Bioorganic & Medicinal Chemistry 20 (2012) 5623–5636

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and pharmacological evaluation of indolinone derivatives as novel ghrelin receptor antagonists

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ARTICLE INFO

Article history:
Received 9 March 2012
Revised 1 June 2012
Accepted 11 July 2012
Available online 24 July 2012

Keywords:
Ghrelin
GHSR1a antagonists
Indolinone derivatives

ABSTRACT

The ghrelin receptor is a G-protein-coupled receptor (GPCR) widely expressed in the brain, stomach and the intestine. It was firstly identified during studies aimed to find synthetic modulators of growth hormone (GH) secretion. GHSR and its endogenous ligand ghrelin were found to be involved in hunger response. Through food intake regulation, they could affect body weight and adiposity. Thus GHSR antagonists rapidly became an attractive target to treat obesity and feeding disorders. In this study we describe the biological properties of new indolinone derivatives identified as a new, chiral class of ghrelin antagonists. Their synthesis as well as the structure–activity relationship will be discussed herein. The in vitro identified compound **14f** was a potent GHSR1a antagonist (IC_{50} = 7 nM). When tested in vivo, on gastric emptying model, **14f** showed an inhibitory intrinsic effect when given alone and it dose dependently inhibited ghrelin stimulation. Compound **14f** also reduced food intake stimulated both by fasting condition (high level of endogenous ghrelin) and by icv ghrelin. Moreover this compound improved glucose tolerance in ipGTT test.

1. Introduction

Ghrelin and its receptor (GHSR) have been shown to affect several important physiological activities such as food intake regulation, energy balance, gastric motility and secretion, as well as glucose metabolism, fat accumulation and cell proliferation.[1,2]

Ghrelin is a 28-amino acid hormone predominantly produced by P/D1 cells of the stomach and small intestine, but is also present in minor quantity in the kidneys, pancreas and hypothalamus.[3] It was discovered in 1999 and recognized as the endogenous bioactive ligand for the Growth Hormone Secretagogue receptor (GHSR).[4] Ghrelin presents an unusual *n*-octanoyl acetylation at serine 3 essential for its biological activity. It has been postulated that this acetylation is also critical for the transport of the ghrelin molecule across the blood–brain barrier into the brain where the receptor is highly expressed.[5]

The ghrelin receptor, GHSR, is a member of the G-protein-coupled receptor (GPCR) superfamily.[6] Two different splice forms of the human GHSR are known; however, only GHSR1a is activated by ghrelin and its mimetics, whereas the role of GHSR1b, a truncated form of GHSR1a, is unknown.[7] GHSR1a is widely expressed in the brain and peripheral tissues, especially in the stomach. It was identified and cloned by Howard et al. in 1996.[8] Its discovery was the result of an extensive research on synthetic modulators of GH secretion seen as a new alternative treatment to growth hormone replacement therapy.[9–11] Interestingly, GHSR1a showed a constitutive activity, independent from the presence of its ligand, being able to change into an active conformation in the absence of the agonist.[12,13]

GHSR1a, with its ligand, ghrelin, is predominantly involved in the hunger perception prior to mealtimes. Ghrelin circulating levels decrease with feeding and increase by fasting achieving concentrations sufficient to stimulate hunger and food intake. Administration of exogenous ghrelin potently stimulates food intake; this effect was more efficient than that of any other molecule, with the exception of neuropeptide Y.[14] Chronic administration of ghrelin in freely feeding mice and rats, increased body weight and adiposity.[15]

Due to its involvement in feeding behavior, in energy homeostasis and body weight regulation, ghrelin has become an attractive target to treat obesity and feeding disorders. Moreover, ghrelin seems to play a direct role on glucose homeostasis through regulation of insulin secretion.[16–18]

Those findings support the potential therapeutic role of ghrelin antagonists in diabetes.

However, while GHSR agonists have been extensively studied, due to their role in GH release, relatively fewer antagonists are described in literature.[19–23] Some of these compounds are shown in Figure 1.

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http://dx.doi.org/10.1016/j.bmc.2012.07.018

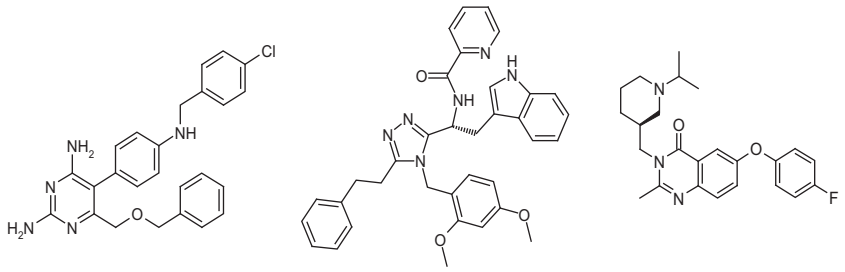


Figure 1. Structure of known GHSR1a antagonists.

As part of our efforts to develop ghrelin receptor antagonists we identified, through HTS screen of the Sanofi-Aventis compound library, an indolinone scaffold as exemplified by the general formula **1** (Fig. 2).

This class of compounds has been previously identified as Vasopressin (V1) receptor antagonists.[24] We designed new molecules devoid of vasopressin activity, with lower molecular weight and improved in vitro ADME profile. We first investigated the influence of *N*-sulphonylation of the indolinone nitrogen atom on GHSR1a antagonism. This group was known to be essential for V1 antagonism. The urea linker was modified with the aim to ameliorate bioavailability. This synthetic strategy also led to the identification of novel, enantiopure compounds.

The hit compound **2** (Fig. 2), with an IC_{50} of 47 nM in functional CreLuc assay, represented an excellent starting point for new leads identification.[25]

Interestingly, during our SAR studies, we could observe that slight modifications in scaffold decoration, could lead to agonist activity. Indeed a series of oxindole derivatives, here represented by SM-130686 (Fig. 3), have already been published by Sumitomo, and reported to be GHSR agonists.[26]

Our compounds, however, basically differ from Sumitomo's class of molecules, especially for the presence of a substituted amide at the indolinone C-3 position and for this carbon configuration.

Looking for new molecules we focused our attention on compounds possessing a full GHSR1a antagonist activity only. Modifications both at the basic moiety and at the aromatic portions were largely evaluated and will be discussed hereafter.

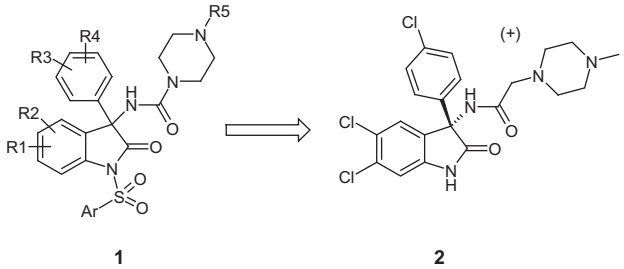


Figure 2. Hit identification. R1, R2, R3, R4 are independently hydrogen, chlorine or methyl groups, R5 is a C 1–3 alkyl group or, at least, a phenyl. Ar represents a benzene substituted with small groups as chlorine or methoxy groups.

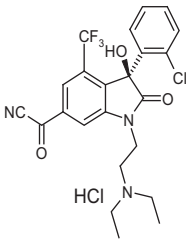


Figure 3. Structure of SM-130686.

2. Chemistry

The synthetic pathway leading to the Hit compound **2** is reported on Scheme 1.

Starting from commercially available 1,2-dichloro-4-fluoro-5-nitrobenzene and methyl-4-chlorophenylacetate, an aromatic S_N reaction provided the intermediate **3**, which was transformed in the halogen derivative **4** by the use of phenyltrimethylammonium tribromide. Other reagents more commonly used for benzylic halogenation, such as NBS, failed in giving the desired product. In some cases the halogenations proceeded also to the aromatic ring positions. Moreover, it was observed that halogen derivative easily undergoes degradation to form several byproducts. Due to its instability it was therefore rapidly used for the next reaction step without further purification.

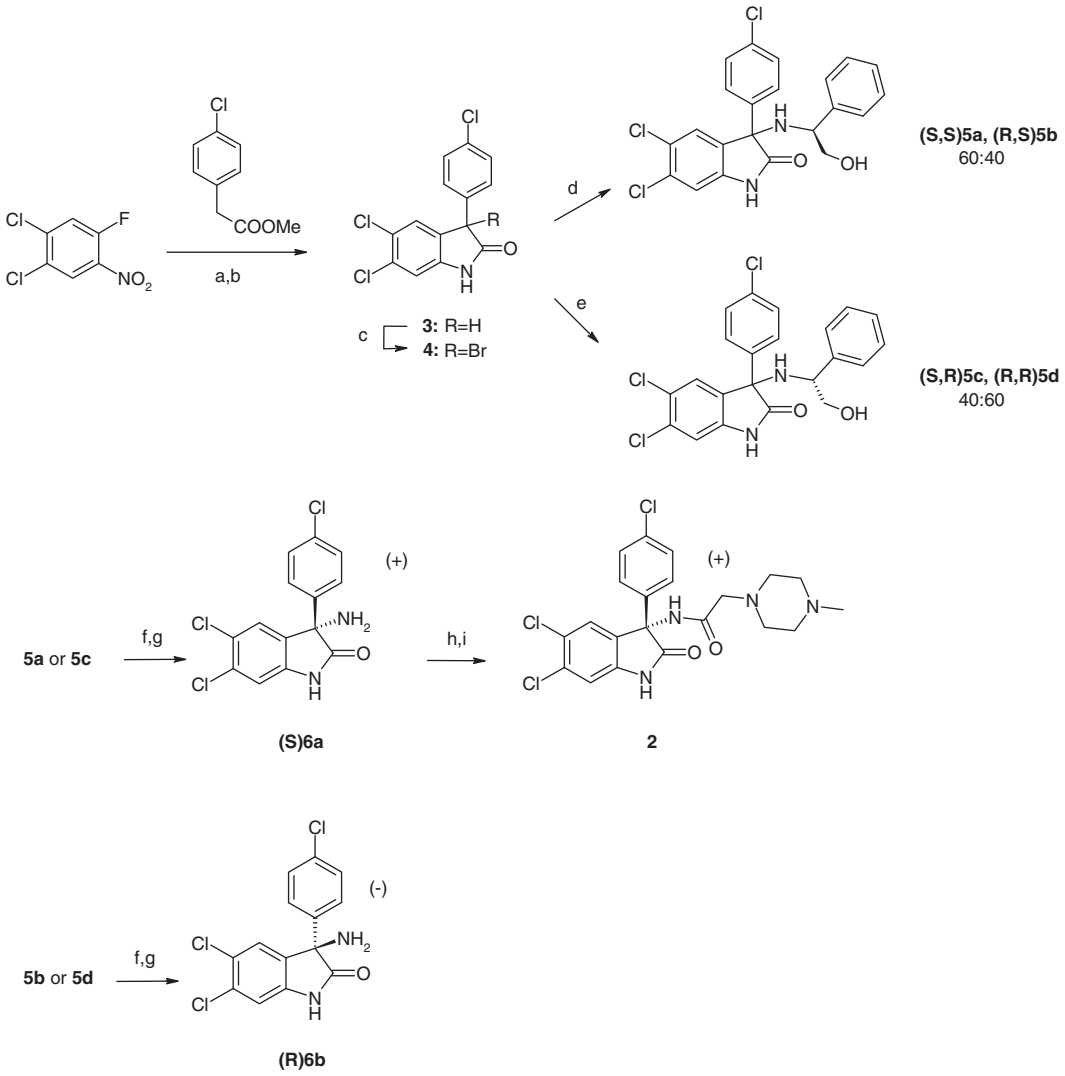
To obtain enantiopure compounds, the synthetic strategy used herein mainly relied on functionalization of racemic indolinone intermediate 4 to form an epimeric pair, through reaction with optically active 2-phenylglycinol. As we aimed to identify which of the two optically active reagents was preferable for our synthetic purposes, we initially performed the reaction with (*R*)-(−)-2-phenylglycinol, thus obtaining the **5c**, **5d** pair. The two compounds were easily separated by flash chromatography. X-ray diffraction of compound **5d** found the absolute configuration at the C-3 position to be (*R*) (Fig. 4).

Subsequent two steps amine dealkylation provided the (−)-3-aminoindolinone **6b**.

The two epimers were well distinguishable each other for their Rf on TLC plate. Also NMR spectra showed several relevant differences between the two diastereoisomers, with a general pattern characterizing each enantiomer. This behavior resulted similar for all couples of diastereoisomeric derivatives synthesized. It was also possible to assess the diastereoisomeric ratio, simply by comparing NMR signal integrals in the mixture of the two compounds. In the same way we controlled the enantioenrichment of separated pairs.

For the **5c**, **5d** pair we found a diastereoisomeric ratio of 40:60, respectively, providing the (*R*)-(−) amine in major quantity after amine deprotection. By performing the same reaction with (*S*)-(+)-2-phenylglycinol we obtained the **5a**, **5b** pair with a 60:40 diastereoisomeric ratio. As both **5b** and **5d** provide the same (*R*)-(−) amine, is easy to identify the C-3 configuration to be (*R*) for **5b and (***S*) for **5a** (Scheme 1). Being mainly interested on the (*S*)-(+) amine, the use of (*S*)-(+)-2-phenylglycinol resulted preferable.

Key intermediate (*S*)**6a** was then opportunely transformed through classical methods into compound **2**.



Scheme 1. Reagents and conditions: (a) NaH, DMF, 3 h, −10 °C/rt, (b) Fe, AcOH, MeOH, 4 h, reflux, (c) $PhMe_3NBr_3$, CH_2Cl_2, rt, 12 h, (d) (*S*)-(+)-2-phenylglycinol, CH_2Cl_2, rt, 18 h, (e) (*R*)-(−)-2-phenylglycinol, CH_2Cl_2, rt, 18 h, (f) $Pb(OAc)_4$, CH_2Cl_2 MeOH, rt, 3 h, (g) MeOH/HCl 3 N, MeOH, rt, 18 h, (h) chloroacetylchloride, pyridine, toluene, 80 °C, 2 h, (i) *N*-methylpiperazine, K_2CO_3, NaI, DMF, 100 °C, 3 h.

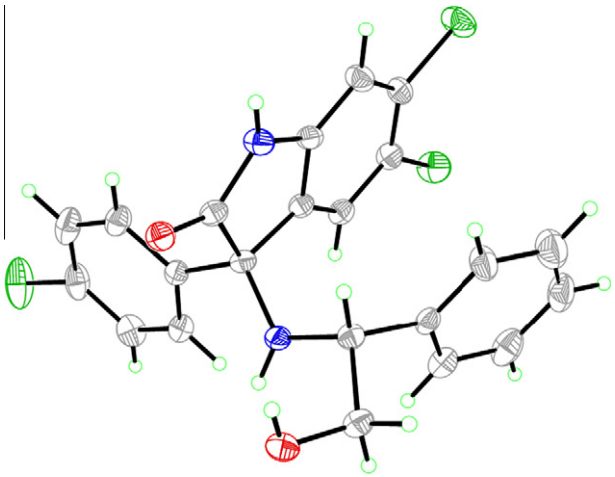


Figure 4. X-ray structure of **5d**.

All compounds **2a–o** listed below, were synthesized according to this synthetic pathway starting from the following precursors, that is, properly substituted 1-fluoro-2-nitro-benzenes and phenylacetates for the synthesis of the scaffold, and the appropriate basic moiety to obtain in particular compounds **2i–o**, as outlined in the following Tables. All enantiopure compounds were prepared by following the previously described strategy. In this way we obtained epimeric pairs with a diastereoisomeric ratio varying from 60:40 to 70:30 according to the substrate (NMR calculation). The enantioenrichment in the final products thus achieved was determined through optical rotation measurements. Unless otherwise noted, following synthetic schemes and SAR tables refers to the (+)-enantiomer. In some other cases the diastereoisomeric mixture was not separated thus isolating a (+/-) mixture at the end of the synthesis, which ratio was not further defined.

This synthetic methodology resulted preferable as this two step indolinone ring formation allow to control from the beginning the desired end position of aromatic substitution. However, an important drawback is the difficult accessibility of suitable substituted 1-fluoro-2-nitro-benzenes, considering both commercial availability and synthesis. In this situation a different pathway, was applied. Compounds **10a–c** were prepared as reported in Scheme 2.

p-Chloromandelic acid was protected at the free oxydrile and transformed into the corresponding acyl chloride. Reaction of **7**

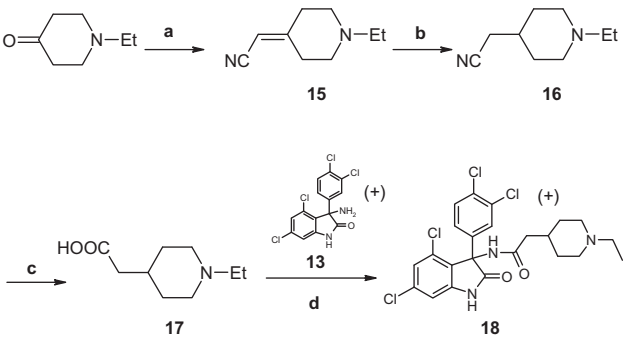
with the required commercially available aniline gave compounds **8a–c** which cyclized under acidic conditions. The symmetrical 3,4,5-trichloroaniline provided, using these conditions, a single product, whereas starting either from 4-chloro-3-methylaniline or from 3-chloro-4-methylaniline a mixture of the two regioisomers is obtained, generally enriched in one of the two regioisomers. Synthesis, accomplished as previously depicted, furnished compounds **10a–c**.

Through substituted isatines commercially available, the synthesis of compounds **14a–l**, was easier. Actually, this useful starting material can be transformed in a few steps in the halogenated key intermediate. Scheme 3 describes the synthesis of intermediate **12** as referring methodology.

The obtained compound was easily functionalized as reported above and used to prepare compounds **14e–l** after reaction with the appropriate base as indicated in the following Tables. Compounds **14a–d** were also obtained through this methodology, starting from suitably substituted isatines and Grignard reagents.

The synthesis of piperidine analogue **18** required the preparation of aminoacid **17**. A Wittig reaction on the *N*-ethyl-piperidone provided the acrylonitril derivative. Hydrogenation of **15** performed with classical methodologies failed in giving the desired product. Compound **16** could be obtained through reduction with magnesium.[27] Further acidic hydrolysis of cyano group led to the carboxylic acid, which was coupled with the previously described amine **13** (Scheme 4).

The use of commercially available 1-methyl-3-piperidone led to compound **19** following the same synthetic procedure.



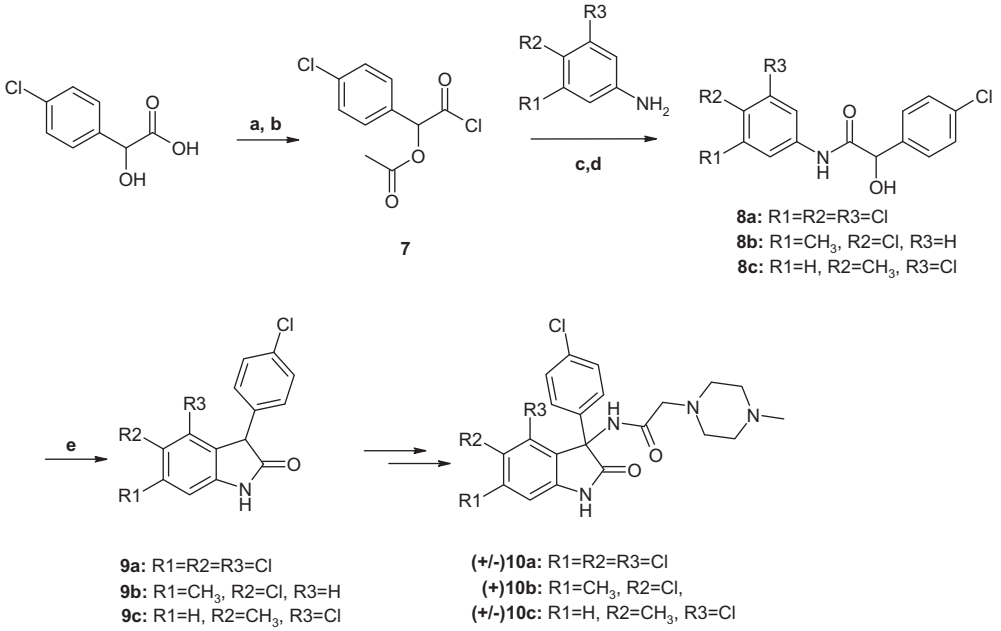
Scheme 4. Reagents and conditions: (a) $(OEt)_2P(O)CH_2CN$, K_2CO_3, THF, 16 h, 70 °C, (b) Mg, MeOH, 4 h, rt, (c) HCl_c, 20 h, 100 °C, (d) **13**, PCl_5, pyridine, CH_2Cl_2, rt, 3 h.

3. Results and discussion

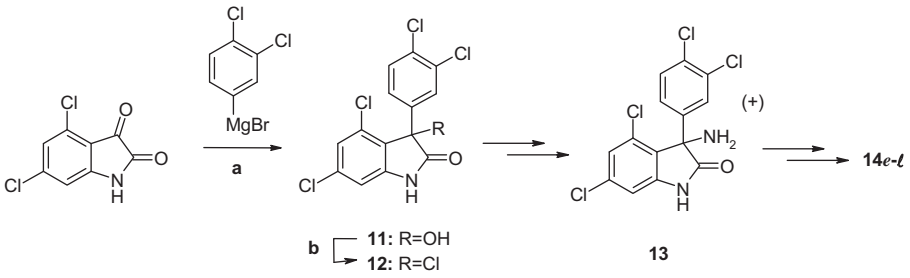
3.1. In vitro SAR studies

Compounds potency and efficacy on GHSR1a were determined by Luciferase reporter assay system employing a hGHSR-1a CHO-Creluc cell line. Binding was performed on membrane preparations from the same cell line in presence of [^{125}I]-ghrelin.

In our preliminary investigation we identified the hit **2**; the (+)-enantiomer showed an IC_{50} of 47 nM, whereas, the corresponding (−)-enantiomer was completely inactive (IC_{50} >10,000 nM). This

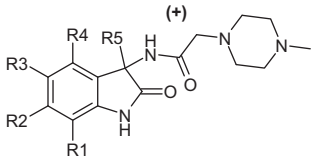


Scheme 2. Reagents and conditions: (a) CH_3COCl, CH_2Cl_2, 50 °C, 3 h, (b) $SOCl_2$, CH_2Cl_2, 65 °C, 2 h, (c) K_2CO_3, CH_2Cl_2, rt, (d) MeOH, toluene, 80 °C, 2 h, (e) H_2SO_4, rt, 4 h.



Scheme 3. Reagents and conditions: (a) THF, rt, 4 h, (b) $SOCl_2$, pyridine, CH_2Cl_2, rt, 1 h.

Table 1
SAR investigation of aromatic substitutions



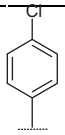
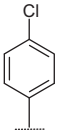
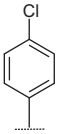
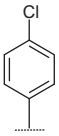
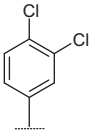
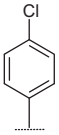
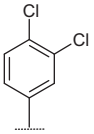
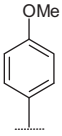
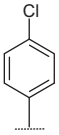
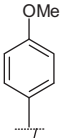
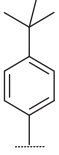
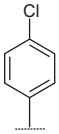
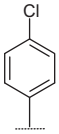
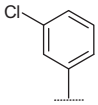
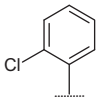
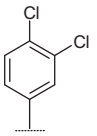
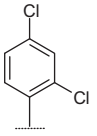
No.	R^1	R^2	R^3	R^4	R^5	Synthetic method (Scheme n°)	GHSR1a antagonism CreLuc[a] (IC_{50}, nM)
2	H	Cl	Cl	H		1(Pathway d)	47 ± 7
14a	H	Cl	H	H		3	140 ± 15
14b	Cl	H	Cl	H		3	1390 ± 180
10a[b]	H	Cl	Cl	Cl		2	273 ± 35
14c	H	Cl	H	H		3	111 ± 12
14d	H	Cl	H	Cl		3	72 ± 14
14e	H	Cl	H	Cl		3	24 ± 7
2a	H	Cl	Cl	H		1(Pathway d)	700 ± 120
10b	H	CH_3	Cl	H		2	251 ± 37
2b	H	Cl	CH_3	H		1(Pathway d)	525 ± 140
2c	H	Cl	Cl	H		1(Pathway d)	1470 ± 220

Table 1 (*continued*)

No.	R^1	R^2	R^3	R^4	R^5	Synthetic method (Scheme n°)	GHSR1a antagonism CreLuc[a] (IC_{50}, nM)
10c[b]	H	H	CH_3	Cl		2	1160 ± 260
2d[b,c]	H	Cl	CH_3	H		1(Pathway d)	213 ± 18
2e[b]	H	Cl	CH_3	H		1(Pathway d)	1180 ± 157
2f[b]	H	Cl	CH_3	H		1(Pathway d)	>10,000
2g[b]	H	Cl	CH_3	H		1(Pathway d)	220 ± 31
2h[b]	H	Cl	CH_3	H		1(Pathway d)	>10,000

[a] Reported in vitro values are an average of at least two replicates.
[b] (+/-) Mixture.
[c] Compound is reported for a better comparison with analogs **2e**–**2h**.

big difference in activity was observed for several pairs of enantiomers and appears to be a general trend for this class of molecules.

Considering substitutions on the aromatic ring a strictly related family of analogs was prepared by varying both the aromatic moieties number and position of chlorine substituents. In addition very few other substituents were used to modulate the molecule electronegativity, that is, alkyl vs methoxy groups. These compounds were then tested for GHSR1a antagonism (Table 1).

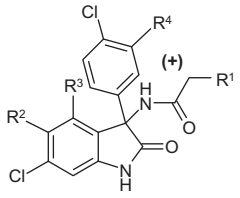
At first glance it is clear that of the limited numbers of substituents examined, chlorine is preferred. In particular substitution at the position 6 of the indolinone core (R2 substituent) is important for potency. Actually its removal causes a major activity loss, as can be observed for compound **14b** and **10c**. When referring to the aromatic in position 3 of the indolinone, Table 1 clearly shows that an *ortho*-substitution causes a dramatic potency loss as shown for both compounds **2f** and **2h**, whereas a *para*-substitution is preferable in comparison to a *meta*-substitution. The double 3, 4 substitution is well tolerated and becomes interesting when coupled with the 3, 4 disubstitution on the other indolinone ring, as was observed for compound **14e**.

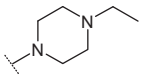
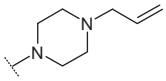
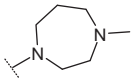
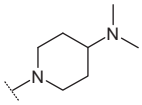
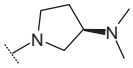
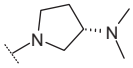
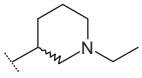
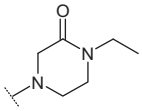
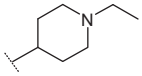
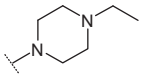
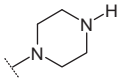
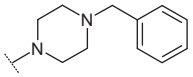
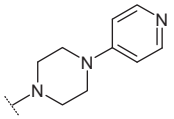
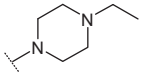
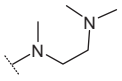
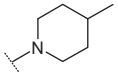
All compounds reported in Table 1, showing nanomolar activity, were further tested for in vitro metabolic stability, using hepatic microsomal fractions from mouse, rat and human. These studies demonstrated that the *N*-methyl piperazinyl moiety is the major site of methabolization (data not shown). The replacement with a series of cyclic or acyclic bases was then explored with the aim to reduce the high rate of metabolization.

Some representative compounds are reported in Table 2.

The data shown in Table 2, demonstrates that the piperazine moiety is optimal for GHSR1a activity, as small structural modifica-

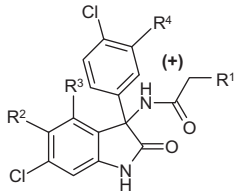
Table 2
SAR investigation at the basic moiety

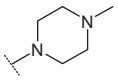
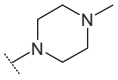
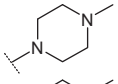
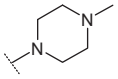
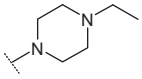
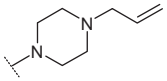
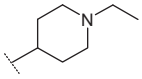
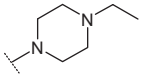
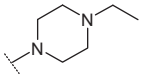


No.	R^1	R^2	R^3	R^4	Synthetic method (Scheme n°)	GHSR1a antagonism CreLuc[a] (IC_{50}, nM)
14f		H	Cl	Cl	3	7 ± 5
14g		H	Cl	Cl	3	38 ± 9
14h		H	Cl	Cl	3	64 ± 4
14i		H	Cl	Cl	3	239 ± 21
14j		H	Cl	Cl	3	25 ± 7
14k		H	Cl	Cl	3	110 ± 27
19		H	Cl	Cl	4	47 ± 11
14l		H	Cl	Cl	3	2780 ± 280
18		H	Cl	Cl	4	1 ± 0.6
2i[b,c]		CH_3	H	H	1(Pathway d)	129 ± 21
2j[b]		CH_3	H	H	1(Pathway d)	>10,000
2k[b]		CH_3	H	H	1(Pathway d)	>10,000
2l[b]		CH_3	H	H	1(Pathway d)	>10,000
2m[b,d]		Cl	H	H	1(Pathway d)	110 ± 12
2n[b]		Cl	H	H	1(Pathway d)	>10,000
2o[b]		Cl	H	H	1(Pathway d)	2540 ± 150

[a] Reported in vitro values are an average of at least two replicates.
[b] (+/-) Mixture.
[c] Compound is reported for a better comparison with analogs **2j**–**2l**.
[d] Compound is reported for a better comparison with analogs **2n**–**2o**.

Table 3
Comparison of GHSR1a CreLuc antagonism and human binding of some selected compounds



No.	R^1	R^2	R^3	R^4	GHSR1a antagonism CreLuc[a] (IC_{50}, nM)	GHSR1a human binding[a] (K_i, nM)
2		Cl	H	H	47 ± 7	249 ± 26
14d		H	Cl	H	72 ± 16	44 ± 8
2d[b]		CH_3	H	H	213 ± 18	392 ± 28
2g[b]		CH_3	H	Cl	220 ± 15	>1000
14f		H	Cl	Cl	7 ± 2	8 ± 2
14g		H	Cl	Cl	38 ± 5	121 ± 10
18		H	Cl	Cl	1 ± 0.8	3 ± 1
2i[b]		CH_3	H	H	129 ± 21	286 ± 25
2m[b]		Cl	H	H	110 ± 12	202 ± 21

[a] Reported in vitro values are an average of at least two replicates.
[b] (+/-) Mixture.

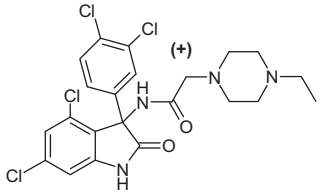
tions causes a substantial loss in activity. In particular ring rigidity is required. The corresponding open chain analogue **2n** is for instance completely inactive. Alkyl substitution on the external nitrogen of the piperazine ring is essential for activity; as shown in compound **2j**, an unsubstituted piperazine results in a loss of activity. Only small alkyl substituents are well tolerated, bulky groups such as benzyl or pyridinyl groups (compounds **2k** and **2l**) cause a significant loss of activity. Replacement of the piperazine with 4-methyl piperidine **2o** or modification of the basicity of the external piperazine nitrogen by introduction of a carbonyl alpha to the nitrogen (compound **14l**) resulted in a dramatic decrease in potency.

On the other hand, compounds like **14j**, and above all **14f** and **18** stand out against other values for their activity. Those results were consistent in all the series checked.

Few representative molecules were compared in the two assays, GHSR1a CreLuc and binding. The results are showed in Table 3. Although, some discrepancies observed were difficult to understand and explain (i.e., **2g** vs **2d**, or **14d** results), data obtained from the two assays where generally in line and it clearly appeared that compounds **14f** and **18**, with a K_i of 8 and 3 nM, respectively, were in vitro potent GHSR-1a antagonists.

The 4-piperidine analogues, exemplified by compound **18**, exhibited excellent potency but they showed an high Pgp affinity

Table 4
In vitro ADME profiling of **14f**



No.	Caco-2/TC-7 permeability		Liver microsomes (5 μM) total metabolisation (%)			Hepatocytes (5 μM) intrinsinc clearance
	% Recovery	P_{total} (10^{-8}cm sec^{-1})	Mouse	Rat	Human	Cl_{int} (mL h^{-1} (10^6hep)$^{-1}$)
14f	51.8 ± 8	38.45 ± 7.5	44	45	41	0.097 ± 0.027

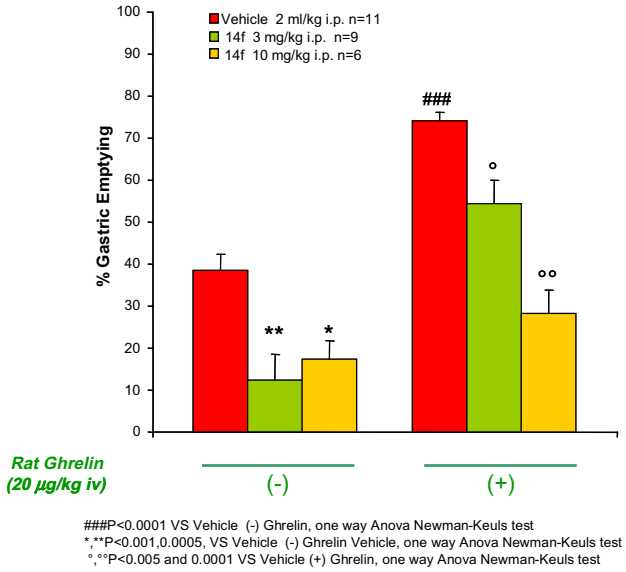


Figure 5. Effect of compounds **14f** on gastric emptying in CD rats in absence and in presence of ghrelin (20 µg/kg iv).

(efflux ratio = 58); this suggests that **18** could display in vivo a low bioavailability and poor brain penetration. Consequently, piperidine series was abandoned despite of the large numbers of compounds synthesized. Piperazines showed a lower efflux ratio and they were chosen as the preferred basic moiety. Indeed **14f** displayed a 10 time lower Pgp value then compound **18** (efflux ratio = 6.6).

As part of our SAR we also explored some modifications at the alkyl linker from the indolinone core to the basic unit. Several compounds from both the piperidine and the piperazine series were prepared to examine the importance of the alkyl chain lenght. However shortening or lengthening of the alkyl chain resulted in a loss of activity. All the reported molecules were devoid of agonist or inverse agonist properties.

On the basis of all those considerations, compound **14f** was selected for further investigation because its selectivity at high concentration over a panel of 33 different receptors (Cerep 33). The compound had no significant binding at 10 µM, including adenosine, adrenergic, cannabinoids, dopamine, histamine, muscarinics, nicotinic, opioids, serotonin, and vasopressin receptors.

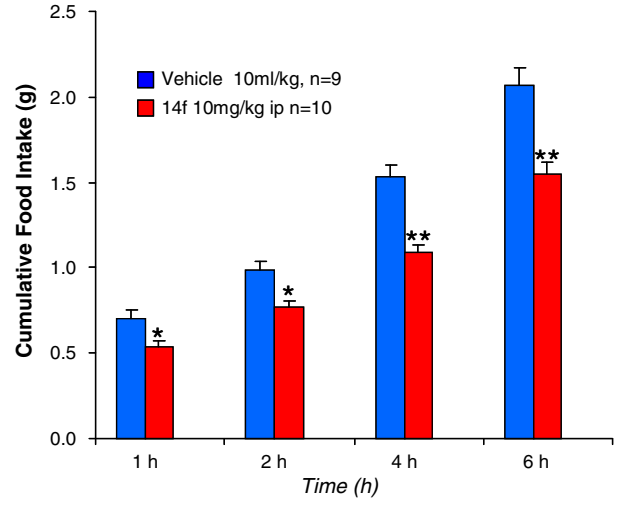


Figure 6. Effect of compound **14f** on food intake in the mouse fasted-refed model (C57/BL6N mice).

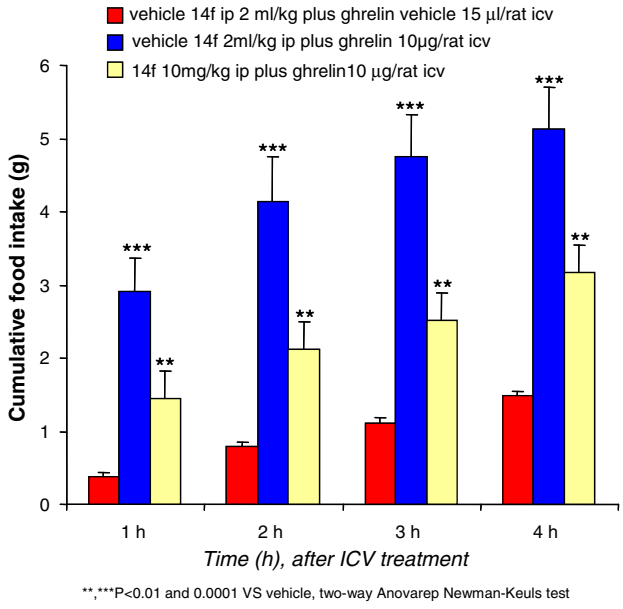


Figure 7. Effect of compound **14f** on rat cumulative food intake stimulated by ICV ghrelin. The compound was dosed 30 min before icv treatment.

Compound **14f** was also investigated for in vitro ADME properties (Table 4). The trans-epithelial intestinal transport (P_{total}) on Caco-2/TC-7 cell monolayers of **14f** was very high. These results suggests that **14f** will show a good bioavailability when oral administered. **14f** also showed an intermediary metabolism, following a 24 h incubation at 5 µM on isolated hepatic microsomal fractions prepared from mouse, rat and humans (see Table 4). In all species the metabolism was similar (range 41–45%). In human hepatocytes the **14f** intrinsic metabolic clearance (Cl_{int}) was also intermediary, 0.097 ± 0.027 mL h^{-1} (10^6 hep)$^{-1}$.

Because the good in vitro profile, **14f** was selected for a further in vivo characterization. The interesting obtained results should be considered as a first activity evaluation of the new indolinone derivative, further studies were commenced to well characterize pharmacokinetic properties for a better contextualization of those results.

3.2. In vivo activity

Ghrelin is a potent stimulator of gastric emptying.[28–30] In 24 h fasted CD male rats the lead GHS-R1a antagonist **14f** (3 and 10 mg/kg, ip) dose dependently inhibited ghrelin effect on gastric emptying by 55% and 62%, respectively. This antagonist also slowed gastric emptying in absence of ghrelin treatment (68% at 3 mg/kg and 55% at 10 mg/kg); this intrinsic, not dose dependently effect, suggests a physiological role of endogenous ghrelin in regulating gastric functions (Fig. 5).[31]

There is strong evidence to suggest that ghrelin plays a critical role in modulating body weight through the control of food intake and/or regulation of fuel substrate efficiency.[32] We tested the effect of compound **14f** in two different models of food intake: the fasted refed mouse model and the icv ghrelin rat model. In the former high plasmatic levels of ghrelin were physiologically induced whereas in the latter ghrelin was injected directly in the central nervous system. As shown in Figure 6, **14f** dosed ip at 10 mg/kg caused a significant reduction in food intake in fasted C57/BL6N mice out to 6 h.

The compound at 10 mg/kg inhibited the food intake stimulated by icv (10 µg/rat) ghrelin injection (Fig. 7) in fed male rats. The ghrelin effect was assessed for 4 h, the compound inhibited the

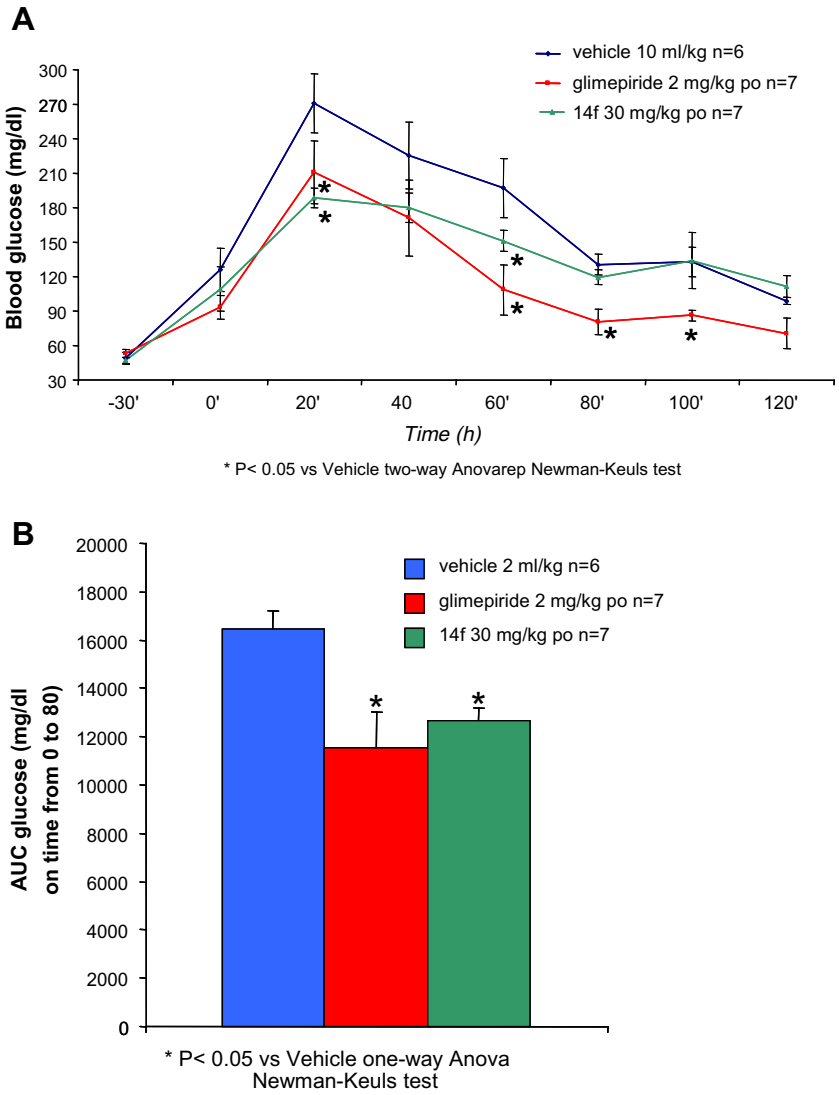


Figure 8. Effect of compound **2** on glucose tolerance in GTT test in C57/BL6N mice.

ghrelin stimulation by 58%, 60%, 61%, 53%, respectively, at 1, 2, 3, 4 h.

The metabolic effect of compound **14f** was assessed on glucose homeostasis in vivo,[28] using an ip Glucose Tolerance Test (ipGTT) in fasted C57/BL6N mice.

Compound **14f**, dosed 30 mg/kg po was shown to significantly improve glucose tolerance (Fig. 8A), reducing the glucose excursion by 32% when compared to vehicle. This was similar in magnitude to the effect of the insulin secretagogue glimepiride, (Fig. 8B).

4. Conclusion

Following the HTS identification of new indolinone derivatives with GHSR-1a receptor antagonist properties, we began a comprehensive synthetic program to improve hits profile and establish a robust structure–activity relationship. Compound **14f** was identified in vitro as a selective and potent lead (7 nM). Thus it was selected for in vivo further characterization. **14f** inhibited the effect of GHSR1a agonist ghrelin on rats gastric emptying and food intake attesting its specificity. It also inhibited food intake in the mouse fasted-refed model in which high plasmatic level of endogenous ghrelin are induced. When tested under ipGTT conditions **14f** showed a significant improvement in glucose tolerance. Encouraged by the initial promising results, **14f** has gone forward for further more detailed pharmacological profiling and a large lead optimization programme has been commenced to build on these results.[33,34]

5. Experimental

5.1. General chemistry

Chemicals were purchased from commercial sources and used as supplied, unless otherwise indicated. All reactions were monitored by TLC on VWR glass plates precoated with silica gel 60 F254; spots were visualized through UV light at 220 nm or by treatment with 1% aq $KMnO_4$. Products were purified by flash chromatography on Biotage® SNAP Cartridge KP-Sil or on Supelco VersaPak™ Cartridges, Spherical Silica (20–45 μm). Melting points have been measured with Buchi B-540 instrument. $[\alpha]_D$ measurements were determined trough a Perkin Elmer polarimeter PE341.

^1H and ^{13}C NMR spectroscopy was performed on 400 MHz Bruker spectrometers. Chemical shifts (δ) are expressed in ppm relative to TMS frequency.

Liquid chromatography mass spectrometry (LC–MS) was performed on a Thermo Electron Surveyor system equipped by a diode array detector and LCQ DecaXP Max ion trap mass spectrometer with electrospray ionization. The mass detection was used in parallel with UV/vis detector and the eluent coming from HPLC was splitted with an intent to optimize both chromatography and ionization at the MS interface.

Two micro liters of a 0.1 mg/ml of considered sample was dissolved in acetonitrile/water (9/1) and analyzed according with the following LC–MS methods.

Method A: The HPLC column was an Xterra C-18 (2.1 × 50 mm, 3.5 μM) The eluents were Milli Q water containing trifluoracetic acid 0.01% (solvent A) and acetonitrile as organic modifier (solvent B).The total run time was 20 min at a flow rate of 0.5 mL/min. with a gradient elution started from 98% of A to 95% of B over 10 min with a final hold at 95% of B of 5 min and additional 5 min of post run. Spectra were scanned from 100 to 1500 amu using a variable ion time according to the number of ions in the source and ionization obtained in electrospray in positive mode (ESI+).

Method B: The HPLC run was preformed with a Phenomenex Gemini C-18 (2.1 × 100 mm, 5.0 μM) column. The eluents were Milli Q water containing 0.005 M ammonium acetate pH 6.5 (solvent A) and acetonitrile as organic modifier (solvent B). The total run time was 27 min at a flow rate of 0.3 mL/min, with a gradient elution started from 95% of A to 90% of B over 17 min with a final hold at 95% of B (5 min), and additional 5 min of post run. Spectra were scanned from 100 to 1500 amu using a variable ion time according to the number of ions in the source and ionization obtained in electrospray in negative mode (ESI−).

The retention time (RT) is reported in min at the apex of the peak as detected at 220 nm by the UV detector.

X-Ray diffraction (XRD) measurements have been conducted on a single crystal sample. Crystals of **5a** were grown in a methanol/acetone mixture. The molecule crystallized in the tetragonal P41/P43 space group, the asymmetric unit in the crystal was found to be made up of two molecules of the compound. The structure did not contain any additional solvate molecule (organic or water). Crystal data, data collection and refinement parameters for $C_{22}H_{17}C_{13}N_2O_2$ are below summarized: MW = 447.73 g/mol, unit cell dimensions; a = 21,636(3) Å, b = 21,636(3) Å, c = 9.0850(18) Å, α = 90,00°, β = 90,00°, γ = 90,00°, V = 4252.8(12) Å^3, Z = 8, ρ_{calcd} = 1.399 mg/m^3, λ = 0.71073 Å, T = 133(2) K, μ = 0.452 mm^{-1}, F(000) = 1840, θ_{range} = 1.33°–25.50°, limiting indices = $-19<h<26$, $-26<k<24$, $-11<l<9$, reflections collected = 24177, independent reflections = 7807 [R(int) = 0.1060], refinement method = full-matrix least-squares on F^2, data/restraints/parameters = 7807/1/524, goodness-of-fit on F^2 = 0.991, final R indices [$I < 2\sigma(I)$]; R1 = 0.0558, wR2 = 0.1242, final R indices (all data) R1 = 0.1270, wR2 = 0.1651, peak/hole = 0.299/−0.254 eÅ^{-3}.

5.2. Synthetic procedures and compound characterization

5.2.1. 5,6-Dichloro-1,3-dihydro-3-(4-chlorophenyl)-indole-2-one (3)

Sodium hydrate 60% (2.85 g, 71.2 mmol) at −10 °C and under nitrogen atmosphere was suspended in *N*,*N*-dimethylformamide (DMF) (45 ml) before being added to a solution of 1,2-dichloro-4-fluoro-5-nitrobenzene (5 g, 23.8 mmol) and methyl-4-chlorophenyl acetate (4.4 g, 23.8 mmol). in DMF (70 ml) The mixture was stirred at −5 °C for 2 h and then allowed to warm up to room temperature. After quenching with ice, an aqueous solution of 10% NH_4Cl was added and the mixture was partitioned between EtOAc and water. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified using column chromatography on silica gel (hexane/EtOAc = 3/1) to give 1,2-dichloro-4-[2-(methy-l-4-chlorophenyl acetate)]-5-nitrobenzene (3.18 g, 36%) as a colourless oil.

A solution of 1,2-dichloro-4-[2-(methy-l-4-chlorophenyl acetate)]-5-nitrobenzene (4,6 g, 11.8 mmol), acetic acid (15 ml), powdered iron (1.7 g, 30.4 mmol) in MeOH (60 ml) was mechanically stirred under nitrogen atmosphere and heated at reflux for 1 h 30. Ice and the mixture was then treated with ice, basified with 10% $NaHCO_{3aq}$ and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. Diisopropylether was then added, and the mixture was further stirred for 10 min. The precipitate was collected by filtration and washed with cold diisopropylether, and the wet solid was dried in vacuo to give 3 (2.65 g, 100%) as a white solid.

Pf: 214–215 °C; ^{1}H NMR (DMSO-d_6, 313 K) δ 4.88 (bs,1H), 7.10 (s,1H), 7.20 (m, 2H), 7.30 (s,1H), 7.42 (m, 2H), 10.76 (s, H), MS (ESI−): m/z = 310 [M$^-$].

5.2.2. 3-Bromo-5,6-dichloro-1,3-dihydro-3-(4-chlorophenyl)-indole-2-one (4)

Compound **3** (2.75 g, 8.8 mmol) was dissolved in CH_2Cl_2 (100 ml) under nitrogen atmosphere. The solution was cooled with ice bath and a solution of $PhMe_3NBr_3$ (3.93 g, 10.5 mmol) in CH_2Cl_2 (100 ml) was added. The reaction was stirred 3 h at room temperature. The mixture was then washed with HCl 1 M and water. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to give 4 (3.4 g, 100%) as a yellow oil, which was used directly for the next step reaction without further purification.

^{1}H NMR (DMSO-d_6, 313 K) δ 7.20 (s, 1H), 7.50 (m, 2H), 7.61 (m, 2H), 7.81 (s, 1H), 11.29 (bs, 1H), MS (ESI+): compound did not ionize under these conditions.

5.2.3. 5,6-Dichloro-3-[(1*S*)-2-hydroxy-1-phenylethyl]amino1,3-dihydro-3-(4-chlorophenyl)-indole-2-one (5a,5b)

3-Bromo-5,6-dichloro-1,3-dihydro-3-(4-chlorophenyl)-indole-2-one (3.4 g, 8.7 mmol) and (*S*)-(+)-2-phenylglycinol (2.9 g, 21.1 mmol) were dissolved in dichloromethane (50 ml) under nitrogen atmosphere. The reaction was stirred 2 h at room temperature. The precipitate was filtered off and the mother liquor was concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc = 7/3) to give **5b** (R_f = 0.35, 1.7 g, 44%) and **5a** (R_f = 0.20, 2.2 g, 56%).

Compound **5a**: pf: 107–109 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 3.30–3.47 (m, 3H), 3.56 (m, 1H), 4.85 (t, 1H, J = 5.7 Hz,), 6.99 (s, 1H), 7.08–7.20 (m, 5H), 7.22 (s, 1H), 7.41 (m, 4H), 10.41 (bs, 1H), LC–MS (ESI+) m/z 447 [M+H]$^+$, RT(min) = 7.2, $[\alpha]_D$: +78° (c 0.1% in MeOH).

Compound **5b**: 1H NMR (DMSO-d_6, 313 K) δ 3.29–3.49 (m, 3H), 3.52–3.63 (m, 1H), 4.94 (m, 1H), 6.48 (s, 1H), 6.88 (s, 1H), 6.99–7.09 (m, 5H), 7.36–7.53 (m, 4H), 10.87 (bs, 1H), MS (ESI+) m/z 447 [M+H]$^+$.

5.2.4. 5,6-Dichloro-3-[(1*R*)-2-hydroxy-1-phenylethyl]amino1,3-dihydro-3-(4-chlorophenyl)-indole-2-one (5c,5d)

The title compounds were prepared from 4 by the method described in the preparation of **5a**,**5b** pairs.

Compound **5c**: 1H NMR (DMSO-d_6, 313 K) δ 10.4 (bs, 1H), 7.41 (m, 4H), 7.22 (s, 1H), 7.14 (m, 5H), 6.99 (s, 1H), 4.85 (m, 1H), 3.56 (m, 1H), 3.34 (d, J = 4 Hz, 1H), 3.4 (m, 2H), MS (ESI+) m/z 447 [M+H]$^+$.

Compound **5d**: 1H NMR (DMSO-d_6, 313 K) δ 10.9 (bs, 1H), 7.44 (m, 4H), 7.0 (m, 5H), 6.87 (s, 1H), 6.48 (s, 1H), 4.94 (bs, 1H), 3.57 (bs, 1H), 3.44 (m, 1H), 3.4 (m. 1H), 3.3 (m, 1H), MS (ESI+) m/z 447 [M+H]$^+$. Single crystal X-ray diffraction allowed to assign to this compound the absolute (*R*,*R*) configuration.

The structure was of good quality, the agreement factor value was R1 = 0.056.

5.2.5. (*S*)-(+)-3-Amino-5,6-dichloro-1,3-dihydro-3-(4-chlorophenyl)-indole-2-one ((*S*)6a)

Compond **5a** (1.8 g, 4 mmol) and lead tetraacetate (1.9 g, 4.3 mmol) were dissolved in CH_2Cl_2 (28 ml) and MeOH (12 ml). The reaction was stirred 3 h at room temperature before being concentrated in vacuo. The residue was then partitioned between EtOAc and aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered and evaporated and the residue was dissolved in MeOH (3.7 ml) and HCl 3 N (36 ml). The mixture was stirred at room temperature overnight, then concentrated in vacuo. After partitioning between EtOAc and water, the organic phase was washed with HCl 1 N. The collected aqueous phase was basified with ammonium hydroxide and extracted with CH_2Cl_2. The organic phase is dried over Na_2SO_4, filtered and dried in vacuo to obtain 6 (0.54 g, 41%) as a white solid, which was used directly for the next step reaction without further purification.

^{1}H NMR (DMSO-d_6, 313 K) δ 2.78 (s, 2H), 7.10 (s, 1H), 7.35 (s, 1H), 7.38 (m, 4H), 10.67 (bs, 1H), MS (ESI+) m/z 327 $[M+H]^+$, $[\alpha]_D$: +32.9° (*c* 0.5% in MeOH).

5.2.6. (*R*)-(−)-3-Amino-5,6-dichloro-1,3-dihydro-3-(4-chlorophenyl)-indole-2-one ((*R*)6b)

The title compound was prepared from **5b** by the method described in the preparation of 6a. The spectral properties of **6b** were identical with those of **6a**. $[\alpha]_D$: −21.9° (*c* 0.3% in MeOH).

5.2.7. (+)-*N*-[5,6-Dichloro-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(4-méthyl-piperazin-1-yl)-acetamide (2)

Compound (*S*)**6a** (1.3 g, 4 mmol) and pyridine (0.32 ml, 4 mmol) were dissolved in toluene (47 ml) under nitrogen atmosphere. After 5 min stirring, chloroacetylchloride (0.31 ml, 4.56 mmol) was slowly added, and the reaction was stirred 2 h at 80 °C. The mixture was quenched with water and extracted with ethyl acetate; the organic layer was dried on Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography (cyclohexane/EtOAc = 8/2) to obtain 2-chloro-*N*-[5,6-dichloro-3-(4-chloro-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-acetamide (0.4 g, 25%).

2-Chloro-*N*-[5,6-dichloro-3-(4-chloro-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-acetamide (0.4 g, 1 mmol), *N*-methylpiperazine (0.11 ml, 1 mmol), K_2CO_3 (0.14 g, 1 mmol) and NaI (70 mg, 0.47 mmol) were dissolved DMF (8 ml). The reaction was stirred 3 h at 100 °C, quenched with water and extracted with ethyl acetate. The organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/MeOH = 8/2) to obtain 2 (40 mg, 80%) as a white solid.

Pf: 207.1–207.6 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.36 (m, 3H), 2.58 (m, 8H), 2.96–3.16 (m, 2H), 7.08 (s, 1H), 7.31 (m, 2H), 7.48 (m, 2H), 7.52 (s, 1H), 8.70 (sb, 1H), 10.70 (bs, 1H), $[\alpha]_D$: +141° (*c* 0.25% in MeOH), LC–MS (ESI+): m/z = 467 $[M+H]^+$, RT(min) = 8.3.

5.2.8. 4-Chloro-*O*-acyl-mandelic-chloride (7)

In a two-necked flask provided with magnetic stirring, 4-chloro-DL mandelic acid (10 g, 53.6 mmol) and acetyl chloride (4.2 ml, 59 mmol) were dissolved in CH_2Cl_2 (80 ml). The reaction was stirred 3 h at 50 °C. Thionyl chloride (7.8 ml, 107.5 mmol) was dropwise added. After 2 h of stirring at reflux the solvent was evaporated under vacuum to obtain 7 (10.7 g, 100%) as an oil, which was used directly for the next step reaction without further purification.

^{1}H NMR ($CDCl_3$, 300 K) δ 2.25 (s, 3H), 6.08 (s, 1H), 7.43–7.48 (m, 4H), MS (ESI−): compound did not ionized under these conditions.

5.2.9. 4-Chloro-*N*-(3,4,5-trichlorophenyl)mandelamide (8a)

In a three-necked flask, provided with mechanical stirrer and under nitrogen atmosphere, 3,4,5-trichloroaniline (4.04 g, 20.6 mmol) were dissolved in toluene (50 ml) and cooled at 0 °C. K_2CO_3 (9.6 g, 69.5 mmol) and a mixture of 7 (6.8 g, 27.5 mmol) in toluene (10 ml) were added and the mixture was stirred for 1 h at room temperature. MeOH (4.15 ml) was added, and the mixture was further stirred 2 h at 80 °C. A solution of HCl 1 N was then added. After extraction with EtOAc, the organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo to obtain **8a (5.7 g,** 95%) as a yellow solid, which was used directly for the next step reaction without further purification.

^{1}H NMR (DMSO-d_6, 313 K) δ 5.16 (d, J = 4.3 Hz, 1H), 6.68 (d, J = 4.3 Hz, 1H), 7.43 (m, 2H), 7.52 (m, 2H), 7.07 (s, 2H), 10.37 (bs, 1H), MS (ESI−): m/z 362 $[M^-]$.

5.2.10. 4,5,6-Trichloro-1,3-dihydro-3-(4-chlorophenyl)-indol-2-one (9a)

At 0 °C, 8a (5.7 g, 15.6 mmol) was gradually added to H_2SO_4 96% (22 ml) and oleum (5 ml). The reaction was stirred 4 h at room temperature then iced water was added. The solution was strongly basified and extracted with CH_2Cl_2 .The organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was crystallized from diethyl ether to obtain **9a** (4.2 g, 77%) as a yellow powder.

^{1}H NMR (DMSO-d_6, 313 K) δ 4.95 (s, 1H), 7.12–7.20 (m, 3H), 7.40 (m, 2H), 10.92 (bs, 1H), LC–MS (ESI−): m/z = 344 $[M-]$, RT(min) = 13.05.

5.2.11. 5-Chloro-6-methyl-1,3-dihydro-3-(4-chlorophenyl)-indol-2-one (9b)

^{1}H NMR (DMSO-d_6, 313 K): δ 2.32 (s, 3H), 4.80 (s, 1H), 6.89 (s, 1H), 7.06(bs, 1H), 7.18 (m, 2H), 7.41 (m, 2H), 10.59 (s, 1H), MS (ESI−): m/z 290 $[M^-]$.

5.2.12. 4-Chloro-5-methyl-1,3-dihydro-3-(4-chlorophenyl)-indol-2-one (9c)

^{1}H NMR (DMSO-d_6, 313 K) δ 2.25 (s, 3H), 4.80 (s, 1H), 6.83 (d, J = 7.8 Hz, 1H) 7.12 (m, 2H), 7.26 (d, J = 7.8 Hz, 1H), 7.39 (m, 2H), 10.56 (bs, 1H), LC–MS (ESI−): m/z = 290 $[M^-]$, RT(min) = 11.

5.2.13. 3-Hydroxy-4,6-dichloro-1,3-dihydro-3-(3,4-dichlorophenyl)-indol-2-one (11)

A solution of 4,6-dichloroisatine (7.2 g, 33 mmol) in THF (120 ml) was dropwise added under nitrogen atmosphere to 3,4-dichlorophenylmagnesium bromide (200 ml, 0.5 M in THF). The mixture was stirred 4 h at 70 °C, then NH_4Cl_{aq} was added. After extraction in EtOAc, the organic phase was washed three times with NaOH 1 N, then dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (cyclohexane/EtOAc = 8/2) to give 11 (6.73 g, 56%) as a white solid.

^{1}H NMR (DMSO-d_6, 313 K) δ 6.95 (d, J = 1.8 Hz, 1H), 7.03 (s, 1H), 7.10 (dd, J = 8.3 and 2.1 Hz, 1H), 7.14 (d, J = 1.8 Hz, 1H), 7.55–7.60 (m, 2H), 10.86 (bs, 1H), MS (ESI+): m/z 345 $[M+H]^+$–H_2O.

5.2.14. 3,4,6-Trichloro-1,3-dihydro-3-(3,4-dichlorophenyl)-indol-2-one (12)

Compound **11** (1.2 g, 3.3 mmol) was dissolved in CH_2Cl_2 (8 ml) under nitrogen atmosphere. The mixture was cooled at 0 °C and pyridine (0.47 ml, 5.8 mmol) was added. After 5 min stirring, a solution of thionyl chloride (0.34 ml, 4.7 mmol) in CH_2Cl_2 (4 ml) was slowly added. The reaction was further stirred 1 h at rt. A saturated solution of ammonium chloride was then added. The organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo

to obtain 12 (1.2 g, 94%) as a yellow oil, which was used directly for the next step reaction without further purification.

^{1}H NMR (DMSO-d_6, 313 K): 7.08 (d, J = 1.7 Hz, 1H), 7.31–7.35 (m, 2H), 7.63 (d, J = 2.3 Hz, 1H), 7.70 (d, J = 8.5 Hz, 1H), 11.47 (bs, 1H), MS (ESI+): compound did not ionize under these conditions.

5.2.15. (1-Ethyl-piperidin-4-ylidene)-acetonitrile (15)

Diethyl(cyanomethyl)phosphonate (8.89 ml, 54.9 mmol) and K_2CO_3 (9.36 g, 67.7 mmol) were dissolved under nitrogen atmosphere in THF (12 ml). The reaction was stirred 15 minute at room temperature and 20 min at reflux, then was cooled to room temperature. 1-Ethyl-4-piperidone (6.5 ml, 55.9 mmol) was added and the mixture was stirred 16 h at reflux. The mixture was partitioned between EtOAc and water. The organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo to obtain 15 (6.8 g, 82%) as a colorless oil, which was used directly for the next step reaction without further purification.

^{1}H NMR (DMSO-d_6, 313 K) δ 1.01 (t, J = 7.2 Hz, 3H), 2.31–2.50 (m, 10H + dmso), 5.45 (m, 1H), MS (ESI+) m/z 151 $[M+H]^+$.

5.2.16. (1-Ethyl-piperidin-4-yl)-acetonitrile (16)

Compound 15 (1 g, 6.7 mmol) was dissolved in MeOH (70 ml) and cooled at 0 °C. Magnesium (7.2 g, 296 mmol) was then added and the mixture was stirred for 4 h before being filtrate. The filtrate phase was concentrated in vacuo and partitioned between CH_2Cl_2 and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to obtain 16 (0.45 g, 44%) as a colorless oil, which was used directly for the next step reaction without further purification.

^{1}H NMR (DMSO-d_6, 313 K) δ 0.98 (t, J = 7.3 Hz, 3H), 2.26 (m, 2H), 1.57 (m, 1H), 1.68 (m, 2H), 1.84 (m, 2H), 2.30 (q, J = 7.3 Hz, 2H), 2.46 (d, J = 6.4 Hz, 2H), 2.85 (m, 2H), MS (ESI+) m/z 153 $[M+H]^+$.

5.2.17. (1-Ethyl-piperidin-4-yl) acetic acid (17)

Compound **16** (3.65 g, 24.3 mmol) was dissolved in HCl_{conc} (47 ml) and stirred at reflux for 20 h. The mixture was partitioned between water CH_2Cl_2. The pH was adjusted to 6 with an aqueous solution of NH_4Cl. The aqueous phase was washed with CH_2Cl_2 and concentrated in vacuo. The residue was dissolved in ethanol and filtrated. The filtrate, concentrated in vacuo, gave 17 (3.6 g, 87%) as a yellow solid.

^{1}H NMR (DMSO-d_6, 313 K) δ 1.06 (t, J = 7.1 Hz, 3H), 1.29 (m, 2H), 1.71 (m, 2H), 2.10–2.25 (m, 3H), 2.55 (m, 2H+DMSO), 3.00 (m, 2H), MS (ESI+) m/z 172 $[M+H]^+$.

5.2.18. (+)*N*-[4,6-Dichloro-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(1-ethyl-piperidin-4-yl)-acetamide (18)

In a two-necked flask under nitrogen atmosphere, PCl_5 (0.2 g, 9.6 mmol) was suspended at 0 °C in CH_2Cl_2 (9 ml), then compound 17 (0.16 g, 9.6 mmol) was added. The suspension was stirred 10 min at 0 °C and 2 h at room temperature (Solution 1). Under nitrogen atmosphere, at 0 °C, compound **13** (0.25 g, 0.7 mmol) and pyridine (0.6 ml, 7.4 mmol) were dissolved in CH_2Cl_2 (9 ml) and stirred 10 min (Solution 2). Solution 1 was then added to Solution 2 at 0 °C and the mixture was stirred 10 min at 0 °C and 3 h at room temperature. The mixture was partitioned between is aqueous $NaHCO_3$ and CH_2Cl_2, and the organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/MeOH = 8/2 to obtain 18 (0.11 g, 28%) as a beige powder.

Pf = 211–213 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 1.17 (m, 3H), 1.36–1.56 (m, 2H), 1.61–1.94 (m, 3H), 2.22 (m, 2H), 2.70–3.03 (m, 4H), 3.11–3.48 (m, 2H + DHO), 6.93 (s, 1H), 7.04 (m, 1H), 7.19 (s, 1H), 7.59 (bs, 1H), 7.65 (d, J = 8.6 Hz, 1H), 9.25 (bs, 1H), 9.99–10.65 (bs, NH+, 1H), 10.86 (bs, 1H) LC–MS (ESI+): m/z = 514 $[M+H]^+$, RT(min) = 8.1.

5.2.19. *N*-[6-Chloro-5-methyl-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(4-methyl-piperazin-1-yl)-acetamide (2g)

White solid, Pf: 229.4–232.6 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.16 (s, 3H), 2.30 (s, 3H), 2.31–2.39 (m, 4H), 2.41–2.55 (m, 4H+dmso), 2.91 (d, J = 15 Hz,1H), 3.03 (d, J = 15 Hz,1H) 6.91 (s, 1H), 7.09 (dd, J_1 = 8.5 Hz, J_2 = 2.3 Hz, 1H), 7.26 (s, 1H), 7.54 (d, J = 2.3 Hz, 1H), 7.64 (d, J = 8.5 Hz, 1H), 8.68 (s, 1H), 10.52 (bs, 1H), LC–MS (ESI+): m/z = 481 $[M+H]^+$, RT(min) = 8.7.

5.2.20. *N*-[6-Chloro-5-methyl-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-piperazinyl-acetamide (2j)

White solid, Pf: 264–265 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.30 (s, 3H), 3.00–4.10 (m, 8H+H_2O), 6.93 (s, 1H), 7.24 (s, 1H), 7.33 (m, 2H), 7.47 (m, 2H), 9.43 (bs, 1H), 9.53 (bs, 1H), 10.59 (s, 1H), LC–MS (ESI+): m/z = 433 $[M+H]^+$, RT(min) = 8.9.

5.2.21. *N*-[6-Chloro-5-methyl-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(4-benzyl-piperazin-1-yl-acetamide (2k)

White solid, Pf: 218.7–220.2 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.28 (s, 3H), 2.36–2.45 (m, 4H), 2.45–2.55 (m, 4H+dmso), 2.93 (d, J = 15 Hz, 1H), 3.02 (d, J = 15 Hz, 1H), 3.47 (m, 2H), 6.88 (s, 1H), 7.20–7.36 (m, 8H), 7.46 (m, 2H), 8.54 (s, 1H), 10.43 (bs, 1H), LC–MS (ESI+): m/z = 523 $[M+H]^+$, RT(min) = 9.2.

5.2.22. *N*-[6-Chloro-5-methyl-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(4-(4-pyridyl)-piperazin-1-yl-acetamide (2l)

White solid, Pf: 314–316 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.30 (s, 3H), 2.55–2.66 (m, 4H), 3.02 (d, J = 15 Hz, 1H), 3.12 (d, J = 15 Hz, 1H), 3.32–3.40 (m, 4H), 6.82 (m, 2H), 6.89 (s, 1H), 7.23 (s, 1H), 7.30 (m, 2H) 7.46 (m, 2H) 8.16 (m, 2H), 8.70 (s, 1H), 10.44 (s, 1H), LC–MS (ESI+): m/z = 510 $[M+H]^+$, RT(min) = 12.4.

5.2.23. *N*-[5,6-Dichloro-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-*N*-methyl-(2-*N*-dimethyl)-ethyl-acetamide (2n)

White solid, Pf: 204.7–205.4 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.01 (s, 6H), 2.26–2.34 (m, 5H), 2.46–2.53 (m, 2H + DMSO), 2.99 (d, J = 16 Hz, 1H), 3.06 (d, J = 16 Hz, 1H), 7.07 (s, 1H), 7.33 (m, 2H), 7.45–7.51 (m, 3H), 9.10 (s, 1H), 10.72 (bs, 1H), LC–MS (ESI+): m/z = 469 $[M+H]^+$, RT(min) = 8.3.

5.2.24. *N*-[4-Chloro-5-methyl-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(1-methyl-piperazin-4-yl)-acetamide (10c)

White solid, Pf: 282.2–284.6 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.15 (s, 3H), 2.28 (s, 3H), 3.33 (m, 4H), 2.45–2.59 (m, 4H+DMSO), 2.98 (d, J = 15 Hz, 1H), 3.06 (d, J = 15 Hz, 1H), 6.80 (d, J = 7.8 Hz, 1H), 7.22 (m, 2H), 7.29 (d, J = 7.8 Hz, 1H), 7.49 (m, 2H), 8.53 (s, 1H), 10.48 (s, 1H), LC–MS (ESI+): m/z = 447 $[M+H]^+$, RT(min) = 7.8.

5.2.25. (+)*N*-[6-Chloro-3-(4-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(1-methyl-piperazin-4-yl)-acetamide (14a)

White solid, Pf: 159–160 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.16 (s, 3H), 2.33 (m, 4H), 2.47 (m, 4H), 2.92 (d, J = 15 Hz, 1H), 3.01(d, J = 15 Hz, 1H), 6.89 (d, J = 2 Hz,1H), 7.08 (dd, J_1 = 8 Hz, J_2 = 2 Hz, 1H), 7.23–7.31 (m, 3H), 7.47 (m, 2H), 8.60 (s, 1H), 10.56 (bs, 1H), LC–MS (ESI+): m/z = 433 $[M+H]^+$, RT(min) = 5.7, $[\alpha]_D$: +134° (c 0.25% in MeOH).

5.2.26. (+)*N*-[4,6-Dichloro-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(1-methyl-piperazin-4-yl)-acetamide (14e)

White solid, Pf: 165–166 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 2.13 (s, 3H), 2.30 (m, 4H), 2.40–2.56 (m, 4H+DMSO), 3.04 (m, 2H), 6.92 (s, 1H), 7.05 (d, J = 8.6 Hz, 1H), 7.20 (s, 1H), 7.54 (s, 1H), 7.66 (d, J = 8.6 Hz, 1H), 8.86 (s, 1H) 10.86 (s, 1H), LC–MS (ESI+): m/z = 501 $[M+H]^+$, RT(min) = 5.2, $[\alpha]_D$: +222° (*c* 0.23% in MeOH).

5.2.27. (+)*N*-[4,6-Dichloro-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(4-*N*-dimethylamino-piperidin-1-yl)-acetamide (14i)

White solid, Pf: 128–130 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 1.37 (m, 2H), 1.70 (m, 2H), 2.00 (m, 1H), 2.05–2.13 (m, 2H), 2.15 (s, 6H) 2.81 (m, 1H), 2.91 (m, 1H), 3.02 (m, 2H), 6.92 (d, 1H), 7.06 (dd, J_1 = 8.5 Hz, J_2 = 2.3 Hz, 1H) 7.20 (d, J = 1.7 Hz, 1H), 7.55 (d, J = 2.3 Hz, 1H), 7.67 (d, J = 8.5 Hz, 1H), 8.81 (s. 1H), 10.88 (bs, 1H), LC–MS (ESI+): m/z = 529 $[M+H]^+$, RT(min) = 5.4, $[\alpha]_D$: +193° (*c* 0.12% in MeOH).

5.2.28. (+)*N*-[4,6-Dichloro-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(*R*)-(3-*N*-dimethylamino-pirrolidin-1-yl)-acetamide (14j)

White solid, Pf: 108–113 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 1.52–1.64 (m, 1H), 1.76–1.87 (m, 1H), 2.08 (s, 6H), 2.41 (m, 1H), 2.53–2.76 (m, 4H), 3.15 (m, 2H), 6.92 (d, J = 1.7 Hz, 1H), 7.06 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz,1H), 7.21 (d, J = 1.7 Hz, 1H), 7.57 (d, J = 2.2 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 8.89 (s, 1H), 10.84 (bs, 1H), LC–MS (ESI+): m/z = 515 $[M+H]^+$, RT(min) = 4.8, $[\alpha]_D$: +230° (*c* 0.13% in MeOH).

5.2.29. (+)*N*-[4,6-Dichloro-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(*S*)-(3-*N*-dimethylamino-pirrolidin-1-yl)-acetamide (14k)

White solid, Pf: 113–115 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 1.53–1.63 (m, 1H), 1.77–1.88 (m, 1H), 2.07 (s, 6H), 2.42 (m, 1H), 2.53–2.59 (m, 1H+DMSO), 2.62–2.76 (m, 3H), 3.11 (d, J = 1.5 Hz, 1H), 3.18 (d, J = 1.5 Hz, 1H), 6.92 (d, J = 1.7 Hz, 1H), 7.06 (dd, J_1 = 8.5 Hz, J_2 = 2.3 Hz, 1H), 7.20 (d, J = 1.7 Hz, 1H), 7.55 (d, J = 2.3 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 8.89 (s, 1H), 10.85 (bs, 1H), LC–MS (ESI+): m/z = 515 $[M+H]^+$, RT(min) = 4.8, $[\alpha]_D$: +205° (*c* 0.11% in MeOH)

5.2.30. (+)*N*-[4,6-Dichloro-3-(3,4-dichlorophenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-2-(4-ethyl-piperazin-3-one)-acetamide (14l)

White solid, Pf: 126–130 °C, ^{1}H NMR (DMSO-d_6, 313 K) δ 1.01 (t, J = 7.2 Hz, 3H), 1.18 (m, 2H), 2.74 (m, 2H), 3.08 (m, 2H), 3.19 (m, 2H), 3.29 (m, 2H + DMSO), 6.92 (d, J = 1.7 Hz, 1H), 7.05 (dd, J_1 = 8.5 Hz, J_2 = 2.3 Hz, 1H), 7.22 (d, J = 1.7 Hz, 1H), 7.58 (d, J = 2.3 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 9.16 (s, 1H), 10.86 (s, 1H), LC–MS (ESI+): m/z = 531 $[M+H]^+$, RT(min) = 6.3, $[\alpha]_D$: +191° (*c* 0.11% in MeOH)

5.3. In vitro assays

5.3.1. Stable CHO double transformants

Stable CHO double transformants were obtained according to the methods previously described.[35] Briefly, human GHSR1a (NM_198407.1) was cloned into a CHO-derived cell line stably transfected with a CRE-Luc reporter gene cassette (six consensus cAMP responsive elements (CRE) linked upstream to the firefly luciferase sequence). Cells were screened for hGHSR1a membrane expression and dose-dependent responsiveness of the reporter gene to human ghrelin (Tocris). Cell lines were therefore subcloned by limited dilution, and the subclone CHO-CREluc-hGHSR1a was selected for functional assays.

5.3.2. Cell stimulation and luciferase assay

Cells were plated onto white 96-well microplates in complete medium (mimimal essential medium supplemented with 10% v/v dialysed foetal bovine serum, 0.5 mM sodium pyruvate 0,01 mg/ml gentamicin, 0,3 mg/ml geneticin, 0,04 mg/ml L-Proline, all products from Gibco) and incubated for 24 h at 37 °C/5%CO_2. The next day, cells were stimulated for 4 h in the presence or absence of various concentrations of compounds in complete medium supplemented with 0.1 μM forskolin (Sigma) and 3 nM human ghrelin (Tocris). Luciferase activities were determined using the Luclite™ assay system (Perkin Elmer) and quantification of light emission was obtained by photon counting in a TopCount™ Microplate Scintillation and Luminescence Counter (Perkin Elmer).

5.3.3. Radioligand binding assay

Radioligand binding assay was performed on crude cell membrane extracts obtained from CHO–CREluc-hGHSR1a as previously described.[36] Cells grown to confluence were collected and spun at 200*g* for 10 min at 4 °C. Crude membranes were prepared by homogenization of cells in TM buffer (10 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$), centrifugation at 1000*g* for 5 min, and further centrifugation of the supernatants at 40,000*g* for 40 min at 4 °C. Pellets were resuspended in TM buffer supplemented with a protease inhibition cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) 2 μM f.c., E-64 14 nM f.c., bestatin 130 nM f.c., leupeptin 1 nM f.c., aprotinin 0.3 nM f.c., and sodium EDTA 1 μM f.c., Sigma), and stored at −80 °C until use. To determine the effect of the selected compounds on displacement of [^{125}I] human ghrelin, membranes (2 μg of protein) were incubated at room temperature for 1 h in TM buffer supplemented with 0.25% BSA w/v and 10% v/v dialysed foetal bovine serum added of 0.25 nM [^{125}I] human ghrelin (Perkin Elmer), in the presence or absence of increasing concentrations of selected compounds. A rapid filtration technique using Whatman GF/B filters (pretreated with 0.5% (w/v) polyethyleneimine) and a 96-well filtration apparatus (Unifilter-96 Harvester, Perkin Elmer) was used to harvest and rinse labeled membranes with cold TM buffer. Following a 90′/60 °C incubation, filter-bound radioactivity was counted upon addition of 50 μl/well Microscint™ 20 liquid scintillation cocktail (Perkin Elmer) using a TopCount™ Microplate Scintillation and Luminescence Counter (Perkin Elmer). Nonspecific binding was determined in the presence of 1 μM human ghrelin (Tocris).

5.4. In vivo assays

5.4.1. Gastric emptying

To assess gastric emptying, a bolus (1 ml/100 g body weight) of a standard meal (BaSO4, Prontobario Esofago, Bracco, Italia; 75% W/V with 0.5% Na–carboxymethylcellulose (CMC) was given by gavage and the rats were euthanized by cervical dislocation immediately (t = 0) or 60 min after the meal.[21] The pylorus and the esophageal junction were clamped before excision of the stomach. The gastric content was assessed by weighing the stomachs before and after removing their contents and then cutting, rinsing and blotting them on dry tissues. At each time, the stomach content remaining after drug treatment was compared to that of control drug-free rats, euthanized at t = 0. The percentage inhibition of gastric emptying was calculated for each rat: % gastric emptying = (content of treated rats/mean content of control rats)−100.

5.4.2. Mouse fasted refed model

Male C57BL/6 mice were fasted overnight and dosed the following day, 1 h before refeeding. The cumulative food intake was recorded for a period of 4 h after food return.

5.4.3. Food intake stimulated by central administration of ghrelin

For icv injection of solutions, adult male CD rats, weighing 200–300 g were implanted with stainless steel cannulae in the lateral ventricle. Animals were anesthetized (sodium pentobarbital, 60 mg/kg body weight, ip injection) and then placed in a stereotaxic frame. A stainless steel guide cannula (550 lm outer diameter, 10 mm length) was implanted stereotaxically at the following coordinates: 0.8 mm posterior to the bregma, 1.4 mm lateral to midline, and 2.0 mm below the surface of the left cortex such that a tip of the cannula was 1.0 mm above the left cerebral ventricle. Two stainless steel anchoring screws were fixed to the skull, and the cannula was secured in place by acrylic dental cement. The animals were then returned to their cages and allowed to recover for at least 7 days. During the recovery period, the animals were handled daily.

For icv injection of ghrelin, or vehicle (sterile 0.9% saline), a stainless steel injector was introduced through the cannula at a depth of 1.0 mm beyond the end of the guide. The total volume of injected solution of ghrelin (10 μg/rat), or saline into the lateral ventricle was 15 μl. Rat ghrelin was purchased from Tocris.

Thirty minutes before icv injection of ghrelin or vehicle, the compound **14f** was dosed at 10 mg/kg ip and animals were put into the cages. The cumulative food intake was measured for 4 h after icv injection of ghrelin or vehicle. The number of rats was 6–7 in each group.

5.4.4. Glucose tolerance test

Male C57/Bl6N were fasted overnight (16–18 h) and then given ghrelin antagonist at 30 mg/kg or vehicle by oral gavage. Thirty minutes after dosing, the fasting blood glucose level was measured from tail-tip blood using a Glucometer (Roche), and after 30 min the animals were given 2 g/kg of glucose by intraperitoneal injection (IPGTT). Blood glucose was measured again after 20, 40, 60, 80, 100, 120 min. The area under the glucose curve (AUC) from 0 to 80 min, was calculated using the trapezoidal method, and the effect of the compound on the AUC was expressed as a percentage of the AUC for the vehicle-treated group.

Acknowledgment

We thank Valentina Ghidelli, Elena Sabbadin, Donatella Picci, Giordano Lesma, Alessandra Silvani, Antonella Larovere, Raffaella Panigada, Massimo Meleri, Elisabetta Ottolina, Giovanni Boccardi, Andrea Bertario, Fausto Gesualdi, Silvia Milanesi, Philippe Ochsenbein, Marc Bianciotto, Laurence Fajas and Sabina Improta for their contribution to this work.

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