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Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28

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Abstract—G-protein coupled receptors encoded by viruses represent an unexplored class of potential drug targets. In this study, we describe the synthesis and pharmacological characterization of the first class of inverse agonists acting on the HCMV-encoded receptor US28. It is shown that replacement of the 4-hydroxy group of lead compound **1** with a methylamine group results in a significant 6-fold increase in affinity. Interestingly, increasing the rigidity of the spacer by the introduction of a double bond also leads to a significant increase in binding affinity compared to **1**. These novel inverse agonists serve as valuable tools to elucidate the role of constitutive signaling in the pathogenesis of viral infection and may have therapeutic potential as leads for new antiviral drugs.

1. Introduction

Chemokines are a group of small (8–14 kDa) soluble proteins that belong to a large family of chemotactic cytokines.[1] They play an important role in the migration and activation of leukocytes in a wide variety of immune-mediated disorders. These proteins are subdivided by structure into four major groups, namely CC, CXC, CX3C and XC chemokines, based on the number and position of conserved cysteine residues in their amino-terminus.[2]

Chemokines mediate their effects by binding to chemokine receptors, which belong to the family of G-protein coupled receptors. These cell surface proteins are major targets for therapeutic intervention and are targeted by more than 40% of all marketed drugs.[3] Binding of chemokines to their cognate receptors appears promiscuous and redundant, as most chemokine receptors can bind more than one chemokine and most chemokines can activate several chemokine receptor subtypes.[4] However, binding to chemokine receptors is often restricted to a single subclass of chemokines; CC chemokine receptors can only be activated by CC chemokines, while CXC chemokines only bind to CXC chemokine receptors.[2] Two exceptions are the promiscuous chemokine binding protein DARC (Duffy antigen receptor for chemokines) and the human cytomegalovirus (HCMV)-encoded receptor US28. DARC binds chemokines of both the CC and CXC subclasses with high affinity,[5] while the viral-encoded receptor US28 binds several CC-chemokines, including CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES as well as the only member of the CX3C chemokine subclass, namely CX3CL1/fractalkine.[6–9]

HCMV is a species-specific β-herpesvirus that persists lifelong in the host without any clinical symptoms in immunocompetent individuals, but the virus can cause severe illness in immunocompromised individuals, like premature neonates, transplant recipients, and HIV-infected people.[10,11] After primary infection, the viral genome establishes a lifelong latent infection within the host. The virus has developed several strategies to evade the immune system, such as the expression of genes that mimic host genes that are involved in the immune system.[12–14] One of these viral genes encodes a G-protein coupled receptor, namely US28, with significant homology to mammalian chemokine receptors.[6,8] US28 shows a 30% amino acid sequence homology with the human CCR1 chemokine receptor,[6] suggesting that HCMV

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exploits chemokine signaling pathways to interfere with the host immune system through chemokine mimicry.[15] The homology of US28 compared to the CC chemokine receptors is even higher within the N-terminus, crucial for chemokine binding, with an amino acid sequence homology of 70% and 52% with the human CCR1 and CCR2 chemokine receptors, respectively.[6]

Currently, the role of US28 is still unknown, but due to its promiscuous chemokine binding profile it is suggested that the receptor acts as a chemokine scavenger by sequestering CC chemokines from the extracellular environment.[16,17] By this means the virus would elude immune surveillance, as chemokines play an important role in the regulation of the immune response. Furthermore, US28 induces the migration of vascular smooth muscle cells upon binding with the chemokines CCL2 and CCL5, which could be exploited by HCMV to disseminate the virus through the human body.[18] This could also provide a possible link between HCMV and the development of vascular diseases, such as arterial restenosis,[19] atherosclerosis,[20] and chronic allograft rejection.[21] Moreover, CCL2 and CCL5 play an important role in the pathogenesis of vascular disease.[22] Like many mammalian chemokine receptors, of which CCR5 and CXCR4 are the primary HIV-1 co-receptors in vivo, US28 can also act as a co-receptor for HIV-1 entry into cells in vitro.[23]

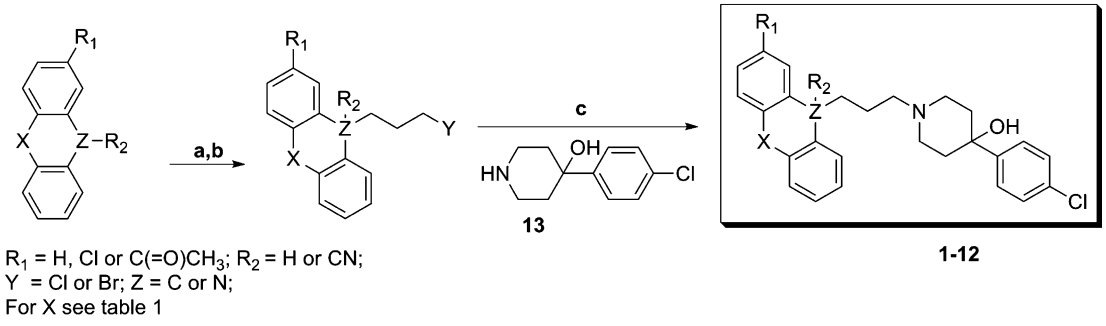
Chemokine receptors have been shown to be involved in the pathophysiology of different diseases.[1,2] The identification of small nonpeptidergic chemokine receptor antagonists that are able to block these receptors proceeds rapidly, with some compounds in clinical trials at the moment.

In contrast to mammalian chemokine receptors, we previously showed that US28 signals in a constitutively active manner and that the receptor affects different signaling pathways, such as phospholipase C and NF-κB,[24] and the transcription factors nuclear factor of activated T-cells (NFAT) and cAMP response element binding protein (CREB).[25–27] The putative role of constitutive activity in viral pathogenesis is not elucidated yet, but it could be a way of the virus to alter the normal homeostasis of a host cell for its own benefit.[24,27] Potent inverse agonists that are able to influence the constitutive signaling of viral-encoded GPCRs could be valuable tools to elucidate the role of constitutive signaling in the pathogenesis of viral infection and may have therapeutic potential as new antiviral drugs acting against pathologies caused by HCMV infection. Screening of a variety of GPCR-directed ligands for their ability to modulate the basal signaling of US28 resulted in the identification of the small nonpeptidergic molecule VUF2274 (**1**) as an inverse agonist.[28] This molecule is not only able to block the basal signaling of US28, but also inhibits 60% of the US28-mediated HIV entry in cells.

Recently, a limited series of analogues of **1** was synthesized to study the very first structure–activity relationships for inverse agonism on a viral-encoded chemokine receptor.[29] To our knowledge, these molecules are currently the only nonpeptidergic inverse agonists acting on US28. In this study, we describe a new series of molecules, in which the rigidity is increased by the introduction of conformationally restrained tricyclic ring systems or rigid fused and nonfused piperidine ring systems. Moreover, we changed the spacer length between the diphenyl acetonitrile group and the piperidine moiety, and introduced rigidity in this part of the molecule. These novel inverse agonists acting on US28 give us more knowledge about the SAR of this class of compounds.

2. Chemistry

Target compounds **1**–**12** were synthesized via an N-alkylation of the appropriate bromide or chloride intermediates with commercially available 4-(4-chlorophenyl)piperidine-4-ol **13** as outlined in Scheme 1.[29–31] Compounds **14**–**21** were synthesized following an analogue synthetic route. Microwave chemistry was used for the N-alkylations to shorten the reaction times. The tricyclic precursors for compounds **2**–**6** were synthesized following methods previously described in the literature.[31–33] 9*H*-carbazole, 10*H*-phenothiazine, 2-chloro-10*H*-phenothiazine, and 1-(10*H*-phenothiazin-2-yl)ethanone were easily deprotonated with NaH at room temperature followed by an alkylation with 1-bromo-3-chloropropane and a reaction with **13** to give target compounds **7** and **10**–**12**.[34,35] However, the deprotonation of 10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine and diphenylamine, which were used for the synthesis of **8**



Scheme 1. Synthetic pathway for the synthesis of **1**–**12**. Reagents and conditions: (a) NaH in DMF or di-*n*-butylether; (b) 1-bromo-3-chloropropane; (c) NaI, Na_2CO_3, CH_3CN, reflux or NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C).

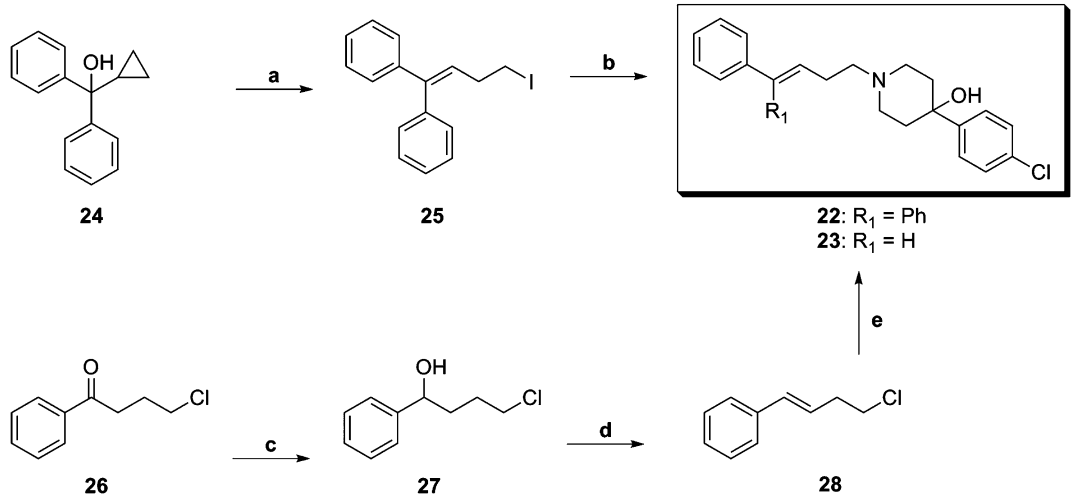
and **9**, could not be accomplished under these reaction conditions and was therefore achieved with NaH as a base in di-*n*-butylether at reflux temperature.[36] Cyclohexyl analogue **14** was synthesized starting from cyclohexylphenylacetonitrile.[31] 1-Benzyl-4-*tert*-butylbenzene, which was used for the synthesis of **15**, was synthesized from the corresponding benzophenone,[29] and 4-(2-benzothiazolyl)propylamine, an intermediate for the synthesis of **16**, was synthesized using *o*-aminothiophenol and 4-chlorobutyryl chloride.[37] The unsaturated compounds **22** and **23** were synthesized according to the procedure shown in Scheme 2. Cyclopropyldiphenylmethanol **24** was reacted with MgI_2, formed in situ from Mg and I_2, to give 4-iodo-1,1-diphenylbut-1-ene **25**, which was reacted with piperidine **13** to give compound **22**.[38,39] A reduction of the carbonyl group of **26** with $NaBH_4$ gave alcohol **27** in a quantitative yield.[40] This alcohol was dehydrated under acidic conditions to give intermediate **28**, which was used for the alkylation of **13** to afford target compound **23**.

Compounds **29**–**31** were synthesized following the same method as described in Scheme 1. Reduction of the carbonyl group of **31** with $NaBH_4$ resulted in target compound **32**. The different fused and non-fused piperidine moieties of compounds **33**–**36** were reacted with the appropriate chloride intermediates in a manner similar to that described for target compounds **1**–**12**. The fused and nonfused ring systems of **33**, **34** and **36** were commercially available, and the six-membered spiropiperidine moiety of **35** was synthesized following a literature procedure.[41] 1-Benzyl-2-methylbenzene, which was used for the synthesis of **34**, was synthesized by the reduction of 2-methylbenzophenone.[29] The synthesis of compounds **37** and **38** is outlined in Scheme 3. Compound **37** was synthesized by reacting *o*-bromobenzoic acid with 2 equiv of *n*-BuLi,[42] followed by a reaction with ketone **39**. Reductive amination of **40** with di-*tert*-butyl 2-(2-aminophenyl)malonate **41**, which was synthesized from the reaction of 2-fluoronitrobenzene with di-*tert*-butyl malonate and subsequent reduction of the nitro group, yielded intermediate **42**. The indoline-2-one compound **38** was obtained by an intramolecular cyclization reaction of **42** by treatment with *p*-toluenesulfonic acid.[43]

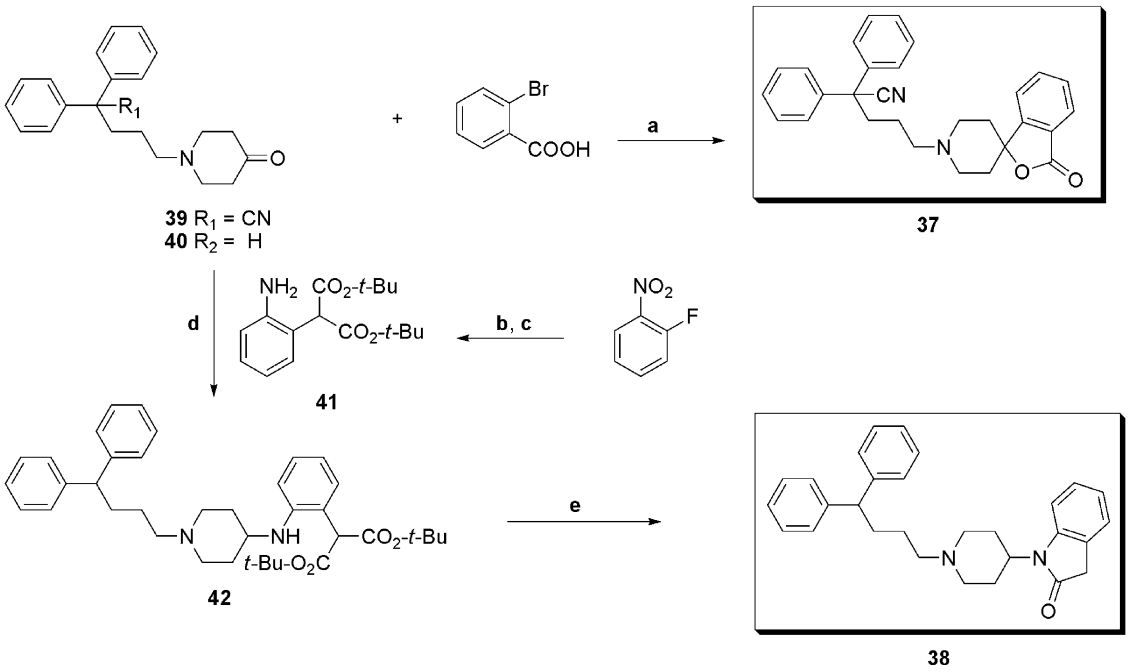
Compounds **43** and **44** were synthesized following a method previously described in the literature.[31] The acetate group of compound **45** was formed by reacting compound **1** with acetylchloride in the presence of triethylamine. Reduction of the methyl ester group of compound **43** with $LiAlH_4$ resulted in alcohol **46**.[31] The synthetic route for the preparation of **47**–**52** is outlined in Schemes 4 and 5. Target compounds **47** and **50** were synthesized by a reaction of 4-chloro-1,1-diphenylbutane[44] with **53** or **54** to give intermediates **55** or **56**, followed by a reduction of the nitrile group in the presence of $AlCl_3$ and $LiAlH_4$. Compound **53**, which was used for the synthesis of **47**, was commercially available as the hydrochloride salt, but **54** needed to be synthesized. Thus, 2-(4-chlorophenyl)acetonitrile was deprotonated by $NaNH_2$ and treated with Boc-protected bis-(2-chloro-ethyl)amine **57** resulting in intermediate **58**, which was deprotected under acidic conditions resulting in the desired piperidine moiety **54**.[45] Substituents were easily introduced on the amine group of **47**, so **48** was synthesized in a high yield by reacting **47** with formic acid in the microwave. The monomethyl-substituted amine **49** was synthesized by refluxing **48** in THF in the presence of $LiAlH_4$. The hydroxy group of **59** was converted to an acetamide group in **51** via a Ritter reaction.[46] In strongly acidic media, a highly electrophilic tertiary carbocation is formed from the tertiary hydroxy group of **59**, and this is followed by an acid-induced nucleophilic addition of the nitrile group of acetonitrile and a hydrolysis resulting in the desired compound **51**. The acetamide group of **51** was hydrolyzed under acidic conditions to the corresponding amine group in **52**.[47]

3. Results and discussion

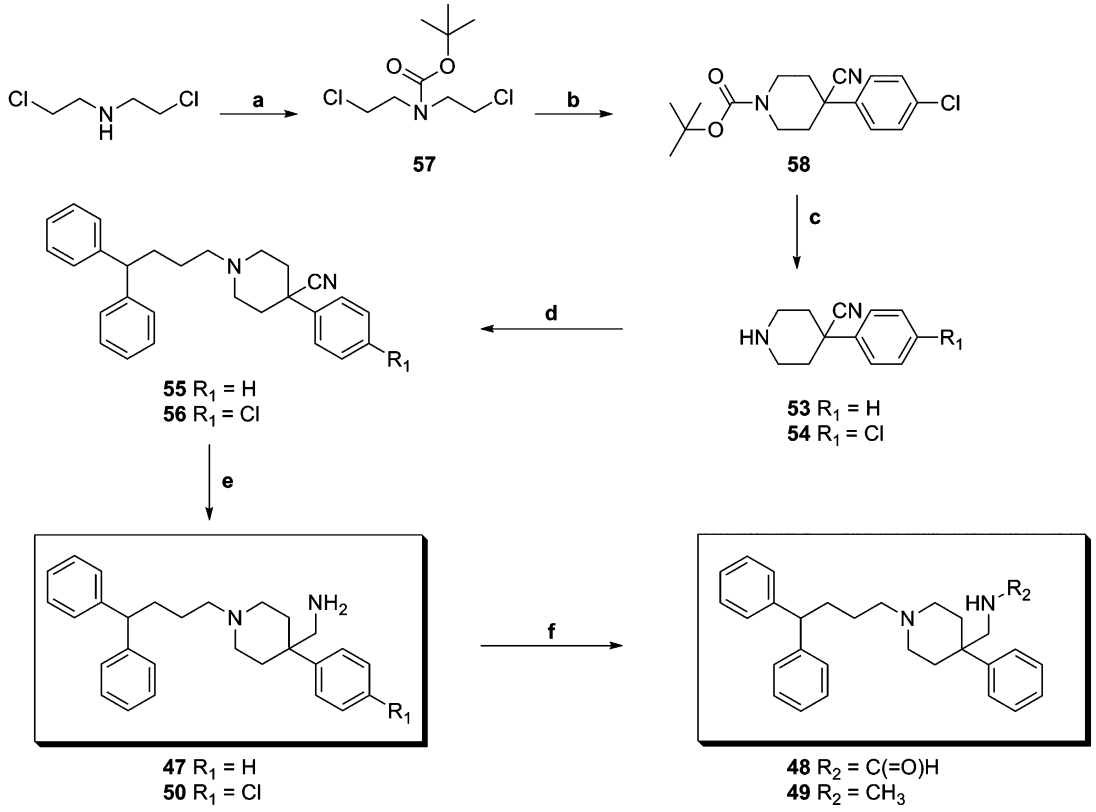
Starting from lead compound **1** we synthesized a new series of compounds, which were evaluated for



Scheme 2. Synthetic pathway for the synthesis of **22** and **23**. Reagents and conditions: (a) MgI_2, Et_2O, reflux; (b) **13**, Na_2CO_3, CH_3CN, reflux; (c) $NaHCO_3$, $NaBH_4$, EtOH; (d) HCl, reflux; (e) **13**, NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C).

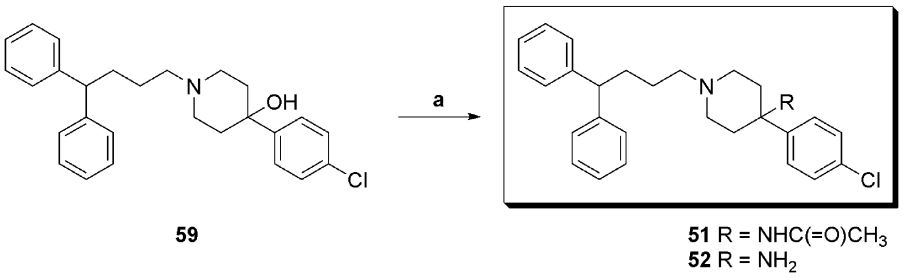


Scheme 3. Synthetic pathway for the synthesis of **37** and **38**. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C; (b) di-*tert*-butylmalonate, NaH, DMF, 90 °C; (c) Pd/C, H_2, EtOAc; (d) AcOH, $NaBH(OAc)_3$, 1,2-dichloroethane; (e) PTSA, toluene, reflux.



Scheme 4. Synthetic pathway for the synthesis of **47**–**50**. Reagents and conditions: (a) di-*tert*-butyl dicarbonate, Et_3N, DCM; (b) $NaNH_2$, 2-(4-chlorophenyl)acetonitrile, toluene, 70 °C; (c) EtOH/HCl; (d) 4-chloro-1,1-diphenylbutane, NaI, Na_2CO_3, CH_3CN, reflux; (e) $AlCl_3$, $LiAlH_4$, THF; (f) formic acid, microwave (5 min, 200 °C) for **48** or formic acid, microwave (5 min, 200 °C) followed by $LiAlH_4$, THF, reflux for **49**.

their potential to dose-dependently displace [^{125}I]CCL5 binding to US28. The inverse agonistic properties of a selection of compounds were investigated by testing their potential to inhibit the US28-mediated constitutive inositol phosphate production in SVEC4-10 cells.



59

51 R = $NHC(=O)CH_3$
52 R = NH_2

Scheme 5. Synthetic pathway for the synthesis of **51** and **52**. Reagents and conditions: (a) H_2SO_4, CH_3CN for **51** followed by HCl, reflux for **52**.

In our previous study,[29] it was shown that a piperidine ring was important for activity and therefore this structural motif was maintained. It was also revealed that the nitrile group in the structure was not essential for affinity and efficacy, so this group could be omitted.[29] In this study, we focussed our chemistry program on other parts of the structure, namely on the diphenyl group (Table 1) and the propyl linker (Table 2). Furthermore, we introduced different substituents at the 4-position of the piperidine ring (Tables 3 and 4).

First, more rigidity was introduced by incorporation of the two phenyl rings in different tricyclic moieties (Table 1). The rotation of the two aromatic phenyl rings is restricted by incorporation of an ethylene group between the two phenyl rings in **3**, a bioisosteric thiomethylene or oxomethylene bridge in **4** and **5** or an unsaturated bridge as in **6**. All these changes were tolerated and did not influence the affinity and potency of the compounds. Interestingly, introduction of a nearly planar tricyclic ring system in **2** also resulted in a compound with a comparable affinity.

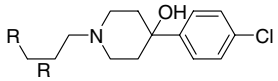
The introduction of a nitrogen atom in the tricyclic system could provide an additional position for hydrogen bonding, but this structural modification did not influence the affinity of the compound. For tricyclic antipsychotics and antidepressants it is known that there is a relationship between the folding of the tricyclic moiety and biological activity. If the sulfur atom of neuroleptic phenothiazine drugs is replaced by an ethylene bridge it results in dibenzazepine derivatives, which have an antidepressant activity.[48] In contrast, replacement of the sulfur atom of phenothiazine analogue **10** into an ethylene bridge, resulting in the tricyclic moiety of dibenzazepine analogue **8**, did not result in any change in affinity or potency on US28. Additionally, introduction of the more rigid and planar carbazole ring system in analogue **7** did not change the affinity for the receptor, as was also shown for compound **2**. The introduction of a chloro or acetyl group in one of the aromatic rings in phenothiazine analogues **11** and **12** resulted in a decrease in binding affinity, so these substitutions are not preferred in the phenothiazine ring.

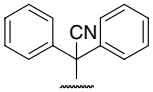
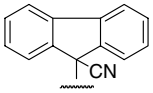
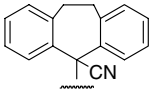
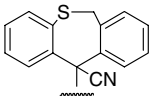
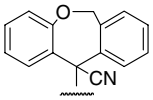
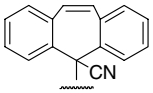
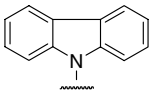
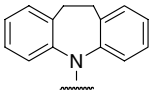
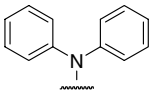
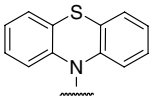
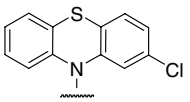
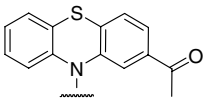
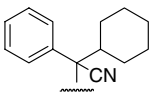
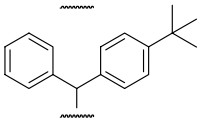
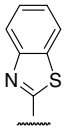
Replacement of one of the phenyl rings by a bulky saturated cyclohexyl ring in **14** is allowed. Previously, we found that introduction of a bulky phenyl substituent at the 4-position of one of the phenyl rings was not allowed to maintain binding affinity.[29] In contrast, the bulky *tert*-butyl group in compound **15** is permitted at this position. Interestingly, replacement of the diphenylacetonitrile group by a benzothiazole ring in compound **16** caused a 3.5-fold drop in binding affinity to the receptor. Taken together, the structural modifications indicate that changes in this part of the molecule are well tolerated and that the conformation of the two phenyl rings does not seem to be important.

Furthermore, the importance of the propyl linker between the diphenylacetonitrile group and the piperidine moiety was investigated by the introduction of different structural modifications in this part of the structure (Table 2). First, we studied the effect of varying the chain length of the linker. Compound **1** has been previously reported as a potent antagonist acting on the human CCR1 chemokine receptor.[34] Interestingly, we recently demonstrated that the SAR of compound **1** and its analogues is completely different on the viral-encoded receptor US28 compared to that of the human chemokine receptor CCR1.[29] Now, the shortening of the propyl spacer with one methylene group in **17** caused a small decrease in affinity on US28, while the addition of one methylene group in the structure of **1**, resulting in compound **18**, did not change the affinity or efficacy. In contrast, for the human CCR1 chemokine receptor it was shown that shortening of the propyl chain with one methylene group caused a large decrease in K_i value, while addition of one or two methylene groups in the structure of **1**, resulting in, respectively, a butyl and a pentyl chain, did not change the affinity on the CCR1 chemokine receptor.[31]

In compounds **19**–**21** one of the phenyl rings was removed and this series of compounds showed a similar trend as demonstrated for compounds **1**, **17**, and **18**, namely that removal of one methylene group in **19** resulted in a decrease of binding affinity, while compounds **20** and **21** have binding affinities comparable to compounds **1** and **18**. Interestingly, the introduction of a more rigid chain in unsaturated analogue **22** resulted in a 3-fold increase in binding affinity compared to lead compound **1**. However, in the functional assay both compounds were equipotent. In our previous study it was shown that removal of a phenyl ring as in **20** resulted in a binding affinity comparable to compound **1**, but this is not true for the more rigid and unsaturated analogues **22** and **23**. Compound **23** has a binding affinity that is more than 10-fold reduced compared to compound **22**.

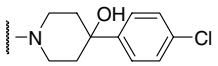
Table 1. Chemical structures and pharmacological properties of compounds **1**–**12** and **14**–**16** for the HCMV-encoded receptor US28

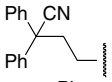
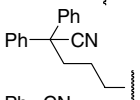
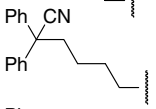
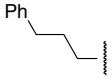
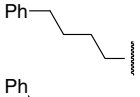
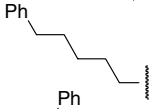
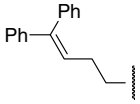
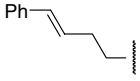


Compound	VUF	R	IC_{50}[a] (μM)	EC_{50}[b] (μM)
1	2274		4.9 (4.4–5.5)	3.2 (2.5–4.0)
2	10004		6.0 (5.5–6.5)	5.7 (4.3–7.1)
3	5713		6.6 (4.8–8.3)	5.7 (3.0–8.5)
4	5727		6.1 (4.6–7.6)	5.4 (4.0–6.9)
5	10007		7.7 (5.9–9.3)	6.2 (3.4–8.9)
6	10003		6.0 (4.7–7.4)	5.1 (2.5–7.8)
7	5932		8.4 (6.2–10.7)	4.5 (2.8–6.2)
8	5982		6.4 (4.6–8.3)	7.1 (3.0–11.2)
9	5983		6.9 (3.5–10.2)	4.4 (2.4–6.3)
10	10005		6.4 (5.2–7.6)	6.8 (5.2–8.3)
11	10006		10.5 (6.5–14.5)	n.d.
12	6902		11.0 (5.5–16.6)	10.7 (6.9–14.5)
14	5892		7.7 (5.5–10.0)	5.2 (3.2–7.1)
15	(±) 5937		5.9 (4.6–7.2)	7.6 (3.2–12.0)
16	5933		18.0 (13.2–22.9)	n.d.

The values are represented as means and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.
[a] [^{125}I]CCL5 displacement.
[b] Inhibition of [^{3}H]inositol phosphate production. n.d., not determined.

Table 2. Chemical structures and pharmacological properties of compounds **1** and **17–23** for the HCMV-encoded receptor US28



Compound	VUF	R	IC_{50}[a] (μM)	EC_{50}[b] (μM)
17	5742		9.2 (7.9–10.5)	4.1 (1.5–6.6)
1	2274		4.9 (4.4–5.5)	3.2 (2.5–4.0)
18	5743		4.6 (3.6–5.5)	5.5 (1.9–9.1)
19	5745		21.1 (15.8–26.3)	n.d.
20	5746		7.8 (6.8–8.9)	n.d.
21	5752		6.6 (4.6–8.7)	n.d.
22	6869		1.7 (1.1–2.3)	4.8 (1.3–8.3)
23	(*E*/*Z*) 6870		20.0 (19.5–20.4)	n.d.

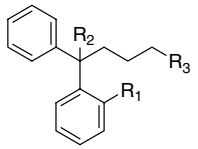
The values are represented as means and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.
[a] [^{125}I]CCL5 displacement.
[b] Inhibition of [^{3}H]inositol phosphate production. n.d., not determined.

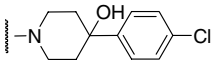
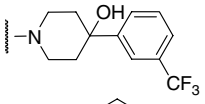
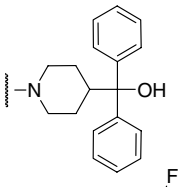
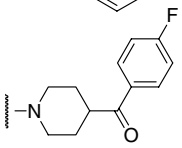
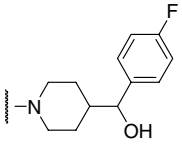
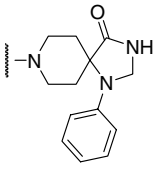
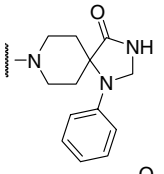
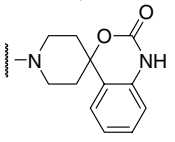
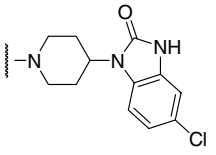
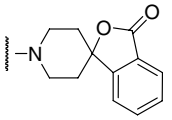
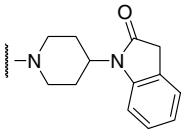
Next, the influence of the substitution pattern at the 4-position of the piperidine ring was investigated to further define the structure–activity relationships on US28 (Tables 3 and 4). We previously showed that a phenyl ring at this position is important,[29] so this was maintained in the structure. In compounds **33–38** the aromatic ring is incorporated in a heterocyclic system, while in compounds **30–32** an additional carbon atom is present between the piperidine ring and the phenyl ring. Both the replacement of the 4-chloro atom in the phenyl ring by a 3-trifluoromethyl group in **29** as well as the introduction of a bulky diphenylmethanol group in compound **30** did not influence the potency of the compounds. In compound **31** a 4-fluorophenylmethanone group was introduced at the 4-position of the piperidine ring and reduction of the carbonyl group resulted in analogue **32**. The introduction of both the 4-fluorophenylmethanone group in compound **31** as well as the 4-fluorophenylmethanol group in analogue **32** did not influence the affinity for US28.

Different fused and nonfused ring systems (heterocyclic-substituted piperidine analogues or bicyclic heterocyclic groups) were introduced at the 4-position of the piperidine ring, because these structural motifs appear frequently in CC chemokine receptor antagonists. Additionally, the heterocyclic groups contain different functional groups, which can act as hydrogen bond acceptor or donor groups. Both compounds **33** and **34** contain a spiropiperidine amide group, but compound **34** was not synthesized with a diphenylacetonitrile group as in compound **33**, but with a ortho methyl-substituted diphenyl group, because recently it was demonstrated that there was a slight preference for this structural motif.[29] However, in this series of compounds the binding affinities of **33** and **34** were comparable and in the same order as lead compound **1**. Additionally, the introduction of the spiropiperidine moieties in both compounds **35** and **37** was allowed to maintain affinity for US28. The benzimidazolone piperidine moiety of **36** is often seen in ligands acting on various G-protein coupled receptors, such as serotonergic and dopaminergic receptors.[43] Interestingly, the introduction of an indolin-2-one group in compound **38** resulted in a compound with an activity compared to **1**, while the introduction of the benzimidazolone piperidine moiety of **36** made the affinity drop slightly.

Moreover, the 4-hydroxy group was replaced by other substituents (Table 4), because a hydroxy group at this position of a piperidine ring is suggested to be a site of potential metabolic toxicity.[49] The introduction of an ester group in **43**, an acetyl group in **44**, an acetate group in **45** or a hydroxy methyl group in **46** did not result in compounds with a higher affinity.

Table 3. Chemical structures and pharmacological properties of compounds **1** and **29–38** for the HCMV-encoded receptor US28



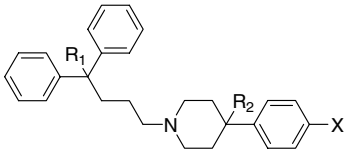
Compound	VUF	R_1	R_2	R_3	IC_{50}[a] (μM)	EC_{50}[b] (μM)
1	2274	H	CN		4.9 (4.4–5.5)	3.2 (2.5–4.0)
29	6984	H	CN		5.2 (4.2–6.3)	4.3 (2.5–6.2)
30	5729	H	CN		4.8 (4.3–5.4)	6.1 (2.3–10.0)
31	6868	H	H		4.8 (3.9–5.6)	n.d.
32	(±) 10010	H	H		6.4 (4.6–8.3)	3.0 (2.8–3.2)
33	5893	H	CN		3.7 (3.5–4.0)	2.0 (1.3–2.8)
34	(±) 5997	Me	H		2.6 (1.2–3.9)	4.2 (1.1–7.4)
35	6967	H	CN		7.6 (6.0–9.1)	n.d.
36	6985	H	CN		7.1 (6.3–7.9)	n.d.
37	6047	H	CN		11.9 (10.0–13.8)	n.d.
38	6048	H	H		5.5 (4.9–6.2)	7.3 (2.2–12.3)

The values are represented as means and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.

[a] [^{125}I]CCL5 displacement.

[b] Inhibition of [^{3}H]inositol phosphate production. n.d., not determined.

Table 4. Chemical structures and pharmacological properties of compounds **1** and **43–52** for the HCMV-encoded receptor US28



Compound	VUF	R_1	R_2	X	IC_{50}[a] (μM)	EC_{50}[b] (μM)
1	2274	CN	OH	Cl	4.9 (4.4–5.5)	3.2 (2.5–4.0)
43	5934	CN	(C=O)OCH_3	H	8.0 (6.0–10.0)	n.d.
44	5984	CN	(C=O)CH_3	H	7.9 (6.3–9.5)	n.d.
45	5936	CN	O(C=O)CH_3	Cl	8.6 (7.4–9.8)	n.d.
46	6881	H	–CH_2OH	H	7.5 (5.6–9.3)	n.d.
47	6046	H	–CH_2NH_2	H	1.6 (1.1–2.0)	3.1 (1.7–4.6)
48	6987	H	–$CH_2NHC(=O)H$	H	2.5 (2.0–3.0)	n.d.
49	6989	H	–CH_2NHCH_3	H	1.3 (1.1–1.5)	3.1 (1.2–4.9)
50	6966	H	–CH_2NH_2	Cl	0.8 (0.7–1.0)	3.6 (2.6–4.6)
51	6981	H	–$NHC(=O)CH_3$	Cl	2.2 (2.0–2.5)	4.1 (1.0–7.1)
52	6993	H	–NH_2	Cl	1.4 (1.3–1.4)	5.7 (2.5–8.9)

The values are represented as means and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.
[a] [^{125}I]CCL5 displacement.
[b] Inhibition of [^{3}H]inositol phosphate production. n.d., not determined.

Furthermore, amine analogue **47** was synthesized to investigate the influence of a primary amine group at the 4-position of the piperidine ring. It was demonstrated earlier that a nitrile group at the 4-position of the piperidine ring was detrimental for both affinity and activity.[29] However, the reduction of this group into a methylamine group of derivative **47** resulted in a 3-fold increase in binding affinity compared to lead compound **1**, while the efficacy of both compounds was comparable. From previous studies[28,29] it was known that removal of the *p*-chloro substituent resulted in a decrease in binding affinity. Thus, a chloro atom was introduced at the 4-position of the phenyl ring in compound **50**, which resulted in a further 2-fold increase of binding affinity compared to unsubstituted analogue **47**. The amine group of compound **47** was substituted, resulting in compounds **48** and **49**. Unfortunately, these substitutions did not cause an increase in the binding affinity on US28. Compound **51** was synthesized to investigate the importance of the position of the amine group and this amine was synthesized starting from compound **52**, in which the amine group is substituted with an acetyl group. Both compounds have an affinity higher than lead compound **1**, but slightly lower than that of our novel lead compound **50**.

4. Conclusion

In summary, we described the synthesis and structure–activity relationships of inverse agonists acting on the viral-encoded GPCR US28. These molecules are considered as valuable tools to investigate the (patho)physiological role of US28 during viral infection. Replacement of the 4-hydroxy group of lead compound **1** into a methylamine group as in compound **50** resulted in the, to our knowledge, highest affinity inverse agonist acting on US28 currently known. Interestingly, the introduction of a double bond in the propyl linker between the diphenyl group and the piperidine moiety in compound **22** caused a significant increase in binding affinity to US28. Currently, these molecules are being used as tools to further investigate the role of constitutive signaling of US28 in the pathogenesis of viral infection. In the future, potent and selective inverse agonists acting on constitutively active viral GPCRs may have therapeutical potential in the treatment of pathologies caused by viral infections.

5. Materials and methods

5.1. General procedures

The solvents were dried according to standard procedures. All reactions were performed under an atmosphere of dry nitrogen. Microwave reactions were performed in a CEM Explorer single mode MW reactor equipped with auto sampler. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. J. T. Baker silica gel was used for flash chromatography. HRMS mass spectra were recorded on a Finnigan MAT 900 mass spectrometer. Melting points were measured on a MPA100 OptiMelt Automated Melting Point System apparatus and are uncorrected. Analytical HPLC-MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV–VIS detector set at 254 nm, with the MS detection performed with a Shimadzu LCMS-2010 liquid chromatograph mass spectrometer. The analyses were performed using the following conditions; condition I: an Alltima(C18)5u column (150 mm × 4.6 mm) with 70% MeOH–30% H_2O–0.1% formic acid (method Ia); 60% MeOH–40% H_2O–0.1% formic acid (method Ib) or 50% MeOH–50% H_2O–0.1% formic acid (method Ic). Flow rate = 1.0 mL/min. Total run time 15 min unless otherwise stated. Condition II: an Alltima(C18)5u

column (150 mm × 4.6 mm) with 50% CH_3CN–50% H_2O–0.1% formic acid (method IIa); 40% CH_3CN–60% H_2O–0.1% formic acid (method IIb) or 30% CH_3CN–70% H_2O–0.1% formic acid (method IIc). Flow rate = 1.0 mL/min. Total run time 20 min. Compounds that were isolated as fumaric acid salts all showed a fumaric acid peak around 2 min. Purities calculated are based on RP HPLC–UV peak surface area using the condition that showed the highest level of impurities (disregarding the fumaric acid peak). Reference compounds **1** and **20** have been described previously and taken from stock.[29] Compounds **3**–**6**, **14**, **17**, **18**, **43**, and **46** were synthesized as previously described in the literature[31] and the characterization data confirmed that the desired compounds had been formed. Compounds **15**, **32**, and **34** were tested as racemic mixtures.

5.2. General method A. 5-(4-Hydroxy-4-(3-(trifluoromethyl)phenyl)piperidin-1-yl)-2,2-diphenylpentanenitrile fumarate (29)

4-(3-(Trifluoromethyl)phenyl)piperidine-4-ol (0.51 g, 2.07 mmol), 5-chloro-2,2-diphenylpentanenitrile[34] (0.54 g, 2.00 mmol), NaI (0.30 g, 2.00 mmol), Na_2CO_3 (0.42 g, 2.00 mmol) and 3 mL CH_3CN were added in a 10 mL microwave and this mixture was reacted during 15 min in the microwave at a temperature of 160 °C (settings: ramp time 5 min, hold time 15 min, power 200 W, pressure 17.2 bar). The solvent was removed in vacuo and the residue was diluted with water (20 mL), followed by an extraction with DCM (3× 15 mL). The combined organic layers were washed with water (3× 40 mL) and brine (40 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (0–50% EtOAc in DCM) gave 772 mg (81%) of oil. This was dissolved in EtOH and converted to the corresponding fumaric salt by the addition of fumaric acid (0.19 g, 1.6 mmol). The fumaric salt was isolated by evaporation in vacuo and recrystallized from EtOH/Et_2O to give 803 mg (68%) of **29** as a white solid. Mp: 123.7–125.3 °C (dec). ^{1}H NMR (DMSO-d_6): δ 1.47–1.78 (m, 5H), 1.98–2.20 (m, 2H), 2.35–2.91 (m, 8H), 6.57 (s, 2H), 7.33–7.81 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 30.74, 35.31, 36.14, 47.91, 51.18, 55.68, 68.78, 121.49, 121.85, 123.64, 126.44, 127.89, 128.20, 128.69, 128.84, 134.72, 134.91, 139.22, 148.43, 169.59. Anal. RP-HPLC Ib: t_R = 6.64 min (purity 100%), IIb: t_R = 11.69 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{29}H_{29}F_3N_2O$: 478.2232; found: 478.22249.

5.3. General method B. 5-(4-(Hydroxydiphenylmethyl)piperidin-1-yl)-2,2-diphenylpentanenitrile (30)

A solution of 4-bromo-2,2-diphenylbutanitrile[31] (0.31 g, 1.00 mmol), diphenyl(piperidin-4-yl)methanol (0.36 g, 1.20 mmol), NaI (0.45 g, 3.00 mmol), and Na_2CO_3 (0.64 g, 6.00 mmol) in CH_3CN (30 mL) was refluxed overnight. The solvent was removed in vacuo, the residue was diluted with water (50 mL) and extracted with DCM (3× 30 mL). The combined organic layers were washed with water (3× 50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (EtOAc) and recrystallization from EtOAc gave 293 mg (59%) of **30** as a white solid. Mp: 77.9–78.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.34–1.69 (m, 5H), 1.85–2.20 (m, 4H) 2.30–2.50 (m, 5H), 2.79–2.99 (m, 2H), 7.11–7.47 (m, 20H). ^{13}C NMR ($CDCl_3$): 22.80, 26.03, 37.25, 43.85, 51.47, 53.71, 57.60, 79.32, 122.22, 125.57, 126.39, 126.69, 127.69, 128.04, 128.71, 129.23, 139.94, 145.70. Anal. RP-HPLC Ib: t_R = 9.48 min (purity 98%), IIa: t_R = 6.11 min (purity 98%). HRMS (EI) *m*/*z* calcd for $C_{35}H_{36}N_2O$: 500.2828; found: 500.28170.

5.4. General method C. 1-(3-(9*H*-Carbazol-9-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (7)

(i) A solution of 9*H*-carbazole (0.84 g, 5.01 mmol) in DMF (20 mL) was cooled to 0 °C and NaH (0.22 g, 5.58 mmol) was added in small portions. After stirring for 1 h at room temperature, the reaction mixture was cooled to 0 °C and 1-bromo-3-chloropropane (0.5 mL, 5.06 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. Water (50 mL) was added and the water layer was extracted with EtOAc (3× 25 mL). The combined organic extracts were washed with water (3× 25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, and filtered. After evaporation under reduced pressure, the residue was purified by flash chromatography (0–15% DCM in hexane) to give 853 mg (70%) of 9-(3-chloropropyl)-9*H*-carbazole. ^{1}H NMR ($CDCl_3$): δ 2.28–2.49 (m, 2H), 3.51 (t, J = 6.0 Hz, 2H), 4.50 (t, J = 6.4 Hz, 2H), 7.21–7.31 (m, 2H), 7.40–7.48 (m, 4H), 8.11 (d, J = 7.7 Hz, 2H).

(ii) Following method B using 9-(3-chloropropyl)-9*H*-carbazole (0.57 g, 2.34 mmol) gave 727 mg (75%) of **7** as a light yellow solid after recrystallization in EtOAc. Mp: 130.1–131.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.54–1.72 (m, 3H), 2.01–2.14 (m, 4H), 2.28–2.41 (m, 4H), 2.68–2.73 (m, 2H), 4.41 (t, J = 6.6 Hz, 2H), 7.17–7.49 (m, 10H), 8.09 (d, J = 7.7 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 25.91, 38.24, 40.54, 49.17, 55.20, 70.92, 108.62, 118.64, 120.17, 122.67, 125.40, 125.97, 128.27, 132.66, 140.33, 146.60. Anal. RP-HPLC Ib: t_R = 9.37 min (purity 100%), IIb: t_R = 10.33 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{26}H_{27}ClN_2O$: 418.1812; found: 418.18153.

5.5. 9-(3-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-propyl)- 9*H*-fluorene-9-carbonitrile hydrochloride (2)

(i) 9*H*-Fluorene-9-carbonitrile[33] (1.03 g, 5.39 mmol) was dissolved in DMF (25 mL) and NaH (0.22 g, 5.58 mmol) was added in small portions. The reaction mixture was heated to 70 °C and after 2 h 1-bromo-3-chloropropane (2.75 mL, 27.8 mmol) was added in one portion. The reaction mixture was allowed to cool to room temperature and stirred overnight. Water (50 mL) was added and the water layer was extracted with Et_2O (3× 50 mL). The combined organic extracts were washed with water (3× 50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo to give 1.06 g of 9-(3-chloropropyl)-9*H*-fluorene-9-car-

bonitrile as a yellow solid. The crude product was used without further purification.

(ii) Following method A using crude 9-(3-chloropropyl)-9*H*-fluorene-9-carbonitrile (0.49 g, 2.16 mmol) gave 445 mg of oil. This was dissolved in Et_2O and dry hydrochloride gas was bubbled through the solution. Isolation of the hydrochloride salt by filtration and recrystallization from MeOH/Et_2O gave 201 mg (17%) of **2** as a white solid. Mp: 156.2–158.1 °C. ^{1}H NMR ($CDCl_3$): δ 1.55–1.94 (m, 5H), 2.33–2.54 (m, 2H), 2.77–3.18 (m, 6H), 3.26–3.40 (m, 2H), 7.35–7.60 (m, 8H), 7.66–7.88 (m, 4H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 18.52, 34.88, 36.00, 47.29, 48.64, 56.16, 68.26, 120.45, 120.68, 124.08, 126.17, 128.22, 128.57, 129.67, 132.92, 139.93, 141.57, 145.12. Anal. RP-HPLC Ib: t_R = 5.10 min (purity 99%), IIb: t_R = 8.39 min (purity 99%). HRMS (EI) *m*/*z* calcd for $C_{28}H_{27}ClN_2O$: 442.1812; found: 442.18072.

5.6. 4-(4-Chlorophenyl)-1-(3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)propyl)piperidin-4-ol (8)

(i) NaH (0.40 g, 10.03 mmol) was added portionwise to a solution of 10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine (1.95 g, 9.99 mmol) in di-*n*-butylether (30 mL). The reaction mixture was heated, refluxed for 3.5 h, and 1-bromo-3-chloropropane (4.00 mL, 40.5 mmol) was added at 100 °C. The reaction mixture was refluxed overnight, water was added (50 mL) and the water layer was extracted with toluene (3× 75 mL). The combined organic extracts were washed with water (3× 100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4, and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (5% DCM in hexane) to give 737 mg (27%) of 5-(3-chloropropyl)-10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.99–2.16 (m, 2H), 3.18 (s, 4H), 3.57 (t, *J* = 6.4 Hz, 2H), 3.91 (t, *J* = 6.5 Hz, 2H), 6.91–6.99 (m, 2H), 7.08–7.24 (m, 6H).

(ii) Following method A using 5-(3-chloropropyl)-10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine (0.42 g, 1.55 mmol) gave 494 mg (71%) of **8** as white crystals after recrystallization from EtOAc. Mp: 116.0–117.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.61–1.84 (m, 5 H), 1.96–2.11 (m, 2H), 2.26–2.47 (m, 4H), 2.62–2.78 (m, 2H), 3.15 (s, 4H), 3.76 (t, *J* = 6.8 Hz, 2H), 6.85–6.93 (m, 2H), 7.06–7.15 (m, 6H), 7.24–7.41 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 25.74, 32.63, 38.67, 49.33, 49.83, 56.95, 71.40, 120.35, 122.86, 126.48, 126.77, 128.79, 130.22, 133.18, 134.64, 147.17, 148.65. Anal. RP-HPLC Ib (total run time 20 min): t_R = 12.71 min (purity 99%), IIa: t_R = 4.76 min (purity 100%), IIb: t_R = 15.55 min (purity 99%). HRMS (EI) *m*/*z* calcd for $C_{28}H_{31}ClN_2O$: 446.2125; found: 446.21311.

5.7. 4-(4-Chlorophenyl)-1-(3-(diphenylamino)propyl)piperidin-4-ol (9)

(i) This was synthesized as described for **8** starting with diphenylamine (1.69 g, 10.00 mmol) to give 683 mg (16% over two steps) of **9** as a white solid after recrystallization from EtOAc. Mp: 109.9–111.9 °C. ^{1}H NMR ($CDCl_3$): δ 1.50–1.95 (m, 5H), 2.03–2.22 (m, 2H), 2.32–2.47 (m, 4H), 2.69–2.87 (m, 2H), 3.77 (t, *J* = 7.3 Hz, 2H), 6.88–7.03 (m, 4H), 7.21–7.45 (m, 10H). ^{13}C NMR ($CDCl_3$): δ 25.33, 38.84, 49.86, 50.54, 56.20, 71.47, 121.38, 121.60, 126.49, 128.83, 129.64, 133.23, 136.18, 148.43. Anal. RP-HPLC Ib: t_R = 8.62 min (purity 100%), IIb: t_R = 11.11 min (purity 99%). HRMS (EI) *m*/*z* calcd for $C_{26}H_{29}ClN_2O$: 420.1968; found: 420.19699.

5.8. 1-(3-(10*H*-Phenothiazin-10-yl)propyl)-4-(4-chlorophenyl)piperidine-4-ol (10)

Following method C starting with 10*H*-phenothiazine gave 462 mg (34% over two steps) of **10** as a light yellow solid. Mp: 59.0–60.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.51–2.11 (m, 7H), 2.21–2.56 (m, 4H), 2.63–2.89 (m, 2H), 3.93 (t, *J* = 6.7 Hz, 2H), 6.87–7.41 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 25.34, 38.86, 45.61, 49.84, 55.99, 71.28, 115.97, 117.14, 122.90, 125.64, 126.47, 127.63, 127.89, 128.82, 133.26, 145.58. Anal. RP-HPLC Ia: t_R = 3.45 min (purity 97%), Ib (total run time 20 min): t_R = 13.93 min (purity 98%), IIb: t_R = 13.46 min (purity 99%). HRMS (EI) *m*/*z* calcd for $C_{26}H_{27}ClN_2OS$: 450.1533; found: 450.15394.

5.9. 1-(3-(2-Chloro-10*H*-phenothiazin-10-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (11)

Following method C starting with 2-chloro-10*H*-phenothiazine gave 376 mg (24% over two steps) of **11** as a light yellow solid. Mp: 66.1–66.7 °C. ^{1}H NMR ($CDCl_3$): δ 1.49–1.80 (m, 3H), 1.87–2.18 (m, 4H), 2.30–2.58 (m, 4H), 2.68–2.85 (m, 2H), 3.91 (t, *J* = 6.8 Hz, 2H), 6.84–7.42 (m, 11H). ^{13}C NMR ($CDCl_3$): δ 24.12, 38.33, 45.62, 49.84, 55.71, 71.01, 116.31, 116.33, 122.76, 123.42, 124.08, 125.35, 126.48, 127.87, 127.97, 128.34, 128.83, 133.30, 133.68, 144.77, 146.89. Anal. RP-HPLC Ib: t_R = 4.27 min (purity 97%), IIa: t_R = 6.45 min (purity 98%). HRMS (EI) *m*/*z* calcd for $C_{26}H_{26}Cl_2N_2OS$: 484.1143; found: 484.11331.

5.10. 1-(10-(3-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)propyl)-10*H*-phenothiazin-2-yl)ethanone (12)

Following method C starting with 1-(10*H*-phenothiazin-2-yl)ethanone gave 410 mg (22% over two steps) of **12** as a light yellow solid. Mp: 108.9–110.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.39–1.68 (m, 5H), 1.81–2.12 (m, 4H), 2.22–2.81 (m, 4H), 2.50 (s, 3H), 3.96 (t, *J* = 6.7 Hz, 2H), 6.79–6.92 (m, 2H), 7.01–7.42 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 23.71, 26.47, 37.90, 45.11, 49.22, 55.29, 70.70, 113.81, 115.76, 122.75, 123.00, 123.71, 125.92, 126.89, 127.33, 127.54, 128.24, 132.13, 132.65, 136.10, 144.19, 145.33, 197.31. Anal. RP-HPLC Ib: t_R = 10.46 min (purity 100%), IIb: t_R = 10.90 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{28}H_{29}ClN_2O_2S$: 492.1638; found: 492.16307.

5.11. 1-(4-(4-*tert*-Butylphenyl)-4-phenylbutyl)-4-(4-chlorophenyl)piperidin-4-ol (15)

This was synthesized following a method previously described[29] starting with 4-*tert*-butylbenzophenone

(1.43 g, 6.01 mmol) to give 801 mg (28% over three steps) of **15** as white crystals after recrystallization from hexane/EtOAc. Mp: 104.0–105.3 °C. ^{1}H NMR ($CDCl_3$): δ 1.26 (s, 9H), 1.48–1.72 (m, 5H), 2.02–2.14 (m, 4H), 2.28–2.44 (m, 4H), 2.64–2.79 (m, 2H), 3.85 (t, J = 7.8 Hz, 1H), 7.12–7.43 (m, 13H). ^{13}C NMR ($CDCl_3$): δ 27.14, 31.21, 33.15, 34.17, 37.31, 49.08, 50.62, 58.24, 70.45, 125.19, 125.89, 125.99, 127.13, 127.66, 128.30, 128.33, 132.91, 135.46, 141.59, 144.64, 148.71. Anal. RP-HPLC Ib: t_R = 5.24 min (purity 100%), IIa: t_R = 9.46 min (purity 99%). HRMS (EI) m/z calcd for $C_{31}H_{38}ClN_2O$: 475.2642; found: 475.26301.

5.12. (1-(3-Benzo[*d*]thiazol-2-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (16)

Following method B using 2-(3-chloropropyl)benzo[*d*]thiazole (0.636 g, 3.00 mmol), which was synthesized according to literature procedure,[37] gave 306 mg (26 %) of **16** as a light yellow solid after recrystallization from EtOAc. Mp: 109.3–110.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.61–1.76 (m, 3H), 2.21–2.39 (m, 4H), 2.50–2.79 (m, 4H), 2.93–3.08 (m, 2H), 3.18 (t, J = 7.3 Hz, 2H), 7.24–7.48 (m, 6H), 7.82–7.96 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 25.76, 31.85, 37.48, 49.11, 57.13, 70.51, 121.41, 122.36, 124.68, 125.86, 125.90, 128.34, 132.83, 134.98, 146.01, 152.97, 170.99. Anal. RP-HPLC Ic: t_R = 8.42 min (purity 99%), IIc: t_R = 10.70 min (purity 99%). HRMS (EI) m/z calcd for $C_{21}H_{23}ClN_2OS$: 386.1220; found: 386.12301.

5.13. 4-(4-Chlorophenyl)-1-(3-phenylpropyl)piperidin-4-ol (19)

Following method B using 1-bromo-3-phenylpropane (0.60 g, 3.03 mmol) afforded 729 mg (73%) of **19** as a light yellow solid. Mp: 95.7–96.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.60–1.95 (m, 5H), 2.03–2.21 (m, 2H), 2.36–2.49 (m, 4H), 2.64 (t, J = 7.7 Hz, 2H), 2.80–2.86 (m, 2H), 7.16–7.31 (m, 7H), 7.42 (d, J = 8.7 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 28.80, 34.12, 38.61, 49.76, 58.44, 71.39, 126.25, 126.49, 128.75, 128.77, 128.83, 133.26, 142.27, 147.06. Anal. RP-HPLC Ic: t_R = 6.17 min (purity 100%), IIc: t_R = 10.14 min (purity 100%). HRMS (EI) m/z calcd for $C_{20}H_{24}ClNO$: 329.1546; found: 329.15489.

5.14. 4-(4-Chlorophenyl)-1-(5-phenylpentyl)piperidin-4-ol (21)

(i) PBr_3 (0.22 mL, 2.34 mmol) was added to a solution of 5-phenyl-1-pentanol (0.82 g, 5.02 mmol) in Et_2O (25 mL) and this was stirred for 24 h at room temperature. Water (50 mL) was added and the solution was basified with K_2CO_3 followed by an extraction with Et_2O (3× 30 mL). The combined organic extracts were washed with water (100 mL) and brine (100 mL), dried over anhydrous $MgSO_4$ and filtered. Evaporation in vacuo and purification by flash chromatography (hexane) gave 555 mg (49%) of 1-bromo-5-phenylpentane as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.36–1.54 (m, 2H), 1.58–1.75 (m, 2H), 1.82–1.98 (m, 2H), 2.61 (t, J = 7.5 Hz, 2H), 3.38 (t, J = 6.8 Hz, 2H), 7.13–7.38 (m, 5H).

(ii) This was synthesized according to method B using 1-bromo-5-phenylpentane (0.55 g, 2.44 mmol) to give 666 mg (77%) of **21** as white crystals after recrystallization from EtOAc. Mp: 103.4–105.2 °C. ^{1}H NMR ($CDCl_3$): δ 1.30–1.42 (m, 2H), 1.50–1.73 (m, 7H), 2.02–2.19 (m, 2H), 2.34–2.42 (m, 4H), 2.60 (t, J = 7.7 Hz, 2H), 2.78–2.84 (m, 2H), 7.15–7.45 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 27.07, 27.66, 31.72, 36.25, 38.66, 49.82, 59.10, 71.41, 126.05, 126.50, 128.65, 128.78, 128.82, 133.22, 142.98, 147.15. Anal. RP-HPLC Ib: t_R = 4.57 min (purity 100%), IIb: t_R = 6.49 min (purity 100%). HRMS (EI) m/z calcd for $C_{22}H_{28}ClNO$: 357.1859; found: 357.18617.

5.15. 4-(4-Chlorophenyl)-1-(4,4-diphenylbut-3-enyl)piperidin-4-ol (22)

Following method B using **25** (1.00 g, 3.00 mmol), which was synthesized according to literature procedure,[38,39] followed by recrystallization from EtOAc/hexane gave 611 mg (49%) of **22** as a white solid. Mp 117.6–118.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.48–1.79 (m, 3H), 1.98–2.20 (m, 2H), 2.29–2.62 (m, 6H), 2.67–2.89 (m, 2H), 6.07 (t, J = 7.2 Hz, 1H), 7.15–7.44 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 27.44, 38.27, 49.13, 58.43, 70.88, 125.97, 126.06, 126.83, 126.89, 127.03, 127.08, 127.96, 128.09, 128.25, 129.66, 132.58, 139.86, 142.40, 142.55, 146.80. Anal. RP-HPLC Ib: t_R = 10.43 min (purity 100%), IIb: t_R = 13.29 min (purity 100%). HRMS (EI) m/z calcd for $C_{27}H_{28}ClNO$: 417.1859; found: 417.18446.

5.16. 4-(4-Chlorophenyl)-1-(4-phenylbut-3-enyl)piperidin-4-ol (23)

(i) A solution of HCl (6 mL) and **27** (0.55 g, 2.98 mmol), which was synthesized as previously described,[40] was heated and refluxed for 4.5 h. The reaction mixture was allowed to cool to room temperature, slowly basified with a saturated solution of Na_2CO_3, and the water layer was extracted with EtOAc (3× 25 mL). The combined organic extracts were washed with water (3× 50 mL) and brine (50 mL), and dried over anhydrous $MgSO_4$. Evaporation in vacuo and purification by flash chromatography (hexane) gave 99 mg (20%) of **28** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 2.61–2.72 (m, 2H), 3.61 (t, J = 6.9 Hz, 2H), 6.12–6.26 (dt, J = 6.9 Hz, 1H), 6.48 (d, J = 15.9 Hz, 1H), 7.19–7.38 (m, 5H).

(ii) Following method A using **28** (0.10 g, 0.59 mmol) gave 101 mg (50%) of **23** as white needles after recrystallization from EtOAc. Mp: 136.3–137.2 °C. ^{1}H NMR ($CDCl_3$): δ 1.51–1.82 (m, 3H), 2.02–2.30 (m, 2H), 2.38–2.70 (m, 6H), 2.79–3.00 (m, 2H), 6.12–6.29 (dt, J = 6.6 Hz, 1H), 6.44 (d, J = 16.0 Hz, 1H), 7.18–7.46 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 30.66, 38.30, 49.21, 58.21, 70.96, 125.83, 125.95, 126.89, 128.27, 128.36, 130.89, 133.01, 137.79. Anal. RP-HPLC Ic: t_R = 8.94 min (purity 100%), IIb: t_R = 4.79 min (purity 99%). HRMS (EI) m/z calcd for $C_{21}H_{24}ClNO$: 341.1546; found: 341.15600.

5.17. [1-(4,4-Diphenylbutyl)-piperidin-4-yl]-(4-fluorophenyl)methanone (31)

This was synthesized according to method B using 4-chloro-1,1-diphenylbutane[44] (1.59 g, 6.50 mmol) and 4-(4-fluorobenzoyl)piperidine toluenesulfonate (2.28 g, 6.00 mmol) to give 1.25 g (50%) of **31** as white crystals after recrystallization from EtOAc. Mp: 88.9–90.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.38–1.53 (m, 2H), 1.69–2.12 (m, 8H), 2.36 (t, J = 7.5 Hz, 2H), 2.80–2.99 (m, 2H), 3.06–3.23 (m, 1H), 3.88 (t, J = 7.8 Hz, 1H), 6.98–7.34 (m, 12H), 7.80–8.00 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 25.34, 28.59, 33.48, 43.64, 51.17, 53.12, 58.61, 115.36, 115.80, 125.94, 127.68, 128.27, 130.61, 130.79, 144.89, 162.90, 167.96, 200.92. Anal. RP-HPLC Ib: t_R = 8.30 min (purity 100%), IIa: t_R = 5.13 min (purity 100%), IIb: t_R = 16.06 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{28}H_{30}FNO$: 415.2311; found: 415.23053.

5.18. (1-(4,4-Diphenylbutyl)piperidin-4-yl)(4-fluorophenyl)methanol fumarate (32)

A solution of **31** (0.16 g, 0.38 mmol) and $NaBH_4$ (0.05 g, 1.32 mmol) in MeOH (20 mL) was stirred overnight at room temperature. The reaction mixture was evaporated in vacuo, water was added (15 mL), and the water layer was extracted with DCM (3× 10 mL). The combined organic extracts were washed with water (3× 25 mL) and brine (25 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo to give 149 mg of oil. This was dissolved in EtOAc and acidified by the addition of a saturated solution of fumaric acid in Et_2O. The fumaric salt was isolated by filtration and recrystallized from IPA/Et_2O to give 161 mg (79%) of **32** as a white solid. Mp: 77.4–79.3 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.29–1.79 (m, 7H), 1.91–2.40 (m, 5H), 2.61–2.83 (m, 2H), 3.08–3.40 (m, 2H), 3.77 (t, J = 7.9 Hz, 1H), 4.12–4.30 (m, 1H), 6.64 (s, 2H), 6.89 (t, J = 8.7 Hz, 2H), 7.05–7.24 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 22.03, 25.75, 32.32, 41.46, 50.48, 56.29, 77.08, 114.81, 115.23, 126.25, 127.51, 127.85, 128.00, 128.45, 135.12, 138.56, 143.89, 159.51, 170.43. Anal. RP-HPLC Ib: t_R = 6.38 min (purity 100%), IIb: t_R = 10.26 min (purity 96%). HRMS (EI) *m*/*z* calcd for $C_{28}H_{32}FNO$: 417.2468; found: 417.24489.

5.19. 5-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-2,2-diphenylpentanenitrile (33)

Following method B using 1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one (0.83 g, 3.61 mmol) afforded 842 mg (60%) of **33** after recrystallization from $CHCl_3$. Mp: 204.1–206.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.60–1.76 (m, 4H), 2.38–2.68 (m, 10H), 4.70 (s, 2H), 6.45 (s, 1H), 6.80–6.92 (m, 3H), 7.24–7.42 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 23.22, 28.84, 37.34, 49.58, 51.49, 57.38, 59.14, 115.11, 118.75, 122.23, 126.70, 127.71, 128.74, 129.11, 140.07, 142.92, 177.93. Anal. RP-HPLC Ib: t_R = 5.41 min (purity 100%), IIb: t_R = 8.17 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{30}H_{32}N_4O$: 464.2576; found: 464.25859.

5.20. 1-Phenyl-8-(4-phenyl-4-*o*-tolylbutyl)-1,3,8-triaza-spiro[4.5]decan-4-one (34)

Following method A using 1-(4-chloro-1-phenylbutyl)-2-methylbenzene[29] (0.16 g, 0.52 mmol) and 1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one (0.14 g, 0.62 mmol) gave 188 mg (80%) of **34** as white crystals after recrystallization from hexane/$CHCl_3$. Mp: 184.3–186.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.39–1.83 (m, 4H), 1.93–2.11 (m, 2H), 2.19 (s, 3H), 2.33–2.81 (m, 8H), 4.11 (t, J = 7.7 Hz, 1H), 4.80 (s, 2H), 6.61 (br s, 1H), 6.86–6.92 (m, 2H), 7.00–7.38 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 19.78, 29.00, 33.97, 41.28, 46.59, 49.57, 58.24, 58.89, 115.36, 118.89, 125.76, 125.85, 126.41, 128.04, 128.15, 129.12, 130.34, 136.14, 177.56. Anal. RP-HPLC Ib: t_R = 8.12 min (purity 100%), IIb: t_R = 11.45 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{30}H_{35}N_3O$: 453.2780; found: 453.27945.

5.21. 5-(2-Oxo-1,2-dihydrospiro[benzo[*d*][1,3]oxazine-4,4′-piperidine]-1′-yl)-2,2-diphenylpentanenitrile (35)

Following method A using spiro[benzo[*d*][1,3]oxazine-4,4′-piperidin]-2(1*H*)one (0.41 g, 1.62 mmol), which was synthesized according to literature procedure,[41] afforded 416 mg (57%) of **35** as a white solid. Mp: 172.7–174.3 °C. ^{1}H NMR ($CDCl_3$): δ 1.56–1.78 (m, 2H), 1.96–2.21 (m, 4H), 2.34–2.83 (m, 8H), 6.83 (d, J = 9.0 Hz, 2H), 6.98–7.56 (m, 12H), 8.89 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 23.65, 35.76, 37.83, 48.59, 52.04, 58.08, 81.91, 114.97, 122.71, 123.66, 124.08, 127.27, 128.28, 129.28, 129.44, 124.71, 140.57, 152.52. Anal. RP-HPLC Ic: t_R = 9.84 min (purity 100%), IIb: t_R = 4.58 min (purity 99%). HRMS (EI) *m*/*z* calcd for $C_{29}H_{29}N_3O_2$: 451.2260; found: 451.22625.

5.22. 5-(4-(5-Chloro-2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazol-1-yl)piperidin-1-yl)-2,2-diphenylpentanenitrile (36)

Following method A using 5-chloro-1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one (0.50 g, 2.00 mmol) gave 734 mg (76%) of **36** as a white solid. Mp: 203.1–204.6 °C. ^{1}H NMR (DMSO-d_6): δ 1.35–1.68 (m, 4H), 1.82–2.09 (m, 2H), 2.20–2.59 (m, 6H), 2.77–2.98 (m, 2H), 4.02–4.23 (m, 1H), 6.98–7.04 (m, 2H), 7.20–7.45 (m, 11H), 11.05 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 23.23, 29.02, 37.27, 50.85, 51.49, 52.98, 57.51, 110.21, 120.87, 122.21, 126.66, 126.72, 127.52, 127.77, 128.61, 128.75, 128.82, 139.99, 155.03. Anal. RP-HPLC Ib: t_R = 5.81 min (purity 100%), IIb: t_R = 8.83 min (purity 100%). HRMS (EI) *m*/*z* calcd for $C_{29}H_{29}ClN_4O$: 484.2030; found: 484.20548.

5.23. 5-(3-Oxo)-3*H*-spiro[isobenzofuran-1,4′-piperidine]-1′-yl-2,2-diphenylpentanenitrile hydrochloride (37)

A solution of *o*-bromobenzoic acid (0.76 g, 3.76 mmol) in THF (20 mL) was cooled to −78 °C and *n*-BuLi (4.7 mL, 1.6 M in hexane, 7.52 mmol) was added slowly in a period of 20 min. The solution was stirred for 2 h at −78 °C and **39** (1.5 g, 4.51 mmol), which was synthesized according to literature procedure,[31] was added slowly in a period of 30 min. The reaction mixture was

allowed to warm to room temperature and water (30 mL) was added. The water layer was extracted with Et_2O (5× 10 mL) and acidified with HCl until a pH of 2. The solution was heated to reflux temperature for 1 h, cooled to room temperature, and stirred overnight. A 10% NaOH solution was added to a pH of 10, and the water layer was rapidly extracted with $CHCl_3$ (5× 20 mL). The combined organic extracts were washed with water (3× 10 mL), brine (20 mL), dried over anhydrous $MgSO_4$, filtered and evaporated in vacuo to afford 1.01 g of a white solid. This was converted to the hydrochloride salt as described for **2** to give 516 mg (31%) of **37** after recrystallization from MeOH/Et_2O. Mp: 129.8–131.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.68–1.92 (m, 4H), 2.48–2.91 (m, 8H), 2.98–3.21 (m, 2H), 7.18–7.60 (m, 12H), 7.66 (d, J = 9.0 Hz, 1H), 7.86 (d, J = 9.0 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 21.60, 34.28, 36.80, 49.27, 51.40, 57.08, 82.78, 121.26, 121.94, 125.00, 125.88, 126.56, 127.86, 128.91, 129.63, 134.49, 139.51, 152.25, 169.01. Anal. RP-HPLC Ic: t_R = 9.86 min (purity 98%), IIb: t_R = 5.64 min (purity 95%). HRMS (EI) m/z calcd for $C_{29}H_{28}N_2O_2$: 436.2151; found: 436.21533.

5.24. 1-(1-(4,4-Diphenylbutyl)piperidin-4-yl)indolin-2-one (38)

(i) NaH (1.96 g, 49.00 mmol) was added in 5 min to a solution of di-*t*-butyl malonate (10 mL, 44.61 mmol) in DMF (250 mL) and this was stirred for 15 min. Subsequently, 2-fluoro-nitrobenzene (4.95 mL, 46.83 mmol) was added in one portion and the reaction mixture was stirred for 8 h at 90 °C and two days at room temperature. Water (300 mL) was added and the water layer was extracted with Et_2O (3× 200 mL). The combined organic extracts were washed with water (4× 150 mL) and brine (150 mL), dried over Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (20% EtOAc in hexane) to give 3.0 g (20%) of di-*t*-butyl 2-(2-nitrophenyl)malonate as a light brown solid. ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 18H), 5.09 (s, 1H), 7.39–7.78 (m, 3H), 8.01 (d, J = 9.1 Hz, 1H).

(ii) This was dissolved in EtOAc (100 mL) and 10% Pd/C (0.35 g) was added. The reaction mixture was hydrogenated with H_2 (5 bar) overnight, filtrated over Hyflo, and washed with EtOAc. After evaporation in vacuo, the residue was purified by recrystallization (Et_2O/hexane) to give 915 mg (34%) of **41** as purple crystals. ^{1}H NMR ($CDCl_3$): δ 1.43 (s, 18H), 4.48 (s, 1H), 6.62–6.78 (m, 2H), 7.03–7.19 (m, 2H).

(iii) Both **41** (0.31 g, 1.00 mmol) and **40** (0.31 g, 1.00 mmol), which was synthesized following literature procedures,[29,31] were dissolved in 1,2-dichloroethane (10 mL). Next, molsieves (4 Å) and AcOH (0.06 mL, 1.00 mmol) were added. The reaction mixture was stirred for 30 min, $NaBH(OAc)_3$ (0.30 g, 1.42 mmol) was added, and this was stirred for another 24 h. A saturated solution of $NaHCO_3$ (15 mL) was added and the water layer was extracted with Et_2O (3× 20 mL). The combined organic extracts were washed with water (3× 25 mL) and brine (25 mL), dried over $MgSO_4$, filtered, and evaporated in vacuo to give 520 mg of **42** as a white solid. The crude product was used without further purification.

(iv) This was dissolved in toluene (20 mL) and *p*-toluene sulfonic acid (0.19 g, 9.99 mmol) was added. The reaction mixture was refluxed for 3 h, quenched with a saturated solution of $NaHCO_3$ (25 mL), and the water layer was extracted with Et_2O (3× 15 mL). The combined organic extracts were washed with water (3× 20 mL) and brine (20 mL), dried over anhydrous $MgSO_4$, and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0–50% EtOAc in DCM) and recrystallized from Et_2O to give 112 mg (26%) of **38** as white crystals. Mp: 139.2–140.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.31–1.78 (m, 4H), 1.91–2.19 (m, 4H), 2.22–2.50 (m, 4H), 2.95 (m, 2H), 3.48 (s, 2H), 3.89 (t, J = 7.7 Hz, 1H), 4.19-4.35 (m, 1H), 7.02–7.31 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 26.04, 28.45, 31.30, 34.01, 36.25, 50.27, 51.73, 53.69, 58.81, 110.91, 122.19, 124.89, 125.19, 126.52, 127.92, 128.24, 128.83, 144.07, 145.42, 175.25. Anal. RP-HPLC Ib: t_R = 5.58 min (purity 99%), IIb: t_R = 9.11 min (purity 99%). HRMS (EI) m/z calcd for $C_{29}H_{32}N_2O$: 424.2515; found: 424.25005.

5.25. 5-(4-Acetyl-4-phenylpiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (44)

Following method A using 1-(4-phenylpiperidin-4-yl)ethanone (0.48 g, 2.00 mmol) gave 728 mg of an oil. This was converted to the hydrochloride salt as described for **2** and recrystallized from MeOH/Et_2O to give 552 mg (54 %) of **44** as a white solid. Mp: 247.1–249.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.75–2.02 (m, 2H), 1.91 (s, 3H), 2.42–3.08 (m, 10H), 3.29–3.51 (m, 2H), 7.11–7.56 (m, 15H), 12.44 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 20.11, 25.13, 27.23, 28.92, 29.61, 34.87, 48.43, 49.59, 51.17, 121.72, 125.25, 125.53, 126.19, 127.39, 127.85, 139.05, 139.42, 207.72. Anal. RP-HPLC Ib: t_R = 4.84 min (purity 100%), IIb: t_R = 10.56 min (purity 100%). HRMS (EI) m/z calcd for $C_{30}H_{32}N_2O$: 436.2515; found: 436.25253.

5.26. 4-(4-Chlorophenyl)-1-(4-cyano-4,4-diphenylbutyl)piperidin-4- yl acetate fumarate (45)

Et_3N (0.12 mL, 0.9 mmol) and acetyl chloride (0.02 mL, 0.28 mmol) were added to a solution of **1** (0.13 g, 0.30 mmol) in DCM (5 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and 1 h at room temperature. Water (10 mL) was added and the water layer was extracted with DCM (3× 10 mL). The combined organic extracts were washed with water (3× 15 mL) and brine (15 mL), dried over anhydrous $MgSO_4$, and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0–25% EtOAc in DCM) to afford 89 mg of a thick oil. This was converted to the fumaric salt as described for **32** and recrystallized from MeOH/Et_2O to give 75 mg (41%) of **45** as white crystals. Mp: 176.4–177.3 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.42–1.69 (m, 2H), 1.81–2.07 (m, 5H), 2.16–2.56 (m, 8H), 2.65–2.83 (m, 2H), 6.59 (s, 2H), 7.11–7.24 (m, 14H). ^{13}C NMR (DMSO-d_6): δ 21.46, 34.51, 35.57, 48.27, 51.19, 56.18, 64.70, 78.96, 122.21,

126.21, 126.38, 127.68, 127.94, 128.82, 131.37, 133.92, 139.79, 143.16, 166.01, 168.76. Anal. RP-HPLC Ib: t_R = 6.42 min (purity 100%), IIa: t_R = 5.39 min (purity 100%), IIb: t_R = 17.41 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{30}H_{31}ClNO_2$: 486.2074; found: 486.20619.

5.27. (1-(4,4-Diphenylbutyl)-4-phenylpiperidin-4-yl)methanamine (47)

(i) Following method B using 4-chloro-1,1-diphenylbutane[44] (1.22 g, 4.98 mmol) and **53** (0.92 g, 4.15 mmol) gave 1.59 g (81%) of **55** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.36–1.71 (m, 2H), 1.99–2.21 (m, 6H), 2.30–2.56 (m, 4H), 2.88–3.05 (m, 2H), 3.88 (t, 1H, J = 7.8 Hz), 7.09–7.51 (m, 15H).

(ii) A suspension of $AlCl_3$ (0.93 g, 6.98 mmol) in THF (10 mL) was added to a suspension of $LiAlH_4$ (0.26 g, 6.85 mmol) in THF (10 mL) at 0 °C and this was stirred for 5 min followed by the dropwise addition of a solution of **55** (1.36 g, 3.45 mmol) in THF (15 mL). The reaction mixture was allowed to warm to room temperature, stirred overnight, cooled with an ice bath, and quenched with a saturated solution of Na_2CO_3 in water until the foaming stopped. Subsequently, the suspension was filtered, and the filtrate was dried over anhydrous $MgSO_4$. Filtration, evaporation in vacuo, and purification by flash chromatography (5% Et_3N in EtOAc) afforded 863 mg (63%) of **47** as a white solid. Mp: 228.2–230.2 °C. ^{1}H NMR (MeOH-d_4): δ 1.53–1.72 (m, 2H), 1.98–2.21 (m, 4H), 2.42–2.99 (m, 6H), 3.15 (s, 2H), 3.21–3.40 (m, 2H), 3.94 (t, J = 7.9 Hz, 1H), 7.02–7.54 (m, 15H). ^{13}C NMR (DMSO-d_6): δ 22.45, 29.81, 31.80, 47.69, 49.87, 55.33, 64.70, 125.87, 126.70, 126.98, 127.31, 127.48, 128.21, 128.79, 144.60. Anal. RP-HPLC Ic: t_R = 3.91 min (purity 100%), IIc: t_R = 4.44 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{28}H_{34}N_2$: 398.2722; found: 398.27414.

5.28. *N*-((1-(4,4-Diphenylbutyl)-4-phenylpiperidin-4-yl)methyl)formamide hydrochloride (48)

Compound **47** (0.11 g, 0.23 mmol) and formic acid (2 mL) were added in a 10 mL microwave vessel and this was reacted during 5 min in the microwave at a temperature of 200 °C (settings: ramp time 5 min, hold time 5 min, power 200 watt, pressure 17.2 bar). The reaction mixture was quenched with a saturated solution of Na_2CO_3 (2 mL), and the water layer was extracted with DCM (3 × 5 mL). The combined organic extracts were dried over $MgSO_4$ and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (5% Et_3N in EtOAc) and the free base was converted to the hydrochloride salt as described for **2** to give 100 mg (94%) of **48** as a white solid. Mp: 52.2–52.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.42–1.74 (m, 2H), 1.86–2.42 (m, 8H), 2.50–2.89 (m, 2H), 3.10–3.29 (m, 2H), 3.39 (d, J = 7.6 Hz, 2H), 3.87 (t, J = 7.8 Hz, 1H), 5.05 (br s, 1H), 7.01–7.61 (m, 15H), 8.03 (s 1H). ^{13}C NMR ($CDCl_3$): δ 24.62, 31.69, 32.24, 33.72, 49.83, 51.45, 58.51, 126.65, 127.42, 128.14, 128.87, 129.00, 129.60, 144.51, 144.96, 161.78. Anal. RP-HPLC Ib: t_R = 5.20 min (purity 100%), IIb: t_R = 6.08 min (purity 93%). HRMS (EI) *m/z* calcd for $C_{29}H_{34}N_2O$: 426.2671; found: 426.26775.

5.29. 1-(1-(4,4-Diphenylbutyl)-4-phenylpiperidin-4-ylmethyl)-*N*- methylmethanamine dihydrochloride (49)

$LiAlH_4$ (0.75 g, 19.76 mmol) was added to a solution of **48** (0.20 g, 0.47 mmol) in THF (10 mL) at 0 °C. The reaction mixture was refluxed for 6 h, quenched with a saturated solution of Na_2CO_3 and the water layer was extracted with DCM (3× 10 mL). The combined organic extracts were dried over anhydrous $MgSO_4$ and evaporated in vacuo. The residue was purified by flash chromatography (5% Et_3N in EtOAc) to give 150 mg of a white solid. This was converted to the corresponding hydrochloride salt as described for **2** to give 104 mg (46%) of **49** as a white solid. Mp: 50.9–52.5 °C. ^{1}H NMR (MeOH-d_4): δ 1.57–1.79 (m, 2H), 1.98–2.27 (m, 4H), 2.59 (s, 3H), 2.60–2.82 (m, 4H), 2.92–3.06 (m, 2H), 3.18–3.30 (m, 2H), 3.40–3.57 (m, 2H), 3.88–4.09 (m, 1H), 7.10–7.33 (m, 10H), 7.36–7.61 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 22.66, 30.45, 35.42, 40.00, 49.34, 51.01, 55.53, 57.59, 61.17, 127.33, 128.08, 128.27, 128.98, 129.03, 130.66, 137.48, 144.28. Anal. RP-HPLC Ic: t_R = 3.52 min (purity 100%), IIc: t_R = 4.63 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{29}H_{36}N_2$: 412.2878; found: 412.28610.

5.30. (4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)piperidin-4-yl)methanamine dihydrochloride (50)

(i) Et_3N (20 mL) was added to a solution of bis-(2-chloroethyl)amine hydrochloride (8.17 g, 45.77 mmol) in DCM (75 mL) and this was stirred for 15 min. After the addition of di-*tert*-butyl dicarbonate (10.00 g, 45.82 mmol) in DCM (50 mL), the reaction mixture was stirred for 20 h and quenched with water (200 mL). The water layer was extracted with DCM (4× 100 mL) and the combined organic extracts were dried over anhydrous Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (30% hexane in DCM) to give 3.90 g (35%) of **57** as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 9H), 3.49–3.70 (m, 8H).

(ii) A suspension of $NaNH_2$ (0.80 g, 20.51 mmol) in toluene (10 mL) was added to a solution of 2-(4-chlorophenyl)acetonitrile in toluene (10 mL) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and added drop wise to a solution of **57** (2.78 g, 10.01 mmol) in toluene (50 mL) at 0 °C. This was heated to 70 °C, stirred overnight, and quenched with water (100 mL). The organic layer was separated and the water layer was extracted with DCM (3× 50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and filtered. Evaporation in vacuo and purification by flash chromatography (DCM) gave 600 mg (19%) of **58** as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.44 (s, 9H), 1.72–2.11 (m, 4H), 3.04–3.29 (m, 2H), 4.13–4.35 (m, 2H), 7.31–7.49 (m, 4H).

(iii) This was dissolved in EtOH (30 mL), which was saturated with hydrochloride gas, and the resulting

reaction mixture was stirred for 5 min. Evaporation in vacuo gave a quantitative yield of **54** as a white solid. ^{1}H NMR (DMSO-d_6): δ 2.31–2.44 (m, 4H), 2.99–3.20 (m, 2H), 3.39–3.48 (m, 2H), 7.49–7.62 (m, 4H).

(iv) Following method B using 4-chloro-1,1-diphenylbutane[44] (0.56 g, 2.28 mmol) and **54** (0.49 g, 1.90 mmol) afforded 500 mg of **56** as a white solid (62%). ^{1}H NMR ($CDCl_3$): 1.32–1.72 (m, 2H), 1.90–2.08 (m, 6H), 2.23–2.59 (m, 4H), 2.72–3.02 (m, 2H), 3.91 (t, J = 7.8 Hz, 1H), 6.99–7.52 (m, 14H).

(v) This was synthesized as described for **47** starting from **56** (0.30 g, 0.47 mmol) to give 130 mg of oil. This was converted to the hydrochloride salt as described for **2** to give 100 mg (42%) of **50** as a white solid. Mp: 257.3–258.9 °C (dec). ^{1}H NMR (MeOH-d_4): δ 1.57–1.80 (m, 2H), 2.01–2.29 (m, 6H), 2.56–2.81 (m, 2H), 2.92–3.20 (m, 4H), 3.32–3.54 (m, 2H), 3.82–4.00 (m, 1H), 7.09–7.59 (m, 14H). ^{13}C NMR (DMSO-d_6): δ 21.80, 29.23, 30.48, 31.64, 47.21, 49.80, 54.98, 60.16, 125.92, 127.30, 128.23, 128.80, 131.97, 135.28, 144.46. Anal. RP-HPLC Ic: t_R = 6.07 min (purity 100%), IIc: t_R = 7.57 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{28}H_{33}ClN_2$: 432.2332; found: 432.23193.

5.31. *N*-(4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)-piperidin-4-yl)acetamide (51)

A solution of 95% H_2SO_4 (3.3 mL, 12.4 mmol) was added dropwise to a solution of **59** (1.37 g, 3.26 mmol) in CH_3CN (16 mL), while the temperature was maintained between 25 °C and 30 °C. The reaction mixture was stirred overnight at room temperature, poured into ice, and neutralized with a 30% solution of NaOH. Water was added (20 mL) and the water layer was extracted with DCM (3× 30 mL). The combined organic layers were washed with water (3× 30 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0–50% MeOH in EtOAc) and recrystallized from EtOH/Et_2O to give 655 mg (75%) of **51** as a white solid. Mp: 189.1–190.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.39–1.83 (m, 6H), 1.89–2.50 (m, 6H), 1.98 (s, 3H), 2.67–2.88 (m, 2H), 3.88 (t, J = 7.8 Hz, 1H), 5.52 (br s, 1H), 7.13–7.27 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 24.02, 24.52, 33.14, 34.32, 49.21, 50.97, 55.54, 57.82, 126.14, 126.47, 127.30, 127.59, 128.38, 132.51, 143.40, 144.45, 169.58. Anal. RP-HPLC Ib: t_R = 5.66 min (purity 100%), IIb: t_R = 8.20 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{29}H_{33}ClN_2O$: 460.2281; found: 460.22991.

5.32. 4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)piperidin-4-amine fumarate (52)

A solution of **51** (0.66 g, 1.42 mmol) in 8% HCl (240 mL) was refluxed for three days. Water was added (40 mL), the water layer was basified with a 20% solution of NaOH and extracted with DCM (3× 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (5% MeOH and 1% Et_3N in EtOAc) to give 290 mg of a white solid. This was converted to the fumaric salt as described for **29** to give 110 mg (19%) of **52** as white crystals after recrystallization from MeOH/Et_2O. Mp: 189.9–190.7 °C. ^{1}H NMR (MeOH-d_4): δ 1.57–1.68 (m, 2H), 1.98–2.08 (m, 2H), 2.08–2.18 (m, 2H), 2.13–2.18 (s, 2H), 2.31–2.42 (m, 2H), 1.82–2.95 (m, 4H), 3.15–3.24 (m, 2H), 3.96 (t, J = 7.8 Hz, 1H), 7.11–7.19 (m, 2H) 7.23–7.32 (m, 8H) 7.44 (d, J = 8.7 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H). ^{13}C NMR (DMSO-d_6): δ 23.88, 35.02, 48.17, 50.04, 52.48, 56.42, 125.78, 127.32, 127.79, 127.90, 128.16, 131.43, 134.70, 144.85, 167.23. Anal. RP-HPLC Ic: t_R = 5.85 min (purity 100%), IIc: t_R = 6.63 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{27}H_{31}ClN_2$: 418.2176; found: 418.21760.

5.33. Pharmacology

5.33.1. Transient and stable expression of US28. COS-7 cells were grown as described previously.[50] Transient transfection of COS-7 cells was performed by DEAE–dextran, using 2 μg of US28-pcDEF3 DNA per million cells.[50] SVEC4-10 (ATCC CRL2181) is a cell line derived by SV40 transformation of endothelial cells from the murine axillary lymph node vessels. SVEC4-10 cells were grown as described previously.[50] SVEC4-10 cells were transfected with US28-pTJE8 using 25 kDa linear polyethylenimine (PEI; Polysciences, Inc.). 'Empty' vector was used for mock transfections. Briefly, 10 μg DNA and 50 μg PEI were separately diluted into 250 μL of 150 mM NaCl solution. The PEI solution was added to the DNA solution, vortexed and incubated for 15 min at room temperature. One million SVEC4-10 cells were seeded in a 10-cm dish the day before transfection. Culture medium was replaced by 6 mL DMEM and the DNA/PEI solution was added dropwise to the cells. The solution was mixed by gently shaking and the dish was incubated at 37 °C for 5 h. The transfection solution was replaced by culture medium and incubated O/N. SVEC4-10 cells stably expressing US28 were selected in culture medium containing 500 μg/mL neomycin G418. Clones were selected based on US28-mediated constitutive IPx accumulation and chemokine binding analysis.

5.33.2. [^{125}I]CCL5 binding experiments. Radioiodination [^{125}I] of CCL5 (Peprotech, Rocky Hill, NJ) was performed using iodogen (Pierce Chemical Co., Rockford, IL) as described previously.[51] Displacement binding experiments were performed on US28-expressing COS-7 cell membranes. Two days after transfection, COS-7 cells were homogenized in ice-cold buffer A (i.e., 15 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA, and 1 mM EGTA) and centrifuged at 200*g* for 10 min. Supernatant was collected and centrifuged at 48,000*g* for 30 min. The pellet was resuspended in buffer B (i.e., 7.5 mM Tris–HCl, pH 7.5, 12,5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, and 250 mM sucrose), aliquoted, and stored at −80 °C until use. Cell membranes (approximately 0.2 μg/sample) were incubated with 0.3 nM [^{125}I]CCL5 in binding buffer (i.e., 50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM NaCl, and 0.2% bovine serum albumin) in the absence or presence of various concentrations of nonpeptidergic

ligands at 37 °C for 1 h. Incubations were terminated by filtration through a UniFilter-96 GF/C filter plate (Perkin-Elmer Life Sciences, Wellesley, MA) presoaked in 0.3% PEI, followed by three rapid washes with ice-cold binding washing buffer (i.e., 50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 500 mM NaCl) using a MicroBeta FilterMate-96 Harvester (PerkinElmer). Non-specific binding was determined on membranes from mock-transfected cells. Radioactivity was quantified with a liquid scintillation using a Wallac MicroBeta TriLux (PerkinElmer).

5.33.3. [^{3}H]inositol phosphate accumulation. SVEC4-10 cells (approximately 4×10^5 cells/well) were seeded into poly-L-lysine-coated 96-well plates in 200 μL culture medium. After approximately 24 h, medium was replaced with 100 μL/well inositol-free Dulbecco's modified Eagle's medium supplemented with 10 μCi/mL *myo*-[2-^{3}H]-inositol (PerkinElmer) and the cells were further incubated. The next day, cells swere washed and preincubated for 10 min with assay buffer (i.e., Dulbecco's modified Eagle's medium containing 10 mM LiCl). Next, cells were incubated in assay buffer in the absence or presence of various concentrations of nonpeptidergic ligands at 37 °C for 2 h. Inositol phosphates were extracted using 10 mM formic acid and quantified using 0.5 mg/well Ysi-RNA-binding SPA beads (Amersham Biosciences), in white clear-bottomed 96-well isoplates (PerkinElmer) using a Wallac MicroBeta TriLux, essentially as previously described.[52]

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