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European Journal of Medicinal Chemistry 38 (2003) 671–686
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Point of view

How a single inversion of configuration leads to a reversal of the binding mode: proposal of a novel arrangement of CCK_2 ligands in their receptor, and contribution to the development of peptidomimetic or non-peptide CCK_2 ligands

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Received 13 January 2003; received in revised form 22 April 2003; accepted 22 April 2003

Abstract

The implication of CCK_2 receptors in crucial physiological functions has driven the search for synthetic ligands of this receptor. A notable rationale starting from CCK-4 (minimal endogenous CCK_2 agonist), the 'dipeptoid' strategy, led to potent CCK_2 antagonists exemplified by CI-988. However, careful examination of the literature enlightened several incompatibilities between the proposed recognition mode of the receptor by such compounds (or peptide analogues) and experimental data. Thus, we hypothesised that CCK_2 'dipeptoid' antagonists bind the receptor in a mode opposite to that previously suggested. The reexamination of numerous published data, supported by the characterisation of new 'hybrid' compounds, brought out strong evidence that this 'reverse' mode truly characterises CCK_2 'dipeptoid' antagonists. These findings renew the perspectives of further chemical development of CCK_2 ligands, e.g. non-peptidic agonists.
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Keywords: Cholecystokinin; CCK_2; Binding mode; Peptidomimetics

1. Introduction

1.1. Preliminary statement

We may here warn the reader that our target in this study was unusual with respect to the field of medicinal chemistry. We did not look forward to fully characterising new CCK_2 antagonists or agonists, but only to further understanding the binding mode of a particular and widely used family of such ligands: 'dipeptoid' [1] antagonists. After a cautious examination of the literature had awoken our interest to intriguing and repeated inconsistencies, we developed a new structural model, which we confirmed by specifically designed molecules, The latter must be considered as *structural tools* or *probes*, but NOT as pharmacologically significant compounds in the limits of the subject debated here. This point needs further characterisation studies, currently under progress. Therefore, the agonist/antagonist profile of the new compounds will not be a topic of special interest, the only relevant parameter, with respect to the model discussed, being their affinity. Likewise, the CCK_1 affinity of the new compounds is a useless parameter that will not be discussed here.

1.2. Generalities

Cholecystokinin (CCK), as the most widely distributed neuropeptide [2], has been a subject of interest for therapeutic research for more than 20 years [3]. Indeed, CCK is implicated in a large range of physiological effects, including regulation of appetite, anxiety, noci-

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doi:10.1016/S0223-5234(03)00112-0

ception, emotional and cognitive processes [4]. These effects are mediated by the interaction of CCK with two seven-transmembrane receptors now designated CCK_1 and CCK_2 (formerly CCK-A and CCK-B, respectively) [5]. The latter is particularly interesting, since it is distributed throughout the brain, and has been shown to be implicated in most central effects of CCK [6].

Naturally, the design of selective ligands of CCK_1 or CCK_2 receptors has been a subject of interest for medicinal chemists since the characterisation of these receptors. The starting points for a rational design of such compounds were the endogenous peptides identified as active on CCK receptors. The sulphated and amidated octapeptide CCK-8S (Asp–Tyr(SO_3^-)–Met–Gly–Trp–Met–Asp–Phe–NH_2) recognises both CCK_1 and CCK_2 receptors while peptides deprived of the sulphated tyrosine residue exhibit a large selectivity for CCK_2 receptors, i.e. CCK-8NS (Asp–Tyr–Met–Gly–Trp–Met–Asp–Phe–NH_2), and CCK-4 (Trp–Met–Asp–Phe–NH_2) which appears as the shortest CCK fragment remaining active on CCK_2 receptors [7].

Amino acid substitutions, peptide bond modifications or cyclizations introduced in the structure of CCK-8S or CCK-4 led to more active and selective CCK_1 and CCK_2 compounds exemplified by the highly selective CCK_2 agonists BC197 (Boc–cyclo(D-Asp–Tyr(SO_3^-)–Nle–DLys)–Trp–Nle–Asp–Phe–NH_2) [8], BC264 (Boc–Tyr(SO_3^-)–gNle–mGly–Trp–NMeNle–Asp–Phe–NH_2) [9], BBL454 (H–$(CH_2)_5$–$COCH_2CO$–Trp–NMeNle–Asp–Phe–NH_2) [10] or RB360 (a macrocyclic derivative of CCK-4) [11], and selective CCK_1 agonists such as JMV-180 (Boc–Asp–Tyr(SO_3^-)–Met–Gly–Trp–Met–Asp–OCH_2CH_2Ph) [12] or A-71623 (Boc–Trp–Lys(*o*-tolylurea)–Asp–NMePhe–NH_2) [13].

1.3. Peptoid CCK_2 antagonists

Whatever their promising in vitro properties, the therapeutic use of such peptide-like compounds remained problematic, essentially in terms of bioavailability. Therefore, the search for smaller and 'less-peptidic' compounds (with a minimal amount of peptide bonds) was naturally the further area of interest for chemists. In the late 80s, Parke–Davis' scientists introduced in this purpose a very nice rationale called the 'dipeptoid strategy' [14] (Fig. 1).

This strategy relies on the assumption that a dipeptide retaining some affinity for the receptor can be extracted from a large peptide of biological interest. Subsequent optimisation of the side chains, N-terminus and C-terminus of this so-called 'dipeptide lead' should allow to design small compounds based on this dipeptide, and endowed with good affinity for the target receptor.

In the case of CCK, Boc–Trp–Phe–NH_2 was identified as the dipeptide lead [15]; optimisation led to CI-988 (PD134,308, ^{2}Adoc–αMe-D-Trp–$NHCH_2CH(Ph)NH$–$CO(CH_2)_2$–CO_2H), which is the first highly potent CCK_2 antagonist resulting from a rational development, and was brought under clinical evaluation [16]. Further evolution from this compound, aiming to reduce its size and improve its solubility and bioavailability, led to CI-1015 [17] (Fig. 1). The 'dipeptoid' rationale was also successfully applied to neurokinin, leading to selective NK-1, NK-2 and NK-3 antagonists [18].

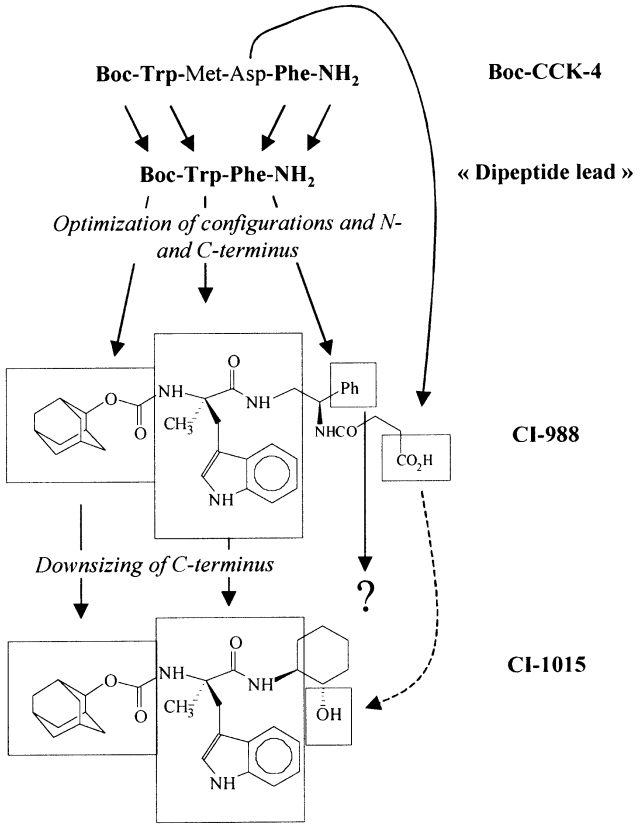


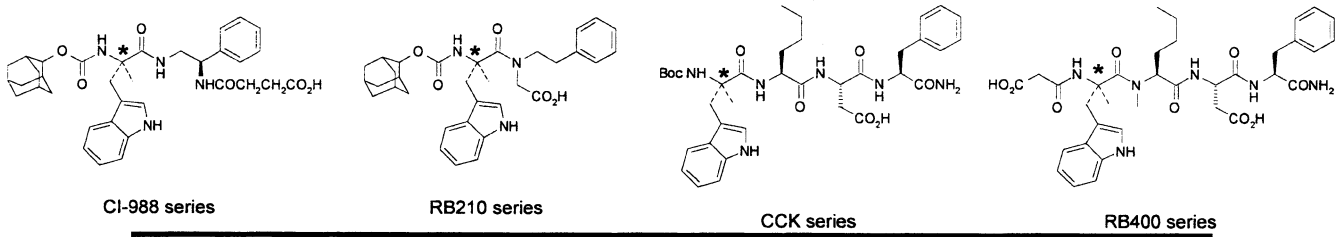
Fig. 1. The 'dipeptoid' strategy.

1.4. Reconsideration of available data on the binding mode of peptoids

Nevertheless, some published data, recalled in Table 1, and concerning the recognition mode of the CCK_2 receptor by 'dipeptoid' antagonists, appeared difficult to understand in this way. As a matter of fact, the pathway followed by their discoverers suggested—and was clearly interpreted in this way—that the central αMe-D-Trp residue took the place of the original Trp, that the aromatic N-terminal group replaced the side chain of the phenylalanine, and that the additional C-terminal acid functionality mimicked the Asp side chain (Fig. 1). Unfortunately, this recognition scheme could not be considered as valid for the new compound CI-1015 (derived from CI-988), which does not bear any aromatic moiety, and where suppressing the C-terminal carboxylate in further derivatives is even tolerated [17].

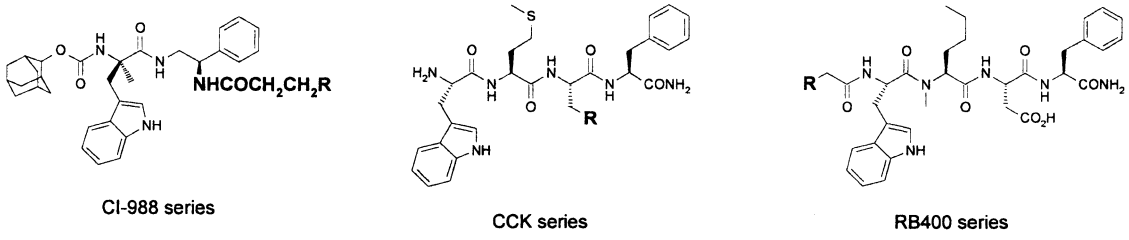
Table 1
Structure–affinity relationships in peptoid and peptide series [a]

- **Configuration of the tryptophan residue**



*	CI-988 series [16a]	RB210 series [16c]	CCK series [23]	RB400 series [26]
R	1.7 nM	6.1 nM	160 nM	6000 nM
S	63 nM	36 nM	3.2 nM	0.75 nM
R/S	**0.027**	**0.17**	**50**	**8000**

- **Acidic moiety : influence of nature**



R	CI-988 series [21a,b]	RB210 series	CCK series [23]	RB400 series [26]
CO_2H	1.7 nM	*data not available*	19 nM	0.75 nM
SO_3H	1.3 nM		> 10000 nM	0.5 nM
CO_2H/ SO_3H	**1.3**		**< 0.001**	**1.5**

Thus, it appeared dubious that the original scheme proposed by Horwell et al. [18] was exact.

1.4.1. Configuration of the tryptophan residue

Several other incompatibilities were enlightened by a reexamination of published structure–affinity relationships on 'dipeptoid' and peptide CCK_2 ligands. The most significant of these inconsistencies are presented on Table 1 and are shortly detailed in the following paragraphs. In fact, it was difficult to understand:

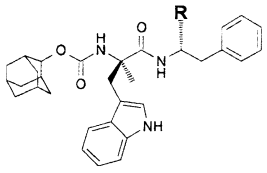
(1) why the preferred configuration of tryptophan should be L in the case of peptides (with a very important selectivity factor against the D-Trp analogues) and D in the case of 'dipeptoids' (the selectivity factor being comparable to that of peptides, but in the opposite way) and

(2) why α-methylation of tryptophan was so important in the case of peptoids. In particular, results from the Parke–Davis team evidence that α-methylation of tryptophan in the CCK-4 series does not improve affinity, and that a L-tryptophan is clearly preferred even after α-methylation [19].

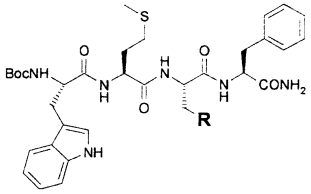
1.4.2. Importance of the C-terminal acid moiety

Besides, 'dipeptoids' show a large tolerance at the level of the acid functionality (in terms of position, nature, even presence or not) [16] whereas, no modification of the Asp side chain (replacement by Glu for instance [20]) in peptides is possible lest the affinity

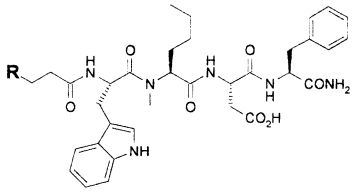
- **Acidic moiety : influence of suppression**



CI-988 series



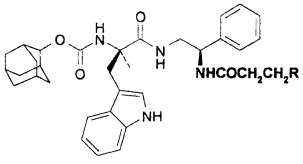
CCK series



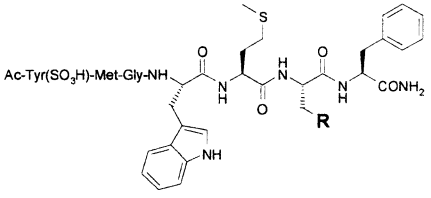
RB400 series

R	CI-988 series [21c]	RB210 series	CCK series [42]	RB400 series [26]
CO_2H	39	*data not available*	42 nM	3 nM
H	32		27000 nM	4.35 nM
CH_3 / CH_2CO_2H	**0.8**		**650**	**1.4**

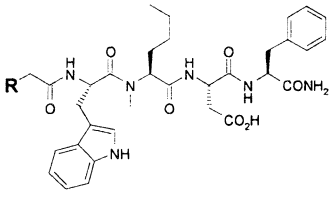
- **Acidic moiety : influence of substitution**



CI-988 series



CCK series



RB400 series

R	CI-988 series [16a, 21c]	RB210 series	CCK series [20b]	RB400 series [26]
CO_2H	1.7 nM	*data not available*	4.4 nM	0.75 nM
CO_2CH_3	15 nM		*data not available*	0.5 nM
$CONH_2$	18 nM		200 nM	2 nM
COR / CO_2H	< 11		**> 45**	< 4

would decrease dramatically (Table 1). Thus, substituting the carboxylate by a sulfonate at this position leads to an equipotent 'dipeptoid' [21] but the sulfoalanine-containing peptide [22] is devoid of affinity ($K_i > 1$ μM on transfected CHO cells; Bischoff, Danascimento, personal communication). Likewise, suppression of the charge at the level of the aspartyl residue in CCK is forbidden, whilst it is clearly possible in peptoids, e.g. esterification of the acidic moiety does not evoke a significant decrease in affinity [16].

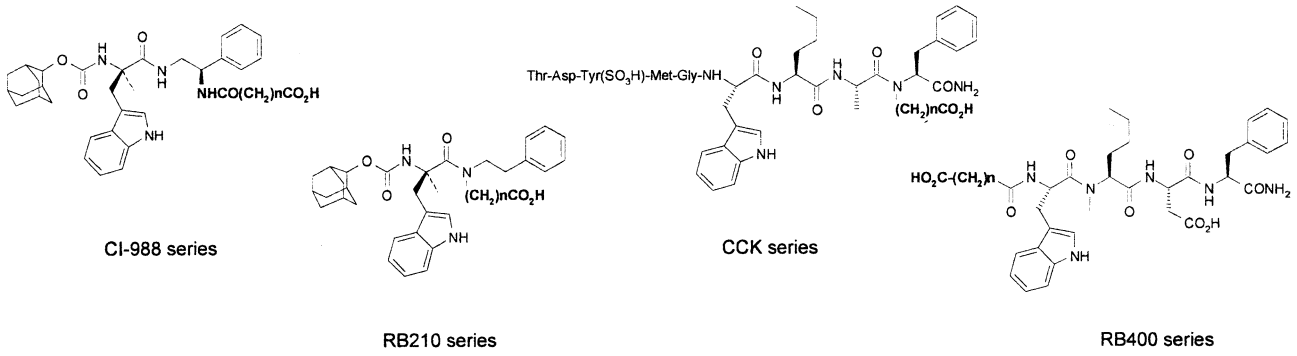
1.4.3. Influence of the N-terminal capping

Lastly, we stated that the influence of the N-terminal group is weak in the peptide series, where a Boc group but also a short propionyl capping are sufficient to allow high-affinity binding to the CCK_2 receptor, which a larger group such as ^{2}Adoc does not improve [23]; on the other hand, in the 'dipeptoid' series, a large hydrophobic group such as ^{2}Adoc or Amoc, is compulsory; indeed, replacing ^{2}Adoc by Boc in CI-988 derivatives leads to a 10-fold decrease in affinity [24].

1.4.4. Incoherence of conformational hypotheses

The conformational basis of the 'dipeptoid' strategy is the putative proximity between the side chains of Trp and Phe in the bioactive conformation of CCK-4. This speculation may not be true, regarding experimental results from conformational analysis and molecular

- **Acidic moiety : influence of chain length**



n	CI-988 series [16a]	RB210 series [16c]	CCK series [20a]	RB400 series [26]
1	0.8 nM	14 nM	0.8 nM	0.75 nM
2	1.7 nM	36 nM	199 nM[b]	3 nM
2 / 1	2.1	2.6	**250**	4

b

[a] The results presented in the tables have been obtained on different biological models from a series to another, but are homogeneous inside a column. The absolute values are much less relevant than the variations due to the same parameter in different columns which are independent from the model used. This variation is explicated in some cases as a ratio between affinity values.

[b] Substitution of Asp by Glu also leads to total loss of activity on a functional array [50].

modelling under NMR-based conformational constraints, which show that an inactive compound (Boc–Trp–*NMePhe*–Asp–Phe–NH_2) is characterised by such a proximity, whereas an active compound (Boc–Trp–*NMeNle*–Asp–Phe–NH_2) adopts a S-shaped conformation, where the Trp and Phe aromatic moieties point to opposite directions, while the Boc moiety and the Nle side chain are located in the vicinity of each other [25].

Indeed, the determined conformations are calculated in solution, and may not be the conformation of the peptide bound to its receptor. However, the design and synthesis of CCK-4 derived cyclic agonists such as RB360, on the basis of these conformational studies, has confirmed that the proposed S-shaped conformation is likely to be the bioactive one [11]. Thus, the proposed 'continuous' binding mode of 'dipeptoids' [14], where the indole and phenyl moieties of CI-988 would be near each other in the bioactive conformation, thus mimicking the behaviour of Trp and Phe side-chains of CCK-4, proves again questionable.

1.4.5. Summary of the previous statements

Thus, the generally admitted superposition of 'dipeptoids' with CCK-8 appears far less than convincing, not only at the light of recent developments (CI-1015 and derivatives can hardly enter the original superposition pattern) but also at the light of a throughout screening of the literature, which suggests a number of inconsistencies, both in terms of structure–affinity relation-

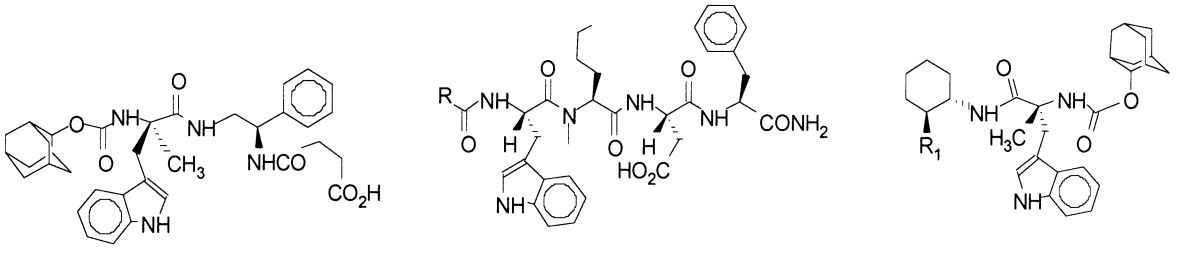


Fig. 2.

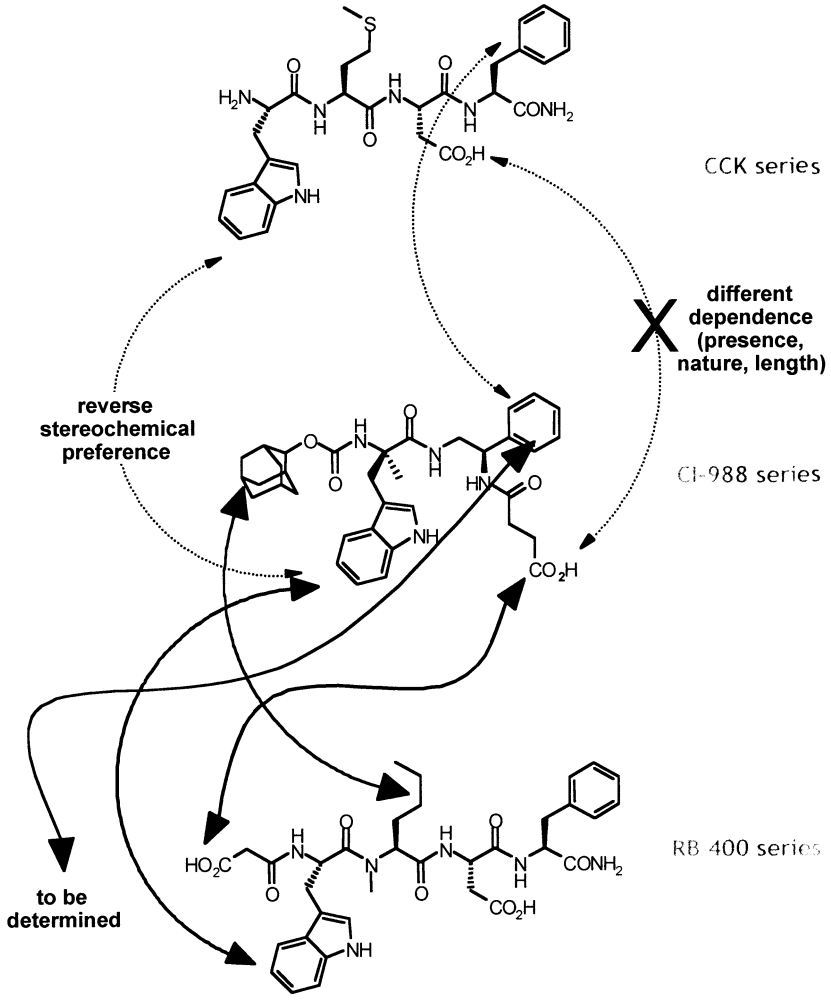


Fig. 3. New analogy proposed between peptoid antagonists and RB400-derived agonists (dashed lines: former model proposed by Parke–Davis (cf. Fig. 1), wide dark lines: hypothesis based on published experimental data (cf. Table 1), thin solid lines: complementary superposable moieties, to be further confirmed).

ships at several levels of the molecule (N-terminus, central Trp residue, C-terminus) and of validity of conformational hypotheses.

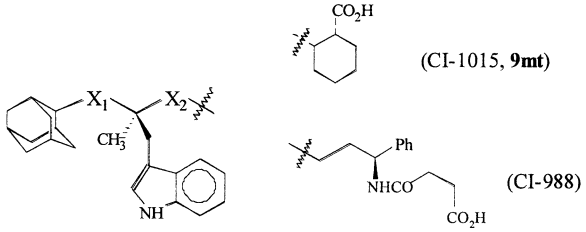
1.5. Hypothesis for an alternative recognition of the CCK_2 receptor by peptoids

To explain these incoherences, it could be simple to propose that 'dipeptoids' and peptides did in fact not recognise the same region of the CCK_2 receptor—but this would mean that the development of 'dipeptoids' was entirely due to chance—which could certainly not be taken as a starting hypothesis at first sight! Fortunately, the recent development of new CCK_2 agonists based on the N-terminal modified analogue of CCK-4, RB400 (HO_2C–CH_2CO–Trp–NMeNle–Asp–Phe–NH_2) [26] brought to our mind the hypothesis that 'dipeptoids' may bind to the receptor in the reverse way as thought before, as shown by the superposition of **1** (the acid analogue of CI-1015) and RB405, a RB400 derivative (Fig. 2). It is most important to see that *the side chain of tryptophan* (which is the most important feature in terms of contribution to the affinity of CCK_2 ligands to the CCK_2 receptor) *has the same orientation in both compounds, when CI-988 is represented from the C-terminus to the N-terminus*, which immediately explains the problem of the preferred configuration at the level of this residue. Available data in the series of RB400 [26] is presented in Table 1, which also summarises the influence of several key features in the series of CCK-4, RB400 and CI-988, depending on the superposition of the latter to the peptidic compounds.

In fact, the C-terminal acid moiety of CI-988 and **1** can alternatively be superposed on the C-terminal aspartate side-chain of RB400 and CCK-4, or on the N-terminal malonate residue of RB400 (Fig. 3). It may be stressed in particular that the D-Trp analogue of RB400, prepared for the aim of this study, shows a 6000-fold decrease in affinity from the original compound. Besides, the N-terminal acidic function of

Table 2
Affinities of the new compounds

Compound	Ki (CHO)[a]
4 : Boc-Trp-NMeNle-(2,4-Cl_2Ph)D-*cis*-Hyp-OH **[2(N)/RB213(C)]**	> 1 μM
5 : HO_2C-CH_2CO-Trp-NMeNle-(2,4-Cl_2Ph)D-*cis*-Hyp-OH **[RB400(N)/RB213(C)]**	> 10 μM
7ta : *trans*-HO_2C-(cHex)-CO-Trp-NMeNle-Asp-Phe-NH_2	1.8 ± 0.8 nM
7tb : *trans*-HO_2C-(cHex)-CO-Trp-NMeNle-Asp-Phe-NH_2[b] **[1(C)/RB400(C)]**	2.9 ± 1.3 nM
8b : HO_2C-$(CH_2)_2$CONHCH*(Ph)CH_2CO-Trp-NMeNle-Asp-Phe-NH_2	(RS)[c] 9 ± 4 nM
[CI-988(C)/RB400(C)]	(S) 8 ± 0.7 nM
9c : *cis*-HO_2C-(cHex)-CO-Trp-$NHCH_2$-(2-adamantyl)[d]	666 ± 98 nM
9t : *trans*-HO_2C-(cHex)-CO-Trp-$NHCH_2$-(2-adamantyl)[d]	420 ± 50 nM
9mt : *trans*-HO_2C-(cHex)-CO-αMe-Trp-$NHCH_2$-(2-adamantyl)[d]	53 ± 5 nM
10a : phenylacetyl-Nle-Gly-Trp-Nle-Asp-PheNH_2	39 ± 6 nM
10b : phenylacetyl-Nle-D-Phg-Trp-Nle-Asp-PheNH_2	132 ± 15 nM
10c : phenylacetyl-D-Asp-Gly-Trp-Nle-Asp-PheNH_2	16 ± 5 nM
10d : phenylacetyl-D-Asp-D-Phg-Trp-Nle-Asp-PheNH_2	98 ± 12 nM



(CI-1015, **9mt**)

(CI-988)

Compound	X_1	X_2	Ki(CHO)[a]	Ki(mouse)[e]
CI-988	OCONH	CONH	7.3 ± 0.7 nM	1.7 nM[f]
CI-1015	OCONH	CONH	n.d.	3 nM[g]
9mt	CH_2NHCO	NHCO	53 ± 5 nM	20 ± 8 nM

(a) K_i values are the means ± S.E.M. of at least three determinations, each performed in triplicate on membranes of CHO cells transfected with the rat CCK_2 receptor. (b) The two diastereomers ((*S*,*S*) and (*R*,*R*) at the level of the cyclohexane ring) could not be characterised. (c) configuration at the * carbon. (d) ca. 1:1 mixture of two diastereomers ((*S*,*S*) and (*R*,*R*) at the level of the cyclohexane ring) which could not be separated. (e) K_i values are the means ± S.E.M. of at least three determinations, each performed in triplicate on mouse brain homogenates (**9mt**) or mouse cortex homogenates (CI) (f) from [16a]. (g) from [17].

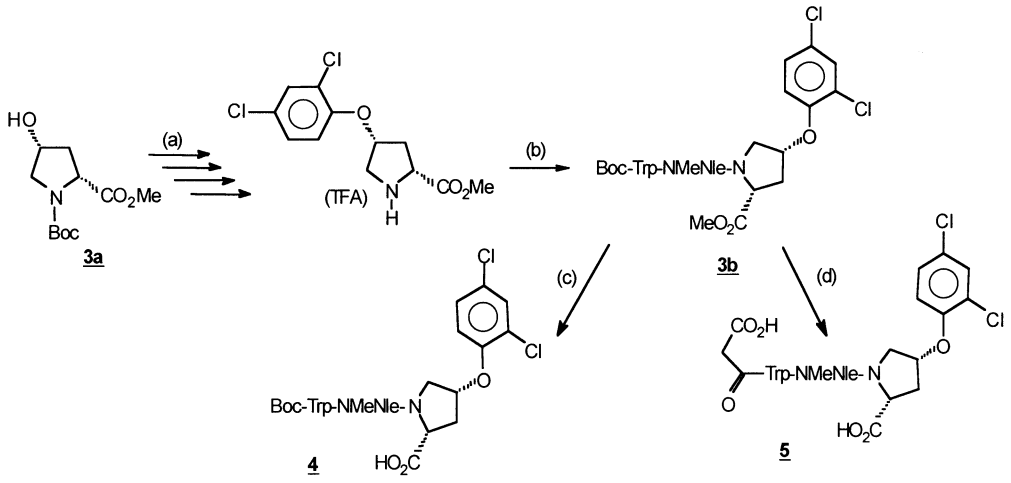
RB400 behaves like the C-terminal analogous moiety of peptoids, since its distance to the peptide backbone influences affinity in the same proportions, and since it can be replaced by a sulfonate or esterified with a minor negative effect.

1.6. Methodology

Starting from this hypothetical 'reverse' superposition of 'dipeptoids' on peptides, we further examined the literature in the field of peptide-derived CCK_2 ligands which could support either binding mode, and we developed new molecules that would ascertain which mode should be regarded as correct.

With this aim in view, 'hybrid' molecules were designed and synthesised (families of compound **4**–**8**; see Ref. [27] for details on the numbering of the compounds), centred on a tryptophan or D-tryptophan residue, and possessing at their N- or C-terminus characteristic features of 'dipeptoids' (CI-988, CI-1015, **1**, and RB213, a constrained 'dipeptoid' developed in our laboratory [28]) or of peptide agonists (RB400, or **2** (Boc–Trp–NMeNle–Asp–Phe–NH_2) [23]). These molecules were used to probe the compatibility between CCK-, RB400-, and 'dipeptoid'-derived fragments, noteworthy to check the possibility of combining two features characteristic for the N-terminus of RB400 and 'dipeptoids' around a central tryptophan residue.

Furthermore, the importance of peptide bonds and the possibility of their inversion was studied in detail,



(a) i - $PhCO_2H$, Ph_3P, DEAD, DMF ; ii - MeOH, NaOH ; iii - $(2,4\text{-}Cl_2)PhOH$, Ph_3P, DEAD, DMF ; iv - TFA, CH_2Cl_2
(b) i - Boc-NMeNle-OH, BOP, DIEA, DMF ; ii - TFA, CH_2Cl_2 ; iii - Boc-Trp-OH, BOP, DIEA, DMF
(c) NaOH, MeOH, H_2O
(d) i - TFA, anisole, CH_2Cl_2 ; ii - $EtO_2C\text{-}CH_2CO_2Na$, BOP, DIEA, DMF ; iii - NaOH, MeOH, H_2O

Fig. 4. Synthesis of hybrid compounds **4** and **5**.

since the proposed docking of 'dipeptoids' in the CCK_2 receptor reverts all peptide bonds compared to the equivalent peptides (see Fig. 2): this was achieved with compounds of the **9** series.

The encouraging results obtained with these 'hybrid' compounds led us into returning to more peptidic compounds to carefully analyse the literature in particular domains (Table 3), especially published data on CCK-4 derivatives. Additional incongruities observed will be presented in Section 4, and led us into designing new probes that could comfort the results obtained with the 'hybrid' series of compounds (**4**–**9**). Briefly, a series of CCK-7-derived compounds (10 series) was prepared, which, this time, hybridised C-terminal features of several CCK-4 analogues, around a central tryptophan residue.

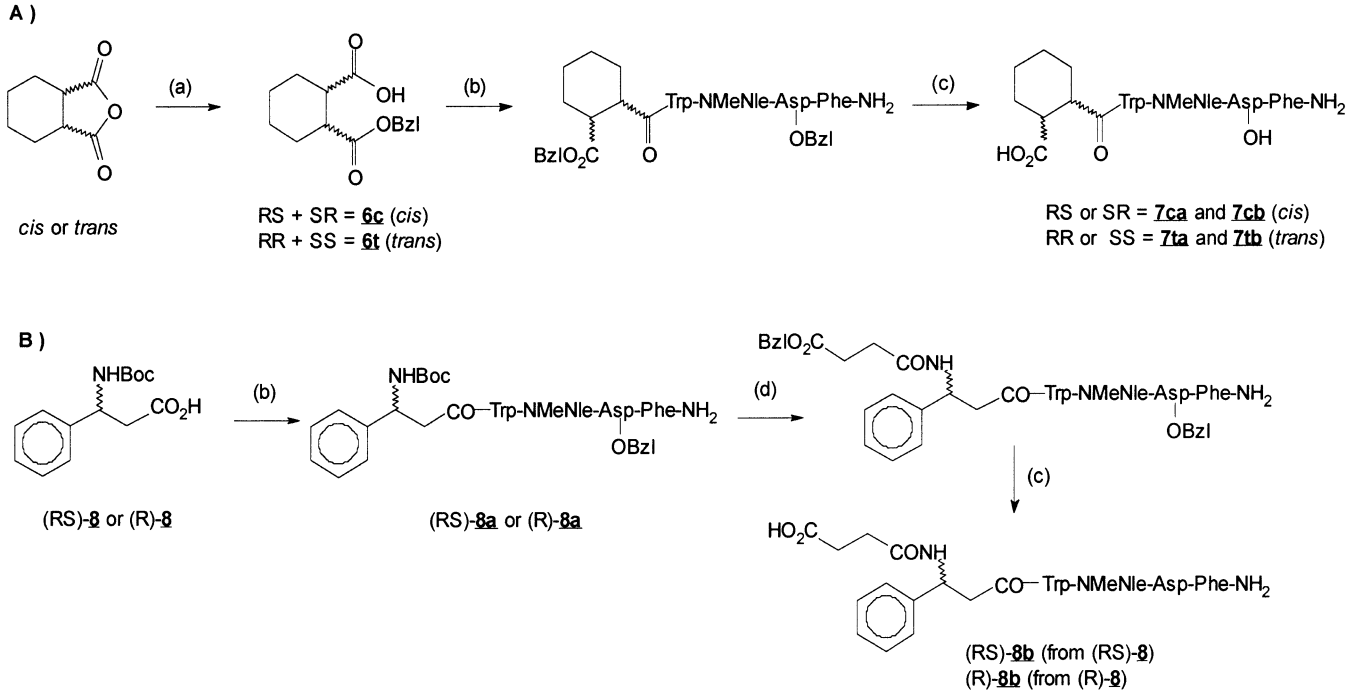
To evaluate the contribution of the characteristic features of these new molecules to the recognition of CCK_2 receptors, they were tested for their capacity to displace the specific binding of [^{3}H]pCCK-8 on membranes of CHO cells stably transfected by the rat CCK_2 receptor [28]. Since our purpose was only to evaluate structural requirements for CCK_2 recognition, and not to develop new pharmacological ligands, neither the agonist or antagonist character of the new molecules, nor their affinity for CCK_1 receptors, mattered to our purposes. In our case, CCK_1 affinity does not interfere for CCK_2 affinity measurements, due to the choice of models where CCK_1 receptors are not present (transfected CHO cells) or in a large minority (murine cerebral homogenates).

2. Chemistry [29]

In the following text, several new molecules (final compounds numbered from **4** to **10** with an adequate letter, see Table 2 for details) resulting from the structural hybridisation of standard CCK_2 agonists and antagonists will be discussed. *For ease of comprehension, they will be both designated by a number and by their composition: for instance, the combination of a C-terminal fragment of CI-988 and a C-terminal fragment of RB400 will be designated CI-988(C)/RB400(C)*. The combination of C-terminal features of 'dipeptoids' with N-terminal sequences of peptides were easily realised by standard liquid-phase peptide synthesis, using BOP as coupling agent and diisopropylethylamine as base. Boc groups were deprotected by trifluoroacetic acid in dichloromethane; saponifications were achieved by NaOH in a methanol/water mixture.

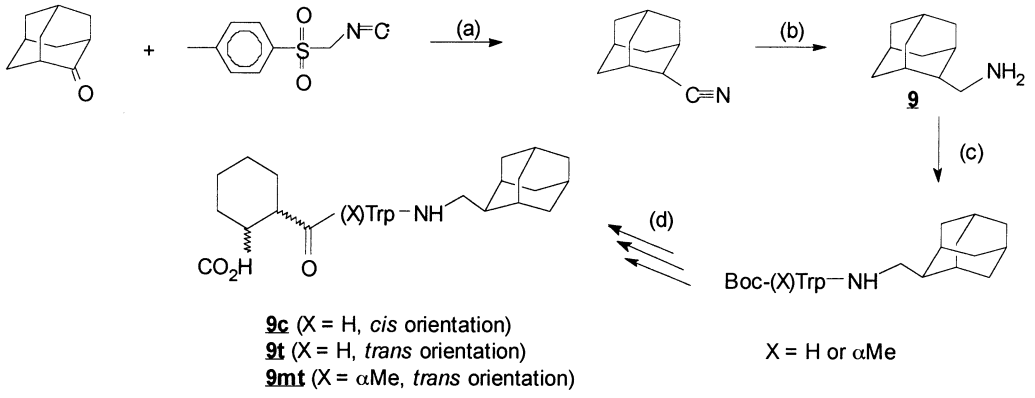
2.1. Synthesis of hybrid compounds *4* and *5*

To introduce a fragment very characteristic for peptoids, we chose to replace the C-terminal dipeptide Asp–Phe–NH_2 of Boc–CCK-4 and of RB400 by the modified amino acid 4-(*o*,*p*-dichlorophenoxy)proline (**3**), a constrained mimic of the C-terminus of CI-988. Boc-*N*-methyl-norleucine was coupled to **3a**, the methyl ester of **3**, prepared following previously described procedures [28], and the resulting dipeptide was deprotected by trifluoroacetic acid. Subsequent coupling of Boc–tryptophan followed by saponification led to **4**, the



(a) $PhCH_2OH$
(b) TFA.Trp-NMeNle-Asp(OBzl)-$PheNH_2$, BOP, DIEA, DMF
(c) H_2, Pd/C, MeOH then separation of diastereomers by semipreparative HPLC
(d) i - TFA, CH_2Cl_2, anisole ; ii - $BzlO_2CCH_2CH_2CO_2H$, BOP, DIEA, DMF

Fig. 5. Synthesis of hybrid compounds **6**–**8**.



a) tBuOK, EtOH ; b) $NaBH_4$, $CoCl_2$, MeOH ; c) Boc-(X)Trp-OH, BOP, DIEA, DMF
d) i - TFA, anisole, CH_2Cl_2 ; ii - **6c** or **6t**, BOP, DIEA, DMF ;
iii - H_2, Pd/C, MeOH followed by separation of diastereomers by semipreparative HPLC

Fig. 6. Synthesis of hybrid compounds **9**.

2(N)/RB213(C) hybrid; alternatively, deprotection of the Boc–Trp followed by condensation with malonic acid ethyl ester and hydrolysis of the two ester functionalities led to **5**, the RB400(N)/RB213(C) hybrid (Fig. 4).

2.2. Synthesis and separation of isomers of hybrid compounds *7c*, *7t*, *8a* and *8b*

The combination of two C-terminal fragments (–NHR_1 and –NHR_2) required that an amine functionality be replaced by a carboxylate one (for instance –NHR_2⟨HO–CO–R_2), to allow the chemical linkage of the fragments by a peptide bond (to yield in this case R_1NHCOR_2). Therefore, analogues of the C-terminal parts of CI-988 (–*NH*CH_2CH(Ph)NH–$COCH_2CH_2CO_2H$) and of **1** (-2-aminocyclohexanecarboxylic acid) were prepared, with a carboxylate moiety replacing the amine function. These analogues are formally cyclohexane-1,2-dicarboxylic acids (in the case of **1**) and $HO_2C$$CH_2$CH(Ph)NH–$COCH_2CH_2CO_2H$ (in the case of CI-988), which is a pseudodipeptide resulting from the coupling of *homo*-phenylglycine and succinic acid.

On the one hand, the preparation of the benzyl monoesters of *cis*- and *trans*-cyclohexane-1,2-dicarboxylic acids (**6c** and **6t**) was achieved in excellent yields by nucleophilic opening of commercially available *cis*- and *trans*-cyclohexane-1,2-dicarboxylic anhydrides by benzyl alcohol.

On the other hand, Boc-*homo*-phenylglycine (**8**) was synthesised en route to CI-988 analogues: racemic **8** was obtained by protection with Boc_2O of commercial *homo*-phenylglycine (sold under the name 'β-phenylalanine'), whilst its pure (*R*)-isomer was obtained by direct homologation of Boc-(*S*)-phenylglycine with diazomethane [30].

Further condensation of **6c**, **6t**, (*RS*)-**8** or (*R*)-**8** with the tetrapeptide TFA. H–Trp–NMeNle–Asp(OBzl)–Phe–NH_2 [26] led to **7c**, **7t**, (*RS*)-**8a** and (*R*)-**8a**, respectively, **7c** and **7t** being mixtures of two diastereoisomers. **7c** and **7t** benzylic protections were removed by catalytic hydrogenation yielding again mixtures of diastereoisomers which could be completely separated by semi-preparative HPLC, leading to four products, **7ca**, **7cb** (*cis* relative orientation of the cyclohexane substituents), **7ta** and **7tb** (*trans* orientation) which are all **1**(C)/RB400(C) hybrids. The Boc moiety of (*R*,*S*)-**8a** and (*R*)-**8a** was deprotected in acidic medium, and the resulting compound was coupled with monobenzyl succinate. Final hydrogenolysis of the benzyl esters led to the hexapeptide analogues (*R*,*S*)-**8b** and (*R*)-**8b**, which are CI-988(C)/RB400(C) hybrids. All these steps are presented on Fig. 5.

2.3. Preparation of retro-inverso compounds *9c*, *9t* and *9mt*

Another series of compounds was derived from **1** by inversion of all peptide bonds to check their importance for the recognition of the CCK_2 receptor. This required again, as for the preceding compounds, the preparation of intermediates where amino groups and carboxylates were exchanged. In the case of non-terminal amino acids, this is immediately obtained by using the enantiomer of the original residue (L-Trp for D-Trp for instance).

Thus, the N-terminal protection of the tryptophan residue in **1**, namely 2-adamantyl*oxycarbonyl*, was replaced by 2-adamantyl*methylamine* **9**, which was prepared in two steps. Adamantanone was first converted via treatment with tosylmethylisocyanide to 2-adamantanenitrile [31], whose reduction with the sodium borohydride-cobalt chloride (II) system [32] yielded the expected amine with a 60% overall yield. This amine was coupled to Boc–tryptophan which was deprotected and coupled to **6c** or **6t**, to give after reduction of the benzyl ester two mixtures of two diastereomers in 1:1 proportions, **9c** (*cis* orientation of the cyclohexane substituents) and **9t**, which are nothing but retro-inverso equivalents of **1**.

In a second series of experiments, Boc–αMe-Trp was introduced instead of Boc–Trp. It was only condensed with the mixture of *trans* isomers **6t**, since this orientation was proved (**9t** having a better affinity than **9c**) to be more favourable for receptor recognition, finally leading to **9mt** the methylated equivalent of **9t**, again a mixture of stereoisomers but whose separation proved impossible by available HPLC techniques (Fig. 6).

2.4. Solid-phase synthesis of modified heptapetides *10a*–*10d*

In a last step, we used the information gathered with compounds **4**–**9** to design modified longer peptide analogues derived from CCK-7. These compounds were prepared by solid-phase synthesis using Fmoc chemistry on a MBHA resin which allows access to C-terminal amidated peptides. For ease of synthesis, the N-terminal amino acid of CCK-7, tyrosine, was replaced by phenylacetic acid, since desamination [33] or introduction of a shorter side chain [34] at this position do not strongly affect the recognition of the CCK_2 receptor; original methionine residues were also replaced by norleucine, which is completely innocuous in terms of biological activity [35]. Thus, four peptides were synthesised, **10a** (Phenylacetyl–Nle–Gly–Trp–Nle–Asp–Phe–NH_2), **10b** (Phenylacetyl–Nle–*D-Phg*–Trp–Nle–Asp–Phe–NH_2), **10c** (Phenylacetyl–*D-Asp*–Gly–Trp–Nle–Asp–Phe–NH_2) and **10d** (Phenylacetyl–*D-Asp*–*D-Phg*–Trp–Nle–Asp–Phe–NH_2).

3. Pharmacology

All compounds relevant to our investigations (see Section 4 for details) were then tested for their ability to displace [^{3}H]pCCK-8 from membrane preparations of CHO cells stably transfected with the rat CCK_2 receptor [28], or—when necessary—from freshly prepared murine cerebral homogenates [36]. Binding results for all new compounds are presented in Table 2.

These results are described and discussed in details in the following section: indeed, as specified in Section 1, these results only become significant when related to previous findings of the literature, and when compared within analogous families of compounds.

Moreover, it must be underlined again that the only parameters discussed here are the structural features responsible for ligand-receptor *binding*, i.e. *structure–affinity relationships* and thus, that the only significant pharmacological value is the affinity of the ligand, expressed as a K_i value. *Structure–activity* relationships, dealing with the agonist or antagonist profile of the new molecules are not relevant to the forthcoming discussion, and will be the subject of another paper.

4. Results and discussion

4.1. Hybrids based on CI-988 and CI-1015

In a first attempt to evaluate the reliability of our new hypothesis, based on the possible superposition of 'dipeptoids' on the N-terminal tripeptide of CCK-5 derivatives like RB400, we prepared hybrid molecules combining C-terminal fragments relevant for 'dipeptoid' antagonists and the C-terminus of CCK-like peptides, the tryptophan residue serving as central common template. It appears that the introduction, at the N-terminal side of the tryptophan moiety in CCK-derived peptides **7ta**, **7tb** and **8b**, of fragments of 'dipeptoids' usually found at its C-terminal side, is favourable in terms of affinity. Indeed, **7ta** and **7tb** are equipotent to RB405 and to **2** (affinities close to 2 nM, see Fig. 2 for structures), to which they are most closely related, and (*RS*)-**8b** is equipotent to its parent CI-988. Interestingly, (*R*)-**8b** has a slightly better affinity for the CCK_2 receptor than the racemate, which indicates that the preferred absolute configuration of the asymmetric carbon atom in the N-terminal fragment is the same as in CI-988.

4.2. Hybrids based on RB213 (containing 4-(*o*,*p*-dichlorophenoxy)-proline)

On the opposite, replacing in **2** and RB400 the original C-terminal dipeptide Asp–Phe–NH_2 by the constrained amino acid *cis*-4-(*o*,*p*-dichlorophenoxy)-proline to yield **4** [RB213(N)/2(C)] and **5** [RB213(N)/RB400(C)] was completely unsuccessful, since **4** had a very weak affinity (1 μM) for the receptor, while **5** was practically devoid of affinity ($K_i > 10$ μM). Thus, while this proline derivative had been thought to mimic the C-terminal dipeptide of CCK-4, its introduction in CCK-4-derived peptides is far from confirming this hypothesis. It is also worth to underline that substituting the malonyl moiety for the N-terminal Boc capping was slightly benefic in the case of RB400 vs. **2**, but is here very unfavourable: this suggests that these fragments might face different regions of the receptor.

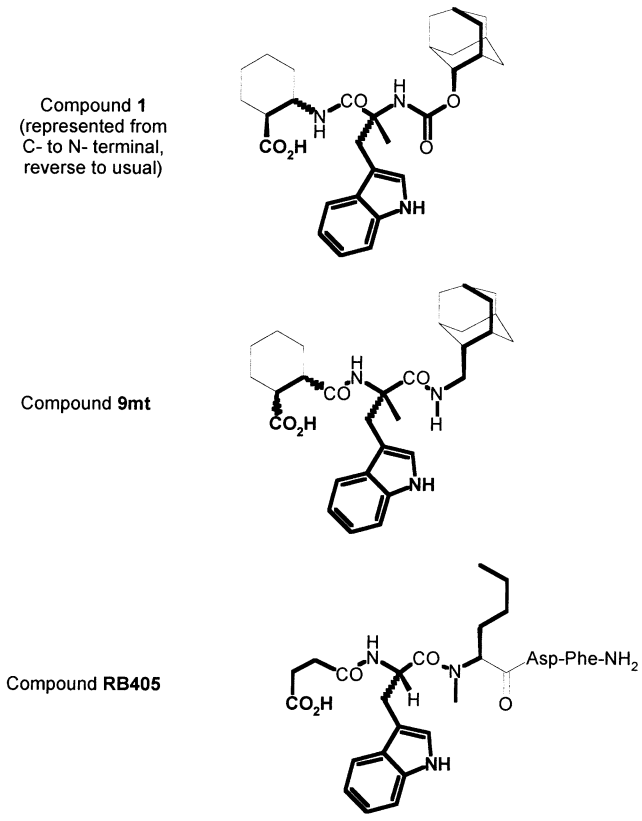


Fig. 7. Structural identity between compounds **1**, **9mt** and RB 405.

These two series of results strongly indicate that the *N-terminal parts of RB400 on the one hand and 'dipeptoids' on the other hand, can be successfully combined around a central tryptophan residue* (compounds **7ta**, **7tb** and **8b**), *whereas the C-terminus of 'dipeptoids' is not compatible with the N-terminus of CCK-peptide derivatives*, even less with the acidic end of RB400 (compounds **4** and **5**).

4.3. Orientation of peptide bonds and substituents of the cyclohexane ring

4.3.1. Conception and assay of CI-1015 retro-inverso analogues

Another major question risen by the new hypothesis formulated here was the role of the peptide bond for the

Table 3
Structure–affinity relationships in the tetrapeptide antagonist series (R–*Trp–AA1–Asp–AA2–NR′2) [23,38]

	R	*	AA1	AA2	R′	K_I (CHO) [a] (nM)	Pharmacological profile
2	Boc	L	NMeNle	Phe	H	0.8	Agonist
	Boc	L	Nle	Phe	H	3.6	Agonist
	Boc	D	Nle	Phe	H	160	Agonist
	Boc	L	Nle	1Nal	H	2.8	Agonist
13	Boc	L	Phg	1Nal	H	14	Agonist
11	Boc	L	Phg	1Nal	Me	39	Antagonist
	CH_3CH_2CO	L	Phg	1Nal	Me	71	Antagonist
	CH_3CH_2CO	αMeD	Phg	1Nal	Me	120	Antagonist
	^{2}Adoc	L	Phg	1Nal	Me	110	Antagonist
	Boc	αMeL	Phg	1Nal	Me	81	Antagonist
	Boc	αMeD	Phg	1Nal	Me	25	Antagonist
12	^{2}Adoc	αMeD	Phg	1Nal	Me	3.5	Antagonist
14	^{2}Adoc	αMeD	Phg	1Nal	H	3.4	Agonist/Antagonist

[a] Affinity measured by displacement of [^{3}H]pCCK-8 on membranes of CHO cells transfected with the rat CCK_2 receptor.

recognition of the receptor. Indeed, suggesting to look at compounds leftwards instead of rightwards meant that peptide bonds would be inverted when docked into the receptor. Therefore, 'retro-inverso' analogues of **1** (see structure on Fig. 2) were prepared, with CONH bonds being replaced by their NHCO equivalents. In the case of the N-terminal carbamate, it was chemically impossible to introduce a O–NHCO system, thus a methylene amide linkage CH_2NHCO was chosen.

In order to investigate the optimal arrangement of the substituents of the cyclohexane ring with limited difficulties of synthesis, we first prepared two compounds containing a non-methylated tryptophan residue, **9t** and **9c**. Both compounds (tested as racemic mixtures) had moderate affinities for the receptor (around 500 nM, see Table 2) but the stereochemical preference at the cyclohexyl moiety was *trans*, as previously observed for the CI-1015 series [17]. Nonetheless, this relatively low affinity was hardly surprising since replacing α-methyl-tryptophan by tryptophan, in all 'dipeptoid' series, is made at a high cost in terms of affinity: for instance, the demethylated analogue of CI-988 suffers a 37-fold decrease in affinity from the parent compound [37]. Therefore, we prepared **9mt**, the α-methylated analogue of the better compound **9t**, whose affinity was much better, as expected, being close to 50 nM on CHO cells. Since this was still 17 times lower than the reference compound CI-1015, we also tested **9mt** on a model closer to that of Parke–Davis' scientists, murine cerebral homogenates, were its affinity was 20 nM, sixfold lower than CI-1015 [17], but satisfactory however.

The loss in affinity observed from reference 'dipeptoids' to their retro-inverso derivative **9mt** may indicate that one or both peptide bonds are no more in their preferred orientation, but another point must be taken into account. Synthetic accessibility forced us to replace the oxy linkage between adamantyl and tryptophan by a methylene, which is not neutral in terms of ligand–receptor interactions; in the CI-988 series, the analogous operation (replacing the carbamate OCONH by CH_2CONH) led to a 10-fold decrease in affinity. Other linkages tested, for instance sulfonamide CH_2SO_2NH, dithiocarbamate SCSNH, led to significant losses in affinity, except the equipotent urea-containing derivative NHCONH [24]. This suggests that this position of the receptor must be very sensitive to changes of the hydrophobicity (mostly) and size of the ligand.

Thus, the observed difference between CI-1015 (or **1**) and **9mt** may only result from this single substitution (O

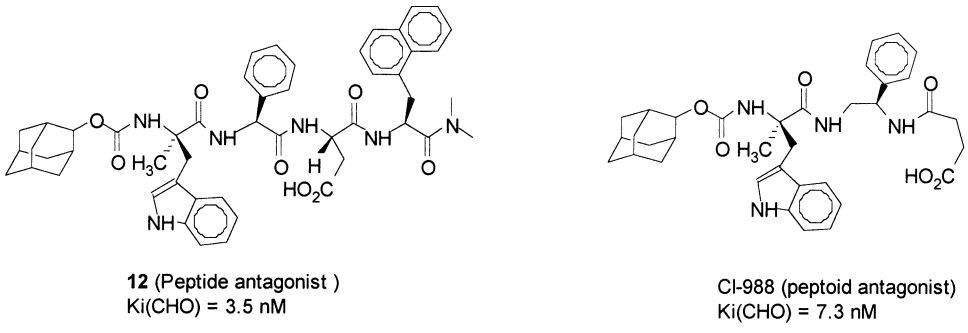


Fig. 8. Structural identity between CI-988 and compound **12**.

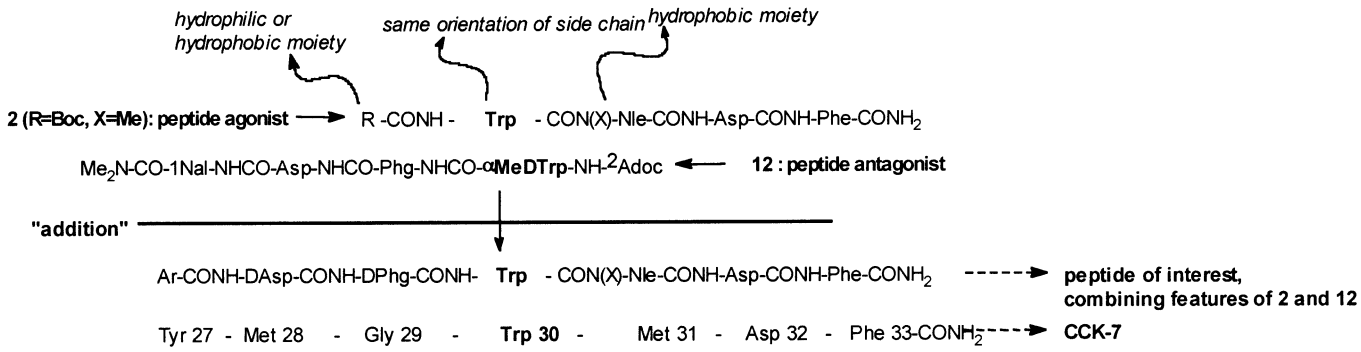


Fig. 9. Design of heptapeptides **10a–d** from **2** and **12**; superposition with CCK-7.

changed to CH_2 in the linkage of the terminal capping), more likely than from any influence of the orientation of the peptide bonds.

In summary, these results indicate that the peptide bonds surrounding the indole side chain may be inverted without inducing a brutal loss in affinity.

4.3.2. Comparison of RB400 and the new compounds (*9mt*)

At this point, superposition of **1** and **9mt** on the one hand, and of **9mt** and RB400 (Fig. 7) on the other hand, shows an extreme degree of similarity between these compounds, both in terms of structure, and of ability to recognise the receptor. This result, which is more visual than the evidence presented earlier, underlines that the alignment of **1** and RB400 in the binding site of the receptor is much more logical in the 'reverse' mode introduced here.

This one-dimensional superimposition supports the idea that *the orientation of the side chain of tryptophan is most important for retaining a good affinity for the receptor*, and that the rest of the molecule arranges around this anchor, thus being directed 'leftwards' or 'rightwards' in a one-dimensional representation.

4.4. Peptide antagonists

4.4.1. Literature data

While reconsidering the available data dealing with CCK ligands in the literature, we observed some unexplained results in a series of peptide agonists and antagonists, resembling the behaviour of 'dipeptoids' to a certain extent. In a first paper [23], an antagonist derived from CCK-4 had been characterised, Boc–Trp–Phg–Asp–1Nal–NMe_2 (compound **11**). Structure–affinity relationships in this series prove that only the dimethylation of the C-terminal amide is responsible for the antagonist character of this compound (compound **13**, Boc–Trp–Phg–Asp–1Nal–NH_2 is a CCK_2 agonist).

Most surprisingly, further development around compound **11** led to some results that could not be satisfactorily explained by the authors [38]. In fact, a L-Trp was very favourable for the binding of agonists (Table 3), while it was most preferred by antagonists, as exemplified by the optimisation of **11**–**12** (^{2}Adoc–αMe-D-Trp–Phg–Asp–1Nal–NMe_2). Moreover, the optimal N-protection of a D-Trp was ^{2}Adoc, the resulting affinities being better than for Boc-containing analogues, while the Ac-protected derivative suffered a considerable loss in affinity. On the opposite, when a L-Trp was introduced, the influence of this protecting group was weak, and Boc or acetyl were superior to ^{2}Adoc whatsoever. These results are recalled in Table 3: so, the behaviour of **12** and other derived antagonists resembles quite much that of peptoids, and we realised that their structure is astonishingly close, and for the C-terminal residue of **12**, as shown on Fig. 8: with respect to the results presented in the upper section, it is most important to stress that the orientation of the side chain of tryptophan is the same for both compounds.

Since 'dipeptoids' and peptides antagonists such as **12** are structurally very close, it seemed possible to extend the conclusions obtained from the work on the binding mode of 'dipeptoids' described earlier. This suggested that **12**, although closely related to the CCK-4 derivative **2** (Boc–Trp–NMeNle–Asp–Phe–NH_2) might in fact not bind to the same region of the receptor. Indeed, if we superimpose **2**, **12**, and their longer parent CCK-7, as shown on Fig. 9, it appears that **12** could in fact mimic the *N-terminal* tetrapeptide of CCK-7! Noteworthy, this implies the possible following substitutions in CCK-8: D-Asp for Met and D-Phg for Gly.

4.4.2. Conception and assay of heptapeptide probes

It is well-known that these two positions are not strictly constrained for CCK_2 receptor recognition: for instance, in CCK-7 series, Gly may be replaced by Ala, D-Ala or even D-Trp with only a moderate decrease in affinity; D residues even seem to confer a higher affinity than their L counterparts [39]. Besides, natural peptides

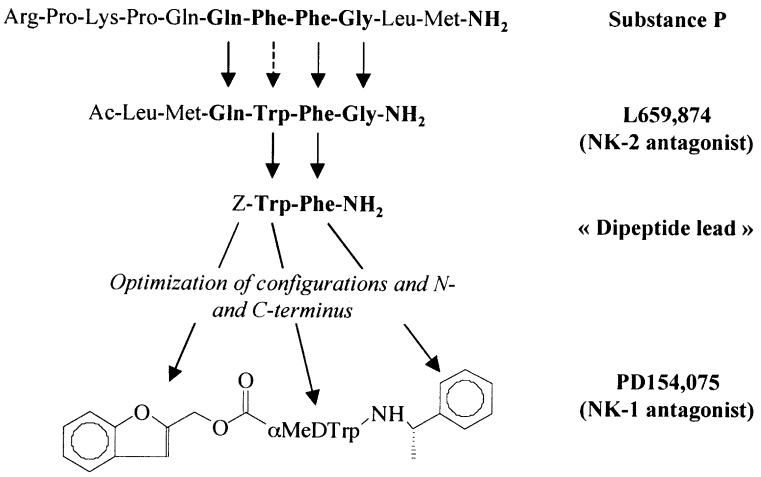


Fig. 10. The dipeptoid strategy applied to neurokinins.

such as gastrin [40] or caerulein [41] show hydrophilic substitutions for Met, respectively, sulphated tyrosine and threonine. The sulphated tyrosine of gastrin is also compatible with that of CCK as in cionin derivatives [42].

To fully ensure that the substitutions suggested by the association of **2** and **11** were compatible with CCK_2 affinity, we prepared peptides **10a**–**d** by solid-phase peptide synthesis techniques. The choice of phenylacetyl as N-terminal capping for CCK-6 derivatives proved not optimal, but the reference compound **10a** was endowed with a good enough affinity (39 nM), consistent for the aims of this study. Compound **10d** showed only a minor (2.7-fold) loss in affinity, resulting from a favourable effect of the Nle (or Met) → D-Asp substitution (seen in **10c**) and a negative impact of the Gly → D-Phg substitution (**10b**). The positive influence of the aspartyl moiety confirms one more time that the receptor displays a hydrophilic subsite at this level, which accounts for the specific properties of RB400 for instance. That heptapeptide compounds as **10a**–**d** have lower affinities for the CCK_2 receptor than their shorter counterparts as **2** and **12** may simply result from a higher rigidity and/or lower adaptability: it is certainly difficult for the longer compounds to fit correctly twice as much side chains into the corresponding pockets of the receptor.

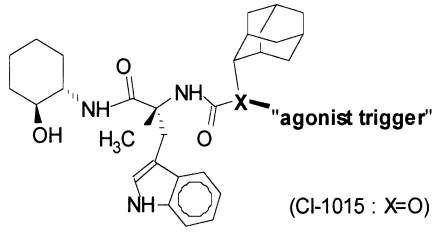


Fig. 11. A possible way to convert CCK_2 antagonists into agonists.

It appears therefrom that tetrapeptides may bind to regions of the receptor recognising either the C-terminal tetrapeptide Trp–Met–Asp–Phe of CCK-8, or its central tetrapeptide Tyr–Gly–Met–Trp; as shown for peptoids, the absolute configuration of the Trp residue is crucial for the orientation towards either binding mode.

5. Conclusions and perspectives

5.1. Conclusion: refinement of the 'dipeptoid' rationale

The compilation of available literature data describing structure–affinity relationships in peptide and 'dipeptoid' series of CCK_2 antagonists has led to the proposal of an alternative arrangement of 'dipeptoids' into the CCK_2 receptor, very different to what had been proposed so far. The preparation of selected compounds displaying characteristic features of both series altogether has allowed to explore and confirm the likeliness of this arrangement. *In this view, 'dipeptoids' thought to mimic a putative 'non-contiguous' Trp–Phe–NH_2 message directed by CCK-4, could rather be superposed to the central part of CCK-8, Gly–Trp–Met.*

Thus, the rationale underlying, *a posteriori*, the relation between CCK and compound **1**, for instance, could be:

- determination of the residue having the larger contribution to affinity of the endogenous peptide (here, Trp—this has been shown for instance in [43])
- construction of a tripeptide mimic centred on this residue, owing to bring sufficient additional features to confer high-affinity binding
- eventually, inverting the configuration of the central residue may open access to other variations (but in

this case, C- and N-terminal substituents should be regarded as reverted, at least in a first attempt)

5.2. Examination of other results based on the 'dipeptoid' strategy

Besides, the results disclosed here could set in jeopardy the real interest of the 'dipeptoid' strategy, since these results underline that the rationale developed by Parke–Davis' scientists was finally little rational at all in this case. A quick survey of NK-1, NK-2 and NK-3 antagonists obtained by the same means was undertaken to see if a generalisation of our restrictions was possible.

The core argument for the suggestion of an alternative binding mode for CCK_2 'dipeptoid' antagonists was the opposite optimal configuration of the crucial tryptophan residue. In the case of neurokinin antagonists derived from substance P, such a contradiction was less evident. Fig. 10 shows the extraction and optimisation of a dipeptide lead Z-Trp–Phe–NH_2, supposed to mimic the central part of substance P (Arg–Pro–Lys–Pro–Gln–Gln–*Phe*–*Phe*–Gly–Leu–Met–NH_2) [44]. Modifications of the first Phe residue in peptide series show that here, a D-Trp is favoured against a L-Trp [45], while in the 'dipeptoid' series, a D-Trp is also the better choice for the NK-1 antagonist PD154,075 [46]: in this case, there is no direct evidence for a similar problem as for CCK_2 antagonists.

5.3. Perspectives

5.3.1. Proposed further validation of our conclusions

A definitive check of our conclusion may come from the cartography of the CCK_2 binding site currently under study, in particular the residues interacting with the C-terminal dipeptide of CCK. Our model suggests that these two residues are not superimposable to any feature of 'dipeptoids', on contrary to the model of Horwell and co-workers. Thus, a mutation at this point abrogating CCK binding should have little influence on 'dipeptoid' binding—unless changing the global conformation of the receptor). Probing the CCK_2 receptor with the RB400 series should also have a great interest to validate our hypothesis, and to understand the seemingly particular importance [26] of the central tripeptide Gly–Trp–Nle of CCK, which peptoids may in fact mimic.

5.3.2. Development of non-peptide CCK_2 agonists

The conclusions of this study are particularly interesting in the field of CCK_2 agonists. The search for such compounds has recently been restarted by accumulating evidence for the pharmacological interest of a subclass of CCK_2 agonists called 'CCK_{2B}', displaying exciting properties such as memory stimulation, absence of anxiogenic effects, and increase of dopamine release [10]. Unfortunately, all compounds belonging to this subclass are peptidic and thus encounter problems of bioavailability.

If a few 'dipeptoids' have proved to be CCK_1 agonists [47], no one has been shown to display significant and reproducible CCK_2 agonist properties so far [48]. This study seems to propose a convenient explanation to this observation: 'dipeptoids' do not possess the essential features for activation of the receptor, which are known to be principally the C-terminal dipeptide. Introduction of an agonist 'trigger', which was achieved for benzodiazepine-based CCK_1 ligands [49], in the structure of peptoids, in their arrangement described here, as proposed on Fig. 11, may bring out a solution for the design of CCK_2—and hopefully CCK_2B—'dipeptoid' agonists.

Acknowledgements

We wish to thank warmly F.E. for multiform technical assistance, N.T. for the gift of (*R*)-**8**, and H.M. for recording of the NMR spectra. M.V. and E.M. are once again acknowledged for their support and fruitful advice.

References

[1] The word "peptoid" will frequently be used in this text in an inconvenient way, to be understood as "peptidomimetic". To support this misuse, we may stress that this family of CCK ligands has been named the "dipeptoid" family for more than ten years and is well-known in the field... but, in any case, the word "dipeptoid" was used between commas to make this misuse clearer to the reader.
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