31] In contrast, allosteric binding leads to insurmountable inhibition in co-incubation experiments, as performed in this study. These data further support our hypothesis that **39** and **43** bind to an allosteric binding site in CCR5, most probably located intracellularly.

Table 4. Effects of compounds 39 and 43 in chemokine-stimulated [^{35}S]GTPγS binding

Receptor	Compound	pEC_{50} ± SEM (EC_{50}, nM)	E_{max} ± SEM (%)[a]
hCCR2	CCL2	8.10 ± 0.06 (8)	107 ± 2
	CCL2 + 10 nM **39**	7.89 ± 0.04 (13)	91 ± 1**
	CCL2 + 30 nM **39**	7.60 ± 0.07 (26)**	75 ± 4****
	CCL2 + 100 nM **39**	7.27 ± 0.10 (56)****	50 ± 3****
	CCL2 + 1 nM **43**	7.91 ± 0.10 (13)	72 ± 4****
	CCL2 + 3 nM **43**	7.53 ± 0.12 (32)***	51 ± 5****
	CCL2 + 10 nM **43**	6.87 ± 0.13 (148)****	33 ± 3****
hCCR5	CCL3	8.42 ± 0.06 (4)	108 ± 2
	CCL3 + 100 nM **39**	8.35 ± 0.09 (5)	79 ± 5****
	CCL3 + 300 nM **39**	8.14 ± 0.12 (8)	56 ± 2****
	CCL3 + 1000 nM **39**	8.14 ± 0.17 (9)	25 ± 4****
	CCL3 + 30 nM **43**	8.30 ± 0.05 (5)	81 ± 3****
	CCL3 + 100 nM **43**	8.21 ± 0.05 (6)	58 ± 1****
	CCL3 + 300 nM **43**	8.05 ± 0.06 (9)*	35 ± 2****

Data represent the mean ± standard error of the mean (SEM) of three independent experiments performed in duplicate. One-way ANOVA with Dunnett's posthoc test was used to analyze differences in pEC_{50} and E_{max} values against CCL2 or CCL3 controls. [a]Maximum effect (E_{max}) of CCL2 or CCL3 measured in the absence or presence of fixed concentrations of compound **39** and **43** in CCR2 or CCR5, respectively.

Docking study

To further investigate the binding mode of triazolo-pyrimidinone derivatives, compound **43** was docked into a CCR2b model based on the crystal structure of CCR2 (PDB 5T1A, **Chapter 3**).[14] Due to the close proximity to the intracellular binding site, several residues from the intracellular loop 3 (ICL3) had to be modelled based on the crystal structure of CCR5 (PDB

4MBS),[32] since they were mutated in the original CCR2 crystal structure to further stabilize the receptor. As seen in Figure 4a, **43** was predicted to adopt a similar binding pose as that of the previously co-crystallized CCR2-RA-[*R*].[14] The di-substituted phenyl group of **43** was constrained to overlap with the corresponding phenyl group of CCR2-RA-[*R*], since the di-substituted aromatic rings of JNJ-27141491 and SD-24 (Figure 1) were also predicted to overlay with the phenyl group of CCR2-RA-[*R*] in a previous study.[20] However, the bromine group of compound **43** is predicted to form a halogen bond with the backbone of Val^{1x53}, which might contribute to the higher affinity of **43** versus CCR2-RA-[*R*] (Figure 4b). This data suggests that all intracellular ligands share similar interactions between the aromatic group and the receptor (Figure 4a), and implies that this aromatic moiety is not responsible for the differences in CCR5 activity observed between the reference ligands[20] (Figure S1b).

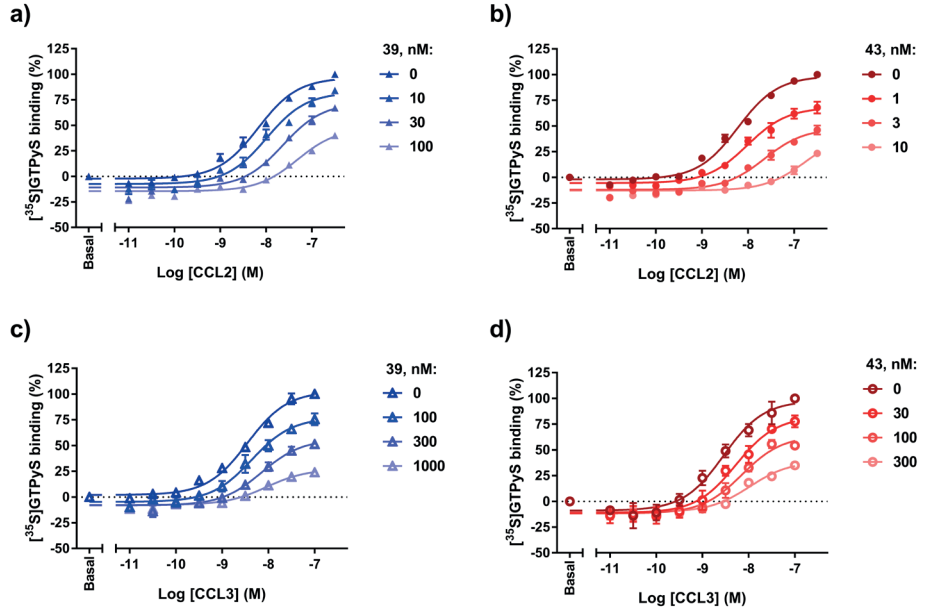


Figure 3. Characterization of compounds 39 and 43 as insurmountable, negative allosteric modulators using a [^{35}S]GTPγS binding assay in hCCR2 and hCCR5. Effect of increasing concentrations of **39** and **43** in a CCL2-stimulated [^{35}S]GTPγS binding in U2OS-CCR2 (a, b), or in a CCL3-stimulated [^{35}S]GTPγS binding in U2OS-CCR5 (c, d), at 25 °C. Parameters obtained from the concentration-response curves (pEC_{50}, E_{max}) are summarized in Table 4. Data are presented as mean ± SEM values of three experiments performed in duplicate.

In addition, the isopropyl group of **43** is predicted to bind in the same position as the cyclohexyl moiety of CCR2-RA-[*R*], although it seems to make less interactions with Val^{6x36} perhaps due to the slightly different ligand orientation (Figure 4b). Previous studies have confirmed the crucial role of Val^{6x36} for binding affinity of some intracellular ligands in CCR2,

as mutation of this residue to alanine completely abolished binding of CCR2-RA-[*R*] to the receptor.[20] Moreover, this residue might be involved in target selectivity, as the main difference between the intracellular pockets of CCR2 and CCR5 is the single substitution of Val^{6x36} by Leu^{6x36}. The steric hindrance introduced by this substitution might be thus responsible for the reduction in affinity of CCR2-RA-[*R*] towards CCR5 compared to CCR2.[20] Indeed, in the case of CCR5, only small aliphatic groups were tolerated in R^3 position, such as cyclopropyl or isopropyl (Table 2), while bigger aliphatic groups resulted in improved selectivity towards CCR2. However, a previous SAR analysis of pyrrolone derivatives in CCR1, which also contains a leucine in position 6x36, showed that aromatic groups in the equivalent R^3 position provide CCR1 selectivity versus CCR2, as aromatic groups are not tolerated in this position in CCR2[18] (Table 2).

The binding pose of **43** seems to be stabilized by a network of hydrogen bonds between the triazolo-pyrimidinone core and residues E^{8x48}, Lys^{8x49}, F^{8x50} and R^{3x50} (Figure 4b). Although the core of CCR2-RA-[*R*] and **43** binds with a different orientation, the carboxy group of both overlay in the same position, interacting with the backbones of Lys^{8x49} and F^{8x50}. Moreover, the secondary and tertiary amino groups present in the triazolo-pyrimidinone core also form hydrogen bonds with the backbones of Lys^{8x49} and Glu^{8x48}, as well as with the side chains of Arg^{3x50}. Finally, the primary amino group in position R^2 of compound **43** also makes an extra hydrogen bond with the side chain of E^{8x48}. Such extended network of hydrogen bond interactions is not present with CCR2-RA-[*R*], and thus it might be responsible for the higher affinity of **43** in CCR2, compared to CCR2-RA-[*R*]. Previous studies have confirmed the importance of residues 8x49 and/or 8x50 in chemokine receptors for the binding of several intracellular ligands. For example, alanine mutations of Lys^{8x49} and F^{8x50} in CCR2 caused a 10-fold reduction or a complete loss of affinity of intracellular ligands, respectively, compared to the wild-type receptor.[20] In CXCR2, alanine mutation of Lys^{8x49} led to a reduced affinity of three different intracellular ligands, while the mutation F^{8x50}A only affected one of the ligands tested, indicating a different binding mode.[33] Moreover, Lys^{8x49} has been suggested as a key residue for target selectivity between CXCR1 and CXCR2, as it is exchanged by Asn^{8x49} in CXCR1.[34] In addition, the crystal structure of CCR9 in complex with vercirnon[15] also shows a binding interaction between the ligand and Arg323^{8x49} and Phe324^{8x50}. Overall, these data suggest that although the intracellular pockets of CCR2 and CCR5 are quite conserved, the design of multitarget compounds is not quite straightforward. Moreover, several of these residues have been shown to be involved in $G\alpha_i$ coupling in recent cryo-electron microscopy (cryo-EM)-derived GPCR structures, including residues 3x50, 6x29, 6x32 to 6x37, 8x47 and 8x49.[35-37] Similarly, homologous residues are also involved in direct interactions between rhodopsin and arrestin,[38] suggesting a direct interference of these intracellular ligands with the $G\alpha_i$ protein and β-arrestin binding site, and the possibility of fine-tuning residue interactions for the design of biased ligands.

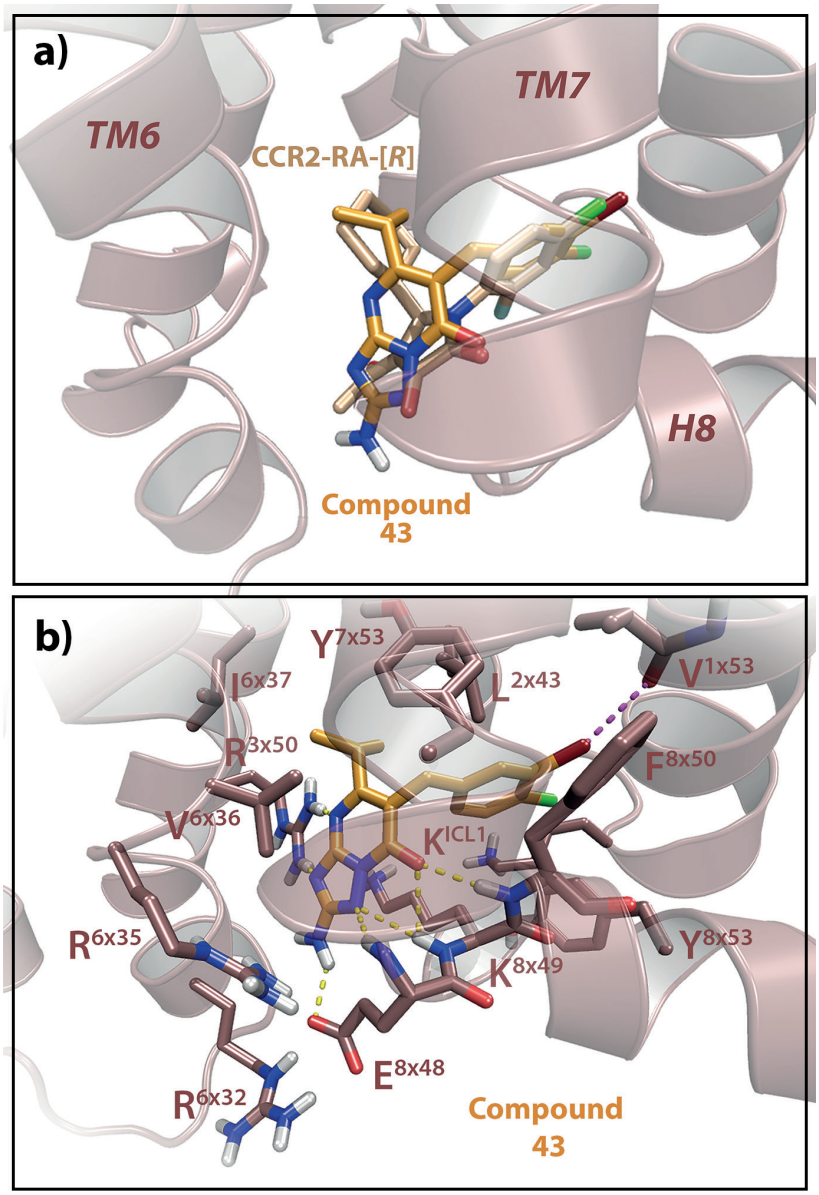


Figure 4. **Proposed binding mode of 43 in hCCR2b.** (a) Overlay of **43** with the CCR2 intracellular ligand CCR2-RA-[*R*], showing that **43** interacts in a similar manner as CCR2-RA-[*R*]. (b) Docking of **43**, displaying the interactions with CCR2. The amino group in R^2 makes an extra hydrogen-bond interaction with E^{8x48}, while the bromine group in R^1 makes an extra halogen bond with the backbone of V^{1x53}, which might contribute to the improved affinity to this receptor. Model of hCCR2 is based on the crystal structure of CCR2 (PDB 5T1A),[14] and amino acid residues are labeled according to their structure-based Ballesteros-Weinstein numbers.[24]

CONCLUSIONS

In this study we first confirmed that the triazolo-pyrimidinone derivative **8** binds to the intracellular pocket of CCR2, in a similar manner as the reference intracellular ligand CCR2-RA-[*R*]. Moreover, compound **8** was also able to inhibit CCR5 in a functional β-arrestin recruitment assay; thus, we took this compound as a starting point for the synthesis of a series of novel and previously described triazolo-pyrimidinone derivatives. Using [^{3}H]-CCR2-RA-[*R*] binding assays and functional β-arrestin recruitment assays, we explored structure-affinity/activity relationships (SAR) in both receptors. Overall, these compounds were mostly selective towards CCR2; however, CCR5 activity was increased with the combination of isopropyl in R^3 position and two halogens placed in adjacent positions at the phenyl group in R^1. Overall, these findings indicate that even though the intracellular pockets of CCR2 and CCR5 are highly conserved, selectivity of intracellular ligands can be fine-tuned, allowing the design of either selective or multitarget ligands. Evaluation of compounds **39** and **43** in a [^{35}S]GTPγS binding assay indicates that both compounds display a noncompetitive, insurmountable mode of inhibition in CCR2 and CCR5, which might represent a therapeutic advantage in inflammatory diseases characterized by a high local concentration of endogenous chemokines, such as multiple sclerosis and rheumatoid arthritis. Thus, in diseases where selective chemokine receptor antagonists have been largely unsuccessful, the development of multitarget, intracellular ligands for CCR2 and CCR5 may represent a novel therapeutic option.

EXPERIMENTAL SECTION

Chemistry

General methods

All solvents and reagents used were of analytical grade and from commercial sources. Demineralized water was used in all cases, unless stated otherwise, and is simply referred to as H_2O. Microwave-based synthesis was carried out using a Biotage Initiator® equipment (Biotage, Sweden). All reactions were monitored by thin-layer chromatography (TLC) using aluminum plates coated with silica gel 60 F_{254} (Merck), and compounds were visualized under ultraviolet light at 254 nm or via $KMnO_4$ staining. Column chromatography for compound purification was performed using silica gel (Merck millipore) with particle size 0.04-0.63 mm. Chemical identity of final compounds was established using ^{1}H NMR and Liquid chromatography–mass spectrometry (LC–MS). ^{1}H NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (^{1}H NMR, 400 MHz) at room temperature (rt). Chemical shifts (δ) are reported in parts per million (ppm), and coupling-constants (J) in Hz. Liquid chromatography–mass spectrometry (LC–MS) of final compounds was performed using a Thermo Finnigan Surveyor LCQ Advantage Max LC-MS system and a Gemini C18 Phenomenex column (50 × 4.6 mm, 3 µm). Analytical purity of the compounds was determined using a Shimadzu high pressure liquid chromatography (HPLC) equipment with a Phenomenex Gemini column (3 x C18 110A column, 50 × 4.6 mm, 3 µm). A flow rate of 1.3 mL/min, and an elution gradient of 10-90% MeCN/H_2O (0.1% TFA) was used. The absorbance of the UV spectrophotometer was set at 254 nm. All compounds tested in biological assays showed a single peak at the designated retention time and were ≥ 95% pure. Sample preparation for HPLC and LC-MS were as follows, unless stated otherwise: 0.3 mg/mL of compound was dissolved in a 1:1:1 mixture of H_2O:MeOH:tBuOH. Of note, some compounds required DMSO and heat to ensure proper dissolution. None of the final compounds were identified as potential pan-assay interference compounds (PAINS) after assessment with the Free ADME-Tox Filtering Tool (FAF-Drugs4),[39, 40] which uses three different PAINS filters based on Baell et al.[41]

General procedure 1: Synthesis of β- keto esters 1f-h,j,k,n.[42]

In a flame-dried round bottom flask under a nitrogen atmosphere, ethyl acetoacetate (2.53 mL, 20.0 mmol, 1.00 eq.) was added drop wise to a suspension of NaH (880 mg, 22.1 mmol 1.10 eq.) in dry THF (5 mL) at 0°C while stirring. After 20 min, n-butyl lithium (20 .0 mmol, 2.50 M solution in pentane,1.00 eq.) was added drop wise to the mixture and stirred for

further 30 min. The respective alkyl halide **2f-h,j,k** or benzyl bromide **2n** (1.20 eq.) was subsequently added drop wise over a period of 10 min to the dianion solution after which the solution was allowed to reach rt. After 14 hours, the reaction was quenched by the addition of saturated NH_4Cl (aq., 80 mL). The mixture was subsequently extracted with diethyl ether (2 X 120 mL). The combined organic fractions were washed with brine (80 mL) and dried over $MgSO_4$ followed by concentration in vacuo. The crude products were purified by flash chromatography (CH_2Cl_2/petroleum ether and/or EtOAc/petroleum ether as the eluent) to give the title compounds **1f-h,j,k,n** as oils. Compounds **1a-e,i,l,m** were commercially available.

Ethyl-3-oxoheptanoate (1f).[42] Synthesized according to general procedure 1. Started from 1-bromopropane (**2f**) (2.00 mL, 22.0 mmol, 1.10 eq.) and purified by silica column chromatography (1% – 30% EtOAc in petroleum ether). Yield: 36% (1.25 g) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (q, *J* = 7.2 Hz, 2H), 3.44 (s, 2H), 2.54 (t, *J* = 7.4 Hz, 2H), 1.65-1.55 (m, 2H), 1.38-1.25 (m, 7H), 0.88 (t, *J* = 6.8 Hz, 3H) ppm.

Ethyl 5-ethyl-3-oxoheptanoate (1g). Synthesized according to the general procedure 1. Started from 3-bromopentane (**2g**) (3.00 mL, 24.2 mmol, 1.21 eq.) and purified by silica column chromatography (1% – 30% EtOAc in petroleum ether). Yield: 18% (630 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.2 Hz, 2H), 3.40 (s, 2H), 2.43 (d, *J* = 6.8 Hz, 2H), 1.88-1.73 (m, 1H), 1.33-1.21 (m, 7H), 0.82 (t, *J* = 7.4 Hz, 6H) ppm.

Ethyl 3-oxooctanoate (1h).[42] Synthesized according to general procedure 1. Started from 1-bromobutane (**2h**) (2.59 mL, 24.1 mmol, 1.21 eq.) and purified by silica column chromatography (1% – 30% EtOAc in petroleum ether). Yield: 38% (1.41 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (q, *J* = 7.2 Hz, 2H), 3.43 (s, 2H), 2.53 (t, *J* = 7.2 Hz, 2H), 1.65-1.55 (m, 2H), 1.37-1.12 (m, 7H), 0.89 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 3-oxononanoate (1j).[42] Synthesized according to the general procedure 1. Started from 1-iodopentane (**2j**) (3.13 mL, 24.0 mmol, 1.20 eq.) and purified by silica column chromatography (1% – 30% EtOAc in petroleum ether). Yield: 44% (1.76 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (q, *J* = 7.2 Hz, 2H), 3.43 (s, 2H), 2.53 (t, *J* = 7.4 Hz, 2H), 1.54-1.64 (m, 2H), 1.33-1.24 (m, 9H), 0.88 (t, *J* = 6.8 Hz, 3H) ppm.

Ethyl 3-oxodecanoate (1k).[43] Synthesized according to the general procedure 1. Started from 1-bromohexane (**2k**) (3.36 mL, 25.0 mmol, 1.24 eq.) and purified by silica column chromatography (30% CH_2Cl_2 in Petroleum ether to 100% CH_2Cl_2). Yield: 15% (625 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (q, *J* = 7.2 Hz, 2H), 3.43 (s, 2H), 2.53 (t, *J* = 7.4 Hz, 2H), 1.64-1.53 (m, 2H), 1.33-1.24 (m, 11H), 0.88 (t, *J* = 7.5 Hz, 3H) ppm.

Ethyl 3-oxo-5-phenylpentanoate (**1n**).[44] Synthesized according to the general procedure 1. Started from benzyl bromide (**2n**) (2.90 mL, 24.4 mmol, 1.22 eq.) and purified by silica column chromatography (1% – 30% EtOAc in petroleum ether). Yield: 20% (1.06 g) as a collorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.23 (m, 2H), 7.23-7.12 (m, 3H), 4.17 (q, *J* = 7.2 Hz, 2H), 3.42 (s, 2H), 2.98-2.81 (m, 4H), 1.26 (t, *J* = 7.2 Hz, 3H) ppm.

General procedure 2. Benzylated β-keto esters 4aa-na, 4bb-bq, 4eq-ev.[22]

LiCl (1.00 eq.) was slurried in anhydrous THF (1 mL/mmol **1a-n**) in a flame dried round bottom flask and under an atmosphere of nitrogen. The desired β-keto ester **1a-n** (1.00 eq.) was added and followed by DIPEA (2.00 eq.) and the respective benzylic halide **3a-v** (1.20 eq.). The reaction mixture was reflux for 20 hours, after which the reaction was completed as indicated by TLC (5-10% EtOAc in Petroleum ether). THF was removed in vacuo, the crude dissolved EtOAc (30 mL) and this organic layer was washed with citric acid (5%, 25 mL) followed by saturated $NaHCO_3$ (25 mL) and brine (25 mL). The organic layer was subsequently dried over $MgSO_4$, concentrated in vacuo to afford the crude product. The crude product was purified by flash column chromatography (5%-10% EtOAc/petroleum ether) to yield the corresponding benzylated β-keto esters **4aa-na, 4bb-bq, 4eq-ev**.

Ethyl 2-(3-chlorobenzyl)-3-oxobutanoate (4aa).[22] Synthesis according to general procedure 2. Reagents: Ethyl 3-oxobutanoate **1a** (0.37 mL, 2.92 mmol, 1.20 eq.), 3-chlorobenzyl bromide **3a** (0.32 mmol, 2.43 mmol, 1.00 eq.), DIPEA (0.85 mL, 4.86 mmol, 2.00 eq.), LiCl (103 mg, 2.43 mmol, 1.00 eq.), 5 mL dry THF. Yield: 70% (433 mg) as a colourless oil. ^{1}H NMR: (400 MHz, $CDCl_3$): δ 7.21-7.14 (m, 3H), 7.09-7.04 (m, 1H), 4.16 (q, *J* = 7.2 Hz, 2H), 3.75 (t, *J* = 8.0 Hz, 1H), 3.17-3.07 (m, 2H), 2.21 (s, 3H), 1.21 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 3-cyclopropyl-2-(3-chlorobenzyl)-3-oxopropanoate (4ba).[22] Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (7.56 mL, 51.2 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (7.06 mL, 53.8 mmol, 1.05 eq.), DIPEA (17.8 mL, 102 mmol, 2.00 eq.), LiCl (2.17 g, 51.22 mmol, 1.00 eq.), 5 mL dry THF. Yield: 71% (10.2 g) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21-7.l6 (m, 3H), 7.10-7.05 (m, 1H), 4.17 (qd, *J* = 7.2, 1.6 Hz, 2H), 3.89 (t, *J* = 7.6 Hz, 1H), 3.15 (dd, *J* = 7.2 Hz, 2H), 2.08-2.02 (m, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.11-1.01 (m, 2H), 0.98-0.88 (m, 2H) ppm.

Ethyl 2-benzyl-3-cyclopropyl-3-oxopropanoate (4bb).[23] Synthesis according to general procedure 2.Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.52 mL, 3.51 mmol, 1.20 eq.), benzylbromide **3b** (0.347 mL, 2.92 mmol, 1.00 eq.), DIPEA (1.02 mL, 5.84 mmol, 2.00 eq.), LiCl (124 mg, 2.92 mmol, 1.00 eq.), 4 mL dry THF. Yield: 26% (105 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29-7.25 (m, 2H), 7.22-7.18 (m, 3H), 4.20-4.12 (m, 2H), 3.91 (t, *J* = 7.6 Hz, 1H), 3.20 (d, *J* = 7.6 Hz, 2H), 2.08-2.02 (m, 1H), 1.20 (t, *J* = 7.2 Hz, 3H), 1.06-1.03 (m, 2H), 0.94-0.84 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(2-methylbenzyl)-3-oxopropanoate (4bc). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.63 mL, 4.27 mmol, 1.20 eq.), 2-methylbenzyl chloride **3c** (0.48 mL, 3.56 mmol, 1.00 eq.), DIPEA (1.24 mL, 7.12 mmol, 2.00 eq.), LiCl (151 mg, 3.56 mmol, 1.00 eq.), 4 mL dry THF. Yield: 80% (742 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15-7.09 (m, 4H), 4.16 (qd, *J* = 7.2, 1.2 Hz, 2H), 3.91 (t, *J* = 7.2 Hz, 1H), 3.20 (d, *J* = 7.6 Hz, 2H), 2.34 (s, 3H), 2.05-2.00 (m, 1H), 1.20 (t, *J* = 7.2 Hz, 3H), 1.07-1.02 (m, 2H), 0.95-0.84 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(2-chlorobenzyl)-3-oxopropanoate (4bd). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.43 mL, 2.92 mmol, 1.20 eq.), 2-chlorobenzyl bromide **3d** (0.32 mL, 2.43 mmol, 1.00 eq.), DIPEA (0.85 mL, 4.86 mmol, 2.00 eq.), LiCl (103 mg, 2.43 mmol, 1.00 eq.), 5 mL dry THF. Yield: 78% (532 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.33 (m, 1H), 7.26-7.24 (m, 1H), 7.18-7.14 (m, 2H), 4.20-4.08 (m, 3H), 3.37-3.22 (m, 2H),

2.10-2.03 (m, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.09-1.01 (m, 2H), 0.97-0.87 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(2-methoxybenzyl)-3-oxopropanoate (4be). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.33 mL, 2.26 mmol, 1.20 eq.), 2-methoxybenzyl bromide[45] **3e** (378 mg, 1.88 mmol, 1.00 eq.), DIPEA (0.66 mL, 3.76 mmol, 2.00 eq.), LiCl (79.7 mg, 1.88 mmol, 1.00 eq.), 5 mL dry THF. Silica column chromatography in 8:1:1 petroleum ether:EtOAc:CH_2Cl_2. Yield: 36% (185 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (td, *J* = 6.4, 1.6 Hz, 1H), 7.13 (dd, *J* = 6.0, 1.2 Hz, 1H), 6.86-6.26 (m, 2H), 4.17-4.09 (m, 2H), 4.04 (dd, *J* = 6.8, 1.6 Hz, 1H), 3.84 (s, 3H), 3.42-3.11 (m, 2H), 2.07-2.02 (m, 1H), 1.19 (t, *J* = 6.0 Hz, 3H), 1.05-1.02 (m, 2H), 0.92-0.85 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(3-methylbenzyl)-3-oxopropanoate (4bf). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.47 mL, 3.24 mmol, 1.20 eq.), 3-methylbenzyl bromide **3f** (0.36 mL, 2.70 mmol, 1.00 eq.), DIPEA (0.94 mL, 5.40 mmol, 2.00 eq.), LiCl (114 mg, 2.70 mmol, 1.00 eq.), 5 mL dry THF. Yield: 74% (521 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (t, *J* = 7.6 Hz, 1H), 7.02-6.96 (m, 3H), 4.19-4.10 (m, 2H), 3.90 (t, *J* = 7.6 Hz, 1H), 3.15 (d, *J* = 7.6 Hz, 2H), 2.30 (s, 3H), 2.07-2.01 (m, 1H), 1.19 (t, *J* = 7.2 Hz, 3H), 1.04-0.99 (m, 2H), 0.93-0.82 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(3-fluorobenzyl)-3-oxopropanoate (4bg).[22] Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.550 mL, 3.73 mmol, 1.31 eq.), 3-fluorobenzylbromide **3g** (0.350 mL, 2.85 mmol, 1.00 eq.), DIPEA (0.940 mL, 5.39 mmol, 1.89 eq.), LiCl (0.140 g, 2.70 mmol, 0.947 eq.), 5 mL dry THF. Yield: 100% (770 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26-7.16 (m, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 6.94-6.84 (m, 2H), 4.19-4.11 (m, 2H), 3.92 (t, *J* = 7.6 Hz, 1H), 3.18 (dd, *J* = 7.6, 1.6 Hz, 2H), 2.10-2.04 (m, 1H), 1.20 (t, *J* = 7.2 Hz, 3H), 1.07-0.98 (m, 2H), 0.93 – 0.83 (m, 2H) ppm.

Ethyl 2-(3-bromobenzyl)-3-cyclopropyl-3-oxopropanoate (4bh). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.35 mL, 2.40 mmol, 1.20 eq.), 3-bromobenzyl bromide **3h** (500 mg, 2.00 mmol, 1.00 eq.), DIPEA (0.70 mL, 4.00 mmol, 2.00 eq.), LiCl (85 mg, 2.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 47% (303 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.32 (m, 2H), 7.17-7.10 (m, 2H), 4.17 (q, *J* = 7.2 Hz, 2H), 3.89 (t, *J* = 7.2 Hz, 1H), 3.15 (d, *J* = 8.0 Hz, 2H), 2.08-2.01 (m, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.11-1.01 (m, 2H), 0.98-0.86 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(3-iodobenzyl)-3-oxopropanoate (4bi). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.550 mL, 3.73 mmol, 1.38 eq.), 3-iodobenzylbromide **3i** (0.802 g, 2.70 mmol, 1.00 eq.), DIPEA (0.940 mL, 5.39 mmol, 2.00 eq.), LiCl (0.150 g, 2.70 mmol, 1.00 eq.), 5 mL dry THF. Yield: quantitative (1.28 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-7.51 (m, 2H), 7.15 (d, *J* = 7.6 Hz, 1H), 6.99 (t, *J* = 7.6 Hz, 1H), 4.16 (qd, *J* = 7.2, 1.6 Hz, 2H), 3.87 (t, *J* = 7.2 Hz, 1H), 3.11 (dd, *J* = 8.0, 2.0 Hz, 2H), 2.06-2.00 (m, 1H), 1.20 (t, *J* = 6.8 Hz, 3H), 1.11-1.00 (m, 2H), 0.98-0.81 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(3-methoxybenzyl)-3-oxopropanoate (4bj).[46] Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.44 mL, 3.00 mmol, 1.20 eq.), 3-methoxybenzyl bromide **3j** (0.35 mL, 2.50 mmol, 1.00 eq.), DIPEA (0.87 mL, 5.00 mmol, 2.00 eq.), LiCl (106 mg, 2.50 mmol, 1.00 eq.), 5 mL dry THF. Silica column chromatography in 8:1:1 petroleum ether:EtOAc:CH_2Cl_2. Yield: 42% (294 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (td, *J* = 7.2 Hz, 1.2 Hz, 1H), 6.79-6.74 (m, 3H), 4.21-4.13 (m, 2H), 3.91 (t, *J* = 7.6 Hz, 1H), 3.78 (s, 3H), 3.17 (d, *J* = 7.6 Hz, 2H), 2.08-2.02 (m, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.06-1.01 (m, 2H), 0.96-0.85 (m, 2H) ppm.

Ethyl 3-cyclopropyl-3-oxo-2-(3-(trifluoromethyl)benzyl)propanoate (4bk).[23] Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.37 mL, 2.51 mmol, 1.20 eq.), 3-(trifluoromethyl)benzyl bromide **3k** (0.32 mL, 2.09 mmol, 1.00 eq.), DIPEA (0.73 mL, 4.18 mmol, 2.00 eq.), LiCl (89 mg, 2.09 mmol, 1.00 eq.), 5 mL dry THF. Yield: 31% (214 mg) as a

colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50-7.44 (m, 2H), 7.42-7.38 (m, 2H), 4.17 (q, *J* = 6.8 Hz, 2H), 3.92 (t, *J* = 8.0 Hz, 1H), 3.25 (d, *J* = 7.2 Hz, 2H), 2.10-2.02 (m, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.12-1.01 (m, 2H), 0.99-0.86 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(4-methylbenzyl)-3-oxopropanoate (4bl). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.48 mL, 3.24 mmol, 1.20 eq.), 4-methylbenzyl bromide **3l** (500 mg, 2.70 mmol, 1.00 eq.), DIPEA (0.94 mL, 5.40 mmol, 2.00 eq.), LiCl (114 mg, 2.70 mmol, 1.00 eq.), 5 mL dry THF. Yield: 74% (521 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (s, 4H), 4.22-4.09 (m, 2H), 3.88 (t, *J* = 7.6 Hz, 1H), 3.15 (d, *J* = 7.6 Hz, 2H), 2.30 (s, 3H), 2.07-2.02 (m, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.07-1.02 (m, 2H), 0.96-0.83 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(4-fluorobenzyl)-3-oxopropanoate (4bm). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.890 mL, 6.04 mmol, 1.14 eq.), 1-(bromomethyl)-4-fluorobenzene **3m** (1.57 g, 5.29 mmol, 1.00 eq.), DIPEA (1.05 mL, 6.00 mmol, 1.13 eq.), LiCl (0.130 g, 3.00 mmol, 0.58 eq.), 5 mL dry THF. Yield: 56% (780 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18-7.12 (m, 2H), 6.96 (tt, *J* = 8.8, 2.0 Hz, 2H), 4.22-4.10 (m, 2H), 3.87 (t, *J* = 8.0 Hz, 1H), 3.16 (d, *J* = 7.6 Hz, 2H), 2.08-2.00 (m, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.09- 0.99 (m, 2H), 0.97-0.84 (m, 2H) ppm.

Ethyl 2-(4-chlorobenzyl)-3-cyclopropyl-3-oxopropanoate (4bn). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.43 mL, 2.92 mmol, 1.20 eq.), 4-chlorobenzyl bromide **3n** (500 mg, 2.43 mmol, 1.00 eq.), DIPEA (0.85 mL, 4.86 mmol, 2.00 eq.), LiCl (103 mg, 2.43 mmol, 1.00 eq.), 5 mL dry THF. Yield: 66% (451 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (dt *J* = 8.8, 2.0 Hz,, 2H), 7.13 (dt, *J* = 8.4, 2.0 Hz, 2H), 4.21-4.11 (m, 2H), 3.87 (dd, *J* = 8.0, 0.8 Hz, 1H), 3.15 (dd, *J* = 6.8, 1.2 Hz, 2H), 2.08-2.01 (m, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.09-1.01 (m, 2H), 0.97-0.86 (m, 2H) ppm.

Ethyl 2-(4-bromobenzyl)-3-cyclopropyl-3-oxopropanoate (4bo). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.880 mL, 5.97 mmol, 1.49 eq.), 1-(bromomethyl)-4-bromobenzene **3o** (1.00 g, 4.00 mmol, 1.00 eq.), DIPEA (1.39 mL, 8.00 mmol, 2.00 eq.), LiCl (0.170 g, 4.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 67% (0.880 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, *J* = 8.4 Hz, 2H), 7.07 (d, *J* = 8.4 Hz, 2H), 4.22-4.10 (m, 2H), 3.87 (t, *J* = 8.0 Hz, 1H), 3.14 (d, *J* = 8.2 Hz, 2H), 2.08-1.96 (m, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.10-0.99 (m, 2H), 0.99-0.85 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(4-methoxybenzyl)-3-oxopropanoate (4bp). Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.57 mL, 3.83 mmol, 1.20 eq.), 4-methoxybenzyl bromide **3p** (0.46 mL, 3.19 mmol, 1.00 eq.), DIPEA (1.11 mL, 6.38 mmol, 2.00 eq.), LiCl (135 mg, 3.19 mmol, 1.00 eq.), 5 mL dry THF. Silica column chromatography in 7:1:2 petroleum ether:EtOAc:CH_2Cl_2. Yield: 52% (454 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (dt, *J* = 8.8, 2.0 Hz, 2H), 6.81 (dt, *J* = 8.8, 2.4 Hz, 2H), 4.20-4.10 (m, 2H), 3.86 (t, *J* = 7.6 Hz, 1H), 3.78 (s, 3H), 3.13 (d, *J* = 7.6 Hz, 2H), 2.07-2.01 (m, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.08-1.01 (m, 2H), 0.95-0.83 (m, 2H) ppm.

Ethyl 3-cyclopropyl-2-(3,4-dichlorobenzyl)-3-oxopropanoate (4bq).[22] Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopropyl-3-oxopropanoate **1b** (0.60 mL, 4.06 mmol, 1.00 eq.), 3,4-dichlorobenzyl bromide **3q** (0.62 mL, 4.27 mmol, 1.05 eq.), DIPEA (1.42 mL, 8.13 mmol, 2.00 eq.), LiCl (172 mg, 4.06 mmol, 1.00 eq.), 5 mL dry THF. Yield: 39% (493 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.4 Hz, 1H), 7.30 (d, *J* = 1.6 Hz, 1H), 7.03 (dd, *J* = 8.4, 2.0 Hz, 1H), 4.22-4.12 (m, 2H), 3.87 (t, *J* = 7.6 Hz, 1H), 3.18-3.09 (m, 2H), 2.09-2.02 (m, 1H), 1.23 (t, *J* = 7.2 Hz, 3H), 1.09-1.01 (m, 2H), 0.95-0.85 (m, 2H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxopentanoate (4ca).[47] Synthesis according to general procedure 2. Reagents: Ethyl 3-oxopentanoate **1c** (0.42 mL, 2.92 mmol, 1.20 eq.), 3-chlorobenzyl bromide **3a** (0.32 mL, 2.43 mmol, 1.00 eq.), NaH (117 mg, 4.86 mmol, 2.00 eq.), 5 mL dry THF. Yield: 52% (340 mg) as a

colourless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.20-7.14 (m, 3H), 7.09-7.03 (m, 1H), 4.15 (qd, *J* = 7.5, 0.8 Hz, 2H), 3.76 (t, *J* = 7.5 Hz, 1H), 3.17-3.08 (m, 2H), 2.60 (dqd, *J* = 18.0, 7.0, 1.0 Hz, 1H), 2.37 (dqd, *J* = 18.5, 7.0, 0.5 Hz, 1H), 1.20 (td, *J* = 7.0, 1.0 Hz, 3H), 1.01 (td, *J* = 7.5, 1.0 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxohexanoate (4da).[22] Synthesis according to general procedure 1. Reagents: Ethyl 3-oxohexanoate **1d** (0.46 mL, 2.92 mmol, 1.20 eq.), 2.43 mmol 3-chlorobenzyl bromide **3a**, DIPEA (0.85 mmol, 4.86 mmol, 2.00 eq.), LiCl (103 mg, 2.43 mmol, 1.00 eq.), 5 mL dry THF. Yield: 76% (522 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22-7.14 (m, 3H), 7.08-7.04 (m, 1H), 4.15 (q, *J* = 7.2 Hz, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.18-3.07 (m, 2H), 2.54 (dt, *J* =17.6, 7.2 Hz, 1H), 2.35 (dt, *J* = 17.2, 7.2 Hz, 1H), 1.57 (sextet, *J* = 7.2 Hz, 2H), 1.21 (t, *J* = 7.2 Hz, 3H), 0.85 (t, *J* = 7.6 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-4-methyl-3-oxopentanoate (4ea).[22] Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (0.51 mL, 3.16 mmol, 1.20 eq.), 3-chlorobenzyl bromide **3a** (0.35 mL, 2.63 mmol, 1.00 eq.), DIPEA (0.95 mL, 5.26 mmol, 2.00 eq.), LiCl (111 mg, 2.63 mmol, 1.00 eq.), 5 mL dry THF. Yield: 86% (640 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.20-7.14 (m, 3H), 7.07-7.04 (m, 1H), 4.14 (qd, *J* = 7.2, 0.8 Hz, 2H), 3.91 (t, *J* = 7.6 Hz, 1H), 3.17-3.07 (m, 2H), 2.66 (septet, *J* = 6.8 Hz, 1H), 1.21 (t, *J* = 7.2 Hz, 3H), 1.07 (d, *J* = 6.8 Hz, 3H), 0.91 (d, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3,4-dichlorobenzyl)-4-methyl-3-oxopentanoate (4eq).[23] Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (5.00 mL, 31.0 mmol, 1.00 eq.), 3,4-dichlorobenzyl bromide **3q** (5.41 mL, 37.2 mmol, 1.20 eq.), DIPEA (10.8 mL, 62.0 mmol, 2.00 eq.), LiCl (1.31 g, 30.9 mmol, 1.00 eq.), 50 mL dry THF. Yield: 33% (3.20 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.4 Hz, 1H), 7.29-7.25 (m, 1H), 7.01 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.15 (qd, *J* = 7.2, 1.6 Hz, 2H), 3.89 (t, *J* = 7.6 Hz, 1H), 3.16-3.04 (m, 2H), 2.69 (heptet, *J* = 6.8 Hz, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.07 (d, *J* = 6.4 Hz, 3H), 0.93 (d, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(2,3-dichlorobenzyl)-4-methyl-3-oxopentanoate (4er). Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (0.670 mL, 4.61 mmol, 1.00 eq.), 2,3-dichlorobenzyl bromide **3r** (1.00 g, 4.17 mmol, 0.90 eq.), DIPEA (1.45 mL, 8.34 mmol, 1.81 eq.), LiCl (180 mg, 0.90 mmol, 1.00 eq.), 5 mL dry THF. Yield: 8% (110 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.15 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.09 (t, *J* = 7.6 Hz, 1H), 4.20-4.03 (m, 3H), 3.34-3.22 (m, 2H), 2.71 (heptet, *J* = 6.8 Hz, 1H), 1.19 (t, *J* = 7.2 Hz, 3H), 1.07 (d, *J* = 6.8 Hz, 3H), 0.90 (d, *J* = 6.8 Hz, 3H) ppm.

Ethyl 2-(2,5-dichlorobenzyl)-4-methyl-3-oxopentanoate (4es). Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (0.204 mL, 1.39 mmol, 1.00 eq.), 2,5-dichlorobenzyl bromide **3s** (500 mg, 2.08 mmol, 1.5 eq.), DIPEA (0.242 mL, 1.39 mmol, 1.00 eq.), LiCl (60 mg, 1.39 mmol, 1.00 eq.), 5 mL dry THF. Yield: 71% (315 mg) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, *J* = 8.8 Hz, 1H), 7.22 (d, *J* = 2.4 Hz, 1H), 7.15 (dd, *J* = 8.4, 2.4 Hz, 1H), 4.18-4.12 (m, 2H), 4.10 (t, *J* = 7.2 Hz, 1H), 3.26-3.16(m, 2H), 2.73 (heptet, *J* = 6.8 Hz, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.93 (d, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3,5-dichlorobenzyl)-4-methyl-3-oxopentanoate (4et).[23] Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (0.480 mL, 3.30 mmol, 1.00 eq.), 3,5-dichlorobenzyl bromide **3t** (0.440 mL, 3.12 mmol, 1.00 eq.), DIPEA (1.05 mL, 6.00 mmol, 1.82 eq.), LiCl (0.310 g, 3.00 mmol, 0.91 eq.), 5 mL dry THF. Yield: 16% (150 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (t, *J* = 2.0 Hz, 1H), 6.99 (d, *J* = 2.0 Hz, 2H), 4.08 (q, *J* = 7.2 Hz, 2H), 3.81 (t, *J* = 7.6 Hz, 1H), 3.07-2.97 (m, 2H), 2.63 (heptet, *J* = 6.8 Hz, 1H), 1.15 (t, *J* = 7.2 Hz, 3H), 1.00 (d, *J* = 6.8 Hz, 3H), 0.87 (d, *J* = 6.8 Hz, 3H) ppm.

Ethyl 2-(3,5-dibromobenzyl)-4-methyl-3-oxopentanoate (4eu). Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (0.480 mL, 3.30 mmol, 1.00 eq.), 3,5-dibromobenzyl bromide **3u** (1.04 g, 3.16 mmol, 0.958 eq.), DIPEA (1.05 mL, 6.00 mmol, 1.82 eq.), LiCl (0.130 g, 3.00 mmol, 0.909 eq.), 5 mL dry. THF. Yield: 29% (0.350 g) as a yellow oil. ^{1}H NMR (400 MHz,

$CDCl_3$) δ 7.27 (s, 3H), 4.15 (q, *J* = 7.2 Hz, 2H), 3.87 (t, *J* = 7.2 Hz, 1H), 3.13-3.05 (m, 2H), 2.70 (heptet, *J* = 6.8 Hz, 1H), 1.24 (t, *J* = 6.4 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.95 (d, *J* = 6.8 Hz, 3H) ppm.

Ethyl 2-(3-bromo-4-chlorobenzyl)-4-methyl-3-oxopentanoate (4ev). Synthesis according to general procedure 2. Reagents: Ethyl 4-methyl-3-oxopentanoate **1e** (0.480 mL, 3.30 mmol, 1.00 eq.), 3-bromo-4-chlorobenzyl bromide **3v** (0.900 g, 3.16 mmol, 0.958 eq.), DIPEA (1.05 mL, 6.00 mmol, 1.82 eq.), LiCl (0.130 g, 3.00 mmol, 0.909 eq.), 5 mL dry THF. Yield: 34% (0.360 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 2.0 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 1H), 7.06 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.15 (qd, *J* = 7.2, 1.6 Hz, 2H), 3.88 (t, *J* = 7.6 Hz, 1H), 3.14-3.04 (m, 2H), 2.69 (heptet, *J* = 6.8 Hz, 1H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxoheptanoate (4fa). Synthesis according to general procedure 2. Reagents: Ethyl 3-oxoheptanoate **1f** (0.47 g, 2.72 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (0.33 mL, 2.51 mmol, 0.922 eq.), DIPEA (1.05 mL, 6.00 mmol, 2.21 eq.), LiCl (0.130 g, 3.00 mmol, 1.10 eq.), 5 mL dry THF. Yield: 67% (0.550 g) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20-7.15 (m, 3H), 7.07-7.03 (m, 1H), 7.17-4.10 (m, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.16-3.06 (m, 2H), 2.55 (dt, *J* = 17.2, 7.2 Hz, 1H), 2.35 (dt, *J* = 17.6, 7.2 Hz, 1H), 1.50 (pentet, *J* = 7.6 Hz, 2H), 1.28-1.19 (m, 5H), 0.85 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-5-ethyl-3-oxoheptanoate (4ga). Synthesis according to general procedure 2. Reagents: Ethyl 5-ethyl-3-oxoheptanoate **1g** (0.600 g, 3.00 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (0.470 mL, 3.57 mmol, 1.19 eq.), DIPEA (1.05 mL, 6.00 mmol, 2.00 eq.), LiCl (0.130 g, 3.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 31% (0.300 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20-7.14 (m, 3H), 7.06 -7.05 (m, 1H), 4.18-4.12 (m, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.17-3.06 (m, 2H), 2.45 (dd, *J* =17.2, 6.8 Hz, 1H), 2.29 (dd, *J* = 17.2, 6.0 Hz, 1H), 1.76 (heptet, *J* = 6.4 Hz, 1H), 1.30-1.17 (m, 7H), 0.78 (t, *J* = 7.6 Hz, 6H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxooctanoate (4ha). Synthesis according to general procedure 2. Reagents: Ethyl 3-oxooctanoate **1h** (0.560 g, 3.01 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (0.470 mL, 3.57 mmol, 1.18 eq.), DIPEA (1.05 mL, 6.00 mmol, 1.99 eq.), LiCl (0.130 g, 3.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 21% (0.197 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.22-7.15 (m, 3H), 7.07-7.04 (m, 1H), 4.15 (q, *J* = 7.2 Hz, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.17-3.07 (m, 2H), 2.54 (dt, *J* = 17.2, 7.2 Hz, 1H), 2.36 (dt, *J* = 17.2, 7.2 Hz, 1H), 1.52 (pentet, *J* = 7.4 Hz, 2H), 1.31-1.13 (m, 7H), 0.86 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-cyclopentyl-3-oxopropanoate (4ia).[22] Synthesis according to general procedure 2. Reagents: Ethyl 3-cyclopentyl-3-oxopropanoate **1i** (0.376 g, 2.04 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (0.240 mL, 1.83 mmol, 0.888 eq.), DIPEA (0.530 mL, 3.03 mmol, 1.47 eq.), LiCl (0.067 g, 1.57 mmol, 0.772 eq.), 5 mL dry THF. Silica column chromatography (20-50% CH_2Cl_2/ Petroleum ether). Yield: 85% (0.483 g) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20-7.15 (m, 3H), 7.08-7.02 (m, 1H), 4.13 (q, *J* = 7.2 Hz, 2H), 3.85 (t, *J* = 7.6 Hz, 1H), 3.15-3.05 (m, 2H), 2.94 (pentet, *J* = 8.0 Hz, 1H), 1.87-1.35 (m, 8H), 1.20 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxononanoate (4ja). Synthesis according to general procedure 2. Reagents: Ethyl 3-oxononanoate **1j** (0.601 g, 3.00 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (0.325 mL, 2.48 mmol, 0.825 eq.), DIPEA (1.05 mL, 6.00 mmol, 2.00 eq.), LiCl (0.130 g, 3.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 97% (0.780 g) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20-7.15 (m, 3H), 7.07-7.01 (m, 1H), 4.14 (q, *J* = 6.8 Hz, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.17-3.06 (m, 2H), 2.53 (dt, *J* =17.6, 7.2 Hz, 1H), 2.35 (dt, *J* = 17.6, 7.2 Hz, 1H), 1.50 (pentet, *J* = 7.2 Hz, 2H), 1.30-1.15 (m, 9H), 0.85 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxodecanoate (4ka). Synthesis according to general procedure 2. Reagents: Ethyl 3-oxodecanoate **1k** (0.650 g, 3.03 mmol, 1.00 eq.), 3-chlorobenzylbromide **3a** (0.475 mL, 3.60 mmol, 1.20 eq.), DIPEA (1.05 mL, 6.00 mmol, 2.00 eq.), LiCl (0.128 g, 3.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 50% (0.511 g) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21-7.16 (m, 3H),

7.07-7.04 (m, 1H), 4.15 (q, *J* = 7.2 Hz, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.18 -3.06 (m, 2H), 2.54 (dt, *J* = 17.6, 7.2 Hz, 1H), 2.36 (dt, *J* = 17.6, 7.6 Hz, 1H), 1.51 (pentet, *J* = 6.8 Hz, 2H), 1.31-1.17 (m, 11H), 0.87 (t, *J* = 6.8 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxo-3-phenylpropanoate (4la).[22] Synthesis according to general procedure 2. Reagents: Ethyl 3-oxo-3-phenylpropanoate **1l** (0.498 mL, 2.92 mmol, 1.20 eq.), 3-chlorobenzyl bromide **3a** (0.320 mL, 2.43 mmol, 1.00 eq.), DIPEA (0.847 mL, 4.86, 2.00 eq.), LiCl (103 mg; 2.43 mmol, 1.00 eq.), 5 mL dry THF. Yield: 85% (686 mg) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 (dd, *J* = 8.0, 0.8, 2H), 7.46 (td, *J* = 7.6, 1.2 Hz, 1H), 7.35 (t, *J* = 7.6 Hz, 2H), 7.20 (s, 1H), 7.10-7.05 (m, 3H), 4.62 (t, *J* = 7.2 Hz, 1H), 4.05-3.95 (m, 2H), 3.31-3.20 (m, 2H), 1.01 (t, *J* = 7.2, 0.8 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxo-3-(*p*-tolyl)propanoate (4ma). Synthesis according to general procedure 2. Reagents: Ethyl 3-oxo-3-(*p*-tolyl)propanoate **1m** (2.30 g, 11.15 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (2.29 mL, 11.15 mmol, 1.00 eq.), DIPEA (3.88 mL, 22.3 mmol, 2.00 eq.), LiCl (473 mg; 11.15 mmol, 1.00 eq.), 30 mL dry THF. Yield: 86% (3.18 g) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (d, *J* = 8.0 Hz, 2H), 7.28-7.22 (m, 3H), 7.19-7.14 (m, 2H), 7.12-7.09 (m, 1H), 4.57 (t, *J* = 7.6 Hz, 1H), 4.13-4.05 (m, 2H), 3.33-3.23 (m, 2H), 2.41 (s, 3H), 1.12 (t, *J* = 7.2 Hz, 3H) ppm.

Ethyl 2-(3-chlorobenzyl)-3-oxo-5-phenylpentanoate (4na). Synthesis according to general procedure 2. Reagents: Ethyl 3-oxo-5-phenylpentanoate **1n** (0.661 g, 3.00 mmol, 1.00 eq.), 3-chlorobenzyl bromide **3a** (0.475 mL, 3.65 mmol, 1.20 eq.), DIPEA (1.05 mL, 6.00 mmol, 2.00 eq.), LiCl (0.128 g, 3.00 mmol, 1.00 eq.), 5 mL dry THF. Yield: 67% (0.690 g) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.26 (m, 2H), 7.24-7.19 (m, 3H), 7.16 (t, *J* = 7.2 Hz, 3H), 4.13 (q, *J* = 7.2 Hz, 2H), 3.75 (t, *J* = 7.6 Hz, 1H), 3.13 (d, *J* = 7.6 Hz, 2H), 2.99-2.85 (m, 3H), 2.75 -2.67 (m, 1H), 1.20 (t, *J* = 7.2 Hz, 3H) ppm.

Procedure for the synthesis of 6-(3-Chlorobenzyl)-5-cyclopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (6).[22, 23] In a sealed microwave tube 3-amino-1,2,4-triazole **5a** (66 mg, 0.78 mmol, 1.1 eq.), **4ba** (200 mg, 0.71 mmol, 1.00 eq.) and H_3PO_4 (96 µl, 1.42 mmol, 2.00 eq.) were heated at 170°C in 1 mL of EtOH in the microwave for 10 hours. The reaction mixture was poured in water (30mL), the pH was adjusted to pH=12 (1M NaOH aq.) and the organics were extracted with EtOAc (3x 30 mL). The combined extracts were dried over $MgSO_4$ and the solvents evaporated in vacuo, resulting in 165 mg crude mixture. The pure product was obtained by column chromatography (5% CH_3OH in CH_2Cl_2) followed by prep HPLC gradient 10-90% CH_3CN/water+0.1%TFA yielding 4% (9 mg) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (s, 1H), 7.28-7.25 (m, 1H), 7.22-7.15 (m, 3H), 7.17-7.08 (m, 3H), 4.16 (s, 2H), 2.22-2.13 (m, 1H), 1.36-1.30 (m, 2H), 1.20-1.15 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{13}ClN_4O$ $[M+H]^+$ 301.09, found 301.1. Purity by HPLC: 97%.

Procedure for the synthesis of 6-(3-Chlorobenzyl)-5-cyclopropyl-2-methyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (7). In a sealed microwave tube 3-amino-5-methyl-1,2,4-triazole **5b** (126 mg, 1.28 mmol, 1.2 eq.), **4ba** (300 mg, 1.07 mmol, 1.0 eq.) and p-toluenesulfonic acid monohydrate (102 mg, 0.53 mmol, 0.5 eq.) were heated for 30 minutes at 180°C in the microwave. As visualised by TLC **4ba** was consumed and mainly one product was formed (Rf 0.5 in 5% CH_3OH in CH_2Cl_2). The crude product was purified by column chromatography (3% CH_3OH in CH_2Cl_2) yielding 31% (96 mg) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$ + drop MeOD): δ 7.23 (s, 1H), 7.21-7.13 (m, 3H), 6.26 (br s, 2H), 4.11 (s, 2H), 2.47 (s, 2H), 2.05-1.96 (m, 1H), 1.14-1.07 (m, 2H), 1.05-1.01 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{15}ClN_4O$ $[M+H]^+$ 315.10, found 315.1. Purity by HPLC: 96%.

General procedure 3. Triazolo-pyrimidinones 8-43.[22]

The synthesis of compounds **8-43** was according to the following procedure: In a microwave tube a mixture of the corresponding benzylated beta keto ester **4aa-na, 4bb-bq, 4eq-ev** (1.00 eq.), triazole **5c** (2.00 eq.) and 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM-PF6, 1 mL or 6.00 eq.) was heated at 200 °C in a microwave reactor for an hour. Afterwards, the reaction was allowed to cool to room temperature and stirred in a mixture of CH_2Cl_2 (30 mL), H_2O (10 mL) and 5 - 10% aqueous citric acid (1 mL) for 20 - 30 min. The resulting mixture was filtered over a glass filter and the residue was washed with hot methanol. Finally, the precipitate was collected and dried *in vacuo* to yield the pure compounds.

2-Amino-6-(3-chlorobenzyl)-5-cyclopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (8).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (216 mg, 2.18 mmol, 2.00 eq.), **4ba** (306 mg, 1.09 mmol, 1.00 eq.) and BMIM-PF6 (1.35 mL, 6.54 mmol, 6.00 eq.). Yield: 36% (125 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.16 (br s, 1H), 7.35-7.24 (m,2H), 7.24-7.14 (m, 2H), 6.24 (br s, 2H), 3.96 (s, 2H), 2.14-2.01 (m, 1H), 1.07-0.94 (m, 2H), 0.94-0.81 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}ClN_5O$ $[M+H]^+$ 316.10, found 316.13. Purity by HPLC: 98%.

2-Amino-6-benzyl-5-cyclopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (9). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (276 mg, 2.84 mmol, 2.00 eq.), **4bb** (350 mg, 1.42 mmol, 1.00 eq.) and BMIM-PF6 (1.75 mL, 8.52 mmol, 6.00 eq.). Yield: 26% (105 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.11 (br s, 1H), 7.26-7.20 (m, 4H), 7.17-7.10 (m, 1H), 6.20 (br s, 2H), 3.96 (s, 2H), 2.10-2.03 (m, 1H), 1.04-0.95 (m, 2H), 0.94-0.88 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}N_5O$ $[M+H]^+$ 282.14, found 282.13. Purity by HPLC: 97%.

2-Amino-5-cyclopropyl-6-(2-methylbenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (10). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (190 mg, 1.96 mmol, 2.00 eq.), **4bc** (255 mg, 0.98 mmol, 1.00 eq.) and BMIM-PF6 (1.21 mL, 5.88 mmol, 6.00 eq.). Yield: 32% (93 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.17 (br s, 1H), 7.15 (d, *J* = 6.8 Hz, 1H), 7.08-6.99 (m, 2H), 6.81 (d, *J* = 6.8 Hz, 1H), 6.21 (br s, 2H), 3.86 (s, 2H), 2.36 (s, 3H), 1.90-1.81 (m, 1H), 1.04-0.94 (m, 2H), 0.88-0.81 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{17}N_5O$ $[M+H]^+$ 296.15, found 296.13. Purity by HPLC: 98%.

2-Amino-6-(2-chlorobenzyl)-5-cyclopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (11). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (173 mg, 1.78 mmol, 2.00 eq.), **4bd** (250 mg, 0.89 mmol, 1.00 eq.) and BMIM-PF6 (1.01 mL, 5.34 mmol, 6.00 eq.). Yield: 37% (104 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.14 (br s, 1H), 7.47-7.42 (m, 1H), 7.24-7.17 (m, 2H), 7.01-6.96 (m, 1H), 6.20 (br s, 2H), 4.00 (s, 2H), 1.90-1.80 (m, 1H), 0.99-0.93 (m, 2H), 0.89-0.80 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}ClN_5O$ $[M+H]^+$ 316.10, found 316.13. Purity by HPLC: 95%.

2-Amino-5-cyclopropyl-6-(2-methoxybenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (12). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (130 mg, 1.34 mmol, 2.00 eq.), **4be** (185 mg, 0.67 mmol, 1.00 eq.) and BMIM-PF6 (0.83 mL, 4.02 mmol, 6.00 eq.). Yield: 43% (89 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.11 (br s, 1H), 7.15 (t, *J* = 6.0 Hz, 1H), 6.95 (d, *J* = 6.4 Hz, 1H), 6.86 (d, *J* = 5.6 Hz, 1H), 6.78 (t, *J* = 5.6 Hz, 1H), 6.18 (br s, 2H), 3.86 (s, 2H), 3.83 (s, 3H), 1.98-1.86 (m, 1H), 1.03-0.91 (m, 2H), 0.90-0.79 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{17}N_5O2$ $[M+H]^+$ 312.15, found 312.2. Purity by HPLC: 98%.

2-Amino-5-cyclopropyl-6-(3-methylbenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (13). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (216 mg, 2.22 mmol, 2.00 eq.), **4bf** (289 mg, 1.11 mmol, 1.00 eq.) and BMIM-PF6 (1.37 mL, 6.66 mmol, 6.00 eq.). Yield: 35% (114 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 11.98 (br s, 1H), 7.12 (t, *J* = 7.6 Hz, 1H), 7.04 (s, 1H), 7.01 (d, *J* = 7.2 Hz, 1H), 6.95 (d, *J* = 7.2 Hz, 1H), 6.17 (br s, 2H), 3.91 (s, 2H), 2.23 (s, 3H), 2.10-2.00 (m, 1H), 1.04-0.94 (m, 2H), 0.94-0.83 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{17}N_5O$ $[M+H]^+$ 296.15, found 296.13. Purity by HPLC: 97%

2-Amino-5-cyclopropyl-6-(3-fluorobenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (14). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (190 mg, 1.92 mmol, 1.45 eq.), **4bg** (350 mg, 1.32 mmol, 1.00 eq.) and BMIM-PF6 (1.14 mL, 5.54 mmol, 4.19 eq.). Yield: 15% (61mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 7.40-7.20 (m, 1H), 7.16-6.85 (m, 3H), 5.81 (br s, 2H), 4.01 (s, 2H), 2.12-1.95 (m, 1H), 1.10-0.85 (m, 4H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}FN_5O$ $[M+H]^+$ 300.13, found 300.1. Purity by HPLC 97%.

2-Amino-6-(3-bromobenzyl)-5-cyclopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (15). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (169 mg, 1.74 mmol, 2.00 eq.), **4bh** (283 mg, 0.87 mmol, 1.00 eq.) and BMIM-PF6 (1.07 mL, 5.22 mmol, 6.00 eq.). Yield: 43% (135 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.21 (br s, 1H), 7.43 (s, 1H), 7.34 (d, *J* = 7.2 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 1H), 7.20 (t, *J* = 7.6 Hz, 1H), 6.26 (br s, 2H), 3.96 (s, 2H), 2.12-2.04 (m, 1H), 1.04-0.94 (m, 2H), 0.94-0.85 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}BrN_5O$ $[M+H]^+$ 360.05, found 360.2. Purity by HPLC: 99%.

2-Amino-5-cyclopropyl-6-(3-iodobenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (16). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (190 mg, 1.92 mmol, 2.32 eq.), **4bi** (320 mg, 0.86 mmol, 1.00 eq.) and BMIM-PF6 (1.14 mL, 4.25 mmol, 6.00 eq.). Yield: 26% (92 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.18 (br s, 1H), 7.62 (s, 4H), 7.51 (d, *J* = 7.6 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 1H), 7.05 (t, *J* = 7.6 Hz, 1H), 6.22 (br s, 2H), 3.92 (s, 2H), 2.12-2.07 (m, 1H), 1.01-0.94 (m, 2H), 0.93-0.86 (m, 2H). LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}IN_5O$ $[M+H]^+$ 408.03, found 408.1. Purity by HPLC: 96%.

2-Amino-5-cyclopropyl-6-(3-methoxybenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (17). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (146 mg, 1.50 mmol, 2.00 eq.), **4bj** (207 mg, 0.75 mmol, 1.00 eq.), BMIM-PF6 (0.93 mL, 4.50 mmol, 6.00 eq.). Yield: 21% (48 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 11.94 (br s, 1H), 7.15 (t, *J* = 8.0 Hz, 1H), 6.82-6.77 (m, 2H), 6.72 (d, *J* = 7.6 Hz, 1H), 6.16 (br s, 2H), 3.93 (s, 2H), 3.69 (s, 3H), 2.11-2.03 (m, 1H), 1.04-0.96 (m, 2H), 0.96-0.85 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{17}N_5O_2$ $[M+H]^+$ 312.15, found 312.13. Purity by HPLC: 96%.

2-Amino-5-cyclopropyl-6-(3-(trifluoromethyl)benzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (18). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (150 mg, 1.54 mmol, 2.00 eq.), **4bk** (242 mg, 0.77 mmol, 1.00 eq.) and BMIM-PF6 (0.95 mL, 4.62 mmol, 6.00 eq.). Yield 6% (17 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.18 (br s, 1H), 7.61 (s, 1H), 7.57-7.46 (m, 3H), 6.24 (br s, 2H), 4.06 (s, 2H), 2.17-2.08 (m, 1H), 1.03-0.95 (m, 2H), 0.94-0.86 (m, 2H) ppm.LC-MS (ESI) *m/z* calcd for $C_{16}H_{14}F_3N_5O$ $[M+H]^+$ 350.13, found 350.27. Purity by HPLC: 97%.

2-Amino-5-cyclopropyl-6-(4-methylbenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (19). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (197 mg, 2.03 mmol, 2.00 eq.), **4bl** (266 mg, 1.02 mmol, 1.00 eq.) and BMIM-PF6 (1.25 mL, 6.09 mmol, 6.00 eq.). Yield: 39% (115 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.06 (br s, 1H), 7.10 (d, *J* = 8.0 Hz, 2H), 7.03 (d, *J* = 8.0 Hz, 2H), 6.18 (br s, 2H), 3.90 (s, 2H), 2.23 (s, 3H), 2.08-2.01 (m, 1H), 1.01-0.94 (m, 2H), 0.94-0.87 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{17}N_5O$ $[M+H]^+$ 296.15, found 296.13. Purity by HPLC: 97%.

2-Amino-5-cyclopropyl-6-(4-fluorobenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (20). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (150 mg, 1.51 mmol, 2.00 eq.), **4bm** (200 mg, 0.756 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 6.39 eq.). Yield: 35% (79 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.13 (s, 1H), 7.28 (dd, *J* = 9.6, 2.4 Hz, 2H), 7.07 (t, *J* = 8.8 Hz, 2H), 6.21 (s br, 2H), 3.95 (s, 2H), 2.15-2.02 (m, 1H), 1.05-0.85 (m, 4H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}FN_5O$ $[M+H]^+$ 300.13, found 300.2. Purity by HPLC: 95%.

2-Amino-6-(4-chlorobenzyl)-5-cyclopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (21). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (179 mg, 1.84 mmol, 2.00 eq.), **4bn** (258 mg, 0.92 mmol, 1.00 eq.) and BMIM-PF6 (1.14 mL, 5.52 mmol, 6.00 eq.). Yield: 33% (105 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.18 (br s, 1H), 7.28 (d, *J* = 8.4 Hz, 2H), 7.25 (d, *J* = 8.4 Hz, 2H), 6.24 (br s, 2H), 3.94 (s, 2H), 2.10-2.00 (m, 1H), 1.04-0.94 (m, 2H), 0.93-0.81 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}ClN_5O$ $[M+H]^+$ 316.10, found 316.13. Purity by HPLC: 96%.

2-Amino-5-cyclopropyl-6-(4-bromobenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (22). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (120 mg, 1.21 mmol, 2.00 eq.), **4bo** (200 mg, 0.615 mmol, 1.00 eq.) and BMIM-PF6 (1,00 mL, 4.86 mmol, 7.90 eq.). Yield: 21% (47 mg) as an off-white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.15 (s, 1H), 7.42 (d, *J* = 8.4 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 2H), 6.24 (br s, 2H), 3.92 (s, 2H), 2.10-2.00 (m, 1H), 1.08-0.75 (m, 4H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{14}BrN_5O$ $[M+H]^+$ 360.05, found 360.1. Purity by HPLC: 96%.

2-amino-5-cyclopropyl-6-(4-methoxybenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (23). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (165 mg, 1.70 mmol, 2.00 eq.), **4bp** (235 mg, 0.85 mmol, 1.00 eq.) and BMIM-PF6 (1.05 mL, 5.10 mmol, 6.00 eq.). Yield: 41% (109 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.07 (br s, 1H), 7.15 (d, *J* = 8.4 Hz, 2H), 6.80 (d, *J* = 8.8 Hz, 2H), 6.19 (br s, 2H), 3.88 (s, 2H), 3.69 (s, 3H), 2.12-2.01 (m, 1H), 1.04-0.95 (m, 2H), 0.94-0.86 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{17}N_5O_2$ $[M+H]^+$ 312.15, found 312.07. Purity by HPLC: 96%.

2-Amino-6-(3-chlorobenzyl)-5-methyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (24).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (94 mg, 0.95 mmol, 2.00 eq.), **4aa** (120 mg, 0.47 mmol, 1.00 eq.) and BMIM-PF6 (0.58 mL, 2.82 mmol, 6.00 eq.). Yield: 57% (77 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): **δ** 12.56 (s, 1H), 7.30-7.21 (m, 3H), 7.17 (d, *J* = 7.2 Hz, 1H), 5.99 (s, 2H), 3.78 (s, 2H), 2.25 (s, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{13}H_{12}ClN_5O$ $[M+H]^+$ 290.08, found 290.1. Purity by HPLC: 99%.

2-Amino-6-(3-chlorobenzyl)-5-ethyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (25).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (141 mg, 1.43 mmol, 2.00 eq.), **4ca** (192 mg, 0.71 mmol, 1.00 eq.) and BMIM-PF6 (0.88 mL, 4.28 mmol, 6.00 eq.). Yield: 21% (46 mg) as a white solid. ^{1}H NMR (500 MHz, DMSO): **δ** 12.57 (s, 1H) 7.29-7.51 (m, 2H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.16 (d, *J* = 7.5 Hz, 1H), 6.02 (s, 2H), 3.81 (s, 2H), 2.57 (q, *J* = 7.5 Hz, 2H), 1.05 (t, J= 7.5 Hz, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{14}H_{14}ClN_5O$ $[M+H]^+$ 304.10, found 304.2. Purity by HPLC: 95%.

2-Amino-6-(3-chlorobenzyl)-5-propyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (26).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (210 mg, 2.12 mmol, 2.00 eq.), **4da** (300 mg, 1.06 mmol, 1.00 eq.) and BMIM-PF6 (1.31 mL, 6.36 mmol, 6.00 eq.). Yield: 22% (76 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.56 (s, 1H), 7.29-7.20 (m, 3H), 7.16 (d, *J* = 7.6 Hz, 1H), 6.01 (s, 2H), 3.81 (s, 2H), 2.53 (t, *J* = 7.6 Hz, 2H), 1.45 (sextet, *J* = 7.6 Hz, 2H), 0.85 (t, *J* = 7.2 Hz, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{16}ClN_5O$ $[M+H]^+$ 318.11, found 318.2. Purity by HPLC: 99%.

2-Amino-6-(3-chlorobenzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (27).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (284 mg, 2.87 mmol, 2.00 eq.), **1ea** (407 mg, 1.44 mmol, 1.00 eq.) and BMIM-PF6 (1.76 mL, 8.63 mmol, 6.00 eq.). Yield: 21% (96 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.33 (s, 1H), 7.28 (t, *J* = 8.0 Hz, 1H),

7.35 (s, 1H), 7.21 (d, *J* = 8.0 Hz, 1H), 7.14 (d, *J* = 7.6 Hz, 1H), 6.04 (s, 2H) 3.89 (s, 2H), 3.18 (septet, J= 6.8 Hz, 1H), 1.12 (d, J= 6.8 Hz, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{16}ClN_5O$ $[M+H]^+$ 318.11, found 318.2. Purity by HPLC: 99%.

2-Amino-5-butyl-6-(3-chlorobenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (28).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (250 mg, 1.51 mmol, 2.14 eq.), **1fa** (210 mg, 0.708 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 6.86 eq.). Yield: 20% (47 mg) as a white solid. Purity: 98%. ^{1}H NMR (400 MHz, DMSO) δ 12.54 (s, 1H), 7.33-7.19 (m, 3H), 7.16 (d, *J* = 7.3 Hz, 1H), 6.02 (s, 2H), 3.82 (s, 2H), 2.60-2.50 (m, 2H), 1.45-1.32 (m, 2H), 1.32-1.20 (m, 2H), 0.81 (t, *J* = 7.1 Hz, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{16}H_{18}ClN_5O$ $[M+H]^+$ 332.13, found 332.3. Purity by HPLC: 98%.

2-Amino-6-(3-chlorobenzyl)-5-(2-ethylbutyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (29). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (130 mg, 1.31 mmol, 2.13 eq.), **4ga** (200 mg, 0.616 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 7.89 eq.) Yield: 9% (20 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.49 (br s, 1H), 7.28 (t, *J* = 7.2 Hz, 1H), 7.24-7.18 (m, 2H), 7.14 (d, *J* = 7.2 Hz, 1H), 5.91 (br s, 2H), 3.82 (s, 2H), 1.58 (s, 2H), 1.30-1.15 (m, 5H), 0.77 (t, *J* = 6.8 Hz, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{18}H_{22}ClN_5O$ $[M+H]^+$ 360.16, found 360.3. Purity by HPLC: 97%.

2-Amino-6-(3-chlorobenzyl)-5-pentyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (30). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (120 mg, 1.21 mmol, 1.88 eq.), **4ha** (200 mg, 0.643 mmol; 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 7.56 eq.). Yield: 24% (52 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.43 (br s, 1H), 7.35-7.10 (m, 4H), 6.02 (br s, 2H), 3.82 (s, 2H), 1.45-1.15 (m, 6H), 0.79 (s, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{17}H_{20}ClN_5O$ $[M+H]^+$ 346.15, found 346.2. Purity by HPLC: 98%.

2-Amino-6-(3-chlorobenzyl)-5-cyclopentyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (31). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (130 mg, 1.31 mmol, 2.00 eq.), **4ia** (200 mg, 0.647 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 7.51 eq.). Yield: 38% (86 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.36 (br s, 1H), 7.38-7.07 (m, 4H), 6.07 (br s, 2H), 3.90 (s, 2H), 3.23 (m, 1H), 1.90-1.45 (m, 8H) ppm. LC-MS (ESI) *m/z* calcd for $C_{17}H_{18}ClN_5O$ $[M+H]^+$ 344.13, found 344.1. Purity by HPLC: 99%.

2-Amino-6-(3-chlorobenzyl)-5-hexyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (32). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (86 mg, 0.86 mmol, 2.00 eq.), **4ja** (140 mg, 0.431 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 11.3 eq.). Yield: 83% (128 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.50 (s, 1H), 7.33-7.19 (m, 3H), 7.15 (d, *J* = 6.8 Hz, 1H), 6.02 (s, 2H), 3.82 (s, 2H), 2.60-2.40 (m, 2H), 1.44-1.32 (m, 2H), 1.30-1.08 (m, 6H), 0.82 (t, *J* = 6.5 Hz, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{18}H_{22}ClN_5O$ $[M+H]^+$ 360.16, found 360.3. Purity by HPLC: 97%.

2-Amino-6-(3-chlorobenzyl)-5-heptyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (33). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (116 mg, 1.18 mmol, 2.00 eq.), **4ka** (200 mg, 0.591 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 8.22 eq.). Yield: 50% (108 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.55 (s, 1H), 7.30-7.19 (m, 3H), 7.15 (d, *J* = 6.4 Hz, 1H), 6.01 (s, 1H), 3.82 (s, 2H), 1.46-1.32 (m, 2H), 1.27-1.10 (m, 8H), 0.83 (t, *J* = 6.4 Hz, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{19}H_{24}ClN_5O$ $[M+H]^+$ 374.18, found 374.3. Purity by HPLC: 98%.

2-Amino-6-(3-chlorobenzyl)-5-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (34). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (177 mg, 1.79 mmol, 2.00 eq.), **4la** (296 mg, 0.89 mmol, 1.00 eq.) and BMIM-PF6 (1.11 mL, 5.36 mmol, 6.00 eq.). Yield: 30% (95 mg) as a white solid. ^{1}H NMR (500 MHz, DMSO): δ 12.83 (s, 1H), 7.53-7.47 (m, 3H), 7.42 (dd, *J* = 8.0, 1.5 Hz, 2H), 7.20 (t, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 7.0 Hz, 1H), 7.03 (s, 1H), 6.94 (d, *J* = 7.0 Hz, 1H), 6.12 (s, 2H), 3.64 (s, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{18}H_{14}ClN_5O$ $[M+H]^+$ 352.10, found 352.2.

Purity by HPLC: 99%.

2-Amino-6-(3-chlorobenzyl)-5-(p-tolyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (35). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (181 mg, 1.82 mmol, 2.00 eq.), **4ma** (314 mg, 0.937 mmol, 1.00 eq.) and BMIM-PF6 (1.5 mL, 7.28 mmol, 7.77 eq.). Yield: 33% (108 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.80 (s, 1H), 7.33-7.28 (m, 4H), 7.26-7.14 (m, 2H), 7.05 (s, 1H), 6.96 (d, *J* = 7.1 Hz, 1H), 6.11 (s, 2H), 3.64 (s, 2H), 2.36 (s, 3H) ppm. LC-MS (ESI) *m/z* calcd for $C_{19}H_{16}ClN_5O$ $[M+H]^+$ 366.20, found 366.2. Purity by HPLC: 98%.

Procedure for the synthesis of 2-Amino-6-(3-chlorobenzyl)-5-phenethyl-[1,2,4]triazolo[1,5-a] pyrimidin-7(4H)-one (36). A mixture of **4na** (213 mg, 0.57 mmol, 1.00 eq.), 3,5-diamino-4*H*-1,2,4-triazole **5c** (116 mg, 1.16 mmol, 2.00 eq.) and ortho-phosphoric acid 85% (59.6 µl, 0.906 mmol,1.58 eq.) in EtOH (1 mL) was stirred for 1 minute at 20°C and then heated at 175°C under microwave irradiation for 3 hours. The reaction mixture was allowed to cool to room temperature and CH_2Cl_2 (30 mL), water (10 mL) and aqueous citric acid (5%, 1 mL) was added. The resulting precipitate was stirred for 20 min, filtered and the residue was washed with hot methanol, collected and dried in vacuo to afford the title compound. Yield: 22% (49 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.67 (s, 1H), 7.32-7.18 (m, 6H), 7.17-7.10 (m, 3H), 6.06 (br s, 2H), 3.76 (s, 2H), 2.84-2.78 (m, 2H), 2.70-2.66 (m, 2H) ppm. LC-MS (ESI) *m/z* calcd for $C_{20}H_{18}ClN_5O$ $[M+H]^+$ 380.13, found 380.2. Purity by HPLC: 97%.

2-Amino-5-cyclopropyl-6-(3,4-dichlorobenzyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (37).[22] Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (157 mg, 1.58 mmol, 2.00 eq.), **4bq** (250 mg, 0.79 mmol, 1.00 eq.) and BMIM-PF6 (1.3 mL, 4.74 mmol, 6.00 eq.). Yield: 31% (86 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.26 (br s, 1H), 7.52-7.47 (m, 2H), 7.22 (d, *J* = 8.4 Hz, 2H), 6.26 (br s, 2H), 3.95 (s, 2H), 2.12-2.01 (m, 1H), 1.01-0.85 (m, 4H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{13}Cl_2N_5O$ $[M+H]^+$ 350.06, found 350.1. Purity by HPLC: 96%.

2-Amino-6-(3,4-dichlorobenzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (38). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (125 mg, 1.26 mmol, 2.00 eq.), **4eq** (200 mg, 0.63 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 7.71 eq.). Yield: 34% (76 mg), as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.23 (br s, 1H), 7.54-7.43 (m, 2H), 7.17 (d, *J* = 8.0 Hz, 1H), 6.09 (s, 2H), 3.88 (s, 2H), 3.22-3.13 (m, 1H), 1.12 (d, *J* = 6.4 Hz, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}Cl_2N_5O$ $[M+H]^+$ 352.08, found 352.3. Purity by HPLC: 97%.

2-Amino-6-(2,3-dichlorobenzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (39). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (125 mg, 1.26 mmol, 2.00 eq.), **4er** (200 mg, 0.630 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 7.71 eq.). Yield: 48% (106 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.27 (br s, 1H), 7.49 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.21 (t, *J* = 8.0 Hz, 1H), 6.96 (d, *J* = 7.2 Hz, 1H), 3.93 (s, 2H), 2.93 (septet, *J* = 6.8 Hz, 1H), 1.14 (d, *J* = 6.8 Hz, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}Cl_2N_5O$ $[M+H]^+$ 352.08, found 352.2. Purity by HPLC: 97%.

2-Amino-6-(2,5-dichlorobenzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (40). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (125 mg, 1.26 mmol, 2.00 eq.), **4es** (200 mg, 0.630 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 7.71 eq.). Yield: 24% (54 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.41 (br s, 1H), 7.70-7.42 (m, 1H), 7.40-7.19 (m, 1H), 7.16-6.85 (m, 1H), 6.08 (br s, 2H), 3.88 (s, 2H), 3.01-2.83 (m, 1H), 1.14 (s, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}Cl_2N_5O$ $[M+H]^+$ 352.08, found 352.2. Purity by HPLC: 96%.

2-amino-6-(3,5-dichlorobenzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (41). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (96 mg, 0.97 mmol, 2 eq.), **4et** (153 mg, 0.48 mmol, 1.00 eq.) and BMIM-PF6 (1.00 mL, 4.86 mmol, 10 eq.) Yield: 18% (31 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.33 (s, 1H), 7.41 (s, 1H), 7.26 (s, 2H), 6.07 (br s, 2H), 3.89 (s, 2H), 1.13 (s, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}Cl_2N_5O$ $[M+H]^+$ 352.08, found 352.1. Purity by HPLC: 98%.

2-Amino-6-(3,5-dibromobenzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin 7(4H)-one (42). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (97 mg, 0.98 mmol, 2.00 eq.), **4eu** (200 mg, 0.49 mmol, 1.00 eq.), and BMIM-PF6 (1.00 mL, 4.86 mmol, 9.91 eq.). Yield: 7% (14 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 12.33 (br s, 1H), 7.63 (s, 1H), 7.42 (s, 2H), 6.07 (br s, 2H), 3.88 (s, 2H), 1.13 (s, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}Br_2N_5O$ $[M+H]^+$ 439.97, found 440.1. Purity by HPLC: 98%.

2-amino-6-(3-bromo-4-chloro-benzyl)-5-isopropyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one (43). Synthesis according to general procedure 3. Reagents: 3,5-diamino-4*H*-1,2,4-triazole **5c** (109 mg, 1.10 mmol, 2.00 eq.), **4ev** (200 mg, 0.553 mmol, 1.00 eq.), and BMIM-PF6 (1.00 mL, 4.86 mmol, 8.83 eq.). Yield: 24% (53 mg) as a white solid. ^{1}H NMR (400 MHz, DMSO): δ 7.60 (d, *J* = 2.0 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 1H), 7.21 (dd, *J* = 8.0, 2.0 Hz, 1H), 6.08 (br s, 2H), 3.87 (s, 2H), 3.22-3.11 (m, 1H), 1.12 (d, *J* = 7.2 Hz, 6H) ppm. LC-MS (ESI) *m/z* calcd for $C_{15}H_{15}BrClN_5O$ $[M+H]^+$ 396.02, found 396.1. Purity by HPLC: 98%.

Biology

Chemicals and reagents

The human recombinant chemokines CCL2 and CCL3 were purchased from PeproTech (Rocky Hill, NJ). TAK-779 was obtained from NIH AIDS reagent program (Germantown, MD, catalogue number 4983). All triazolo-pyrimidinone derivatives were synthesized in-house. Guanosine 5'-*O*-[gamma-thio]triphosphate ([^{35}S[GTPγS) (specific activity 1250 Ci/mmol) was purchased from PerkinElmer (Waltham, MA), while [^{3}H]-CCR2-RA-[*R*] (specific activity 59.6 Ci mmol^{-1}) was custom-labelled by Vitrax (Placentia, CA). Bovine serum albumin was purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) and Pierce™ BCA protein assay kit were purchased from Pierce Biotechnology (Thermo Scientific, Rockford, IL). Tango™ U2OS cells stably expressing human CCR2b (U2OS-CCR2) or human CCR5 (U2OS-CCR5) were purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from standard commercial sources.

Cell culture

Both U2OS-CCR2b and U2OS-CCR5 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 25 mM HEPES, 1 mM sodium pyruvate, 200 IU/mL penicillin, 200 µg/mL streptomycin, 100 µg/mL G418, 40-50 µg/mL hygromycin and 125 µg/mL zeocin. Cells were grown until 80% confluence and cultured twice-weekly on 10 or 15 cm Ø plates by trypsinization. Dialyzed fetal calf serum was used when culturing cells for functional assays or as a last step before membrane preparation.

Membrane preparation

Membranes from U2OS-CCR2 or U2OS-CCR5 cells were prepared as previously described for CCR2.[19] Briefly, U2OS-CCR2 or U2OS-CCR5 cells were scraped from confluent 15 cm Ø plates using phosphate-buffered saline (PBS) and subsequently centrifuged at 3000 rpm for 5 minutes. Pellets were then resuspended in ice-cold Tris buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) before homogenization with an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Membranes and cytosolic contents were separated using an Optima LE-80 K ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) at 31000 rpm for 20 minutes at 4 °C. After a second cycle of homogenization and centrifugation, the final pellet was resuspended and homogenized in ice-cold Tris buffer, aliquoted and stored at -80 °C. Finally, membrane protein concentrations were determined using a BCA protein determination assay, as described by the manufacturer (Pierce™ BCA protein assay kit).[48]

[^{3}H]-CCR2-RA-[*R*] binding assays

For [^{3}H]-CCR2-RA-[*R*] displacement assays, U2OS-CCR2b membrane homogenates (15 - 20 µg of total protein) were incubated with ~6 nM [^{3}H]-CCR2-RA-[*R*] and at least 6 increasing concentrations of competing ligand in a final volume of 100 µL assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1% CHAPS, pH 7.4). Ligands were diluted to the desired concentration with an HP D300 digital dispenser (Tecan, Giessen, The Netherlands). Total radioligand binding did not exceed 10% of the amount added to prevent ligand depletion, and nonspecific binding was determined using 10 µM CCR2-RA-[*R*]. After 2 hours at 25 °C, incubation was terminated by rapid filtration through a 96-well GF/B filterplate on a PerkinElmer FilterMate harvester, using ice-cold wash buffer (50 mM Tris-HCl buffer supplemented with 5 mM $MgCl_2$ and 0.05% CHAPS, pH 7.4). Filters were washed 10 times with ice-cold wash buffer, and subsequently dried at 55 °C for 30 min. After addition of 25 µl Microscint scintillation cocktail (PerkinElmer), the filter-bound radioactivity was measured by scintillation spectrometry using the P-E 2450 Microbeta^2 counter (PerkinElmer).

Tango β-arrestin recruitment assay

β-arrestin recruitment was measured using the Tango™ CCR2-*bla* or CCR5-*bla* U2OS cell-based assay (Invitrogen) according to the manufacturer's protocol. Briefly, U2OS-CCR2b or U2OS-CCR5 cells were grown until approximately 80% confluence and detached by trypsinization. Cells were recovered by centrifugation at 1000 rpm for 5 minutes before resuspending in assay medium (FreeStyle™ Expression Medium, Invitrogen) to a density

of 10,000 cells per well and seeded into black-wall, clear-bottom, 384-well assay plates (Corning). For agonist assays, cells were exposed to increasing concentrations of CCL2 or CCL3, for CCR2 or CCR5 respectively, for 16 hours at 37 °C and 5% CO_2. For antagonist assays, compounds were first diluted in assay medium containing a final dimethylsulfoxide (DMSO) concentration of 0.5% or lower. Cells were then pre-incubated with either 1 µM (for single-point inhibition experiments) or increasing concentrations of antagonist for 30 minutes at room temperature, before a 16 hour co-incubation with an EC_{80} concentration of CCL2 (5 nM) or CCL3 (14 nM) at 37 °C and 5% CO_2. After 16h cells were loaded in the dark with 8 µl of LiveBLAzer™-FRET B/G substrate (Invitrogen) and incubated for 2 hours at room temperature. Finally, fluorescence emission at 460 nm and 535 nm was measured in an EnVision multilabel plate reader (PerkinElmer) after excitation at 400 nm. The ratio of emission at 460 and 535 nm was calculated for each well.

[^{35}S]GTPγS binding assay

To determine the mechanism of inhibition [^{35}S]GTPγS binding assays were performed. In CCR2 the [^{35}S]GTPγS binding assay was performed as previously described.[18, 19] In the case of CCR5 10 µg U2OS-CCR5 membranes with 0.25 mg/mL saponin were pre-incubated with 5 µM GDP, increasing concentrations of CCL3 and three different antagonist concentrations for 30 minutes at 25 °C. All dilutions were made in assay buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA, and 0.05% BSA. [^{35}S]GTPγS (0.3 nM) was added and the mixture was co-incubated for an additional 90 minutes at 25 °C before harvesting. Incubation was stopped by dilution with ice-cold 50 mM Tris-HCl buffer with 5 mM $MgCl_2$. Separation of bound and unbound radioligand was performed by rapid filtration through a 96-well GF/B filter plate as described in "[^{3}H]-CCR2-RA-[*R*] binding assays".

Data analysis

All experiments were analyzed using non-linear regression curve fitting program Prism 7 (Graphpad, San Diego, CA). EC_{50}, EC_{80}, E_{max} and IC_{50} values from functional assays were obtained by nonlinear regression analysis. All values obtained are means ± S.E.M. of at least three separate experiments performed in duplicate, unless stated otherwise. For radioligand binding assays, K_i values were determined using the Cheng-Prussoff equation using a K_D of 6.3 nM for the radioligand.[18]

Computational modelling

The inactive crystal structure of hCCR2b with BMS-681 and CCR2-RA-[*R*] (PDB: 5T1A)[14] was used as the basis for docking compound **43**. Docking was performed in the Schrodinger suite,[49] as previously described for the docking of CCR2-RA-[*R*].[18] Before docking, the CCR2b crystal structure was prepared by replacing the residues between L226^{5x62} and R240^{6x32}, which correspond to the M2 muscarinic acetylcholine receptor, with the CCR2b sequence using prime[50-52] and CCR5 as template (PDB IB: 4MBS).[32] Induced fit docking, with a substructure restraint on the right hand phenyl (R^1, SMARTS: "c1ccccc1") was used to dock compound **43** in the hCCR2b model.[53, 54] Figures 4a and 4b were rendered using PyMOL.[55]

REFERENCES

1. Bachelerie, F.; Ben-Baruch, A.; Burkhardt, A. M.; Combadiere, C.; Farber, J. M.; Graham, G. J.; Horuk, R.; Sparre-Ulrich, A. H.; Locati, M.; Luster, A. D.; Mantovani, A.; Matsushima, K.; Murphy, P. M.; Nibbs, R.; Nomiyama, H.; Power, C. A.; Proudfoot, A. E.; Rosenkilde, M. M.; Rot, A.; Sozzani, S.; Thelen, M.; Yoshie, O.; Zlotnik, A. International union of basic and clinical pharmacology. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol. Rev.* **2014,** 66, 1-79.
2. Lopez-Cotarelo, P.; Gomez-Moreira, C.; Criado-Garcia, O.; Sanchez, L.; Rodriguez-Fernandez, J. L. Beyond chemoattraction: multifunctionality of chemokine receptors in leukocytes. *Trends Immunol* **2017,** 38, 927 - 941.
3. Schall, T. J.; Proudfoot, A. E. Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat Rev Immunol* **2011,** 11, 355-363.
4. Zweemer, A. J.; Toraskar, J.; Heitman, L. H.; IJzerman, A. P. Bias in chemokine receptor signalling. *Trends Immunol* **2014,** 35, 243-252.
5. Koelink, P. J.; Overbeek, S. A.; Braber, S.; de Kruijf, P.; Folkerts, G.; Smit, M. J.; Kraneveld, A. D. Targeting chemokine receptors in chronic inflammatory diseases: an extensive review. *Pharmacol Ther* **2012,** 133, 1-18.
6. White, G. E.; Iqbal, A. J.; Greaves, D. R. CC chemokine receptors and chronic inflammation—therapeutic opportunities and pharmacological challenges. *Pharmacol. Rev.* **2013,** 65, 47-89.
7. Woollard, S. M.; Kanmogne, G. D. Maraviroc: a review of its use in HIV infection and beyond. *Drug Des. Devel. Ther.* **2015,** 9, 5447-5468.
8. Horuk, R. Chemokine receptor antagonists: overcoming developmental hurdles. *Nat Rev Drug Discov* **2009,** 8, 23-33.
9. Horuk, R. Promiscuous drugs as therapeutics for chemokine receptors. *Expert Rev. Mol. Med.* **2009,** 11, e1.
10. Friedman, S. L.; Ratziu, V.; Harrison, S. A.; Abdelmalek, M. F.; Aithal, G. P.; Caballeria, J.; Francque, S.; Farrell, G.; Kowdley, K. V.; Craxi, A. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology* **2018,** 67, 1754-1767.
11. Zhao, Q. Dual targeting of CCR2 and CCR5: therapeutic potential for immunologic and cardiovascular diseases. *J. Leukoc. Biol.* **2010,** 88, 41-55.
12. Junker, A.; Kokornaczyk, A. K.; Strunz, A. K.; Wünsch, B. Selective and dual targeting of CCR2 and CCR5 receptors: a current overview. In *Chemokines: Chemokines and Their Receptors in Drug Discovery*, Tschammer, N., Ed. Springer International Publishing: Cham, 2015; pp 187-241.
13. Kothandan, G.; Gadhe, C. G.; Cho, S. J. Structural insights from binding poses of CCR2 and CCR5 with clinically important antagonists: a combined in silico study. *PLoS One* **2012,** 7, e32864.
14. Zheng, Y.; Qin, L.; Ortiz Zacarías, N. V.; de Vries, H.; Han, G. W.; Gustavsson, M.; Dabros, M.; Zhao, C.; Cherney, R. J.; Carter, P.; Stamos, D.; Abagyan, R.; Cherezov, V.; Stevens, R. C.; IJzerman, A. P.; Heitman, L. H.; Tebben, A.; Kufareva, I.; Handel, T. M. Structure of CC chemokine receptor 2 with orthosteric and allosteric antagonists. *Nature* **2016,** 540, 458-461.
15. Oswald, C.; Rappas, M.; Kean, J.; Dore, A. S.; Errey, J. C.; Bennett, K.; Deflorian, F.; Christopher, J. A.; Jazayeri, A.; Mason, J. S.; Congreve, M.; Cooke, R. M.; Marshall, F. H. Intracellular allosteric antagonism of the CCR9 receptor. *Nature* **2016,** 540, 462-465.
16. Turner, M. D.; Nedjai, B.; Hurst, T.; Pennington, D. J. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta* **2014,** 1843, 2563-2582.
17. Ortiz Zacarias, N. V.; Lenselink, E. B.; IJzerman, A. P.; Handel, T. M.; Heitman, L. H. Intracellular receptor modulation: novel approach to target GPCRs. *Trends Pharmacol Sci* **2018,** 39, 547-559.

18. Ortiz Zacarías, N. V.; van Veldhoven, J. P. D.; Portner, L.; van Spronsen, E.; Ullo, S.; Veenhuizen, M.; van der Velden, W. J. C.; Zweemer, A. J. M.; Kreekel, R. M.; Oenema, K.; Lenselink, E. B.; Heitman, L. H.; IJzerman, A. P. Pyrrolone derivatives as intracellular allosteric modulators for chemokine receptors: selective and dual-targeting inhibitors of CC chemokine receptors 1 and 2. *J. Med. Chem.* **2018,** 61, 9146-9161.

19. Zweemer, A. J.; Nederpelt, I.; Vrieling, H.; Hafith, S.; Doornbos, M. L.; de Vries, H.; Abt, J.; Gross, R.; Stamos, D.; Saunders, J.; Smit, M. J.; IJzerman, A. P.; Heitman, L. H. Multiple binding sites for small-molecule antagonists at the CC chemokine receptor 2. *Mol. Pharmacol.* **2013,** 84, 551-561.

20. Zweemer, A. J.; Bunnik, J.; Veenhuizen, M.; Miraglia, F.; Lenselink, E. B.; Vilums, M.; de Vries, H.; Gibert, A.; Thiele, S.; Rosenkilde, M. M.; IJzerman, A. P.; Heitman, L. H. Discovery and mapping of an intracellular antagonist binding site at the chemokine receptor CCR2. *Mol. Pharmacol.* **2014,** 86, 358-368.

21. Andrews, G.; Jones, C.; Wreggett, K. A. An intracellular allosteric site for a specific class of antagonists of the CC chemokine G protein-coupled receptors CCR4 and CCR5. *Mol. Pharmacol.* **2008,** 73, 855-867.

22. Bengtsson, B. A.; Blackaby, W.; Cumming, J.; Faull, A. W.; Larsson, J.; Nash, I. A.; Oldham, K.; Pape, A. 4H-[1, 2, 4] Triazolo [5, 1-b] Pyrimidin-7-one Derivatives As CCR2b Receptor Antagonists. Patent WO 2011/114148-A1. **2011**.

23. Boyd, J. W.; Meo, P.; Higginbottom, M.; Simpson, I.; Mountford, D.; Savory, E. D. 7-Hydroxy-Pyrazolo [1,5-a] Pyrimidine Compounds And Their Use As CCR2 Receptor Antagonists. Patent WO 2012/041817-A1. **2012**.

24. Isberg, V.; de Graaf, C.; Bortolato, A.; Cherezov, V.; Katritch, V.; Marshall, F. H.; Mordalski, S.; Pin, J.-P.; Stevens, R. C.; Vriend, G.; Gloriam, D. E. Generic GPCR residue numbers – aligning topology maps while minding the gaps. *Trends Pharmacol. Sci.* **2015,** 36, 22-31.

25. Baba, M.; Nishimura, O.; Kanzaki, N.; Okamoto, M.; Sawada, H.; Iizawa, Y.; Shiraishi, M.; Aramaki, Y.; Okonogi, K.; Ogawa, Y.; Meguro, K.; Fujino, M. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc. Natl. Acad. Sci. USA* **1999,** 96, 5698-5703.

26. Thum, S.; Kokornaczyk, A. K.; Seki, T.; De Maria, M.; Ortiz Zacarias, N. V.; de Vries, H.; Weiss, C.; Koch, M.; Schepmann, D.; Kitamura, M.; Tschammer, N.; Heitman, L. H.; Junker, A.; Wunsch, B. Synthesis and biological evaluation of chemokine receptor ligands with 2-benzazepine scaffold. *Eur. J. Med. Chem.* **2017,** 135, 401-413.

27. Corbisier, J.; Gales, C.; Huszagh, A.; Parmentier, M.; Springael, J. Y. Biased signaling at chemokine receptors. *J Biol Chem* **2015,** 290, 9542-9554.

28. Prinz, H. Hill coefficients, dose-response curves and allosteric mechanisms. *J. Chem. Biol.* **2010,** 3, 37-44.

29. Zou, D.; Zhai, H.-X.; Eckman, J.; Higgins, P.; Gillard, M.; Knerr, L.; Carre, S.; Pasau, P.; Collart, P.; Grassi, J. Novel, acidic CCR2 receptor antagonists: from hit to lead. *Lett. Drug. Des. Discov.* **2007,** 4, 185-191.

30. Buntinx, M.; Hermans, B.; Goossens, J.; Moechars, D.; Gilissen, R. A.; Doyon, J.; Boeckx, S.; Coesemans, E.; Van Lommen, G.; Van Wauwe, J. P. Pharmacological profile of JNJ-27141491 [(S)-3-[3, 4-difluorophenyl)-propyl]-5-isoxazol-5-yl-2-thioxo-2, 3-dihydro-1H-imidazole-4-carboxyl acid methyl ester], as a noncompetitive and orally active antagonist of the human chemokine receptor CCR2. *J. Pharmacol. Exp. Ther.* **2008,** 327, 1-9.

31. Vauquelin, G.; Szczuka, A. Kinetic versus allosteric mechanisms to explain insurmountable antagonism and delayed ligand dissociation. *Neurochem. Int.* **2007,** 51, 254-260.

32. Tan, Q.; Zhu, Y.; Li, J.; Chen, Z.; Han, G. W.; Kufareva, I.; Li, T.; Ma, L.; Fenalti, G.; Li, J. Structure of the CCR5 chemokine receptor–HIV entry inhibitor maraviroc complex. *Science* **2013,** 341, 1387-1390.

33. Salchow, K.; Bond, M. E.; Evans, S. C.; Press, N. J.; Charlton, S. J.; Hunt, P. A.; Bradley, M. E. A common intracellular allosteric binding site for antagonists of the CXCR2 receptor. *Br J Pharmacol* **2010,** 159, 1429-1439.

34. Nicholls, D. J.; Tomkinson, N. P.; Wiley, K. E.; Brammall, A.; Bowers, L.; Grahames, C.; Gaw, A.; Meghani, P.; Shelton, P.; Wright, T. J.; Mallinder, P. R. Identification of a putative intracellular allosteric antagonist binding-site in the CXC chemokine receptors 1 and 2. *Mol. Pharmacol.* **2008,** 74, 1193-1202.

35. Draper-Joyce, C. J.; Khoshouei, M.; Thal, D. M.; Liang, Y.-L.; Nguyen, A. T. N.; Furness, S. G. B.; Venugopal, H.; Baltos, J.-A.; Plitzko, J. M.; Danev, R.; Baumeister, W.; May, L. T.; Wootten, D.; Sexton, P. M.; Glukhova, A.; Christopoulos, A. Structure of the adenosine-bound human adenosine A1 receptor–Gi complex. *Nature* **2018,** 558, 559-563.

36. Koehl, A.; Hu, H.; Maeda, S.; Zhang, Y.; Qu, Q.; Paggi, J. M.; Latorraca, N. R.; Hilger, D.; Dawson, R.; Matile, H.; Schertler, G. F. X.; Granier, S.; Weis, W. I.; Dror, R. O.; Manglik, A.; Skiniotis, G.; Kobilka, B. K. Structure of the μ-opioid receptor–Gi protein complex. *Nature* **2018,** 558, 547-552.

37. Kang, Y.; Kuybeda, O.; de Waal, P. W.; Mukherjee, S.; Van Eps, N.; Dutka, P.; Zhou, X. E.; Bartesaghi, A.; Erramilli, S.; Morizumi, T.; Gu, X.; Yin, Y.; Liu, P.; Jiang, Y.; Meng, X.; Zhao, G.; Melcher, K.; Ernst, O. P.; Kossiakoff, A. A.; Subramaniam, S.; Xu, H. E. Cryo-EM structure of human rhodopsin bound to an inhibitory G protein. *Nature* **2018,** 558, 553-558.

38. Kang, Y.; Zhou, X. E.; Gao, X.; He, Y.; Liu, W.; Ishchenko, A.; Barty, A.; White, T. A.; Yefanov, O.; Han, G. W.; Xu, Q.; de Waal, P. W.; Ke, J.; Tan, M. H.; Zhang, C.; Moeller, A.; West, G. M.; Pascal, B. D.; Van Eps, N.; Caro, L. N.; Vishnivetskiy, S. A.; Lee, R. J.; Suino-Powell, K. M.; Gu, X.; Pal, K.; Ma, J.; Zhi, X.; Boutet, S.; Williams, G. J.; Messerschmidt, M.; Gati, C.; Zatsepin, N. A.; Wang, D.; James, D.; Basu, S.; Roy-Chowdhury, S.; Conrad, C. E.; Coe, J.; Liu, H.; Lisova, S.; Kupitz, C.; Grotjohann, I.; Fromme, R.; Jiang, Y.; Tan, M.; Yang, H.; Li, J.; Wang, M.; Zheng, Z.; Li, D.; Howe, N.; Zhao, Y.; Standfuss, J.; Diederichs, K.; Dong, Y.; Potter, C. S.; Carragher, B.; Caffrey, M.; Jiang, H.; Chapman, H. N.; Spence, J. C.; Fromme, P.; Weierstall, U.; Ernst, O. P.; Katritch, V.; Gurevich, V. V.; Griffin, P. R.; Hubbell, W. L.; Stevens, R. C.; Cherezov, V.; Melcher, K.; Xu, H. E. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* **2015,** 523, 561-567.

39. Lagorce, D.; Douguet, D.; Miteva, M. A.; Villoutreix, B. O. Computational analysis of calculated physicochemical and ADMET properties of protein-protein interaction inhibitors. *Sci. Rep.* **2017,** 7, 46277.

40. Lagorce, D.; Sperandio, O.; Baell, J. B.; Miteva, M. A.; Villoutreix, B. O. FAF-Drugs3: a web server for compound property calculation and chemical library design. *Nucleic Acids Res.* **2015,** 43, W200-W207.

41. Baell, J. B.; Holloway, G. A. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* **2010,** 53, 2719-2740.

42. Lager, E.; Nilsson, J.; Østergaard Nielsen, E.; Nielsen, M.; Liljefors, T.; Sterner, O. Affinity of 3-acyl substituted 4-quinolones at the benzodiazepine site of GABAA receptors. *Bioorg. Med. Chem.* **2008,** 16, 6936-6948.

43. Liu, Y.; Zhang, Q.; Chen, L. H.; Yang, H.; Lu, W.; Xie, X.; Nan, F. J. Design and synthesis of 2-alkylpyrimidine-4,6-diol and 6-alkylpyridine-2,4-diol as potent GPR84 agonists. *ACS Med Chem Lett* **2016,** 7, 579-583.

44. Bowman, R. K.; Johnson, J. S. Nickel-catalyzed rearrangement of 1-acyl-2-vinylcyclopropanes. A mild synthesis of substituted dihydrofurans. *Org Lett* **2006,** 8, 573-576.

45. Doni, E.; Mondal, B.; O'Sullivan, S.; Tuttle, T.; Murphy, J. A. Overturning established chemoselectivities: selective reduction of arenes over malonates and cyanoacetates by photoactivated organic electron donors. *J. Am. Chem. Soc.* **2013,** 135, 10934-10937.

46. Amupitan, J. A.; Beddoes, R. L.; Mills, O. S.; Sutherland, J. K. 3-Methylcyclohex-2-enone derivatives as initiators of cyclisation. Part 4. Some bicyclisations. *J. Chem. Soc. Perkin Trans 1* **1983**, 759-763.

47. Sun, X.; Tymianski, M.; Garman, D. Agents And Methods For Treating Ischemic And Other Diseases. Patent WO 2012/174488-A2. **2012**.

48. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985,** 150, 76-85.

49. *Maestro Release 2017-1*; Schrödinger, LLC, New York, 2017.

50. *Prime Release 2017-1*; Schrödinger, LLC, New York, 2017.

51. Jacobson, M. P.; Friesner, R. A.; Xiang, Z.; Honig, B. On the role of the crystal environment in determining protein side-chain conformations. *J. Mol. Biol.* **2002,** 320, 597-608.

52. Jacobson, M. P.; Pincus, D. L.; Rapp, C. S.; Day, T. J.; Honig, B.; Shaw, D. E.; Friesner, R. A. A hierarchical approach to all-atom protein loop prediction. *Proteins* **2004,** 55, 351-367.

53. *Glide Release 2017-1; Schrödinger Suite Prime 2017-1 Induced Fit Docking protocol*; Schrödinger, LLC, New York, 2017.

54. Sherman, W.; Day, T.; Jacobson, M. P.; Friesner, R. A.; Farid, R. Novel procedure for modeling ligand/receptor induced fit effects. *J. Med. Chem.* **2006,** 49, 534-553.

55. *The PyMOL Molecular Graphics System*, version 1.8; Schrödinger, LLC, New York, 2015.

SUPPORTING INFORMATION

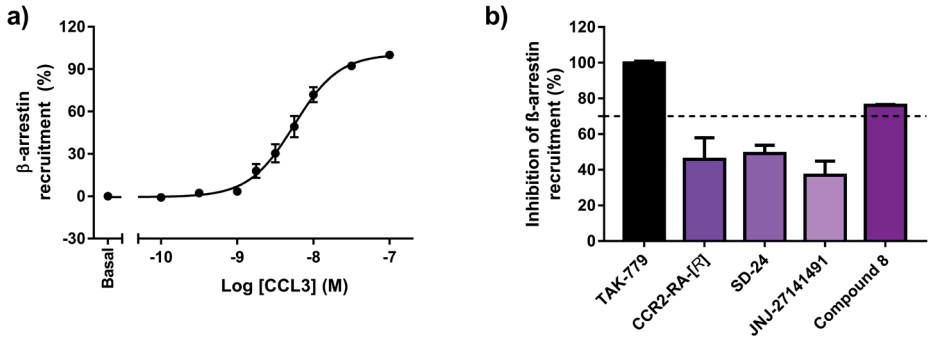


Figure S1. Characterization of intracellular ligands in a U2OS-CCR5 β-arrestin-recruitment assay. (a) Increasing concentrations of CCL3-induced β-arrestin recruitment in U2OS-CCR5 cells, with a pEC_{50} value of 8.3 ± 0.08 (6 nM) and a pEC_{80} of 7.9 ± 0.08 (14 nM). (b) Inhibition of β-arrestin recruitment in U2OS-CCR5 by the orthosteric compound TAK-779 and several intracellular ligands with different chemical structures, all tested at 1 µM, after stimulation with an EC_{80} concentration of CCL3. The dashed line indicates 70% inhibition. Only TAK-779 and compound **8** were able to inhibit CCL3-induced β-arrestin recruitment more than 70%.

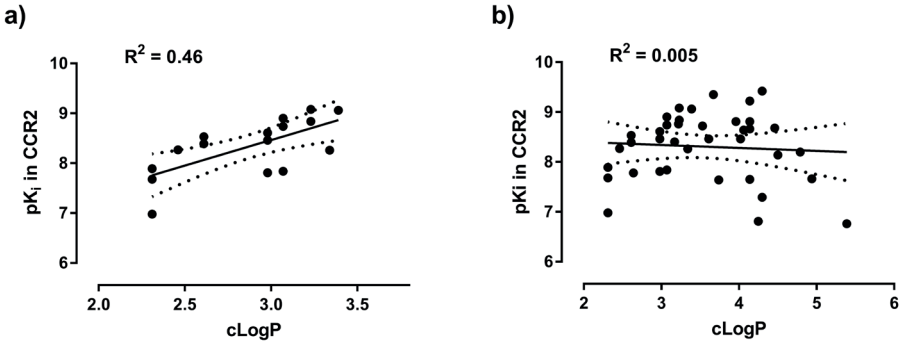


Figure S2. **Correlation between log P (cLogP) and affinity (pK_i) values in CCR2.** (a) Correlation shown for compounds **8** – **23** (Table 1), with R^1 modifications. (b) Correlation shown for all triazolo-pyrimidinone derivatives. In all cases, cLogP values were calculated using the calculator plugins in MarvinSketch, version 19.1.0, 2019, developed by ChemAxon (http://www.chemaxon.com). pK_i values were determined from [^{3}H]-CCR2-RA-[*R*] displacement assays in U2OS-CCR2 and are shown in Tables 1 – 3.

Table S1. List of intermediate compounds 4aa-na, 4bb-bq, 4eq-ev.

Compound	R^3	R^1
4aa	Me	3-Cl
4ba	*c*Pr	3-Cl
4bb	*c*Pr	H
4bc	*c*Pr	2-Me
4bd	*c*Pr	2-Cl
4be	*c*Pr	2-OMe
4bf	*c*Pr	3-Me
4bg	*c*Pr	3-F
4bh	*c*Pr	3-Br
4bi	*c*Pr	3-I
4bj	*c*Pr	3-OMe
4bk	*c*Pr	3-CF3
4bl	*c*Pr	4-Me
4bm	*c*Pr	4-F
4bn	*c*Pr	4-Cl
4bo	*c*Pr	4-Br
4bp	*c*Pr	4-OMe
4bq	*c*Pr	3,4-diCl
4ca	Et	3-Cl
4da	Pr	3-Cl
4ea	*i*Pr	3-Cl
4eq	*i*Pr	3,4-diCl
4er	*i*Pr	2,3-diCl
4es	*i*Pr	2,5-diCl
4et	*i*Pr	3,5-diCl
4eu	*i*Pr	3,5-diBr
4ev	*i*Pr	3-Br, 4-Cl
4fa	Bu	3-Cl
4ga	2-EtBu	3-Cl
4ha	Pent	3-Cl
4ia	*c*Pent	3-Cl
4ja	Hex	3-Cl
4ka	Hept	3-Cl
4la	Ph	3-Cl
4ma	4-MePh	3-Cl
4na	CH_2CH_2Ph	3-Cl

Table S2. Functional activity of TAK-779 and CCR2-RA-[*R*] in hCCR5, using a CCL3-induced β-arrestin recruitment assay.

Compound	pIC_{50} ± SEM (IC_{50}, nM)	Hill slope
TAK-779	8.32 ± 0.17 (6)	-1.1 ± 0.1
CCR2-RA-[*R*]	6.15 ± 0.02 (703)	-2.4 ± 0.2**

Data represent the mean ± standard error of the mean (SEM) of three independent experiments performed in duplicate. **p < 0.01 (p = 0.0038) versus Hill slope (n_H) of TAK-779, determined with a two-tailed, unpaired Student's t-test.

Table S3. Functional activity of compounds 39 and 43 in hCCR2, using a CCL2-induced β-arrestin recruitment assay.

Compound	pIC_{50} ± SEM (IC_{50}, nM)	Hill slope
39	7.68 ± 0.05 (21)	-2.5 ± 0.2
43	8.40 ± 0.01 (4)	-3.4 ± 0.4

Data represent the mean ± standard error of the mean (SEM) of three independent experiments performed in duplicate.